Aging-Delaying Plant Extracts Cause Age-Related Changes in Cellular and Organellar Lipidomes of the Chronologically Aging Yeast *Saccharomyces Cerevisiae*

Veronika Svistkova

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_____ Chair

Dr. …

_____ External Examiner

Dr. …

_____ Examiner

Dr. Reginald Storms

_____ Examiner

Dr. Jinsuk Lee

_____ Supervisor

Dr. Vladimir Titorenko

Approved by _____

Chair of Department or Graduate Program Director

_____

Dean of Faculty of Arts and Science

Date _____

ABSTRACT

Aging-Delaying Plant Extracts Cause Age-Related Changes in Cellular and Organellar Lipidomes of the Chronologically Aging Yeast *Saccharomyces Cerevisiae*

Veronika Svistkova, M.Sc.

The yeast *Saccharomyces cerevisiae* has been successfully used to identify genes, signaling pathways and chemical compounds that delay cellular and organismal aging in evolutionarily distant eukaryotes. These findings provided evidence that the mechanisms of biological aging on a cellular level have been conserved in the course of evolution. Recent studies revealed 6 plant extracts that delay chronological aging in *S. cerevisiae*. All these plant extracts are more efficient aging-delaying interventions than any of the currently known longevity-extending chemicals. As a first step towards uncovering molecular mechanisms through which the 6 plant extracts delay yeast chronological aging, I used quantitative mass spectrometry to compare the concentrations of different classes of lipids in cells and cellular organelles of chronologically aging yeast exposed to each of these extracts or remained untreated. I demonstrate that each of the 6 aging-delaying plant extracts causes age-related changes in cellular and organellar lipidomes of chronologically aging yeast. My findings suggest that each of these extracts differently alters the relative rates of phosphatidic acid flow into the biosynthetic pathways for triacylglycerols in the endoplasmic reticulum, glycerol phospholipids in the endoplasmic reticulum and mitochondria, and cardiolipin in mitochondria. Such re-wiring of phosphatidic acid conversion into triacylglycerols, glycerol phospholipids and cardiolipin delays aging by mechanisms that remain to be established. My study provides important knowledge on which aspects of lipid metabolism are essential for the ability of the 6 plant extracts to delay aging.

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List of Abbreviations

AMPK/TOR, the AMP-activated protein kinase/target of rapamycin signaling pathway; cAMP/PKA, the cAMP/protein kinase A signaling pathway; CFU, colony forming units; CL, cardiolipin; CLS, chronological lifespan; CR, caloric restriction; D, diauxic growth phase; DAG, diacylglycerol; DR, dietary restriction; ER, endoplasmic reticulum; ERCs, extrachromosomal rDNA circles; FFA, free fatty acids; L, logarithmic growth phase; LCA, lithocholic acid; LD, lipid droplet; m/z, mass/ion charge ratio; mDNA, mitochondrial DNA; MFQL, Molecular Fragmentation Query Language; MS, mass spectrometry; MS/MS, tandem mass spectrometry; nDNA, nuclear DNA; non-CR, non-caloric restriction; OD_{600}, optical density at 600 nm; PD, post-diauxic growth phase; PA; phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PKA, protein kinase A; ppm, parts per million; RLS, replicative lifespan; ROS, reactive oxygen species; Sch9, serine-threonine protein kinase Sch9; SE, sterol esters; ST, stationary growth phase; TAG, triacylglycerols; TCA cycle, tricarboxylic acid cycle; TORC1, target of rapamycin complex 1; UPR, unfolded protein response; WT, wild-type strain.

Introduction

1.1 The yeast *Saccharomyces cerevisiae* is a beneficial model for uncovering mechanisms of cellular aging in multicellular eukaryotic organisms

Aging affects all biological organisms, from simple eukaryotes to human beings. The budding yeast *Saccharomyces cerevisiae* is a simple, unicellular eukaryote that has been used as a model organism for studying aging. [1] It has a short lifespan, its genome has already been sequenced, and it can be easily cultured under laboratory conditions. These benefits make *S. cerevisiae* an ideal organism to discover novel genes or signaling pathways that regulate aging. [1] Studies confirmed that the mechanisms that control cellular aging are maintained in eukaryotes across phyla. [1] *S. cerevisiae* has helped in the identification of many genes involved in regulating aging. [2, 3] It is also commonly used to screen extensive libraries of numerous chemical compounds for those that can delay aging. [2] A few anti-aging drugs have already been found using yeast. Those drugs include resveratrol, rapamycin and spermidine. [4] Resveratrol is a chemical compound initially found in red wine; it has been shown to extend the lifespan of yeast, nematodes, flies and short-lived fish. [4] However, studies in mice didn't show an increase in lifespan. [4] Resveratrol was found to activate sirtuins, a family of NAD^+-dependent deacetylases. [4] Activation of sirtuins was already found to increase the lifespan of yeast, nematodes and flies. [4] Resveratrol is currently being clinically tested as a treatment for type 2 diabetes and metabolic syndrome. [4, 6] Another drug whose aging-delaying effects have been initially discovered in yeast is rapamycin, also called sirolimus. [4] It is a chemical with antifungal properties and is a macrocyclic lactone made by the bacterium *Streptomyces hygroscopicus.* [7, 8] Rapamycin has been used

with other drugs as an immunosuppressant in renal transplantation and it was found to reduce the level of malignancies in renal-transplant recipients. [9] Recent studies have shown that rapamycin prevents deficits in memory in mouse models of Alzheimer's disease. [7] Spermidine, another molecule with anti-aging properties, can interact with RNA, DNA, and lipids due to the presence of cations in its structure. [10] Research has shown that when yeast, nematodes, fruit flies and human peripheral blood mononuclear cells where exposed to this chemical agent, their lifespan increased. [10] Spermidine can also alter the lipid metabolism in the fruit fly *Drosophila melanogaster.* [10] Resveratrol, rapamycin and spermidine are only few examples of compounds that have been discovered to be aging-delaying compounds in studies of the yeast *S. cerevisiae.* Aging-delaying compounds discovered in studies of yeast have also been also used for the discovery of several key protein components of the anti-aging and pro-aging pathways; these key protein components are sirtuins and the TOR (target of rapamycin) protein kinases, respectively. [4] The abilities of both these proteins to define the pace of aging have been conserved in the course of evolution. [4]

1.2 Two different modes of yeast aging

When referring to the aging of yeast, some keys concepts need to be defined. Two types of aging are known in yeast; they are called replicative and chronological aging (**Figure 1.1**). [3] The ways for monitoring these two types of aging differ. [3] Some proteins are master regulators of only the replicative mode of aging, others regulate only the chronological mode of aging, whereas some proteins play essential roles in regulating both modes of yeast aging. [3]

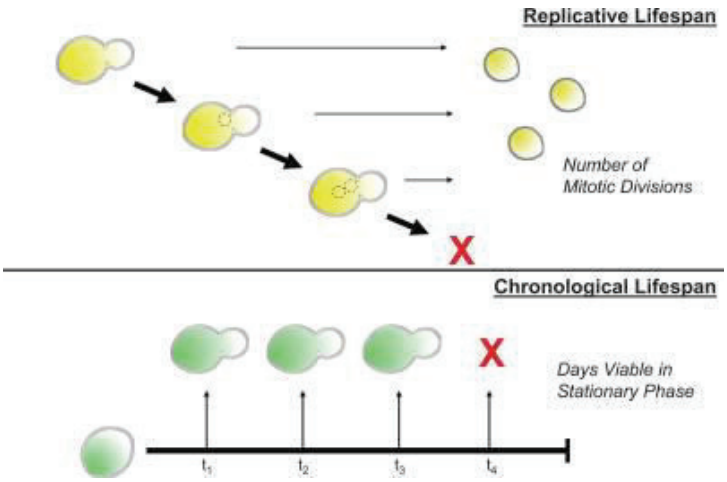

Figure 1.1 Two different modes of yeast aging. Replicative aging is monitored by assessing the number of mitotic divisions a mother cell can undergo before becoming senescent. Chronological aging is monitored by assessing a number of days a yeast cell remains viable after entering stationary phase of growth that is after becoming senescent. [11]

Yeast replicative aging occurs because *S. cerevisiae* proliferates by budding of a small daughter cell from a large mother cell. [3] A mother yeast cell can divide a limited number of time, and the higher this number is, the higher the replicative lifespan (RLS) of this particular yeast cell. A single yeast cell can form not more than 20 to 25 cells, and then becomes unable to replicate by budding. [3] The cell that gives rise to the new cells is referred to as "mother" cell and the new cells are called "daughter" cells. Yeast RLS can be used as a model for mitotically active mammalian cells. [4] Measuring RLS in the laboratory involves a standard tetrad dissection microscope with a micromanipulator that can remove the daughter cells. This would allow to keep track of how many times a cell undergoes budding. [3] Other methods of monitoring yeast RLS have recently emerged. For example, the mother cell can be coated with biotin, and the daughter cells that originate from the buds of newly synthesized membranes (and, thus, lack a biotin coat)

can be counted. [3] Research has shown that the capacity of a yeast cell to replicate a finite number of times depends on various cellular processes. [2] These processes take place in different cellular locations, occur at different stages of the RLS (i.e. at the early, intermediate or late stage of the RLS), and contribute to the accumulation of so-called aging factors. [2] Before taking a look into the cellular processes that define yeast RLS, the different stages of replicative aging need to be outlined. The early stage of the RLS lasts from cell birth to the completion of first 10 cell divisions, the intermediate stage refers to the period of time between cell divisions 10 and 20, and the late-age stage involves the period of time between cell division 20 and cell death. [2] One of the cellular processes that drives yeast replicative aging is a gradual decline of vacuolar functionality. Vacuoles in yeast play the role of lysosomes in mammals. [12] Vacuoles undergo an increase in pH during the early stage of the RLS, increase in size during the intermediate stage, and further enlargement during the late stage. [2] Another aging factor in replicating yeast cells is the age-related accumulation of protein aggregates, which suggests a gradual impairment of cellular proteostasis in replicatively aging yeast. [2] From the early stage to the late stage of the RLS, there is a gradual increase in the abundancies of oxidatively damaged and aggregated proteins. [2] The build-up of such aggregates is one of the hallmarks of replicative aging in yeast. [2] Several factors related to mitochondrial functionality can also serve as hallmark events of yeast replicative aging. [2] These factors become noticeable during the intermediate and late stages of the RLS. [2] During the intermediate stage, mitochondrial membrane potential is decreased and reactive oxygen species (ROS) accumulate; both these processes continue during the subsequent late stage of the RLS. [2] At this stage, individual mitochondria form large

aggregates and lose their DNA. [2] Some changes in replicatively aging yeast are known to affect the nucleus. During the intermediate stage of replicative aging, histones in the subtelomeric regions of the chromatin undergo acetylation and dissociate, thereby allowing transcription of these chromatin regions. [2] During this stage, there is also a significant increase in the number of extrachromosomal rDNA circles (ERCs) within the nucleus; such increase in the abundance of ERCs marks a transition from the intermediate stage to the late stage of the RLS. [2] Recent findings indicate that the rise in the number of ERCs is one of the key indicators of aging. [2] Mutants that exhibit an accelerated accumulation of ERCs (as compared to a wild-type strain) age prematurely and have shortened replicative lifespan. [13] Other type of organelles undergoing characteristic changes during yeast replicative aging are peroxisomes. [3] These organelles accommodate such important metabolic processes as fatty acid oxidation and decomposition of hydrogen peroxide, a ROS molecule. [3] Peroxisomes also contribute to the maintenance of ROS homeostasis in the cell by housing ROS-producing oxidases. [14] Moreover, protein import into peroxisomes is known to gradual decrease during replicative aging in yeast. [2]

The RLS-defining cellular processes mentioned above are regulated by 2 groups of mechanisms that can slow down replicative aging by operating within a mother cell. The first group of such mechanisms allows to prevent or decelerate the buildup of different aging factors in the mother cell. [3] An example of a pathway that belongs to this group of aging-delaying mechanisms is the unfolded protein response (UPR) signaling. [3] As mentioned earlier in the text, aggregates of proteins becoming more abundant when the mother cell ages replicatively. To attenuate such protein aggregation,

the UPR signaling pathway is triggered in the endoplasmic reticulum (ER); this initiates a cascade of events that ultimately activate transcription of many UPR target genes, including protein chaperones confined to the ER. [15, 16] Another example of a pathway from the first group of replicative aging-delaying mechanisms is a specialized form of autophagy. This form of autophagy is activated in response to accumulation of oxidatively damaged lipids within the mother cell. It involves a translocation of a lipase from the ER to the vacuoles and subsequent lipolytic degradation of oxidatively damaged membranes within autophagosomes. [3, 17] This ensures degradation of dysfunctional and/or damaged organelles, as well as aggregates of organelles and proteins. [3, 17]

The second group of mechanisms that can slow down replicative aging by operating within a mother cell is the one which prevents a transmission of accumulated aging factors from the mother cell to the daughter cell. This ensures that the daughter cell remains replicatively "young" and does not inherit the aging factors accumulated in the replicatively "old" mother cell. [3] An example of such mechanisms is the association of protein aggregates with the actin cytoskeleton. Such association prevents a transfer of the protein aggregates from the mother cell to the daughter cell during budding. [2] Another example for the second group of aging-delaying mechanisms operating within a mother cell is a mechanism which prevents a transfer of dysfunctional mitochondria from the mother cell to the daughter bud. [18] Such transfer of mitochondria is driven by motor proteins that walk on the bundles of actin filaments. Mitochondria that are functional are known to move faster on these actin cables than dysfunctional ones. [18]

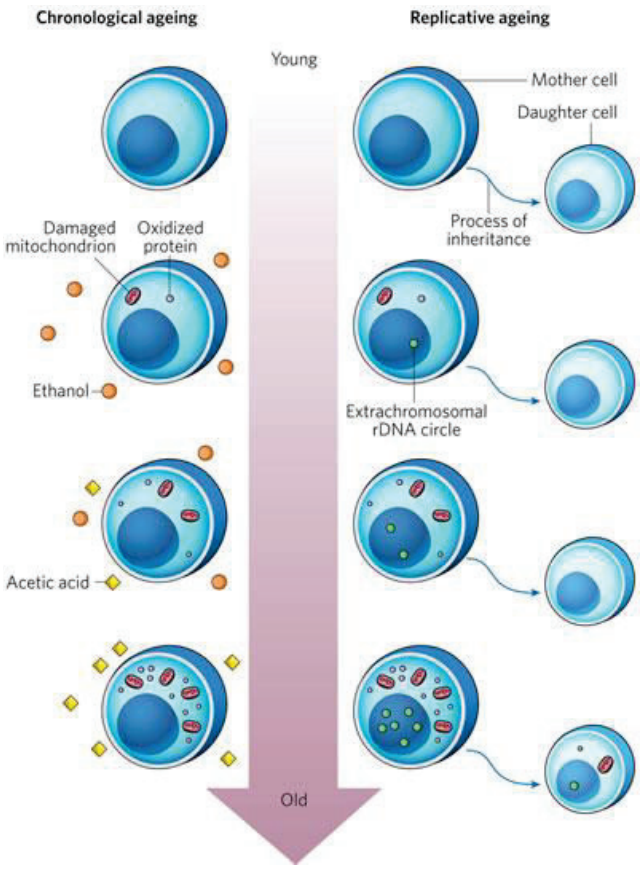

Figure 1.2 An age-related accumulation a distinct set of aging factors promotes both replicative and chronological aging in yeast. An age-related accumulation of damaged mitochondria and oxidized proteins causes both replicative and chronological aging in yeast. Extrachromosomal rDNA circles gradually accumulate in the mother cell, thereby promoting its replicative aging. [4]

The chronological lifespan (CLS) of yeast differs from yeast RLS by definition and by the way it is measured under laboratory conditions. It refers to the ability of non-dividing (quiescent and senescent) yeast cells to remain viable for a certain time period during stationary growth phase. [3] The longer a cell remains viable, the longer the CLS of such cell. [3] When yeast cells are cultured in a liquid medium initially containing glucose as a sole carbon source, they entirely consume glucose after approximately 24 hours and reach so-called post diauxic growth phase. During this phase, yeast cells slowly grow using ethanol, a product of glucose fermentation, as a source of carbon. [3]

After the end of the post diauxic phase, yeast cells enter stationary growth phase. In stationary phase, the majority of cells exits the cell cycle and remains in a viable yet non-dividing state. [3] This non-dividing state of yeast cells undergoing chronological aging is being used as a model to study aging of non-diving cells of higher eukaryotic organisms. [3] Moreover, this non-dividing state of chronologically aging yeast cells is also considered as a model for organismal aging of higher eukaryotes. [1] The common method to measure CLS is a so-called clonogenic assay. [1] In this assay, the viability of a yeast cell is tested by plating this cell on the surface of a solid nutrient-rich medium. [1] If a cell is able to form a colony, it is considered to be viable because it is capable to reproduce by budding. [1] In the clonogenic assay of yeast CLS, the percentage of viable cells in the liquid medium is calculated at different consecutive time points. [1]

Certain cellular processes and a distinct set of proteins influence the length of yeast CLS. [1] Some of these processes and proteins have been shown to define yeast CLS by acting prior to the entry into the non-dividing state, whereas others do so only after a yeast cell enters this non-proliferative state. [1] Thus, the process of chronological aging in yeast can be divided into two equally important consecutive phases. The initial phase of yeast chronological aging occurs in chronologically “young” cells, whereas the subsequent phase takes place in chronologically “old” cells. [1]

Cellular processes that define the progression through the two consecutive phases of yeast chronological aging and define yeast CLS are subjected to complex spatiotemporal regulation. [1] These cellular processes are involved in metabolism, growth, proliferation, macromolecular homeostasis and stress resistance. [1] One of the key players that influence proteostasis during yeast chronological aging is trehalose. [1]

The intracellular concentrations of this disaccharide in both chronologically "young" and "old" yeast cells play an important role in defining longevity of chronologically aging yeast. [1]

It has been found that in chronologically "young" yeast cells, trehalose can bind to newly synthesized proteins. Such binding to trehalose stabilizes these newly synthesized proteins by preventing their improper folding and aggregation through the protection of exposed hydrophobic parts of misfolded proteins, as well as by limiting their oxidation through the shielding of oxidation-prone protein domains. [1] In chronologically "old" yeast cells, the effect of trehalose on yeast CLS is quite different. In fact, if the concentration of trehalose in this "old" cells exceeds a threshold, yeast CLS is shortened. [1] This is because in chronologically "old" yeast cells, trehalose binding to hydrophobic regions of misfolded proteins impairs their proper folding by competing with molecular chaperones that are needed for such folding. [1] These findings imply that the intracellular concentration of trehalose can define yeast CLS by differently influencing protein homeostasis in chronologically "young" and "old" cells. [1]

As mentioned earlier, protein import into peroxisomes gradually deteriorates in yeast cells that age replicatively. [2] Such age-related deterioration of peroxisomal protein import has been also observed in chronologically aging yeast. In chronologically "young" yeast cells, protein import into peroxisomes is highly efficient. [1] Proteins that are efficiently imported into the peroxisome in these chronologically "young" yeast include enzymes needed to break down hydrogen peroxide and other ROS, enzymes involved in the β-oxidation of fatty acids, and enzymes needed to synthesize intermediates of Krebs cycle. [1] Peroxisomal import of these enzymes is facilitated by

the proteins called Pex5p and Pex7p. [1] It has been shown that when the efficiency of peroxisomal import of these proteins decreases below a certain threshold, a pro-aging cellular pattern is established. [1] Indeed, in chronologically "young" yeast cells the efficiency of such peroxisomal protein import exceeds the threshold level, whereas and in chronologically "old" yeast cells such efficiency drops below the threshold. [1] Recent evidence supports the notion that the establishment of the pro-aging pattern in chronologically "old" yeast cells with reduced peroxisomal protein import is due to the following equally important processes: 1) an elevation of intracellular concentrations of hydrogen peroxide and other ROS, which increases the extent of oxidative damage to macromolecules; 2) a reduction in peroxisomal fatty acids oxidation, which promotes a buildup of free fatty acids known to trigger a so-called liponecrotic form of programmed cell death; and 3) a decrease in the production of TCA cycle intermediates, which triggers a cascade of events involving a reduced efficiency of the electron transport chain in mitochondria, mitochondrial fragmentation, and the efflux of cytochrome *c* (all these factors are known to elicit an apoptotic form of programmed cell death). [1]

As a result of the above findings, yeast CLS is viewed as a progression through several consecutive "checkpoints". [1, 2] The efficiency of progression through each of these "checkpoints" defines longevity of chronologically aging yeast and involves a distinct set of cellular processes. [1, 2] Several of these checkpoints are known to occur in chronologically "young" cells progressing through logarithmic, diauxic and post diauxic phases of growth. [1] The rates and efficiencies of progression of longevity-defining cellular processes through each of these checkpoints define longevity of chronologically aging yeast. [1, 2] When the efficiencies of these cellular processes

decrease below a threshold, molecular damage begins to accumulate. [2] Such age-related accumulation of damage to cellular molecules accelerates yeast chronological aging. [2]

Moreover, yeast CLS is also known to be influenced by the acidity of the medium in which yeast cells are cultured. [4] Indeed, yeast cells that underwent a diauxic transition from logarithmic phase to stationary phase are metabolizing ethanol as the secondary energy source. [4] This favors the release of acetic acids and other organic acids in the medium and, thus, promoting an acidification of this medium. [4] Acetic acid can enter the cell by simple diffusion and lower the intracellular pH. [19] This acidification has been shown to hinder vesicle trafficking and affect cellular metabolism. [19] Importantly, increasing pH of the medium by the use of certain buffers can increase yeast CLS. [4]

Taken together, both RLS and CLS are important for understanding mechanisms of the aging process in yeast. Yeast RLS mimics aging of mitotically active human cells, whereas yeast CLS is a benefitial model for studying aging on non-dividing human cells. [4] Until recently, the two types of yeast aging have been studied separately from each other because they were considered to be independent processes. [4] However, recent studies have provided evidence that RLS and CLS are partially overlapping and mutually dependent processes in yeast. [4] Indeed, a caloric restriction diet has been shown to extend both these types of lifespan in yeast. [2] Furthermore, an attenuation of the pro-aging TOR/Sch9 pathway (see below) extends both RLS and CLS in yeast. [2] It needs to be emphasized that CLS and RLS do not fully overlap in yeast; indeed, some single-gene-deletion mutations can increase yeast RLS but do not affect yeast CLS. [2]

1.3 A network of integrated signaling pathways and protein kinases defines the rate of yeast chronological aging

Aging is a complex process, which is regulated by a distinct set of master regulator proteins integrated into a network of overlapping nutrient-sensing pathways of signal transduction. [20]

One of these master regulator proteins is a nutrient-sensing serine/threonine protein kinase, TOR (target of rapamycin). Yeast, algae, slime mold, plants, worms, flies and mammals all possess a *TOR* gene. [20] Two TOR complexes are presently known, TORC1 (referred to as mTORC1 for mammals) and TORC2 (referred as mTORC2 in mammals). [21] While TORC2 is known mainly for its essential role in controlling the actin cytoskeleton dynamics, TORC1 has been linked to many cellular processes that impact cellular and organismal aging in evolutionarily distant eukaryotes. [21] TORC1 regulates translation, transcription, ribosome formation, nutrient transport across membranes and autophagy. [11, 21] The TORC1 pathway is considered to be a pro-aging pathway since mutants with reduced TORC1 activity exhibit an increase in both RLS and CLS not only in yeast but also in such evolutionarily distant eukaryotic organisms as worms, fruit flies and mice. [11] In order to regulate translation, it is believed that TORC1 interacts with the translation initiation factor eIF4E. [22] The exact mechanism of translational regulation by TORC1 remains to be defined. [22] The TORC1 pathway is able to coordinate the formation of new ribosomes by favouring the transcription of genes that encode mRNAs for ribosomal proteins, rRNAs and tRNAs. [11, 22] TORC1 can also activate the import of amino acids across the plasma membrane into the cell. [11] Amino acids are used by yeast cells as sources of nitrogen and they get inside the cell with the

help of various permeases. [22] TORC1-driven signaling is known to activate amino acid import by stimulating activities of amino acid permeases. [22] Indeed, the inhibition of the TORC1 pathway by rapamycin has been shown to decrease the import of exogenous amino acids across the plasma membrane. [22] Inhibition of TORC1 by rapamycin also stimulates autophagy, thus suggesting that TORC1 inhibits autophagic degradation of cellular organelles and macromolecules. [22]

TORC1 signaling is known to inhibit transcription driven by such transcription factors as Msn2, Msn4 (both of which activate transcription of stress-response genes under caloric restriction conditions) and bHLH/Zip (which induces expression of genes encoding enzymes involved in synthesis of some metabolic intermediates). [22]

TORC1 can respond to changes in nutrient availability to yeast cells by phosphorylating and activating the nutrient-sensing protein kinases Sch9 (an ortholog of S6K in mammals) and protein kinase A (PKA). [11, 23] It needs to be emphasized that, similarly to attenuation of TORC1, a mutational impairment of Sch9 or a decreased activity of PKA have been shown to extend both RLS and CLS in yeast. [23] TORC1, Sch9 and PKA are considered to be nutrient-responsive kinases that promote aging. [23] Their combined action is believed to cause a global suppression of the ability of yeast to respond to various age-related stresses. TORC1, Sch9 and PKA suppress stress response by preventing the import of Msn2/4 heterodimers into the nucleus, thereby attenuating the Msn2/4-dependent transcription of numerous stress-response genes. [23]

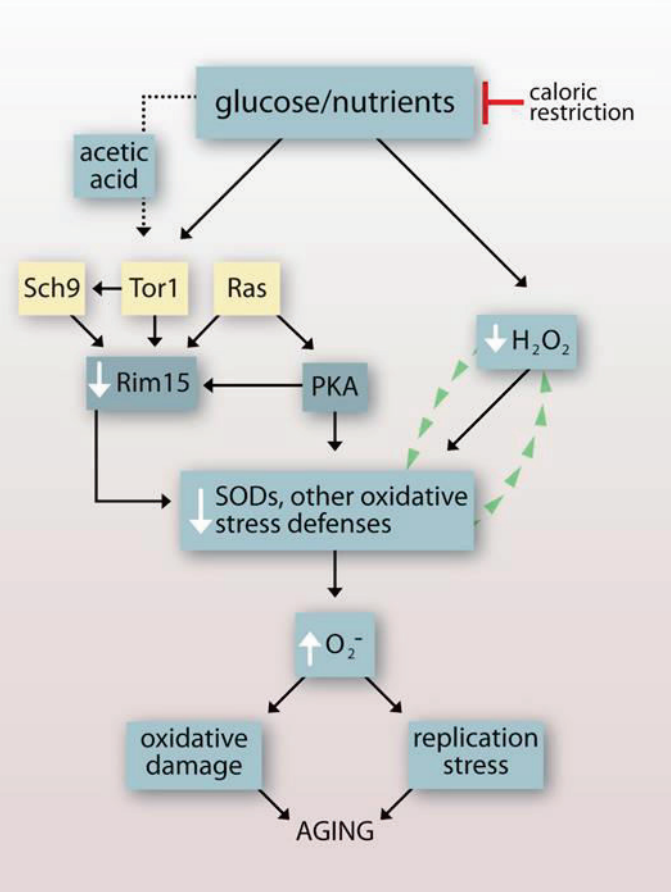

Figure 1.3 The ability of yeast to respond to the availability of exogenous nutrients define CLS. Sch9, Tor1 and Ras respond to nutrients availability by preventing the Rim15-dependent activation of oxidative stress response. This elicits an increase in O_2^- concentration, which in turn causes oxidative macromolecular damage and accelerates yeast chronological aging. [24]

