Lithocholic Acid Extends Yeast Chronological Lifespan, Drives the Evolution of Longevity Regulation Mechanisms in Ecosystems and Suppresses Mitochondrial Deficiency Causing a Neurological Disorder in Humans

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ABSTRACT

Lithocholic acid extends yeast chronological lifespan, drives the evolution of longevity regulation mechanisms in ecosystems and suppresses mitochondrial deficiency causing a neurological disorder in humans

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This thesis describes studies in which the budding yeast *Saccharomyces cerevisiae* was used as a model organism for uncovering mechanisms underlying aging of eukaryotic cells. We identified lithocholic acid (LCA), a bile acid, as a natural compound that increases the chronological lifespan of yeast cultured under longevity-extending caloric restriction (CR) conditions by targeting lipid metabolism. Our findings revealed mechanisms by which LCA extends yeast chronological lifespan, drives the evolution of longevity regulation within ecosystems, and suppresses mitochondrial deficiency known to cause a neurological disorder in humans. We demonstrated that the age-dependent dynamics of the mitochondrial tubular network regulates longevity of chronologically aging yeast by modulating age-related apoptosis. This mitochondria-controlled form of programmed apoptotic death is elicited by the efflux of the pro-apoptotic proteins cytochrome *c*, Aif1p and Nuc1p from mitochondria in reproductively mature yeast cells that enter stationary growth phase; furthermore, this form of age-related apoptotic death depends on the metacaspase Yca1p. We provided evidence that the CR diet delays the fragmentation of the mitochondrial tubular network during early stationary phase. This, in turn, slows down the age-related exit of pro-apoptotic proteins from mitochondria, attenuates apoptotic cell death, and ultimately prolongs lifespan. Our findings also

revealed that LCA further increases the chronological lifespan of CR yeast by preventing mitochondrial fragmentation during late stationary phase, thus averting the age-related exit of pro-apoptotic proteins from mitochondria and inhibiting programmed apoptotic cell death. Moreover, findings reported in this thesis imply that LCA extends longevity of chronologically aging yeast only if added at certain critical periods of their lifespan. Based on these findings, we propose a hypothesis of a biomolecular longevity network undergoing a stepwise progression through a series of checkpoints in chronologically aging yeast. In this thesis we also propose a hypothesis in which LCA - as well as other interspecies chemical signals released into the environment - create xenohormetic, hormetic and cytostatic selective forces driving the ecosystemic evolution of longevity regulation mechanisms. Moreover, findings described in this thesis suggest a previously unknown mechanism by which LCA suppresses mitochondrial deficiency causing the late-onset Leigh syndrome, a severe neurological disorder in humans.

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List of Abbreviations

ACO, aconitase; AMPK/TOR, AMP-activated protein kinase/target of rapamycin; cAMP/PKA, cAMP/protein kinase A; C/EBPα, CCAAT/enhancer-binding protein; CCO, cytochrome c oxidase; CFU, colony forming units; CNS, central nervous system; CL, cardiolipin; CLS, chronological life span; CR, caloric restriction; DAG, diacylglycerols; DHAP, dihydroxyacetone phosphate; DHR, dihydrorhodamine 123; DR, dietary restriction; EE, ergosteryl esters; ESI/MS, electrospray ionization mass spectrometry; ER, endoplasmic reticulum; ERG, ergosterol; FA-CoA, CoA esters of fatty acids; FFA, free fatty acids; FoxO, Forkhead box type O; GC/MS, gas chromatography followed by mass spectrometry; HPLC, high performance liquid chromatography; IGF-1, insulin/insulin-like growth factor 1; LBs, lipid bodies; LCA, lithocholic acid; LOD, limit of detection; LOQ, limit of quantitation; LPA, lysophosphatidic acid; MCA, metabolic control analysis; NB, neuroblastoma; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PMBC, peripheral blood mononuclear cells; rDNA, ribosomal DNA; ROS, reactive oxygen species; RLS, replicative life span; SD, standard deviation; SDH, succinate dehydrogenase; TAG, triacylglycerols; TLC, thin-layer chromatography; TORC1, TOR complex 1; WAT, white adipose tissue.

