Mechanisms Underlying Regulation of Yeast Longevity by Genetic and Pharmacological Interventions that Alter Mitochondrial Membrane Lipidome and Remodel Mitochondrial Respiratory Supercomplexes

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ABSTRACT

Mechanisms Underlying Regulation of Yeast Longevity by Genetic and Pharmacological Interventions that Alter Mitochondrial Membrane Lipidome and Remodel Mitochondrial Respiratory Supercomplexes

Olivia Roseline Koupaki, M.Sc.

Recent studies in our laboratory demonstrated that in chronologically aging yeast grown under CR conditions LCA, a natural anti-aging compound, alters the age-related dynamics of changes in mitochondrial abundance and morphology, respiration, membrane potential, and ROS production. Cardiolipin (CL), a dimeric glycerophospholipid that is synthesized and almost exclusively localized in the inner mitochondrial membrane, has been shown to modulate mitochondria-governed processes whose dysfunction underlies aging and age-related pathologies. Phosphatidylethanolamine (PE) is another glycerophospholipid that is almost exclusively synthesized in the inner mitochondrial membrane, from which it is then distributed to various other cellular membranes. Hence, it is likely that the synthesis and stability of CL and, perhaps, PE in the inner mitochondrial membrane are important targets of longevity-extending and health-improving interventions. Because of the plausible importance of mitochondrially synthesized CL and PE in the longevity-extending effect of LCA, other graduate students in our laboratory elucidated how mutations eliminating nucleus-encoded mitochondrial proteins involved in the synthesis of CL and PE within the inner mitochondrial membrane influence the lifespan-extending efficacy of LCA in chronologically aging yeast grown under CR conditions. The results of this genetic analysis suggested that the synthesis of both these membrane lipids in mitochondria plays an essential role in the ability of LCA to extend longevity of yeast placed on

a CR diet. All these findings prompted us to elucidate how LCA influences the composition of mitochondrial membrane lipids in chronologically aging yeast grown under CR conditions. To attain this objective, in experiments described in my thesis we used mass spectrometry (MS)-based lipidomics to elucidate the effect of LCA on the repertoire and quantities of membrane lipids in mitochondria that were purified from wild-type (WT) strain and from various long- and short-lived mutant strains impaired in different aspects of CL and PE metabolism. By correlating the effects of LCA on the age-related dynamics of changes in the composition and quantities of membrane lipids in mitochondria of these strains to the effects of this anti-aging compound on their chronological lifespan, we concluded that under CR conditions LCA extends yeast longevity by remodeling the composition of mitochondrial membrane lipids and thereby modulating longevity-defining processes confined to and governed by mitochondria. Specifically, findings described in my thesis strongly suggest that LCA extends longevity of WT yeast by (1) elevating the level of phosphatidylserine (PS; a precursor for the synthesis of PE in mitochondria) in the mitochondrial membrane, thereby enhancing its positive effect on longevity-defining processes in this membrane; (2) reducing the level of PE in the mitochondrial membrane, thereby weakening its negative effect on longevity-defining processes in this membrane; and (3) proportionally decreasing the levels of PE and CL in the mitochondrial membrane, thereby increasing PS/CL and PS/PE ratios but maintaining PE/CL ratio of mitochondrial membrane lipids and causing some longevity-extending changes in this membrane. It is important to emphasize that these LCA-induced alterations in mitochondrial membrane lipids can satisfactorily explain the observed implications of LCA treatment on mitochondrial structure and function, including (1) the ability of LCA to cause dramatic changes in the length and curvature of the inner mitochondrial membrane; and (2) the ability of LCA to activate protein machines involved in mitochondrial respiration, the maintenance of

mitochondrial membrane potential, ROS production in mitochondria and mitochondrial fusion.

Based on our recent findings and data of other researchers working in the field of mitochondrial biology, we hypothesized that, by altering the level of CL and other glycerophospholipids within the mitochondrial membrane, LCA could modulate the stoichiometry, composition and/or functional state of respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane. To test the validity of this hypothesis, in studies described in my thesis we used a multistep method for recovery of intact respiratory complexes and supercomplexes from purified yeast mitochondria, their subsequent first-dimension electrophoretic separation using so-called blue-native gel electrophoresis (BN-PAGE), their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the mass spectrometry (MS)-based identification of each of these individual protein components. Findings described in my thesis validate our hypothesis. Specifically, these findings revealed several ways of rearranging respiratory supercomplexes in the inner mitochondrial membrane of cells exhibiting altered mitochondrial membrane lipidome in response to LCA treatment or genetic manipulations impairing the synthesis of CL and other glycerophospholipids within the inner membrane of mitochondria. First, by altering the level of CL and other glycerophospholipids synthesized and residing in the inner mitochondrial membrane, LCA modulates the abundance of several major respiratory supercomplexes (respirasomes) in this membrane. Second, LCA- and genetic manipulations-driven changes in the inner mitochondrial membrane lipidome cause a recruitment of a number of new mitochondrial protein components, not previously known for being permanently associated with the ETC, into the remodeled respirasomes. Importantly, many of the proteins newly recruited into the remodeled respirasomes are known for their essential roles in mitochondria-confined processes that define longevity.

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List of Abbreviations

1D, first dimension electrophoresis; 2D, second dimension electrophoresis; ACO, aconitase; AMPK/TOR, AMP-activated protein kinase/target of rapamycin; ABC, ammonium bicarbonate;

APS, ammonium persulfate; BN, blue native; BN-PAGE, blue native polyacrylamide gel electrophoresis; cAMP/PKA, cAMP/protein kinase A; CCO, cytochrome c oxidase; CFU, colony forming units; CL, cardiolipin; CLS, chronological life span; CR, caloric restriction; DAF-16, dauer formation protein 16; DR, dietary restriction; DVE-1, defective proventriculus protein 1; ESI/MS, electrospray ionization mass spectrometry; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; IGF-1, insulin-like growth factor 1; LCA, lithocholic acid; LC/MS, liquid chromatography followed by mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PHA-4, pharynx development protein 4; PI, phosphatidylinositol; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; RLS, replicative life span; SD, standard deviation; SDH, succinate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TAG, triacylglycerols; TLC, thin-layer chromatography; TORC1, target of rapamycin protein complex 1; UBL-5, ubiquitin-like protein 5; UPR^{mt}, mitochondria-specific unfolded protein response.

1 Introduction

1.1 Programmed complexity of a multistep process of biological aging

Aging of multicellular and unicellular eukaryotic organisms is a complex biological process that affects numerous cellular activities [1 - 16]. At the organismal level, aging manifests itself as a progressive decline in the ability of an organism to resist stress, repair damage and battle disease [2, 3, 9, 10, 13 - 15]. At the demographic level, aging can be defined as an exponential increase in the mortality rate with the age of the cohort [2, 3, 5, 12 - 14].

Two contradictory views of biological aging exist. One view foresees that aging of multicellular and unicellular eukaryotic organisms is due to a "passive" lifelong build-up of unrepaired damage to cellular macromolecules and organelles [3, 12 - 14, 17]. By impairing normal functioning of the entire organism, progressive, age-related accumulation of such damage increases a risk of disease and death [11 - 15]. Hence, in this view of biological aging, it is simply a result of the time-dependent, unavoidable accumulation of unrepaired cellular and molecular damage [3, 12, 17]. Yet, recent evidence strongly suggests that aging is the final step of a developmental program. According to this alternative view, aging is an "active" route, in which a limited number of evolutionarily conserved nutrient- and energy-sensing signaling pathways (often called "master regulators") orchestrate numerous cellular processes; each of these cellular processes is considered as a functional module integrated with other modules into a biomolecular network [18 - 22]. In this view of aging as an "active" route, a synergistic action of individual modules constituting the biomolecular network could define

longevity by establishing the rate of cellular and organismal aging; furthermore, the relative impact of each module on the rate of aging in a particular organism or cell type could differ at various stages of its lifetime and could also vary in different organisms and cell types [19 - 25]. The recently intensified reconstruction of various biomolecular networks of longevity and aging on the cellular and organismal levels is based on the application of a network biology approach, which has been initially devised and implemented by Witten for theoretical modeling of the replicative senescence process in mammalian organisms [26 - 28]. The use of system biological and computational analyses of protein-protein interactions, age-related changes in gene expression and/or longevity-defining metabolic alterations has resulted in numerous models of such longevity and aging networks [18, 25, 29 - 43].

The conducted by our laboratory analysis of the metabolic history of chronologically aging yeast provided evidence that their longevity is programmed by the level of metabolic capacity and organelle organization that yeast developed, in a diet-specific fashion, before entering a quiescent state - and, thus, that chronological aging in yeast is likely to be the final step of a developmental program progressing through at least one checkpoint prior to entry into quiescence [12]. Moreover, our recent investigation of how lithocholic acid (LCA) - a synthesized and secreted by mammals bile acid whose potent anti-aging ability we discovered [44] - influences longevity and several longevity-defining cellular processes in chronologically aging yeast if added to growth medium at different periods of lifespan has revealed that LCA can extend longevity only if added at either of two lifespan periods [45]. One of these critical lifespan periods includes logarithmic and diauxic growth phases, whereas the other

period exists in early stationary phase [45]. Our findings suggested a mechanism linking the ability of LCA to increase the lifespan of CR yeast only if added at either of the two periods to its differential effects on various longevity-defining processes [45]. In this mechanism, LCA controls these processes at three checkpoints that exist in logarithmic/diauxic, post-diauxic and early stationary phases. We therefore proposed a hypothesis in which a biomolecular longevity network in chronologically aging yeast progresses through a series of checkpoints; at each of these checkpoints (1) genetic, dietary and pharmacological anti-aging interventions modulate a distinct set of longevity-defining processes (modules) comprising the network; and (2) checkpoint-specific master regulators monitor and govern the functional states of these processes (modules) in order to limit the age-related accumulation of molecular and cellular damage [45]. In our hypothesis, the resulting changes in the dynamics of individual processes (modules) constituting the network and in its general configuration are critically important for defining longevity by establishing the rate of cellular and organismal aging [45].

Recent findings support the validity of our hypothesis on a stepwise progression of a biomolecular longevity network through a series of checkpoints. In fact, studies in chronologically aging yeast revealed that, by promoting coupled respiration in mitochondria, elevating the mitochondrial membrane potential and increasing mitochondrial reactive oxygen species (ROS) production during logarithmic growth phase, a CR diet and some pharmacological interventions extend longevity by causing changes in several longevity-defining processes during the subsequent diauxic, post-diauxic and stationary phases. These changes include (1) increased intracellular levels of trehalose and glycogen, the two major glucose stores of yeast; (2) a remodeling of lipid

metabolism in the endoplasmic reticulum, lipid bodies and peroxisomes; (3) reduced cell susceptibility to age-related forms of mitochondria-controlled apoptotic and lipid-induced necrotic death; (4) an attenuation of mitochondrial fragmentation; (5) a reduction in the mitochondrial membrane potential and mitochondrial ROS production; (6) elevated stability of nuclear and mitochondrial genomes; and (7) enhanced resistance to chronic oxidative and thermal stresses [12, 44, 46 - 49]. It seems that in chronologically aging yeast TORC1 (the target of rapamycin protein complex 1) operates as one of the predicted by our hypothesis checkpoint-specific master regulators that at a checkpoint in logarithmic growth phase can monitor and govern a functional state of mitochondria [46 - 49].

Furthermore, recent studies in chronologically aging yeast suggest the existence of two checkpoints at which the intracellular level of trehalose defines longevity by modulating cellular proteostasis throughout lifespan [12, 50]. At one of these lifespan checkpoints in post-diauxic phase, trehalose operates as an anti-aging compound that (1) stabilizes the native state of proteins and thereby reduces the formation of their aberrantly folded species; (2) reduces the formation of insoluble protein aggregates by shielding the contiguous exposed hydrophobic side chains of amino acids that are abundant in misfolded, partially folded and unfolded protein species and promote their aggregation; and (3) protects cellular proteins from oxidative carbonylation by interacting with their carbonylation-prone misfolded and unfolded species [50]. At another lifespan checkpoint in stationary phase, trehalose functions as a pro-aging compound that shields the contiguous exposed hydrophobic side chains of amino acids abundant in misfolded, partially folded and unfolded protein species. By competing with molecular chaperones

for binding with these patches of hydrophobic amino acid residues, trehalose interferes with the essential longevity-extending process of chaperone-assisted refolding of aberrantly folded protein species [50].

Moreover, a confirmation of our hypothesis on a stepwise progression of a biomolecular longevity network through a series of checkpoints, each being monitored by a limited set of checkpoint-specific master regulators, comes from studies that revealed distinctive timing requirements for modulating the pace of organismal aging in the nematode *Caenorhabditis elegans*. This roundworm devotes at least three independent regulatory systems that, by monitoring a particular cellular process or processes during a specific stage of life, use this information to establish the rate of cellular and organismal aging. The first of these three regulatory systems influences lifespan by operating only during larval development [51 - 53]. By monitoring mitochondrial respiration, electrochemical membrane potential and ATP production early in life, during the L3/L4 larval stage, it establishes the rate of aging that persists during adulthood [51 - 54]. Mechanisms underlying the essential role of this first regulatory system in defining longevity of *C. elegans* may involve (1) a remodeling of mitochondria-confined ATP production pathways during larval development, which may establish a specific configuration of the longevity-defining metabolic network in a cell-autonomous manner [55, 56]; and (2) a retrograde signalling pathway that in response to mild mitochondrial impairment and stress during the L3/L4 larval stage activates UBL-5 (ubiquitin-like protein 5)/DVE-1 (defective proventriculus protein 1)-driven expression of the mitochondria-specific unfolded protein response (UPR^{mt}) genes in the nucleus, thereby stimulating synthesis of a subset of UPR^{mt} proteins that can extend longevity not only

cell-autonomously but also in a cell-non-autonomous fashion [57, 58]. The second regulatory system operates exclusively during adulthood, mainly during early adulthood, to influence the lifespan of *C. elegans* via the insulin/IGF-1 (insulin-like growth factor 1) longevity signaling pathway and the transcription factor DAF-16 (dauer formation protein 16) [59 - 61]. Of note, the magnitude of the effect of this second regulatory system on lifespan declines with age, becoming insignificant after several days of adulthood [59]. The third regulatory system influences the lifespan of *C. elegans* in a diet-restriction-specific fashion by operating exclusively during adulthood [62]. This system regulates longevity via the transcription factor PHA-4 (pharynx development protein 4) only in response to reduced food intake. Importantly, the PHA-4-mediated regulatory system operates independently of the other two regulatory systems modulating the rate of aging in *C. elegans* [62]. In our hypothesis for a stepwise development of a longevity network configuration at a series of checkpoints, a genetic, dietary or pharmacological anti-aging intervention may modulate the key cellular process or processes that are monitored at a particular checkpoint by a master regulator of the longevity control system. In *C. elegans*, UBL-5/DVE-1, DAF-16 and PHA-4 may function as the checkpoint-specific master regulators of longevity by governing progression through the three consecutive checkpoints operating during larval development and early adulthood [57, 59 - 62].

In addition, our hypothesis on a stepwise progression of a biomolecular longevity network through a series of checkpoints, each being monitored by a limited set of checkpoint-specific master regulators, could be applicable to laboratory mice and rats. Indeed, although a CR diet considerably extends lifespan in these organisms even if it is

implemented at the age at which skeletal development is complete, the maximal benefit of this low-calorie diet for longevity can be achieved only if CR is initiated during the rapid growth period [63 - 65]. These findings suggest that laboratory mice and rats (1) could employ a CR-dependent longevity control system that, by monitoring some key, longevity-defining cellular processes, can establish a particular rate of cellular and organismal aging; and (2) could have at least two checkpoints, one in early adulthood and another in late adulthood, at which the proposed CR-dependent longevity control system senses the rate and/or efficiency of the critical cellular processes that define longevity. It is likely therefore that the proposed CR-dependent longevity control system in laboratory mice and rats can extend longevity even if the rate and efficiency of the critical, CR-modulated cellular processes are appropriate only at the late-adulthood checkpoint, but not as markedly as if they are suitable already at the checkpoint in early adulthood.

Taken together, these findings support the validity of our hypothesis that aging in organisms across phyla is the final step of a developmental program whose progression through several lifespan checkpoints in a genotype-specific fashion is modulated by environmental cues (such as caloric and dietary intake, environmental stresses, endocrine factors, etc.) and is both monitored and governed by an evolutionarily conserved set of checkpoint-specific master regulators. Noteworthy, some theoretical considerations in support of the validity of our hypothesis on programmed aging progressing through several lifespan checkpoints have been also discussed in recent years [66 - 75].

The major challenge now is to get a greater insight into mechanisms that in chronologically aging yeast underly (1) a stepwise progression of the biomolecular longevity network through a series of checkpoints; (2) a modulation of various longevity-

defining processes comprising the longevity network by genetic, dietary and pharmacological anti-aging interventions administered at different checkpoints; and (3) a monitoring of these longevity-defining processes at each checkpoint by specific master regulators. To address this challenge, several important questions need to be answered. How will genetic, dietary and pharmacological anti-aging interventions known to directly target specific longevity-extending or longevity-shortening processes alter the age-related dynamics of changes in the proteomes, lipidomes and metabolomes of chronologically aging yeast? How will these interventions affect the chronology of other longevity-defining processes that comprise the longevity network but are known not to be directly modulated by these interventions? How will genetic and pharmacological anti-aging interventions that specifically modulate the functional states of several currently known master regulators of yeast longevity influence a timeline of changes in the proteomes, lipidomes and metabolomes of chronologically aging yeast and what will be their effects on the age-related dynamics of various longevity-defining processes comprising the longevity network? We shall have to answer these important questions if we want to understand the complexity of the biomolecular network whose progression through a series of checkpoints is modulated by various environmental cues and is governed by checkpoints-specific master regulators.

1.2 The budding yeast *Saccharomyces cerevisiae* is a beneficial model organism for understanding mechanisms of cellular aging in multicellular eukaryotes

The baker's yeast *Saccharomyces cerevisiae*, a unicellular eukaryotic organism with annotated genome, is amenable to comprehensive biochemical, genetic, cell biological,

chemical biological and system biological analyses [76 - 79]. This yeast embodies a beneficial model for studying the basic biology of aging and understanding longevity regulation mechanisms in multicellular eukaryotes [4, 9, 18, 80 - 85].

There are two different ways to measure aging in yeast. Yeast replicative aging is monitored by counting the maximum number of daughter cells that a mother cell can produce before reaching a quiescent/senescent state; replicative aging in yeast is known to mimic aging of dividing, "mitotic" cells in a multicellular eukaryotic organism [4, 18, 81, 85]. In studies presented in this thesis, we elucidated mechanisms underlying yeast chronological aging. This kind of aging is measured by the length of time during which a yeast cell remains viable following entry into a quiescent/senescent state [4, 18, 81, 85, 86]. Chronological aging in yeast is known as an advantageous model for studying aging of non-dividing, "post-mitotic" cells (such as neurons) in a multicellular eukaryotic organism [4, 18, 81, 85, 86]. To monitor the progress of yeast chronological aging, a simple clonogenic assay measures the percentage of yeast cells that remain viable at different time points following entry into a quiescent/senescent state upon reaching the non-proliferative stationary phase [4, 12, 18, 81, 85, 86]. Both replicative and chronological aging of yeast can be significantly delayed by caloric restriction (CR), a low-calorie dietary regimen that extends lifespan in various multicellular eukaryotic organisms and delays the onset or reduces the incidence of many age-related diseases in mice and primates [9, 12, 18, 85 - 93].

Because longevity signaling pathways and mechanisms of their modulation by genetic, dietary and pharmacological interventions have been conserved during the evolution [3, 9, 10, 23, 80 - 82, 94], the relatively short and easily measured lifespan of

yeast - in combination with the amenability of this unicellular eukaryote with annotated genome to comprehensive biochemical, genetic, cell biological, chemical biological and system biological analyses - enabled to (1) identify numerous novel longevity genes, all of which have been later implicated in regulating longevity of multicellular eukaryotic organisms; (2) establish the chemical nature of molecular damage that causes aging and accelerates the onset of age-related diseases across phyla; and (3) reveal several longevity-extending small molecules, all of which have been later shown to slow down aging, improve health, attenuate age-related pathologies and delay the onset of age-related diseases in eukaryotic organisms across phyla [4, 65, 81, 83, 85, 89, 91, 95].

1.3 Some dietary and pharmacological interventions can extend longevity and improve health by attenuating age-related pathologies and delaying the onset of age-related diseases

Nutrient intake is known to play an important role in defining longevity and improving health [96 - 99]. The dietary regimens known as caloric restriction (CR) and dietary restriction (DR) have been shown not only to extend longevity of evolutionarily distant organisms ranging from yeast to rhesus monkeys, but also to improve health by attenuating age-related pathologies and delaying the onset of age-related diseases across phyla [97 - 104]. A CR dietary regimen has been designed to reduce only calorie intake without compromising the supply of amino acids, vitamins and other nutrients [98 - 101]. In contrast, various DR diets have been developed to reduce the intake of nutrients (but not necessarily of calories) by limiting food supply without causing malnutrition [97, 102 - 104].

In organisms across phyla, the beneficial effects of CR and DR diets on longevity and health are governed by a limited number of evolutionarily conserved signaling pathways. In a so-called "TOR-centric" view of longevity regulation, the AMPK/TOR (AMP-activated protein kinase/target of rapamycin) pathway is considered as the only signaling pathway underlying longevity-extending and health-improving effects of CR and DR [105 - 110]. In this "radical" view of longevity regulation, TORC1 - a core protein complex within the AMPK/TOR pathway - modulates the beneficial effects of CR and DR on longevity and health by performing the following three functions. First, TORC1 integrates the flow of information on the organismal and intracellular nutrient and energy status from AMPK (a protein kinase in the AMPK/TOR pathway), PKA (a protein kinase in the cAMP/PKA pathway), PKB/AKT (a protein kinase in the insulin/IGF-1 pathway), ERK1/2 (a protein kinase in the Raf/MEK/ERK signaling cascade) and $P66^{Shc}$ (a mitochondria-confined sensor of the redox status within this organelle) [105 - 107]. Second, TORC1 operates in an AMPK-independent fashion as a sensor of the intracellular levels of amino acids [105 - 107]. Third, TORC1 functions in a sirtuin-independent manner as a control center that modulates a number of longevity-related processes by gathering and processing the information on the organismal and intracellular nutrient, energy and redox status and on amino acid availability [105 - 107]. The validity of the TOR-centric view on TORC1-governed modulation of the longevity-extending effects of CR and DR has been confirmed by studies in roundworms and replicatively aging yeast. Specifically, it has been demonstrated that various DR diets are unable to extend longevity of the nematode *C. elegans* under conditions that reduce TOR signaling [109, 110]. Furthermore, the beneficial effect of a CR diet on longevity of

replicatively aging yeast can be eliminated through the impairment of any of the key components comprising the TOR signaling pathway [108].

Although in replicatively aging yeast the TOR pathway alone mediates the longevity benefit associated with CR, the lifespan-extending effect of this diet in chronologically aging yeast relies on a signaling network in which the protein kinase Rim15p functions as a nutritional integrator of the TOR and cAMP/PKA (cAMP/protein kinase A) signaling pathways; this network also includes some other signaling pathways that are not converged on Rim15p and remain to be identified [111]. Moreover, considering the comprehensive evidence for the convergence of the AMPK/TOR, cAMP/PKA and insulin/IGF-1 pathways in worms, fruit flies and mammals into a complex network regulating longevity [96, 112 - 115], it is believed that these three divergent and multiply branched signaling pathways equally contribute to the lifespan-extending effects of CR and DR in these organisms [112, 116 - 125]. Recent studies in the nematode *C. elegans* support the validity of the view in which a signaling network, rather than the AMPK/TOR pathway alone, mediates the longevity benefit associated with CR and DR by coordinating information flow along the convergent, divergent and multiply branched signaling pathways comprising this network [126].

Longevity in organisms across phyla can be extended, health improved, age-related pathologies attenuated and the onset of age-related diseases delayed not only by CR and DR diets. Certain chemical compounds, many of which are natural molecules synthesized and released into the environment by some organisms within an ecosystem, exhibit beneficial effects on longevity and health of other organisms comprising this ecosystem [127]. Many of these longevity-extending and health-improving pharmacological

compounds target the AMPK/TOR signaling pathway that regulates longevity in evolutionarily distant organisms by responding to their intracellular nutrient and energy status. The type 2 diabetes therapeutic compound metformin has been shown to activate AMPK, thereby reducing TORC1 signaling and extending longevity in roundworms and mice [128, 129]. The glutamine synthetase inhibitor methionine sulfoximine is known to extend longevity of chronologically aging yeast by decreasing the intracellular concentration of the TORC1 activator glutamine, thereby reducing TORC1 signaling [130, 131]. By inhibiting upstream TORC1 activators PI3K and MEK, the compounds called LY294002 and U0126 (respectively) attenuate TORC1 signaling to increase the replicative lifespan of cultured human fibrosarcoma cells [132]. Furthermore, the macrocyclic lactone rapamycin acts through FKBP12 to inhibit TORC1, thereby extending (1) longevity in fruit flies and mice [133, 134]; (2) the replicative lifespans of cultured rodent fibroblasts, human epithelium cells and human fibrosarcoma cells [135]; and (3) the replicative and chronological lifespans of yeast [130, 136, 137]. Moreover, the xanthine alkaloid caffeine reduces the catalytic activity of Tor1p to extend longevity of chronologically aging yeast [138].

Several longevity-extending compounds delay aging by targeting the insulin/IGF-1 signaling pathway, known to monitor the nutritional status of the whole organism. First, the serotonin receptor antagonist mianserin extends longevity of roundworms by modulating an upstream step in this pathway, thereby inhibiting neurotransmission related to food sensing and mimicking a DR-like physiological state [139]. Second, valproic acid targets a downstream step in the insulin/IGF-1 signaling pathway to increase lifespan in roundworms by promoting the translocation of the pro-longevity

FoxO transcriptional factor DAF-16 into the nucleus [140].

Some natural longevity-extending compounds delay aging, improve health and attenuate age-related pathologies by modulating longevity-defining processes controlled by proteins that currently are not considered as components of the AMPK/TOR, cAMP/PKA and insulin/IGF-1 signaling pathways but are known to be dynamically integrated into a longevity regulation network (see section 1.1) that they govern. Resveratrol is one of these natural anti-aging compounds. This small polyphenol produced by plants has been shown to modulate activities of numerous proteins by interacting with them [141, 142] and thereby inhibiting many longevity-shortening processes and activating a number of longevity-extending processes [143 - 149]. Due to its capability to modulate activities of numerous longevity-defining proteins and processes that these proteins govern, resveratrol exhibits a potent ability to (1) extend the replicative (but not chronological) lifespan of yeast [143]; (2) increase the replicative lifespan of cultured human fibroblasts [150]; and (3) extend longevity of roundworms, fruit flies, fishes and mice [144, 145, 151, 152]. Similar to resveratrol, sodium nitroprusside extends longevity by promoting sirtuin-dependent protein deacetylation. However, unlike resveratrol, sodium nitroprusside can activate expression of the gene encoding sirtuin SIRT1, thereby enhancing SIRT1-driven deacetylation of lysine 16 in histone H4 to establish an anti-aging pattern of transcription that ultimately leads to replicative lifespan extension in cultured human peripheral blood mononuclear cells (PMBC) [153]. A longevity-extending process of the reduction of histone acetylation can be promoted not only by sodium nitroprusside, but also by the natural polyamine called spermidine. By inhibiting histone acetyltransferases and promoting histone H3

deacetylation, spermidine activates transcription of numerous autophagy-related genes, suppresses age-related necrotic cell death and ultimately extends the chronological lifespan of yeast, the replicative lifespan of cultured human PMBC, and longevity of roundworms and fruit flies [154, 155]. The process of histone modification is also a target of another longevity-extending compound, the alkali metal ion Li^+. Li^+ has been shown to cause the age-related chromatin reorganization by altering transcription of genes involved in histone methylation, nucleosome composition and chromatin structure; these properties of Li^+ are believed to underlie its beneficial properties as a mood stabilizer in humans and an anti-aging compound in roundworms [156].

Several anti-aging compounds extend longevity by influencing longevity-defining processes confined to or governed by mitochondria, cellular organelles known for their essential role in longevity regulation across phyla [113, 157 - 159]. One of these longevity-extending compounds is the plastoquinone derivate SkQ1. Following its sorting to mitochondria, SkQ1 functions as an antioxidant that attenuates oxidative damage to mitochondrial proteins and lipids, changes mitochondrial morphology, and impairs mitochondria-controlled forms of apoptotic and necrotic cell death known to be caused by an excessive accumulation of mitochondria-produced hydrogen peroxide [160]. By modulating these longevity-defining processes confined to mitochondria, SkQ1 not only extends longevity in fungi, daphnias, fruit flies and mice, but also improves health and delays the onset of age-related diseases in mice [160]. In roundworms and fruit flies, the detoxification of mitochondria-produced free radicals and the resulting enhancement of resistance to mitochondria-controlled oxidative stress underly longevity extension by such thermal stress mimetics as lipoic acid, propyl gallate, trolox and taxifolin [161, 162].