The network of nutrient-sensing signaling pathways and protein kinases shown in Figure 1.3 has been shown to coordinate an abundance of longevity-defining cellular processes in chronologically aging yeast. These processes include stress response, protein synthesis in the cytosol and mitochondria, maintenance of nuclear and mitochondrial genomes, autophagy, mitochondrial respiration, peroxisome biogenesis, gluconeogenesis, lipid metabolism, glyoxylate cycle, glycogen synthesis and degradation, and the synthesis of amino acids and fatty acids. [1, 3, 5]

1.4 Metabolism of different lipid classes in yeast cells

Lipids in yeast cells are known for their essential roles in the assembly and fusion of membrane bilayers, the stabilization and vesicular trafficking of membrane proteins, signal transduction, apoptosis, and energy storage. [25, 26, 27] Because most of the aspects of lipid metabolism and function have been conserved in the course of evolution, the budding yeast *S. cerevisiae* have been successfully used as an advantageous model organism for uncovering mechanisms underlying all these vital processes in cells of multicellular eukaryotic organisms. [25]

The metabolic pathways for the biosynthesis, degradation and storage of different classes of lipids have been extensively studied in yeast. [25, 26, 27] One of these lipid classes is the class of phospholipids, the most abundant lipid constituents of organellar and plasma membranes in yeast. They include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). Of note, membrane bilayers of different organelles in yeast cells differ from each other in the relative levels of PC, PE, PI and PS; it is believed that such difference in phospholipid composition is one of the key reason for the differences in functions of these organelles. [25, 26, 27] Moreover, some lipid species of the phospholipid class can be found only in the inner and outer mitochondrial membranes; in yeast mitochondria, these lipid species are phosphatidylglycerol (PG) and cardiolipin (CL). [25, 26, 27]

Two classes of lipids in yeast cells, namely triacylglycerols (TAG) and steryl esters (SE), belong to so-called neutral lipids. [25, 26, 27] TAG and SE are deposited inside of the lipid droplets (LD), an organelle surrounded by a monolayer of

phospholipids and a limited number of proteins. [26] In addition to playing essential roles as a source of energy and precursors for membrane lipid synthesis, TAG and SE serve as a chemical form for the detoxification of such lipid classes as free fatty acids (FFA) and sterols. [26]

Although the synthesis of phospholipids and neutral lipids in yeast is confined to various organelles (including the ER, mitochondria and LD), a phospholipid called phosphatidic acid (PA) serves as a common precursor molecule for all of these lipid classes. [25, 26, 27] All phospholipids are synthesized via the CDP-DAG (CDP-diacylglycerol) branch of PA conversion, whereas TAG and SE are formed through the DAG (diacylglycerol) of PA conversion. (**Figure 1.4**) [25, 26, 27] CDP-DAG is a liponucleotide intermediate which serves as a precursor for the synthesis of PI and PS in the ER. (**Figure 1.4**) [25, 26, 27] Further metabolic conversion of PS occurs in mitochondria and results in the formation of PE. (**Figure 1.4**) [25, 26, 27] The subsequent conversion of PE to PC occurs in the ER. (**Figure 1.4**) [25, 26, 27] CDP-DAG also serves as a precursor in the synthesis of PG and then CL in the inner mitochondrial membrane. (**Figure 1.4**) [25, 26, 27] Thus, the biosynthesis of phospholipids in yeast is a highly organized process which involves a synthesis of various lipid intermediates in the ER and mitochondria and a bidirectional transport of these intermediates between these organelles. (**Figure 1.4**) [25, 26, 27] Two phospholipids, PC and PE, can also be formed from DAG via the Kennedy pathway, which requires exogenous choline and ethanolamine. (**Figure 1.4**) [25, 26, 27]

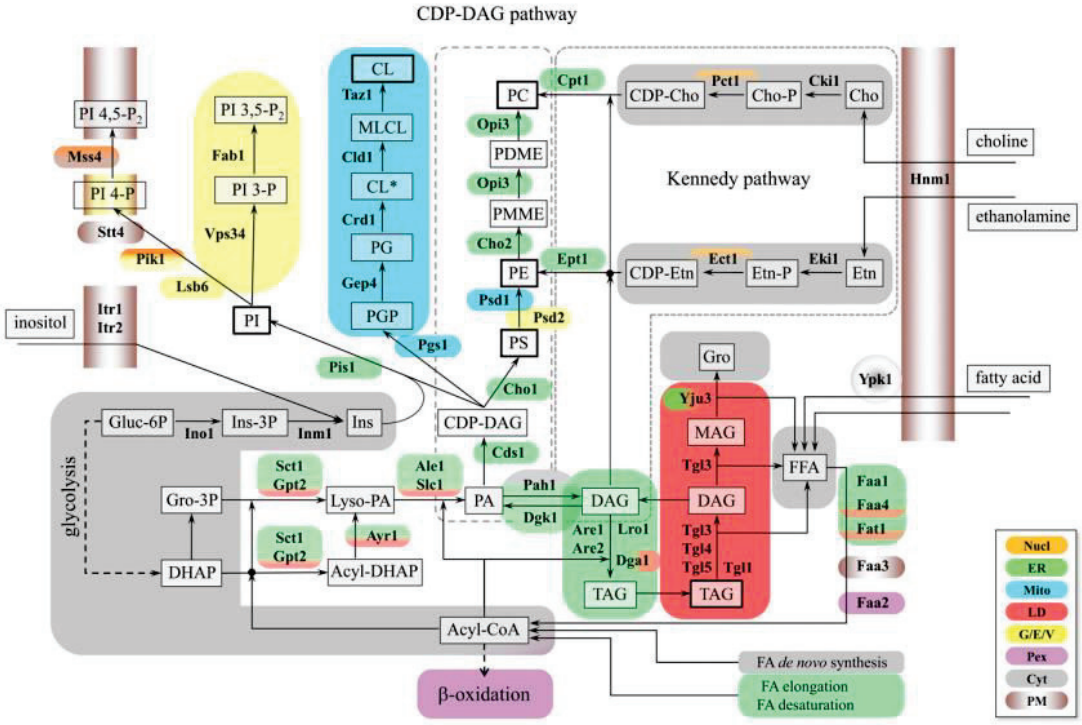

Figure 1.4 Pathways for lipid synthesis, interorganellar transport and degradation in a yeast cell. PA is a precursor for the synthesis of both phospholipids and neutral lipids. Phospholipids PC, PE, PI, PS, PG and CL are synthesized via the CDP-DAG branch of PA conversion in the ER and mitochondria. An alternative route for the synthesis of PC and PE is the Kennedy pathway, which uses DAG as a precursor and requires exogenous choline and ethanolamine. Neutral lipids TAG and SE are first synthesized via the DAG branch of PA conversion in the ER, and are then deposited in the LD. Both neutral lipids can be used as a source for replenishing the pools of PA, DAG and FFA for lipid synthesis or degradation. [25]

TAG and SE are synthesized via the DAG (diacylglycerol) of PA conversion in the ER of yeast cells. (**Figure 1.4**) [25, 26, 27] These two neutral lipids are then deposited in the LD which forms by budding from the ER. (**Figure 1.4**) [25, 26, 27] TAG and SE can be used as an abundant source for the replenishment of the pools of PA, DAG and FFA, which can be then used for lipid synthesis or degradation. (**Figure 1.4**) [25, 26, 27]

1.5 Some dietary and pharmacological interventions delay yeast chronological aging by altering lipid metabolism

Various changes in the environmental conditions, including changes in nutrients availability, can dramatically change the cellular and organellar lipidomes of yeast cells by re-wiring the network of overlapping metabolic pathways schematically depicted in **Figure 1.4.** [28] Some of these changes can delay chronological aging in yeast, as outlined below.

An aging-delaying dietary intervention which has been shown to remodel lipid metabolism is so-called caloric restriction (CR). [30] CR is a robust longevity-extending dietary intervention known to delay aging not only in yeast but also in worms, fruit flies and mice. [30] In chronologically aging yeast, CR can be administered by lowering the final concentration of glucose in growth medium from 2% (non-CR conditions) to 0.2%. [30] It has been shown that under non-CR conditions chronologically aging yeast accumulates ethanol, the major product of glucose fermentation. [30, 31] Ethanol suppresses peroxisomal oxidation of FFA, which under non-CR conditions are formed due to the lipolytic degradation of TAG deposited in the LDs. [30, 31] This causes an accumulation of FFA in the LDs, thus enabling FFA to initiate several negative feedback loops that elicit the build-up of DAG and FFA in the ER. [31] The excessive accumulation of these two lipid classes accelerates yeast chronological aging by triggering an age-related form of programmed cell death called liponecrosis. [29, 30, 31] The CR diet delays chronological aging in yeast by significantly lowering the intracellular concentration of ethanol and, thus, by preventing the suppression of FFA oxidation in peroxisomes. [31] This, in turn, prevents the build-up of FFA and DAG in

the ER, thus delaying the onset of liponecrotic cell death and slowing down yeast chronological aging. [29, 30, 31]

An aging-delaying pharmacological intervention which has been shown to remodel lipid metabolism is lithocholic acid (LCA), the most hydrophobic bile acid. [31, 32] This LCA-driven re-wiring of lipid metabolism in chronologically aging yeast involves the following three processes. First, LCA promotes mitochondrial transport of acetyl-CoA to limits its availability for the synthesis of FFA in the ER. [31] Second, LCA stimulates TAG synthesis from FFA and DAG in the ER. [31] Third, LCA decelerates the lipolysis of TAG to FFA and DAG. [31] Because of these three effects of LCA on lipid metabolism and transport, LCA causes a decrease in the intracellular concentrations of FFA and DAG. This, in turn, delays the onset of liponecrosis which can be triggered by these two lipids and slows down chronological aging. [31]

LCA delays yeast chronological aging also by influencing mitochondrial composition, structure and function. This bile acid has been shown to cross the plasma membrane and then to be sorted to the inner mitochondrial membrane. [33] The accumulation of LCA in the inner mitochondrial membrane causes a characteristic remodeling of phospholipid metabolism in and phospholipid transport across this membrane. [33] This causes significant changes in the relative concentrations of several phospholipids within both inner and outer membranes of mitochondria. Specifically, the concentrations of PA, PC, PS and PG are increased, whereas the concentrations of PE and CL are decreased. [33] Such global changes in phospholipid concentrations trigger a significant enlargement of mitochondria, decrease the number of mitochondria, and increase the abundance of mitochondrial cristae. [33] These LCA-driven changes in

mitochondrial morphology intensify mitochondrial respiration, increase membrane potential and stimulate mitochondrial ATP synthesis in chronologically "old" yeast cells, thus delaying their aging and increasing their lifespan. [33]

1.6 The objectives of studies described in this thesis

Research in the Titorenko laboratory is aimed at using *S. cerevisiae* as a model organism to unveil molecular mechanisms of cellular aging in multicellular eukaryotic organisms. Recent studies in the laboratory discovered 6 plant extracts that greatly delay chronological aging in this yeast. My objective was to make a first step towards uncovering molecular mechanisms through which each of these plant extracts delays yeast chronological aging. To make such initial step, in studies described here I used quantitative mass spectrometry to compare the concentrations of different classes of lipids in whole cells and cellular organelles of chronologically aging yeast exposed to each of these extracts or remained untreated.

2 An aging-delaying plant extract PE21 remodels cellular and organellar lipidomes of chronologically aging yeast

2.1 Abstract

Recently published studies conducted in the Titorenko laboratory have identified 6 plant extracts that slow down the chronological mode of aging in *S. cerevisiae*. All these plant extracts are significantly more efficient aging-delaying interventions than any of the currently known anti-aging chemicals. One of these plant extracts, called PE21, is the most potent aging-delaying pharmacological intervention yet described. Recent unpublished findings of the Titorenko laboratory suggest that some aspects of lipid metabolism and interorganellar transport may play essential roles in the delay of aging by PE21. In studies described in this chapter of my Thesis, I have used mass-spectrometric quantitative analysis to compare the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE21 or remained untreated. My findings imply that PE21 alters the relative rates of phosphatidic acid (PA) flow into the synthesis of other lipids as follows: 1) it decreases the rate of PA flow into the synthesis of triacylglycerols (TAG) in the endoplasmic reticulum (ER); 2) it increases the rate of PA flow into the synthesis of glycerol phospholipids in the ER and mitochondria; and 3) it decreases the rate of PA flow into the synthesis of cardiolipin (CL) in mitochondria. Such re-wiring of PA conversion into TAG, glycerol phospholipids and CL delays aging by a mechanism that remains to be established.

2.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) from Thermo Scientific/Open Biosystems was grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1. The stock solution of aging-delaying plant extract 21 (PE21) in ethanol was made on the day of adding this PE to cell cultures. This stock solution was added to growth medium immediately following cell inoculation into the medium. The final concentration of PE21 in growth medium was 0.1%.

Lipid extraction from yeast cells [34]

Yeast cells were harvested by centrifugation in a Centra CL2 clinical centrifuge at 3,000 × g for 5 min at room temperature. The cell pellet was resuspended in ice-cold 155 mM ammonium bicarbonate (pH 8.0), and the cells were subjected to centrifugation at 16,000 × g for 1 min at 4^{o}C. The cell pellet was stored at -80^{O}C until lipid extraction. For lipid extraction, the pelleted cells kept at -80^{O}C were thawed on ice before being resuspended in 1 mL of ice-cold nanopure water. The volume that contained 5×10^{7} cells was transferred to a 15-mL high-strength glass screw top centrifuge tube with a Teflon lined cap (#0556912; Fisher Scientific). The volume of each sample was topped off to 1 mL

with ice-cold nanopure water. To each tube the following was added: 20 µL of the internal standard mix prepared in Chromasolv HPLC (>99.9%) chloroform (Sigma-Aldrich) (**Table 2.1**), 800 µL of 425-600 µM acid-washed glass beads to break open the cells (#G8772; Sigma-Aldrich) and 3 mL of a Chromasolv HPLC (>99.9%) chloroform-methanol mixture (both from Sigma-Aldrich) at a 17:1 ratio. The samples were then vortexed vigorously for 2 h at 4^{O}C and subjected to centrifugation in a Centra CL2 clinical centrifuge at 3,000 × g for 5 min at room temperature; this centrifugation allowed to separate the upper aqueous phase from the lower organic phase, which contained nonpolar lipids TAG, PC, PE and PG. The lower organic phase was then transferred to another 15-mL high-strength glass screw top centrifuge tube using a glass Pasteur pipette with careful attention not to disrupt the glass beads or upper aqueous phase. 1.5 mL of chloroform-methanol (2:1) solution was added to the remaining upper aqueous phase to allow the separation of polar lipids PA, PS, PI and CL. The samples were again vortexed vigorously at 4^{O}C for 1 h. The initial separated organic band was kept at 4^{O}C for the duration of the second vortexing. At the end of 1 h, the samples were again centrifuged for 5 min at 3,000 × rpm at room temperature; the lower organic phase was then separated and added to the corresponding initial organic phase with a glass Pasteur pipette. With both lower organic phases combined, the solvent was evaporated off by nitrogen gas flow. Once all solvent was evaporated, the remaining lipid film was dissolved in 100 µL of chloroform-methanol (1:2) and immediately transferred into 2-mL glass vials with Teflon screw tops to avoid evaporation until samples were analysed by mass spectrometry. Samples were then stored at -80^{O}C and ran on the LTQ Orbitrap Mass Spectrometer within one week of the extraction.

Table 2.1. Internal standard mix composition (modified from [34])

Detection mode	Class of lipid standard	Lipid chain composition (number carbons: number double bonds on fatty acid chain)	Exact mass molecular weight (g/mol)	M/Z (mass/ion charge)	Concentration in mix (mg/µL)
Negative	CL	14:0 / 14:0 / 14:0 /14:0	1274.9000	619.4157	0.10
	FFA	19:0	298.2872	297.2711	0.02
	PA	14:0 / 14:0	614.3920	591.4026	0.10
	PE	14:0 / 14:0	635.4526	634.4448	0.10
	PG	14:0 / 14:0	688.4290	665.4394	0.10
	PS	14:0 / 14:0	701.4240	678.4271	0.02
Positive	TAG	13:0 / 13:0 / 13:0	680.5955	698.6299	0.10
	PC	13:0 / 13:0	649.4683	650.4761	0.10

Internal standards CL, PA, PE, PG, PS and PC were all from Avanti Polar Lipid, Alabaster, AL, USA. TAG internal standard originates from Larodan, Malmo, Sweden.

Lipid identification and quantitation using mass spectrometry

Samples were diluted (1:1) with 1:2 chloroform-methanol supplemented with 0.1% ammonium hydroxide for improved ionization efficiency. Samples were injected one at a time using a Thermo Orbitrap Velos Mass Spectrometer equipped with HESI-II ion source (Thermo Scientific) at a flow rate of 5 µL/ min. The instrument settings for the Orbitrap used the optimized settings, as described by Richard et al., 2014 (**Table 2.2**).

Table 2.2. Thermo Orbitrap Velos mass spectrometer's tune file instrument settings (from [34])

Instrument polarity	Positive	Negative

Source voltage (kV)	3.9	4
Capillary temperature (^{O}C)	275	275
Sheath gas flow	5	5
Aux gas flow	1	1
FT-MS injection time (ms)	100	500
FT-MS microscans	3	1

Data were acquired according to the Instrument Method for data-dependent acquisition for 5 min in both positive and negative modes by the FT-MS analyser at a resolution of 100,000 for both MS and MS/MS data. (**Table 2.3**)

Table 2.3. Instrument method for data-dependent acquisition (from [34])

Acquisition time	5 min (with a 0.25 minute delay)	
Instrument polarity	Positive	Negative
MS (Segment I)		
Analyzer	FTMS	FTMS
Mass range	Normal	Normal
Resolution	100,000	100,000
Data type	Centroid	Centroid
Scan range	400-1,200	400-1,200
Data-dependent MS/MS (segments 2-10)		
Analyzer	FTMS	FTMS
Resolution	30,000	30,000
Data type	Centroid	Centroid
Activation	HCD	HCD
Activation time (ms)	0.1	0.1
Isolation width	1	1
Collision energy	35	65
Mass range	Normal	Normal
Data type	Centroid	Centroid
Scan range	-	-

Between each sample, the line was flushed with chloroform-methanol (1:2) mixture until the ion detection steadied and returned back into the baseline level. Diluted internal standard mix was injected multiple times throughout the acquisition to ensure no sensitivity loss throughout the run.

Once all data was acquired, raw files were converted to open format mzXML using ProteoWizard MSConvert software (http://proteowizard.sourceforge.net/), the file format used by the Lipid Identification Software LipidXplorer (https://wiki.mpi-cbg.de/lipidx/Main_Page). Data files were then imported into this software using settings described in **Table 2.4**; all lipids in the PA, PC, PE, PI, PS, CL and TAG lipid classes were identified with the help of Molecular Fragmentation Query Language (MFQL) files. MFQL files were obtained from the LipidXplorer page listed above and aided in the identification of lipids by their m/z ratio as well as their fragmentation patterns.

LipidXplorer's output data was then opened under a Microsoft Excel file and all detected lipids were quantified by comparison with the intensity of the corresponding lipid class' internal standard's known concentration in the sample. Each quantified lipid had a corresponding internal standard from the same lipid class, allowing the calculation of molar percentage of each identified lipid species and, therefore, of each lipid class.

Table 2.4. Lipid identification by LipidXplorer import settings for data acquired under positive and negative mode (from [34])

	Positive mode	Negative mode
Selection Window (Da)	1	1
Time range (sec.)	0-350	0-350
Calibration masses		
MS	0	0
MS/MS	0	0
m/z range (m/z-m/z)		
MS	140-1,200	100-400
MS/MS	200-1,400	200-1200
Resolution (FMHW)		
MS	100,000	100,000
MS/MS	30,000	30,000
Tolerance (ppm)		
MS	10	10

MS/MS	10	10
Resolution Gradient (res/(m/z))		
MS	0	-55
MS/MS	0	0
Minimum Occupation (0<1)		
MS	0.5	0.5
MS/MS	0	0
MS1 offset (Da)	0	0

Purification of the endoplasmic reticulum (ER) from yeast cells

Reagents and solutions

1. TSD reduction buffer: 0.1 M Tris/Sulfate (pH 9.4), 10 mM DTT

2. HEPES lysis buffer: 20 mM HEPES/KOH, pH 6.8, 50 mM KCl, 200 mM sorbitol, 2 mM EDTA, 1 mM DTT

3. Spheroplast medium A (pH 7.5): 0.67% yeast nitrogen base (w/o) amino acids, 2 % (w/v) glucose, 1 M sorbitol, 20 mM Tris/HCl (pH 7.5)

4. Spheroplast medium B: 0.67% yeast nitrogen base (w/o) amino acids, 2 % (w/v) glucose, 1 M sorbitol

5. 1.2 M sucrose/ HEPES, 36% (w/w): 7.2 g sucrose + 12.8 ml HEPES lysis buffer

6. 1.5 M sucrose/ HEPES, 43% (w/w): 8.6 g sucrose + 11.4 ml HEPES lysis buffer

7. MES buffer 1 (MES breaking buffer): 10 mM MES/Tris (pH 6.9), 12 % (w/w) Ficoll 400, 0.2 mM EDTA

8. MES buffer 2: 10 mM MES/Tris (pH 6.9), 8 % (w/w) Ficoll 400, 0.2 mM EDTA

9. MES buffer 3: 10 mM MES/Tris (pH 6.9), 0.6 M sorbitol, 8 % (w/w) Ficoll 400, 0.2 mM EDTA

10. MES buffer 4: 10 mM MES/Tris (pH 6.9), 0.25 M sorbitol, 0.2 mM EDTA

11. KPi buffer (pH 7.4): 20 mM KH_2PO_4/KOH (pH 7.4), 1.2 M sorbitol

Procedure

Wild-type cells were grown in YEPD medium initially containing 2% glucose as carbon source, with 0.1% PE21 or without it. Culture was harvested at diauxic phase, checked for contamination by bright-field microscopy and used to measure cell density at OD_{600}. The non-contaminated wild-type cells were pelleted at 4,000 × g for 5 min at room temperature. Cells were then resuspended at 10 OD_{600} units/ml in TSD reduction buffer, incubated for 10 min at room temperature and centrifuged for 5 min at 4,000 × g at room temperature. Pelleted cells were then resuspended at 20 OD_{600} units/ml in Spheroplast medium A and supplemented with Zymolyase 100T at a concentration of 7.5 µg per OD_{600} units of cells. 10 µl of each cell suspension was then removed, diluted in 990 µl of H_2O and used to measure the OD_{600}. The remaining cell suspensions were incubated at 30°C for 30 min and the efficiency of cell wall removal was monitored by measuring the OD_{600}. Cell wall digestion was allowed to proceed until the OD_{600} measurement of the diluted cell suspension became 5% of the original value, with the total digestion time not exceeding 1 hour. Spheroplasts were then harvested by centrifugation at 1,500 × g for 5 min at room temperature, followed by resuspending at 1 to 5 OD_{600} units/ml in Spheroplast medium B by gentle swirling of the tube or gentle stirring with a glass rod. Spheroplasts were then harvested by centrifugation at 1,500 × g for 5 min at 4°C and then resuspended at a concentration of 1,000 OD_{600} units/ml of ice-cold HEPES lysis buffer with freshly-added DTT. Spheroplasts were then homogenized using 20 strokes and resulting lysates were centrifuged at 1, 000 × g for 10 min at 4°C. The supernatants (S_{1000}) were subjected to another round of centrifugation at 1, 000 × g for 10 min at 4°C,

and resulting supernatants were further centrifuged at 27,000 × g for 10 min at 4°C. The pelleted membranes ($P_{27,000}$) were resuspended in 1.0 ml of HEPES lysis buffer (5,000 OD_{600} equivalents per ml) using a trimmed 1-ml pipette tip and carefully layered on top of a sucrose gradient prepared in advance (2.1 ml of 1.5 M sucrose/HEPES solution was deposited to the bottom of a Beckman Ultra-Clear centrifuge tube for the Beckman MLS-50 rotor, and then overlaid with 2.1 ml of 1.2 M sucrose/HEPES solution). The gradient tubes were then placed in the pre-chilled swinging bucket Beckman MLS-50 rotor and centrifuged at 100,000 × g (36,000 rpm) for 1 hr at 4°C using the slow acceleration and deceleration setting to minimize disruption of gradients. 18 gradient fractions of 227 µl each were then collected starting from the top of the sucrose gradient and stored at -20°C for further analyses.

Isolation of crude mitochondrial fraction from yeast cells

Reagents

1. Dithiothreitol (DTT) buffer [100 mM Tris-H_2SO_4, 10 mM dithiothreitol]
2. Zymolyase 100T from *Arthrobacter luteus* (MP Biomedicals)
3. Zymolyase buffer [1.2 M sorbitol, 20 mM potassium phosphate]
4. Homogenization buffer [0.6 M sorbitol, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2% (w/v) BSA]
5. SEM buffer [250 mM sucrose, 1 mM EDTA, 10 mM Mops (pH 7.2)]

Procedure

Cell cultures were combined in pre-weighed centrifuge bottles and cells were pelleted at 3,000 × g for 5 min at room temperature using a Beckman JA-10 rotor. The cells were washed twice with distilled water, followed by the determination of their wet weight. The cell pellets were resuspended in 2 ml/g DTT buffer and incubated on a shaker at 80 rpm for 20 min at 30ºC. The cells were pelleted as per initial centrifugation, washed in 7 ml/g Zymolyase buffer without Zymolyase and pelleted once more. The cells were incubated on a shaker at 80 rpm for 45 min at 30ºC with 1 mg/g (wet weight) of Zymolyase-100T in 7 ml/g Zymolyase buffer. Zymolyase was used because of its well-known strong lytic activity required to digest yeast cell wall. The spheroplasts obtained were then spun down at 2,200 × g for 8 min at 4ºC. All subsequent steps were carried out on ice or at 4°C with the use of cut pipette tips to avoid breaking organelles. The spheroplasts were resuspended in 6.5 ml/g ice-cold homogenization buffer and washed by centrifugation at 2,200 × g for 8 min at 4ºC. The spheroplasts were then mechanically homogenized with 15 strokes in 6.5 ml/g ice-cold homogenization buffer to disrupt yeast plasma membrane for releasing organelles and cytoplasm. Following the homogenization, the cell debris was pelleted by centrifuging at 1,500 × g for 5 min at 4ºC using a Beckman JA-17 rotor. The resulting lysate supernatant was subjected to centrifugation twice at 3000 × g for 5 min at 4ºC to pellet the nuclei and 12,000 × g for 15 min at 4ºC. The newly obtained pellet contains mostly mitochondria, but also the endoplasmic reticulum (ER), Golgi, peroxisomes, lysosomes and vacuoles, whereas the supernatant contains the cytosol, microsomes from the ER and vacuoles. The pellet was resuspended in 6.5 ml/g in ice-cold homogenizing buffer, spun down for 5 min at 3,000 × g at 4ºC to obtain a supernatant containing mitochondria, which was then subjected to a spin at 12, 000 × g

for 15 min at 4ºC. The resulting pellet was resuspended in 3 ml of SEM to be overlaid onto a sucrose gradient.

Purification of mitochondria devoid of microsomal and cytosolic contaminations

Reagents

1. SEM buffer [250 mM sucrose, 1 mM EDTA, 10 mM Mops (pH 7.2)]

2. EM buffer [10 mM Mops (pH 7.2), 1 mM EDTA]

Procedure

In order to purify yeast mitochondria from the crude mitochondrial fraction, an equilibrium density-gradient centrifugation was performed. Yeast mitochondria have a density of 1.18 g/cm^3 whereas 10% and 50% sucrose respectively have a density of 1.10 g/cm^3 and 1.30 g/cm^3. To prepare a sucrose density gradient for the purification of mitochondria, 1.5 ml of 60% sucrose in EM buffer was overlaid with 4 ml of 32%, 1.5 ml of 23% and 1.5 ml of 15% sucrose in EM buffer, followed by 3 ml of the crude mitochondrial suspension. The sucrose density gradient containing the mitochondrial suspension was subjected to centrifugation in a Beckman SW41 Ti swinging-bucket rotor at 134,000 × g for 60 min at 4ºC. The mitochondrial band, which was easily distinguishable, appeared at the interface between 60% and 32% sucrose. Fractions of 1 ml were recovered using a cut pipette tip and placed in 1.5 ml Eppendorf tubes and frozen at -80ºC until use. In order to quickly freeze the mitochondrial fractions, the fractions were immersed, with the aid of long tweezers, in a beaker of isopropyl alcohol kept in the -80ºC freezer.