1.1 Introduction

The budding yeast *Saccharomyces cerevisiae* is a beneficial model for uncovering fundamental mechanisms and biological principles that underlie the complexity of cellular aging in multicellular eukaryotes [1 - 6]. This unicellular eukaryotic organism is amenable to comprehensive biochemical, genetic, cell biological, chemical biological, system biological and microfluidic dissection analyses [7 - 12]. Therefore, the use of *S. cerevisiae* as a model in aging research provided deep mechanistic insights into cellular processes that play key roles in regulating longevity of evolutionarily distant eukaryotic organisms. Because *S. cerevisiae* possesses relatively short and easily monitored chronological and replicative lifespans, this unicellular eukaryote played a key role in discovering: (1) many genes that control cellular aging and define organismal longevity not only in yeast but also in other eukaryotic organisms across species; (2) several crucial nutrient- and energy-sensing signaling pathways orchestrating an evolutionarily conserved compendium of longevity-defining cellular processes across phyla; and (3) some longevity-extending chemical compounds that slow down aging, improve health, attenuate age-related pathologies and delay the onset of age-related diseases in evolutionarily distant multicellular eukaryotic organisms [1 - 6, 13 - 22]. These studies provided comprehensive evidence that the signaling pathways orchestrating cellular aging and mechanisms of their fine-tuning by longevity-extending genetic, dietary and pharmacological interventions are conserved across species.

Two different paradigms of yeast aging are known, each being investigated separately from each other with the help of robust assays. These assays are conducted under manageable laboratory conditions and have been automated to facilitate a systemic

analysis of the aging process in a high-throughput format [9, 10, 11, 12, 23 - 29]. In the chronological aging paradigm, yeast aging is defined by the length of time during which a cell remains viable after its growth and division have been arrested [24, 30, 31]. Yeast chronological aging under laboratory conditions is elucidated with the help of a simple clonogenic assay. The assay examines the proportion of yeast cells that in liquid cultures remain viable at different time points following entry of a cell population into the non-proliferative stationary phase; cell viability in the clonogenic assay is measured by monitoring the ability of a cell to form a colony on the surface of a solid nutrient-rich medium [3, 28, 30]. Chronological aging in yeast not only mimics aging of non-dividing, post-mitotic cells (such as neurons) in a multicellular eukaryotic organism but also provides a simple model for organismal aging [32, 33]. Replicative aging of yeast is defined by the maximum number of daughter cells that a mother cell can make before becoming senescent [26, 34, 35]. Because *S. cerevisiae* reproduces by asymmetric cell division, its replicative aging under laboratory conditions is elucidated using a micromanipulator to remove the budding progeny of a mother cell and counting the total number of asymmetric mitotic divisions the mother cell could undertake [26, 35]. Yeast replicative aging is believed to mimic aging of dividing, mitotically active cells (such as lymphocytes) within a multicellular eukaryotic organism [2, 32]. The use of vigorous assays for elucidating longevity regulation in chronologically or replicatively aging yeast under laboratory conditions has significantly advanced our understanding of cell-autonomous mechanisms underlying longevity-defining processes within an individual cell in eukaryotic organisms across phyla [1 - 3, 5, 6, 10].

Moreover, studies in yeast also advanced fundamental knowledge about cell-non-autonomous mechanisms that regulate longevity. Such mechanisms operate within organized populations of yeast cells attached to solid surfaces to form a colony or a biofilm. Such cells: (1) communicate with each other and cells in surrounding colonies or biofilms; (2) age chronologically and replicatively; and (3) undergo spatially organized growth, differentiation, aging or death, depending on their position within the colony [4, 36 - 43].

It needs to be emphasized that cell-autonomous and cell-non-autonomous intraspecies mechanisms regulating yeast longevity have evolved in the process of natural selection within an ecosystem [44 - 46]. It has been suggested that this process: (1) is orchestrated by ecosystemic, interspecies mechanisms of lifespan regulation acting within the ecosystem; and (2) is driven by the ability of yeast cells to undergo certain pro-survival changes to their metabolism and physiology in response to some chemical compounds that, after being released to the ecosystem by other groups of organisms, may trigger a hormetic and/or cytostatic response in yeast [44 - 48].

Aging of multicellular and unicellular eukaryotic organisms is a multifactorial biological phenomenon that has various causes and affects a number of cellular activities [1, 49]. These numerous activities are modulated by only a few nutrient- and energy-sensing signaling pathways that are conserved across phyla and include the insulin/insulin-like growth factor 1 (IGF-1), AMP-activated protein kinase/target of rapamycin (AMPK/TOR) and cAMP/protein kinase A (cAMP/PKA) pathways (Figure 1.1) [1, 49 - 52]. By sharing a compendium of protein kinases and adaptor proteins, the insulin/IGF-1, AMPK/TOR and cAMP/PKA pathways in yeast, worms, fruit flies and

mammals converge into a network regulating longevity [50 - 54]. By sensing the nutritional status of the whole organism as well as the intracellular nutrient and energy status, functional state of mitochondria, and concentration of reactive oxygen species (ROS) produced in mitochondria, the longevity network regulates lifespan across species by coordinating information flow along its convergent, divergent and multiply branched signaling pathways.