It is important to emphasize that all longevity-extending compounds known prior to our recent study [44] have been called "CR mimetics" and "DR mimetics" because they: (1) similar to CR and DR dietary regimens, increase lifespan and/or improve health under non-CR or non-DR conditions; (2) unlike CR and DR diets, do not restrict caloric and nutrient intake; and (3) mimic longevity-extending and health-improving effects of CR and DR on metabolic pathways, physiological processes, stress response and gene expression [163 - 165]. Furthermore, almost all longevity-extending compounds known prior to our recent study [44] target the AMPK/TOR and insulin/IGF-1 pathways and impact the sirtuin-governed protein deacetylation module of the integrating these pathways longevity signaling network that defines longevity only in response to the organismal and intracellular nutrient and energy status. Moreover, such CR mimetics and DR mimetics as resveratrol, metformin and mianserin have been shown to increase lifespan and beneficially influence health only under non-CR or non-DR conditions, but do not extend longevity or improve health if the supply of calories or nutrients is limited [128, 139, 143, 144, 151]. We therefore introduced the term "adaptable" to define the AMPK/TOR, cAMP/PKA and insulin/IGF-1 signaling pathways and sirtuin-governed protein deacetylation module [44], all of which are integrated into the longevity regulation network targeted by the currently known longevity-extending CR mimetics and DR mimetics. By coining this term, we emphasized the fact that all these pathways and the sirtuin-governed module exhibit the beneficial effects on longevity and attenuate age-related pathologies only in response to certain changes in the extracellular and intracellular nutrient and energy status of an organism [44]. Of note, recent studies revealed that Li^+ in roundworms and rapamycin in fruit flies can extend lifespan even

under DR conditions; however, the longevity-extending efficacies of both these chemical compounds under such nutrient-limited conditions has been shown to be significantly lower than that under non-DR conditions [133, 156]. Based on these observations, we proposed that some longevity assurance pathways could be "constitutive" or "housekeeping" by nature, assuming that they (1) modulate longevity irrespective of the extracellular and intracellular nutrient and energy status – in contrast to the adaptable AMPK/TOR, cAMP/PKA and insulin/IGF-1 signaling pathways and sirtuin-governed protein deacetylation module; and (2) do not overlap (or only partially overlap) with the adaptable pathways that are under the stringent control of calorie and nutrient availability [44]. To identify such housekeeping longevity pathway(s) operating in chronologically aging yeast, we carried out a chemical genetic screen for anti-aging small molecules that can extend longevity even under CR conditions [44]. We thought that, because under CR conditions the adaptable pro-aging pathways would be fully suppressed and the adaptable anti-aging pathways would be fully activated, a chemical compound that can further increase yeast chronological lifespan could target the housekeeping longevity pathway(s) that we were trying to identify. Our chemical genetic screen identified lithocholic acid (LCA), a bile acid, as one of these compounds. One of the major objectives of studies described in my thesis was to establish molecular and cellular mechanisms underlying the potent longevity-extending effect of LCA. Because our unpublished findings imply that LCA enters yeast cells and accumulates mainly in the inner mitochondrial membrane (Beach et al., manuscript in preparation), in studies described in my thesis we investigated how this bile acid influences mitochondrial membrane lipidome and how it impacts the composition of mitochondrial respiratory complexes and supercomplexes.

1.4 Mechanisms underlying the essential role of lipid metabolism in longevity regulation

It is well established that the deposition and mobilization of neutral lipids and the maintenance of their intracellular homeostasis in evolutionarily distant organisms play a pivotal role in longevity regulation [166 - 169]. However, the molecular and cellular mechanisms linking the metabolism of neutral lipids and longevity remain to be established. Recent studies carried out in our laboratory and findings by other researchers in the field suggest several of such mechanisms operating in chronologically aging yeast and multicellular eukaryotic organisms, as outlined in this section of my thesis.

The two major species of neutral lipids in yeast cells are triacylglycerols (TAG) and ergosteryl esters (EE); they are known to be deposited in so-called lipid bodies (LBs) [170 - 172]. TAG and EE are the main storage molecules for free fatty acids (FFA), diacylglycerols (DAG) and ergosterol (ERG). Lipolysis of TAG produces FFA, which then can be incorporated into newly synthesized phospholipids and cardiolipins (CL) or, alternatively, degraded through β-oxidation within yeast peroxisomes [171, 172]. Lipolysis of TAG also generates various DAG species, which (1) serve as building blocks for synthesis of the phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC); and (2) act as potent signaling molecules modulating a protein kinase C (PKC)-dependent signal transduction network that governs multiple stress response-related and longevity-defining processes [173, 174]. Lipolysis of EE in yeast cells not only generates FFA but also leads to the formation of ERG, a sterol lipid known for its important role in numerous essential functions of cellular membranes [170 - 172,

175, 176]. In sum, a fine-tuned modulation of the biosynthesis and degradation of the neutral lipids TAG and EE plays a pivotal role in regulating numerous processes in yeast cells, including (1) energy homeostasis; (2) phospholipid and cardiolipin biosynthesis; (3) neutralization of excessive amounts of membrane-perturbing species of FFA, ERG and TAG; (4) the FFA- and DAG-induced lipoapoptotic and necrotic modes of cell death; and (5) a DAG-modulated, PKC-dependent signal transduction network that governs multiple stress response- and longevity-related processes [170 - 176]. All these cellular processes are known for their essential roles in defining longevity of yeast and multicellular eukaryotic organisms [166 - 169, 173, 174, 177 - 179].

As in cells of higher eukaryotes, neutral lipids in yeast cells are synthesized in the endoplasmic reticulum (ER) [171, 172, 175, 180]. Lysophosphatidic acid (LPA) is the earliest precursor lipid molecule in the biosynthesis of TAG, a process that progresses through several acylation and dephosphorylation steps. The two alternative ways of synthesizing LPA include (1) acylation of glycerol-3-phosphate in a reaction catalyzed by either Gpt2p or Sct1p protein; and (2) acylation of dihydroxyacetone phosphate (DHAP), also in a Gpt2p- or Sct1p-dependent manner, followed by formation of DHAP from 1-acyl-DHAP in a reaction catalyzed by Ayr1p [171, 172, 175, 180]. Following a transfer of an acyl group to the *sn*-2 position of LPA and the resulting formation of phosphatidic acid (PA), PA is dephosphorylated by one of three dephosphorylases, Pah1p, Dpp1p or Lpp1p, to form DAG. An alternative way of synthesizing DAG consists in the removal of the head group-phosphate from a glycerophospholipid molecule [171, 172, 175, 180]. Acylation of the *sn*-3 position in a DAG molecule, either with acyl-CoA (in a reaction catalyzed by Dga1p, Are1p or Are2p) or with an acyl group transferred from a

glycerophospholipid (in a reaction catalyzed by Lro1p), ultimately results in TAG formation. The synthesis of EE, another neutral lipid in yeast cells, is confined to the ER and involves the transfer of an acyl group from acyl-CoA to ERG in a reaction catalyzed by Are1p or Are2p [171, 172, 175, 180].

The degradation of TAG in yeast cells is confined to LBs, where this neutral lipid undergoes lipolysis catalyzed by the Tgl2p, Tgl3p, Tgl4p and Tgl5p lipases [171, 172, 175, 180]. EE is degraded either within the plasma membrane in a Yeh2p-catalyzed reaction or within LBs in a reaction driven by the Tgl1p lipase [171, 172, 175, 180].

A body of evidence supports the view that the accumulation and lipolytic degradation of neutral lipids in LBs of fat storage tissues define longevity of multicellular eukaryotic organisms [181 - 183]. LBs have been found in most eukaryotic and some prokaryotic cells; to reflect differences in their origin, biochemical content, morphology and other features observed in different biological systems, LBs are also often called lipid droplets, lipid particles and oil bodies [184, 185].

Until recently, LBs have been considered as relatively inert organellar compartments whose only function is to store excess energy, FFA, phospholipids and sterols that are deposited mainly in the forms of TAG and steryl esters [184, 186 - 189]. In LBs, these neutral lipids form a dense core, which is surrounded by a monolayer of phospholipids and associated proteins. The neutral lipid core of LBs can be hydrolyzed in a regulated fashion by several types of lipases, thereby enabling a retrieval of stored FFA, phospholipids and sterols that then can be used to (1) provide energy via fatty acid oxidation during times of nutrient scarcity; (2) maintain the homeostasis of membrane lipids during cell growth and division; and (3) modulate the levels of free sterols inside

and outside of the cell [184 - 189].

Recent findings contradict a traditional view of LBs as relatively static organellar compartments that function only in the deposition of neutral lipids. A growing body of evidence implies that LBs constitute dynamic organellar compartments whose protein and lipid composition, *de novo* formation from the ER template, growth, fragmentation, shrinkage due to lipolysis, movement, and association with other organelles are regulated by numerous proteins and support essential processes within a cell [187, 189, 190 - 202]. Hence, the new paradigm is that LBs are dynamically integrated into vital cellular processes and can serve both as an intracellular signaling compartment and as an organizing platform orchestrating these processes. For example, studies in yeast revealed that the phosphorylation and activation of the LBs-associated lipase Tgl4p by cyclin-dependent kinase 1 is required for mobilization of LBs-deposited TAG, thereby providing fatty acids and phospholipids for the secretory vesicle-dependent bud formation that stimulates the G1/S phase transition to enable cell-cycle progression [203]. Furthermore, it has been demonstrated that LBs in white adipose tissue (WAT) of mice modulate the synthesis and secretion of adipokines and the lipokine C16:1n7-palmitoleate, thereby being integrated into a regulatory network that maintains metabolic homeostasis whose impairment is associated with obesity, insulin resistance, type 2 diabetes, atherosclerosis and inflammatory disorders [195, 197, 204 - 208]. Moreover, LBs have been shown to play important roles in the regulation of many vital processes within mammalian cells by recruiting proteins from other cellular locations, temporally housing them, managing their availability and modulating their activities [187, 197, 201]. Specifically, the recruitment of certain proteins to LBs causes their conversion into an organizing platform that (1)

removes excess proteins from other organellar compartments, inactivates these proteins and/or stores them for later use; (2) promotes the refolding of unfolded proteins targeted to LBs by recruiting molecular chaperones from the cytosol; (3) provides a surface for the ordered degradation of partially unfolded and misfolded LBs-bound proteins that otherwise can form toxic aggregates in the cytosol; and (4) delivers some LBs-associated proteins to their target organellar compartments via permanent or transient contact sites [187, 197, 201, 209 - 211].

It should be emphasized that recent findings in evolutionarily distant organisms unveiled the essential roles for LBs in longevity regulation. For example, it has been shown that various interventions that inhibit the deposition of neutral lipids in LBs or activate their lipolytic degradation in fat storage tissues of the nematode *C. elegans*, the fruit fly *Drosophila melanogaster* or laboratory mice also extend their longevity [212 - 217]. In *C. elegans*, certain humoral signals produced and secreted by somatic cells in the gonad stimulate nuclear import of the forkhead transcription factor DAF-16 in intestinal cells, known to function as the principal site of neutral lipid storage in this organism [212]. Following its translocation into the nucleus, DAF-16 has been shown to induce the expression of a gene encoding a specific lipase, thereby promoting the lipolysis of neutral lipids stored in LBs of intestinal cells and extending *C. elegans* longevity by a mechanism that remains to be defined [212]. Furthermore, the elimination of LBs-associated lipase Brummer in the fat body of flies or the abolition of ATGL (adipose triacylglycerol lipase) in WAT of mice has been demonstrated to shorten their lifespans [213, 214]. Moreover, the elimination of WAT-specific insulin receptor or the replacement of the C/EBPα (CCAAT/enhancer-binding protein) protein with its

paralogue C/EBPβ has been shown to extend longevity in mice by greatly reducing the levels of neutral lipids stored in WAT [215 - 217].

As it has been discussed in section 1.3 of this thesis, a CR diet extends longevity in a wide spectrum of organisms and delays the onset of age-related diseases in mice and rhesus monkeys. The longevity-extending effects of this low-calorie diet in worms and flies are mediated by sirtuins, a family of NAD^+-dependent protein deacetylases and ADP ribosylases [204, 216]. In mice, a CR diet is known to elevate the level of the sirtuin SIRT1, which has been shown to repress transcription of genes essential for the LBs-confined accumulation of neutral lipids in WAT [204, 218]. Based on these findings, it has been proposed that the beneficial effect of CR on longevity in mice is due in part to elevated abundance of SIRT1, which can cause longevity extension by shifting a balance between the opposing processes of LBs' formation and degradation in WAT towards lipolytic degradation of neutral lipids in this fat storage tissue [204, 218].

In sum, these findings strongly support the view that the deposition and lipolytic degradation of neutral lipids confined to LBs in fat storage tissues define longevity of multicellular eukaryotic organisms. Recent studies suggested the following three mechanisms by which a delicate balance between the opposing processes of neutral lipids accumulation and their lipolytic degradation can regulate longevity in these organisms.

First, the size of LBs in WAT of mice has been proposed to define the efficacy with which the ER in this tissue can synthesize and despatch for secretion various anti-hyperglycaemic and pro-hyperglycaemic protein hormones known to control metabolic homeostasis in other tissues [204, 205, 219, 220]. Of note, LBs that accumulate in intestine (a fat storage tissue) of roundworms have been shown to contribute sterols for

the biosynthesis in the ER and subsequent secretion of the lipophilic hormones dafachronic acids and pregnenolone, both of which are known to delay cellular aging in other tissues [220]. It is likely therefore that the dynamics of deposition and lipolytic degradation of neutral lipids in LBs residing in fat storage tissues defines the efficacy with which these tissues can synthesize in the ER and then secrete humoral signals controlling longevity-extending and longevity-shortening processes in other tissues [181].

Second, it has been found that the lipolytic hydrolysis of neutral lipids deposited in LBs results in the release of the lipokine C16:1n7-palmitoleate, an unsaturated FFA, from WAT of mice [207]. C16:1n7-palmitoleate functions as a lipid hormone that regulates systemic carbohydrate and lipid homeostasis in muscle and liver by promoting muscle insulin sensitivity and attenuating hepatic steatosis [207]. It is conceivable therefore that, following its synthesis in and secretion by WAT of mice, C16:1n7-palmitoleate acts as a signaling molecule that plays an important role in longevity regulation [181].

Third, it has been shown that WAT of mice and the fat body of flies can respond to enhanced lipolysis of neutral lipids deposited in LBs residing in these tissues by activating certain regulatory networks known to stimulate mitochondrial biogenesis and β-oxidation of FFA in mitochondria [208, 217, 221]. The resulting decline of the levels of saturated FFA secreted by these fat storage tissues can reduce the risk of lipotoxicity in other tissues, thereby preventing muscle insulin resistance and hepatic steatosis [206 - 208, 222]. It should be stressed that the stimulation of mitochondrial biogenesis and β-oxidation of FFA in WAT of mice expressing the β paralogue of C/EBP has been shown to extend longevity [217]. It is likely therefore that this third mechanism, which is initiated in fat storage tissues, could play an important role in delaying cellular aging in

peripheral tissues and increasing longevity of the entire organism [181].

Longevity can be extended and health can be improved not only by genetic, dietary and pharmacological interventions that specifically modulate the age-related dynamics of changes in the deposition and mobilization of neutral lipids. Cardiolipin (CL), a dimeric glycerophospholipid residing mainly in the inner mitochondrial membrane, has been shown to modulate mitochondria-governed processes whose dysfunction underlies aging and age-related pathologies [223 - 230]. Therefore, the synthesis and stability of CL and other glycerophospholipids in the inner mitochondrial membrane are important targets of longevity-extending and health-improving interventions. CL contains three glycerol backbones and four acyl chains [223 - 230]. It is well established that various molecular species of this signature lipid of the inner mitochondrial membrane control a number of vital processes by: (1) being associated with all five complexes of the mitochondrial respiratory chain, modulating their activities and promoting their organization into supramolecular assemblies; (2) binding to and modulating the activity of cytochrome c, a heme-containing mobile component of the mitochondrial respiratory chain also known for its essential role in mitochondria-controlled apoptosis; (3) being associated with and regulating adenine nucleotide translocator activity; (4) playing an important role in maintaining the electrochemical gradient across the inner mitochondrial membrane; (5) conferring fluidity and stability to the inner membrane of mitochondria; (6) being primary targets of ROS-inflicted damage because of their highly unsaturated acyl chains; and (7) regulating protein import into mitochondria [223 - 230]. Because of its propensity to associate via non-covalent interactions with numerous unrelated proteins in the inner mitochondrial membrane, CL acts as a molecular chaperone that governs the assembly of

a number of protein assemblies involved in wide spectrum of essential biological processes [224 - 230]. In addition, the conversion of CL to phosphatidic acid (PA) is one of the processes driving mitochondrial fission and fusion [231 - 233].

Specific changes in the content and/or composition of various molecular forms of CL have been revealed in pathological states characteristic of various disorders; many of these disorders impair mitochondria-confined processes known to be modulated by CL. For example, by lowering total CL levels, elevating concentrations of a monolyso form of CL and reducing the levels of its unsaturated fatty acyl species, certain mutations in human gene for tafazzin (an enzyme involved in the remodeling of CL acyl chains) cause an X-linked recessive disorder called Barth Syndrome [223, 225, 228]. Clinically, the Barth Syndrome manifests itself as cardioskeletal myopathy, neutropenia and abnormal growth [223, 225]. Furthermore, characteristic features of early stages of aging in mice and humans include reduced levels of CL and the resulting accumulation of dysfunctional mitochondria [224, 225, 228]. Noteworthy, due to the high content of vulnerable to oxidative damage unsaturated fatty acids in CL and because of the proximity of CL to the sites of ROS generation in mitochondria, this signature lipid of the inner mitochondrial membrane exhibits high susceptibility to mitochondrially generated ROS - whose levels are known to increase progressively with age [224, 226 - 229]. Other disorders associated with certain changes in the content and/or composition of CL include Tangier disease, diabetic cardiomyopathy, dilated cardiomyopathy with ataxia and non-alcoholic fatty liver disease [225, 228].

The molecular and cellular mechanisms underlying the essential role of CL in regulating numerous mitochondrial processes that are affected in aging and age-related

pathologies remain to be determined. One of such mechanisms may involve the CL-driven modulation of mitochondrial membrane proteins called prohibitins. The prohibitins Phb1p and Phb2p are evolutionarily conserved proteins that form multimeric, high-molecular weight complexes with each other in the inner mitochondrial membrane; these large complexes of Phb1p and Phb2p are known for their essential scaffolding function in maintaining the organization and functional integrity of this membrane [227, 230, 234 - 237]. A body of evidence implies that prohibitins perform such scaffolding function by modulating activities of their numerous physically and/or functionally interacting protein partners, including: (1) proteins that are actively involved in the assembly of the respiratory chain; (2) proteins that are essential for both the maintenance of mitochondrial morphology and the assembly of β-barrel proteins in the outer membrane; (3) the *m*-AAA protease, an ATP-dependent protease with a critical role in protein quality control within the inner mitochondrial membrane; and (4) proteins that regulate, in a highly coordinated fashion, the levels of CL and the glycerophospholipid phosphatidylethanolamine (PE) [227, 230, 234 - 237]. One of the major objectives of my studies described in this thesis was to establish molecular mechanisms underlying the ability of LCA, a natural longevity-extending compound, to delay yeast chronological aging by influencing the concentrations of CL (and, perhaps, of other mitochondrial membrane glycerophospholipids) in the inner mitochondrial membrane.

1.5 Mitochondrial electron transfer chain (ETC) and mitochondrial theory of aging

The mitochondrion is a versatile organelle that houses the synthesis of ATP via the

oxidative phosphorylation pathway and modulates the progression of programmed apoptotic cell death, thereby governing a plethora of cellular activities that are vital for a multicellular eukaryotic organism [238 - 241]. Initiated as early as in the beginning of 1950s, studies aimed at understanding the importance of mitochondria in maintaining essential cellular processes provided the comprehensive evidence for the fundamental role of these organelles in regulating longevity of evolutionarily distant organisms [238, 240]. For example, studies in chronologically aging yeast revealed that, by stimulating mitochondrial respiration, increasing the mitochondrial membrane potential and promoting mitochondrial ROS formation during logarithmic growth phase, a CR diet and some pharmacological interventions (including LCA) extend longevity by causing changes in a distinct set of longevity-defining processes during the subsequent diauxic, post-diauxic and stationary phases [25, 44, 46 - 49, 182]. Moreover, studies in the nematode *C. elegans* demonstrated that the efficacy of mitochondrial coupled respiration, the value of the electrochemical potential across the inner mitochondrial membrane and the extent of mitochondrial ATP production early in life, during the L3/L4 larval stage, define longevity by (1) causing the establishment of a specific configuration of the longevity-defining metabolic network later in life, during adulthood [51 - 56]; and (2) activating a retrograde signalling pathway that in response to mild mitochondrial impairment and stress during the L3/L4 larval stage activates expression of the mitochondria-specific unfolded protein response (UPR^{mt}) genes in the nucleus, thereby stimulating synthesis of a subset of UPR^{mt} proteins known for their essential roles in longevity extension [57, 58]. In sum, by defining the efficacy of oxidative phosphorylation, electrochemical potential, ATP synthesis and ROS production in

mitochondria, the electron transport chain (ETC) in the inner mitochondrial membrane plays a pivotal role in regulating longevity of organisms across phyla.

Two models have been proposed for the organization of the mitochondrial ETC. One of these models, known as the random diffusion model or the fluid model, suggests that respiratory complexes move in a random fashion within the inner mitochondrial membrane [242]. Another model, which is currently accepted by most researchers in the field of mitochondrial bioenergetics, is known as the solid model. This model suggests that the respiratory complexes are assembled into larger supercomplexes within the inner mitochondrial membrane; these larger supramolecular structures are also called respirasomes [243 - 252]. Several important advantages of the supramolecular organization of respiratory complexes into supercomplexes have been proposed, including substrate channeling, catalytic enhancement and sequestration of reactive intermediates [243, 245 - 249, 252]. Substrate channeling is an efficient and beneficial process because it guides various intermediates of a biochemical process from one enzyme to another specific enzyme, thereby protecting every intermediate from being trapped by other enzymes; a body of evidence imply that this advantage is applicable to a mobile form of cytochrome c in yeast, bacterial and mammalian ETCs [243, 247, 248, 251]. Thus, a supramolecular arrangement of individual respiratory complexes into supercomplexes/respirasomes enables a substantial catalytic enhancement of the ETC in the inner mitochondrial membrane [243 - 252].

It is well established that the first component of the mitochondrial ETC, NADH dehydrogenase (complex I) is responsible for the transfer of two electrons from NADH to a lipid-soluble carrier, ubiquinone (CoQ) [238, 240, 241, 253]. This process is

accompanied by the shuttling of four protons from the mitochondrial matrix to the intermembrane space [240, 252, 253]. Succinate dehydrogenase (complex II) uses FAD to transfer two more electrons from succinate to the CoQ pool in the inner mitochondrial membrane [240, 241, 245, 253]. The cytochrome bc1 complex (complex III) then removes two electrons from the fully reduced CoQH2 at the Q0 site and carries them over to cytochrome c in the intermembrane space [240, 248 - 250, 253]. The other two electrons are carried over to the Qi site, where they are used to reduce the fully oxidized CoQ. In addition, complex III allows a shuttling of two protons to the intermembrane space as well as a pumping of two proteins from a full CoQ cycle [240, 242, 249, 253]. Cytochrome c oxidase (complex IV) then removes electrons from the reduced cytochrome c molecules in the exoplasmic face of the mitochondrial membrane and transfers them to oxygen in the mitochondrial matrix through a series of electron transfers to produce water [240, 253]. Complex IV also allows the translocation of two additional protons out of the matrix [241, 253]. The efflux of ten protons per a single reduced NADH molecule creates an electrochemical gradient that provides energy for ATP synthesis by the F0F1 complex (Complex V) in a process known as oxidative phosphorylation [240 -242, 249, 253].

It is well established that ROS, mainly produced as a by-product of the electron transfer through mitochondrial respiratory chain, play a pivotal role in longevity regulation in organisms across phyla [238, 240, 241, 253]. In 1972, Denham Harman proposed the mitochondrial free radical theory of aging, which was inspired by a body of evidence for the progressive accumulation of mitochondrially generated ROS with cell and organismal age [254]. This theory was based on numerous findings convincingly

demonstrated ROS toxicity in living cells [255] and on the discovery that the ROS-generating respiratory chain is confined solely to the inner mitochondrial membrane [256, 257]. Most of the known targets for the oxidative macromolecular damage caused by ROS are mitochondrial proteins, DNA and lipids, including (1) aconitase, a [4Fe-4S] cluster enzyme of the tricarboxylic acid (TCA) cycle; (2) Lys4p, a [4Fe-4S] cluster enzyme of lysine biosynthesis that takes place in the mitochondrion; (3) succinate dehydrogenase (SDH), a [3Fe-3S] cluster- and heme-containing enzyme of the TCA that also functions as complex II of the ETC in the inner mitochondrial membrane; (4) cytochrome c, a heme-containing mobile component of the mitochondrial ETC; (5) cytochrome c oxidase, a heme-containing complex IV of the mitochondrial ETC; (6) the mitochondrial DNA (mtDNA) nucleoid that, in contrast to chromatin in the nucleus, lacks histones; and (7) saturated fatty acids of membrane lipids [182, 240, 253, 258 - 261]. According to the mitochondrial free radical theory of aging, the gradual accumulation of macromolecular damage caused by mitochondrially produced ROS throughout lifespan accelerates cellular dysfunction and later in life leads to a functional decline and increased mortality [254, 255]. Although a body of evidence does not validate the core statement of this theory on a casual role of ROS generation in aging, the importance of ROS in mediating a longevity-extending stress response to age-related cellular damage is supported by numerous findings [262 -267]. Indeed, while high intracellular and intraorganellar levels of ROS can cause oxidative damage to various cellular constituents, low concentrations of intracellular ROS can activate cellular processes that protect cellular macromolecules from oxidative damage [182, 240, 253, 262 - 267]. Furthermore, such low concentrations of ROS activate a protein team that protects the cell from an age-

related form of programmed apoptotic cell death [182, 240, 253, 262 - 267]. A term "hormesis" has been coined to describe longevity extension in response to a mild stress caused by low levels of mitochondrially produced ROS [262 - 267].

One of the major objectives of studies described in my thesis was to investigate how LCA, a natural anti-aging compound, as well as genetic interventions altering the synthesis of CL and other glycerophospholipids in the inner mitochondrial membrane influence the supramolecular organization of mitochondrial ETC. Our recent unpublished findings suggested that LCA could modulate the stoichiometry, composition and/or functional state of respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane, perhaps by altering the level of CL and other glycerophospholipids within this membrane (Koupaki et al., manuscript in preparation). We hypothesized that such LCA- and CL-dependent remodeling of the mitochondrial respiratory supercomplexes (1) may rely on a distinct set of protein components of the respiratory complexes that compose the respirasomes; (2) may alter the age-dependent dynamics of mitochondrial respiration, oxidative phosphorylation, ADP/ATP exchange, ATP synthesis, membrane potential and/or ROS generation; (3) may occur in an age-dependent fashion; and (4) may extend longevity by increasing the efficiency of ATP synthesis in chronologically aging yeast and/or specifically altering the age-dependent dynamics of changes in mitochondrially produced ROS, thereby protecting yeast from chronic oxidative stress.

1.6 Thesis outline and contributions of colleagues

Chapter 2 of my thesis describes how we used mass spectrometry (MS)-based lipidomics to elucidate the effect of LCA on the repertoire and quantities of membrane lipids in

mitochondria that were purified from wild-type (WT) strain and from various long- and short-lived mutant strains impaired in different aspects of cardiolipin (CL) and phosphatidylethanolamine (PE) metabolism. By correlating the effects of LCA on the age-related dynamics of changes in the composition and quantities of membrane lipids in mitochondria of these strains to the effects of this anti-aging compound on their chronological lifespan, we concluded that under caloric restriction (CR) conditions LCA extends yeast longevity by remodeling the composition of mitochondrial membrane lipids and thereby modulating longevity-defining processes confined to and governed by mitochondria. Specifically, findings described in Chapter 2 strongly suggest that LCA extends longevity of WT yeast by (1) elevating the level of phosphatidylserine (PS; a precursor for the synthesis of PE in mitochondria) in the mitochondrial membrane, thereby enhancing its positive effect on longevity-defining processes in this membrane; (2) reducing the level of PE in the mitochondrial membrane, thereby weakening its negative effect on longevity-defining processes in this membrane; and (3) proportionally decreasing the levels of PE and CL in the mitochondrial membrane, thereby increasing PS/CL and PS/PE ratios but maintaining PE/CL ratio of mitochondrial membrane lipids and causing some longevity-extending changes in this membrane. These LCA-induced alterations in mitochondrial membrane lipids can satisfactorily explain the observed implications of LCA treatment on mitochondrial structure and function, including (1) the ability of LCA to cause dramatic changes in the length and curvature of the inner mitochondrial membrane; and (2) the ability of LCA to activate protein machines involved in mitochondrial respiration, the maintenance of mitochondrial membrane potential, ROS production in mitochondria and mitochondrial fusion.