Purification of lipid droplets (LDs) from yeast cells

Reagents and solutions

1. TSD reduction buffer: 0.1 M Tris/Sulfate (pH 9.4), 10 mM DTT

2. HEPES lysis buffer: 20 mM HEPES/KOH, pH 6.8, 50 mM KCl, 200 mM sorbitol, 2 mM EDTA, 1 mM DTT

3. Spheroplast medium A (pH 7.5): 0.67% yeast nitrogen base (w/o) amino acids, 2 % (w/v) glucose, 1 M sorbitol, 20 mM Tris/HCl (pH 7.5)

4. Spheroplast medium B: 0.67% yeast nitrogen base (w/o) amino acids, 2 % (w/v) glucose, 1 M sorbitol

5. 1.2 M sucrose/ HEPES, 36% (w/w): 7.2 g sucrose + 12.8 ml HEPES lysis buffer

6. 1.5 M sucrose/ HEPES, 43% (w/w): 8.6 g sucrose + 11.4 ml HEPES lysis buffer

7. MES buffer 1 (MES breaking buffer): 10 mM MES/Tris (pH 6.9), 12 % (w/w) Ficoll 400, 0.2 mM EDTA

8. MES buffer 2: 10 mM MES/Tris (pH 6.9), 8 % (w/w) Ficoll 400, 0.2 mM EDTA

9. MES buffer 3: 10 mM MES/Tris (pH 6.9), 0.6 M sorbitol, 8 % (w/w) Ficoll 400, 0.2 mM EDTA

10. MES buffer 4: 10 mM MES/Tris (pH 6.9), 0.25 M sorbitol, 0.2 mM EDTA

11. KPi buffer (pH 7.4): 20 mM KH_2PO_4/KOH (pH 7.4), 1.2 M sorbitol

Procedure

Wild-type cells were grown in YEPD medium initially containing 2% glucose as carbon source, with 0.1% PE21 or without it. Cultures were harvested at diauxic phase, checked

for contamination by bright-field microscopy and used to measure cell density at OD_{600}. The non-contaminated wild-type cells were pelleted at 4,000 × g for 5 min at room temperature. The cells were then washed once with distilled water and resuspended in the TSD reduction buffer at 10 x OD_{600} units/ml. Following a 10-min incubation at room temperature, the cells were pelleted by centrifugation at 4,000 × g for 5min at room temperature and then washed once in Spheroplasts medium A. The cells were then resuspended in Spheroplasts medium A at 20 × OD_{600} units/ml, supplemented with Zymolyase 100T at a concentration of 2.5 μg per OD_{600} units of cells, and incubated at 30°C for 45 min on a shaker set at 75 rpm. Spheroplasts were then pelleted by 5min centrifugation at 1,500 × g at room temperature, resuspended in the ice-cold Spheroplasts medium B at 5 × OD_{600} units/ml using pipettes with cut tips, and centrifuged for 5 min at 1,500 × g at 4°C. Spheroplasts were then washed twice by resuspending the pellets in ice-cold KPi buffer at a concentration of 5 OD_{600} units/ml followed by centrifugation for 5 min at 1,500 × g at 4°C. After the second wash and centrifugation step, clean spheroplasts were resuspended in ice-cold MES Buffer 1 at a concentration of 1,000 OD_{600} units/ml using pipettes with non-cut tips. Spheroplasts were then lysed in a homogenizer using 20 strokes at 4°C. Lysates were centrifuged for 5 min at 5,000 × g at 4°C and the resulting supernatants ($S_{5,000}$) were transferred to pre-chilled centrifuge tubes kept on ice. LDs were then purified by subjecting the spheroplast lysates to a series of flotation gradient centrifugations using a MLS 50 swinging bucket rotor (Beckman). The first flotation gradient was prepared by placing 2.5 ml of the $S_{5,000}$ fraction to the bottom of a centrifuge tube (Ultra-Clear Beckman tubes for MLS 50 rotor) and overlaying it with 2.5 ml of ice-cold MES Buffer 1. Following 1 hour centrifugation at 100,000 × g (36,000 rpm) at 4°C,

the floating layer from the top portions of the gradients was collected with a cut tip and transferred to the bottom of a new centrifuge tube, which was then supplemented with MES buffer 2 to a total volume of 2.5 ml and gently mixed by pipetting. Resulting suspension was overlaid with 2.5 ml of ice-cold MES buffer 2 and then centrifuged for 1 hr at 100,000 × g (36,000 rpm) at 4°C. The floating layer from the top portions of the gradients was again collected with a cut tip and transferred to the bottom of a new centrifuge tube, which was then supplemented with MES buffer 3 to a total volume of 2.5 ml and gently mixed by pipetting. Resulting suspension was overlaid with 2.5 ml of ice-cold MES buffer 4 and then centrifuged for 1 hr at 100,000 × g (36,000 rpm) at 4°C. The floating layer from the top portions of the gradients was collected with a cut tip and purified lipid droplets were stored -20°C for further analyses.

Statistical analysis

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean ± SEM. The *p* values were calculated with the help of GraphPad *t* test calculator using an unpaired two-tailed *t* test.

2.3 Results

Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae* involve numerous steps, each catalyzed by a known enzyme or several enzymes (**Figure 2.1**).

2.3.1 PE21 decreases the concentration of triacylglycerol (TAG) in chronologically aging yeast

Using mass-spectrometric quantitative analysis, I compared the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE21 or remained untreated. I found that PE21 decreases the concentration of the neutral lipid TAG in chronologically aging yeast (**Figure 2.2**). There are three different ways to decrease the concentration of TAG in yeast treated with PE21. These ways include the following: 1) decrease Are1p and/or Are2p concentration and activity; 2) decrease Dga1p and/or Are2p concentration and activity; or 3) increase Tgl1p, Tgl3p, Tgl4p and/or Tgl5p concentration and activity (**Figure 2.1**). Future studies should address the importance of the observed decreased TAG concentration in the aging-delaying ability of PE21 by answering the following three questions: 1) Will single-gene-deletion mutations eliminating Are1p, Are2p, Dga1p or Lro1p (each of which is expected to further decrease TAG concentration) enhance the aging-delaying effect of PE21? 2) Will single-gene-deletion mutations eliminating Tgl1p, Tgl3p, Tgl4p or Tgl5p (each of which is expected to increase TAG concentration) weaken/eliminate the aging-delaying effect of PE21? 3) What are the concentrations of TAG (and other lipid classes) in cells of each of these single-gene-deletion mutants?

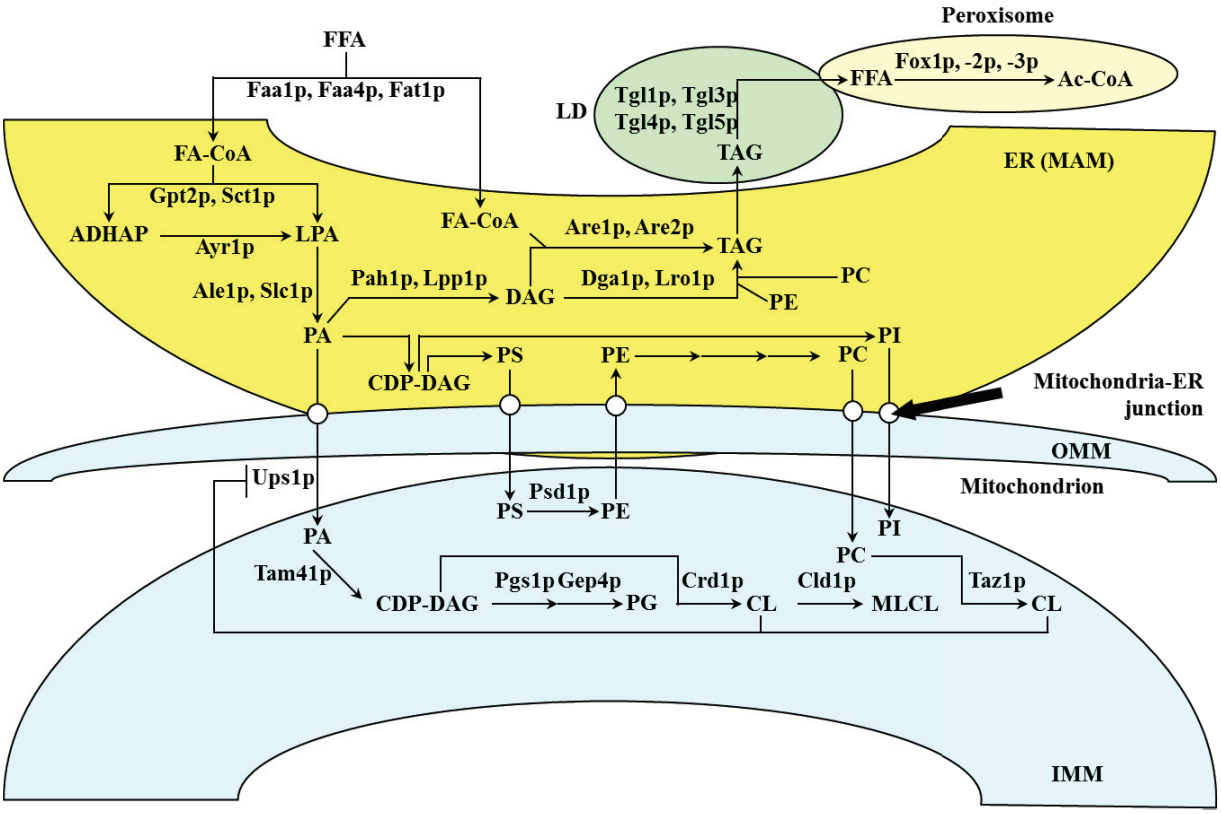

Figure 2.1. Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae*. [25, 26, 27]

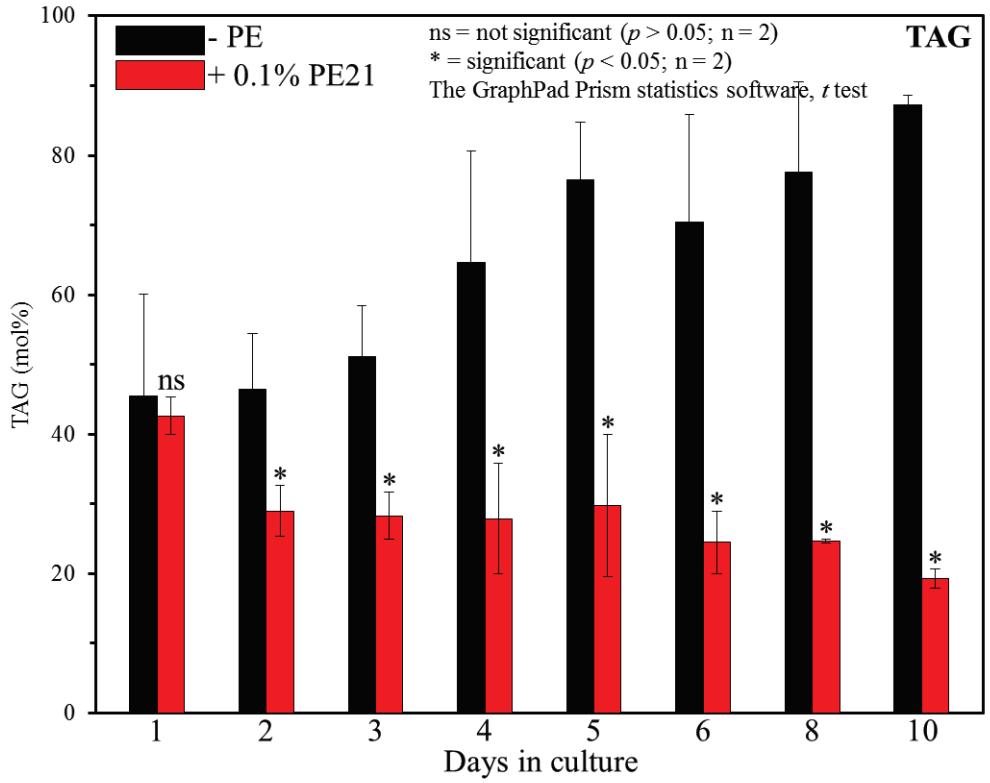

Figure 2.2. PE21 decreases the concentration of the neutral lipid triacylglycerol (TAG) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of TAG were calculated in mol%. Data are presented as means ± SEM (n = 2).

2.3.2 PE21 does not alter the concentration of phosphatidic acid (PA) in chronologically aging yeast

I found that PE21 does not change the concentration of PA in chronologically aging yeast (**Figure 2.3**). There are three different ways to keep PA concentration unchanged in yeast treated with PE21. These ways include the following: 1) not to change Ale1p, Slc1p, Pah1p, Lpp1p, Cds1p and/or Ups1p concentration/activity; 2) increase Ale1p and/or Slc1p and increase Pah1p, Lpp1p, Cds1p and/or Ups1p concentration/activity; or 3) decrease Ale1p and/or Slc1p and decrease Pah1p, Lpp1p,

Cds1p and/or Ups1p concentration/activity (**Figure 2.1**). Future studies should address the importance of the observed lack of the effect of PE21 on PA concentration in the aging-delaying ability of this plant extract by answering the following three questions: 1) Will single-gene-deletion mutations eliminating any of the enzymes involved in PA synthesis (i.e. Ale1p or Slc1p; each of which is expected to decrease PA concentration) influence the aging-delaying effect of PE21? 2) Will single-gene-deletion mutations eliminating any of the enzymes involved in PA consumption or transport to mitochondria (i.e. Pah1p, Lpp1p, Cds1p and/or Ups1p; each of which is expected to increase PA concentration) influence the aging-delaying effect of PE21? 3) What are the concentrations of PA (and other lipid classes) in cells of each of these single-gene-deletion mutants?

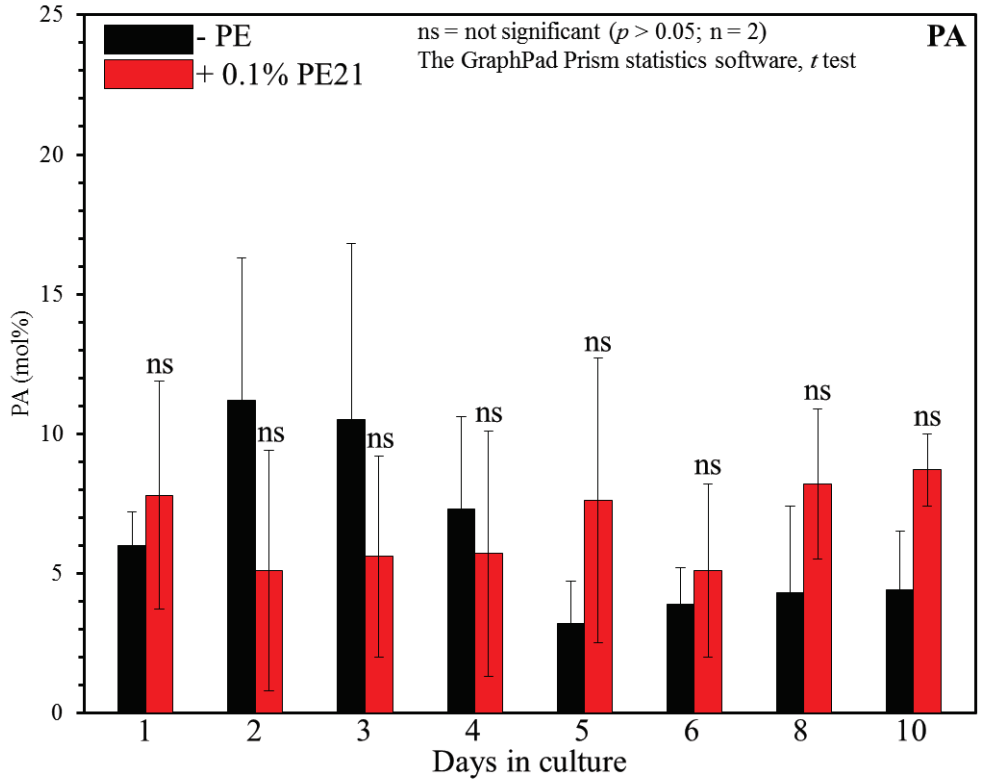

Figure 2.3. PE21 does not change the concentration of phosphatidic acid (PA) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-

rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of PA were calculated in mol%. Data are presented as means ± SEM (n = 2).

2.3.3 PE21 increases the concentration of phosphatidylserine (PS) in chronologically aging yeast

I found that PE21 increases the concentration of PS in chronologically aging yeast (**Figure 2.4**). There are two different ways to increase PS concentration in yeast treated with PE21. These ways include the following: 1) increase Cds1p and/or Cho1p concentration/activity; or 2) decrease Psd1p concentration/activity (**Figure 2.1**). Future studies should address the importance of the observed increased PS concentration in the aging-delaying ability of PE21 by answering the following three questions: 1) Will single-gene-deletion mutations eliminating any of the enzymes involved in PS synthesis (i.e. Cds1p or Cho1p; each of which is expected to decrease PS concentration) weaken/eliminate the aging-delaying effect of PE21? 2) Will a single-gene-deletion mutation eliminating an enzyme involved in PS consumption (i.e. Psd1p; which is expected to further increase PS concentration) enhance the aging-delaying effect of PE21? 3) What are the concentrations of PS (and other lipid classes) in cells of each of these single-gene-deletion mutants?

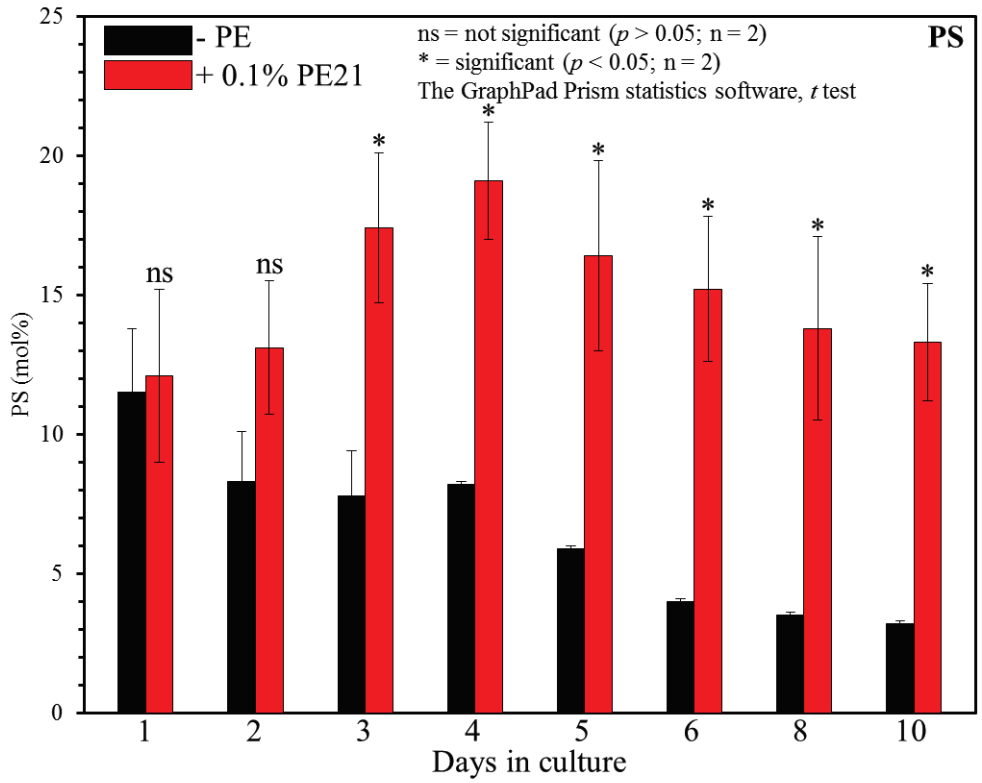

Figure 2.4. PE21 increases the concentration of phosphatidylserine (PS) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of PS were calculated in mol%. Data are presented as means ± SEM (n = 2).

2.3.4 PE21 increases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast

I found that PE21 increases the concentration of PE in chronologically aging yeast (**Figure 2.5**). There are two different ways to increase PE concentration in yeast treated with PE21. These ways include the following: 1) increase Psd1p concentration/activity; or 2) decrease Cho2p and/or Opi3p concentration/activity (**Figure 2.1**). Future studies should address the importance of the observed increased PE concentration in the aging-delaying ability of PE21 by answering the following three questions: 1) Will a single-

gene-deletion mutation eliminating an enzyme involved in PE synthesis (i.e. Psd1p; which is expected to decrease PE concentration) weaken/eliminate the aging-delaying effect of PE21? 2) Will single-gene-deletion mutations eliminating any of the enzymes involved in PE consumption (i.e. Cho2p and/or Opi3p; each of which is expected to increase PE concentration) enhance the aging-delaying effect of PE21? 3) What are the concentrations of PE (and other lipid classes) in cells of each of these single-gene-deletion mutants?

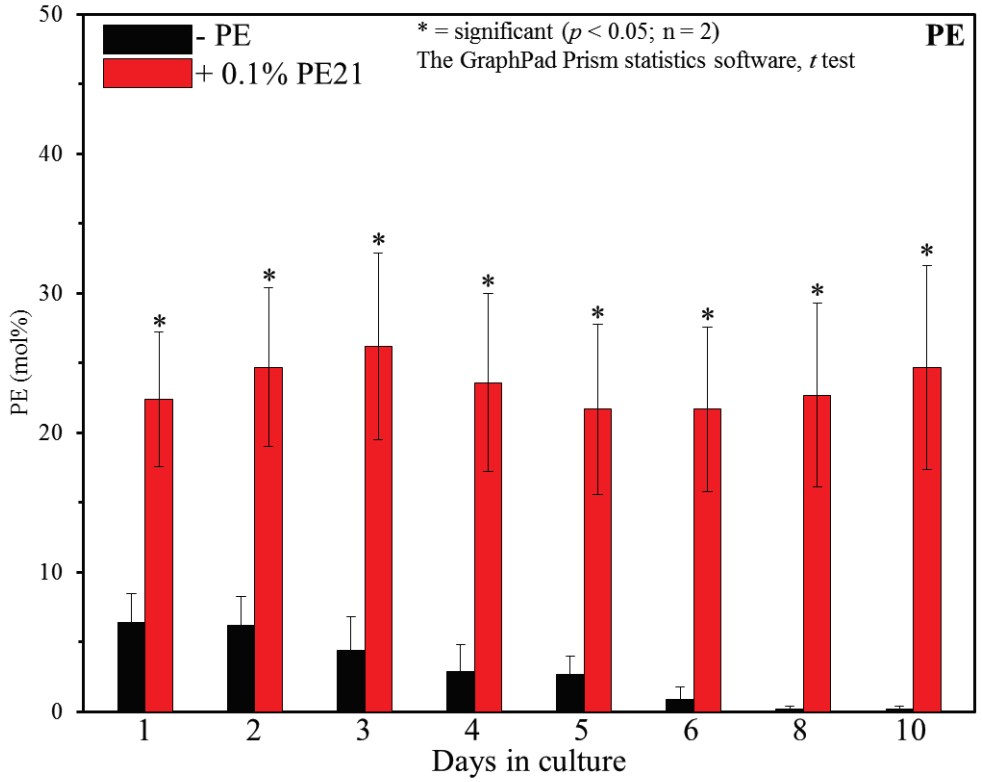

Figure 2.5. PE21 increases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of PE were calculated in mol%. Data are presented as means ± SEM (n = 2).

2.3.5 PE21 increases the concentration of phosphatidylcholine (PC) late in life of chronologically aging yeast

I found that PE21 increases the concentration of PC late in life of chronologically aging yeast (**Figure 2.6**). There are two different ways to increase PC concentration in yeast treated with PE21. These ways include the following: 1) increase Cho2p and/or Opi3p concentration/activity; or 2) decrease Taz1p concentration/activity (**Figure 2.1**). Future studies should address the importance of the late-in-life increased PC concentration in the aging-delaying ability of PE21 by answering the following three questions: 1) Will single-gene-deletion mutations eliminating enzymes involved in PC synthesis (i.e. Cho2p and/or Opi3p; each of which is expected to decrease PC concentration) weaken/eliminate the aging-delaying effect of PE21? 2) Will a single-gene-deletion mutation eliminating an enzyme involved in PC consumption (i.e. Taz1p; which is expected to increase PC concentration) enhance the aging-delaying effect of PE21? 3) What are the concentrations of PC (and other lipid classes) in cells of each of these single-gene-deletion mutants?

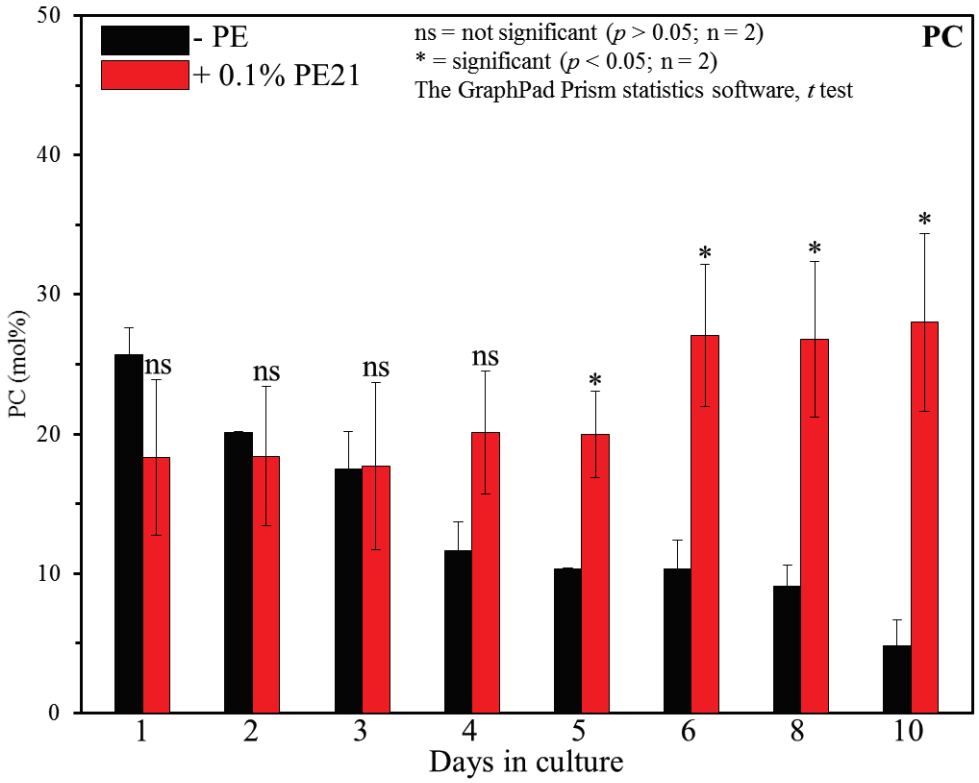

Figure 2.6. PE21 increases the concentration of phosphatidylcholine (PC) late in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of PC were calculated in mol%. Data are presented as means ± SEM (n = 2).

