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Genetic re-engineering of polyunsaturated phospholipid profile of *Saccharomyces cerevisiae* identifies a novel role for Cld1 in mitigating the effects of cardiolipin peroxidation

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Abstract

Cardiolipin (CL) is a unique phospholipid localized almost exclusively within the mitochondrial membranes where it is synthesized. Newly synthesized CL undergoes acyl remodeling to produce CL species enriched with unsaturated acyl groups. Cld1 is the only identified CL-specific

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AUTHOR CONTRIBUTIONS

W.L.*, H.T.*, C.A.R.*, V.E.K., and M.L.G. designed the experiments. W.L., C.A.R., and Z.L. performed yeast growth experiments, J.J. and W.Y. performed the measurement of respiration and membrane potential, Y.L. performed the qRT-PCR experiment, H.T. and Y.Y.T. performed the LC-MS analytical protocols identification and quantitation of lipids and their oxidation products, W.L., C.A.R., H.T., Y.Y.T., V.E.K., and M.L.G. analyzed the data, D.S. synthesized and characterized mito-AAPH, M.F. and P.W. synthesized cardiolipins, H.B. and J.G. participated in the design of the experiments, interpretation of results and discussion of the project, W.L., C.A.R., V.E.K., and M.L.G. wrote the manuscript.

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phospholipase in yeast and is required to initiate the CL remodeling pathway. In higher eukaryotes, peroxidation of CL, yielding CL_{OX}, has been implicated in the cellular signaling events that initiate apoptosis. CL_{OX} can undergo enzymatic hydrolysis, resulting in the release of lipid mediators with signaling properties. Our previous findings suggested that *CLDI* expression is upregulated in response to oxidative stress, and that one of the physiological roles of CL remodeling is to remove peroxidized CL. To exploit the powerful yeast model to study functions of *CLDI* in CL peroxidation, we expressed the *H. brasiliensis* ¹²-desaturase gene in yeast, which then synthesized PUFAs that are incorporated into CL species. Using LC-MS-based redox phospholipidomics, we identified and quantified the molecular species of CL and other phospholipids in *clDI* vs. WT cells. Loss of *CLDI* led to a dramatic decrease in chronological lifespan, mitochondrial membrane potential, and respiratory capacity, and increased levels of mono-hydroperoxy-CLs, particularly among the highly unsaturated CL species, including tetralinoleoyl-CL. In addition, purified Cld1 exhibited a higher affinity for CL_{OX}, and treatment of cells with H₂O₂ increased *CLDI* expression in the logarithmic growth phase. These data suggest that *CLDI* expression is required to mitigate oxidative stress. The findings from this study contribute to our overall understanding of CL remodeling and its role in mitigating oxidative stress.

Keywords

cardiolipin; remodeling; yeast; polyunsaturated fatty acid (PUFA); lipid peroxidation; mass spectrometry (MS); Cld1

INTRODUCTION

The dimeric mitochondrial phospholipid cardiolipin (CL) is most abundant in the mammalian heart, where it was first discovered [1]; however, it is present in nearly all mammalian tissues and throughout the eukaryotic kingdom [2]. The cellular and subcellular functions of CL have been studied for decades using the powerful yeast genetic model, *Saccharomyces cerevisiae* [3]. Yeast genes encoding the CL biosynthetic enzymes (Fig. 1) have been identified, and well-characterized mutants are available for each step of the pathway, enabling *in vivo* studies of CL function that are not easily carried out in mammalian cells [4]. The yeast model has, thus, been pivotal in demonstrating the critical role of CL in mitochondrial bioenergetics [5–9], mitochondrial protein import [6, 10], mitochondrial dynamics [11, 12], cell cycle progression [13], cellular aging [14], and mitophagy [15] among other essential functions.

CL that is synthesized *de novo* undergoes remodeling, in which it is deacylated to mono-lyso-CL (MLCL), which is reacylated by the transacylase, tafazzin, thus forming CL species that are more unsaturated [16, 17]. Polyunsaturated tetralinoleoyl-CL (TLCL) is the major CL species detected in human and other mammalian heart, skeletal muscle and liver cells [17]. Despite the obvious energetic expenditures required for the biosynthesis of a unique homo-acylated TLCL, the physiological significance of TLCL remains unclear. It is absent from cells of patients with Barth syndrome (BTHS), an X-linked recessive disorder associated with variable cardiomyopathy, skeletal myopathy, neutropenia, and organic

aciduria [17, 18]. BTHS is caused by mutations in the *TAZ* gene that codes for tafazzin [19]. Accordingly, BTHS patient cells accumulate MLCL and are devoid of TLCL, owing to a loss in the final step of the CL remodeling pathway [20]. In yeast, unremodeled and remodeled CL are thought to be functionally indistinguishable [21], and deletion of the CL-specific phospholipase, Cld1, was found to rescue the defects caused by loss of the CL-transacylase, Taz1 [22]. This raises the question of why cells remodel CL, and how CL enriched with PUFAs are functionally distinct from those containing saturated or monounsaturated fatty acyl CL species. Recent studies indicate that peroxidation of CL is a powerful cell signaling event that can initiate apoptosis [23–28]. Furthermore, the enzymatic hydrolysis of oxidized CL (CL_{OX}), leading to the release of lipid mediators, may have diverse signaling properties [29, 30]. In the wild, yeast cells proliferate in diverse environments [31–34], from which PUFAs may be readily incorporated into CL. We previously reported that yeast cells supplemented with PUFAs linoleic acid (C18:2) or arachidonic acid (C20:4) readily incorporate these fatty acids into CL [35]. Cipak and co-workers demonstrated that *S. cerevisiae* cells expressing the *H. brasiliensis* ¹²-desaturase synthesize PUFAs and exhibit sensitivity to oxidative stress [36, 37]. However, the effect of ¹²-desaturase-expression on CL biosynthesis and remodeling or CL peroxidation has not been explored.