Based on our findings described in Chapter 2 of my thesis and data of other researchers working in the field of mitochondrial biology, we hypothesized that, by altering the level of CL and other glycerophospholipids within the mitochondrial membrane, LCA could modulate the stoichiometry, composition and/or functional state of respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane. To test the validity of this hypothesis, in studies described in Chapter 3 of my thesis we used a multistep method for recovery of intact respiratory complexes and supercomplexes from purified yeast mitochondria, their subsequent first-dimension electrophoretic separation using so-called blue-native gel electrophoresis (BN-PAGE), their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the mass spectrometry (MS)-based identification of each of these individual protein components. Findings described in this chapter of my thesis validate our hypothesis. Specifically, these findings revealed several ways of rearranging respiratory supercomplexes in the inner mitochondrial membrane of cells exhibiting altered mitochondrial membrane lipidome in response to LCA treatment or genetic manipulations impairing the synthesis of CL and other glycerophospholipids within the inner membrane of mitochondria. First, by altering the level of CL and other glycerophospholipids synthesized and residing in the inner mitochondrial membrane, LCA modulates the abundance of several major respiratory supercomplexes (respirasomes) in this membrane. Second, LCA- and genetic manipulations-driven changes in the inner mitochondrial membrane lipidome cause a recruitment of a number of new mitochondrial protein components, not previously known for being permanently associated with the ETC, into the remodeled respirasomes. Importantly, many of the

proteins newly recruited into the remodeled respirasomes are known for their essential roles in mitochondria-confined processes that define longevity.

Findings described in Chapter 2 are presented in the manuscript of a paper [Bourque, S.D.*, Koupaki, O.*, Richard, V.R., Beach, A., Burstein, M.T., Kyryakov, P. and Titorenko, V.I. Lithocholic acid extends yeast longevity in part by altering the composition of mitochondrial membrane lipids; * *Equally contributed first co-authors*] that is currently in preparation for submission to *Current Biology*. I expect this manuscript to be submitted for publication in late September or early October of 2012. I carried out more than 30% of the work described in this publication and prepared the first draft of sections relevant to my work. I am an equally contributed first co-author on this publication. Dr. V. Titorenko provided intellectual leadership of this project and is currently editing the manuscript.

All abbreviations, citations, and the numbering of figures and tables that have been used in the published papers and in the manuscripts in preparation have been changed to the format of this thesis.

2 Lithocholic acid (LCA) extends longevity of chronologically aging yeast by altering the composition of mitochondrial membrane lipids

2.1 Abstract

Recent studies in our laboratory demonstrated that in chronologically aging yeast grown under CR conditions LCA, a natural anti-aging compound, alters the age-related dynamics of changes in mitochondrial abundance and morphology, respiration, membrane potential, and ROS production. Cardiolipin (CL), a dimeric glycerophospholipid that is synthesized and almost exclusively localized in the inner mitochondrial membrane, has been shown to modulate mitochondria-governed processes whose dysfunction underlies aging and age-related pathologies [223 - 230]. Phosphatidylethanolamine (PE) is another glycerophospholipid that is almost exclusively synthesized in the inner mitochondrial membrane, from which it is then distributed to various other cellular membranes [268, 269]. Hence, it is likely that the synthesis and stability of CL and, perhaps, PE in the inner mitochondrial membrane are important targets of longevity-extending and health-improving interventions. Because of the plausible importance of mitochondrially synthesized CL and PE in the longevity-extending effect of LCA, other graduate students in our laboratory elucidated how mutations eliminating nucleus-encoded mitochondrial proteins involved in the synthesis of CL and PE within the inner mitochondrial membrane influence the lifespan-extending efficacy of LCA in chronologically aging yeast grown under CR conditions. The results of this genetic analysis suggested that the synthesis of both these membrane lipids in mitochondria plays an essential role in the ability of LCA to extend longevity of yeast

placed on a CR diet. All these findings prompted us to elucidate how LCA influences the composition of mitochondrial membrane lipids in chronologically aging yeast grown under CR conditions. To attain this objective, in experiments described in this chapter of my thesis we used mass spectrometry (MS)-based lipidomics to elucidate the effect of LCA on the repertoire and quantities of membrane lipids in mitochondria that were purified from wild-type (WT) strain and from various long- and short-lived mutant strains impaired in different aspects of CL and PE metabolism. By correlating the effects of LCA on the age-related dynamics of changes in the composition and quantities of membrane lipids in mitochondria of these strains to the effects of this anti-aging compound on their chronological lifespan, we concluded that under CR conditions LCA extends yeast longevity by remodeling the composition of mitochondrial membrane lipids and thereby modulating longevity-defining processes confined to and governed by mitochondria. Specifically, findings described here strongly suggest that LCA extends longevity of WT yeast by (1) elevating the level of phosphatidylserine (PS; a precursor for the synthesis of PE in mitochondria) in the mitochondrial membrane, thereby enhancing its positive effect on longevity-defining processes in this membrane; (2) reducing the level of PE in the mitochondrial membrane, thereby weakening its negative effect on longevity-defining processes in this membrane; and (3) proportionally decreasing the levels of PE and CL in the mitochondrial membrane, thereby increasing PS/CL and PS/PE ratios but maintaining PE/CL ratio of mitochondrial membrane lipids and causing some longevity-extending changes in this membrane. It is important to emphasize that these LCA-induced alterations in mitochondrial membrane lipids can satisfactorily explain the observed implications of LCA treatment on mitochondrial

structure and function, including (1) the ability of LCA to cause dramatic changes in the length and curvature of the inner mitochondrial membrane; and (2) the ability of LCA to activate protein machines involved in mitochondrial respiration, the maintenance of mitochondrial membrane potential, ROS production in mitochondria and mitochondrial fusion.

2.2 Introduction

It is well established that various molecular species of CL, a signature lipid of the inner mitochondrial membrane, control a number of vital processes by: (1) being associated with all five complexes of the mitochondrial respiratory chain, modulating their activities and promoting their organization into supramolecular assemblies; (2) binding to and modulating the activity of cytochrome c, a heme-containing mobile component of the mitochondrial respiratory chain also known for its essential role in mitochondria-controlled apoptosis; (3) being associated with and regulating adenine nucleotide translocator activity; (4) playing an important role in maintaining the electrochemical gradient across the inner mitochondrial membrane; (5) conferring fluidity and stability to the inner membrane of mitochondria; (6) being primary targets of ROS-inflicted damage because of their highly unsaturated acyl chains; and (7) regulating protein import into mitochondria [223 - 230]. Because of its propensity to associate via non-covalent interactions with numerous unrelated proteins in the inner mitochondrial membrane, CL acts as a molecular chaperone that governs the assembly of a number of protein assemblies involved in wide spectrum of essential biological processes [224 - 230]. In

addition, the conversion of CL to phosphatidic acid (PA) is one of the processes driving mitochondrial fission and fusion [231 - 233].

Specific changes in the content and/or composition of various molecular forms of CL have been revealed in pathological states characteristic of various disorders; many of these disorders impair mitochondria-confined processes known to be modulated by CL [223 - 225, 228]. Furthermore, characteristic features of early stages of aging in mice and humans include reduced levels of CL and the resulting accumulation of dysfunctional mitochondria [224, 225, 228]. Noteworthy, due to the high content of vulnerable to oxidative damage unsaturated fatty acids in CL and because of the proximity of CL to the sites of ROS generation in mitochondria, this signature lipid of the inner mitochondrial membrane exhibits high susceptibility to mitochondrially generated ROS - whose levels are known to increase progressively with age [224, 226 - 229]. Moreover, we recently found that a longevity-extending CR diet reduces the level of CL in the mitochondrial membrane of chronologically aging yeast [25]. In sum, it is quite likely that the synthesis and stability of CL (and, perhaps, PE and other glycerophospholipids) in the inner mitochondrial membrane play an essential role in regulating various mitochondria-confined processes whose dysfunction underlies aging and age-related pathologies. However, the molecular mechanisms underlying such essential role for CL and other mitochondrial membrane glycerophospholipids in modulating longevity-defining mitochondrial processes are yet to be established. As a first step towards defining these mechanisms, in experiments described in this chapter of my thesis we used MS-based lipidomics to elucidate the effect of LCA on the composition and quantities of membrane lipids in mitochondria purified from WT strain and from various long- and short-lived

mutant strains impaired in different aspects of the metabolism of CL and other mitochondrial membrane glycerophospholipids.

2.3 Materials and Methods

Strains and media

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and mutant strains *ups1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ups1Δ::kanMX4*), *taz1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 taz1Δ::kanMX4*), *gep1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gep1Δ::kanMX4*), *psd1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 psd1Δ::kanMX4*) and *crd1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 crd1Δ::kanMX4*) were used in this study. Yeast were grown in in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose as carbon source. 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.

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 *S. cerevisiae* 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.

Analysis of lipids by mass spectrometry

4 ml of SEM buffer were added to a 1-ml sucrose density gradient peak fraction containing highly purified mitochondria. The mitochondria were pelleted by centrifugation in a Beckman MLS50 swinging-bucket rotor at 100,000 × g for 30 min at 4ºC. The lipids were extracted by resuspending the pellet of mitochondria in 3.8 ml of chloroform - methanol - water (1:2:0.8) and by vortexing the suspension with glass beads three times for 1 min. 1 ml of chloroform was then added, and the extract was incubated for 5 min with occasional mixing. 1 ml of water was then added, and the extract was incubated for 5 min with occasional mixing. The extract was centrifuged and the entire supernatant was collected. The pellet was resuspended in 1.6 ml of chloroform, and the lipids were extracted by vortexing the suspension with glass beads two times for 1 min. The extract was then centrifuged and the entire supernatant was collected. The pooled supernatants were centrifuged and the organic phase was collected. The lipid extract was dried under nitrogen. The lipid film was dissolved in 400 µl of chloroform - methanol (1:1) containing either 3 mM ammonium hydroxide for positive-ion analyses or no additive for negative-ion measurements.

Mass spectrometric analyses were performed with a Micromass Q-TOF 2 mass spectrometer that was used in the single-stage mass spectrometry mode for positive- or negative-ion analyses as follows:

1. Prior to injection, combine 10 µl of a sample with 10 µl of the standard mix of

lipids in 200 µl of methanol - chloroform (1:1) with 0.1% ammonium hydroxide. The standard to sample ratio can be changed as needed.

2. Resolve lipids using a Micromass Q-TOF 2 mass spectrometer equipped with a nano-electrospray source operating at a flow rate of 1 µl/min. Although we have taken advantage of the high resolution of a Q-TOF type of mass spectrometer, it is not a mandatory requirement. Exact instrument parameters will vary from instrument to instrument. See Table 2.1 for the settings for a Micromass Q-ToF 2 (Waters, Milford, MA, USA) equipped with a nano-electrospray source. To analyze PC and TAG, the cone voltage was set to 28 v and the capillary voltage to 3.0 kv in positive mode. For the analysis of FFA, CL, PE, PS, PA and PI, the cone voltage was set to 30 v and the capillary voltage to 3.2 kv in negative mode. In both modes, the collision gas was set to 10 (arbitrary units). Survey MS data were acquired for up to 3 minutes or until the ion count of the standards was above 2×10^3.

Table 2.1. Instrument settings for a Micromass Q-ToF 2 (Waters, Milford, MA, USA) equipped with a nano-electrospray source.

	Flow rate	Cone voltage	Capillary voltage	Collision gas
Positive mode	1µl/min	-28 v	3.0 kv	10
Negative mode	1µl/min	30 v	-32 kv	10

For quantification of individual lipid molecular species, the commercially available lipids with non-natural fatty acid compositions were used as internal standards that are

known to be absent in yeast cells (see Table 2.2). Various molecular forms of the glycerophospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA), as well as of cardiolipin (CL), were from Avanti Polar Lipid (Alabaster, AL, USA). Different species of free fatty acids (FFA) and triacylglycerols (TAG) were from Larodan (Malmo, Sweden).

Table 2.2. Internal lipid standards, their concentrations in the standard mix and the MS mode for their analysis.

Lipid class	Standard chain composition	Mass of standard	Concentration (μg/ml)	MS mode
Phosphatidic acid	14:0/14:0	591.40	100	Negative
Phosphatidylethanolamine	14:0/14:0	634.45	200	Negative
Phosphatidylinositol	N/A	N/A	N/A	Negative
Phosphatidylserine	14:0/14:0	622.37	40	Negative
Cardiolipin	4×14:0	619.92	100	Negative
Free fatty acids	19:0	297.28	100	Negative
Phosphatidylcholine	14:0/14:0	650.48	100	Positive
Triacylglycerols	13:0/13:0/13:0	698.63	200	Positive

After acquisition, the mass spectra were smoothed and background subtracted and centred using the MassLynx software package (Waters, Milford, MA, USA). The peak list was then imported into Microsoft Excel for deconvolution and deisotoping. The list

was first searched using equation 2.1 to determine the peaks of interest. The resulting peak list was then deconvoluted and deisotoped using a lookup table, which represented the relative intensities of the isotope peaks. The peak list was first scanned specifically for CL species and, using the I_1 isotope, the contribution of these lipid species to isobaric peaks was determined and subtracted. The peak list was then scanned for other lipid classes from low to high mass, the peaks deisotoped and the contribution to isobaric isotope peaks subtracted. Peak intensities were then normalized to the internal standard of the appropriate lipid class. The molecular forms of various lipid classes detected following lipid extraction from whole yeast cells are listed in Table 2.3.

Equation 2.1. The following Excel function was used to search the column LookUpVector and return the corresponding value in the column ResultsVector, which are within the range LookUpValue +/- Error:

=IF(ISNA(LOOKUP(1,1/(ABS(LookUpVector-LookUpValue)
<=Error),ResultsVector)),0,LOOKUP(1,1/(ABS(LookUpVector-LookUpValue)
<=Error),ResultsVector))

Table 2.3. The molecular forms of various lipid classes detected following lipid extraction from whole yeast cells and their exact masses.

Lipid Class	Chain Composition	Mass
PA	38:0	731.55
	38:1	729.54

	38:2	727.52
	38:4	723.49
	36:0	703.52
	36:1	701.51
	36:2	699.49
	34:0	675.49
	34:1	673.48
	34:2	671.46
	32:0	647.46
	32:1	645.44
	32:2	643.43
	30:0	619.43
	30:1	617.42
	30:2	615.40
	28:0	591.40
	28:1	589.39
	28:2	587.37
PE	38:0	774.60
	38:1	772.59
	38:2	770.57
	38:4	766.54
	36:0	746.57
	36:1	744.55
	36:2	742.53
	34:0	718.53
	34:1	716.52
	34:2	714.51
	32:0	690.51
	32:1	688.49
	32:2	686.48
	30:0	662.48

	30:1	660.46
	30:2	658.45
	28:0	634.45
	28:1	632.43
	28:2	630.41
	38:0	893.61
	38:1	891.60
	38:2	889.58
	38:4	885.55
	36:0	865.58
	36:1	863.56
	36:2	861.55
	34:0	837.55
	34:1	835.53
PI	34:2	833.52
	32:0	809.52
	32:1	807.50
	32:2	805.49
	30:0	781.49
	30:1	779.47
	30:2	777.46
	28:0	753.46
	28:1	751.44
	28:2	749.42
	38:0	790.56
	38:1	788.54
	38:2	786.53
PS	36:0	762.53
	36:1	760.51
	36:2	758.50
	34:0	734.50

	34:1	732.48
	34:2	730.47
	32:0	706.47
	32:1	704.45
	32:2	702.43
	30:0	678.43
	30:1	676.42
	30:2	674.40
	28:0	650.40
	28:1	648.39
	28:2	646.37
	26:0	622.37
FFA	C10:0	171.13
	C10:1	169.12
	C12:0	199.17
	C12:1	197.15
	C14:0	227.20
	C14:1	225.19
	C16:0	255.23
	C16:1	253.22
	C18:0	283.26
	C18:1	281.25
	C20:0	311.29
	C20:1	309.28
	C22:0	339.33
	C22:1	337.31
CL	56:0	619.92
	64:4	671.95
	64:3	672.96
	64:2	673.96
	64:1	674.97

	64:0	675.98
	66:4	685.96
	66:3	686.97
	66:2	687.98
	66:1	688.99
	66:0	689.99
	68:4	699.99
	68:3	700.99
	68:2	701.99
	68:1	703.00
	68:0	704.01
	70:4	713.99
	70:3	715.00
	70:2	716.01
	70:1	717.01
	70:0	718.02
	72:4	728.01
	72:3	729.02
	72:2	730.03
	72:1	731.0335
	72:0	732.0413
PC	38:0	818.6637
	38:1	816.6481
	38:2	814.6325
	36:0	790.6325
	36:1	788.6169
	36:2	786.6013
	34:0	762.6013
	34:1	760.5857
	34:2	758.5701
	32:0	734.5701

	32:1	732.5545
	32:2	730.5389
	30:0	706.5389
	30:1	704.5233
	30:2	702.5077
	28:0	678.5077
	28:1	676.4921
	28:2	674.4765
	26:0	650.4765
	39:0	698.6298
	42:0	740.6765
	48:0	824.7706
	48:1	822.755
	48:2	820.7394
	48:3	818.7238
	50:0	852.8018
	50:1	850.7862
	50:2	848.7706
	50:3	846.755
	52:0	880.833
TAG	52:1	878.8174
	52:2	876.8018
	52:3	874.7862
	54:0	908.8642
	54:1	906.8486
	54:2	904.833
	54:3	902.8174
	56:0	936.8954
	56:1	934.8798
	56:2	932.8642
	56:3	930.8486

58:0	964.9266
58:1	962.911
58:2	960.8954
58:3	958.8798
60:0	992.9578
60:1	990.9422
60:2	988.9266
60:3	986.911

2.4 Results

2.4.1 LCA alters the concentrations of membrane lipids and their relative abundance in mitochondria of WT cells

The individual steps of the partially overlapping biosynthetic pathways for CL and PE in the inner membrane of yeast mitochondria, as well as mitochondrial proteins catalyzing or regulating each of these steps, are well established (Figure 2.1) [268 - 273]. To assess a role of CL and other mitochondrial membrane lipids in the longevity-extending ability of LCA, other graduate students in our laboratory recently tested if LCA can increase the CLS of various single-gene-deletion mutants lacking nuclear genes that encode different mitochondrial proteins involved in the synthesis of CL and PE within the inner mitochondrial membrane (Figure 2.1). These studies revealed that mutations eliminating the Crd1p, Taz1p, Psd1p, Ups1p or Gep1p mitochondrial proteins involved in the synthesis of CL and PE within the mitochondrial inner membrane differentially alter the ability of LCA to extend the CLS of yeast grown under CR conditions on 0.2% glucose. Specifically, it was found that LCA is unable to extend the CLS of the *ups1*Δ mutant lacking a protein that regulates the biosynthesis of PG, CL and PE (Figure 2.1). It was

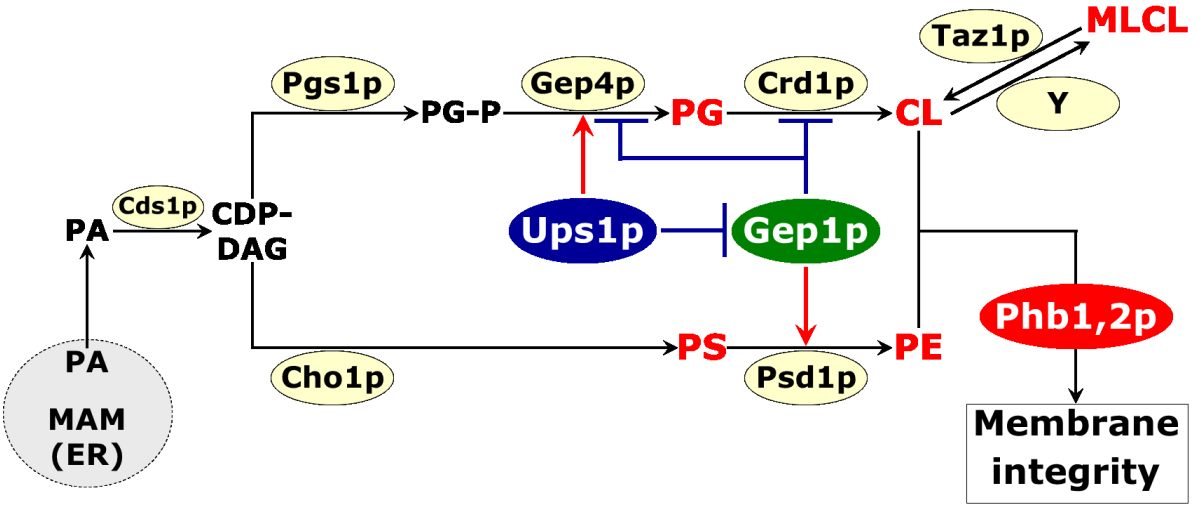

Figure 2.1. Outline of the biosynthesis of cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylserine (PS) from phosphatidic acid (PA) in the inner mitochondrial membrane. PA is delivered to the mitochondrial inner membrane from the mitochondria-associated membrane (MAM) domain of the endoplasmic reticulum (ER), where it is synthesized. PA is then converted to cytidine diphosphodiacylglycerol (CDP-DAG), which serves as a precursor for synthesis of both CL and PE in the inner mitochondrial membrane. It has been proposed that both CL and PE modulate the ability of prohibitins Phb1p and Phb2p - two evolutionarily conserved proteins that form large complexes with each other in the mitochondrial inner membrane - to maintain the organization and functional integrity of this membrane [268 - 273]. Other abbreviations: PG-P, phosphatidylglycerol phosphate; MLCL, monolysocardiolipin.

also found that LCA is unable to extend the CLS of the *taz1*Δ mutant, which lacks an enzyme catalyzing the conversion of MLCL to CL (Figure 2.1). Furthermore, these studies also revealed that the *gep1*Δ mutation, which eliminates a protein regulating the biosynthesis of CL and PE (Figure 2.1), enhances the ability of LCA to extend the CLS of yeast under CR conditions. Moreover, it was showed that the *psd1*Δ mutation, which eliminates an enzyme catalyzing the biosynthesis of PE (Figure 2.1), enhances the ability

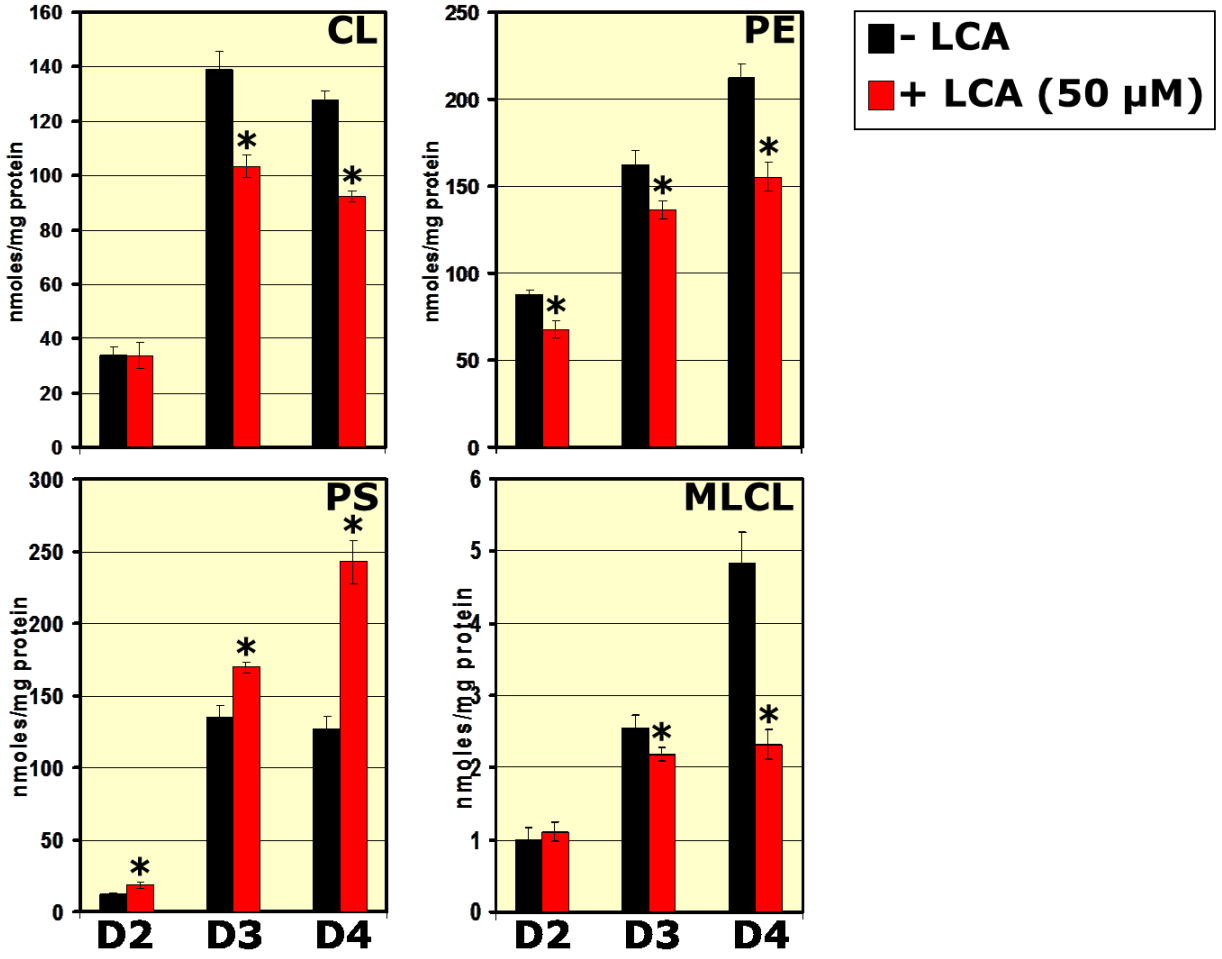

Figure 2.2. Effect of LCA on the age-related dynamics of changes in the total levels of CL, PE, PS and MLCL in the membranes of mitochondria purified from WT cells. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 µM), and collected for the purification of mitochondria at days 2, 3 or 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. *$p < 0.05$.

of LCA to extend longevity of chronologically aging yeast grown under CR conditions. In addition, these studies demonstrated that the *crd1*Δ mutation, which eliminates an enzyme catalyzing the biosynthesis of CL (Figure 2.1), enhances the ability of LCA to

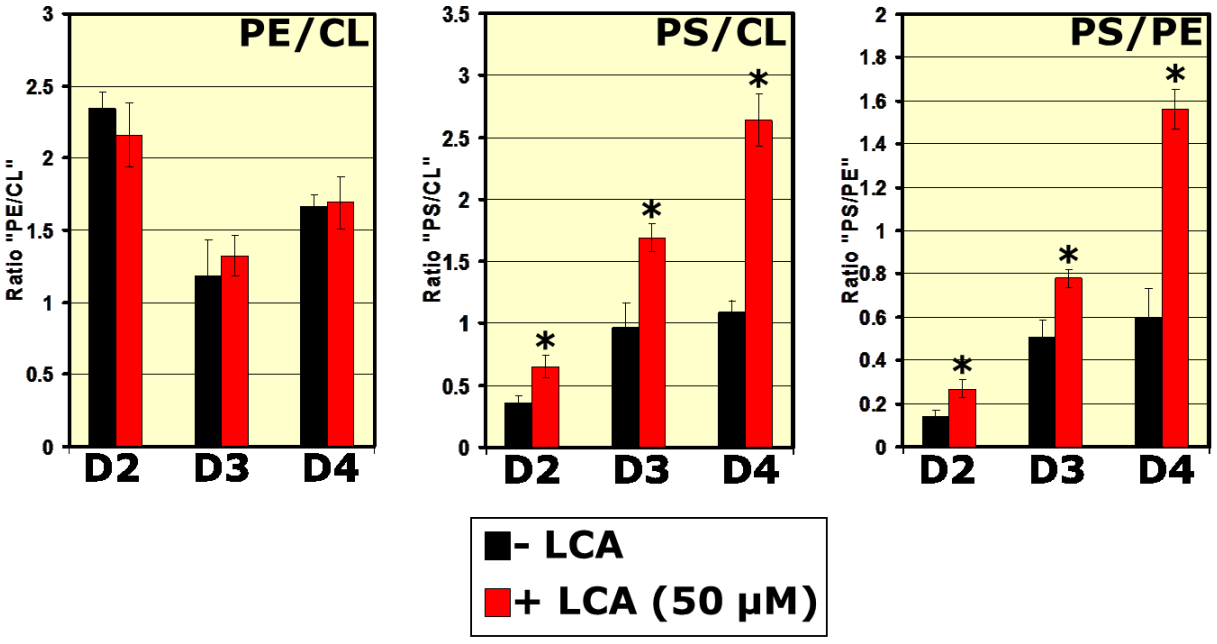

Figure 2.3. Effect of LCA on the age-related dynamics of changes in PE/CL, PS/CL and PS/PE ratios in the membranes of mitochondria purified from WT cells. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 µM), and collected for the purification of mitochondria at days 2, 3 or 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. *$p < 0.05$.

extend the CLS of yeast grown under CR conditions.