2.3.6 PE21 increases the concentration of phosphatidylinositol (PI) late in life of chronologically aging yeast

I found that PE21 increases the concentration of PI late in life of chronologically aging yeast (**Figure 2.7**). The only way to increase PC concentration in yeast treated with PE21 consists in increasing Cds1p and/or Pis1p concentration/activity (**Figure 2.1**). Future studies should address the importance of the late-in-life increased PI concentration in the aging-delaying ability of PE21 by answering the following two questions: 1) Will single-gene-deletion mutations eliminating enzymes involved in PI synthesis (i.e. Cds1p

and/or Pis1p; each of which is expected to decrease PI concentration) weaken/eliminate the aging-delaying effect of PE21? 2) What are the concentrations of PI (and other lipid classes) in cells of each of these single-gene-deletion mutants?

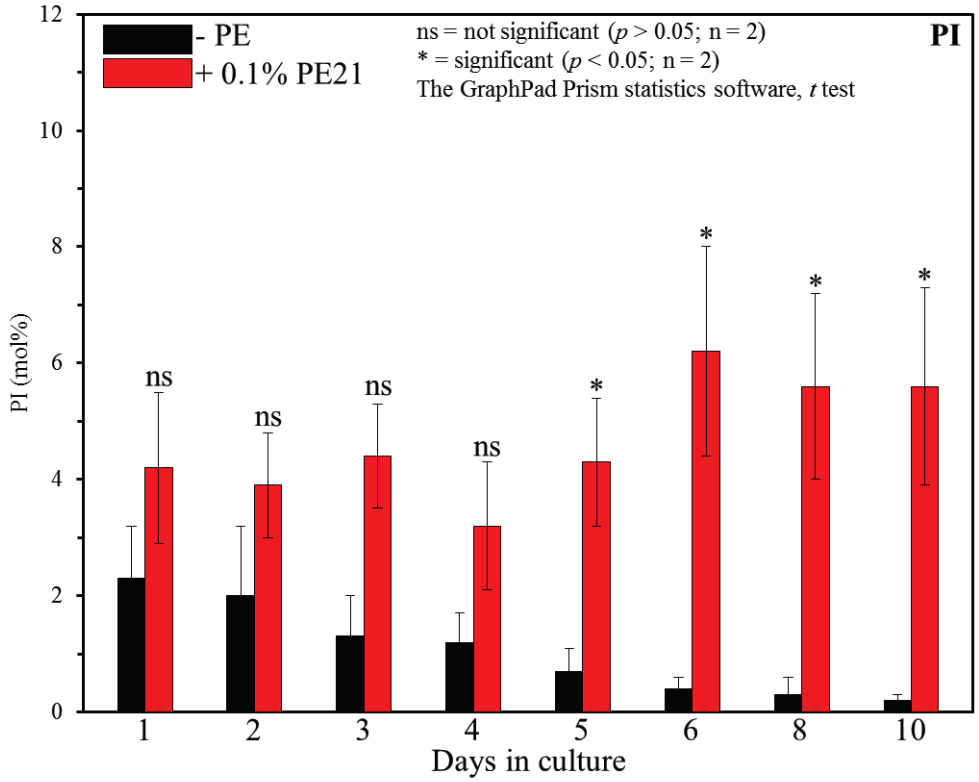

Figure 2.7. PE21 increases the concentration of phosphatidylinositol (PI) late in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of PI were calculated in mol%. Data are presented as means ± SEM (n = 2).

2.3.7 PE21 decreases the concentration of cardiolipin (CL) in chronologically aging yeast

I found that PE21 decreases the concentration of CL (a signature lipid of the inner mitochondrial membrane) in chronologically aging yeast (**Figure 2.8**). The only way to increase CL concentration in yeast treated with PE21 consists in decreasing Ups1p,

Tam41p, Pgs1p, Gep4p, Crd1p, Cld1p and/or Taz1p concentration/activity (**Figure 2.1**). Future studies should address the importance of the decreased CL concentration in the aging-delaying ability of PE21 by answering the following two questions: 1) Will single-gene-deletion mutations eliminating enzymes involved in CL synthesis (i.e. Ups1p, Tam41p, Pgs1p, Gep4p, Crd1p, Cld1p and/or Taz1p; each of which is expected to further decrease CL concentration) enhance the aging-delaying effect of PE21? 2) What are the concentrations of CL (and other lipid classes) in cells of each of these single-gene-deletion mutants?

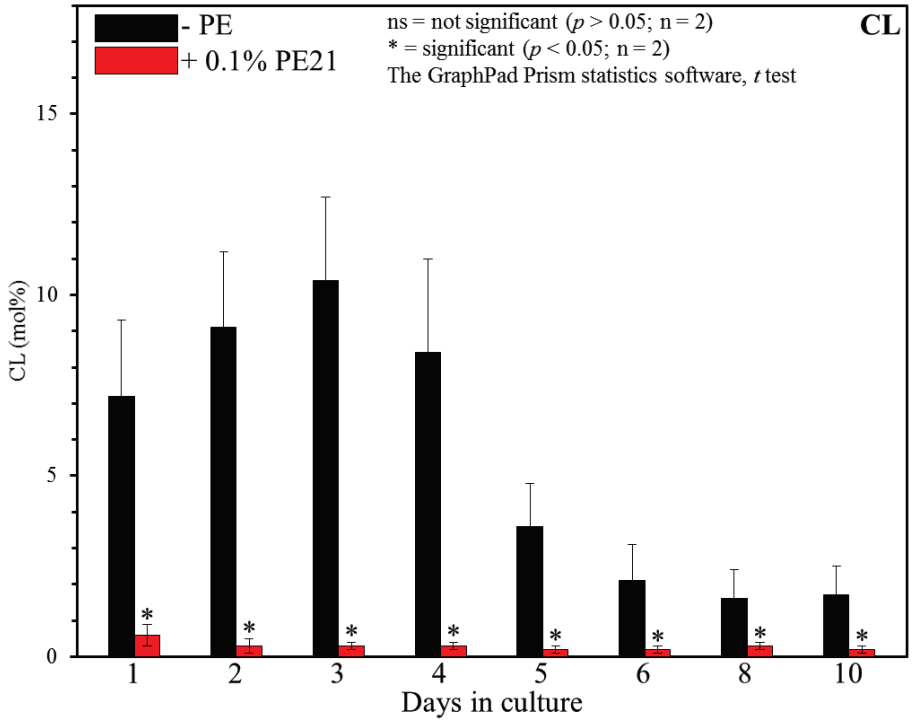

Figure 2.8. PE21 decreases the concentration of cardiolipin (CL) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of CL were calculated in mol%. Data are presented as means ± SEM (n = 2).

2.3.8 PE21 differently affect the concentrations of various classes of lipids in the ER, mitochondria and LDs of chronologically aging yeast

Only CL is confined to a single type of organelles, being a signature lipid class of mitochondria (**Figure 2.1**). All other classes of lipids reside in several types of organelles. Indeed, glycerol phospholipids other than CL can be found in the ER, mitochondria and LDs (**Figure 2.1**), whereas TAG can be detected in the ER and LDs (**Figure 2.1**). Therefore, I used quantitative mass spectrometry to assess how PE21 impacts lipidomes of the ER, mitochondria and LDs purified from chronologically aging yeast. Each of these types of organelles was purified from yeast cells that were recovered on day 2 of culturing, at diauxic growth phase. I found that PE21 increases the concentration of PE, decreases the concentration of TAG and does not alter the concentrations of other lipid classes in the ER (**Figure 2.9**). PE21 also differently affected the abundance of several lipid classes in mitochondria by increasing the concentration of PE, decreasing the concentrations of PG and CL, and not altering the concentrations of other lipid classes (**Figure 2.10**). I found no effect of PE21 on the lipidome of LDs, which contains mainly TAG and traces of PC (**Figure 2.11**).

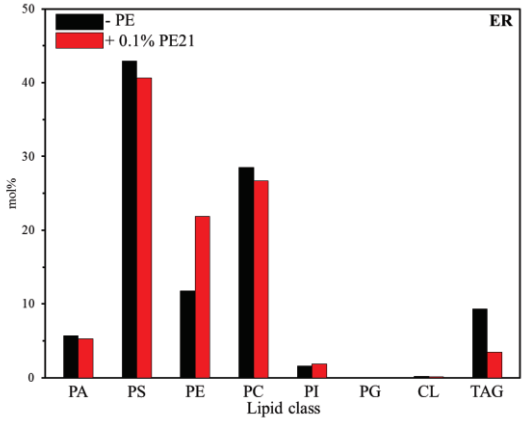

Figure 2.9. PE21 alters the concentrations of some lipid classes in the ER of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-

rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on day 2 of culturing. The ER was purified as described in Materials and Methods. Extraction of lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of different lipid classes were calculated in mol%.

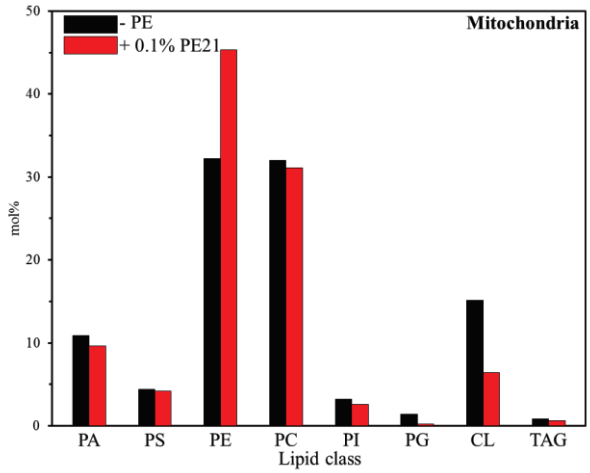

Figure 2.10. PE21 alters the concentrations of some lipid classes in mitochondria of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on day 2 of culturing. Mitochondria were purified as described in Materials and Methods. Extraction of lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of different lipid classes were calculated in mol%.

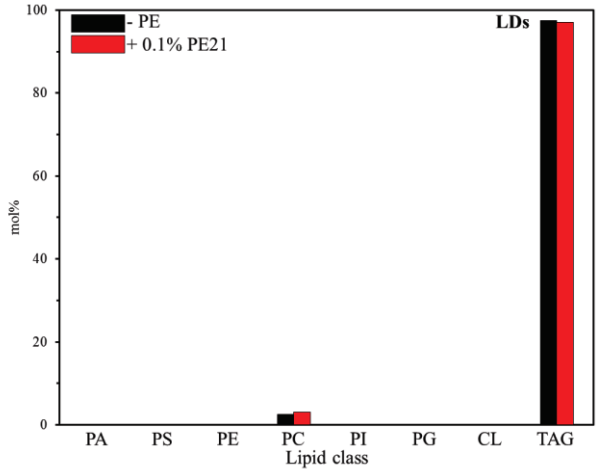

Figure 2.11. PE21 does not alter the concentrations of lipids in LDs of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-

rich YP medium initially containing 2% glucose with or without PE21. Cells were recovered on day 2 of culturing. LDs were purified as described in Materials and Methods. Extraction of lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods. Based on these data, the concentrations of different lipid classes were calculated in mol%.

2.4 Discussion

My data on how PE21 influences the concentrations of different lipid classes in cells of chronologically aging yeast suggest that this aging-delaying plant extract alters the relative rates of PA flow into the synthesis of other lipid classes as follows: 1) PE21 decreases the rate of PA flow into the synthesis of TAG in the ER; 2) PE21 increases the rate of PA flow into the synthesis of glycerol phospholipids (i.e. PS, PE, PC and PI) in the ER and mitochondria; and 3) PE21decreases the rate of PA flow into the synthesis of CL in mitochondria (**Figure 2.12**). Such re-wiring of PA conversion into TAG, glycerol phospholipids and CL delays aging by a mechanism that remains to be established. I speculate that this mechanism may affect several longevity-defining processes in chronologically aging yeast, as described below.

First, the observed reduction in the concentration of CL in mitochondria of yeast cells treated with PE21 may cause the specific changes in the age-related chronology of mitochondrial functionality seen in these cells. [35] These changes affect mitochondrial respiration, mitochondrial membrane potential, mitochondrially produced ROS and stability of mitochondrial DNA. [35] To validate this hypothesis, it needs to be assessed how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentration of CL in the mitochondrial membrane impact mitochondrial functionality and the ability of PE21 to delay aging.

Second, the observed increase in the concentrations of glycerol phospholipids in the ER of yeast cells treated with PE21 may cause the increased resistance of these cells to chronic oxidative and thermal stresses. [35] To test the validity of this hypothesis, it is necessary to examine how genetic manipulations (such as single-gene-deletions and elevated gene expression) that decrease or increase the concentration of glycerol phospholipids in the ER membrane affect stress resistance and the efficiency with which PE21 delays aging.

Third, one of the outcomes of the observed re-wiring of lipid metabolism and transport in yeast cells treated with PE21 may be a decrease in the cellular concentrations of FFA, a class of lipids whose attachment to PA initiates all three major routes of lipid metabolism in the ER, mitochondria and LDs (**Figure 2.12**). FFA are known to trigger an age-related form of programmed cell death called liponecrosis. [36, 37] To validate this hypothesis, the concentrations of FFA in yeast cells treated with PE21 need to be compared to those in cells that remained untreated. Furthermore, it needs to be examined how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentrations of FFA in yeast cells impact the efficiency of liponecrosis and the ability of PE21 to delay aging.

My quantitative mass-spectrometric analysis of various lipid classes from three different types of purified organelles (such as the ER, mitochondria and LDs) was performed for yeast cells that were recovered on day 2 of culturing, at diauxic growth phase. This analysis has revealed that an increase in the concentration of PE observed in the whole-cell lipidome of PE21-treated cells on day 2 of culturing was due, in part, to the increase of PE abundance in both the ER and mitochondria. It remains to be

determined if the increase in the concentration of total cellular PE was also due to an increase (if any) of PE abundance in the plasma membrane (PM). Furthermore, it needs to be assessed what portion of total lipids are contributed by the ER, mitochondria and PM. Furthermore, this analysis of purified organelles has revealed that on day 2 of cell culturing PE21 causes a decrease in TAG in the ER, but not in LDs. Moreover, this analysis of purified organelles has confirmed that on day 2 of cell culturing PE21 causes a decrease in CL in the mitochondrial membranes. I therefore concluded that the quantitative mass-spectrometric analysis of various lipid classes from different types of purified organelles is a powerful tool for revealing the age-related chronology of changes in lipid dynamics in yeast cells treated with PE21. Because my findings imply that various lipid classes undergo different kinds of changes in their concentrations in these cells at different stages of the aging process, in the future this powerful tool is expected to be used intensively for uncovering mechanisms that underlie the essential role of lipid dynamics in the ability of PE21 to delay yeast chronological aging.

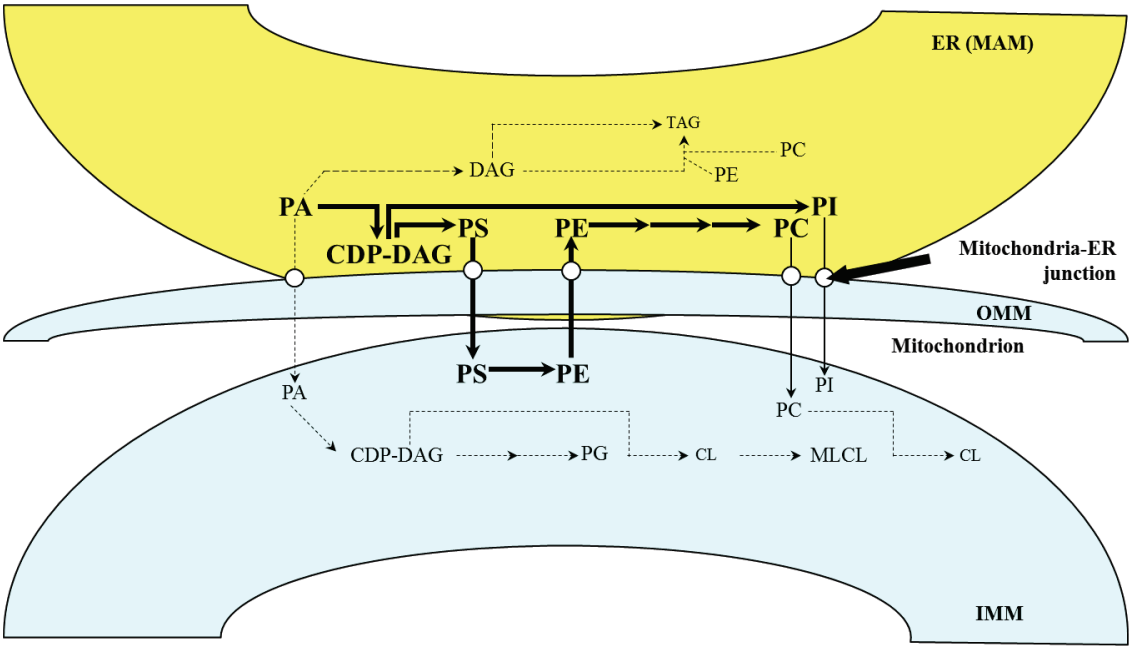

Figure 2.12. A hypothetical model of how PE21 alters the relative rates of PA flow into the synthesis of other lipid classes. PE21: 1) decreases the rate of PA flow into the synthesis of TAG in the ER; 2) increases the rate of PA flow into the synthesis of glycerol phospholipids (i.e. PS, PE, PC and PI) in the ER and mitochondria; and 3) decreases the rate of PA flow into the synthesis of CL in mitochondria.

3 An aging-delaying plant extract PE4 remodels cellular lipidome of chronologically aging yeast

3.1 Abstract

Recent studies from the Titorenko laboratory have identified 6 plant extracts that delay chronological aging of *S. cerevisiae*. All these plant extracts are significantly more efficient aging-delaying interventions than any of the currently known anti-aging chemicals. One of these plant extracts is called PE4. Recent unpublished findings of the Titorenko laboratory suggest that some aspects of lipid metabolism and interorganellar transport may play essential roles in the delay of aging by PE4. In studies described in this chapter of my Thesis, I have used mass-spectrometric quantitative analysis to compare the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE4 or remained untreated. My findings indicate that PE4 alters the relative rates of phosphatidic acid (PA) formation and of PA flow into the synthesis of other lipids as follows: 1) it intensifies PA synthesis from free fatty acids (FFA) in the endoplasmic reticulum (ER); 2) it decreases the rate of PA flow into the synthesis of triacylglycerols (TAG) in the ER; 3) it increases the rate of PA flow into the synthesis of phosphatidylethanolamine (PE) in mitochondria; 4) it increases the rate of PA flow into the synthesis of phosphatidylinositol (PI) in the ER; and 5) it increases the rate of PA flow into the synthesis of cardiolipin (CL) in mitochondria. Such re-wiring of PA synthesis and its conversion into TAG, PE, PI and CL delays aging by a mechanism that remains to be established.

3.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) from Thermo Scientific/Open Biosystems was grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1. The stock solution of aging-delaying plant extract 4 (PE4) in ethanol was made on the day of adding this PE to cell cultures. This stock solution was added to growth medium immediately following cell inoculation into the medium. The final concentration of PE4 in growth medium was 0.5%.

Lipid extraction, identification and quantitation using mass spectrometry

These experimental procedures have been described in chapter 2 of my thesis.

Statistical analysis

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean ± SEM. The *p* values were calculated with the help of GraphPad *t* test calculator using an unpaired two-tailed *t* test.

3.3 Results

Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae* involve numerous steps, each catalyzed by a known enzyme or several enzymes (**Figure 3.1**).

3.3.1 PE4 decreases the concentration of triacylglycerol (TAG) in chronologically aging yeast

Using mass-spectrometric quantitative analysis, I compared the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE4 or remained untreated. I found that PE4 decreases the concentration of the neutral lipid TAG in chronologically aging yeast (**Figure 3.2**).

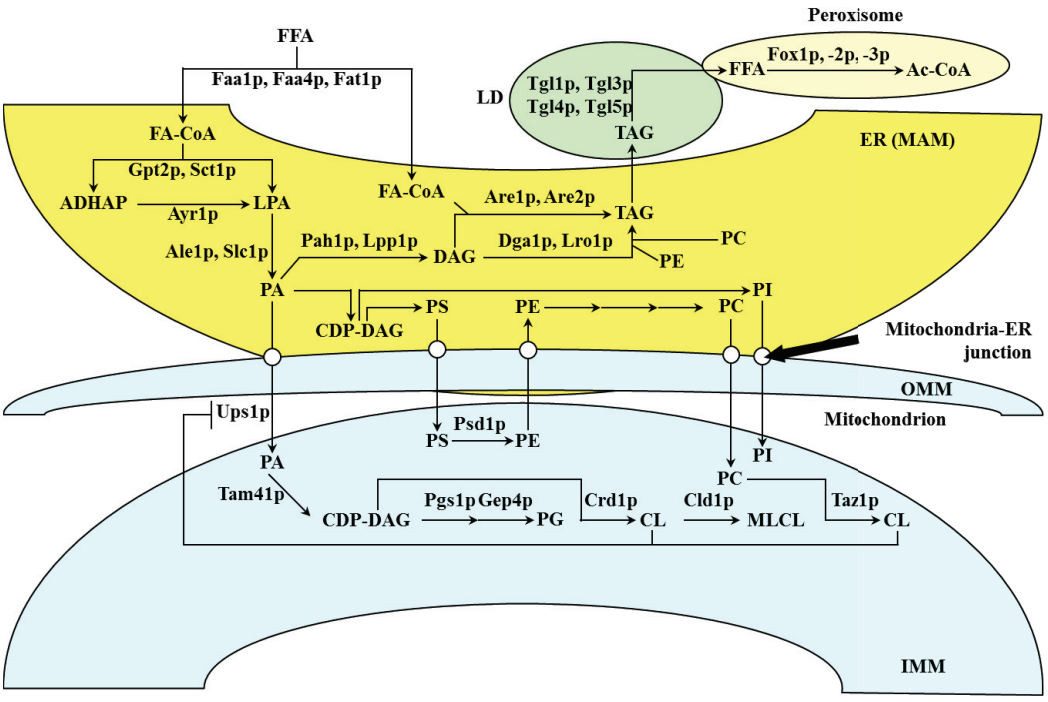

Figure 3.1. Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae*. [25, 26, 27]

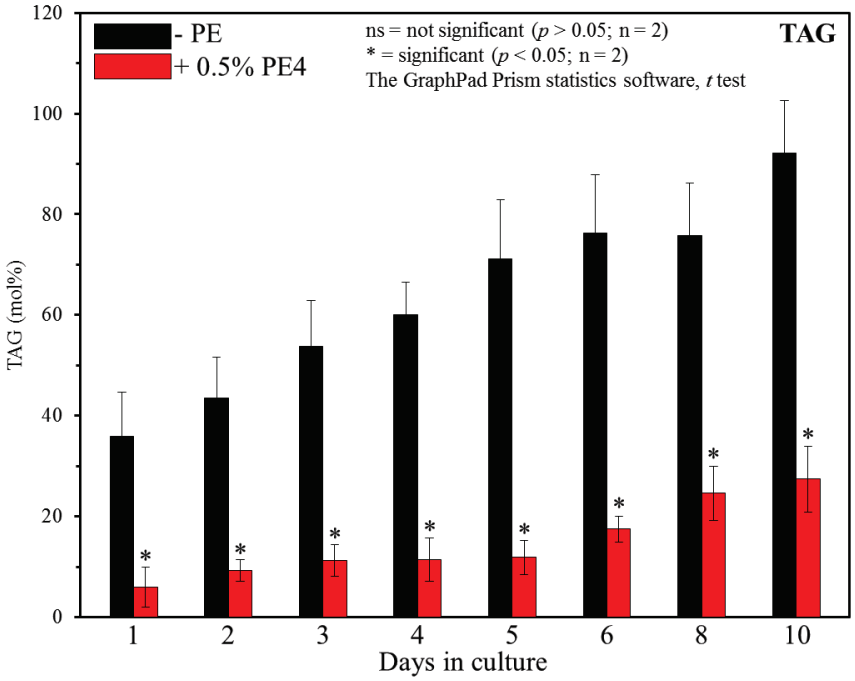

Figure 3.2. PE4 decreases the concentration of the neutral lipid triacylglycerol (TAG) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE4. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of TAG were calculated in mol%. Data are presented as means ± SEM (n = 2).

3.3.2 PE4 increases the concentration of phosphatidic acid (PA) in chronologically aging yeast

I found that PE4 elevates the concentration of PA in chronologically aging yeast (**Figure 3.3**).

3.3.3 PE4 does not alter the concentration of phosphatidylserine (PS) in chronologically aging yeast

I found that PE4 does not change the concentration of PS in chronologically aging yeast (**Figure 3.4**).

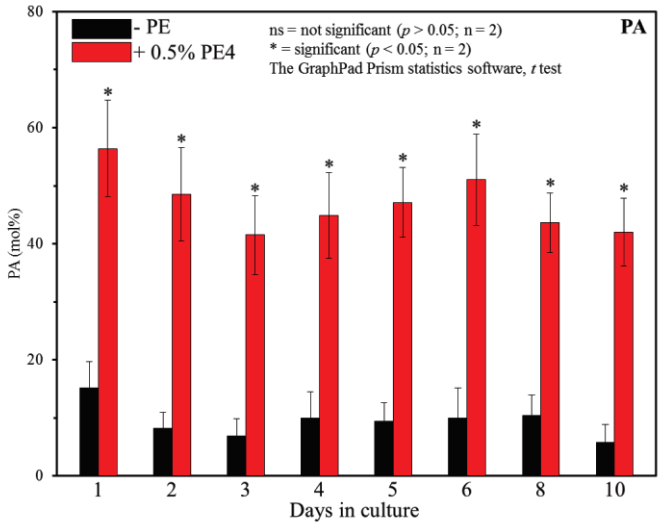

Figure 3.3. PE4 increases the concentration of phosphatidic acid (PA) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE4. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PA were calculated in mol%. Data are presented as means ± SEM (n = 2).

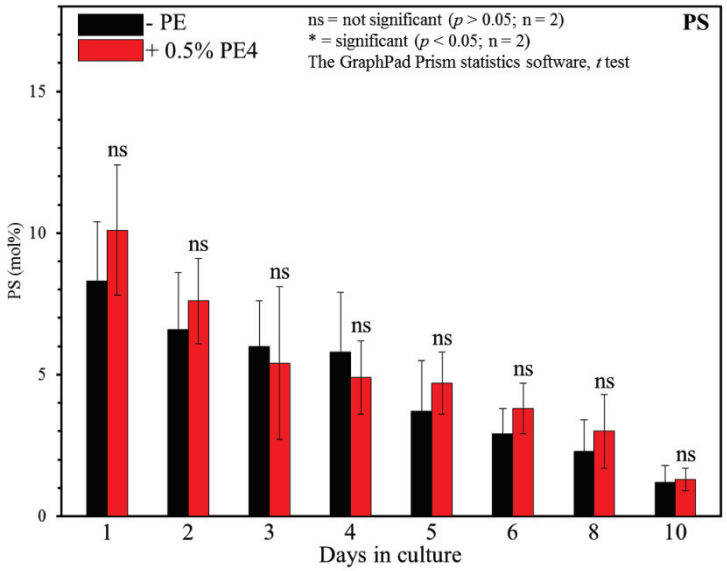

Figure 3.4. PE4 does not alter the concentration of phosphatidylserine (PS) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE4. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PS were calculated in mol%. Data are presented as means ± SEM (n = 2).

3.3.4 PE4 increases the concentration of phosphatidylethanolamine (PE) late in life of chronologically aging yeast

I found that PE4 increases the concentration of PE late in life of chronologically aging yeast (**Figure 3.5**).