In the current study, multiple species of CL_{OX}, including mono-hydroperoxy-CL and di-hydroperoxy-CL, were also detected in these cells. Further, oxidized species of two additional phospholipids abundant in mitochondria, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were also detected. However, the oxidized form of phosphatidylserine (PS), which is lacking from mitochondria, was not detected. Thus, our findings suggest that yeast cells synthesizing PUFAs generate CL_{OX} species within the mitochondria. We compared the CL profiles of ¹²-desaturase-expressing WT and *cld1* cells. ¹²-Desaturase-expressing *cld1* cells contain increased levels of di-hydroperoxy-CLs, particularly among the highly unsaturated CL species, including TLCL. Furthermore, we observed that *CLD1* expression is upregulated in response to treatment with H₂O₂, and purified Cld1 protein exhibits higher activity towards hydroperoxy-TLCL than non-peroxidized TLCL. The presence of PUFA-containing CL and the production of CL_{OX} were not associated with major growth defects under optimum conditions. Interestingly, loss of *CLD1* in ¹²-desaturase-expressing cells led to decreased chronological lifespan. These results suggest a novel role for Cld1 and CL remodeling in mitigating the effects of oxidative stress and CL peroxidation. Importantly, these findings provide a powerful genetic model that can be exploited to elucidate mechanisms underlying the generation and function of CL peroxidation.

METHODS AND MATERIALS

Strains, Media, and Growth Conditions—

The *S. cerevisiae* strain used in this work is BY4741(*MATa his3⁻1 leu2⁻0 met15⁻0 ura3⁻0*). Plasmids pYES2-EV and pYES2-*desa* were kindly given by Dr. Ana Cipak Gasparovic (Rudjer Boskovic Institute, Zagreb, Croatia). Expression of the ¹²-desaturase is controlled by a Gal promoter and is induced by growth on galactose-containing medium. Complex

medium contained yeast extract (1%), peptone (2%), and glucose (2%) (YPD). The solid medium contained agar (2%) in addition to the ingredients mentioned above. Synthetic complete (SC) *ura⁻* medium contained adenine (20.25 mg/liter), histidine (20 mg/liter), arginine (20 mg/liter), lysine (200 mg/liter), leucine (60 mg/liter), methionine (20 mg/liter), tryptophan (20 mg/liter), threonine (300 mg/liter), all the essential components of yeast nitrogen base without amino acids (Difco), 0.2% ammonium sulfate, and glucose/galactose (2%). Yeast strains were grown at 30°C. *E. coli* strain DH5- α was used for plasmid maintenance and amplification. Bacteria were grown at 37°C in LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl), supplemented with ampicillin (100 μ g/mL) for selection purposes. For growth on plates, media were supplemented with 1.5 and 2% agar for *E. coli* and yeast, respectively. Growth in liquid cultures was monitored spectrophotometrically. For lipidomic analysis, cells were harvested and centrifuged at 4°C (4,300 rpm, 5 min). The supernatant was discarded, and glass beads (0.3 g) were added to the centrifuge tubes. Samples were vortexed (5 $\times$ 1 min intervals) and centrifuged again at 4°C (4,300 rpm, 5 min). Supernatants were collected and frozen at -80°C for lipidomic analysis.

Construction of Plasmids and Expression of Yeast His₆-tagged Cld1 in *S. cerevisiae*—

To construct a His₆-tagged *CLD1* overexpression plasmid, a 1373-bp sequence containing the entire open reading frame without the stop codon of *CLD1* was amplified from yeast genomic DNA using an EcoRI-tagged forward primer *CLD1_EcoRI_F3* (5'-TGATTAATAAGAATTCAACACAATGTTCAAGTCAACTTTAACTC-3') and an XbaI-tagged reverse primer *CLD1_XbaI_R3* (5'-ATTTTGAGATTCTAGATATTTTTGCATTCTTTTCG-3'). The PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega). The purified DNA fragments were ligated into the pYES2/CT vector cut with *EcoRI* and *XbaI* downstream of the GAL1 promoter. All the plasmids were amplified and extracted using standard protocols. The plasmids were transformed into yeast strains using a one-step transformation protocol.

Purification of His₆-tagged Cld1—

S. cerevisiae BY4741 was used to express the recombinant His₆-tagged Cld1. Cells were grown at 30°C in synthetic complete raffinose medium lacking uracil. Galactose (2%) was used to induce overexpression of the recombinant protein. Cell extracts were prepared by disrupting cells with glass beads (0.5 mm diameter), vortexing for 5 min intermittently. The disruption buffer contained 50 mM Tris-Cl (pH 8.0) and 300 mM NaCl. Protease inhibitor was added to the disruption buffer before breaking the cells. His₆-tagged Cld1 was purified with Millipore® PureProteome nickel magnetic beads following the manufacturer's instructions. Millipore® UFC503024 Amicon® Ultra-0.5 centrifugal filter concentrator (30 KD) was used to concentrate the protein and remove imidazole. Protein concentration was determined with BIO-RAD DC protein assay kit using bovine serum albumin as the standard.

Synthesis of CLs—

Commercially available CLs: 1,1',2,2'-Tetramyristoyl-CL (TMCL, CL (14:0)₄); 1,1',2,2'-Tetralinoleoyl-CL (TLCL, CL (18:2)₄) and 1,2-Dioleoyl-sn-glycerol-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). 1,1',2,2'-

Tetrapalmitoyl-CL and (TPCL, CL (16:0)₄) was synthesized and purified as previously described [35]. Hydroperoxy-TLCL (TLCL-OOH, CL(18:2)₃/18:2-OOH) was prepared in a soybean lipoxygenase-catalyzed reaction (Cayman Chemical Company, Ann Arbor, MI) in 50 mM HEPES buffer, containing 100 μ M DTPA. To prevent the conversion of hydroperoxy-groups to secondary products during incubation, HEPES buffer was saturated with oxygen. After CL oxygenation, the crude product was purified by HPLC. Internal MLCL (14:0)₃ and reference standards MLCL (16:0)₃ and MLCL (18:2)₃ were prepared from the respective CLs by a PLA2 (porcine pancreatic, Sigma) driven reaction as described by Kim and Hoppel [38] with slight modifications.

Chronological Lifespan—

Yeast chronological lifespan was determined by survival of non-dividing cells in a prolonged stationary culture [39]. A protocol modified from the one previously described [40] was followed to assess the chronological lifespan. In brief, individual colonies were inoculated in 5 mL of SC galactose medium and incubated overnight. The cultures were then diluted in 50 mL of SC galactose medium, and cells were allowed to grow until saturation during the first 24 hours. At this time, cells stopped dividing and reached a maximum number. Viability was considered to be 100% at this time, and viable cells were measured by counting colonies that were formed following dilution and plating on YPD plates. Viable cells were measured every day in the same volume of cell culture and the percentage of cells that formed colonies on day one was determined.

Spotting Assay—

Cells were pre-cultured in SC galactose medium to the early stationary growth phase at 30°C and washed with sterile water. 2.5 μ L aliquots of a series of 10-fold dilutions of 0.5 units of A₅₅₀ cells were spotted onto the indicated plates and incubated at 30°C for 3 days.