In sum, these data suggested that the rates of synthesis of CL and PE from their precursors PG and PS (respectively), as well as the rate of remodeling of acyl chains of CL by the sequential action of a phospholipase A (converting CL to MLCL) and a transacylation reaction catalyzed by Taz1p (Figure 2.1), within the inner mitochondrial membrane define the longevity-extending efficacy of LCA. We therefore hypothesized that one of the mechanisms underlying the ability of LCA to extend yeast longevity consists in the LCA-driven remodeling of the mitochondrial membrane by altering the concentrations of CL, PE, PG, PS and/or MLCL and, perhaps, by changing the relative

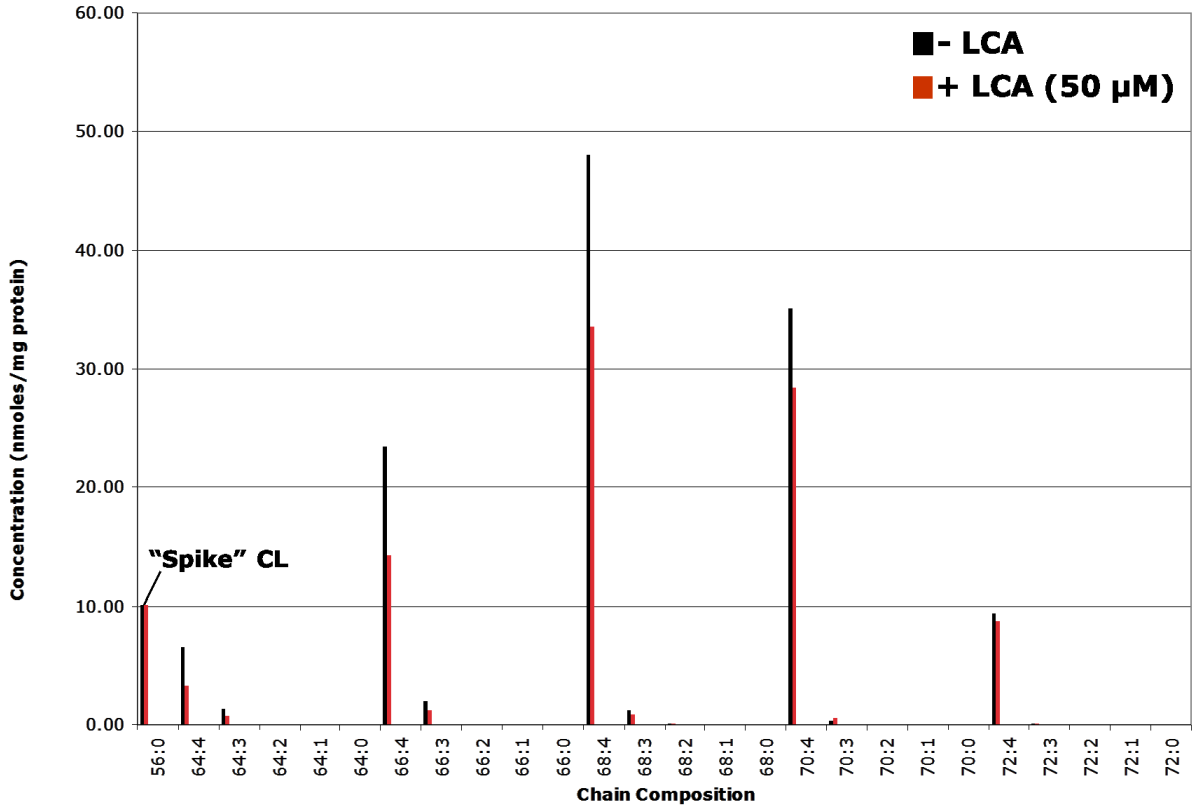

Figure 2.4. Effect of LCA on the levels of various molecular forms of CL in the membranes of mitochondria purified from WT cells. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 µM), and collected for the purification of mitochondria at day 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. The term "spike" refers to a commercially available non-yeast molecular form of CL that was used for the MS-based quantitation of CL species in purified yeast mitochondria.

abundance of certain molecular species of these membrane lipids. In our hypothesis, such LCA-driven remodeling of the composition of mitochondrial membrane lipids extends the CLS of CR yeast by modulating longevity-defining processes confined to and/or governed by mitochondria. To test the validity of this hypothesis, we used MS-based lipidomics to monitor how LCA influences the age-related dynamics of changes in the

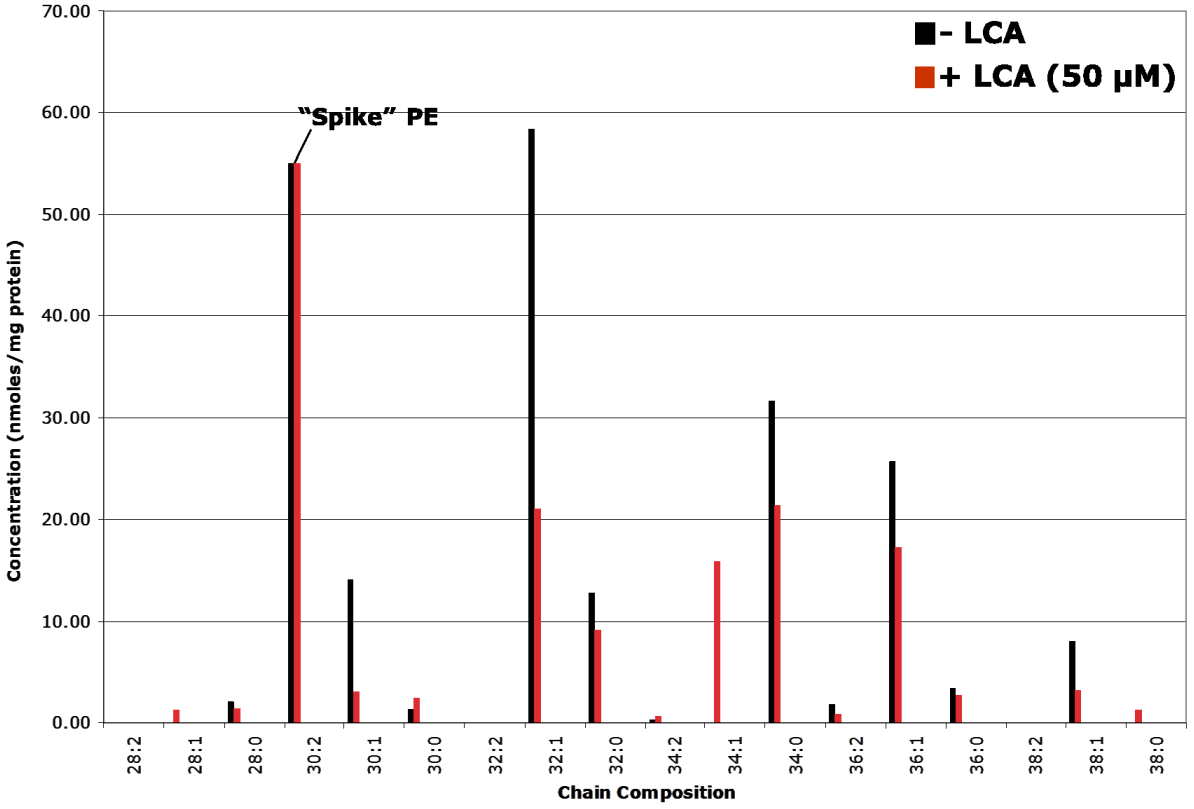

Figure 2.5. Effect of LCA on the levels of various molecular forms of PE in the membranes of mitochondria purified from WT cells. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 µM), and collected for the purification of mitochondria at day 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. The term "spike" refers to a commercially available non-yeast molecular form of PE that was used for the MS-based quantitation of PE species in purified yeast mitochondria.

levels of CL, PE, PS and MLCL in the membrane of mitochondria purified from WT cells (Of note, because CDP-DAG, PG-P and PG do not accumulate in significant amounts in mitochondrial membranes under normal conditions [268, 269], these important intermediates for the synthesis of CL, PE, PS and MLCL within the inner mitochondrial membrane (Figure 2.1) were not detectable even by MS in

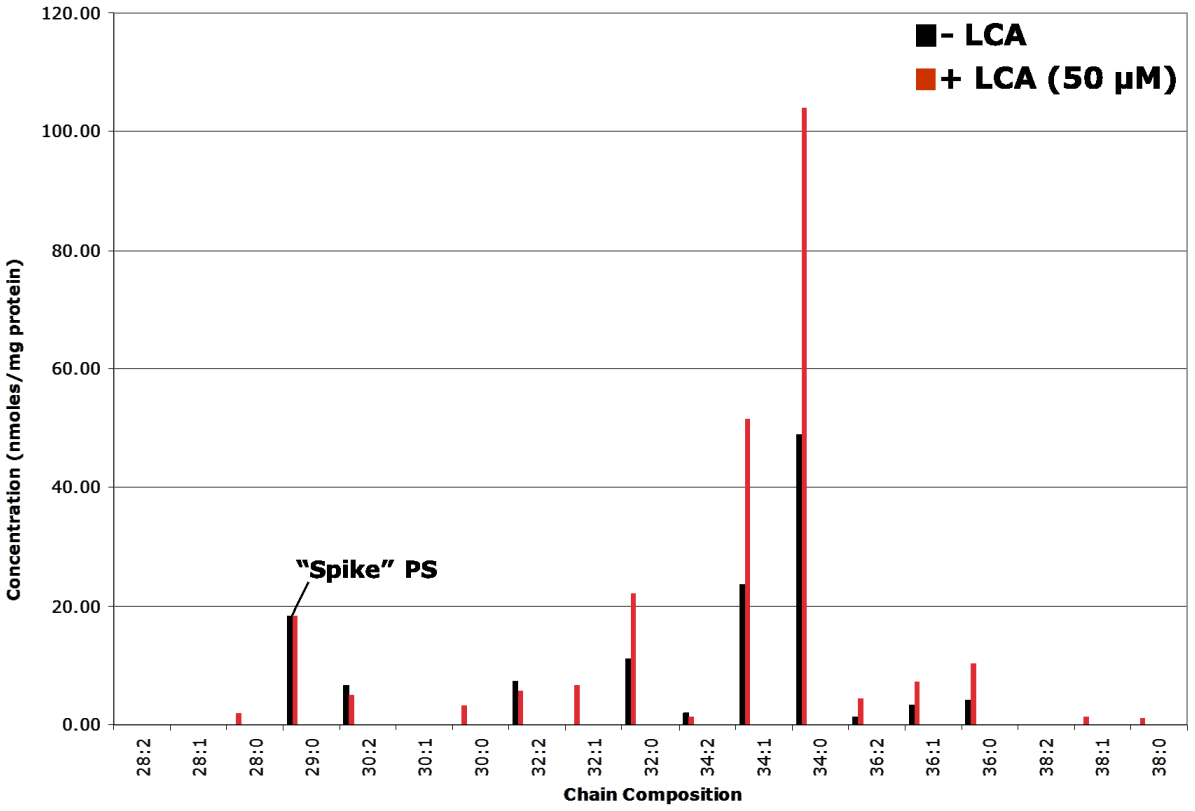

Figure 2.6. Effect of LCA on the levels of various molecular forms of PS in the membranes of mitochondria purified from WT cells. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 µM), and collected for the purification of mitochondria at day 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. The term "spike" refers to a commercially available non-yeast molecular form of PS that was used for the MS-based quantitation of PS species in purified yeast mitochondria.

mitochondria purified from WT cells; we were able to detect PG accumulation by MS only in mitochondria purified from the *crd1*Δ mutant, which lacks an enzyme catalyzing the synthesis of CL from PG and CDP-DAG). We found that LCA reduces the total levels and the levels of several molecular forms of CL (Figures 2.2 and 2.4), PE (Figures 2.2 and 2.5) and MLCL (Figures 2.2 and 2.7) but significantly elevates the total level and

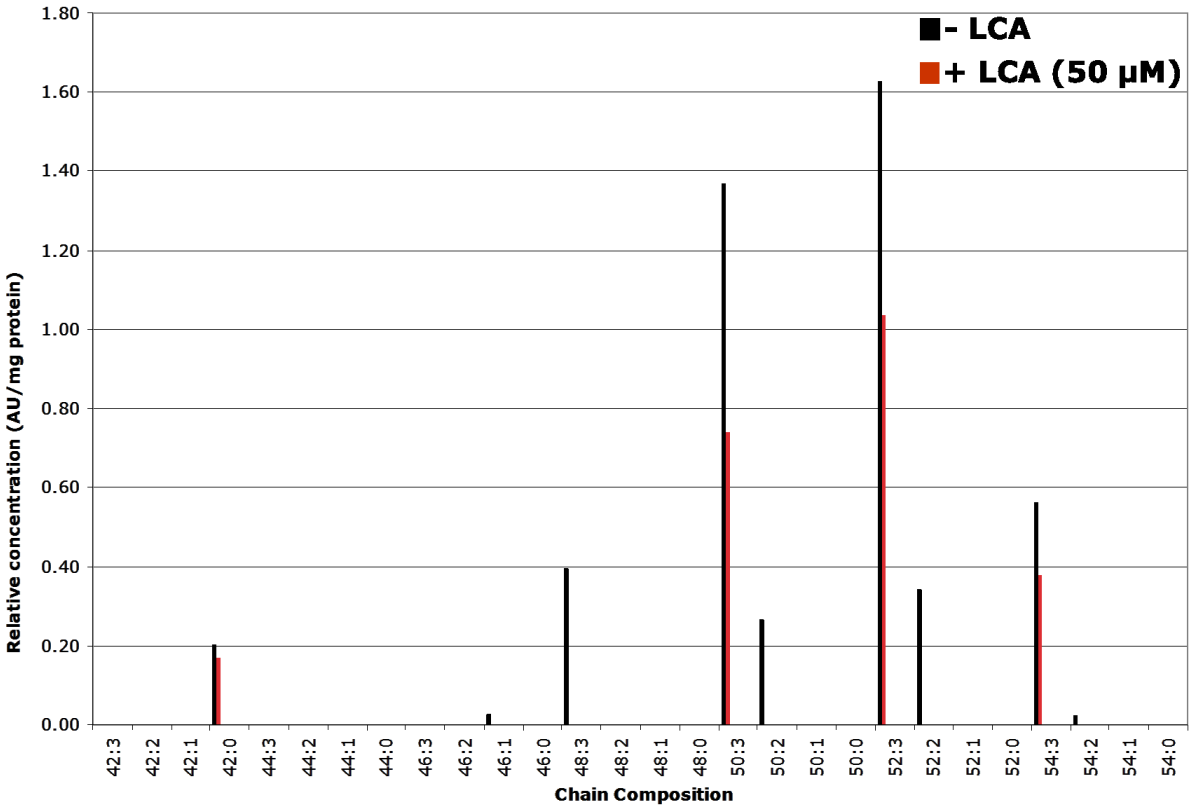

Figure 2.7. Effect of LCA on the levels of various molecular forms of MLCL in the membranes of mitochondria purified from WT cells. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 µM), and collected for the purification of mitochondria at day 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods.

the levels of several molecular forms of PS (Figures 2.2 and 2.6). The observed effect of LCA on the quantities of CL, PE and PS enables to maintain PE/CL ratio in the mitochondrial membrane (Figure 2.3). Furthermore, our MS-based lipidomic analysis revealed that, by elevating the total level of PS, LCA greatly increases PS/CL and PS/PE ratios in the membrane of WT mitochondria (Figure 2.3). Altogether, these findings suggest a hypothesis in which LCA inhibits the Crd1p- and Psd1p-driven reactions of the

biosynthesis of CL and PE (respectively) in the inner mitochondrial membrane, thereby reducing the quantities of these two lipid classes and increasing the quantity of PS, a precursor of PE (Figure 2.8).

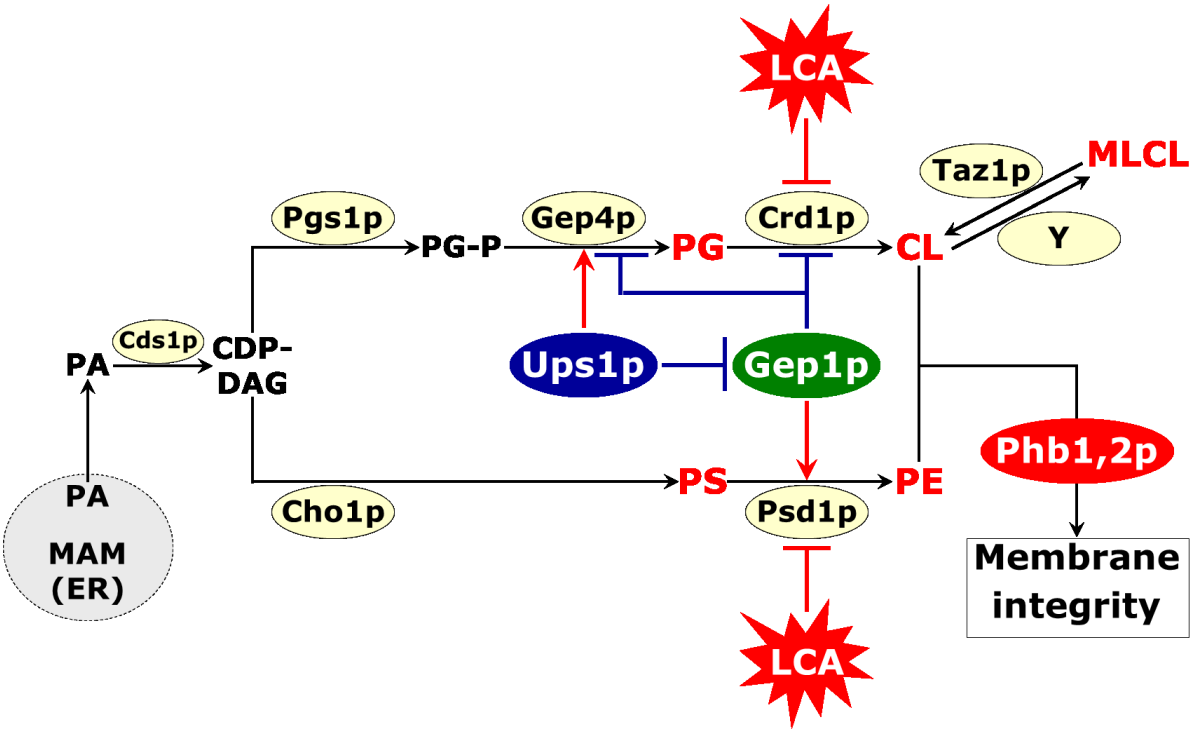

Figure 2.8. Findings described in section 2.4.1 of my thesis suggest a hypothesis in which LCA inhibits the Crd1p- and Psd1p-driven reactions of the biosynthesis of CL and PE (respectively) in the inner mitochondrial membrane, thereby reducing the quantities of these two lipid classes and increasing the quantity of PS, a precursor of PE.

2.4.2 The LCA-driven remodeling of the mitochondrial membrane by altering the concentrations and relative abundances of CL, PE and PS is essential for the longevity-extending ability of LCA and defines its efficacy

According to our hypothesis on the inhibition by LCA of the Crd1p- and Psd1p-driven reactions of the biosynthesis of CL and PE (respectively) in the inner mitochondrial

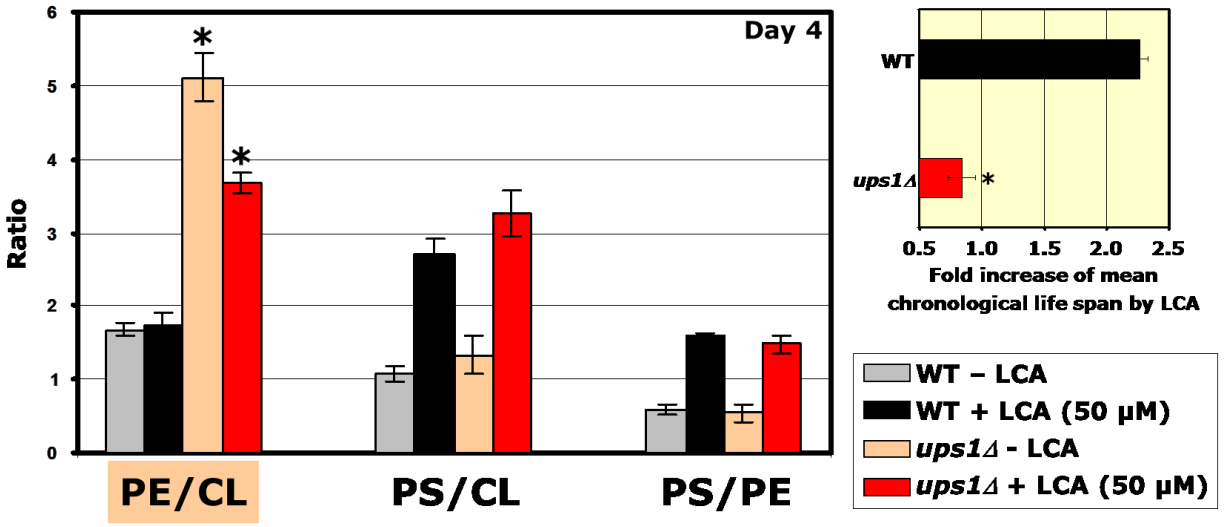

Figure 2.9. LCA cannot extend the chronological lifespan of the *ups1*Δ mutant whose mitochondrial membrane has greatly elevated (as compared to WT strain) PE/CL ratio, in spite of the ability of LCA to reduce this ratio in the mitochondrial membranes of *ups1*Δ. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 μM), and collected for the purification of mitochondria at day 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. *$p < 0.05$.

membrane (Figure 2.8), the caused by LCA reduction of the levels of CL and PE and increase of the quantity of PS is essential for the longevity-extending ability of this bile acid and defines its efficacy as an anti-aging compound. We tested the validity of this hypothesis by using MS-based lipidomics for the identification and quantitation of various species of CL, PE and PS extracted from purified mitochondria of *ups1*Δ, *taz1*Δ, *psd1*Δ or *gep1*Δ mutant cells grown in medium without LCA or with LCA (used in the concentration of 25 μM or 50 μM) and collected at days 2, 3 or 4. Our comparative MS-assisted lipidomic analysis leads to the following conclusions. First, although LCA reduces PE/CL ratio in the mitochondrial membranes of *ups1*Δ (Figure 2.9), this

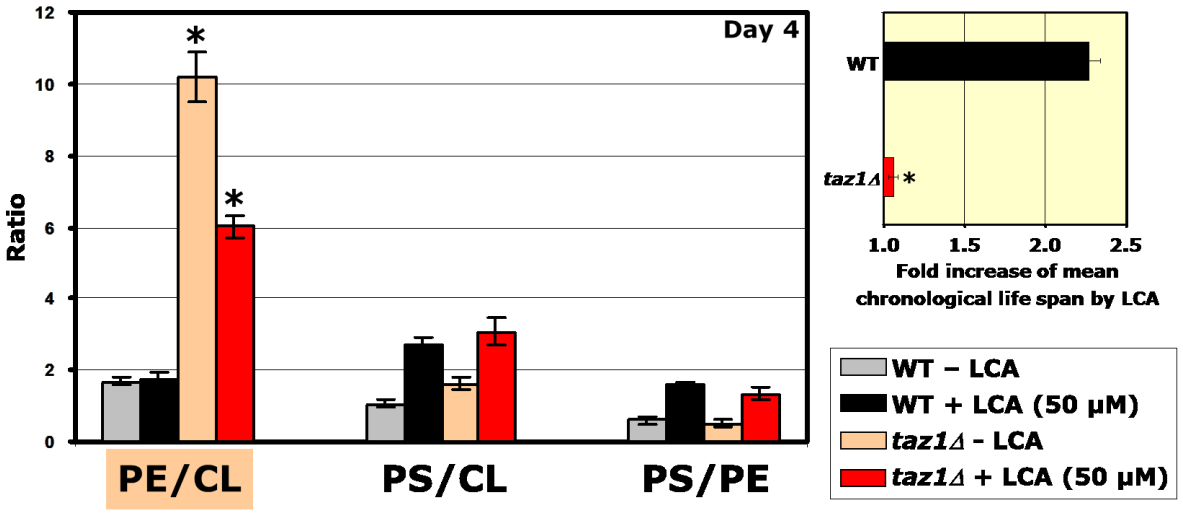

Figure 2.10. LCA cannot extend the chronological lifespan of the *taz1*Δ mutant whose mitochondrial membrane has greatly elevated (as compared to WT strain) PE/CL ratio, in spite of the ability of LCA to reduce this ratio in the mitochondrial membranes of *taz1*Δ. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 μM), and collected for the purification of mitochondria at day 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. *$p < 0.05$.

ratio in the mitochondrial membrane of LCA-treated *ups1*Δ mutant cells remains significantly elevated as compared to that in the mitochondrial membrane of WT cells (Figure 2.9); importantly, LCA has been found to be unable to extend longevity of the *ups1*Δ mutant strain (Figure 2.9). Second, although LCA reduces PE/CL ratio in the mitochondrial membranes of *taz1*Δ (Figure 2.10), this ratio in the mitochondrial membrane of LCA-treated *taz1*Δ mutant cells remains significantly elevated as compared to that in the mitochondrial membrane of WT cells (Figure 2.10); importantly, LCA is unable to extend longevity of the *taz1*Δ mutant strain (Figure 2.10). Third, PE/CL ratio in the inner mitochondrial membrane of LCA-treated *psd1*Δ mutant cells is very similar to

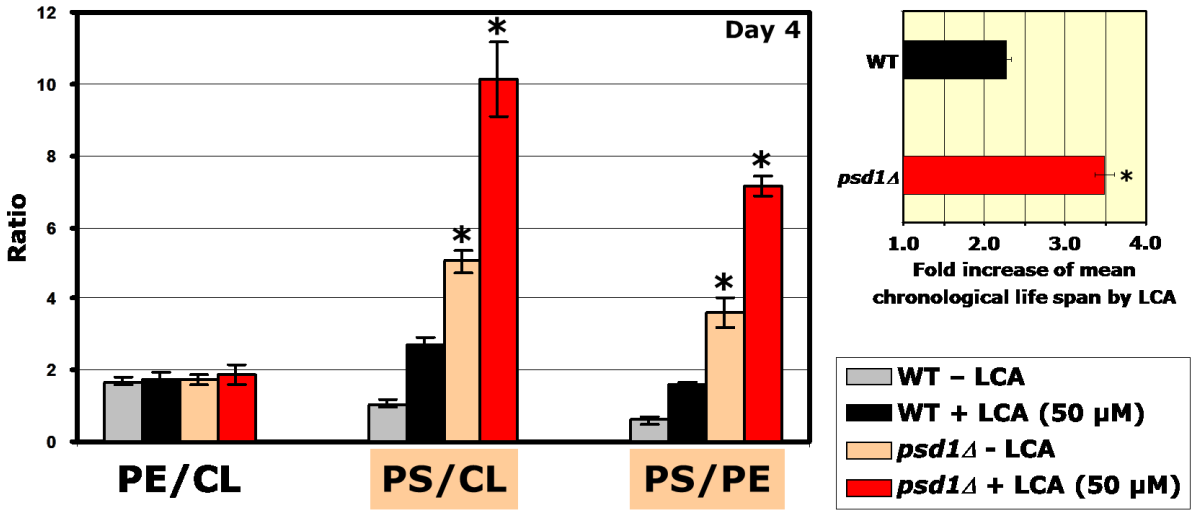

Figure 2.11. The ability of LCA to extend chronological lifespan is enhanced in the *psd1*Δ mutant strain in which (1) PE/CL ratio in the mitochondrial membrane is at the same level as that in WT strain; and (2) both PS/CL and PS/PE ratios in the mitochondrial membrane are significantly increased (as compared to those in WT strain) in LCA-treated and untreated cells. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 μM), and collected for the purification of mitochondria at day 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. *$p < 0.05$.

that in WT cells (Figure 2.11), whereas both PS/CL and PS/PE ratios in the inner mitochondrial membrane of LCA-treated *psd1*Δ cells are significantly elevated as compared to those in the mitochondrial membrane of WT cells (Figure 2.11); importantly, we found that the longevity-extending efficacy of LCA is enhanced in the *psd1*Δ mutant strain (Figure 2.11). Fourth, PE/CL ratio in the inner mitochondrial membrane of LCA-treated *gep1*Δ mutant cells is at the same level as that in WT cells (Figure 2.12), whereas both PS/CL and PS/PE ratios in the inner mitochondrial membrane of LCA-treated and untreated *gep1*Δ cells are significantly elevated as

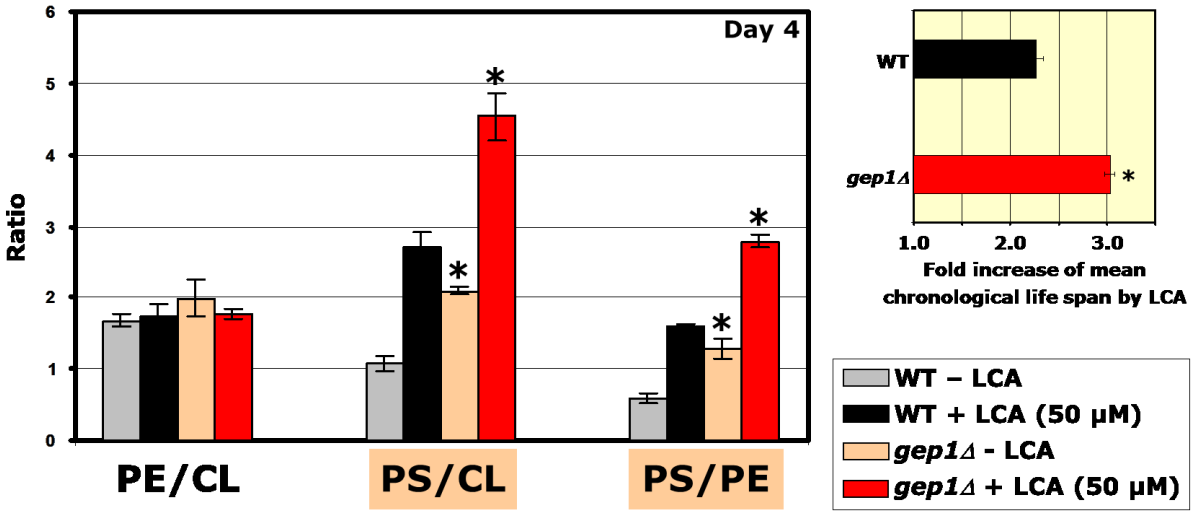

Figure 2.12. The ability of LCA to extend chronological lifespan is enhanced in the *gep1*Δ mutant strain in which (1) PE/CL ratio in the mitochondrial membrane is at the same level as that in WT strain; and (2) both PS/CL and PS/PE ratios in the mitochondrial membrane are significantly increased (as compared to those in WT strain) in LCA-treated and untreated cells. Yeast were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 μM), and collected for the purification of mitochondria at day 4. Mitochondrial membrane lipids were identified and quantitated by MS as described in Materials and Methods. *$p < 0.05$.

compared to those in the mitochondrial membrane of WT cells exposed to LCA or remained untreated (Figure 2.12); importantly, the longevity-extending efficacy of LCA is enhanced in the *gep1*Δ mutant strain (Figure 2.12).