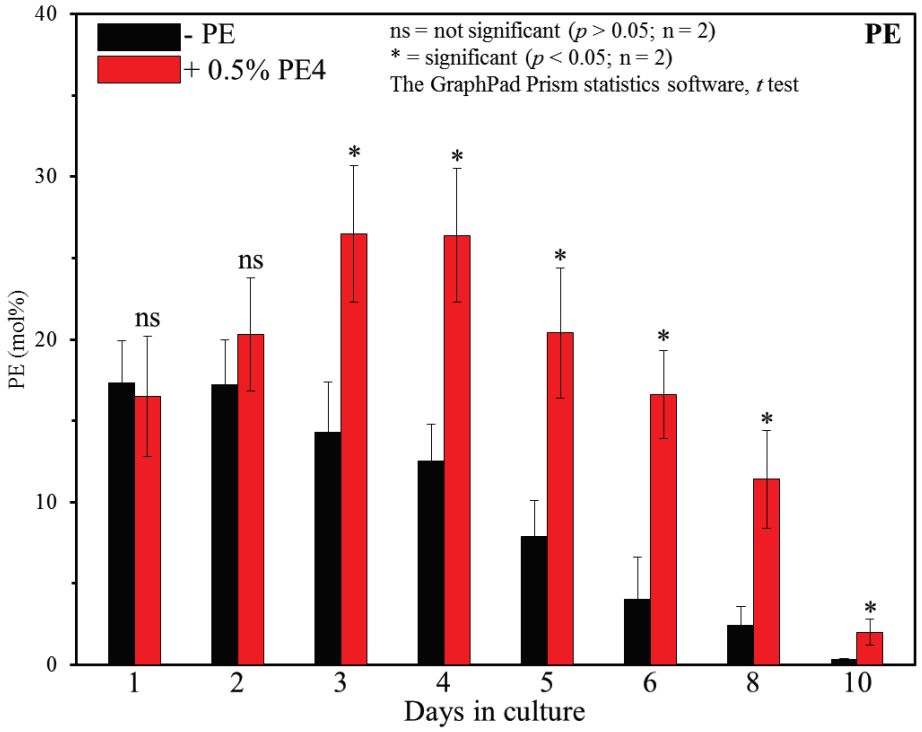

Figure 3.5. PE4 increases the concentration of phosphatidylethanolamine (PE) late in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE4. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PE were calculated in mol%. Data are presented as means ± SEM (n = 2).

3.3.5 PE4 does not alter the concentration of phosphatidylcholine (PC) in chronologically aging yeast

I found that PE4 has no effect on the concentration of PC in chronologically aging yeast (**Figure 3.6**).

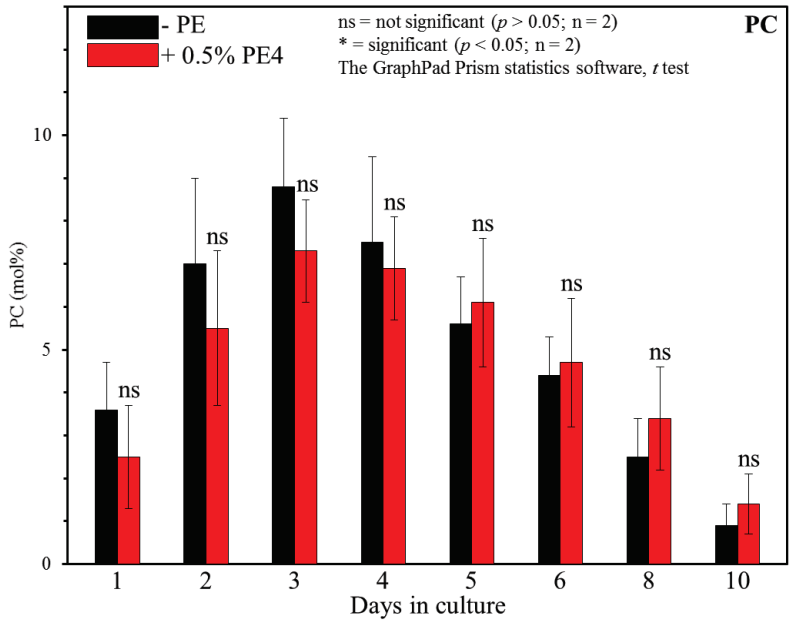

Figure 3.6. PE4 does not alter the concentration of phosphatidylcholine (PC) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE4. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PC were calculated in mol%. Data are presented as means ± SEM (n = 2).

3.3.6 PE4 increases the concentration of phosphatidylinositol (PI) in chronologically aging yeast

I found that PE4 causes an increase in the concentration of PI in chronologically aging yeast (**Figure 3.7**).

3.3.7 PE4 increases the concentration of cardiolipin (CL) in chronologically aging yeast

I found that PE4 increases the concentration of CL (a signature lipid of the inner mitochondrial membrane) in chronologically aging yeast (**Figure 3.8**).

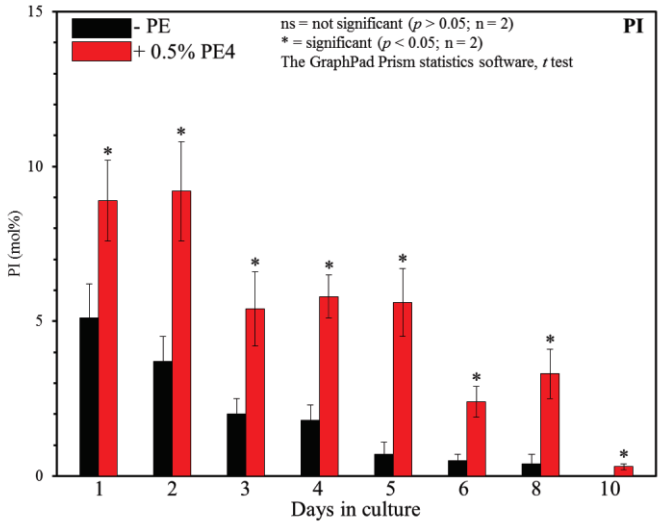

Figure 3.7. PE4 increases the concentration of phosphatidylinositol (PI) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE4. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PI were calculated in mol%. Data are presented as means ± SEM (n = 2).

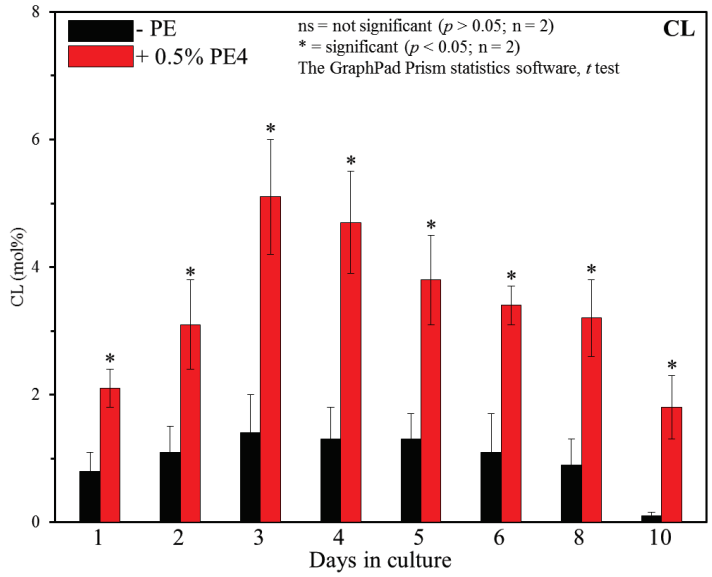

Figure 3.8. PE4 increases the concentration of cardiolipin (CL) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE4. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of CL were calculated in mol%. Data are presented as means ± SEM (n = 2).

3.4 Discussion

My data on how PE4 influences the concentrations of different lipid classes in cells of chronologically aging yeast suggest that this aging-delaying plant extract alters the relative rates of phosphatidic acid (PA) formation and of PA flow into the synthesis of other lipids as follows: 1) it intensifies PA synthesis from FFA in the ER; 2) it decreases the rate of PA flow into the synthesis of TAG in the ER; 3) it increases the rate of PA flow into the synthesis of PE in mitochondria; 4) it increases the rate of PA flow into the synthesis of PI in the ER; and 5) it increases the rate of PA flow into the synthesis of CL in mitochondria (**Figure 3.9**). Such re-wiring of PA synthesis and its conversion into TAG, PE, PI and CL delays aging by a mechanism that remains to be established. I speculate that this mechanism may affect several longevity-defining processes in chronologically aging yeast, as described below.

First, the observed increase in the concentration of CL in mitochondria of yeast cells treated with PE4 may cause certain changes in the age-related chronology of mitochondrial functionality seen in these cells. [35] These changes involve alterations in mitochondrial respiration, mitochondrial membrane potential, mitochondrially produced ROS and stability of mitochondrial DNA. [35] To validate this hypothesis, it needs to be assessed how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentration of CL in the mitochondrial membrane impact mitochondrial functionality and the ability of PE4 to delay aging.

Second, the observed increase in the concentrations of two glycerol phospholipids, PE and PI, in yeast cells treated with PE4 may cause the increased resistance of these cells to chronic oxidative and thermal stresses. [35] To test the validity

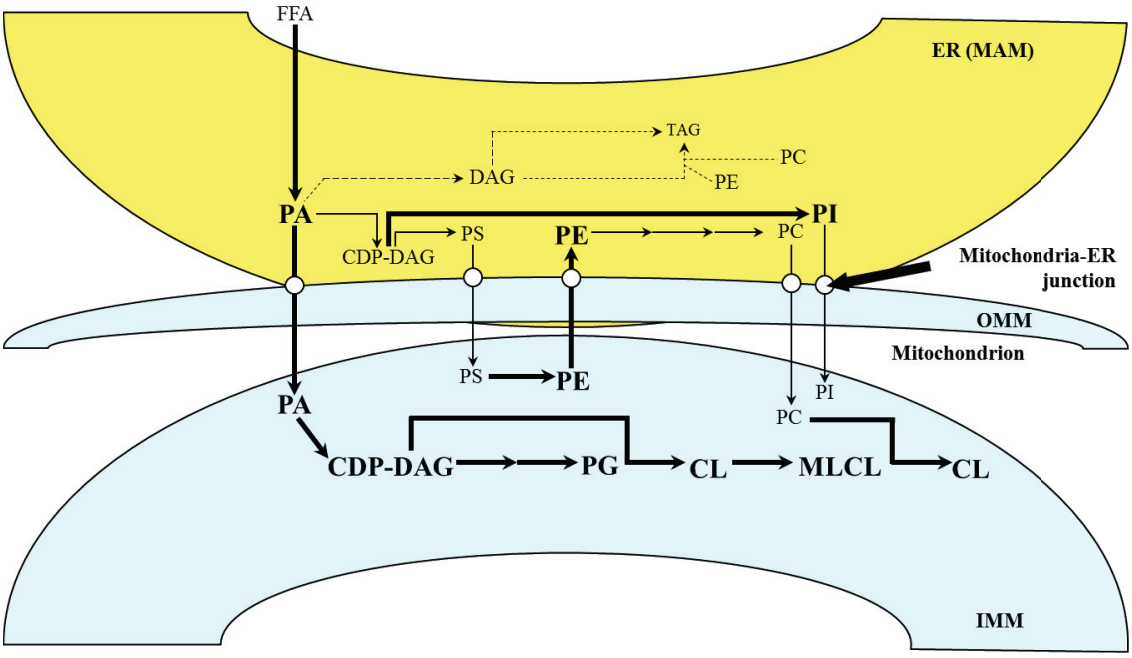

Figure 3.9. A hypothetical model of how PE4 alters the relative rates of PA synthesis and of PA flow into the synthesis of other lipid classes. PE4: 1) intensifies PA synthesis from FFA in the ER; 2) decreases the rate of PA flow into the synthesis of TAG in the ER; 3) increases the rate of PA flow into the synthesis of PE in mitochondria; 4) increases the rate of PA flow into the synthesis of PI in the ER; and 5) increases the rate of PA flow into the synthesis of CL in mitochondria.

of this hypothesis, it is necessary to examine how genetic manipulations (such as single-gene-deletions and elevated gene expression) that decrease or increase the concentration of PE and PI affect stress resistance and the efficiency with which PE4 delays aging.

Third, one of the outcomes of the observed re-wiring of lipid metabolism and transport in yeast cells treated with PE4 may be a decrease in the cellular concentrations of FFA, a class of lipids whose incorporation into PA initiates all three major routes of lipid metabolism in the ER, mitochondria and LDs (**Figure 3.9**). FFA are known to trigger an age-related form of programmed cell death called liponecrosis. [36, 37] To validate this hypothesis, the concentrations of FFA in yeast cells treated with PE4 need to be compared to those in cells that remained untreated. Furthermore, it needs to be

examined how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentrations of FFA in yeast cells impact the efficiency of liponecrosis and the ability of PE4 to delay aging.

Future studies also need to use quantitative mass spectrometry to monitor an age-related chronology of changes in various lipid classes extracted from three different types of purified organelles (such as the ER, mitochondria and LDs). These studies will allow a critical validation of my hypothesis on how PE4 alters the relative rates of PA formation and of PA flow into the synthesis of other lipids in these organelles (**Figure 3.9**).

4 An aging-delaying plant extract PE5 remodels cellular lipidome of chronologically aging yeast

4.1 Abstract

Another aging-delaying plant extract recently discovered by the Titorenko laboratory is called PE5. Recent unpublished studies in the Titorenko laboratory suggest that some aspects of lipid metabolism and interorganellar transport may play essential roles in the delay of aging by PE5. In studies described in this chapter of my Thesis, I have used mass-spectrometric quantitative analysis to compare the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE5 or remained untreated. My findings indicate that PE5 alters the relative rates of phosphatidic acid (PA) formation and of PA flow into the synthesis of other lipids as follows: 1) it promotes PA synthesis from free fatty acids (FFA) in the endoplasmic reticulum (ER); 2) it decreases the rate of PA flow into the synthesis of triacylglycerols (TAG) in the ER; 3) it decreases the rates of PA flow into the synthesis of phosphatidylcholine (PC) and phosphatidylinositol (PI) in the ER, as well as into the synthesis of phosphatidylethanolamine (PE) in mitochondria; and 4) it increases the rate of PA flow into the synthesis of cardiolipin (CL) in mitochondria. Such re-wiring of PA synthesis and its conversion into TAG, PC, PI, PE and CL delays aging by a mechanism that remains to be established.

4.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) from Thermo Scientific/Open Biosystems was grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1. The stock solution of aging-delaying plant extract 5 (PE5) in ethanol was made on the day of adding this PE to cell cultures. This stock solution was added to growth medium immediately following cell inoculation into the medium. The final concentration of PE5 in growth medium was 0.5%.

Lipid extraction, identification and quantitation using mass spectrometry

These experimental procedures have been described in chapter 2 of my thesis.

Statistical analysis

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean ± SEM. The *p* values were calculated with the help of GraphPad *t* test calculator using an unpaired two-tailed *t* test.

4.3 Results

Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae* involve numerous steps, each catalyzed by a known enzyme or several enzymes (**Figure 4.1**).

4.3.1 PE5 decreases the concentration of triacylglycerol (TAG) in chronologically aging yeast

Using mass-spectrometric quantitative analysis, I compared the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE5 or remained untreated. I found that PE5 decreases the concentration of the neutral lipid TAG in chronologically aging yeast (**Figure 4.2**).

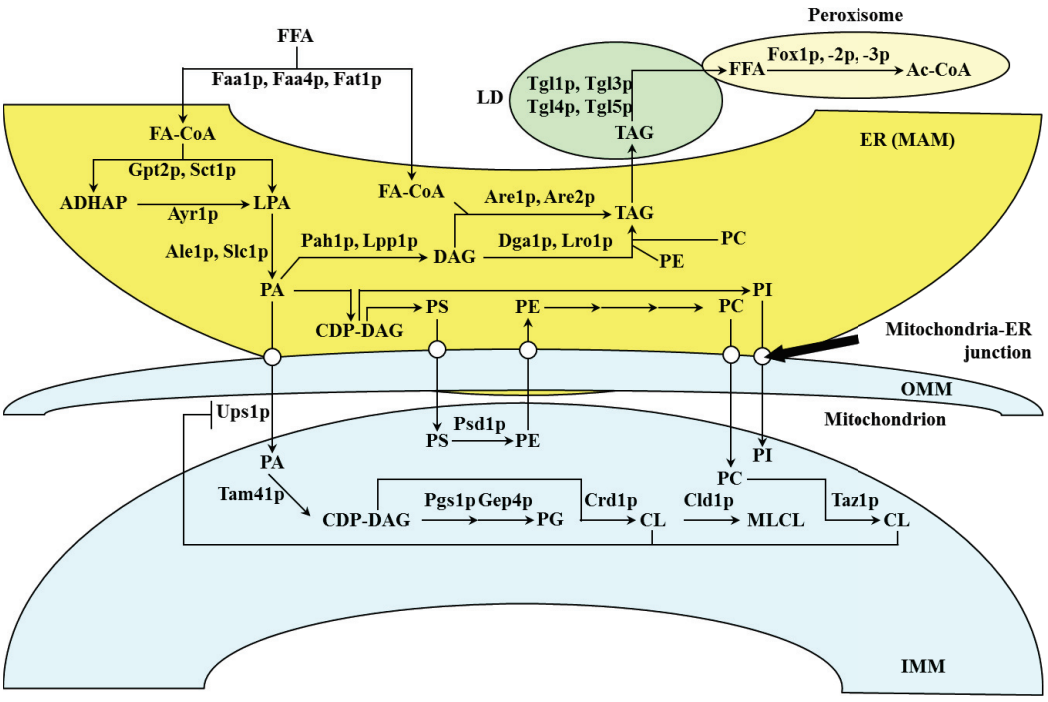

Figure 4.1. Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae*. [25, 26, 27]

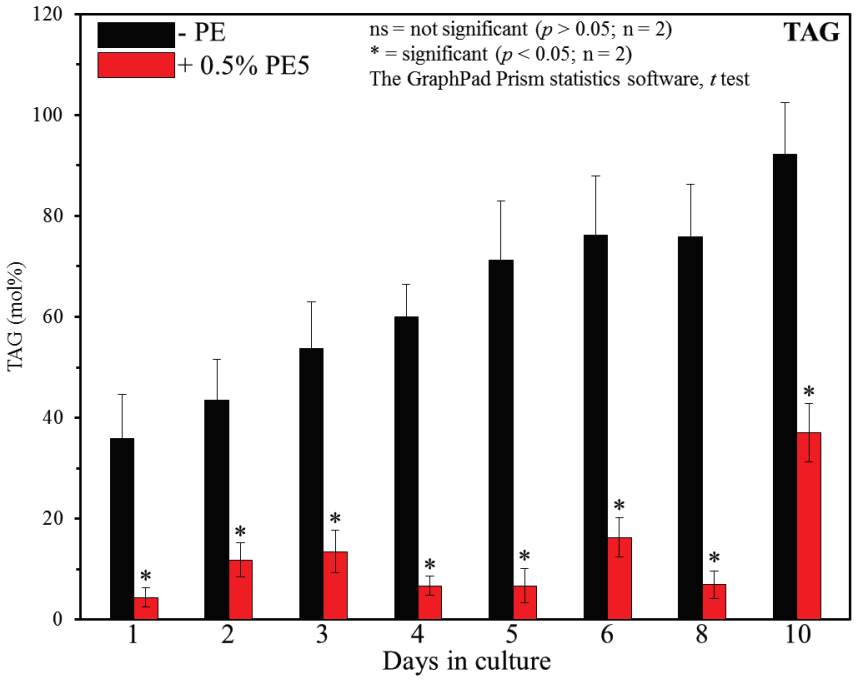

Figure 4.2. PE5 decreases the concentration of the neutral lipid triacylglycerol (TAG) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE5. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of TAG were calculated in mol%. Data are presented as means ± SEM (n = 2).

4.3.2 PE5 causes an increase in the concentration of phosphatidic acid (PA) in chronologically aging yeast

I found that PE5 increases the concentration of PA in chronologically aging yeast (**Figure 4.3**).

4.3.3 PE5 does not change the concentration of phosphatidylserine (PS) in chronologically aging yeast

I found that PE5 has no effect on the concentration of PS in chronologically aging yeast (**Figure 4.4**).

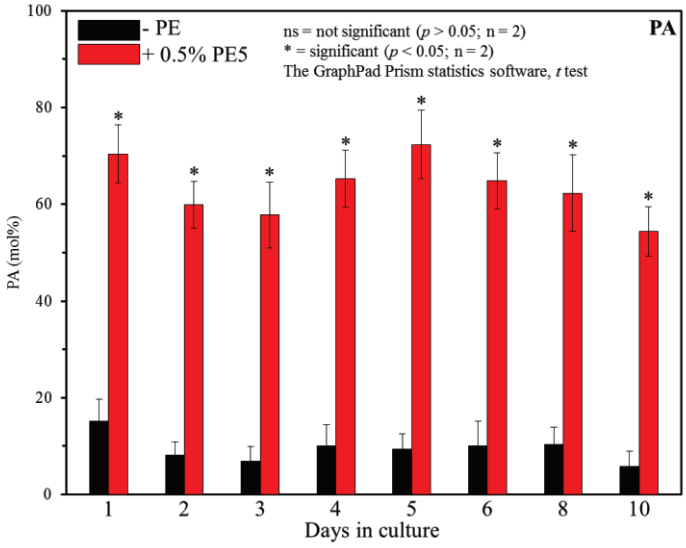

Figure 4.3. PE5 increases the concentration of phosphatidic acid (PA) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE5. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PA were calculated in mol%. Data are presented as means ± SEM (n = 2).

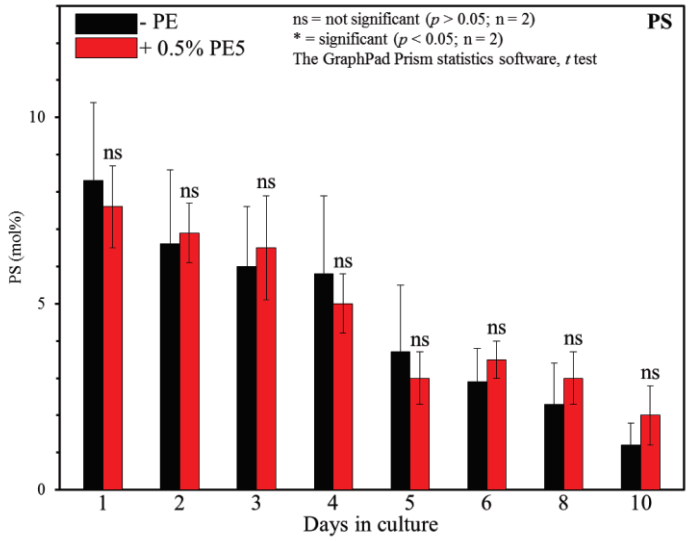

Figure 4.4. PE5 does not alter the concentration of phosphatidylserine (PS) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE5. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PS were calculated in mol%. Data are presented as means ± SEM (n = 2).

4.3.4 PE5 decreases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast

I found that PE5 causes a decrease of the concentration of PE in chronologically aging yeast (**Figure 4.5**).

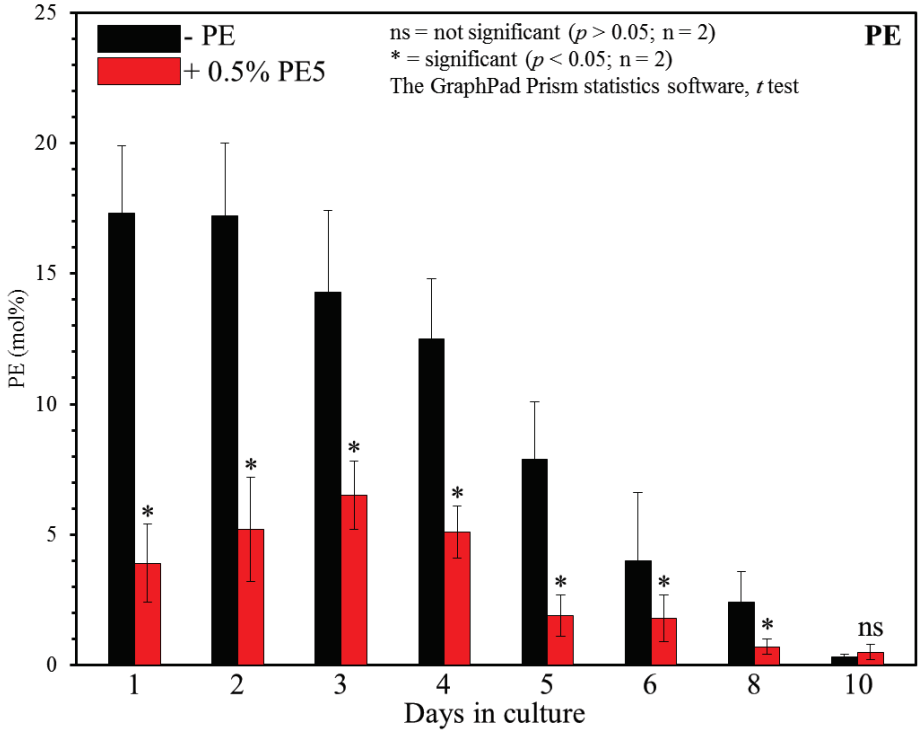

Figure 4.5. PE5 decreases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE5. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PE were calculated in mol%. Data are presented as means ± SEM (n = 2).

4.3.5 PE5 decreases the concentration of phosphatidylcholine (PC) in chronologically aging yeast

I found that PE5 reduces the concentration of PC in chronologically aging yeast (**Figure 4.6**).

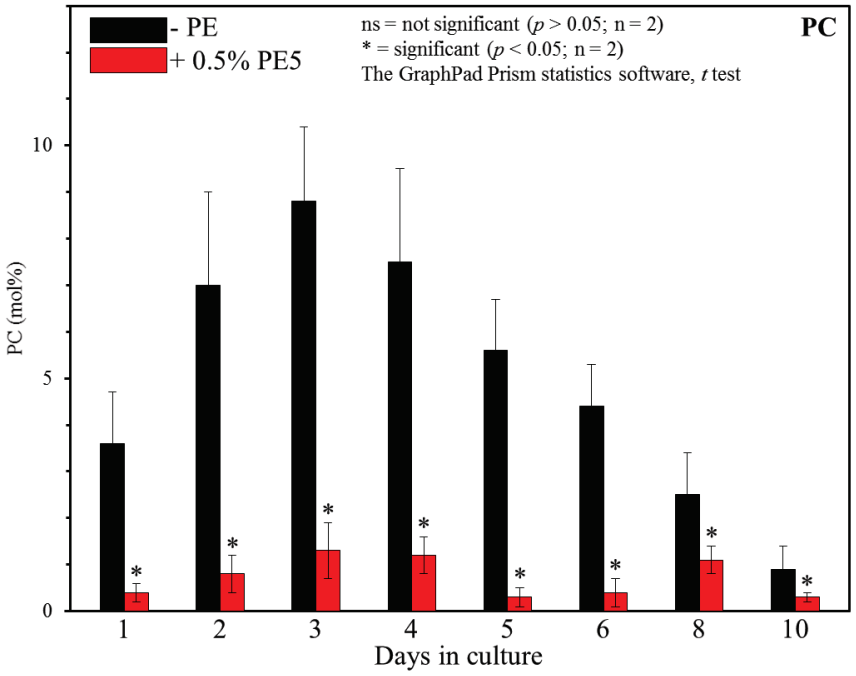

Figure 4.6. PE5 decreases the concentration of phosphatidylcholine (PC) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE5. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PC were calculated in mol%. Data are presented as means ± SEM (n = 2).

4.3.6 PE5 reduces the concentration of phosphatidylinositol (PI) in chronologically aging yeast

I found that PE5 decreases the concentration of PI in chronologically aging yeast (**Figure 4.7**).

4.3.7 PE5 increases the concentration of cardiolipin (CL) in chronologically aging yeast

I found that PE5 causes a rise in the concentration of CL (a signature lipid of the inner mitochondrial membrane) in chronologically aging yeast (**Figure 4.8**).