Real-Time Quantitative PCR (RT-qPCR) Analysis—

Cells were grown to the indicated growth phase and harvested at 4°C. Total RNA was extracted and purified using the RNeasy Mini Plus kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized using the first strand cDNA synthesis kit (Roche Applied Science) according to the manufacturer's manuals. RT-qPCRs were performed in a 20 μ L volume using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA). Triplicates were included for each reaction. The primers for *CLDI* and *ACT1* RT-qPCR were described previously [22]. RNA levels were normalized to *ACT1*. Relative values of mRNA transcripts were shown as fold change relative to indicated controls.

Synthesis of Mito-AAPH-

2-[(E)-(2-Amino-2-imino-1,1-dimethyl-ethyl)azo]-2-methyl-propanimidamide hydrochloride (2**; yield, 82%):** In ice-cold methanol (100 mL), 2,2'-azobis-2-methyl-propanimidamide (AAPH; 2,2'-azobis-2-methyl-propanimidamide, **1**; 1 g; 3.7 mmol) was neutralized with NaOH (0.3 g; 7.4 mmol). The solvent was rotor-evaporated (25 °C; 10 torr) and the crystals formed were suspended in chloroform (100 mL). Undissolved **1** and NaCl were filtered off. Rotor-evaporation of the filtrate afforded **2** (0.6 g; 3 mmol), which was used immediately for the preparation of **5**.

(4-Chloro-4-oxo-butyl)-triphenyl-phosphonium bromide (4**; yield, 65%):** 3-carboxypropyl(triphenyl)phosphonium bromide (**3**, 1 g; 2.3 mmol) was dissolved in chloroform (50 mL) containing thionyl chloride (3 g; 25 mmol) and dimethylformamide (DMF; 0.05 g; 0.68 mmol), and the reaction solution was refluxed for 4 hours. The volume of the solvent was reduced to ~5 mL (rotor-evaporation; 25 °C; 150 torr) and trituration with n-hexane afforded **4** (0.68 g), which was dried under vacuum (40 °C; 10 torr) for 1 hour.

2-[(E)-[2-Imino-1,1-dimethyl-2-(4-triphenylphosphoniumylbutanoylamino)ethyl]azo]-2-methyl-propanimidoyl]ammonium chlorobromide (5**; yield 45%):** Chloroform (50 mL) containing **2** (0.5 g; 2.5 mmol) and **4** (1.12 g; 2.5 mmol) was incubated for 24 hours at 0 °C. Thereafter, the solvent was rotor-evaporated (20 °C; 10 torr) and the dry residue was redissolved in minimal volume of ethanol. Trituration of the solution with diethyl ether afforded **5** (0.69 g), which was filtered and dried under vacuum (20 °C; 1 torr) for 30 min. ESI MS calculated for the triphenylphosphonium cation of **5** (C₃₀H₃₈N₆OP⁺), 529.64; found, 529.80.

Mito-AAPH Treatment—

Mito-AAPH was diluted with ethanol to make a 50 mM stock. For the spotting assay, 50 μM Mito-AAPH was directly spread on the plate before the experiment. For liquid culture, 50 μM Mito-AAPH was added at log phase ($A_{550} \approx 0.5$).

Determination of mitochondrial membrane potential (MMP)—

Cells were grown in SC Gal Ura⁻ medium to log phase and incubated with 500 nM TMRM for 30 min at 30°C. Cells were washed, resuspended in warm medium and divided in half. One group was treated with 6 μ M FCCP and the other group was untreated. The TMRM fluorescence intensity was determined in 30,000 cells from each group using an LSR II flow cytometer in the Wayne State University Microscopy, Imaging and Cytometry Resources Core. Median fluorescence intensity was calculated after correcting for background TMRM fluorescence (values obtained after treatment with FCCP) using FlowJo v10.2.

Respiration measurements—

Oxygen consumption rate (ORC) of intact yeast cells was analyzed via the polarographic method in a closed 500 μ L chamber equipped with a micro Clark-type oxygen electrode (Oxygraph Plus System, Hansatech Instruments) at 30°C. Yeast cells were grown in synthetic complete medium until log phase. Cells were mixed in fresh medium with a cell density normalized to a protein concentration of 0.5 mg/mL followed by the measurement of respiration. KCN (0.2 mM) was added at the end of the experiment to inhibit cytochrome c oxidase, to correct for cytochrome c oxidase-independent oxygen utilization. Oxygen consumption was recorded on a computer and analyzed with Oxygraph plus software. Oxygen consumption rate (ORC) is defined as consumed O₂ (nmol)/min/total protein (mg).

Statistical Analysis—

The results are presented as mean $\pm$ S.D. values from at least three experiments, and statistical analyses were performed by paired/unpaired Student's t-test. The statistical significance of differences was set at $p < 0.05$.

LC/MS assessment of phospholipids—

Lipids were extracted from cells, and the LC/MS analysis of phospholipids was performed as previously described [35]. Briefly, phospholipids were analyzed in negative mode using a Dionex UltimateTM 3000 RSLCnano system coupled online to a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA). Lipids were separated on a normal phase column (Silica Luna 3 μ m, 100A, 150 $\times$ 2 mm, (Phenomenex, Torrance CA)) with flow rate 0.2 mL/min using gradient solvents containing 5 mM CH₃COONH₄ (A - *n*-hexane:2-propanol: water, 43:57:1 (v/v/v) and B - *n*-hexane:2-propanol: water, 43:57:10 (v/v/v)). MS lipid standards used in this study were from Avanti Polar Lipids (Alabaster, AL). Analysis of LC-MS data was performed using software package Compound DiscovererTM (ThermoFisher Scientific) with an in-house generated analysis workflow and peroxidized phospholipid database.