In sum, findings described in this section of my thesis strongly suggest that LCA extends longevity of chronologically aging yeast under CR conditions by (1) increasing the level of PS (a precursor for the synthesis of PE in mitochondria) in the mitochondrial membrane, thereby enhancing its positive effect on longevity-defining processes in this membrane; (2) decreasing the level of PE in the mitochondrial membrane, thereby weakening its negative effect on longevity-defining processes in this membrane; and (3)

proportionally decreasing the levels of PE and CL in the mitochondrial membrane, thereby increasing PS/CL and PS/PE ratios but maintaining PE/CL ratio of mitochondrial membrane lipids and causing some longevity-extending changes in this membrane.

2.4.3 LCA alters the levels of the inverted cone-shaped and cone-shaped species of PS, CL and PE known to affect membrane curvature

Our MS analysis revealed that LCA alters the levels of various molecular forms of CL (Figure 2.4), PE (Figure 2.5) and PS (Figure 2.6) in the mitochondrial membrane of WT cells grown under CR conditions. These findings suggest that by causing these changes LCA is likely to alter the curvature of the inner mitochondrial membrane, thereby affecting its ability to form cristae, contact sites or remain flat and, as a result, specifically modulating the longevity-defining morphology and functional state of mitochondria. Indeed, LCA decreases the level of the cone-shaped species of CL enriched in unsaturated fatty acids, and thus is expected to decrease the number of contact sites by reducing negative curvature of the inner mitochondrial membrane; perhaps by causing such effect, LCA may shift the equilibrium of membrane curvature towards the enhancement of cristae formation by promoting positive curvature of the inner mitochondrial membrane (Figure 2.13). Furthermore, LCA reduces the level of the cone-shaped species of PE enriched in unsaturated fatty acids, and thus is expected to decrease the number of contact sites by reducing negative curvature of the inner mitochondrial membrane (Figure 2.14). Moreover, LCA increases the level of the inverted cone-shaped species of PS enriched in saturated fatty acids and, thus, is expected to enhance cristae formation by promoting positive curvature of the inner mitochondrial

membrane (Figure 2.15).

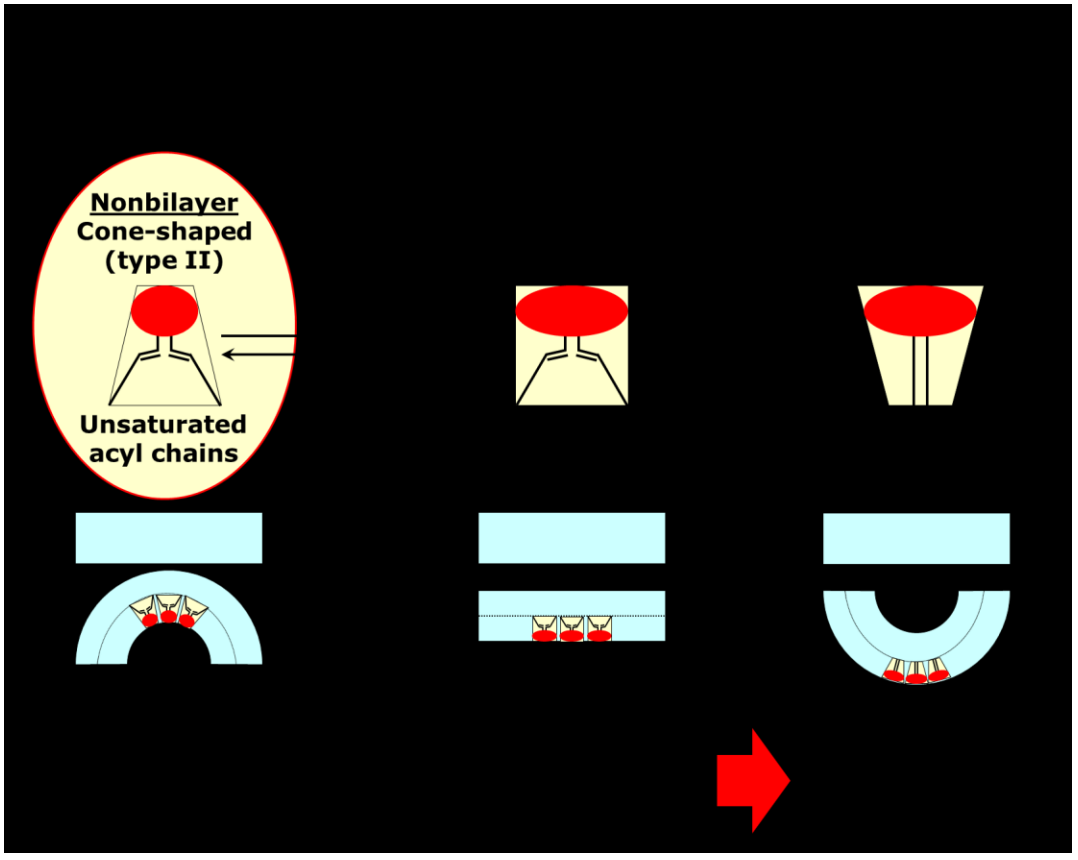

Figure 2.13. LCA reduces the level of the cone-shaped species of CL enriched in unsaturated fatty acids, and thus is expected to decrease the number of contact sites by reducing negative curvature of the inner mitochondrial membrane; perhaps by causing such effect, LCA may shift the equilibrium of membrane curvature towards the enhancement of cristae formation by promoting positive curvature of the inner mitochondrial membrane.

Taken together, these findings suggest that, by enhancing the ability of the inner mitochondrial membrane to form cristae, LCA may alter the dynamics of mitochondrial respiration, electrochemical membrane potential maintenance and ROS production; ultimately, these changes in longevity-defining processes confined to mitochondria may

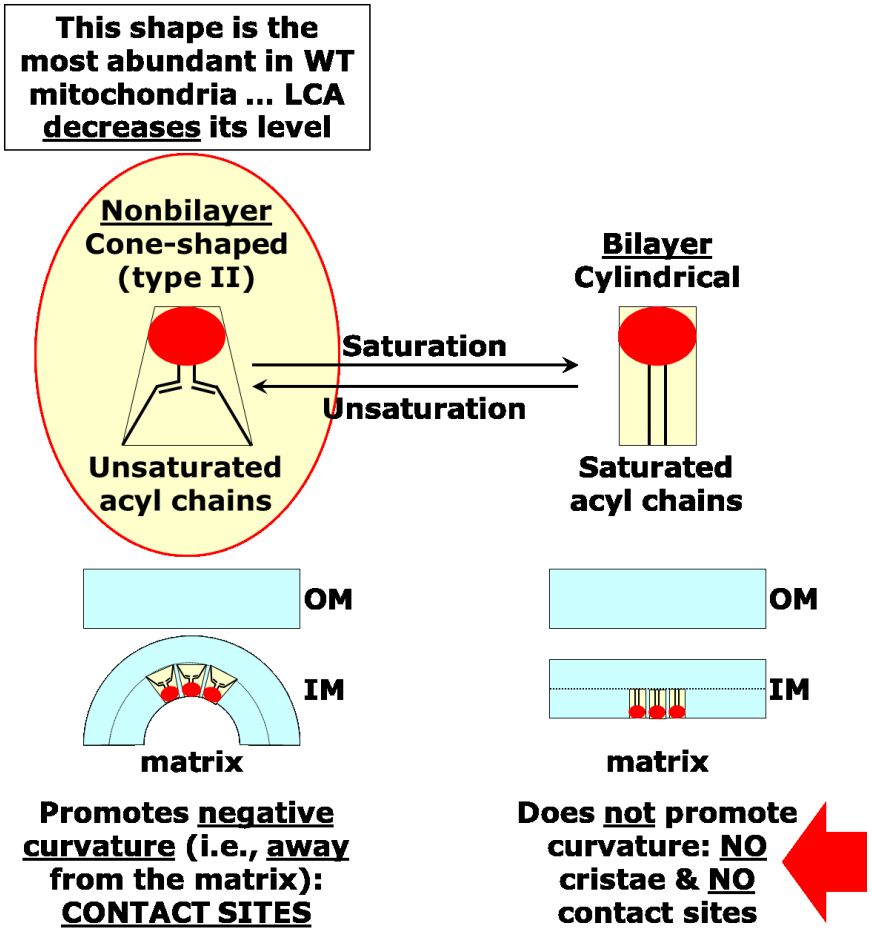

Figure 2.14. LCA reduces the level of the cone-shaped species of PE enriched in unsaturated fatty acids, and thus is expected to decrease the number of contact sites by reducing negative curvature of the inner mitochondrial membrane.

be responsible for the observed longevity extension by LCA. Recent findings by other graduate students in our laboratory support this assumption by demonstrating that LCA (1) causes profound changes in the size and number of mitochondria as well as in the length and morphology of mitochondrial cristae in WT cells grown under CR conditions; and (2) alters the age-related dynamics of several longevity-defining processes confined to mitochondria, including respiration, membrane potential maintenance and ROS generation.

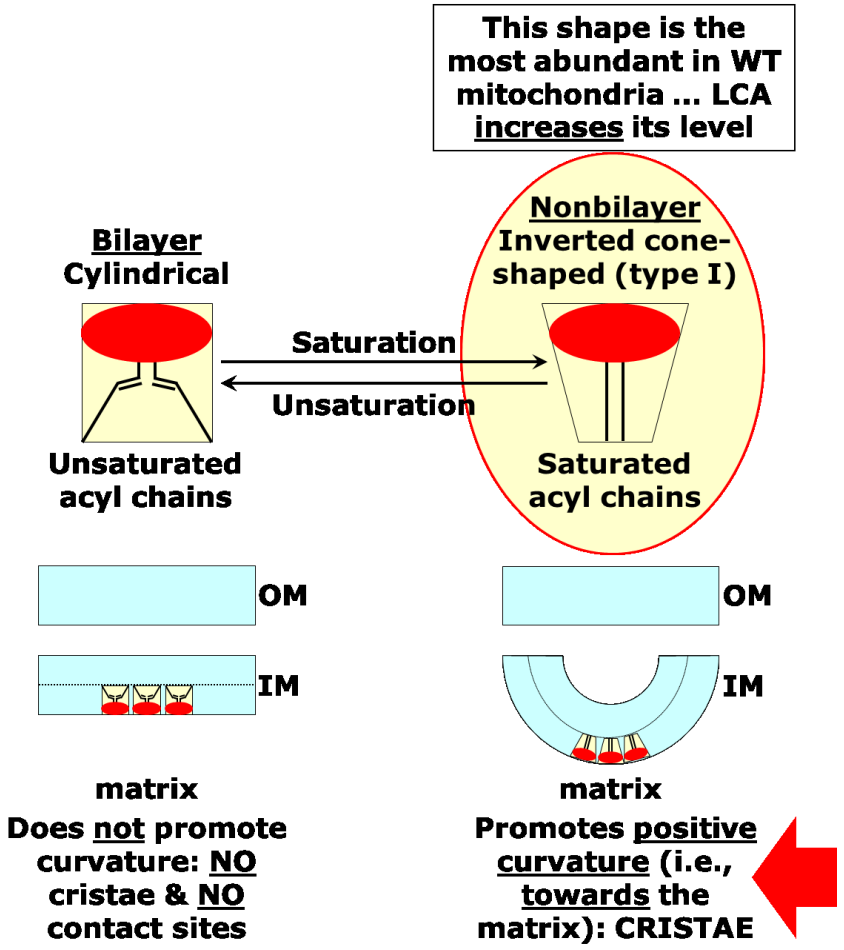

Figure 2.15. LCA elevates the level of the inverted cone-shaped species of PS enriched in saturated fatty acids, and thus is expected to enhance cristae formation by promoting positive curvature of the inner mitochondrial membrane.

2.5 Discussion

Findings described in this chapter of my thesis strongly suggest that LCA extends longevity of chronologically aging yeast cultured under CR conditions in part by altering the composition of mitochondrial membrane lipids. Indeed, LCA causes the remodeling of the mitochondrial membrane by proportionally reducing the quantities of PE and CL and simultaneously increasing the quantity of PS, a precursor of PE. It is plausible that

such LCA-driven remodeling of the repertoire of mitochondrial membrane lipids is due to an inhibiting effect of LCA on the Crd1p- and Psd1p-driven reactions of the biosynthesis of CL and PE (respectively) in the inner mitochondrial membrane. LCA not only alters the total levels of CL, PE and PS in the mitochondrial membrane, but also has a specific effect in the relative abundance of their various molecular forms that differ from each other by the extent of saturation of the hydrophobic tails and their length. Indeed, we found that LCA (1) reduces the concentrations of the cone-shaped species of CL and PE enriched in unsaturated fatty acids, thereby shifting the equilibrium of membrane curvature towards the enhancement of cristae formation by promoting positive curvature of the inner mitochondrial membrane and (2) elevates the concentration of the inverted cone-shaped species of PS enriched in saturated fatty acids, thereby enhancing cristae formation by promoting positive curvature of the inner mitochondrial membrane. As other graduate students in our laboratory demonstrated, such LCA-driven remodeling of differently shaped molecular forms of PE, CL and PS reduces the number of mitochondria and increases their size. One could assume that the observed effect of LCA on the abundance of mitochondria is caused by the ability of this bile acid to shift a balance between the opposing processes of mitochondrial fission and fusion towards fusion, thereby preventing the fragmentation of mitochondria and the resulting efflux of cytochrome c and several other pro-apoptotic proteins from the mitochondrial intermembrane space. Ultimately, such effect of LCA on mitochondrial morphology may cause a delay of the age-related form of mitochondria-controlled apoptosis and longevity extension. Another likely outcome of the observed LCA-driven remodeling of differently shaped molecular forms of PE, CL and PS may consist in significant expansion of the

mitochondrial inner membrane, which could be manifested in a great increase of the length of mitochondrial cristae and in a change of their morphology. This prediction has been recently confirmed by other graduate students in our laboratory. It is plausible that such LCA-driven enhancement of the ability of the mitochondrial inner membrane to form cristae is responsible for the observed proficiency of LCA in altering the age-related dynamics of changes in several longevity-defining processes confined to mitochondria, including respiration, membrane potential maintenance and ROS generation.

2.6 Conclusions

Findings described here imply that LCA extends longevity of chronologically aging yeast by (1) elevating the level of PS (a precursor for the synthesis of PE in mitochondria) in the mitochondrial membrane, thereby enhancing its positive effect on longevity-defining processes in this membrane; (2) reducing the level of PE in the mitochondrial membrane, thereby weakening its negative effect on longevity-defining processes in this membrane; and (3) proportionally decreasing the levels of PE and CL in the mitochondrial membrane, thereby increasing PS/CL and PS/PE ratios but maintaining PE/CL ratio of mitochondrial membrane lipids and causing some longevity-extending changes in this membrane. These LCA-induced alterations in mitochondrial membrane lipids can satisfactorily explain the important implications of LCA treatment on mitochondrial structure and function, including (1) the ability of LCA to cause dramatic changes in the length and curvature of the inner mitochondrial membrane; and (2) the ability of LCA to activate protein machines involved in mitochondrial respiration, the maintenance of

mitochondrial membrane potential, ROS production in mitochondria and mitochondrial fusion.

3 By altering mitochondrial membrane lipidome, lithocholic acid (LCA) remodels mitochondrial respiratory supercomplexes

3.1 Abstract

Based on our findings described in Chapter 2 of my thesis and data of other researchers working in the field of mitochondrial biology, we hypothesized that, by altering the level of CL and other glycerophospholipids within the mitochondrial membrane, LCA could modulate the stoichiometry, composition and/or functional state of respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane. To test the validity of this hypothesis, in studies described in this Chapter of my thesis we used a multistep method for recovery of intact respiratory complexes and supercomplexes from purified yeast mitochondria, their subsequent first-dimension electrophoretic separation using so-called blue-native gel electrophoresis (BN-PAGE), their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the mass spectrometry (MS)-based identification of each of these individual protein components. Findings described in this chapter of my thesis validate our hypothesis. Specifically, these findings revealed several ways of rearranging respiratory supercomplexes in the inner mitochondrial membrane of cells exhibiting altered mitochondrial membrane lipidome in response to LCA treatment or genetic manipulations impairing the synthesis of CL and other glycerophospholipids within the inner membrane of mitochondria. First, by altering the level of CL and other glycerophospholipids synthesized and residing in the inner mitochondrial membrane, LCA modulates the abundance of several major respiratory supercomplexes (respirasomes) in this membrane. Second, LCA- and genetic

manipulations-driven changes in the inner mitochondrial membrane lipidome cause a recruitment of a number of new mitochondrial protein components, not previously known for being permanently associated with the ETC, into the remodeled respirasomes. Importantly, many of the proteins newly recruited into the remodeled respirasomes are known for their essential roles in mitochondria-confined processes that define longevity.

3.2 Introduction

A growing body of evidence supports the view that the electron transport chain (ETC) in the inner mitochondrial membrane plays a pivotal role in regulating longevity of evolutionarily distant organisms by defining the efficacy of oxidative phosphorylation, electrochemical potential, ATP synthesis and reactive oxygen species (ROS) production in mitochondria as well as by modulating mitochondrial retrograde signaling and mitochondria-specific unfolded protein response [25, 51 - 58, 182, 238 - 241]. Two models have been proposed for the organization of the mitochondrial ETC. One of these models, known as the random diffusion model or the fluid model, suggests that respiratory complexes move in a random fashion within the inner mitochondrial membrane [242]. Another model, which is currently accepted by most researchers in the field of mitochondrial bioenergetics, is known as the solid model. This model suggests that the respiratory complexes are assembled into larger supercomplexes within the inner mitochondrial membrane; these larger supramolecular structures are also called respirasomes [243 - 252]. Several important advantages of the supramolecular organization of respiratory complexes into supercomplexes have been proposed, including substrate channeling, catalytic enhancement and sequestration of reactive

intermediates [243, 245 - 249, 252]. Substrate channeling is an efficient and beneficial process because it guides various intermediates of a biochemical process from one enzyme to another specific enzyme, thereby protecting every intermediate from being trapped by other enzymes; a body of evidence implies that this advantage is applicable to a mobile form of cytochrome c in yeast, bacterial and mammalian ETCs [243, 247, 248, 251]. Thus, a supramolecular arrangement of individual respiratory complexes into supercomplexes/respirasomes enables a substantial catalytic enhancement of the ETC in the inner mitochondrial membrane [243 - 252]. Based on findings described in Chapter 2 of this thesis, we concluded that under CR conditions LCA extends yeast longevity by remodeling the repertoire of mitochondrial membrane lipids and thereby modulating longevity-defining processes confined to and governed by mitochondria. Specifically, our findings presented in Chapter 2 of this thesis imply that LCA extends longevity of WT yeast by (1) elevating the level of PS (a precursor for the synthesis of PE in mitochondria) in the mitochondrial membrane, thereby enhancing its positive effect on longevity-defining processes in this membrane; (2) reducing the level of PE in the mitochondrial membrane, thereby weakening its negative effect on longevity-defining processes in this membrane; and (3) proportionally decreasing the levels of PE and CL in the mitochondrial membrane, thereby increasing PS/CL and PS/PE ratios but maintaining PE/CL ratio of mitochondrial membrane lipids and causing some longevity-extending changes in this membrane. It is important to emphasize that various molecular species of CL are known to control a number of vital mitochondria-confined processes by: (1) being associated with all five complexes of the mitochondrial respiratory chain, modulating their activities and promoting their organization into supramolecular assemblies termed

respiratory supercomplexes or respirasomes; (2) binding to and modulating the activity of cytochrome c, a heme-containing mobile component of the mitochondrial respiratory chain also known for its essential role in mitochondria-controlled apoptosis; (3) being associated with and regulating adenine nucleotide translocator activity; (4) playing an important role in maintaining the electrochemical gradient across the inner mitochondrial membrane; (5) conferring fluidity and stability to the inner membrane of mitochondria; (6) being primary targets of ROS-inflicted damage because of their highly unsaturated acyl chains; and (7) regulating protein import into mitochondria [223 - 230]. Because of its propensity to associate via non-covalent interactions with numerous unrelated proteins in the inner mitochondrial membrane, CL (likely in collaboration with PE) acts as a molecular chaperone that governs the assembly of a number of protein assemblies involved in wide spectrum of essential biological processes [224 - 230].

Based on all these findings, we hypothesized that, by altering the level of CL and other glycerophospholipids within the mitochondrial membrane, LCA could modulate the stoichiometry, composition and/or functional state of respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane. In our hypothesis, such LCA- and CL-dependent remodeling of the mitochondrial respiratory supercomplexes (1) may rely on a distinct set of protein components of the respiratory complexes that compose the respirasomes; (2) may recruit new mitochondrial protein components, not previously known for being permanently associated with the ETC, into the remodeled respirasomes; (3) may alter the age-dependent dynamics of mitochondrial respiration, oxidative phosphorylation, ADP/ATP exchange, ATP synthesis, membrane potential and/or ROS generation; (4) may occur in an age-dependent fashion; and (5) may extend longevity by

increasing the efficiency of ATP synthesis in chronologically aging yeast, altering the age-dependent dynamics of changes in mitochondrially produced ROS (thereby protecting yeast from chronic oxidative stress), and/or modulating the longevity-defining processes that in mitochondria are governed by proteins newly recruited into the remodeled respirasomes (even although some or all of these newly recruited proteins have not been traditionally viewed as proteins permanently associated with the ETC).

To test the validity of this hypothesis, in studies described in this Chapter of my thesis we used a multistep method [243 - 253] for recovery of intact respiratory complexes and supercomplexes from purified yeast mitochondria, their subsequent first-dimension electrophoretic separation using so-called blue-native gel electrophoresis (BN-PAGE), their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the mass spectrometry (MS)-based identification of each of these individual protein components. To do this, we first purified mitochondria by separating them from other cellular organelles. We then quantified the protein in each sample of highly purified mitochondria. These samples were fractionated in the first dimension with the help of BN-PAGE, a method known for its propensity in recovering intact mitochondrial respiratory complexes and supercomplexes ranging in their molecular weights from 10 kDa to 10 MDa [243 - 253]. The supercomplexes were then fractionated in the second dimension using denaturing Tricine-SDS-PAGE to yield individual protein components of various respiratory complexes and supercomplexes; the high-ionic strength Tricine-SDS gel was used for this purpose instead of a traditional SDS gel to ensure high protein recovery during electrophoretic run, which yields sharp protein bands on the gel [243 - 253]. Finally, the resulting protein bands were excised out

of the gel, washed and digested with trypsin into individual peptides. The peptides were then analyzed using MS to identify all of the protein constituents of each of the isolated respiratory complexes and supercomplexes.

3.3 Materials and Methods

Strains and media

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and mutant strains *ups1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ups1Δ::kanMX4*), *taz1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 taz1Δ::kanMX4*), *gep4Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gep4Δ::kanMX4*), *psd1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 psd1Δ::kanMX4*) and *crd1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 crd1Δ::kanMX4*) were used in this study. Yeast were cultured in in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose as carbon source. The stock solution of LCA in DMSO or water (as indicated in the text) was made on the day of adding this compound to cell cultures. LCA was added to growth medium in DMSO or water (as indicated in the text) at the final concentration of 50 μM immediately following cell inoculation into the medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with drug vehicle) was 1% (v/v). 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.

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 *S. cerevisiae* 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.

Blue-native gel electrophoresis (BN-PAGE)

Equipment

1. Hoefer SE 400 vertical slab gel electrophoresis unit

2. Bio Rad Power Pac 3000 Power supply

Reagents

1. 4% acrylamide running gel [1.5 ml AB (acrylamide/bis-acrylamide)-3 mix, 6 ml gel buffer 3×, 10.4 ml distilled water, 100 µl APS, 10 µl TEMED]

2. 13% acrylamide running gel

3. 3.5% acrylamide sample gel

4. Solubilization buffer A [50 mM sodium chloride, 50 mM imidazole/HCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA, pH 7.0]

5. Coomassie blue G-250 dye stock [5% wt/vol in 500 mM 6-aminohexanoic acid]

6. Cathode buffer B [50 mM Tricine, 7.5 mM imidazole, 0.02% Coomassie blue G-250, pH 7.0]

7. Cathode buffer B/10 [50 mM Tricine, 7.5 mM imidazole, 0.002% Coomassie blue G-250, pH 7.0]

8. Anode buffer [25 mM imidazole, pH 7.0]

Procedure

Mitochondria protein aliquots of 400 μg were pelleted by centrifugation at 50,000 × g for 30 min using a Beckman MLS-50 rotor. The mitochondria pellet was resuspended in 40 μl of solubilization buffer A and the mitochondria membranes were homogenized by twirling a tiny spatula. The detergent digitonin at 20% (3.0 g/g; *i.e.*, 6μl) was added to the homogenate, which was incubated for 10 min at room temperature. The sample was subsequently centrifuged at 20,000 × g for 20 min at room temperature. To the resulting supernatant, 5 μl of 5% Coomassie blue G-250 dye in 50% glycerol was added at an 8 g/g detergent/dye ratio (*i.e.*, 3 μl). The sample containing solubilized mitochondrial membrane was loaded to a 0.16 × 0.5 cm well of a gradient separation gel with dimensions 1.5 × 14 × 15 cm composed of 4% and 13% acrylamide mixture with a sample gel of 3.5% acrylamide. Urease at 1mg/ml was also loaded on an adjacent well and used as a molecular marker (10 μl urease, 10 μl buffer A, 2.4 μl 50 % glycerol, 1.6 μl 5% Coomassie blue G-250). BN-PAGE was performed at 4°C under the following conditions: 300 ml of Cathode buffer B in the upper chamber and 300 ml of Anode buffer in the lower chamber were used and the power supply set at constant 100 V, 15 mA, 400 W until the sample entered the gradient gel. Electrophoresis was then continued at 500 V

and 15 mA until the blue running front moved about a third of the total running distance. At this point, Cathode Buffer B was replaced with 300 ml of Cathode buffer B/10 for better detection of faint protein bands and electrophoresis was continued for a total of 4 h or until the blue running front was 1 cm from the bottom of the gel. Once electrophoresis was completed the blue native gel stained with Coomassie blue G-250 was photographed, documented and stored at 4°C.

Tricine-SDS-PAGE

Equipment

1. Hoefer SE 400 vertical slab gel electrophoresis unit
2. Bio Rad Power Pac 3000 Power supply

Reagents

1. 10%T, 3%C Tricine-SDS running gel
2. 10% acrylamide native stacking gel
3. Anode buffer 10× [1.0 M Tris, 0.225 M HCl, pH 8.9]
4. Cathode buffer 10× [1.0 M Tris, 1.0 M Tricine, 1% SDS, pH 8.25]
5. Precision Plus Protein™ Unstained standards 10 - 250 kD (Bio-Rad)

Procedure

A 0.5-cm gel strip was cut from the 1st dimension (1D) BN-PAGE gel and placed on a glass plate with the top of the strip to the left and the bottom of the strip to the right. The strip was soaked with 5 ml of a solution containing 1% SDS and 1% mercaptoethanol for

2 h at room temperature. Following the incubation, the strip was thoroughly rinsed with water and squeezed in between 1.5-mm spacers and 18 × 16 cm glass plates. A 30-ml separating gel mixture composed of 10%T, 3%C Tricine-SDS was poured in between the glass plates leaving a gap of 0.5 cm from the native gel strip. After polymerization, a 0.5-cm spacer was inserted between the plates, and placed to the right of the gel to create a well to load protein markers. In order to fill the 0.5-cm gap between the running gel and the native strip, as well as the space between the native gel and the spacers, 5 ml of a 10% acrylamide native gel mixture was used, and Unstained protein molecular markers (Bio-Rad) were loaded to the far right of the gel for reference. Electrophoresis was performed at constant 200 V, 50 mA, 400 W for 3 h at room temperature.