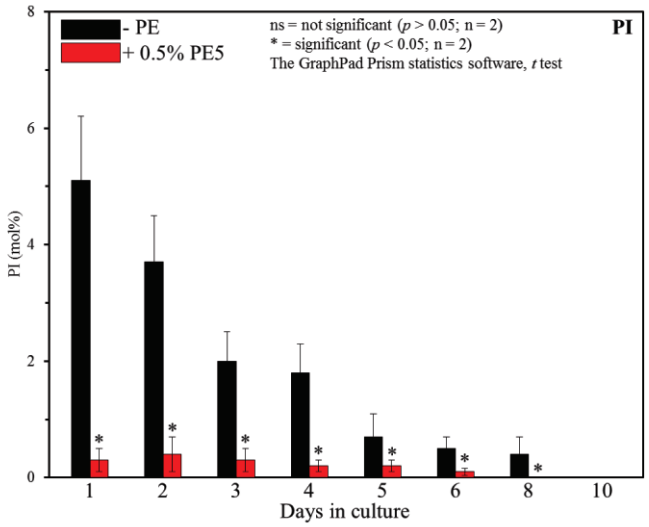

Figure 4.7. PE5 decreases the concentration of phosphatidylinositol (PI) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE5. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PI were calculated in mol%. Data are presented as means ± SEM (n = 2).

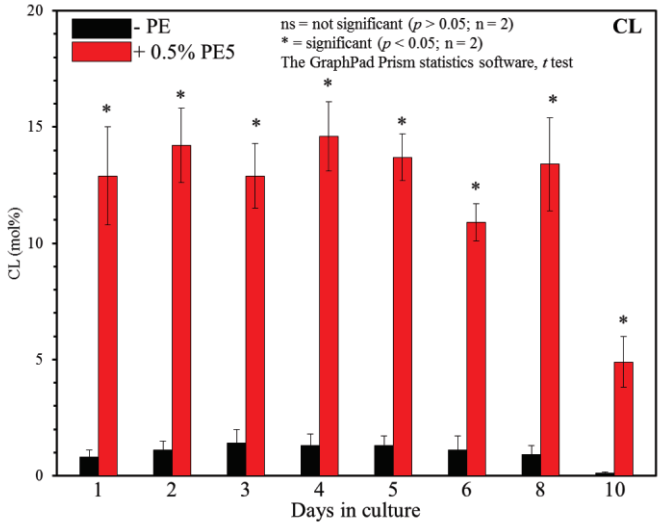

Figure 4.8. PE5 increases the concentration of cardiolipin (CL) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE5. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of CL were calculated in mol%. Data are presented as means ± SEM (n = 2).

4.4 Discussion

My data on how PE5 influences the concentrations of different lipid classes in cells of chronologically aging yeast suggest that this aging-delaying plant extract alters the relative rates of phosphatidic acid (PA) formation and of PA flow into the synthesis of other lipids as follows: 1) it promotes PA synthesis from FFA in the ER; 2) it decreases the rate of PA flow into the synthesis of TAG in the ER; 3) it decreases the rate of PA flow into the synthesis of PE in mitochondria; 4) it decreases the rate of PA flow into the synthesis of PC and PI in the ER; and 5) it increases the rate of PA flow into the synthesis of CL in mitochondria (**Figure 4.9**). Such re-wiring of PA synthesis and its conversion into TAG, PE, PC, PI and CL delays aging by a mechanism that remains to be established. I speculate that this mechanism may affect several longevity-defining processes in chronologically aging yeast, as described below.

First, the observed increase in the concentration of CL in mitochondria of yeast cells treated with PE5 may cause certain changes in the age-related chronology of mitochondrial functionality seen in these cells. [35] These changes involve alterations in mitochondrial respiration, mitochondrial membrane potential, mitochondrially produced ROS and stability of mitochondrial DNA. [35] To validate this hypothesis, it needs to be assessed how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentration of CL in the mitochondrial membrane impact mitochondrial functionality and the ability of PE5 to delay aging.

Second, the observed decrease in the concentrations of three glycerol phospholipids, PE, PC and PI, in yeast cells treated with PE5 may cause the increased resistance of these cells to chronic oxidative and thermal stresses. [35] To test the validity

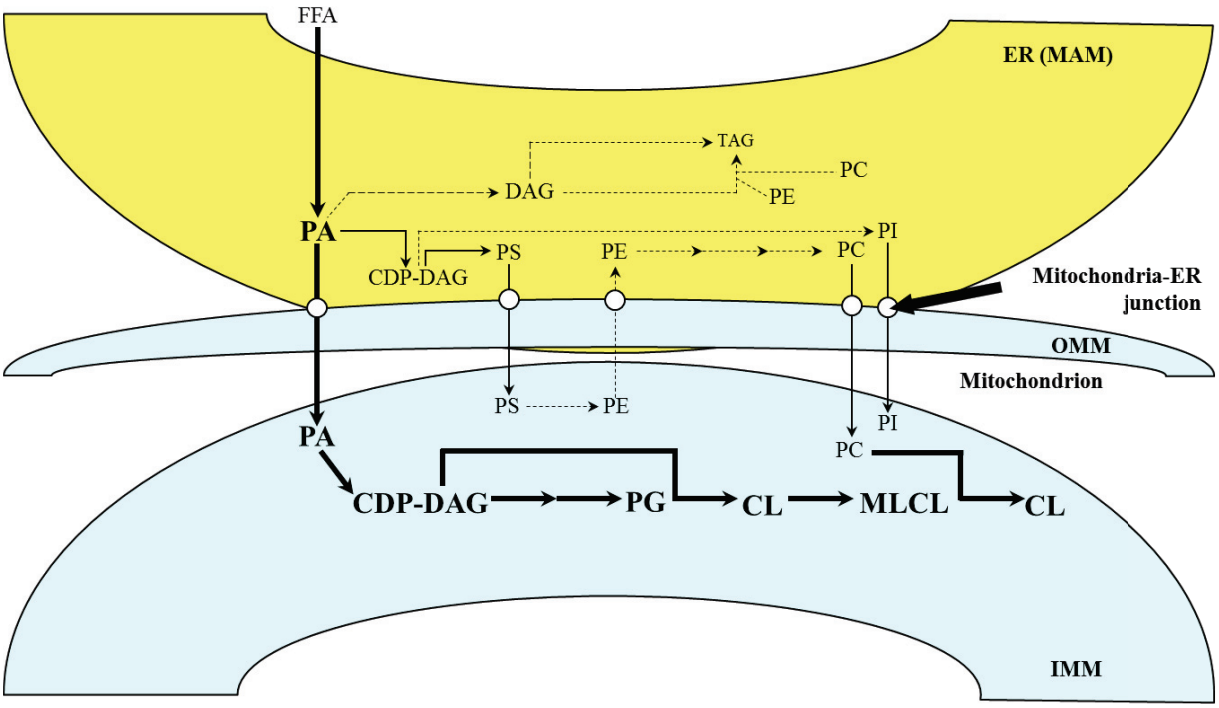

Figure 4.9. A hypothetical model of how PE5 alters the relative rates of PA synthesis and of PA flow into the synthesis of other lipid classes. PE5: 1) promotes PA synthesis from FFA in the ER; 2) decreases the rate of PA flow into the synthesis of TAG in the ER; 3) decreases the rate of PA flow into the synthesis of PE in mitochondria; 4) decreases the rate of PA flow into the synthesis of PC and PI in the ER; and 5) increases the rate of PA flow into the synthesis of CL in mitochondria

of this hypothesis, it is necessary to examine how genetic manipulations (such as single-gene-deletions and elevated gene expression) that decrease or increase the concentration of PE, PC and PI affect stress resistance and the efficiency with which PE5 delays aging.

Third, one of the outcomes of the observed re-wiring of lipid metabolism and transport in yeast cells treated with PE5 may be a decrease in the cellular concentrations of FFA, a class of lipids whose incorporation into PA initiates all three major routes of lipid metabolism in the ER, mitochondria and LDs (**Figure 4.9**). FFA are known to trigger an age-related form of programmed cell death called liponecrosis. [36, 37] To validate this hypothesis, the concentrations of FFA in yeast cells treated with PE5 need to

be compared to those in cells that remained untreated. Furthermore, it needs to be examined how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentrations of FFA in yeast cells impact the efficiency of liponecrosis and the ability of PE5 to delay aging.

Future studies also need to use quantitative mass spectrometry to monitor an age-related chronology of changes in various lipid classes extracted from three different types of purified organelles (such as the ER, mitochondria and LDs). These studies will allow a critical validation of my hypothesis on how PE5 alters the relative rates of PA formation and of PA flow into the synthesis of other lipids in these organelles (**Figure 4.9**).

5 An aging-delaying plant extract PE6 remodels cellular lipidome of chronologically aging yeast

5.1 Abstract

Among the aging-delaying plant extract discovered by the Titorenko laboratory is PE6. Recent unpublished studies in the Titorenko laboratory suggest that some aspects of lipid metabolism and interorganellar transport may play essential roles in the delay of aging by PE6. In studies described in this chapter of my Thesis, I have used mass-spectrometric quantitative analysis to compare the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE6 or remained untreated. My findings indicate that PE6 alters the relative rates of phosphatidic acid (PA) formation and of PA flow into the synthesis of other lipids as follows: 1) it promotes PA synthesis from free fatty acids (FFA) in the endoplasmic reticulum (ER) late in life of chronologically aging yeast; 2) it decreases the rate of PA flow into the synthesis of triacylglycerols (TAG) in the ER late in life of chronologically aging yeast; 3) it increases the rates of PA flow into the synthesis of phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylinositol (PI) in the ER; 4) it decreases the rates of PA flow into the synthesis of phosphatidylethanolamine (PE) in mitochondria; and 5) it increases the rate of PA flow into the synthesis of cardiolipin (CL) in mitochondria. Such re-wiring of PA synthesis and its conversion into TAG, PS, PC, PI, PE and CL delays aging by a mechanism that remains to be established.

5.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) from Thermo Scientific/Open Biosystems was grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1. The stock solution of aging-delaying plant extract 6 (PE6) in ethanol was made on the day of adding this PE to cell cultures. This stock solution was added to growth medium immediately following cell inoculation into the medium. The final concentration of PE6 in growth medium was 1.0%.

Lipid extraction, identification and quantitation using mass spectrometry

These experimental procedures have been described in chapter 2 of my thesis.

Statistical analysis

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean ± SEM. The *p* values were calculated with the help of GraphPad *t* test calculator using an unpaired two-tailed *t* test.

5.3 Results

Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae* involve numerous steps, each catalyzed by a known enzyme or several enzymes (**Figure 5.1**).

5.3.1 PE6 decreases the concentration of triacylglycerol (TAG) late in life of chronologically aging yeast

Using mass-spectrometric quantitative analysis, I compared the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE6 or remained untreated. I found that PE6 decreases the concentration of the neutral lipid TAG late in life of chronologically aging yeast (**Figure 5.2**).

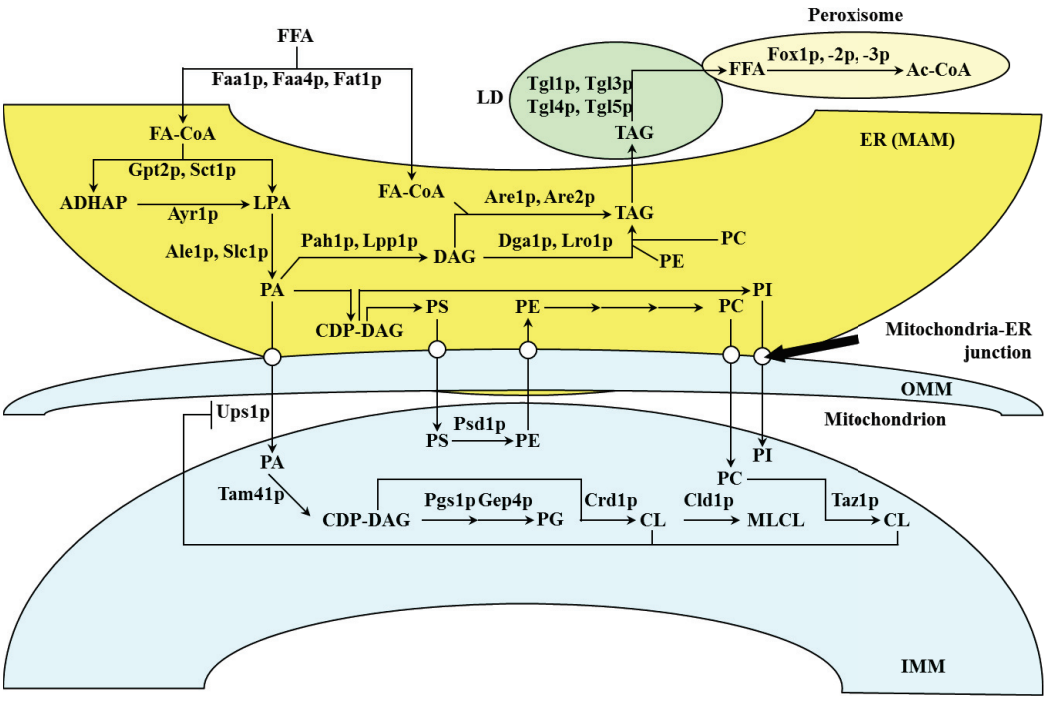

Figure 5.1. Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae*. [25, 26, 27]

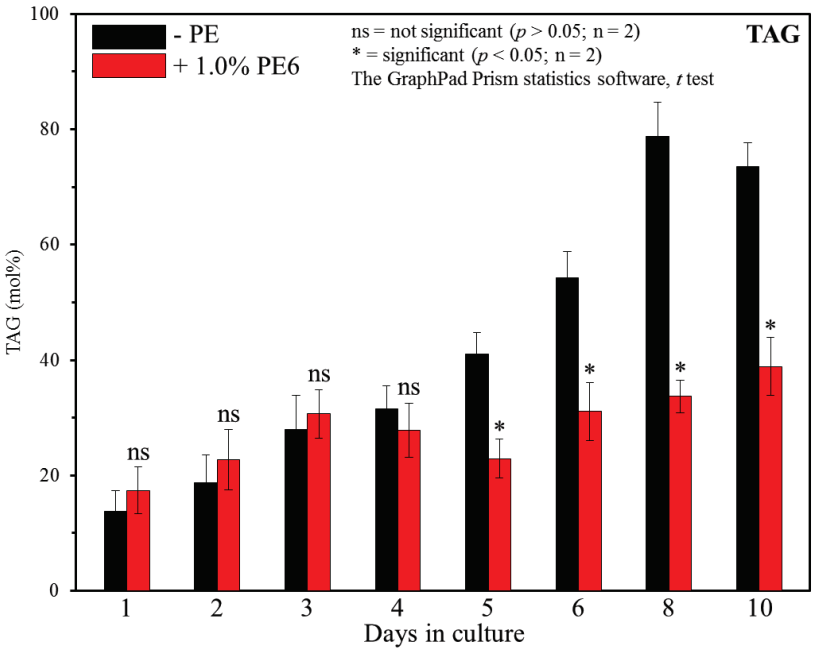

Figure 5.2. PE6 decreases the concentration of the neutral lipid triacylglycerol (TAG) late in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE6. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of TAG were calculated in mol%. Data are presented as means ± SEM (n = 2).

5.3.2 PE6 causes an increase in the concentration of phosphatidic acid (PA) late in life of chronologically aging yeast

I found that PE6 increases the concentration of PA late in life of chronologically aging yeast (**Figure 5.3**).

5.3.3 PE6 increases the concentration of phosphatidylserine (PS) in chronologically aging yeast

I found that PE6 causes a rise in the concentration of PS in chronologically aging yeast (**Figure 5.4**).

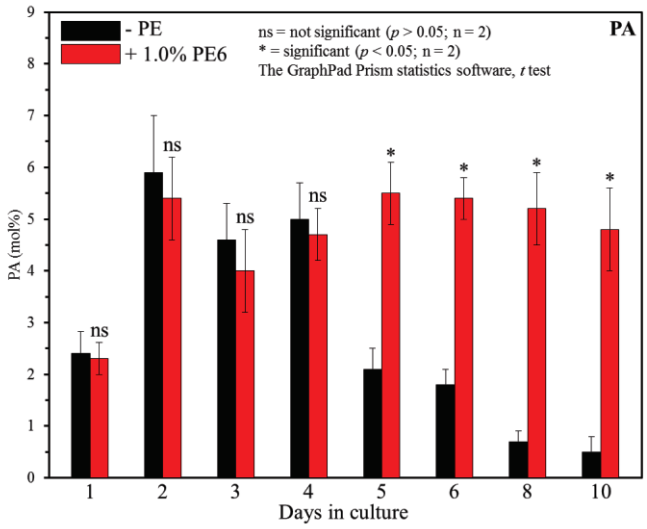

Figure 5.3. PE6 increases the concentration of phosphatidic acid (PA) late in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE6. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PA were calculated in mol%. Data are presented as means ± SEM (n = 2).

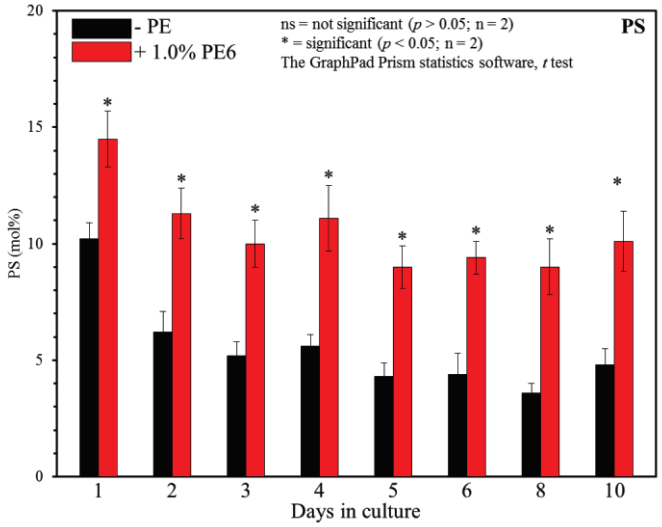

Figure 5.4. PE6 increases the concentration of phosphatidylserine (PS) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE6. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PS were calculated in mol%. Data are presented as means ± SEM (n = 2).

5.3.4 PE6 decreases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast

I found that PE6 causes a decrease of the concentration of PE in chronologically aging yeast (**Figure 5.5**).

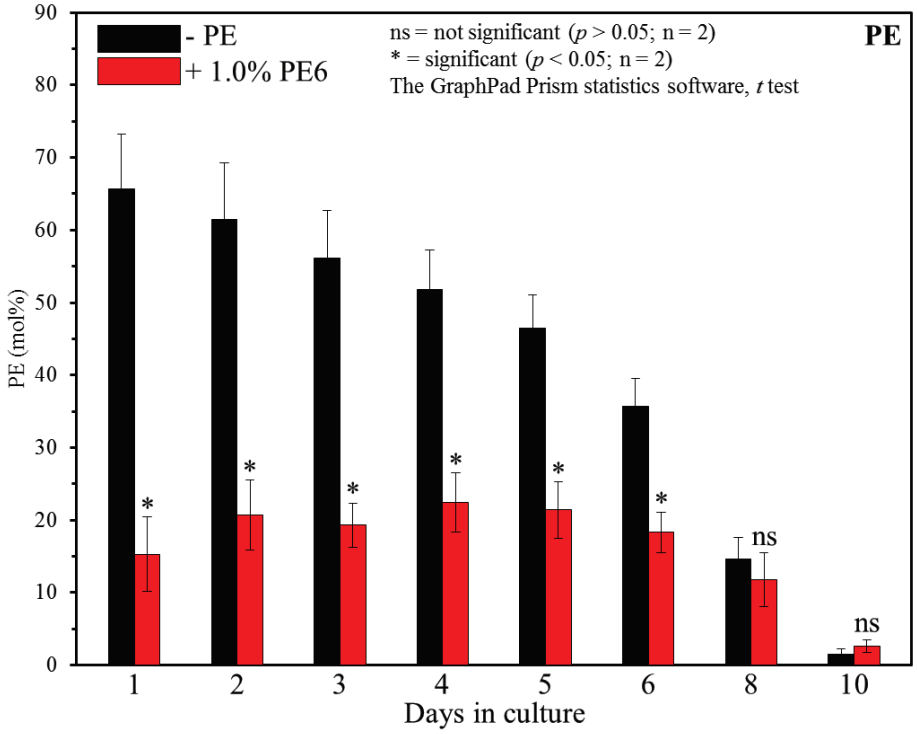

Figure 5.5. PE6 decreases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE6. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PE were calculated in mol%. Data are presented as means ± SEM (n = 2).

5.3.5 PE6 increases the concentration of phosphatidylcholine (PC) in chronologically aging yeast

I found that PE6 causes a rise in the concentration of PC in chronologically aging yeast (**Figure 5.6**).

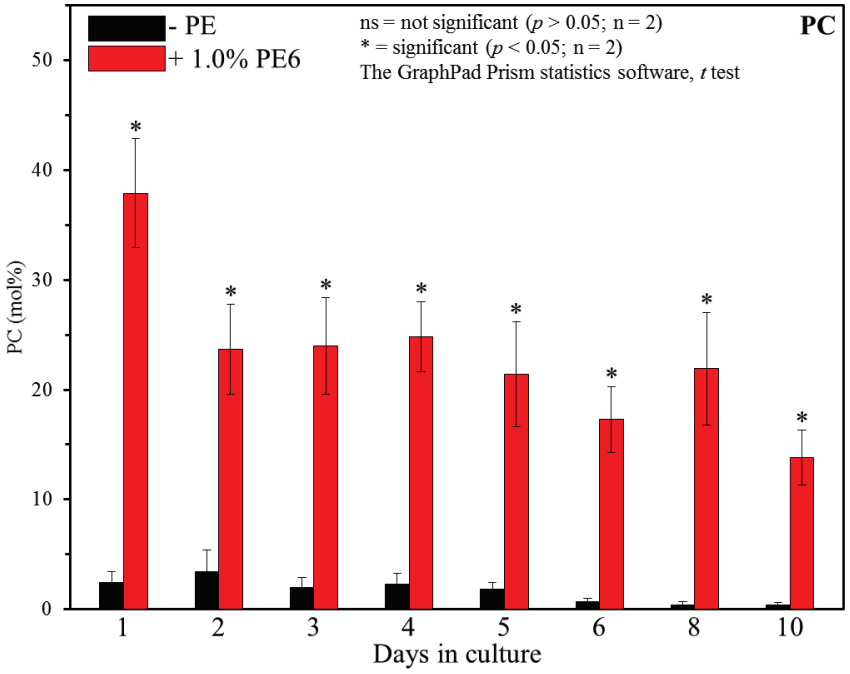

Figure 5.6. PE6 increases the concentration of phosphatidylcholine (PC) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE6. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PC were calculated in mol%. Data are presented as means ± SEM (n = 2).

5.3.6 PE6 elevates the concentration of phosphatidylinositol (PI) in chronologically aging yeast

I found that PE6 increases the concentration of PI in chronologically aging yeast (**Figure 5.7**).

5.3.7 PE6 increases the concentration of cardiolipin (CL) in chronologically aging yeast

I found that PE6 causes a rise in the concentration of CL (a signature lipid of the inner mitochondrial membrane) in chronologically aging yeast (**Figure 5.8**).

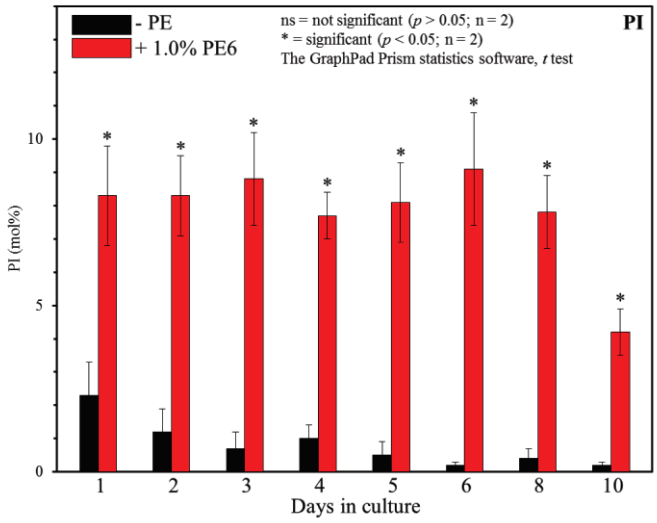

Figure 5.7. PE6 increases the concentration of phosphatidylinositol (PI) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE6. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PI were calculated in mol%. Data are presented as means ± SEM (n = 2).

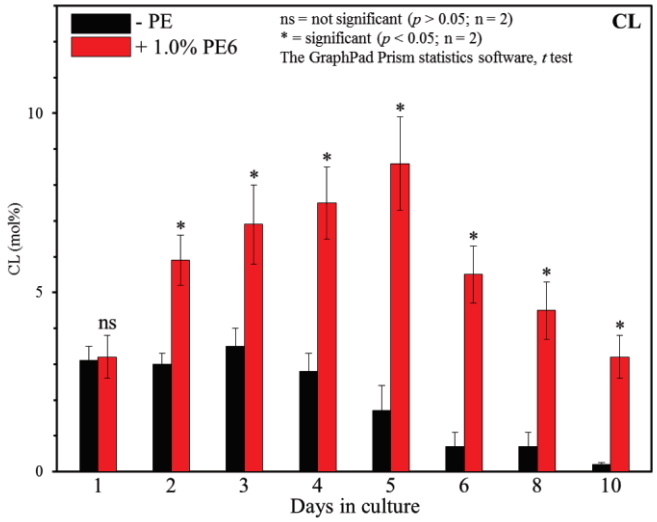

Figure 5.8. PE6 increases the concentration of cardiolipin (CL) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE6. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of CL were calculated in mol%. Data are presented as means ± SEM (n = 2).

5.4 Discussion

My data on how PE6 influences the concentrations of different lipid classes in cells of chronologically aging yeast suggest that this aging-delaying plant extract alters the relative rates of phosphatidic acid (PA) formation and of PA flow into the synthesis of other lipids as follows: 1) it promotes PA synthesis from FFA in the ER late in life of chronologically aging yeast; 2) it decreases the rate of PA flow into the synthesis of TAG in the ER late in life of chronologically aging yeast; 3) it decreases the rate of PA flow into the synthesis of PE in mitochondria; 4) it increases the rate of PA flow into the synthesis of PS, PC and PI in the ER; and 5) it increases the rate of PA flow into the synthesis of CL in mitochondria (**Figure 5.9**). Such re-wiring of PA synthesis and its conversion into TAG, PS, PE, PC, PI and CL delays aging by a mechanism that remains to be established. I speculate that this mechanism may affect several longevity-defining processes in chronologically aging yeast, as described below.

First, the observed increase in the concentration of CL in mitochondria of yeast cells treated with PE6 may cause certain changes in the age-related chronology of mitochondrial functionality seen in these cells. [35] These changes involve alterations in mitochondrial respiration, mitochondrial membrane potential, mitochondrially produced ROS and stability of mitochondrial DNA. [35] To validate this hypothesis, it needs to be assessed how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentration of CL in the mitochondrial membrane impact mitochondrial functionality and the ability of PE6 to delay aging.