RESULTS

Expression of the *H. brasiliensis* Δ^{12} -desaturase alters the molecular speciation of CL in *S. cerevisiae* cells -

Yeast cells that are not supplemented with exogenous fatty acids produce only saturated and ⁹-monounsaturated fatty acyl residues, and cannot synthesize PUFAs [35]. To construct a

yeast model with PUFA-containing CL, we expressed the *H. brasiliensis* Δ^6 -desaturase gene in yeast [36] and established its effect on the phospholipidome of these cells. As described in Figure 1, yeast readily produce 16:0-CoA and 18:0-CoA, which can be converted to 16:1-CoA and 18:1-CoA via endogenous Δ^9 -desaturase activity. These acyl-CoA species can be incorporated into phosphatidic acid (PA) and used in the *de novo* synthesis of CL via the CL synthase (Crd1) -catalyzed reaction to form nascent CL, which contains predominately unsaturated fatty acyl chains [41–44]. Nascent CL is deacylated by the phospholipase, Cld1, to form monolysocardiolipin (MLCL) [16, 45]. The transacylase, Taz1, utilizes donor phospholipids, particularly PC and PE, to acylate MLCL to form CL, with the concomitant formation of lyso-PC (LPC) and lyso-PE (LPE) [46, 47]. Multiple cycles of deacylation-reacylation lead to the synthesis of mature CL containing mainly unsaturated acyl chains [46, 47]. As we showed previously, Cld1 has a greater affinity for C16- than for C18-containing acyl chains [35]. Accordingly, the predominant CL species detected in yeast contain C18 acyl chains.

The expression of Δ^{12} -desaturase results in the production of 16:2-CoA and 18:2-CoA, which are incorporated into CL through *de novo* synthesis or via CL remodeling. As shown in Figure 2A expression of the Δ^{12} -desaturase caused dramatic changes in CL composition. Wild-type (WT) yeast cells expressing empty vector (EV) have predominantly species containing saturated or monounsaturated fatty acyls such as CL64:3, CL66:4, CL68:4, CL70:4 and CL72:4, while WT cells expressing the Δ^{12} -desaturase contained predominantly CL66:5, CL68:5, CL70:6 and CL72:7. The appearance of PUFA-CL species are readily observed in differential LC-MS spectra obtained by subtracting the spectrum corresponding to EV-expressing WT cells from the spectrum of Δ^{12} -desaturase-expressing WT cells (red spectrum). Similarly, the loss of saturated and monounsaturated CL is apparent when subtracting the spectrum of Δ^{12} -desaturase-expressing WT cells from EV-expressing WT cells (blue spectrum) (Figure 2B). The quantitative assessment of individual CL species indicates that Δ^{12} -desaturase expression resulted in the production of multiple PUFA-containing CL species and a concomitant reduction in the abundance of CL species containing saturated or monounsaturated fatty acids (Figure 2C and 2D).

Δ^{12} -desaturase expression leads to accumulation of CL_{OX} -

The accumulation of CL_{OX} was readily observed in WT cells expressing Δ^{12} -desaturase (Figure 3A–C). Mono-hydroperoxy-CL and di-hydroperoxy-CL species were observed in these cells. As anticipated, oxidized CL species were not detected in EV-expressing WT cells. Oxidized MLCL species were not found, but oxidized PG, particularly PG36:4+2[O], was readily detectable in the Δ^{12} -desaturase-expressing WT cells (Figure 4A). The accumulated CL_{OX} could be synthesized from either pre-oxidized PG (containing peroxidized fatty acid residues), or generated from oxidizable PUFA-CL species via direct peroxidation or via re-acylation with the transfer of peroxidized PUFA residues.

Fragmentations of non-peroxidized PUFA-CL, including 16:2 and 18:2 fatty acids, were found in our analysis, but the structural information related to the location of dienes was not available. In yeast, exogenous plant Δ^{12} -desaturase utilized 16:1-CoA and 18:1-CoA that were generated by endogenous Δ^9 -desaturase as substrates [36], indicating that the location

of dienes are at position 9 and position 12 in the 18:2 and 16:2 aliphatic chain. In addition, the fragmentations of CL monohydroperoxides and dihydroperoxides were examined to precisely identify CL peroxidation and locate the oxygen within the species. However, the fragmentations were not able to be detected due to insufficient levels of CL peroxidation products (the ratio of oxidized CL in the analysis was about 0.13 mol% of total phospholipids and 2.5 mol% of non-oxidized CL). In fact, the position of oxygen in CL monohydroperoxides and dihydroperoxides was already identified via mass spectrometry in previous studies when the CL peroxidation was performed *in vitro*. Previously, the position of oxygen was structurally identified in CL generated from TLCL incubated with cytochrome *c* (cyt *c*) and H₂O₂, in which hydroperoxidation at position 9 and position 13 was detected [48]. In addition, soybean lipoxygenase incubated with CL specifically generated 13-hydroperoxy-CL and a very low amount of 9-hydroperoxy-CL [49]. Recently, Shu-Ping Hui and coworkers identified the position of oxygen in CL monohydroperoxides generated from oxidation of commercial bovine heart CL and HepG2 lipid extracts treated with CuSO₄/H₂O₂, showing that the oxygen atom was located at position 13 of linoleoyl chain [50]. However, the location of oxygens of CL peroxidation occurring within cells is still unknown. Further investigation is needed.

Detection of other oxidized phospholipids in ¹²-desaturase-expressing WT cells -

The effects of ¹²-desaturase expression on PC (Figure 4B and S1), PE (Figure 4C and S2), and PS (Figure 4D and S3) were determined. All phospholipids analyzed in these cells contained C16:2 and/or C18:2 fatty acyl chains. Oxidized species of PC (PC_{OX}) and PE (PE_{OX}) were detected in ¹²-desaturase-expressing cells. Interestingly, however, oxidized PS (PS_{OX}) species were not found. Given that PC and PE species are enriched within mitochondrial membranes while PS species contribute minimally to the mitochondrial phospholipidome [51, 52], this finding suggests that phospholipid peroxidation observed in ¹²-desaturase-expressing cells likely occurs within the mitochondrial subcellular compartment. This conclusion is further supported by the comparative data on the absolute amounts of oxidized phospholipids and their relative content (mol %) normalized to either total amounts of all phospholipids or the amounts of individual phospholipid classes (Table 1). The results show that the total oxidized phospholipid species constituted ~1% of the total PLs whereby PC_{OX} products were the most abundant. Notably, the content of CL_{OX} constituted ~2.5% of the CL species, compared to the levels of PC_{OX} and PE_{OX}, which were ~1.1% and 0.9% as normalized to PC and PE, respectively.