Silver stain after 1D-BN-PAGE followed by 2D-Tricine-SDS-PAGE

Protein bands are then visualized by silver staining using the Silver Stain Plus Kit from Bio-Rad.

Reagents

1. Fixative solution [200 ml methanol, 40 ml acetic acid, 40 ml Fixative Enhancer solution, 120 ml distilled water]
2. Staining solution [35 ml distilled water, 5 ml Silver complex solution, 5 ml Reduction Moderator solution, 5 ml Image Development solution, 50 ml Development Accelerator solution]
3. Stop solution [5% acetic acid]

Procedure

Once electrophoresis was completed, the gel was incubated in 400 ml of 50% methanol on a shaker at 55 rpm for 2 h. The gel was then incubated in 200 ml of the Fixative solution for 20 min and washed twice with 200 ml of distilled water for 10 min. The gel was then immersed in 100 ml of the Staining Solution composed of Silver complex, Reduction Moderator, Image Development and Development Accelerator solutions. Once the desired staining intensity was reached, development was immediately stopped by incubating the silver stained gel in 400 ml of 5% acetic acid for 15 min, which was followed by a rinse with 400 ml of distilled water for 5 min. The gel was then photographed, documented and stored at 4°C in 400 ml of distilled water.

In-gel digestion of proteins separated by 2D-Tricine-SDS-PAGE

Reagents

1. 30 mM Potassium Ferricyanide
2. 100 mM Sodium Thiosulfate
3. 100mM Ammonium Bicarbonate (ABC)
4. 10 mM Dithiothreitol (DTT) [in 5ml of 100mM Ammonium Bicarbonate (ABC)]
5. 55 mM Iodoacetamide (IAA) [in 5ml of 100mM Ammonium Bicarbonate (ABC)]
6. Trypsin, Proteomics Grade (Sigma Aldrich)

Procedure

Once the individual mitochondrial proteins were separated, each band on the 1D BN-2D Tricine-SDS gel was cut out with a razor blade and the gel piece was placed in 0.5-ml

siliconized Eppendorf tubes. The bands were washed with distilled water and destained with 50 µl of a 1:1 mixture of 20 mM potassium ferricyanide and 100 mM sodium thiosulfate for 20 min in the dark with occasional vortexing. The destaining solution was removed and the bands were washed twice with distilled water. The bands were then incubated in 50 µl of acetonitrile (ACN) for 5 min at 37°C, after which ACN was removed and the bands were dried at 37°C. Next, the destained bands were incubated in 50 µl of 10 mM dithiothreitol for 30 min at 37°C to reduce thiol groups in peptides. DTT was discarded and the bands were incubated in 50 µl of 55 mM iodoacetamide for 20 min at 37°C in the dark to remove the residual DTT. IAA was removed and the bands were incubated in 50 µl of a 1:1 mixture of 100 mM ABC and 50% acetonitrile for 10 min at 37°C. The mixture was discarded and the bands were incubated twice in 50 µl of CAN under the same conditions and dried at 37°C. The trypsin and trypsin buffer were prepared as follows: (1) 1.6 ml of a 1:1 mixture of 100 mM ABC and 10mM $CaCl_2$ were used to resuspend 20 µg of trypsin; and (2) for protein digest, 50 µl of trypsin solution (1 mg/ml) was added to the bands, which were then incubated overnight at 37°C.

Extracting peptides from yeast mitochondrial proteins digested with trypsin

The following day, the samples were spun down and the supernatants containing peptides were transferred to new 0.5-ml siliconized Eppendorf tubes. To extract more peptides, the gel pieces were subjected to several washes and treatments at room temperature; the supernatants were conserved and combined with the first set to extracted peptides. For the first extraction, the bands were initially incubated in 50 µl of 25 mM ABC for 10 min and then in 50 µl of ACN for 10 min. The samples were spun down and the supernatant were

added to the first set of extracted peptides. For the second extraction, the bands were incubated in 50 µl of 5% formic acid for 10 min and then in 50 µl of ACN for 10 min. The samples were spun down and the supernatant were combined with the first set of extracted peptides. The gel pieces were no longer used and discarded. To prevent possible oxidation during storage, 12.5 µl of 100 mM DTT was added to each set of peptides. The peptides were completely dried in a Speed-Vac at medium temperature settings (37°C) for 2 h and stored at -20°C until MS analysis.

Sample preparation for MS analysis

Dried peptides were resuspended in 20 µl of 5% ACN. For each recovered protein band, an aliquot of 10 µl of dried peptides in 5% ACN was diluted 2-fold in Nano pure water for MS analysis. Samples can be stored at -20°C until being subjected to MS analysis.

MS analysis

Individual proteins composing each band were then identified by reverse phase high performance liquid chromatography coupled to mass spectrometry (RP-HPLC/MS) using an LTQ Orbitrap. 3-µl aliquots of peptides were separated in ACN gradient using a 100-µM capillary column packed with C18 mobile phase.

Analysis using the Thermo Proteome Discoverer application and SEQUEST

Once acquiring time was completed using the LTQ Orbitrap, the raw mass spectrometry data file obtained by Xcalibur were analyzed using the Thermo Scientific Xcalibur Proteome Discoverer application (version 1.3) hereafter referred to as the Proteome

Discoverer. The Proteome Discoverer was used to identify individual protein components of the isolated mitochondrial respiratory complexes and supercomplexes by comparing the raw data of mass spectra of digested fragments to the mass spectra of peptides within the Uniprot FASTA database. The analysis by the Proteome Discoverer coupled to the FASTA database was enabled by using the peak-finding search engine SEQUEST. The SEQUEST engine processes MS data using a peak-finding algorithm to search the raw data for generating a peak probability list with relative protein abundances.

SEQUEST search wizard within the Proteome Discoverer application

Raw file and Scan Range Selection Parameters

The raw file was selected and the Base peak ion chromatogram appeared to reveal: (1) the "Intensity (counts)" corresponding to the intensity of the largest peak in the spectrum; and (2) the "Time (min)" showing the retention time (RT). The following algorithm and settings were used:

Lower RT Limit (min)

The beginning of the RT of the scan range of interest: 10 min.

Upper RT Limit (min)

The end of the retention time of the scan range of interest: 18 min.

Scan Extraction Parameters

First mass

The mass of the first precursor ion in the range of ion fragments to search for in the database: 350 Daltons (Da).

Last mass

The mass of the last precursor ion in the range of ion fragments to search for in the database: 5000 Daltons (Da).

Activation type

The fragmentation method to use for activating the scan: Collision Induced Dissociation (CID).

Unrecognized charge replacement

Specifies the charge number of the precursor ions: Automatic (Default), assigns a charge number of +2 and +3 to the spectrum.

Intensity threshold

Specifies the intensity threshold below which ions are filtered out. The default value of 0.0 was used.

Minimum ion count

The minimum ion count corresponds to the minimum number of ions that must be present in an MS/MS spectrum for it to be included in a search. The default value of 1 was used.

S/N threshold

The signal-to-noise threshold is the intensity of the signal to the intensity of the background noise. The use of this threshold filters out low-intensity ions that function as a noise. The value of 1.5 was used.

Database

Uniprot_sprot FASTA

Enzyme

Trypsin

Missed Cleavages

The maximum number of internal cleavage sites per peptide fragment that is acceptable for an enzyme to miss during proteolytic digest. The default value of 2 was used.

Precursor mass tolerance

The precursor mass tolerance value used for finding peptide candidates. The possible range of values is 0.01 to 5000 ppm. The default value of 10 ppm was used.

Fragment mass tolerance

The default mass tolerance value of 0.8 Da was used for matching fragment peaks. The possible range of values was 0.0001 to 2.0 Da.

Ions series calculated

Specifies the ion factors for a, b, c, x, y, and z ions for the experiment type. The possible range is 0 through 1.0 for all ion factors. The ion factors used are b ions: 1 and y ions :1.

Search against decoy database

Specifies if the application uses a decoy database in the search; the "yes" was used.

Target FDR (strict)

Specifies a strict target false discovery rate (FDR) for peptide matches with high confidence. The possible value range of 0.0 to 1.0 was used. The default value of 0.01 (1% FDR) was used.

Target FDR (relaxed)

Specifies a relaxed target false discovery rate (FDR) for peptide matches with moderate confidence. The possible value range from 0.0 to 1.0 was used. The default value of 0.05 (5% FDR) was used.

Identification of yeast mitochondria complexes and supercomplexes using the Thermo Proteome Search Results Report

Protein Results Parameters

The protein page displays all the proteins and their corresponding peptides found in the sample during the database search. For each protein, the report shows the following results:

Accession

The unique identifier was assigned to the protein by the Uniprot FASTA database.

Description

The name and description of the protein of the identifier appeared in the corresponding Accession column.

Score

The SEQUEST protein score is the sum of all peptide XCorr values above the specified score threshold. The score threshold was calculated as "0.8 + *peptide charge* * *peptide relevance factor*", where *peptide relevance factor* has a default value of 0.4. For each spectrum, only the highest-scoring match was used. For each spectrum and sequence, the Proteome Discoverer application uses only the highest scored peptide.

Coverage

Represents the percentage of the protein sequence covered by the identified peptides.

 # Proteins

Represents the number of identified proteins in the protein group of a master protein. When the Protein Grouping setting is disabled, the #Proteins is always equal to 1.

 # Unique Peptides

Represents the number of peptide sequences unique to a protein.

 # Peptides

Represents the number of different peptide sequences identified in the protein.

 # PSMs

The peptide spectrum matches (PSMs) value corresponds to the total number of identified peptide sequences for the protein, including those that have been identified redundantly.

 # AAs

Represents the number of amino acid in the sequence length of the protein.

MW [kDa]

Represents the calculated molecular weight of the protein.

Calc. pI

Represents the theoretically calculated isoelectric point, *i.e.* the pH value at which a particular molecule carries no net electrical charge.

Peptides Results Parameters

For each identified peptide, the report shows the following results:

Protein Descriptions

Identifies a protein associated with the peptides. This description is taken from the Uniprot FASTA file.

 # Proteins

Represents the total number of proteins in which this peptide can be found.

Probability

Represents the probability score for the peptide.

SpScore (search-dependent)

Represents the raw value of the preliminary score of the SEQUEST algorithm.

XCorr (search-dependent)

Scores the number of fragment ions that are common to two different peptides with the same precursor mass; calculates the cross-correlation score for all candidate peptides queried from the database.

Δ Score

A measure of the difference between the top two scores for the peptides identified by that spectrum; the Proteome Discoverer application calculates this score as follows: ΔScore = Score(Rank N Peptide) – Score(Rank 1 Peptide)/Score(Rank 1 Peptide).

ΔCn

Represents the normalized score difference between the currently selected PSM and the highest-scoring PSM for that spectrum.

 # Missed Cleavages

Represents the number of cleavage sites in a peptide sequence that trypsin did not cleave, excluding the cases where the presence of proline prevents trypsin from cleaving the peptide bond.

Peptides Matched

Represents the number of peptides included in the precursor mass tolerance window set for the search.

Charge

Represents the charge state of the peptide.

Intensity

Represents the intensity of the precursor ion.

MH+ [Da]

Represents the protonated monoisotopic mass of the peptide, in Daltons.

ΔM [ppm]

Represents the difference between the theoretical mass of the peptide and the experimental mass of the precursor ion.

RT [min]

Represents the retention time when the peptide was observed, in minutes.

Xcalibur is a registered trademark of Thermo Fisher Scientific Inc. in the United States. SEQUEST is a registered trademark of the University of Washington in the United States.

3.4 Results

3.4.1 LCA and genetic manipulations changing the makeup of mitochondrial membrane lipids alter the abundance of respiratory supercomplexes in the inner mitochondrial membrane

Based on our previous findings and data of other researchers working in the field of mitochondrial bioenergetics, we hypothesized that, by altering the level of CL and other glycerophospholipids within the mitochondrial membrane, LCA could modulate the abundance of some of the respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane (see section 3.2 of this thesis). To test the validity of this

hypothesis, we first used a method for recovery of intact respiratory complexes and supercomplexes from purified yeast mitochondria followed by their first-dimension electrophoretic separation using BN-PAGE [243 - 253]. Using this method, we assessed the relative abundance of respiratory supercomplexes in the inner membranes of mitochondria that were purified from cells of WT strain and cells of various long- and short-lived mutant strains impaired in different aspects of CL, PE and PS metabolism, either exposed to LCA or remained untreated. Cells of WT and mutant strains were cultured under CR conditions on 0.2% glucose, and mitochondria were purified from cells recovered at day 4 of culturing. We found that LCA and mutations changing the makeup of mitochondrial membrane lipids (see Chapter 2 of this thesis) alter the abundance of respiratory supercomplexes in the inner mitochondrial membrane (Figure 3.1).

Specifically, our BN-PAGE analysis revealed that exposure of WT cells to LCA significantly increases the level of a respiratory supercomplex with the highest molecular weight (likely in a range of several MDa), which is marked by a red arrow in Figure 3.1. Our MS-based analysis described below (see section 3.4.2 of this Chapter on my thesis) revealed that this respiratory supercomplex in the inner mitochondrial membrane of LCA-treated WT cells consists of (1) complexes II, III, IV and V of the mitochondrial ETC; (2) the ADP-ATP carrier protein (called AAC2 or ADT2); (3) the mitochondrial external NADH dehydrogenase NDE1, a type II NAD(P)H:quinone oxidoreductase that catalyzes the oxidation of cytosolic NADH to provide cytosolic NADH to the mitochondrial ETC; and (4) the mitochondrial protein MMF1 that is required for transamination of isoleucine, may regulate specificity of branched-chain transaminases

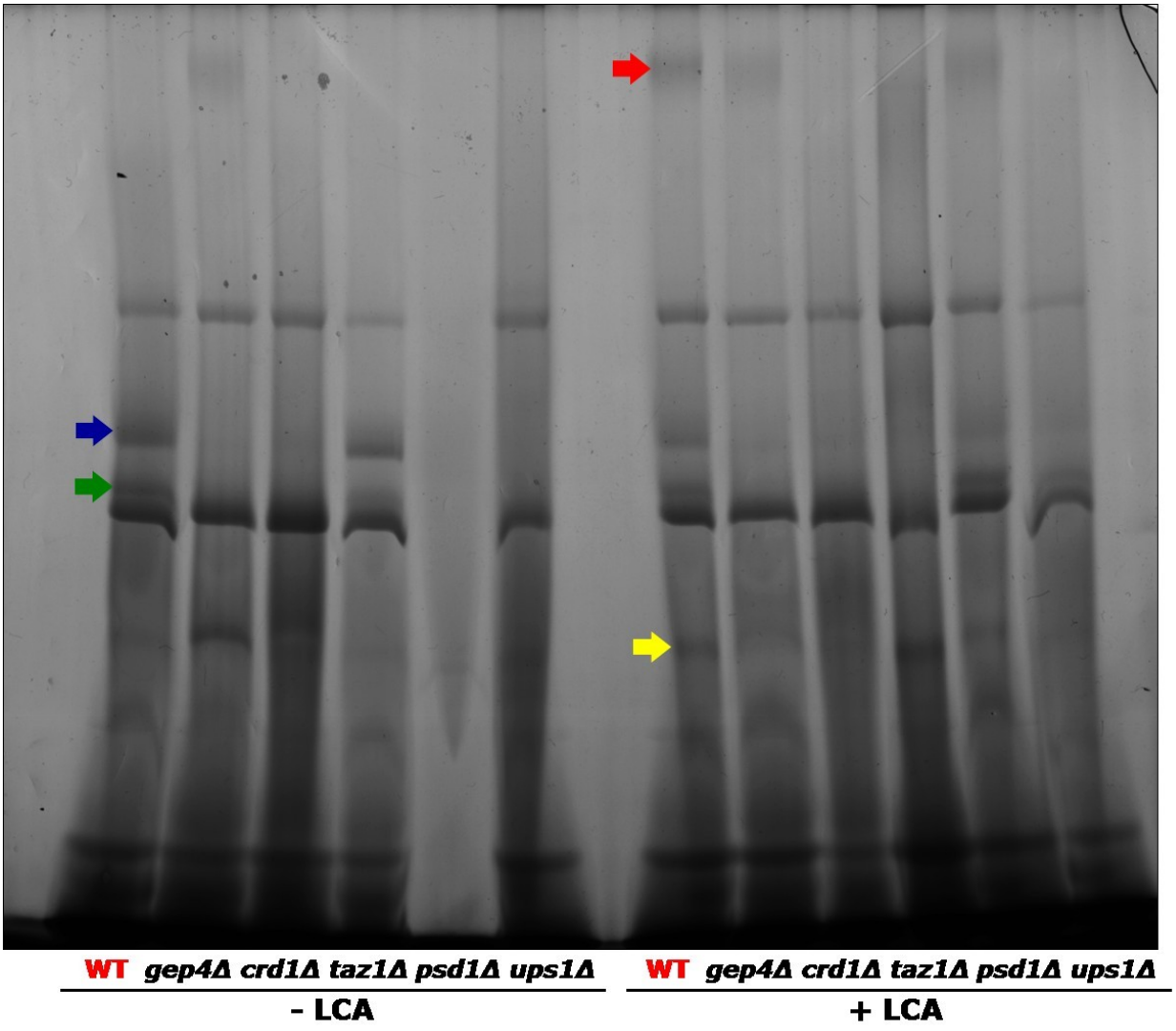

Figure 3.1. LCA and genetic manipulations changing the composition of mitochondrial membrane lipids alter the abundance of respiratory supercomplexes in the inner mitochondrial membrane. Cells of WT strain, as well as cells of various long- and short-lived mutant strains impaired in different aspects of CL, PE and PS metabolism, were grown in YP medium initially containing 0.2% glucose as carbon source, without LCA or with this bile acid (used in the concentration of 50 μM). Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory supercomplexes and their first-dimension BN-PAGE separation were performed as described in Materials and Methods. The respiratory supercomplexes marked with arrows are described in the text.

Bat1p and Bat2p, and is known to interact genetically with mitochondrial ribosomal protein genes.

Our BN-PAGE analysis also demonstrated that exposure of WT cells to LCA increases the level of a respiratory supercomplex of a very high molecular weight (likely in an MDa range), which is marked by a yellow arrow in Figure 3.1. Our MS-based analysis described below (see section 3.4.2 of this Chapter on my thesis) showed that this respiratory supercomplex in the inner mitochondrial membrane of LCA-treated WT cells consists of (1) complexes III and V of the mitochondrial ETC; (2) the ALD4 isoform of acetaldehyde dehydrogenase, an enzyme involved in the conversion of acetaldehyde to acetyl-CoA in yeast mitochondria; (3) the IDH1 and IDH2 isoforms of isocitrate dehydrogenase, an enzyme involved in the TCA cycle; and (4) the mitochondrial porin POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability.

We found that each of the tested mutations impairing different aspects of CL, PE and PS metabolism in the inner mitochondrial membrane affects the abundance of one or more respiratory supercomplexes in this membrane. Such effects of these mutations were observed in cells exposed to LCA or remained untreated, as described below.

In cells not exposed to LCA, the *gep4Δ* mutation (known to impair the synthesis of phosphatidylglycerol from phosphatidylglycerol phosphate [see Figure 2.1] and to shorten yeast longevity [Beach et al., manuscript in preparation]) significantly increases the level of a respiratory supercomplex with the highest molecular weight (likely in a range of several MDa), which is marked by a red arrow in Figure 3.1. Our MS-based analysis described below (see section 3.4.2 of this Chapter on my thesis) showed that this

respiratory supercomplex in the inner mitochondrial membrane of *gep4Δ* cells not exposed to LCA consists of (1) complex V of the mitochondrial ETC; (2) the mitochondrial external NADH dehydrogenase NDE1, a type II NAD(P)H:quinone oxidoreductase that catalyzes the oxidation of cytosolic NADH to provide cytosolic NADH to the mitochondrial ETC; (3) the mitochondrial succinate-fumarate transporter SFC1, which is known to transport succinate into and fumarate out of the mitochondrion and to be required for ethanol and acetate utilization; (4) the lipid-binding protein PIL1, a mitochondrial member of the BAR domain protein family; and (5) the mitochondrial porin POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability.

Furthermore, in cells not exposed to LCA, the *gep4Δ* mutation also increases the level of a respiratory supercomplex of a very high molecular weight (likely in an MDa range), which is marked by a yellow arrow in Figure 3.1. Our MS-based analysis described below (see section 3.4.2 of this Chapter on my thesis) showed that this respiratory supercomplex in the inner mitochondrial membrane of *gep4Δ* cells not exposed to LCA consists of (1) complexes III and V of the mitochondrial ETC; (2) the IDH1 and IDH2 isoforms of isocitrate dehydrogenase, an enzyme involved in the TCA cycle; and (3) the ADP-ATP carrier protein AAC2.

Moreover, in cells not exposed to LCA, the *gep4Δ* mutation greatly reduces the levels of two high-molecular-weight (both likely in an MDa range) respiratory supercomplexes that are marked by blue and green arrows in Figure 3.1.

Of note, an exposure of *gep4Δ* cells to LCA, reduces (not elevates, as in LCA-treated WT cells) the level of a respiratory supercomplex of a very high molecular weight

(likely in an MDa range), which is marked by a yellow arrow in Figure 3.1. Our MS-based analysis described below (see section 3.4.2 of this Chapter on my thesis) revealed that this respiratory supercomplex in the inner mitochondrial membrane of LCA-treated WT cells consists of (1) complexes III and V of the mitochondrial ETC; (2) the ALD4 isoform of acetaldehyde dehydrogenase, an enzyme involved in the conversion of acetaldehyde to acetyl-CoA in yeast mitochondria; (3) the IDH1 and IDH2 isoforms of isocitrate dehydrogenase, an enzyme involved in the TCA cycle; and (4) the mitochondrial porin POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability.

We also found that in cells not exposed to LCA, the *crd1Δ* mutation (known to impair the synthesis of CL from PG [see Figure 2.1] and to shorten yeast longevity [Beach et al., manuscript in preparation]) greatly reduces the levels of two high-molecular-weight (both likely in an MDa range) respiratory supercomplexes that are marked by blue and green arrows in Figure 3.1. This effect of the *crd1Δ* mutation was reminiscent to the changes in the abundance of these two respiratory supercomplexes seen in *gep4Δ* mutant cells not exposed to LCA (Figure 3.1). Furthermore, the *crd1Δ* mutation impairs the observed in LCA-treated WT cells rise in the levels of two rrespiratory supercomplexes marked by red and yellow arrows in Figure 3.1.

Notably, although in the absence of LCA the *taz1Δ* mutation (known to impair the conversion of CL to MLCL [see Figure 2.1] and not to affect yeast longevity [Beach et al., manuscript in preparation]) does not alter the abundance of any respiratory supercomplex found in WT cells not exposed to this bile acid, in the presence of LCA

this mutation greatly reduces the levels of three high-molecular-weight respiratory supercomplexes that are marked by red, blue and green arrows in Figure 3.1. It should be emphasized that, as we found, the *taz1Δ* mutation eliminates the longevity-extending effect of LCA (Beach et al., manuscript in preparation).

Noteworthy, in the absence of LCA the *psd1Δ* mutation (known to impair the conversion of PS to PE [see Figure 2.1] and to significantly shorten yeast longevity [Beach et al., manuscript in preparation]) entirely abrogates the assembly of all of the respiratory supercomplexes found in WT cells not exposed to LCA (Figure 3.1). However, an exposure of *psd1Δ* mutant cells to LCA (1) suppresses the caused by the *psd1Δ* mutation defect in the assembly of each of these respiratory supercomplexes; and (2) just like in LCA-treated WT cells, increases the levels of two high-molecular-weight respiratory supercomplexes that are marked by red and yellow arrows in Figure 3.1. It should be emphasized that, as we found, the *psd1Δ* mutation enhances the longevity-extending efficacy of LCA (Beach et al., manuscript in preparation).

Finally, in cells not exposed to LCA, the *ups1Δ* mutation (known to eliminate a mitochondrial membrane protein that stimulates the synthesis of CL and inhibits the synthesis of PE [see Figure 2.1] and to shorten yeast longevity [Beach et al., manuscript in preparation]) greatly reduces the levels of two high-molecular-weight (both likely in an MDa range) respiratory supercomplexes that are marked by blue and green arrows in Figure 3.1. This effect of the *ups1Δ* mutation was reminiscent to the changes in the abundance of these two respiratory supercomplexes seen in *gep4Δ* and *crd1Δ* mutant cells not exposed to LCA (Figure 3.1). Furthermore, the *ups1Δ* mutation impairs the observed in LCA-treated WT cells rise in the levels of three rrespiratory supercomplexes marked

by red, blue and yellow arrows in Figure 3.1.

3.4.2 In WT cells, LCA alters the composition of several respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane

In our hypothesis, by altering the level of CL and other glycerophospholipids within the mitochondrial membrane in WT cells, LCA could modulate not only the abundance of some of the respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane, but also their protein composition (see section 3.2 of this thesis). According to our hypothesis, such LCA-dependent remodeling of the mitochondrial respiratory supercomplexes in WT cells may recruit new mitochondrial protein components, not previously known for being permanently associated with the ETC, into the remodeled respirasomes. We also hypothesized that LCA may alter the composition of some non-respiratory protein supercomplexes in the inner mitochondrial membrane or stimulate their *de novo* formation. To test the validity of this hypothesis, we used a multistep method [243 - 253] for recovery of intact respiratory complexes and supercomplexes from purified yeast mitochondria, their subsequent first-dimension electrophoretic separation using BN-PAGE, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components. Cells of WT strain were cultured under CR conditions on 0.2% glucose, and mitochondria were purified from cells recovered at day 4 of culturing. We found that LCA alters the composition of several respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane (Figures 3.2 and 3.3). Specifically, LCA causes a remodeling of three respiratory supercomplexes, eliminates

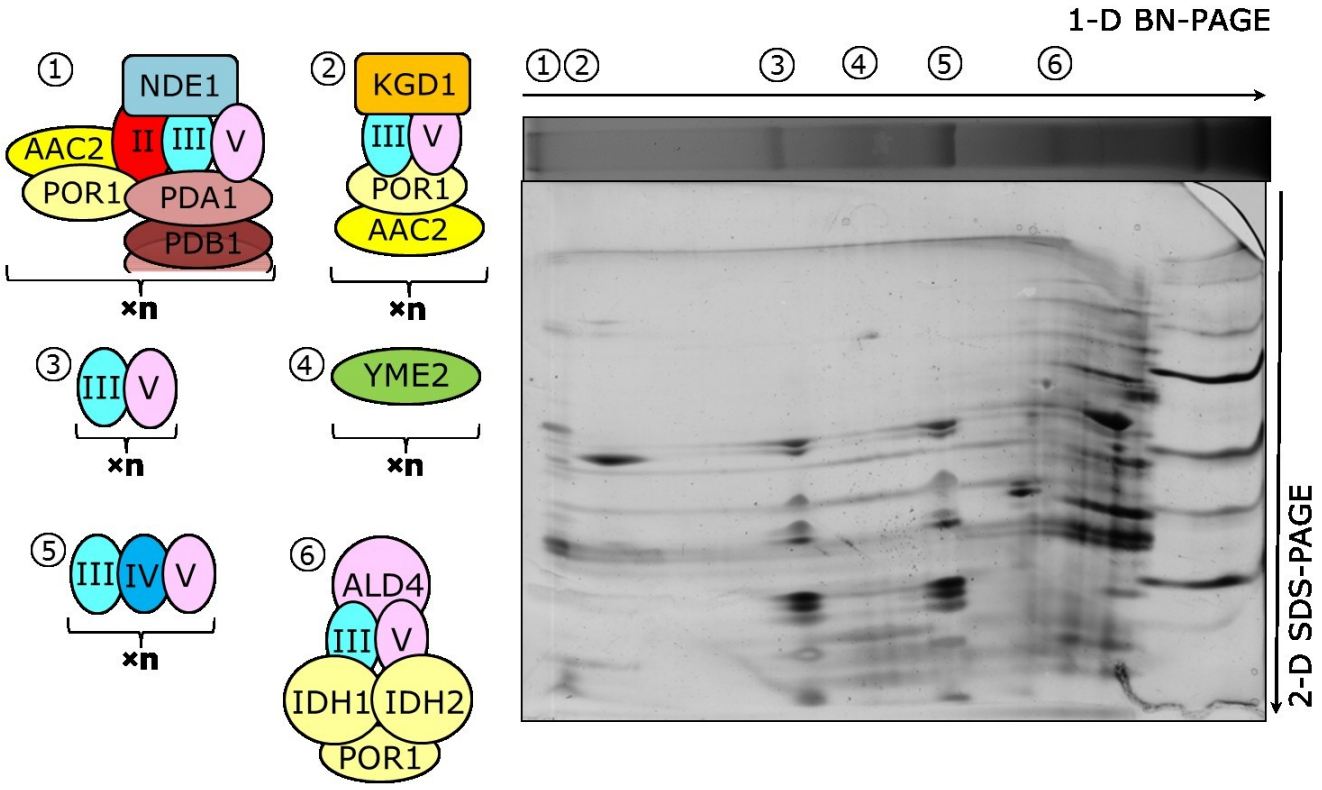

Figure 3.2. The composition of respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane of WT cells not exposed to LCA. Respiratory and non-respiratory protein supercomplexes recovered using a first-dimension BN-PAGE were dissociated into individual protein components with the help of a second-dimension Tricine-SDS-PAGE and identified using MS-based proteomics. Cells of WT strain were grown in YP medium initially containing 0.2% glucose as carbon source without LCA. Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory and non-respiratory protein supercomplexes, their first-dimension BN-PAGE separation, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components were performed as described in Materials and Methods.