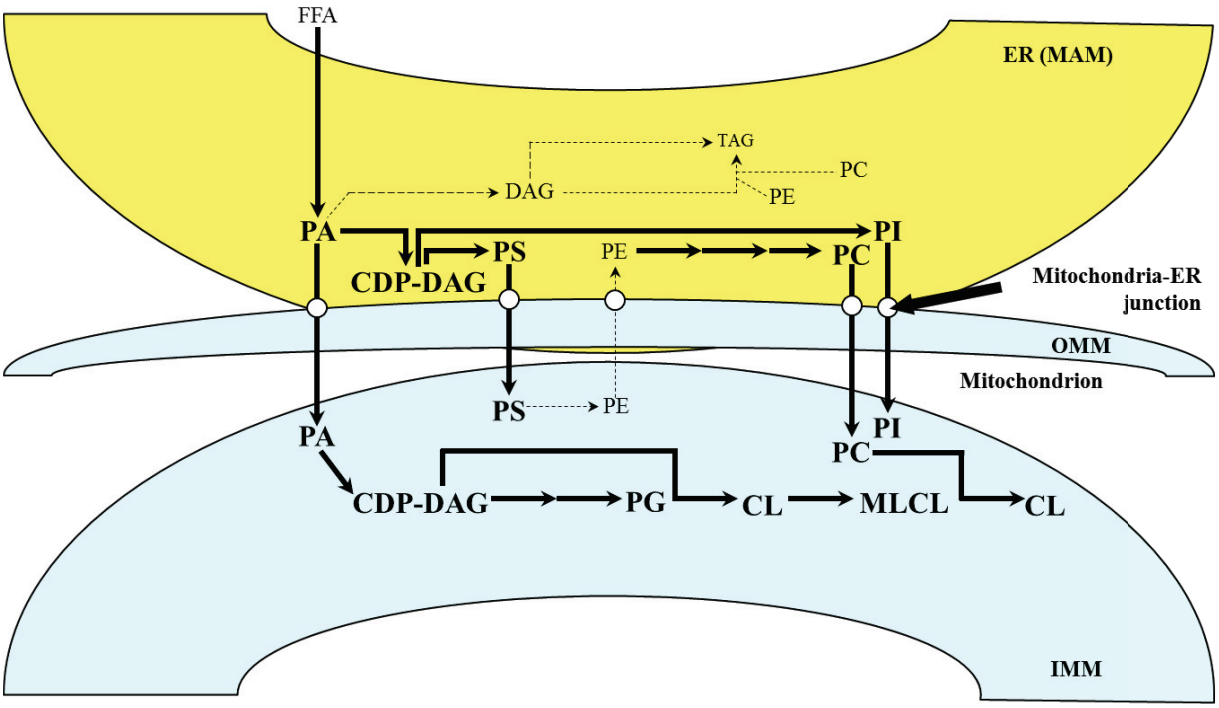

Figure 5.9. A hypothetical model of how PE6 alters the relative rates of PA synthesis and of PA flow into the synthesis of other lipid classes. PE6: 1) promotes PA synthesis from FFA in the ER late in life of chronologically aging yeast; 2) decreases the rate of PA flow into the synthesis of TAG in the ER late in life of chronologically aging yeast; 3) decreases the rate of PA flow into the synthesis of PE in mitochondria; 4) increases the rate of PA flow into the synthesis of PS, PC and PI in the ER; and 5) increases the rate of PA flow into the synthesis of CL in mitochondria

Second, the observed increase in the concentrations of three glycerol phospholipids, PS, PC and PI, in yeast cells treated with PE6 may cause the increased resistance of these cells to chronic oxidative and thermal stresses. [35] To test the validity of this hypothesis, it is necessary to examine how genetic manipulations (such as single-gene-deletions and elevated gene expression) that decrease or increase the concentration of PS, PC and PI affect stress resistance and the efficiency with which PE6 delays aging.

Third, one of the outcomes of the observed re-wiring of lipid metabolism and transport in yeast cells treated with PE6 may be a late-life decrease in the cellular concentrations of FFA, a class of lipids whose incorporation into PA initiates all three

major routes of lipid metabolism in the ER, mitochondria and LDs (**Figure 5.9**). FFA are known to trigger an age-related form of programmed cell death called liponecrosis. [36, 37] To validate this hypothesis, the concentrations of FFA in yeast cells treated with PE6 need to be compared to those in cells that remained untreated. Furthermore, it needs to be examined how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentrations of FFA in yeast cells impact the efficiency of liponecrosis and the ability of PE6 to delay aging.

Future studies also need to use quantitative mass spectrometry to monitor an age-related chronology of changes in various lipid classes extracted from three different types of purified organelles (such as the ER, mitochondria and LDs). These studies will allow a critical validation of my hypothesis on how PE6 alters the relative rates of PA formation and of PA flow into the synthesis of other lipids in these organelles (**Figure 5.9**).

6 An aging-delaying plant extract PE8 remodels cellular lipidome of chronologically aging yeast

6.1 Abstract

Among the aging-delaying plant extract discovered by the Titorenko laboratory is PE8. Recent unpublished studies in the Titorenko laboratory suggest that some aspects of lipid metabolism and interorganellar transport may play essential roles in the delay of aging by PE8. In studies described in this chapter of my Thesis, I have used mass-spectrometric quantitative analysis to compare the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE8 or remained untreated. My findings indicate that PE8 alters the relative rates of PA flow into the synthesis of other lipids as follows: 1) it decreases the rate of PA flow into the synthesis of triacylglycerols (TAG) in the ER; 2) it increases the rate of PA flow into the synthesis of phosphatidylethanolamine (PE) in mitochondria; 3) it decreases the rates of PA flow into the synthesis of phosphatidylserine (PS) and phosphatidylcholine (PC) in the ER early in life of chronologically aging yeast; 4) it increases the rate of PA flow into the synthesis of phosphatidylinositol (PI) in the ER; and 5) it decreases the rate of PA flow into the synthesis of cardiolipin (CL) in mitochondria early in life of chronologically aging yeast. Such re-wiring of PA conversion into TAG, PS, PC, PI, PE and CL delays aging by a mechanism that remains to be established.

6.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) from Thermo Scientific/Open Biosystems was grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1. The stock solution of aging-delaying plant extract 8 (PE8) in ethanol was made on the day of adding this PE to cell cultures. This stock solution was added to growth medium immediately following cell inoculation into the medium. The final concentration of PE8 in growth medium was 0.3%.

Lipid extraction, identification and quantitation using mass spectrometry

These experimental procedures have been described in chapter 2 of my thesis.

Statistical analysis

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean ± SEM. The *p* values were calculated with the help of GraphPad *t* test calculator using an unpaired two-tailed *t* test.

6.3 Results

Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae* involve numerous steps, each catalyzed by a known enzyme or several enzymes (**Figure 6.1**).

6.3.1 PE8 decreases the concentration of triacylglycerol (TAG) in chronologically aging yeast

Using mass-spectrometric quantitative analysis, I compared the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE8 or remained untreated. I found that PE8 decreases the concentration of the neutral lipid TAG in chronologically aging yeast (**Figure 6.2**).

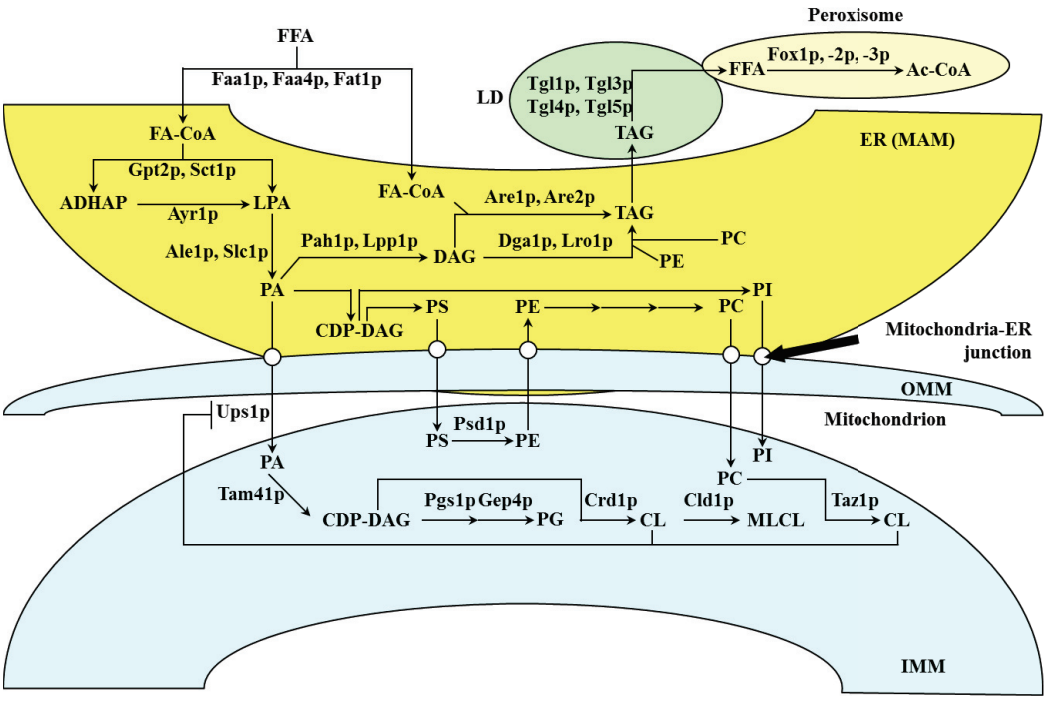

Figure 6.1. Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae*. [25, 26, 27]

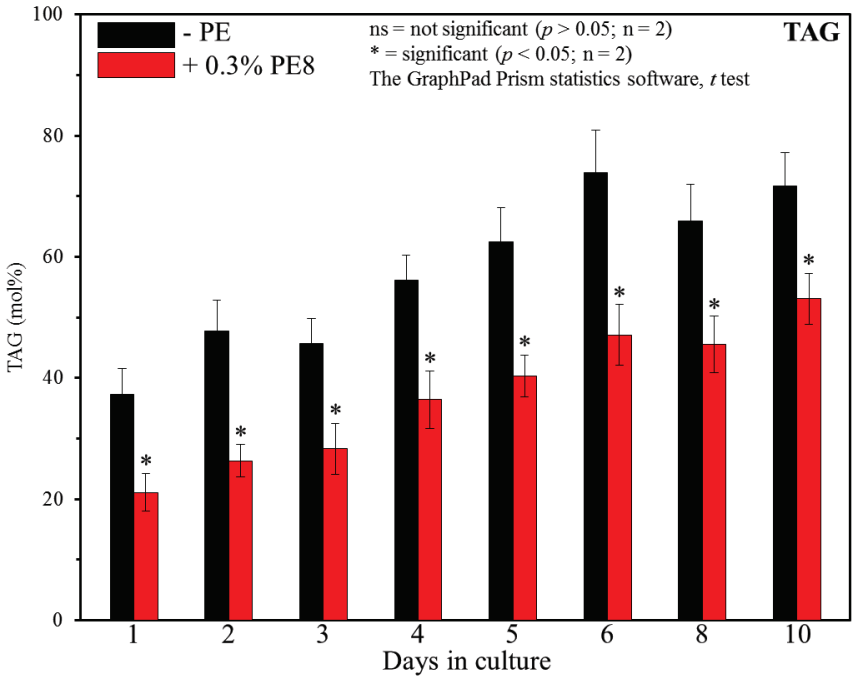

Figure 6.2. PE8 decreases the concentration of the neutral lipid triacylglycerol (TAG) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE8. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of TAG were calculated in mol%. Data are presented as means ± SEM (n = 2).

6.3.2 PE8 causes a decrease in the concentration of phosphatidic acid (PA) in chronologically aging yeast

I found that PE8 decreases the concentration of PA in chronologically aging yeast (**Figure 6.3**).

6.3.3 PE8 decreases the concentration of phosphatidylserine (PS) early in life of chronologically aging yeast

I found that PE8 causes a decrease in the concentration of PS early in life of chronologically aging yeast (**Figure 6.4**).

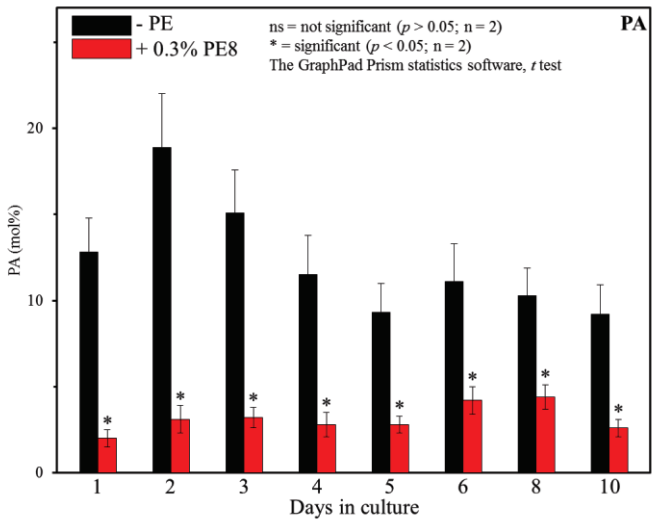

Figure 6.3. PE8 decreases the concentration of phosphatidic acid (PA) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE8. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PA were calculated in mol%. Data are presented as means ± SEM (n = 2).

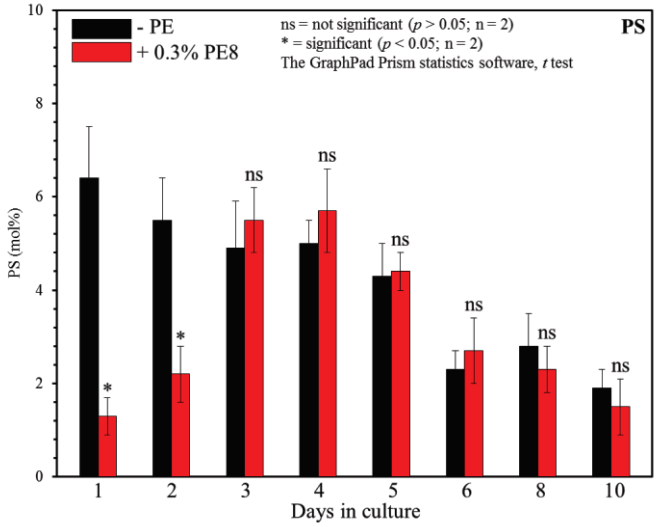

Figure 6.4. PE8 decreases the concentration of phosphatidylserine (PS) early in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE8. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PS were calculated in mol%. Data are presented as means ± SEM (n = 2).

6.3.4 PE8 increases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast

I found that PE8 causes a rise of the concentration of PE in chronologically aging yeast (**Figure 6.5**).

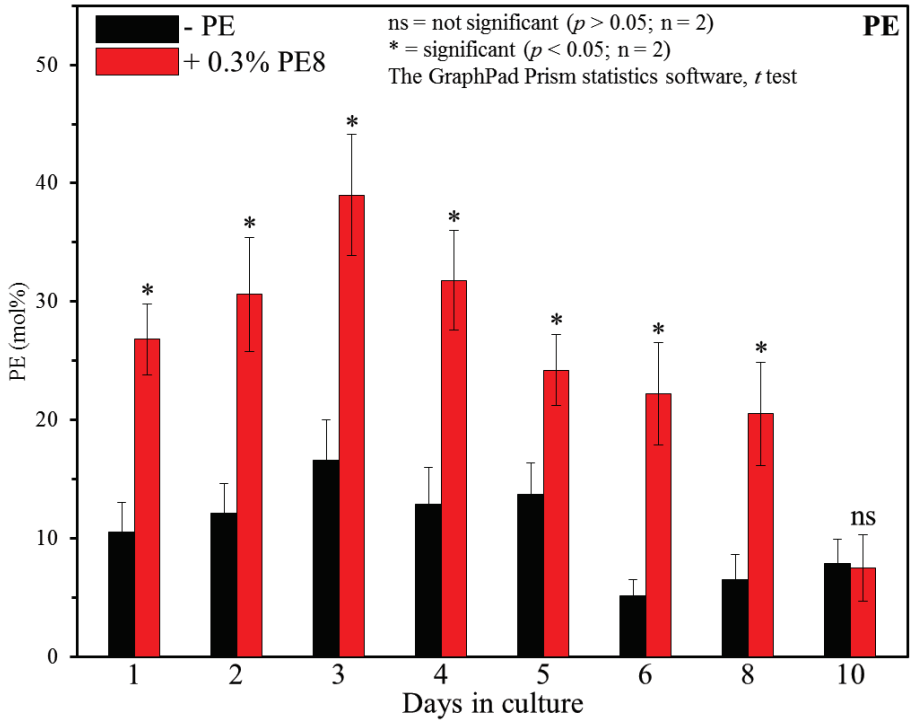

Figure 6.5. PE8 increases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE8. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PE were calculated in mol%. Data are presented as means ± SEM (n = 2).

6.3.5 PE8 decreases the concentration of phosphatidylcholine (PC) early in life and increases it late in life of chronologically aging yeast

I found that PE8 causes a decrease in the concentration of PC early in life and increases it late in life of chronologically aging yeast (**Figure 6.6**).

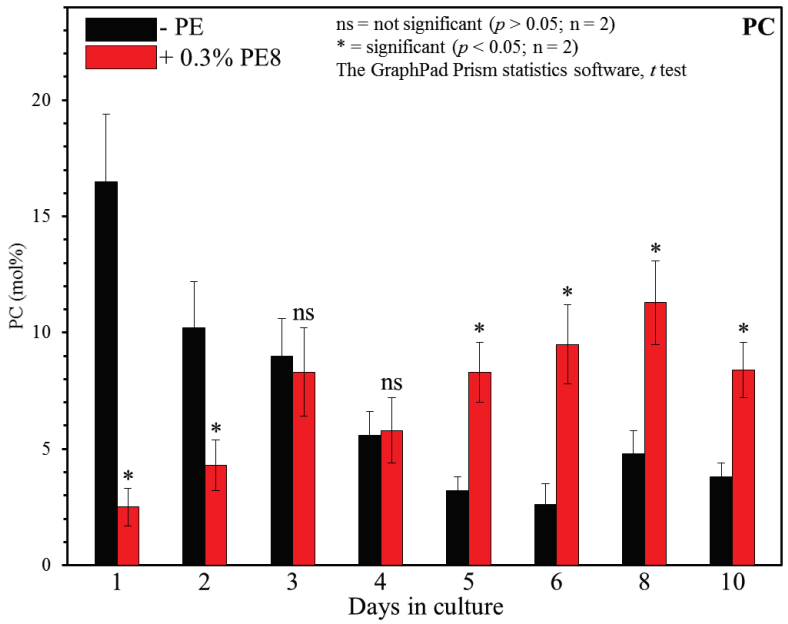

Figure 6.6. PE8 decreases the concentration of phosphatidylcholine (PC) early in life and increases it late in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE8. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PC were calculated in mol%. Data are presented as means ± SEM (n = 2).

6.3.6 PE8 elevates the concentration of phosphatidylinositol (PI) in chronologically aging yeast

I found that PE8 increases the concentration of PI in chronologically aging yeast (**Figure 6.7**).

6.3.7 PE8 decreases the concentration of cardiolipin (CL) early in life of chronologically aging yeast

I found that PE8 causes a decrease in the concentration of CL early in life of chronologically aging yeast (**Figure 6.8**).

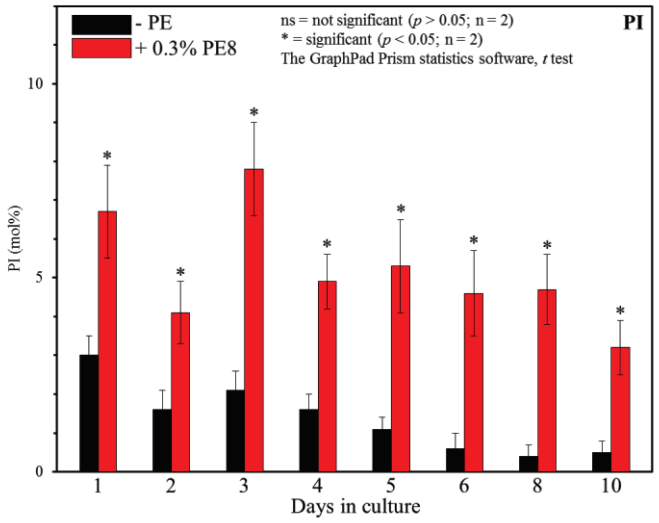

Figure 6.7. PE8 increases the concentration of phosphatidylinositol (PI) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE8. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PI were calculated in mol%. Data are presented as means ± SEM (n = 2).

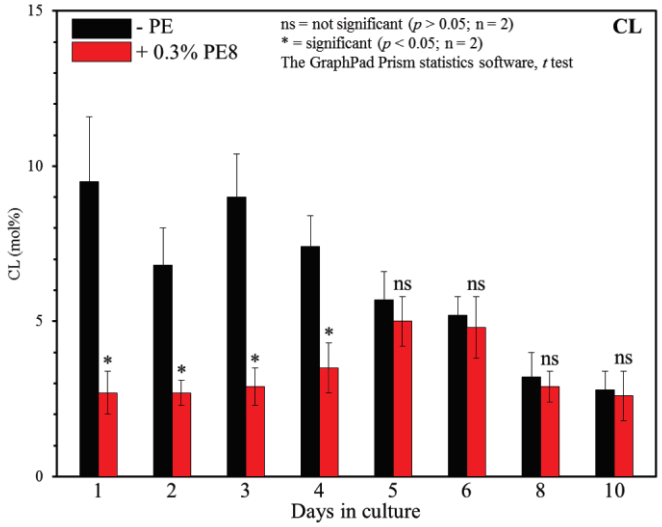

Figure 6.8. PE8 decreases the concentration of cardiolipin (CL) early in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE8. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of CL were calculated in mol%. Data are presented as means ± SEM (n = 2).

6.4 Discussion

My data on how PE8 influences the concentrations of different lipid classes in cells of chronologically aging yeast suggest that this aging-delaying plant extract alters the relative rates of phosphatidic acid (PA) flow into the synthesis of other lipids as follows: 1) it decreases the rate of PA flow into the synthesis of TAG in the ER; 2) it increases the rate of PA flow into the synthesis of PE in mitochondria; 3) it decreases the rates of PA flow into the synthesis of PS and PC in the ER early in life of chronologically aging yeast; 4) it increases the rate of PA flow into the synthesis PI in the ER; and 5) it decreases the rate of PA flow into the synthesis of CL in mitochondria early in life of chronologically aging yeast (**Figure 6.9**). Such re-wiring of PA conversion into TAG, PS, PE, PC, PI and CL delays aging by a mechanism that remains to be established. I speculate that this mechanism may affect several longevity-defining processes in chronologically aging yeast, as described below.

First, the observed decrease in the concentration of CL in mitochondria of yeast cells treated with PE8 may cause certain changes in the age-related chronology of mitochondrial functionality seen in these cells. [35] These changes involve alterations in mitochondrial respiration, mitochondrial membrane potential, mitochondrially produced ROS and stability of mitochondrial DNA. [35] To validate this hypothesis, it needs to be assessed how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentration of CL in the mitochondrial membrane impact mitochondrial functionality and the ability of PE8 to delay aging.

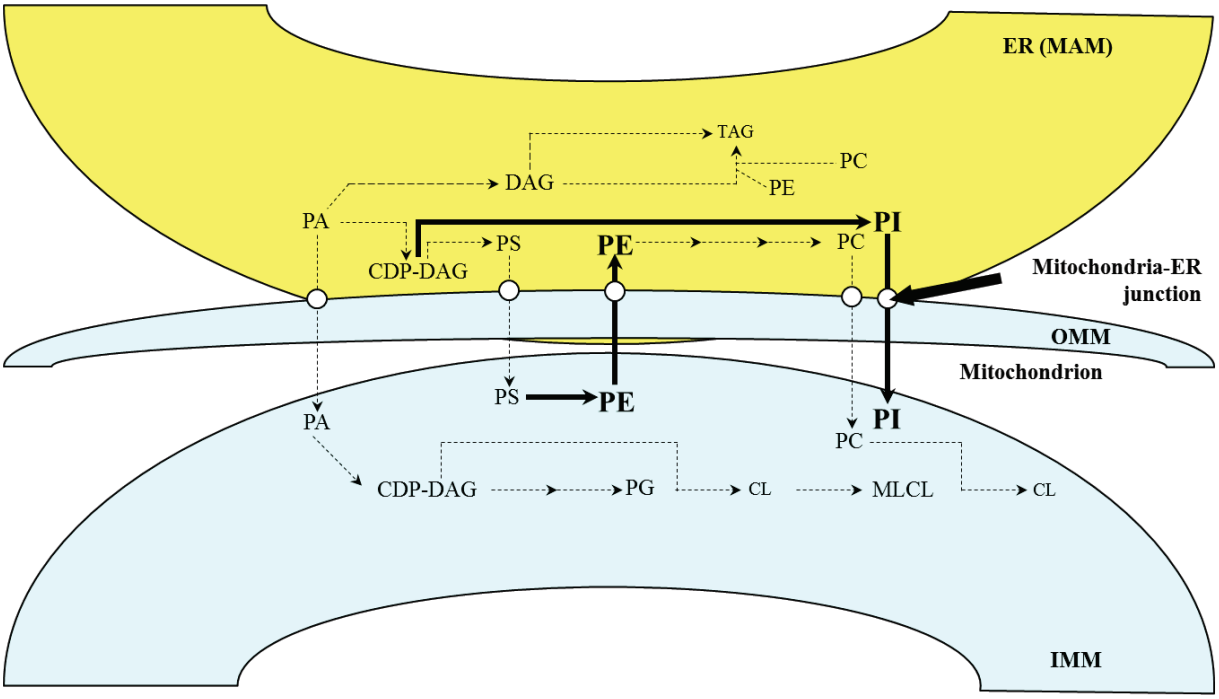

Figure 6.9. A hypothetical model of how PE8 alters the relative rates of PA synthesis and of PA flow into the synthesis of other lipid classes. PE8: 1) decreases the rate of PA flow into the synthesis of TAG in the ER; 2) increases the rate of PA flow into the synthesis of PE in mitochondria; 3) decreases the rates of PA flow into the synthesis of PS and PC in the ER early in life of chronologically aging yeast; 4) increases the rate of PA flow into the synthesis PI in the ER; and 5) decreases the rate of PA flow into the synthesis of CL in mitochondria early in life of chronologically aging yeast.

Second, the observed changes in the concentrations of four glycerol phospholipids, PS, PE, PC and PI, in yeast cells treated with PE8 may cause the increased resistance of these cells to chronic oxidative and thermal stresses. [35] To test the validity of this hypothesis, it is necessary to examine how genetic manipulations (such as single-gene-deletions and elevated gene expression) that decrease or increase the concentration of PS, PE, PC and PI affect stress resistance and the efficiency with which PE8 delays aging.

Third, one of the outcomes of the observed re-wiring of lipid metabolism and transport in yeast cells treated with PE8 may be a late-life decrease in the cellular

concentrations of FFA, a class of lipids whose incorporation into PA initiates all three major routes of lipid metabolism in the ER, mitochondria and LDs (**Figure 6.9**). FFA are known to trigger an age-related form of programmed cell death called liponecrosis. [36, 37] To validate this hypothesis, the concentrations of FFA in yeast cells treated with PE8 need to be compared to those in cells that remained untreated. Furthermore, it needs to be examined how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentrations of FFA in yeast cells impact the efficiency of liponecrosis and the ability of PE8 to delay aging.

Future studies also need to use quantitative mass spectrometry to monitor an age-related chronology of changes in various lipid classes extracted from three different types of purified organelles (such as the ER, mitochondria and LDs). These studies will allow a critical validation of my hypothesis on how PE8 alters the relative rates of PA formation and of PA flow into the synthesis of other lipids in these organelles (**Figure 6.9**).