¹²-Desaturase expression results in an increased sensitivity to mitochondria-targeted lipid peroxidation -

We next used a mitochondria-targeted azo-initiator of peroxy radicals, mito-AAPH, to examine how CL peroxidation and growth are affected in ¹²-desaturase-expressing WT cells. We determined that ¹²-desaturase-expressing WT cells exhibit increased sensitivity to mito-AAPH (Figure 5A). As mito-AAPH partitions into mitochondria, it spontaneously decomposes at a constant rate to generate carbon-centered radicals, which, in aerobic conditions, are converted into peroxy radicals that stimulate lipid peroxidation [53]. Exposure to mito-AAPH resulted in increased generation of specific mono-hydroperoxy-CL species, including CL62:6+2[O], CL 64:8+2[O], CL66:7+2[O], CL66:8+2[O],

CL68:8+2[O], and CL72:8+2[O] (Figure 5B). PC36:4+2[O] (Figure 5C), PE32:4+2[O], PE34:4+2[O] (Figure 5D), and PG36:4+2[O] (Figure 5E) were also increased. CL66:7+2[O] and PE34:3+2[O] were found only in desaturase-expressing WT cells exposed to mito-AAPH. Oxidized MLCL and PS were not found in desaturase-expressing WT cells exposed to mito-AAPH. These results suggest that the increased mitochondrial lipid peroxidation contributes to the overall growth defects observed in the $\Delta 12$ -desaturase-expressing WT cells.

***cld1* cells that express $\Delta 12$ -desaturase exhibit decreased levels of PUFA-CLs including L4CL but increased levels of specific peroxidized CL species -**

We have previously shown that expression of *CLD1* is highest when cells are actively respiring [22]. These conditions lead to increased generation of ROS and a pro-oxidant environment conducive to enhanced PUFA lipid peroxidation. Increased *CLD1* in these conditions suggests the possibility that Cld1 deacylates peroxidized CL, in which case the loss of *CLD1* would lead to increased content of peroxidized CL. To investigate the role of Cld1 in deacylating peroxidized CL, we expressed the $\Delta 12$ -desaturase in *cld1* cells. PUFA-containing CL species were detected in the $\Delta 12$ -desaturase-expressing *cld1* cells, likely because some incorporation of PUFA into CL occurs during *de novo* CL biosynthesis.

In *cld1* +desa, (Figure 6A), the LC-MS spectrum contained the signals from CL species formed through the biosynthesis. By subtracting this spectrum from the spectrum corresponding to the WT cells expressing $\Delta 12$ -desaturase we obtained the differential LC-MS spectrum (Figure 6B) where the increasing red signals correspond to the remodeling products whereas the decreasing blue signals are derived from the substrates of the remodeling pathway. In other words, CL species containing saturated and monounsaturated acyl chains were susceptible to remodeling, and the remodeling process yielded CL species with polyunsaturated fatty acyls. Quantitatively, these data are summarized in the heat-map (Figure 6C). PUFA-containing CL species were detected in the $\Delta 12$ -desaturase-expressing *cld1* cells, likely because some incorporation of PUFA into CL occurs via *de novo* CL biosynthesis. Peroxidized CL species were also detected in *cld1* cells expressing $\Delta 12$ -desaturase. Surprisingly, although $\Delta 12$ -desaturase-*cld1* cells had decreased total levels of peroxidizable CL (Figure 6D), they contained significantly more CL_{OX} species within PUFA-CL species (CL64:7, CL64:8, CL66:7, CL66:8, CL68:7, CL68:8, CL70:8, and CL72:8) (Figure 7A–B). Notably, mono-hydroperoxy-CLs were mainly found in *cld1* (Figure 7A–B), whereas di-hydroperoxy-CLs were predominately generated in WT cells (Figure 7A).

Cld1 mitigates the effects of CL peroxidation -

As both non-oxidized as well as peroxidized PUFA-CL species may be hydrolyzed by Cld1, we performed *in vitro* experiments which showed that Cld1 exhibits greater affinity for the latter than for the former (Figure 8A). Incubation of DOPC/CL (16:0)₄; DOPC/CL (18:2)₄ and DOPC/CL (18:2)₃/18:2-OOH liposomes with Cld1 isolated from *S. cerevisiae* resulted in the accumulation of two molecular species of MLCL respectively. The signals at m/z 1113.7 and 1185.7 corresponded to 16:0/16:0/16:0 and 18:2/18:2/18:2 molecular species. Quantitatively, the larger signals were formed from TPCL and TLCL-OOH respectively and

a much weaker signal (also at m/z 1185.7) from TLCL (Figure 8B). These data indicate that CL (18:2)₄ was a relatively “poor” substrate for Cld1, whereas both CL (16:0)₄ and CL (18:2)₃/18:2-OOH were readily hydrolyzed by the enzyme (Figure 8B). We have previously shown that *CLDI* expression is increased during respiratory growth and regulated by the HAP complex [22]. It is plausible that increased *CLDI* expression during respiratory growth mitigates the effects of superoxide anions and their dismutation product H₂O₂ generated by respiratory complex III. This would likely contribute to the enhanced peroxidation of CL. Consistent with this possibility, WT cells treated in the log phase with H₂O₂ for 1 hr exhibited a 2-fold increase in *CLDI* expression (Figure 8E). The chronological life span of *cld1* was dramatically decreased by expression of ¹²-desaturase (Figure 9A), while the lifespan of WT cells expressing the ¹²-desaturase was not affected. Interestingly, while desaturase expression increased the mitochondrial membrane potential and respiratory capacity in WT cells, the mitochondrial membrane potential and respiratory capacity in *cld1* cells were decreased (Figure 9B–C). Taken together, these findings support a role for Cld1 in mitigating the effects of CL peroxidation.

DISCUSSION

This study demonstrates for the first time that ¹²-desaturase expression in yeast promotes the formation of PUFA-containing CL species that are readily peroxidized *in vivo* without any pro-oxidant stimulation. To date, the study of processes governing CL peroxidation and the various cellular signaling functions of CL_{OX} and CL_{OX}-derived lipid mediators has not been possible using yeast model systems. We have confirmed that 16:2-CoA and 18:2-CoA produced by yeast expressing the ¹²-desaturase are incorporated into CL. This likely occurs, in part, during *de novo* synthesis of CL from PUFA-containing PG and CDP-diacylglycerol by CL synthase, with subsequent enrichment of CL with PUFAs occurring during CL remodeling.