one high-molecular-weight (likely in an MDa range) non-respiratory protein supercomplex, increases the molecular weight of a respiratory supercomplex, and stimulates the formation of a high-molecular-weight non-respiratory protein

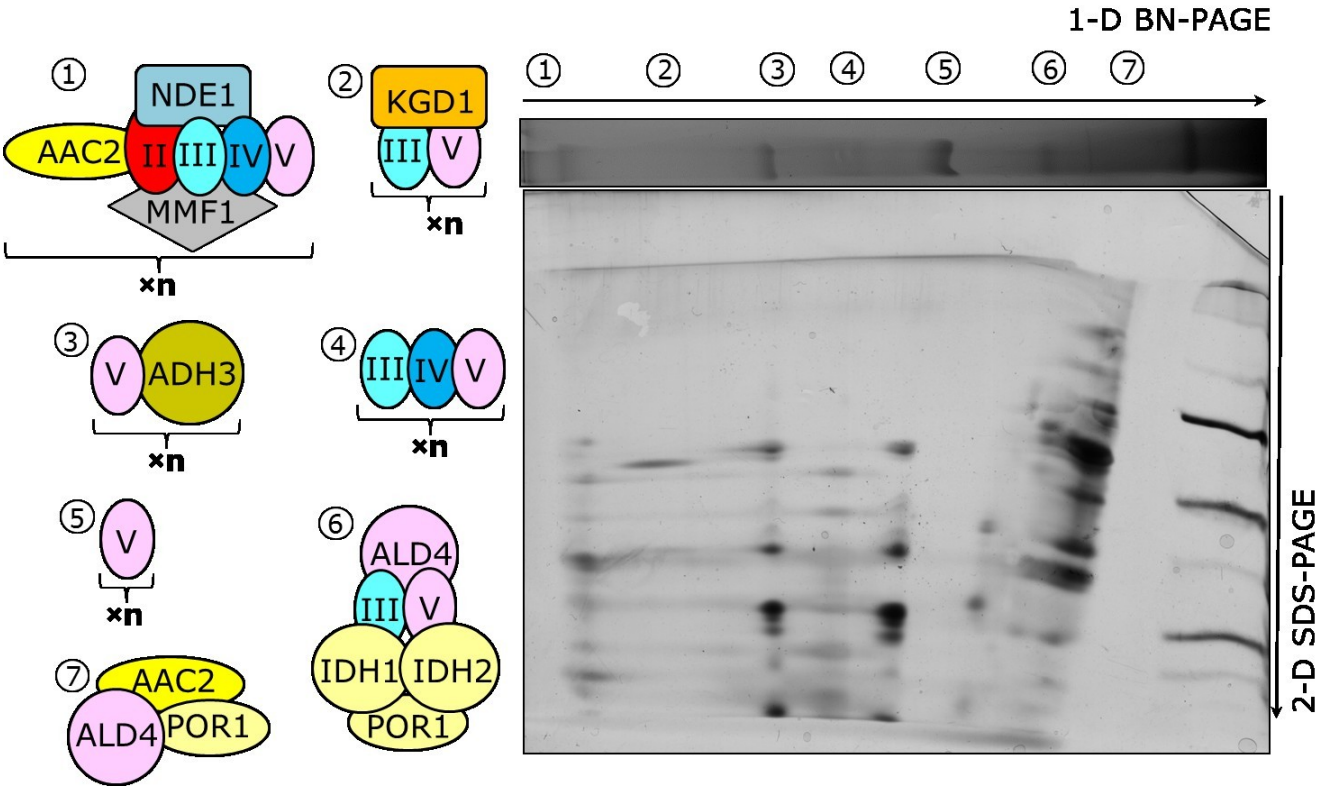

Figure 3.3. In WT cells, LCA alters the composition of several respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane. Respiratory and non-respiratory protein supercomplexes recovered using a first-dimension BN-PAGE were dissociated into individual protein components with the help of a second-dimension Tricine-SDS-PAGE and identified using MS-based proteomics. Cells of WT strain were grown in YP medium initially containing 0.2% glucose as carbon source in the presence of 50 µM LCA. Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory and non-respiratory protein supercomplexes, their first-dimension BN-PAGE separation, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components were performed as described in Materials and Methods.

supercomplex that cannot be detected in WT cells not exposed to LCA (Figures 3.2 and 3.3).

A detailed description of LCA-induced changes in the composition of respiratory

and non-respiratory protein supercomplexes in the inner mitochondrial membrane of WT cells is presented below.

LCA causes a remodeling of respiratory supercomplex 1 by: (1) promoting the incorporation of respiratory complex IV into this supercomplex; (2) stimulating the insertion of the mitochondrial protein MMF1 (which is required for transamination of isoleucine, may regulate specificity of branched-chain transaminases Bat1p and Bat2p, and is known to interact genetically with mitochondrial ribosomal protein genes) into this supercomplex; and (3) causing a release of PDA1 (an E1 alpha subunit of the pyruvate dehydrogenase complex, which catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA), PDB1 (an E1 alpha subunit of the pyruvate dehydrogenase complex, which catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA) and POR1 (a voltage-dependent anion channel, which is called porin and is required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability) from this supercomplex (Figures 3.2 and 3.3).

LCA causes a remodeling of respiratory supercomplex 2 by causing a release of POR1 (porin, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability) and AAC2 (an ADP-ATP carrier protein) from this supercomplex (Figures 3.2 and 3.3).

LCA causes a remodeling of respiratory supercomplex 3 by: (1) stimulating the insertion of ADH3 (a mitochondrial alcohol dehydrogenase isozyme III involved in the shuttling of mitochondrial NADH to the cytosol and ethanol production); and (2) causing a release of respiratory complex III from this supercomplex (Figures 3.2 and 3.3).

LCA eliminates a high-molecular-weight non-respiratory protein supercomplex

formed by YME2 (an integral inner mitochondrial membrane protein with a role in maintaining mitochondrial nucleoid structure and number); this supercomplex is given number 4 in Figure 3.2.

LCA increases the molecular weight of a respiratory supercomplex composed of respiratory complexes III, IV and V; this supercomplex is given number 5 in Figure 3.2 and number 4 in Figure 3.3.

Finally, LCA stimulates the formation of a high-molecular-weight non-respiratory protein supercomplex 7 that cannot be detected in WT cells not exposed to LCA (Figures 3.2 and 3.3). This supercomplex consists of (1) AAC2, an ADP-ATP carrier protein; (2) the ALD4 isoform of acetaldehyde dehydrogenase, an enzyme involved in the conversion of acetaldehyde to acetyl-CoA in yeast mitochondria; and (3) the porin POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability (Figures 3.2 and 3.3).

3.4.3 Genetic manipulations changing the makeup of mitochondrial membrane lipids alter the composition of respiratory supercomplexes in the inner mitochondrial membrane

According to our hypothesis, genetic manipulations that alter the level of CL and other glycerophospholipids within the mitochondrial membrane could modulate not only the abundance of some of the respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane, but also their protein composition (see section 3.2 of this thesis). We hypothesized that such remodeling of the mitochondrial respiratory supercomplexes in mutant cells may recruit new mitochondrial protein components, not

previously known for being permanently associated with the ETC, into the remodeled respirasomes. We also assumed that genetic manipulations changing the makeup of mitochondrial membrane lipids may alter the composition of some non-respiratory protein supercomplexes in the inner mitochondrial membrane or stimulate their *de novo* formation. To test the validity of this hypothesis, we used a multistep method [243 - 253] for recovery of intact respiratory complexes and supercomplexes from purified yeast mitochondria, their subsequent first-dimension electrophoretic separation using BN-PAGE, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components. Cells of the *gep4Δ*, *crd1Δ* and *taz1Δ* mutant strains were cultured under CR conditions on 0.2% glucose (with or without LCA), and mitochondria were purified from cells recovered at day 4 of culturing. We found that each of these mutations alters the composition of several respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane, regardless of the presence of LCA (Figures 3.5 - 3.9).

A detailed description of changes in the composition of respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane of *gep4Δ*, *crd1Δ* and *taz1Δ* mutant cells, exposed to LCA or remained untreated, is presented below.

In *gep4Δ* mutant cells not exposed to LCA, the respiratory supercomplex 1: (1) lacks respiratory complexes II and III of the ETC; (2) lacks AAC2, an ADP-ATP carrier protein; (3) lacks PDA1 (an E1 alpha subunit of the pyruvate dehydrogenase complex, which catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA) and PDB1 (an E1 alpha subunit of the pyruvate dehydrogenase complex, which catalyzes the

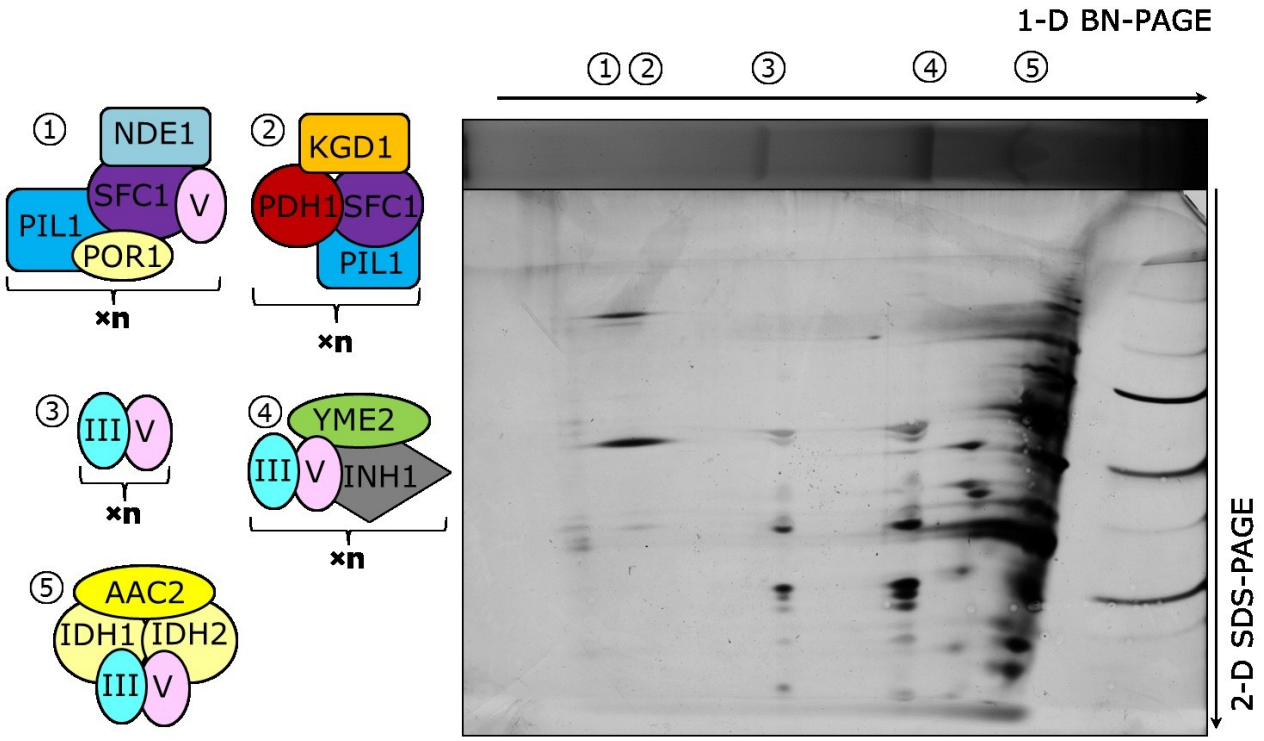

Figure 3.4. The composition of respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane of *gep4Δ* mutant cells not exposed to LCA. Respiratory and non-respiratory protein supercomplexes recovered using a first-dimension BN-PAGE were dissociated into individual protein components with the help of a second-dimension Tricine-SDS-PAGE and identified using MS-based proteomics. Cells of *gep4Δ* mutant strain were grown in YP medium initially containing 0.2% glucose as carbon source without LCA. Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory and non-respiratory protein supercomplexes, their first-dimension BN-PAGE separation, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components were performed as described in Materials and Methods.

direct oxidative decarboxylation of pyruvate to acetyl-CoA); (4) acquires the lipid-binding protein PIL1, a mitochondrial member of the BAR domain protein family; and (5) acquires the mitochondrial succinate-fumarate transporter SFC1, which is known to transport succinate into and fumarate out of the mitochondrion and to be required for

ethanol and acetate utilization (compare Figures 3.2 and 3.4).

In *gep4Δ* mutant cells not exposed to LCA, the respiratory supercomplex 2: (1) lacks respiratory complexes III and V of the ETC; (2) lacks AAC2, an ADP-ATP carrier protein; (3) lacks the mitochondrial porin POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability; (4) acquires the mitochondrial succinate-fumarate transporter SFC1, which is known to transport succinate into and fumarate out of the mitochondrion and to be required for ethanol and acetate utilization; (5) acquires the lipid-binding protein PIL1, a mitochondrial member of the BAR domain protein family; and (6) acquires PDH1, a protein that participates in respiration through a mechanism that remains to be established (compare Figures 3.2 and 3.4).

In *gep4Δ* mutant cells not exposed to LCA, the respiratory supercomplex 4: (1) acquires complexes III and V of the mitochondrial ETC; and (2) acquires INH1, a calmodulin-binding protein that inhibits ATP hydrolysis by respiratory complex V (compare Figures 3.2 and 3.4).

Importantly, *gep4Δ* mutant cells not exposed to LCA lack a respiratory supercomplex that in untreated with LCA WT cells is called respiratory supercomplex 5 and consists of respiratory complexes III, IV and V (compare Figures 3.2 and 3.4).

In *gep4Δ* mutant cells not exposed to LCA, the respiratory supercomplex 5: (1) lacks the ALD4 isoform of acetaldehyde dehydrogenase, an enzyme involved in the conversion of acetaldehyde to acetyl-CoA in yeast mitochondria; (2) lacks the mitochondrial porin POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane

permeability; and (3) acquires AAC2, an ADP-ATP carrier protein (compare Figures 3.2 and 3.4).

In *gep4Δ* mutant cells treated with LCA, the respiratory supercomplex 1: (1) lacks respiratory complexes II and IV; (2) lacks the mitochondrial external NADH dehydrogenase NDE1, a type II NAD(P)H:quinone oxidoreductase that catalyzes the oxidation of cytosolic NADH to provide cytosolic NADH to the mitochondrial ETC; (3) lacks the mitochondrial protein MMF1 (which is required for transamination of isoleucine, may regulate specificity of branched-chain transaminases Bat1p and Bat2p, and is known to interact genetically with mitochondrial ribosomal protein genes) into this supercomplex; (4) acquires citrate synthase CIT1, a rate-limiting enzyme of the TCA cycle which catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate; (5) acquires aconitase ACO1, which is required for the TCA cycle and also independently required for mitochondrial genome maintenance; (6) acquires PDH1, a protein that participates in respiration through a mechanism that remains to be established; (7) acquires PRX1, a mitochondrial peroxiredoxin with thioredoxin peroxidase activity; (8) acquires SOD2, a mitochondrial manganese superoxide dismutase that protects cells against extremely toxic superoxide radicals; (9) acquires MIR1, a mitochondrial phosphate carrier that imports inorganic phosphate into mitochondria; (10) acquires the lipid-binding protein PIL1, a mitochondrial member of the BAR domain protein family; (11) acquires AIM46, a protein required for mitochondrial DNA maintenance; and (12) acquires FMP46, a protein protecting mitochondria from oxidative damage (compare Figures 3.3 and 3.5).

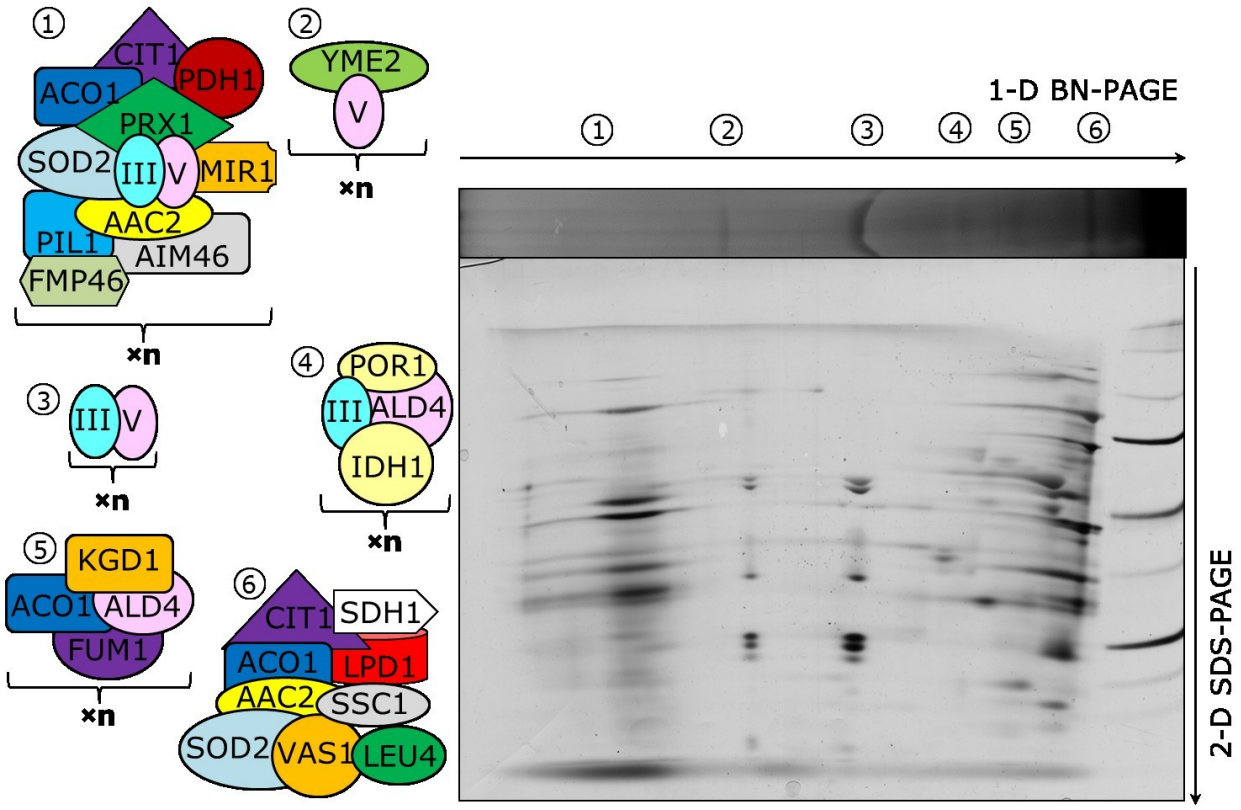

Figure 3.5. In *gep4Δ* cells, LCA alters the composition of several respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane. Respiratory and non-respiratory protein supercomplexes recovered using a first-dimension BN-PAGE were dissociated into individual protein components with the help of a second-dimension Tricine-SDS-PAGE and identified using MS-based proteomics. Cells of *gep4Δ* mutant strain were grown in YP medium initially containing 0.2% glucose as carbon source in the presence of 50 μM LCA. Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory and non-respiratory protein supercomplexes, their first-dimension BN-PAGE separation, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components were performed as described in Materials and Methods.

In *gep4Δ* mutant cells treated with LCA, the respiratory supercomplex 2: (1) lacks ADH3, a mitochondrial alcohol dehydrogenase isozyme III involved in the shuttling of mitochondrial NADH to the cytosol and ethanol production; and (2) acquires YME2, an

integral inner mitochondrial membrane protein with a role in maintaining mitochondrial nucleoid structure and number (compare Figures 3.3 and 3.5). In WT cells treated with LCA, this respiratory supercomplex is called supercomplex 3 (compare Figures 3.3 and 3.5).

In *gep4Δ* mutant cells treated with LCA, the respiratory supercomplex 3 acquires respiratory complex III; in WT cells exposed to LCA, this respiratory supercomplex is called supercomplex 5 (compare Figures 3.3 and 3.5).

Importantly, three respiratory and non-respiratory supercomplexes are formed *de novo* in *gep4Δ* mutant cells treated with LCA; indeed, none of them can be found in WT cells exposed to LCA (compare Figures 3.3 and 3.5). A description of these protein supercomplexes is provided below.

In *gep4Δ* mutant cells treated with LCA, the *de novo* formed respiratory supercomplex 4 includes the following proteins: (1) complex III of the ETC; (2) POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability; (3) the ALD4 isoform of acetaldehyde dehydrogenase, an enzyme involved in the conversion of acetaldehyde to acetyl-CoA in yeast mitochondria; and (4) the IDH1 isoform of isocitrate dehydrogenase, an enzyme involved in the TCA cycle (Figure 3.5).

In *gep4Δ* mutant cells treated with LCA, the *de novo* formed non-respiratory supercomplex 5 includes the following proteins: (1) KGD1, a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the TCA cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA; (2) aconitase ACO1, which is required for the TCA cycle and also independently

required for mitochondrial genome maintenance; (3) the ALD4 isoform of acetaldehyde dehydrogenase, an enzyme involved in the conversion of acetaldehyde to acetyl-CoA in yeast mitochondria; and (4) fumarase FUM1 that converts fumaric acid to L-malic acid in the TCA cycle (Figure 3.5).

Finally, in *gep4Δ* mutant cells treated with LCA, the *de novo* formed non-respiratory supercomplex 5 includes the following proteins: (1) citrate synthase CIT1, a rate-limiting enzyme of the TCA cycle that catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate; (2) aconitase ACO1, which is required for the TCA cycle and also independently required for mitochondrial genome maintenance; (3) SDH1, a flavoprotein subunit of succinate dehydrogenase that couples the oxidation of succinate to the transfer of electrons to ubiquinone as part of the TCA cycle and the mitochondrial ETC; (4) dihydrolipoamide dehydrogenase LPD1, a lipoamide dehydrogenase component (E3) of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multi-enzyme complexes; (5) AAC2, an ADP-ATP carrier protein; (6) SSC1, an HSP70 family ATPase that is involved in mitochondrial protein translocation and folding; (7) SOD2, a mitochondrial manganese superoxide dismutase that protects cells against extremely toxic superoxide radicals; (8) VAS1, a valyl-tRNA synthetase for protein synthesis in mitochondria; and (9) alpha-isopropylmalate synthase (2-isopropylmalate synthase) LEU4, the main isozyme responsible for the first step in the leucine biosynthesis pathway (Figure 3.5).

In *crd1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 1: (1) lacks respiratory complex II of the ETC; (2) lacks PDA1 (an E1 alpha subunit of the pyruvate dehydrogenase complex, which catalyzes the direct oxidative decarboxylation

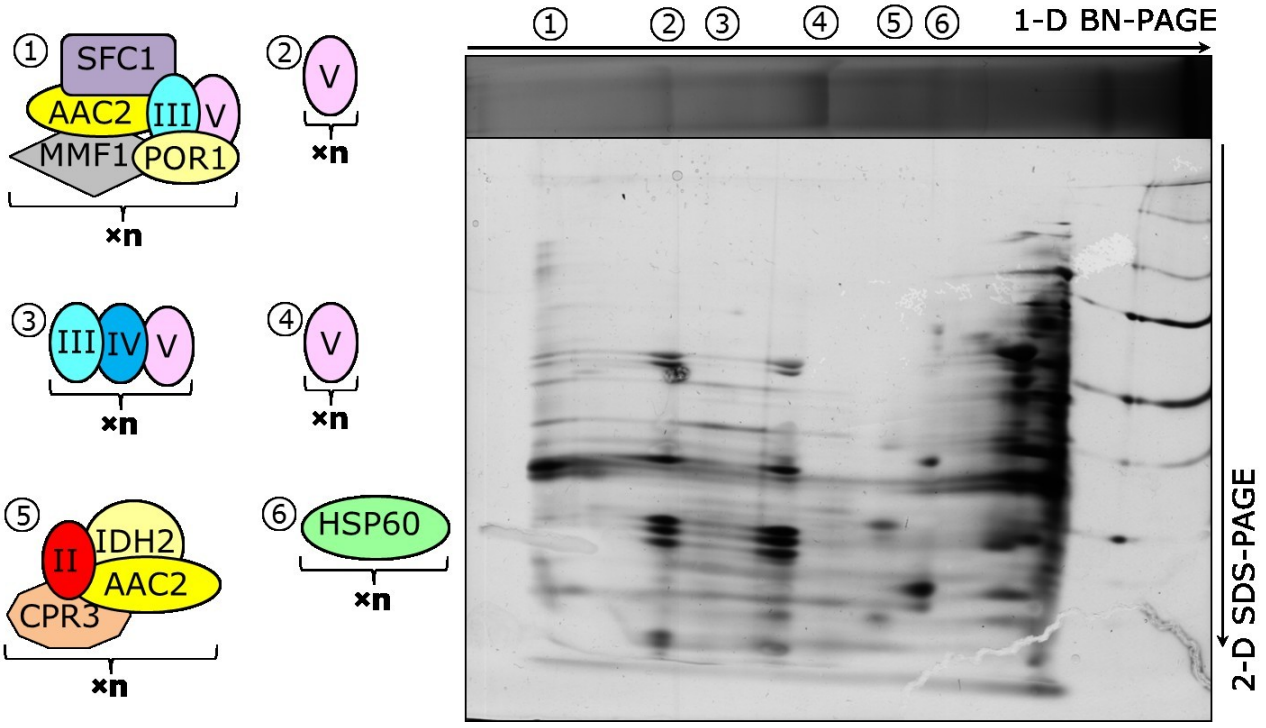

Figure 3.6. The composition of respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane of *crd1Δ* mutant cells not exposed to LCA. Respiratory and non-respiratory protein supercomplexes recovered using a first-dimension BN-PAGE were dissociated into individual protein components with the help of a second-dimension Tricine-SDS-PAGE and identified using MS-based proteomics. Cells of *crd1Δ* mutant strain were grown in YP medium initially containing 0.2% glucose as carbon source without LCA. Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory and non-respiratory protein supercomplexes, their first-dimension BN-PAGE separation, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components were performed as described in Materials and Methods.

of pyruvate to acetyl-CoA) and PDB1 (an E1 alpha subunit of the pyruvate dehydrogenase complex, which catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA); (3) lacks the mitochondrial external NADH dehydrogenase NDE1, a type II NAD(P)H:quinone oxidoreductase that catalyzes the oxidation of

cytosolic NADH to provide cytosolic NADH to the mitochondrial ETC; (4) acquires the mitochondrial succinate-fumarate transporter SFC1, which is known to transport succinate into and fumarate out of the mitochondrion and to be required for ethanol and acetate utilization; and (5) acquires the mitochondrial protein MMF1 (which is required for transamination of isoleucine, may regulate specificity of branched-chain transaminases Bat1p and Bat2p, and is known to interact genetically with mitochondrial ribosomal protein genes) into this supercomplex (compare Figures 3.2 and 3.6).

In *crd1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 2 found in untreated WT cells (Figure 3.2) is absent (Figure 3.6); this respiratory supercomplex consists of respiratory complexes III and V of the ETC, KGD1, POR1, and AAC2.

In *crd1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 2 lacks respiratory complex III; in untreated WT cells, this respiratory supercomplex is called supercomplex 3 (compare Figures 3.2 and 3.6).

In *crd1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 4 found in untreated WT cells (Figure 3.2) is absent (Figure 3.6); this respiratory supercomplex consists of YME2 protein molecules.

The molecular weight of the respiratory supercomplex 3 found in *crd1Δ* mutant cells not exposed to LCA is significantly higher (Figure 3.6) than that of the supercomplex 5 seen in untreated WT cells (Figure 3.2); both these respiratory supercomplexes composed of respiratory complexes III, IV and V.

In *crd1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 4 lacks respiratory complexes III and IV; in untreated WT cells, this respiratory supercomplex is called supercomplex 5 (compare Figures 3.2 and 3.6).

In *crd1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 6 found in untreated WT cells (Figure 3.2) is absent (Figure 3.6); this respiratory supercomplex consists of respiratory complexes III and V of the ETC, ALD4, IDH1, IDH2, and POR1.

Importantly, one respiratory and one non-respiratory supercomplexes are formed *de novo* in *crd1Δ* mutant cells not exposed to LCA; indeed, none of them can be found in untreated WT cells (compare Figures 3.2 and 3.6). A description of these protein supercomplexes is provided below.

In *crd1Δ* mutant cells not exposed to LCA, the *de novo* formed respiratory supercomplex 5 includes the following proteins: (1) respiratory complex II of the ETC; (2) the IDH2 isoform of isocitrate dehydrogenase, an enzyme involved in the TCA cycle; (3) AAC2, an ADP-ATP carrier protein; and (4) CPR3, a mitochondrial peptidyl-prolyl cis-trans isomerase (cyclophilin), which catalyzes the cis-trans isomerization of peptide bonds N-terminal to proline residues and is involved in protein refolding after import into mitochondria (Figure 3.6).