7 An aging-delaying plant extract PE12 remodels cellular lipidome of chronologically aging yeast

7.1 Abstract

Among the aging-delaying plant extract discovered by the Titorenko laboratory is PE12. Recent unpublished studies in the Titorenko laboratory suggest that some aspects of lipid metabolism and interorganellar transport may play essential roles in the delay of aging by PE12. In studies described in this chapter of my Thesis, I have used mass-spectrometric quantitative analysis to compare the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE12 or remained untreated. My findings indicate that PE12 alters the relative rates of PA flow into the synthesis of other lipids as follows: 1) it decreases the rate of PA flow into the synthesis of triacylglycerols (TAG) in the ER; 2) it increases the rate of PA flow into the synthesis of phosphatidylethanolamine (PE) in mitochondria; 3) it increases the rate of PA flow into the synthesis of phosphatidylserine (PS) in the ER early in life of chronologically aging yeast; 4) it increases the rate of PA flow into the synthesis of phosphatidylcholine (PC) and phosphatidylinositol (PI) in the ER late in life of chronologically aging yeast; and 5) it decreases the rate of PA flow into the synthesis of cardiolipin (CL) in mitochondria early in life of chronologically aging yeast. Such re-wiring of PA conversion into TAG, PS, PC, PI, PE and CL delays aging by a mechanism that remains to be established.

7.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) from Thermo Scientific/Open Biosystems was grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1. The stock solution of aging-delaying plant extract 12 (PE12) in ethanol was made on the day of adding this PE to cell cultures. This stock solution was added to growth medium immediately following cell inoculation into the medium. The final concentration of PE12 in growth medium was 0.1%.

Lipid extraction, identification and quantitation using mass spectrometry

These experimental procedures have been described in chapter 2 of my thesis.

Statistical analysis

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean ± SEM. The *p* values were calculated with the help of GraphPad *t* test calculator using an unpaired two-tailed *t* test.

7.3 Results

Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae* involve numerous steps, each catalyzed by a known enzyme or several enzymes (**Figure 7.1**).

7.3.1 PE12 decreases the concentration of triacylglycerol (TAG) in chronologically aging yeast

Using mass-spectrometric quantitative analysis, I compared the concentrations of different classes of lipids in cells of chronologically aging yeast exposed to PE12 or remained untreated. I found that PE12 decreases the concentration of the neutral lipid TAG in chronologically aging yeast (**Figure 7.2**).

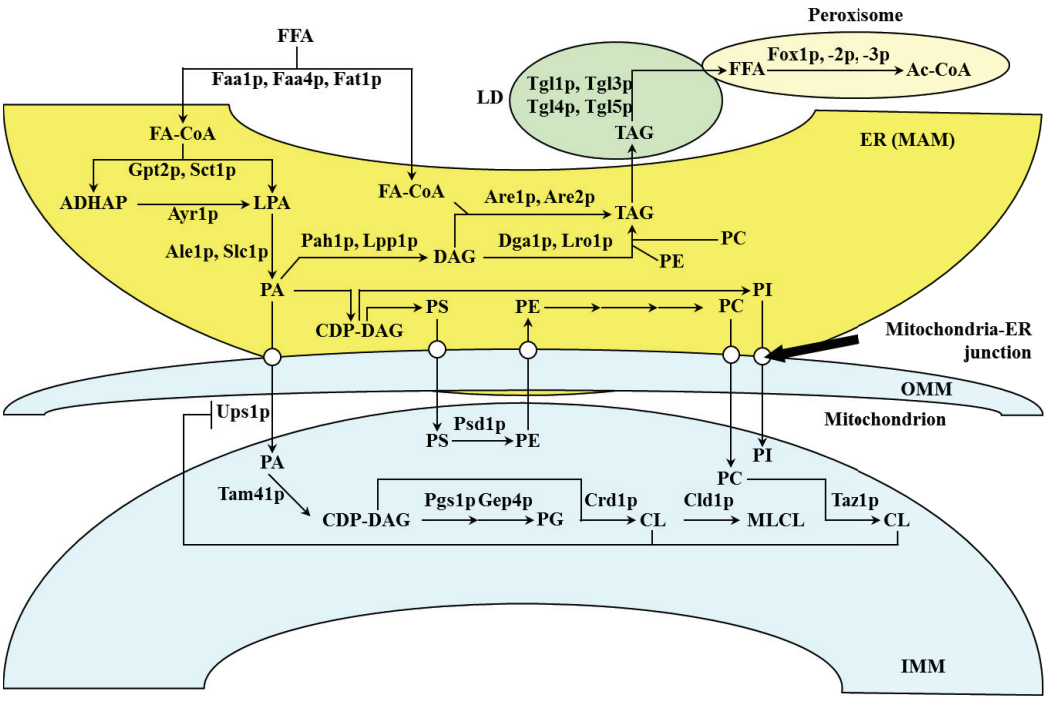

Figure 7.1. Lipid metabolism and interorganellar transport in mitochondria, the endoplasmic reticulum (ER), lipid droplets (LD) and peroxisomes of the yeast *S. cerevisiae*. [25, 26, 27]

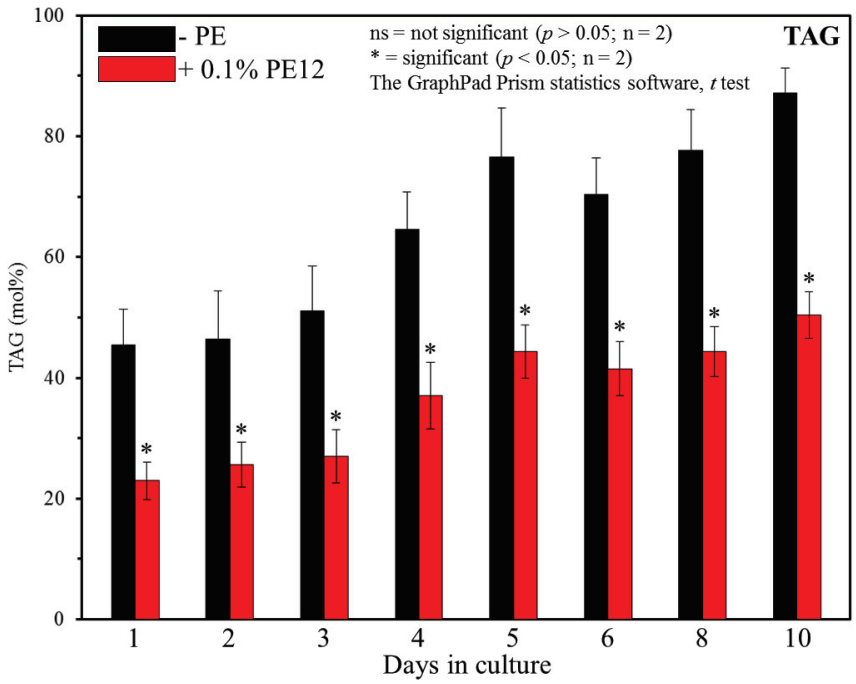

Figure 7.2. PE12 decreases the concentration of the neutral lipid triacylglycerol (TAG) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE12. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of TAG were calculated in mol%. Data are presented as means ± SEM (n = 2).

7.3.2 PE12 causes a decrease in the concentration of phosphatidic acid (PA) early in life of chronologically aging yeast

I found that PE12 decreases the concentration of PA early in life of chronologically aging yeast (**Figure 7.3**).

7.3.3 PE12 increases the concentration of phosphatidylserine (PS) early in life of chronologically aging yeast

I found that PE12 causes a rise in the concentration of PS early in life of chronologically aging yeast (**Figure 7.4**).

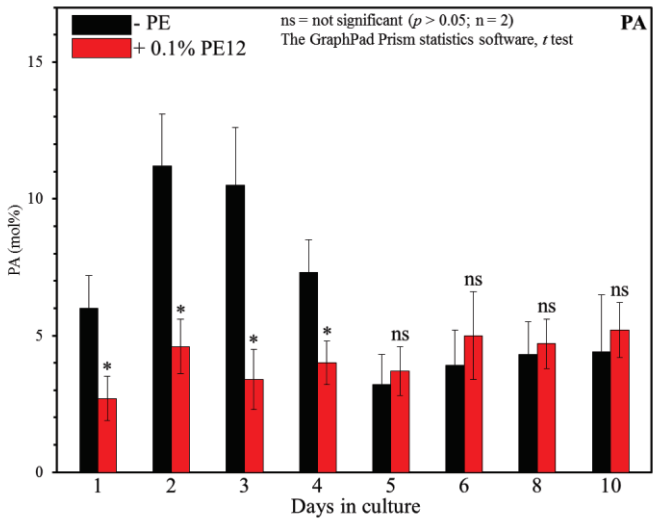

Figure 7.3. PE12 decreases the concentration of phosphatidic acid (PA) early in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE12. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PA were calculated in mol%. Data are presented as means ± SEM (n = 2).

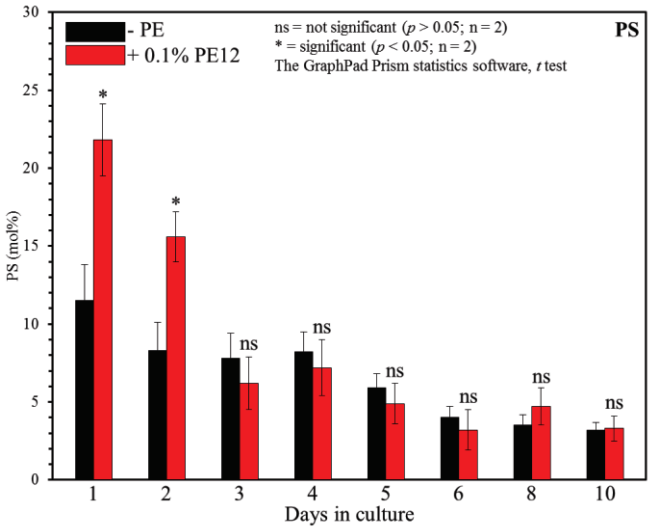

Figure 7.4. PE12 increases the concentration of phosphatidylserine (PS) early in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE12. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PS were calculated in mol%. Data are presented as means ± SEM (n = 2).

7.3.4 PE12 increases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast

I found that PE12 causes a rise of the concentration of PE in chronologically aging yeast (**Figure 7.5**).

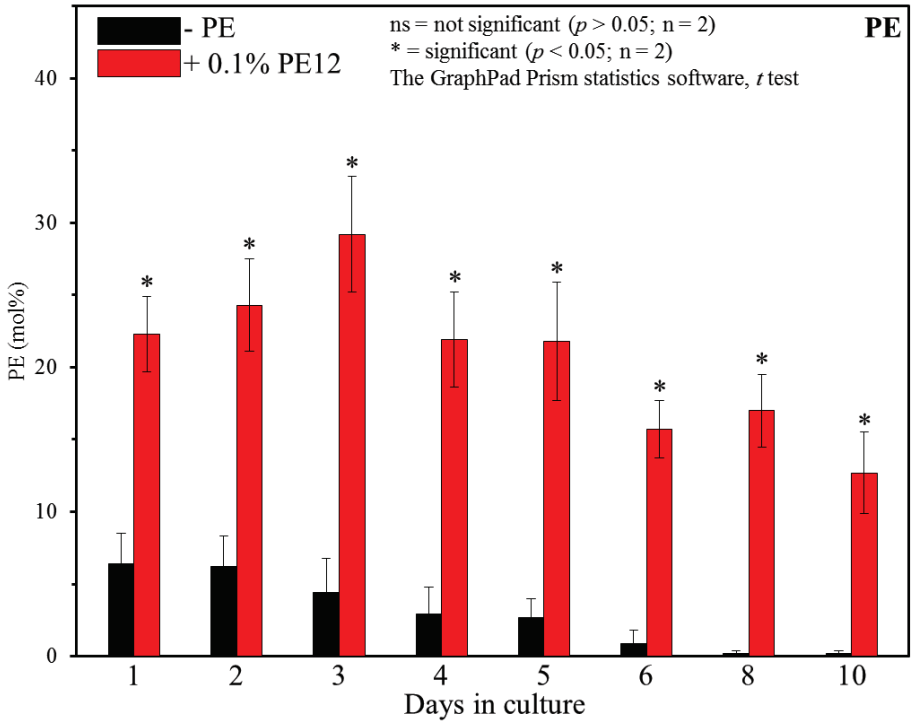

Figure 7.5. PE12 increases the concentration of phosphatidylethanolamine (PE) in chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE12. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PE were calculated in mol%. Data are presented as means ± SEM (n = 2).

7.3.5 PE12 increases the concentration of phosphatidylcholine (PC) late in life of chronologically aging yeast

I found that PE12 causes a rise in the concentration of PC late in life of chronologically aging yeast (**Figure 7.6**).

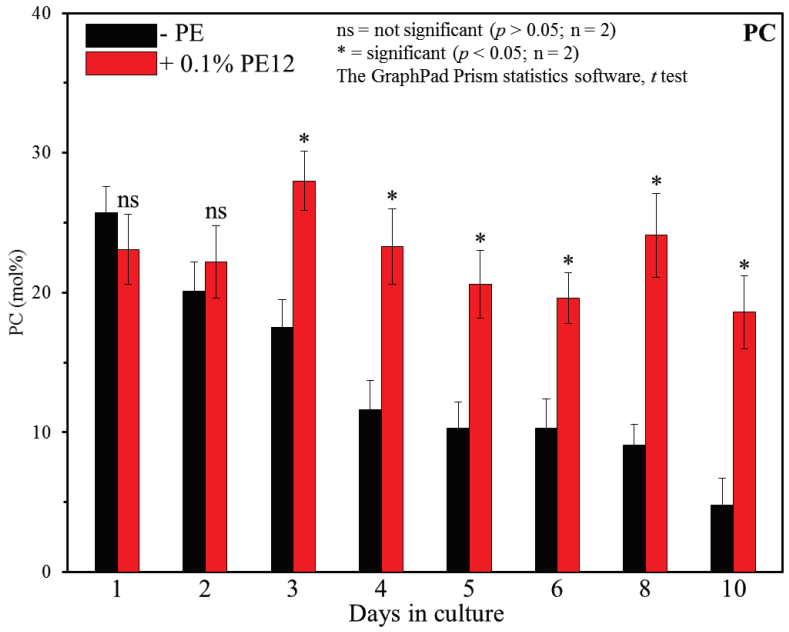

Figure 7.6. PE12 increases the concentration of phosphatidylcholine (PC) late in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE12. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PC were calculated in mol%. Data are presented as means ± SEM (n = 2).

7.3.6 PE12 elevates the concentration of phosphatidylinositol (PI) late in life of chronologically aging yeast

I found that PE12 increases the concentration of PI late in life of chronologically aging yeast (**Figure 7.7**).

7.3.7 PE12 decreases the concentration of cardiolipin (CL) early in life of chronologically aging yeast

I found that PE12 causes a decrease in the concentration of CL early in life of chronologically aging yeast (**Figure 7.8**).

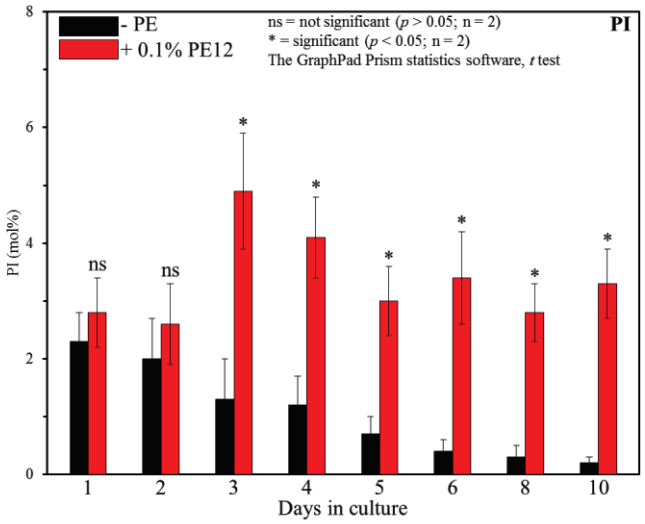

Figure 7.7. PE12 increases the concentration of phosphatidylinositol (PI) late in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE12. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of PI were calculated in mol%. Data are presented as means ± SEM (n = 2).

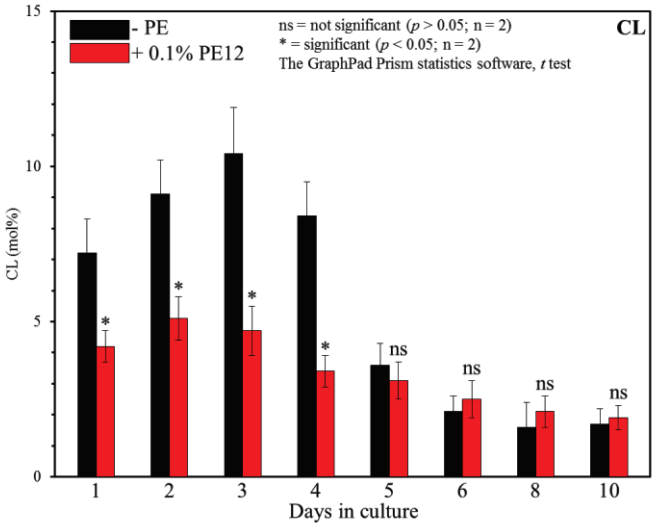

Figure 7.8. PE12 decreases the concentration of cardiolipin (CL) early in life of chronologically aging yeast. The wild-type strain of yeast was cultured in the nutrient-rich YP medium initially containing 2% glucose with or without PE12. Cells were recovered on days 1, 2, 3, 4, 5, 6, 8 or 10 of culturing. Extraction of cellular lipids, as well as their mass spectrometric identification and quantitation, were carried out as described in Materials and Methods of chapter 2. Based on these data, the concentrations of CL were calculated in mol%. Data are presented as means ± SEM (n = 2).

7.4 Discussion

My data on how PE12 influences the concentrations of different lipid classes in cells of chronologically aging yeast suggest that this aging-delaying plant extract alters the relative rates of phosphatidic acid (PA) flow into the synthesis of other lipids as follows: 1) it decreases the rate of PA flow into the synthesis of triacylglycerols (TAG) in the ER; 2) it increases the rate of PA flow into the synthesis of phosphatidylethanolamine (PE) in mitochondria; 3) it increases the rate of PA flow into the synthesis of phosphatidylserine (PS) in the ER early in life of chronologically aging yeast; 4) it increases the rate of PA flow into the synthesis of phosphatidylcholine (PC) and phosphatidylinositol (PI) in the ER late in life of chronologically aging yeast; and 5) it decreases the rate of PA flow into the synthesis of cardiolipin (CL) in mitochondria early in life of chronologically aging yeast (**Figure 7.9**). Such re-wiring of PA conversion into TAG, PS, PC, PI, PE and CL delays aging by a mechanism that remains to be established. I speculate that this mechanism may affect several longevity-defining processes in chronologically aging yeast, as described below.

First, the observed decrease in the concentration of CL in mitochondria of yeast cells treated with PE12 may cause certain changes in the age-related chronology of mitochondrial functionality seen in these cells. [35] These changes involve alterations in mitochondrial respiration, mitochondrial membrane potential, mitochondrially produced ROS and stability of mitochondrial DNA. [35] To validate this hypothesis, it needs to be assessed how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentration of CL in the mitochondrial membrane impact mitochondrial functionality and the ability of PE12 to delay aging.

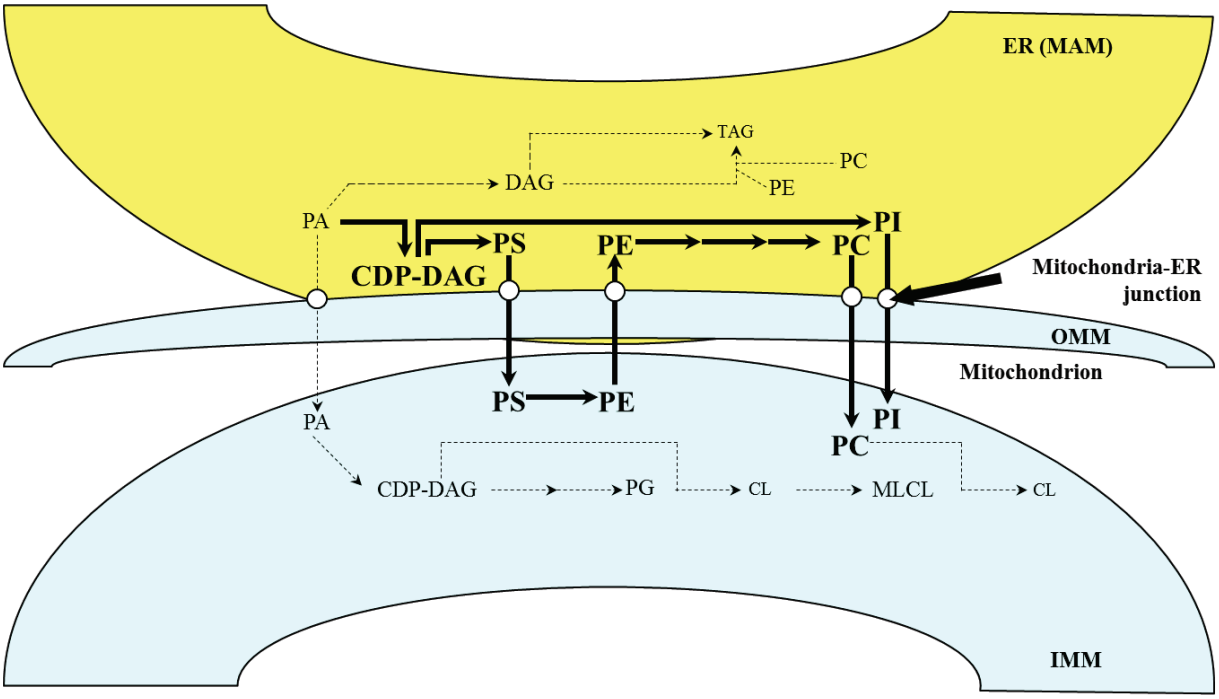

Figure 7.9. A hypothetical model of how PE12 alters the relative rates of PA synthesis and of PA flow into the synthesis of other lipid classes. PE12: 1) decreases the rate of PA flow into the synthesis of TAG in the ER; 2) increases the rate of PA flow into the synthesis of PE in mitochondria; 3) increases the rate of PA flow into the synthesis of PS in the ER early in life of chronologically aging yeast; 4) increases the rate of PA flow into the synthesis of PC and PI in the ER late in life of chronologically aging yeast; and 5) decreases the rate of PA flow into the synthesis of CL in mitochondria early in life of chronologically aging yeast

Second, the observed changes in the concentrations of four glycerol phospholipids, PS, PE, PC and PI, in yeast cells treated with PE12 may cause the increased resistance of these cells to chronic oxidative and thermal stresses. [35] To test the validity of this hypothesis, it is necessary to examine how genetic manipulations (such as single-gene-deletions and elevated gene expression) that decrease or increase the concentration of PS, PE, PC and PI affect stress resistance and the efficiency with which PE12 delays aging.

Third, one of the outcomes of the observed re-wiring of lipid metabolism and transport in yeast cells treated with PE12 may be a late-life decrease in the cellular

concentrations of FFA, a class of lipids whose incorporation into PA initiates all three major routes of lipid metabolism in the ER, mitochondria and LDs (**Figure 7.9**). FFA are known to trigger an age-related form of programmed cell death called liponecrosis. [36, 37] To validate this hypothesis, the concentrations of FFA in yeast cells treated with PE8 need to be compared to those in cells that remained untreated. Furthermore, it needs to be examined how genetic manipulations (such as single-gene-deletions and amplified gene expression) that decrease or increase the concentrations of FFA in yeast cells impact the efficiency of liponecrosis and the ability of PE12 to delay aging.

Future studies also need to use quantitative mass spectrometry to monitor an age-related chronology of changes in various lipid classes extracted from three different types of purified organelles (such as the ER, mitochondria and LDs). These studies will allow a critical validation of my hypothesis on how PE12 alters the relative rates of PA formation and of PA flow into the synthesis of other lipids in these organelles (**Figure 7.9**).

8 General Discussion

My findings imply that each of the 6 aging-delaying plant extracts causes age-related changes in cellular lipidomes of chronologically aging yeast. I demonstrated that each of these extracts differently alters the relative rates of PA formation and PA flow into the biosynthetic pathways for TAG in the ER, glycerol phospholipids in the ER and mitochondria, and CL in mitochondria. The summary of changes in the concentrations of various lipid classes in yeast cells treated with different aging-delaying plant extracts is presented in **Table 8.1**. Three consecutive stages of the chronological aging process can be distinguished with regard to such changes. They include the early-age (days 1-2 of culturing), intermediate-age (days 3-4 of culturing) and late-age (days 5-10 of culturing) stages.

The only common feature of age-related changes in the cellular lipidomes of yeast treated with each of the 6 aging-delaying plant extracts is a decrease in the concentration of TAG. In yeast treated with PE4, PE5, PE8, PE12 or PE21, such decrease occurs during each of the three stages of the chronological aging process (**Table 8.1**). In yeast treated with PE6, the decrease in the concentration of cellular TAG can be seen only during the late-age (days 5-10 of culturing) stage (**Table 8.1**).

Different aging-delaying plant extracts differently impact the concentration of PA. PE21 does not alter PA concentration at any of the stages of the chronological aging process (**Table 8.1**), PE4 and PE5 increase it during each of the three stages (**Table 8.1**), PE8 decrease PA concentration during each of the three stages (**Table 8.1**), PE6 increases PA concentration only during the late-age (days 5-10) stage (**Table 8.1**), whereas PE12

decreases PA concentration during the early-age (days 1-2) and intermediate-age (days 3-4) stages (**Table 8.1**).

Table 8.1. Summary of changes in the concentrations of various lipid classes in yeast cells treated with different aging-delaying plant extracts at the early-age (days 1-2), intermediate-age (days 3-4) and late-age (days 5-10) stages of the chronological aging process.

0.1% PE21:

	Days 1-2	**Days 3-4**	**Days 5-10**
TAG	↓	↓	↓
PA	NS	NS	NS
PS	NS	↑	↑
PE	↑	↑	↑
PC	NS	NS	↑
PI	NS	NS	↑
CL	↓	↓	↓

0.5% PE4:

	Days 1-2	**Days 3-4**	**Days 5-10**
TAG	↓	↓	↓
PA	↑	↑	↑
PS	NS	NS	NS
PE	NS	↑	↑
PC	NS	NS	NS
PI	↑	↑	↑
CL	↑	↑	↑

0.5% PE5:

	Days 1-2	**Days 3-4**	**Days 5-10**
TAG	↓	↓	↓
PA	↑	↑	↑
PS	NS	NS	NS
PE	↓	↓	↓
PC	↓	↓	↓
PI	↓	↓	↓
CL	↑	↑	↑

1.0% PE6:

	Days 1-2	**Days 3-4**	**Days 5-10**
TAG	NS	NS	↓
PA	NS	NS	↑
PS	↑	↑	↑

PE	⬇	⬇	⬇
PC	⬆	⬆	⬆
PI	⬆	⬆	⬆
CL	⬆	⬆	⬆

0.3% PE8:

	Days 1-2	**Days 3-4**	**Days 5-10**
TAG	⬇	⬇	⬇
PA	⬇	⬇	⬇
PS	⬇	NS	NS
PE	⬆	⬆	⬆
PC	⬇	NS	⬆
PI	⬆	⬆	⬆
CL	⬇	⬇	NS

0.1% PE12:

	Days 1-2	**Days 3-4**	**Days 5-10**
TAG	⬇	⬇	⬇
PA	⬇	⬇	NS
PS	⬆	NS	NS
PE	⬆	⬆	⬆
PC	NS	⬆	⬆
PI	NS	⬆	⬆
CL	⬇	⬇	NS

Different aging-delaying plant extracts differently impact the concentrations of glycerol phospholipids that are synthesized in the ER (i.e. PS, PC and PI) or in mitochondria (i.e. PE and CL) (**Table 8.1**).

My findings for the first time demonstrate that different aging-delaying pharmacological interventions can cause different types of a re-wiring of lipid metabolism and transport in yeast cells. It remains to be seen how important is each of these different types of changes in lipid dynamics for the ability of each of the 6 plant extracts to delay yeast chronological aging. Moreover, mechanisms through which such changes in lipid dynamics can delay aging remain to be established.

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