This is the first study to identify a role for Cld1 in mitigating the effects of CL peroxidation. Previously, we determined that deletion of *CLDI* rescues the respiratory growth defect of tafazzin-deficient yeast cells, indicating that an increased MLCL/CL ratio, not a decrease in CL containing unsaturated fatty acyl chains, is detrimental to tafazzin-deficient cells [22]. Similarly, a study by Baile and colleagues illustrated that unremodeled and remodeled CL are functionally indistinguishable in yeast [21]. An interesting question arises from these observations: why do yeast require CL remodeling if there is no adaptive advantage of accumulating CL dominated by unsaturated fatty acyl chains? A clue to answering this question comes from our current findings, which indicate that expression of *CLDI* is increased in response to respiratory growth conditions, and this increase is mediated by the HAP and Mig1 transcriptional factors. Baile and coworkers revealed that Cld1 protein level and function are decreased when cells are grown on glucose and increased when grown on the non-fermentable carbon source, lactate [45]. They observed that CL remodeling is regulated by carbon source and mitochondrial membrane potential. Mitochondria are the major producer of cellular ROS, and CL-hydroperoxides are known to reduce oxidative metabolism by inactivating complex IV [54]. Similarly, respiratory chain defects have been shown to increase ROS production and decrease the membrane potential [55, 56]. These findings support a model in which decreased membrane potential resulting from the

accumulation of CL-hydroperoxides serves as a signal to activate CL remodeling. This model is further supported by the notion that CL remodeling may function to remove peroxidized fatty acyl residues from CL, thereby mitigating the downstream consequences of mitochondrial CL- hydroperoxide accumulation, possibly including mitophagy or apoptosis. In mammalian cells, Liu et al. demonstrated that iPLA2 γ , a gene product whose function is analogous to Cld1, is the major enzyme responsible for deacylation of oxidized aliphatic chains from CL [57]. Deletion of iPLA2 γ results in the accumulation of peroxidized CL in mouse mitochondria when subjected to oxidative stress. However, it is unclear if this model applies to yeast cells because the loss of *CLD1* does not cause any detectable functional defects [21]. CL-hydroperoxides are not produced in either WT or *cld1* cells unless PUFA is supplied in the growth medium or an exogenous desaturase is expressed [35]. WT yeast cells readily incorporate PUFAs into CL, which suggests that CL enriched with PUFAs may influence cellular function in *S. cerevisiae*.

In the current study, we used 12 -desaturase-expressing cells to study the functions of Cld1 and CL remodeling in the presence of PUFA-containing CL, and reveal that loss of Cld1 caused an accumulation of CL_{OX} species among the C18:2-containing CL and decreased chronological lifespan. These results suggest that Cld1 is involved in removing C18:2-hydroperoxide acyl moieties from CL_{OX} species. This is supported by the demonstration that purified Cld1 has a higher affinity for peroxidized (C18:2)₄CL than non-peroxidized (C18:2)₄CL. This may explain the previous observation that *CLD1* expression is upregulated during the stationary phase and that *CLD1* upregulation is respiration dependent [22]. Consistent with this model, H₂O₂ treatment, which leads to increased oxidative stress, upregulates *CLD1* expression.

In the current study, we found that 12 -desaturase-expressing WT cells exhibit increased sensitivity to mito-AAPH, and treatment with mito-AAPH was associated with an increase in C18:2-containing CL_{OX} species. Increased sensitivity to lipid peroxidation was previously reported using this genetic yeast model [37]. However, our comprehensive lipidomics approach provides compelling evidence to implicate mitochondrial redox reactions and CL peroxidation in contributing to the growth defects observed in 12 -desaturase-expressing WT cells. Excessive generation of CL_{OX} in response to mitochondrial oxidative stress likely contributes to the small but significant growth defect observed under these conditions.

The absence of oxidized PS supports a model of mitochondria-localized phospholipid peroxidation. PS species are confined almost exclusively to the cell membrane and secretory vesicles [58]. Although PS is imported into mitochondrial membranes, its intra-mitochondrial transport is linked to enzymatic conversion to PE by PS decarboxylase [59–61]. Thus, the relative abundance of PS within the mitochondrial membranes is minimal when compared to PG, PC and PE [58]. Peroxidation of CL, PG, PC, and PE but not PS suggests that phospholipid peroxidation observed in 12 -desaturase-expressing cells likely occurs within the mitochondrial subcellular compartment. In yeast, beta-oxidation exclusively occurs in the peroxisome, but in mammalian cells, it occurs within both mitochondria and peroxisomes. To be specific, short chain fatty acids (C6-C20) are degraded by mitochondrial beta-oxidation, but long chain fatty acids (>C20) are oxidized in the peroxisome. Hydrogen peroxide resulting from oxygen in the first step of beta- oxidation is

the major source of ROS in the peroxisome. Mitochondria, especially the electron transport chain, have long been considered the major source of ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radicals [62]. In mammalian cells, ROS are predominantly generated from Complex I and Complex III, while in *Saccharomyces cerevisiae* ROS are generated only by Complex III due to the lack of Complex I [63, 64]. Indeed, hydrogen peroxide can easily permeate through the membrane, and peroxisomal catalase deletion in *Saccharomyces cerevisiae* leads to a high level of intracellular hydrogen peroxide [65]. It is possible that ROS generated from peroxisomes contribute to mitochondrial CL peroxidation, and PC/PE peroxidation in yeast. However, the exact contribution of this “trans-organellar” process is not well-understood. The yeast cells used during this study were in stationary phase, during which respiratory activity is relatively high. Respiration takes place in mitochondria, suggesting that the ROS-mediated oxidation of CL, PC, and PE is primarily of mitochondrial origin.

While substantial evidence exists to link the accumulation of mitochondrial hydroperoxy-CLs with cytotoxicity and apoptosis, the precise catalyst of CL peroxidation remains to be fully elucidated. CL peroxidase activity of cytochrome *c* (cyt *c*) has been identified as the primary enzymatic mechanism leading to the accumulation of CL_{OX} in mammalian mitochondria [66, 67]. Thus, it is tempting to speculate that cyt *c* is responsible for peroxidation of CL in yeast mitochondria. This is compatible with the indications of the less stringent barrier and easier activation of yeast vs. mammalian cyt *c* into a peroxidase [68, 69]. Importantly, cyt *c* does not exhibit appreciable peroxidase activity toward phospholipid species other than CL [70]. Our findings in the ¹²-desaturase-expressing WT cells indicate that yeast mitochondria readily accumulate oxidized species of PC and PE, which suggests that enzymatic peroxidation by cyt *c* may not be the sole mechanism contributing to the formation of oxidized phospholipids in yeast mitochondria. In mammalian cells, 15-lipoxygenase (15LOX) enzymes are major catalysts of PE peroxidation that participate in ferroptotic cell death [71]. As 15LOX enzymes have not been identified in the yeast genome, the catalysts of phospholipid peroxidation in yeast cells remain to be identified.