Finally, in *crd1Δ* mutant cells not exposed to LCA, the *de novo* formed respiratory supercomplex 6 includes protein molecules of HSP60, a tetradecameric mitochondrial chaperonin that not only is required for ATP-dependent folding of precursor polypeptides and complex assembly, but also prevents aggregation and mediates protein refolding after heat shock (Figure 3.6).

In *crd1Δ* mutant cells treated with LCA, the respiratory supercomplex 1: (1) lacks respiratory complexes II and IV; (2) lacks the mitochondrial external NADH dehydrogenase NDE1, a type II NAD(P)H:quinone oxidoreductase that catalyzes the oxidation of cytosolic NADH to provide cytosolic NADH to the mitochondrial ETC; (3)

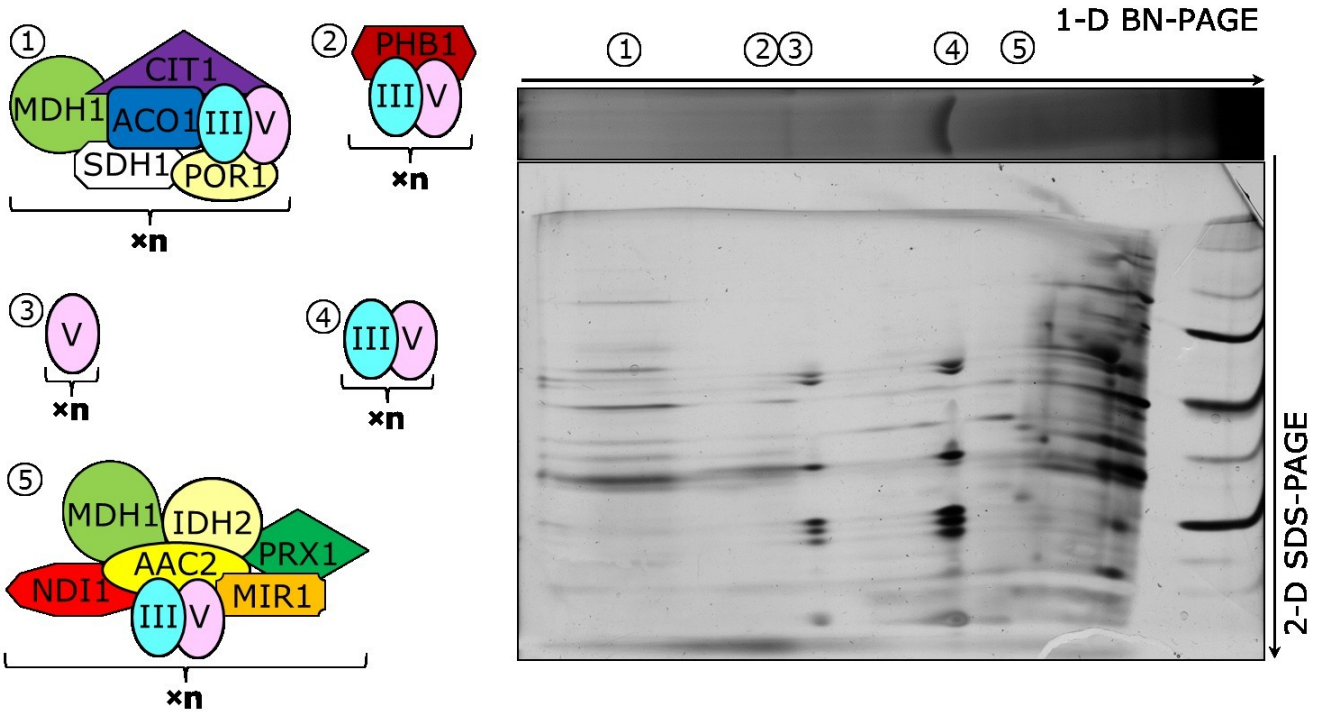

Figure 3.7. In *crd1Δ* cells, LCA alters the composition of several respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane. Respiratory and non-respiratory protein supercomplexes recovered using a first-dimension BN-PAGE were dissociated into individual protein components with the help of a second-dimension Tricine-SDS-PAGE and identified using MS-based proteomics. Cells of *crd1Δ* mutant strain were grown in YP medium initially containing 0.2% glucose as carbon source in the presence of 50 μM LCA. Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory and non-respiratory protein supercomplexes, their first-dimension BN-PAGE separation, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components were performed as described in Materials and Methods.

lacks the mitochondrial protein MMF1 (which is required for transamination of isoleucine, may regulate specificity of branched-chain transaminases Bat1p and Bat2p, and is known to interact genetically with mitochondrial ribosomal protein genes) into this supercomplex; (4) lacks AAC2, an ADP-ATP carrier protein; (5) acquires citrate

synthase CIT1, a rate-limiting enzyme of the TCA cycle which catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate; (6) acquires aconitase ACO1, which is required for the TCA cycle and also independently required for mitochondrial genome maintenance; (7) acquires malate dehydrogenase MDH1, an enzyme that catalyzes interconversion of malate and oxaloacetate as part of the TCA cycle; (8) acquires SDH1, a flavoprotein subunit of succinate dehydrogenase that couples the oxidation of succinate to the transfer of electrons to ubiquinone as part of the TCA cycle and the mitochondrial ETC; and (9) acquires POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability (compare Figures 3.3 and 3.7).

In *crd1Δ* mutant cells treated with LCA, the respiratory supercomplex 2: (1) lacks KGD1, a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the TCA cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA; and (2) acquires PHB1, a subunit of the prohibitin complex that functions as a membrane chaperone stabilizing newly synthesized proteins (compare Figures 3.3 and 3.7).

The molecular weight of the respiratory supercomplex 3 found in *crd1Δ* mutant cells treated with LCA is significantly higher (Figure 3.7) than that of the supercomplex 5 seen in WT cells exposed to LCA (Figure 3.3); both these respiratory supercomplexes composed of numerous molecules of respiratory complex V.

In *crd1Δ* mutant cells treated with LCA, the respiratory supercomplex 4 found in WT cells exposed to LCA (Figure 3.3) is absent (Figure 3.7); this respiratory supercomplex consists of respiratory complexes III, IV and V.

In *crd1Δ* mutant cells treated with LCA, the respiratory supercomplex 4 acquires respiratory complex IV; in WT cells exposed to LCA, this respiratory supercomplex is called supercomplex 5 (compare Figures 3.2 and 3.6).

Importantly, one respiratory supercomplex are formed *de novo* in *crd1Δ* mutant cells treated with LCA; indeed, this supercomplex cannot be found in WT cells exposed to LCA (compare Figures 3.3 and 3.7). This respiratory supercomplex includes the following proteins: (1) respiratory complexes I, III and V of the ETC; (2) the IDH2 isoform of isocitrate dehydrogenase, an enzyme involved in the TCA cycle; (3) AAC2, an ADP-ATP carrier protein; (4) MDH1, an enzyme that catalyzes interconversion of malate and oxaloacetate as part of the TCA cycle; (5) PRX1, a mitochondrial peroxiredoxin with thioredoxin peroxidase activity; and (6) MIR1, a mitochondrial phosphate carrier that imports inorganic phosphate into mitochondria (Figure 3.7).

In *taz1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 1: (1) lacks respiratory complex II of the ETC; (2) lacks PDA1 (an E1 alpha subunit of the pyruvate dehydrogenase complex, which catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA) and PDB1 (an E1 alpha subunit of the pyruvate dehydrogenase complex, which catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA); (3) lacks AAC2, an ADP-ATP carrier protein; and (4) acquires KGD 1, a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the TCA cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA (compare Figures 3.2 and 3.8).

In *taz1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 2: (1) lacks AAC2, an ADP-ATP carrier protein; (2) lacks POR1, a voltage-dependent anion

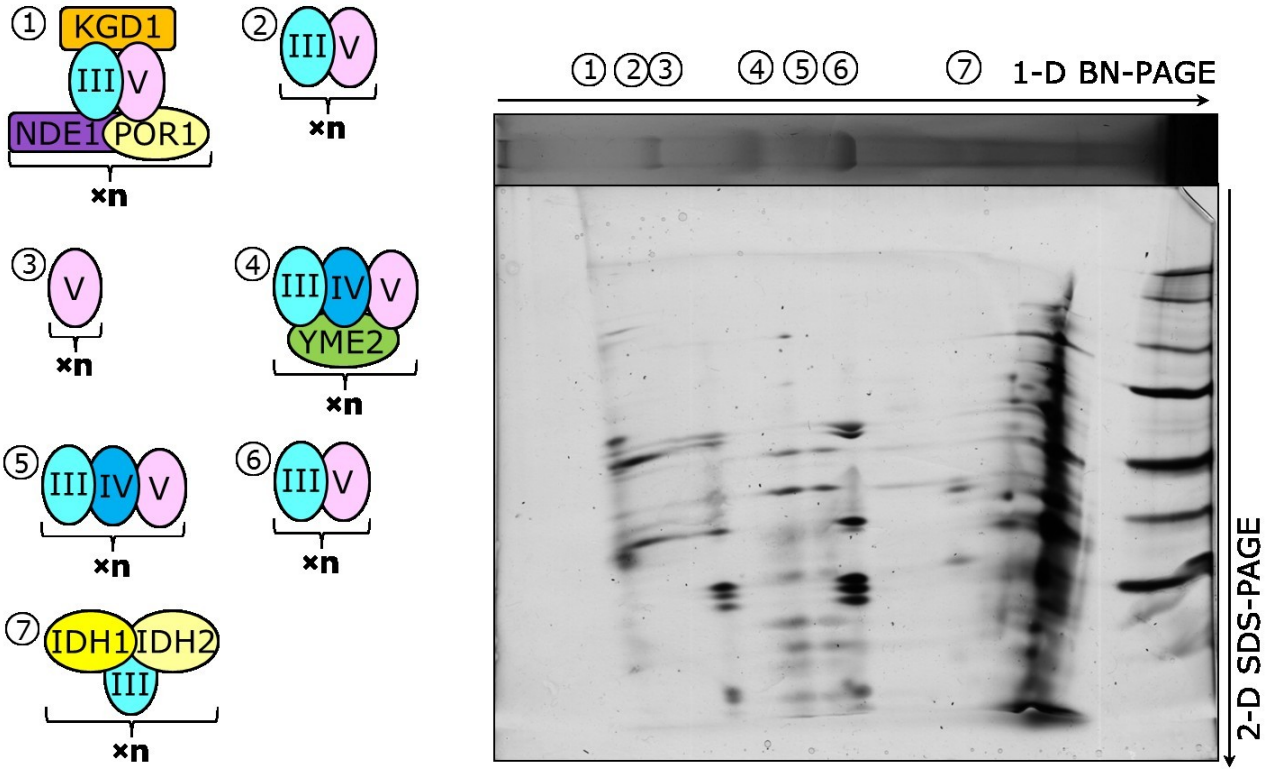

Figure 3.8. The composition of respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane of *taz1Δ* mutant cells not exposed to LCA. Respiratory and non-respiratory protein supercomplexes recovered using a first-dimension BN-PAGE were dissociated into individual protein components with the help of a second-dimension Tricine-SDS-PAGE and identified using MS-based proteomics. Cells of *taz1Δ* mutant strain were grown in YP medium initially containing 0.2% glucose as carbon source without LCA. Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory and non-respiratory protein supercomplexes, their first-dimension BN-PAGE separation, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components were performed as described in Materials and Methods.

channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability; and (3) lacks KGD1, a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in

the TCA cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA (compare Figures 3.2 and 3.8).

In *taz1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 3 lacks respiratory complex III (compare Figures 3.2 and 3.8).

In *taz1Δ* mutant cells not exposed to LCA, the respiratory supercomplex 4 acquires respiratory complexes III, IV and V (compare Figures 3.2 and 3.8).

Of note, instead of a single respiratory supercomplex 5 composed of respiratory complexes III, IV and V found in untreated WT cell (Figure 3.2), there are two respiratory supercomplexes of very similar molecular weights in *taz1Δ* mutant cells not exposed to LCA (compare Figures 3.2 and 3.8). One of them (which is called the respiratory supercomplex 5) consists of respiratory complexes III, IV and V, whereas the other supercomplex (which is termed the respiratory supercomplex 6) includes respiratory complexes III and V (Figure 3.8).

Importantly, one respiratory supercomplex is formed *de novo* in *taz1Δ* mutant cells not exposed to LCA; indeed, this supercomplex cannot be found in untreated WT cells (compare Figures 3.2 and 3.8). This respiratory supercomplex 7 includes the following proteins: (1) respiratory complex III of the ETC; and (2) the IDH1 and IDH2 isoforms of isocitrate dehydrogenase, an enzyme involved in the TCA cycle (Figure 3.8).

In *taz1Δ* mutant cells treated with LCA, the respiratory supercomplex 1: (1) lacks respiratory complexes II and IV; (2) lacks the mitochondrial protein MMF1 (which is required for transamination of isoleucine, may regulate specificity of branched-chain transaminases Bat1p and Bat2p, and is known to interact genetically with mitochondrial ribosomal protein genes) into this supercomplex; and (3) acquires POR1, a voltage-

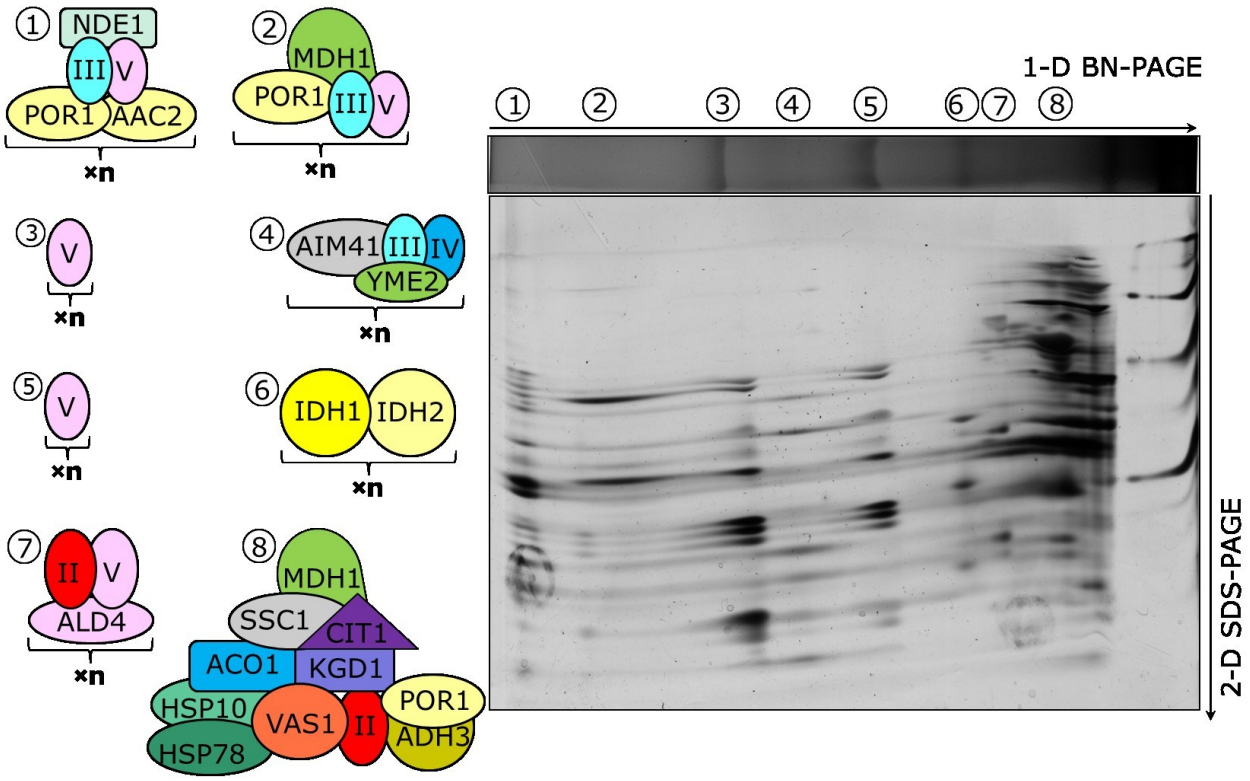

Figure 3.9. In *taz1Δ* cells, LCA alters the composition of several respiratory and non-respiratory protein supercomplexes in the inner mitochondrial membrane. Respiratory and non-respiratory protein supercomplexes recovered using a first-dimension BN-PAGE were dissociated into individual protein components with the help of a second-dimension Tricine-SDS-PAGE and identified using MS-based proteomics. Cells of *taz1Δ* mutant strain were grown in YP medium initially containing 0.2% glucose as carbon source in the presence of 50 μM LCA. Cells were collected for the purification of mitochondria at day 4. The purification of mitochondria, solubilization of native respiratory and non-respiratory protein supercomplexes, their first-dimension BN-PAGE separation, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components were performed as described in Materials and Methods.

dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability (compare Figures 3.3 and 3.9).

In *taz1Δ* mutant cells treated with LCA, the respiratory supercomplex 2: (1) lacks KGD1, a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the TCA cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA; (2) acquires MDH1, an enzyme that catalyzes interconversion of malate and oxaloacetate as part of the TCA cycle; and (3) acquires POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability (compare Figures 3.3 and 3.9).

In *taz1Δ* mutant cells treated with LCA, the respiratory supercomplex 3 lacks ADH3, a mitochondrial alcohol dehydrogenase isozyme III involved in the shuttling of mitochondrial NADH to the cytosol and ethanol production (compare Figures 3.3 and 3.9).

In *taz1Δ* mutant cells treated with LCA, the respiratory supercomplex 4: (1) lacks respiratory complex V of the ETC; (2) acquires AIM41, a protein required for mitochondrial DNA maintenance; and (3) acquires YME2, an integral inner mitochondrial membrane protein with a role in maintaining mitochondrial nucleoid structure and number (compare Figures 3.3 and 3.9).

In *taz1Δ* mutant cells treated with LCA, the respiratory supercomplex 6: (1) lacks respiratory complexes III and V of the ETC; (2) lacks the ALD4 isoform of acetaldehyde dehydrogenase, an enzyme involved in the conversion of acetaldehyde to acetyl-CoA in yeast mitochondria; and (3) lacks POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability (compare Figures 3.3 and 3.9).

In *taz1Δ* mutant cells treated with LCA, the respiratory supercomplex 7: (1) lacks POR1, a voltage-dependent anion channel required for the maintenance of mitochondrial osmotic stability and mitochondrial membrane permeability; (2) lacks AAC2, an ADP-ATP carrier protein; and (3) acquires respiratory complexes II and V of the ETC (compare Figures 3.3 and 3.9).

Importantly, one respiratory supercomplex are formed *de novo* in *taz1Δ* mutant cells treated with LCA; indeed, this supercomplex cannot be found in WT cells exposed to LCA (compare Figures 3.3 and 3.9). This respiratory supercomplex includes the following proteins: (1) respiratory complex II of the ETC; (2) MDH1, an enzyme that catalyzes interconversion of malate and oxaloacetate as part of the TCA cycle; (3) SSC1, an HSP70 family ATPase that is involved in mitochondrial protein translocation and folding; (4) CIT1, a rate-limiting enzyme of the TCA cycle which catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate; (5) aconitase ACO1, which is required for the TCA cycle and also independently required for mitochondrial genome maintenance; (6) KGD1, a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the TCA cycle, the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA; (7) HSP10, a co-chaperonin that inhibits the ATPase activity of HSP60, a mitochondrial chaperonin, and is involved in protein folding and sorting in the mitochondria; (8) HSP78, a chaperone that cooperates with SSC1 in mitochondrial thermotolerance after heat shock ad is also known to prevent the aggregation of misfolded proteins and to resolubilize protein aggregates; (9) VAS1, a valyl-tRNA synthetase for protein synthesis in mitochondria; (10) POR1, a voltage-dependent anion channel required for the maintenance of

mitochondrial osmotic stability and mitochondrial membrane permeability; and (11) ADH3, a mitochondrial alcohol dehydrogenase isozyme III involved in the shuttling of mitochondrial NADH to the cytosol and ethanol production (Figure 3.9).

3.5 Discussion

Based on our findings described in Chapter 2 of my thesis and data of other researchers working in the field of mitochondrial biology, we hypothesized that, by altering the level of CL and other glycerophospholipids within the mitochondrial membrane, LCA could modulate the stoichiometry, composition and/or functional state of respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane. In our hypothesis, such LCA- and CL-dependent remodeling of the mitochondrial respiratory supercomplexes (1) may rely on a distinct set of protein components of the respiratory complexes that compose the respirasomes; (2) may recruit new mitochondrial protein components, not previously known for being permanently associated with the ETC, into the remodeled respirasomes; (3) may alter the age-dependent dynamics of mitochondrial respiration, oxidative phosphorylation, ADP/ATP exchange, ATP synthesis, membrane potential and/or ROS generation; (4) may occur in an age-dependent fashion; and (5) may extend longevity by increasing the efficiency of ATP synthesis in chronologically aging yeast, altering the age-dependent dynamics of changes in mitochondrially produced ROS (thereby protecting yeast from chronic oxidative stress), and/or modulating the longevity-defining processes that in mitochondria are governed by proteins newly recruited into the remodeled respirasomes (even although some or all of these newly recruited proteins have not been traditionally viewed as proteins permanently associated with the ETC).

To test the validity of this hypothesis, in studies described in this Chapter of my thesis we used a multistep method [243 - 253] for recovery of intact respiratory complexes and supercomplexes from purified yeast mitochondria, their subsequent first-dimension electrophoretic separation using BN-PAGE, their resolution into individual protein components with the help of denaturing Tricine-SDS-PAGE, and the MS-based identification of each of these individual protein components.

Findings described in this chapter of my thesis validate our hypothesis. These findings revealed several ways of rearranging respiratory supercomplexes in the inner mitochondrial membrane of cells exhibiting altered mitochondrial membrane lipidome in response to LCA treatment or genetic manipulations impairing the synthesis of CL and other glycerophospholipids within the inner membrane of mitochondria. First, by altering the level of CL and other glycerophospholipids synthesized and residing in the inner mitochondrial membrane, LCA modulates the abundance of several major respiratory supercomplexes (respirasomes) in this membrane. Second, LCA- and genetic manipulations-driven changes in the inner mitochondrial membrane lipidome cause a recruitment of a number of new mitochondrial protein components, not previously known for being permanently associated with the ETC, into the remodeled respirasomes. It should be emphasized that many of the proteins newly recruited into the remodeled respirasomes are known for their essential roles in mitochondria-confined processes that define longevity. Specifically, the observed recruitment of AAC2 to the remodelled respiratory supercomplexes suggests a mechanism in which the ability of AAC2 to sense the rates/efficacies with which electrons are transferred through the ETC and ATP is synthesized in mitochondria enables this transport protein to modulate accordingly the

rate of ATP-ADP exchange between mitochondria and cytosol. The resulting increase of the efficiency of ATP synthesis in mitochondria of chronologically aging yeast is likely to extend their longevity. Furthermore, the observed recruitment of IDH1, IDH2, ALD4 and HSP60 as well as of ACO1, KGD1, ALD4 and SSC1 to the remodelled respiratory supercomplexes is expected to provide an efficient way of coordinating the rates of NADH and acetyl-CoA synthesis with functional states of numerous proteins governed by the chaperone and protein import activities of HSP60 and SSC1 in mitochondria, thereby extending yeast longevity. Moreover, the observed recruitment of VAS1 (a valyl-tRNA synthetase for mitochondrial protein synthesis) to the remodelled respiratory supercomplexes is expected to provide an efficient way of coordinating the rates of electron transher through the ETC, ATP generation and NADH synthesis with the synthesis of mitochondrial DNA-encoded proteins. Again, this remodeling of respiratory supercomplexes is likely to extend longevity of chronologically aging yeast.

3.6 Conclusions

Findings described here imply that, by altering the level of CL and other glycerophospholipids within the mitochondrial membrane, LCA modulates the abundance and composition of respiratory supercomplexes (respirasomes) in the inner mitochondrial membrane. These findings revealed several ways of rearranging respiratory supercomplexes in the inner mitochondrial membrane of cells exhibiting altered mitochondrial membrane lipidome in response to LCA treatment or genetic manipulations impairing the synthesis of CL and other glycerophospholipids within the inner membrane of mitochondria. Many of these ways of respiratory supercomplexes

rearrangement are likely to delay yeast aging by beneficially influencing mitochondria-confined processes known for their essential role in defining longevity.

4 References

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5 List of my publications and manuscripts in preparation

Published papers

1. Goldberg, A.A., Richard, V.R., Kyryakov, P., Bourque, S.D., Beach, A., Burstein, M.T., Glebov, A., **Koupaki, O.**, Boukh-Viner, T., Gregg, C., Juneau, M., English, A.M., Thomas, D.Y. and Titorenko, V.I. Chemical genetic screen identifies lithocholic acid as an anti-aging compound that extends yeast chronological life span in a TOR-independent manner, by modulating housekeeping longevity assurance processes. *Aging* (2010) 2:393-414.

   This article was highlighted in the news media, including Radio-Canada (http://www.radio-canada.ca/nouvelles/science/2010/09/16/002-longevite- bile.shtml); **TFI News France** (http://lci.tf1.fr/science/sante/2010-09/la-bile-un- espoir-contre-le-vieillissement-6071272.html); **The McGill Daily** (http://hotink.theorem.ca/system/ mcgilldaily/issues/000/004/689/ vol100iss7_screen_quality.pdf?1285709690); **Science Daily** (http://www.sciencedaily.com/releases/2010/09/100915100935.htm); **EurekAlert!** (http://www.eurekalert.org/pub_releases/2010-09/cu-foy091510.php); **Now Concordia** (http://now.concordia.ca/what-we-do/research/20100921/fountain-of-youth-in-bile-longevity-molecule-identified.php); **Media Relations Concordia** (http://mediarelations. concordia.ca/pressreleases/archives/2010/09/fountain_of_youth_in_bile_long.php?&print=1); **Bio Ethics Hawaii** (http://www.bioethicshawaii.org/s-science/the-key-to-human-longevity-in-yeast-could-be/); **Fight Aging!** (http://www.fightaging.org/archives/2010/09/bile-acids-and-yeast-longevity.php); **Xenophilia** (https://xenophilius.wordpress.com/2010/09/16/fountain-of-youth-in-bile-longevity-molecule-identified/); **Thaindian** (http:// www.thaindian.com/newsportal/health/bile-may-harbour-human-fountain-of-youth_ 100429315.html); **DNA India** (http://www.dnaindia.com/scitech/report_bile-may-harbour-human-fountain-of-youth_1438869); **India Vision** (http://www.indiavision.com/news/article/scitech/103189/); **REVLET** (http://www.revleft.com/vb/fountain-youth-bilei-t141779/index.html?s=1294a5663f51df1055ad3ff2b53db082&p=1865643); **Stop Aging Solutions** (http://stopagingsolutions.com/?p=704); **Longevity Medicine** (http://www.longevitymedicine.tv/longevity-as-housekeeping-and-a-role-for-bile-acids/); **News Guide US** (http://newsguide.us/education/science/Fountain-of-youth-in-bile-Longevity-molecule-identified/?date=2010-03-26); **Dallas News** (http://topics. dallasnews.com/quote/06AP1cA3HLa9j?q=Diabetes); **e! Science News** (http:// esciencenews.com/articles/2010/09/15/fountain.youth.bile.longevity.molecule.identified);**TENDENCIAS CIENTIFICAS** (http://www.tendencias21.net/La-clave-de-la-longevidad-humana-podria-estar-en-la-levadura_a4848.html?utm_source= feedburner& utm_medium=feed); **Canadian Health Reference Guide** (http://www.chrgonline.com/ news_detail. asp?ID=140067);

Techno-Science (http://www.chrgonline.com/news_detail.asp?ID=140067); **METRO** (http://www.journalmetro.com/plus/article/672613--la-bile-fontaine-de-jouvence) **and others.**

2. Burstein, M.T., Beach, A., Richard, V.R., **Koupaki, O.**, Gomez-Perez, A., Goldberg, A.A., Kyryakov, P., Bourque, S.D., Glebov, A. and Titorenko, V.I. Interspecies chemical signals released into the environment may create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms. *Dose-Response* (2012) 10:75-82.

3. Burstein, M.T., Kyryakov, P., Beach, A., Richard, V.R., **Koupaki, O.**, Gomez-Perez, A., Leonov, A., Levy, S., Noohi, F. and Titorenko, V.I. Lithocholic acid extends longevity of chronologically aging yeast only if added at certain critical periods of their lifespan. *Cell Cycle* (2012); in press.

Manuscripts in preparation

1. Bourque, S.D.*, **Koupaki, O.***, Richard, V.R., Beach, A., Burstein, M.T., Kyryakov, P. and Titorenko, V.I. Lithocholic acid extends yeast longevity in part by altering the composition of mitochondrial membrane lipids. In preparation for submission to *Current Biology*

2. Kyryakov, P., Gomez-Perez, A., **Koupaki, O.**, Beach, A., Burstein, M.T., Richard, V.R., Leonov, A. and Titorenko, V.I. Mitophagy is a longevity assurance process that in chronologically aging yeast sustains functional mitochondria and maintains lipid homeostasis. In preparation for submission to *Autophagy*.