Mammalian cells effectively reduce hydroperoxy-phospholipids to hydroxy-phospholipids via a glutathione peroxidase 4 (GPX4)-dependent enzymatic mechanism [71, 72]. However, the yeast glutathione peroxidase enzymes appear to be incapable of reducing hydroperoxy-phospholipids. The predominant accumulation of hydroperoxy-CL, hydroperoxy-PC, and hydroperoxy-PE species in the absence of corresponding hydroxy-phospholipids is indicative of relative inefficiency of this pathway in yeast. *S. cerevisiae* has three glutathione peroxidase enzymes encoded by *GPX1*, *GPX2*, and *GPX3*, all of which are believed to have hydroperoxy-phospholipid reducing glutathione-dependent peroxidase activity. While the loss of *GPX1/2/3* confers sensitivity to supplementation with linolenate [73, 74], our findings in the ¹²-desaturase-expressing WT cells indicate that yeast glutathione peroxidase enzymes do not readily reduce endogenously produced hydroperoxy-phospholipids *in vivo*. Given the recently established role of GPX4 in thwarting ferroptotic programmed death via reduction of hydroperoxy-phospholipids [29, 71, 75], it is interesting to speculate if this newly discovered cell death program can be realized in yeast cells [76].

CL peroxidation provides a critical signal for the initiation of apoptosis [66, 77, 78], and therapeutic reduction of CL peroxidation is being explored to treat human diseases associated with apoptosis [79–81]. Hydrolysis of CL_{OX} likely serves as a key mitochondrial source of oxidized fatty acid lipid signaling molecules [82]. The expression of exogenous desaturase enzymes in yeast has variable effects on cellular function and viability depending on the yeast strain, and growth conditions used [36, 37, 83, 84]. Johansson and co-workers observed severe growth defects in cells expressing both ¹² and ⁶-desaturase enzymes [83]. Cipak and co-workers report distinct effects of ¹²-desaturase expression depending on the yeast strain used [36, 37, 84]. The current study is the first to perform unbiased lipidomic analysis of yeast cells expressing an exogenous desaturase enzyme. Our findings indicate that CL_{OX} is readily detected in ¹²-desaturase-expressing yeast under normal growth conditions, suggesting that CL peroxidation may occur in the absence of cytotoxic stressors. The ¹²-desaturase-expressing yeast provides a powerful tool for the study of CL peroxidation and CL_{OX} signaling. Future studies will explore potential inducers of CL peroxidation as well as functions of CL_{OX} using this model system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- 12 -desaturase expression leads to accumulation of peroxidized cardiolipin
- Cld1 has a higher affinity for peroxidized cardiolipin
- *cld1* cells expressing 12 -desaturase exhibit increased peroxidized L₄CL
- 12 -desaturase expression in *cld1* cells leads to decreased mitochondrial functions
- 12 -desaturase expression in *cld1* cells leads to decreased chronological lifespan

Figure 1. CL biosynthesis and remodeling in *S. cerevisiae*.

Yeast cells readily produce 16:0-CoA and 18:0-CoA, which can be converted to 16:1-CoA and 18:1-CoA via endogenous Δ^9 -desaturase activity. 16:1-CoA and 18:1-CoA can be further converted to 16:2-CoA and 18:2-CoA by the expressed Δ^{12} -desaturase. All available acyl-CoA species can be incorporated into phosphatidic acid (PA) and used in the *de novo* synthesis of CL via CL synthase (Crd1). Newly synthesized CL contains predominantly unsaturated fatty acyl chains. Nascent CL may undergo Cld1-catalyzed deacylation to form mono-lyso-CL (MLCL). The transacylase, Taz1, utilizes donor phospholipids, particularly phosphatidylcholine (PC) and phosphatidylethanolamine (PE), to reacylate MLCL to CL, accompanied by the formation of the hydrolysis products of PC and PE, lyso-PC (LPC) and lyso-PE (LPE). PC and PE are also synthesized from PA. Multiple cycles of deacylation-reacylation generate CL that contains mainly unsaturated acyl chains.

Figure 2. CL species synthesized in empty vector (EV)- and ¹²-desaturase (desa)-expressing WT cells.

(A) Typical mass spectra of CL obtained from wild-type (WT) yeast cells expressing the empty vector (EV) (upper panel) or desa (lower panel). Major CL molecular species are identified as: CL58:3 (m/z 1261.8241); CL60:3 (m/z 1289.8578); CL62:3 (m/z 1317.8895); CL64:3 (m/z 1345.9180); CL66:4 (m/z 1371.9358); CL68:4 (m/z 1399.9666); CL70:4 (m/z 1427.9991); CL72:4 (m/z 1456.0305); CL60:4 (m/z 1287.8410); CL62:4 (m/z 1315.8712); CL64:4 (m/z 1343.9119); CL66:5 (m/z 1369.9176); CL68:5 (m/z 1397.9476); CL70:6 (m/z 1423.9635); CL72:7 (m/z 1449.9794). (B) A differential mass spectra of CL species were obtained to highlight individual CL species that were increased [+(desa-EV)] or decreased [-(desa-EV)].

(desa-EV)] by the expression of desa. (C) Heat map of CL quantified from WT yeast cells expressing EV or desa. (D) Quantitative assessment of individual CL molecular species in WT cells expressing the EV or desa.

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Figure 3. Peroxidized CL (CL_{OX}) in desa-expressing WT cells.

(A) Heat map of CL_{OX}. (B) Quantitative assessment of total CL_{OX}. (C) Quantitative assessment of individual CL_{OX} molecular species.

Figure 4. Heat maps of (A) PG and PG_{OX}, (B) PC and PC_{OX}, (C) PE and PE_{OX}, (D) PS in WT cells expressing empty vector (EV) or desaturase (desa).

Figure 5. Effect of mito-AAPH on EV- and desa-expressing WT cells.

(A) Serial 10-fold dilutions of cells were spotted on SC medium containing 2% galactose as the carbon source. Mito-AAPH was spread on the plates immediately before spotting. Plates were incubated at 30°C for 3 days. (B) Heat map and quantitative assessment of individual CL_{OX} molecular species in WT cells expressing ¹²-desaturase (desa) treated with or without mito-AAPH. (C) Heat map and quantitative assessment of individual PC_{OX} molecular species in WT cells expressing desa treated with or without mito-AAPH. (D) Heat map and quantitative assessment of individual PE_{OX} molecular species in WT cells expressing desa treated with or without mito-AAPH. (E) Heat map and quantitative assessment of individual PG_{OX} molecular species in WT cells expressing desa treated with or without mito-AAPH.

Figure 6. CL profile analysis of desa-expressing WT and *cld1* yeast cells.

(A) Typical mass spectra of CL species obtained from WT cells expressing desaturase (*desa*) and *cld1* cells expressing *desa* (*cld1* +*desa*). (B) Differential mass spectral analysis of CL profiles highlights individual CL species that were increased (product of remodeling) or decreased (substrate for remodeling) by the deletion of *CLD1*. Major CL molecular species are identified as: CL60:3 (m/z 1289.8560); CL62:4 (m/z 1315.8712); CL64:4 (m/z 1371.9329); CL66:4 (m/z 1371.9329); CL70:6 (m/z 1423.9635); CL72:7 (m/z 1449.9794); CL60:2 (1291.8715); CL62:2 (m/z 1319.9023); CL66:3 (m/z 1373.9482); CL68:3 (m/z 1401.9790); CL72:6 (m/z 1451.9932). (C) Heat map of CL molecular species of WT or *cld1* cells expressing *desa*. (D) Quantitative assessment of peroxidizable CLs in *desa*-expressing WT and *cld1* yeast cells.

A

CN:DB	# of oxygen	m/z	desa	<i>cld1Δ</i> +desa
60:3	4	1353.8276	65.18	0.86
60:4	4	1351.8127	47.71	N.D.
62:4	4	1379.8433	55.26	1.85
62:5	4	1377.8282	23.97	N.D.
62:6	2	1343.8262	155.68	174.35
64:4	4	1407.8744	116.35	36.15
64:5	4	1405.8598	63.41	1.87
64:6	4	1403.8439	16.13	0.28
64:7	2	1369.8411	0.45	51.30
64:8	2	1367.8262	0.23	40.00
66:3	2	1405.9315	3.28	5.48
66:5	4	1433.8898	196.28	5.33
66:6	4	1431.8749	97.57	3.68
66:7	2	1397.8716	N.D.	91.36
66:7	4	1429.8594	14.68	1.46
66:8	2	1395.8561	13.15	95.41
68:6	4	1459.9053	178.56	3.90
68:7	2	1425.9031	N.D.	36.50
68:7	4	1457.8900	52.42	4.60
68:8	2	1423.8878	9.32	51.64
70:3	4	1493.9848	9.88	N.D.
70:6	4	1487.9366	92.07	0.25
70:7	4	1485.9211	42.02	0.61
70:8	2	1451.9182	0.43	78.85
72:8	2	1479.9482	0.40	164.76

pmol/μmol PLs

N.D. – not detected

B

Figure 7. CL and CLOX in *desa*-expressing WT and *cld1* yeast cells.

(A) Heat map of CLOX molecular species of WT or *cld1* yeast cells expressing *desa* (B) Quantitative assessment of specific CL and mono-hydroperoxy-CL species (with 7 or 8 double bonds) including tetralinoleoyl-CL (TLCL) in *desa*-expressing WT and *cld1* yeast cells.

Figure 8. Cld1 exhibits greater affinity for oxidized than non-oxidized CL *in vitro* and its expression is increased by supplementation of H₂O₂.

(A) A mixture of 10 μ M CL that contains TLCL and oxidized TLCL (TLCL_{OX}) in HEPES buffer pH 7.4 was incubated at 37 °C for 45 min with Cld1. Total TLCL was extracted by the Folch procedure, and TLCL, TLCL_{OX}, and MLCL were quantified by LC-MS. Hydrolysis of CL_{OX} was evident by an accumulation of MLCL and concomitant reduction of CL_{OX}. $n=3$, * $p = 0.020$, and ** $p = 0.007$, respectively. (B) Liposomes containing DOPC and CL (10 μ M, 1:1) - DOPC/TPCL; DOPC/TLCL and DOPC/TLCL-OOH were prepared by sonication (Ultrasonic Homogenizer 4710 series, Cole-Parmer Instrument Co., Chicago, IL) and treated with Cld1 (0.5 μ g of protein) in 50 mM HEPES at pH 7.4, containing 100 μ M DTPA for 20 min at 37°C. After extraction, CL hydrolysis was detected by reduction of CL content and accumulation of MCL: ((16:0)₄ > (16:0)₃; (18:2)₄ > (18:2)₃ and (18:2)₃/(C18:2-OOH) > (18:2)₃). $n=3$, * $p = 0.030$, and ** $p = 0.027$, respectively. (C) Isolated Cld1 was incubated with TLCL or with a mixture of TLCL + TLCL_{OX} (ratios 1:1). (D) Isolated Cld1 was incubated with TLCL, TLCL containing one mono-hydroperoxy-linoleic acid (LA) residue (TLCL+2[O]), or TLCL containing two mono-hydroperoxy-LA residues (TLCL+4[O]). In the presence of CL_{OX} (TLCL+2[O] or TLCL+4[O]) Cld1 generated significantly increased free mono-hydroperoxy-LA (m/z 311) than non-oxidized-LA (m/z 279). (E) WT cells were grown to the mid-logarithmic phase and treated with 1 mM H₂O₂ for 1 hour. *CLD1* expression in H₂O₂-treated cells and non-treated cells was determined using RT-qPCR. * $p = 0.050$ vs WT.

Figure 9. Analysis of chronological lifespan and mitochondrial functions of desa-expressing yeast cells.

(A) The chronological lifespan of WT and *cld1* yeast cells expressing desa or EV. The data depicted are representative of three experiments. (B) Mitochondrial membrane potential is increased in WT+desa but reduced in *cld1* +desa. Membrane potential of growing cells was determined by fluorescence quantification of the membrane potential dependent dye TMRM. (C) Mitochondrial respiratory capacity is decreased in *cld1* +desa compared with *cld1* +EV. Cells were grown in synthetic complete medium with galactose as the carbon source. Respiration of intact cells was analyzed via the polarographic method. * $p < 0.05$ versus control (n=3).

Table 1.

The absolute amounts of oxidized phospholipids and their relative content (mol%) normalized to either the amounts of total phospholipids or the amounts of individual phospholipid class in desaturase (desa)-expressing WT cells.

PL class	# of PL _{ox}	mol% to total PLs	mol% to individual PL class
CL	23	0.13	2.49
PC	19	0.68	1.08
PE	12	0.15	0.85
PG	2	0.01	0.13
PS	0	0	0