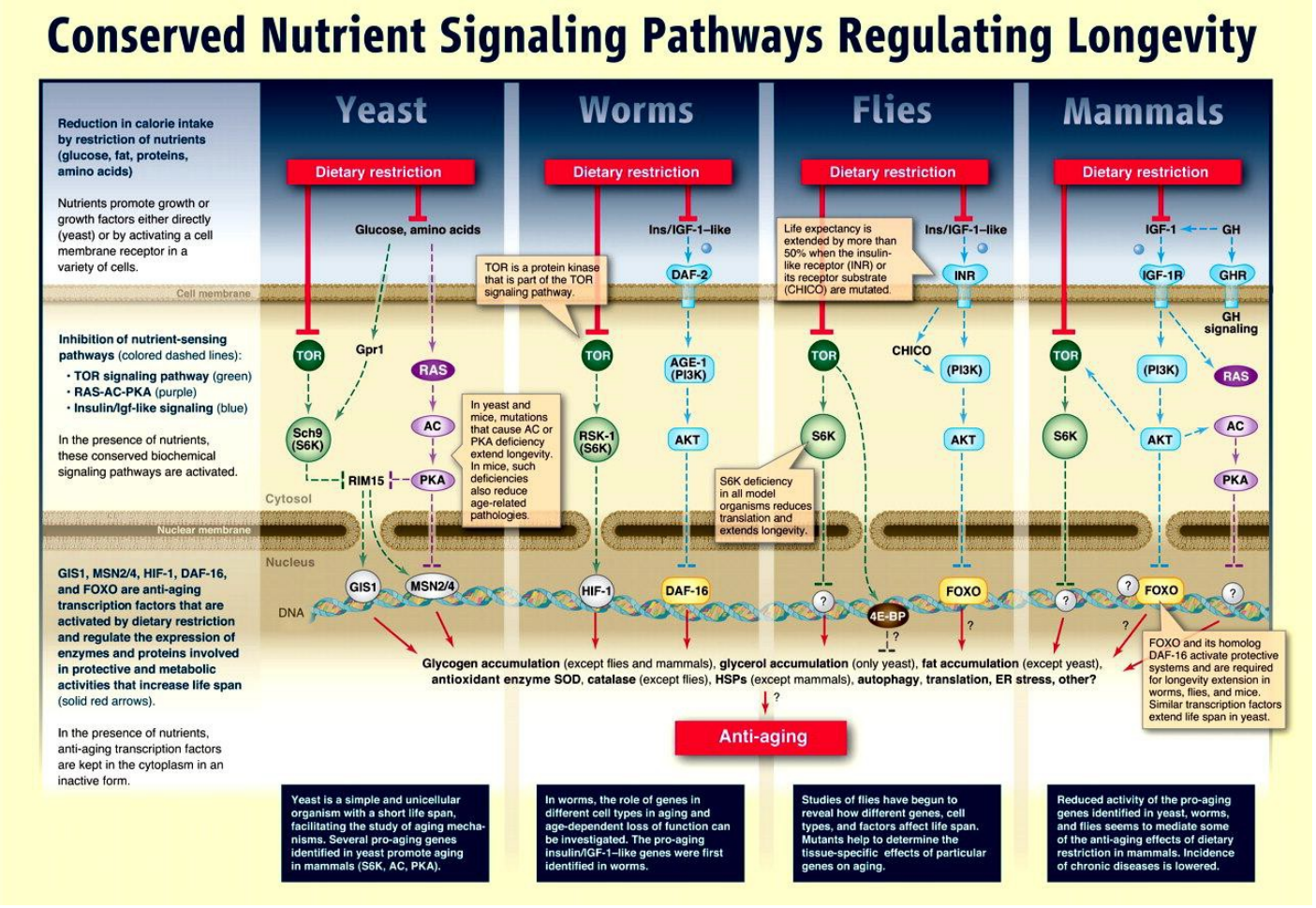

Figure 1.1. The functional states of numerous longevity-defining processes and their spatiotemporal organization are modulated by only a few nutrient- and energy-sensing signaling pathways that are conserved across phyla and include the AMP-activated protein kinase/target of rapamycin (AMPK/TOR), cAMP/protein kinase A (cAMP/PKA) and insulin/insulin-like growth factor 1 (IGF-1) pathways (see text in section 1.2 for

details). Reproduced from Fontana, L., Partridge, L. and Longo, V.D. (2010). Extending healthy life span - from yeast to humans. Science 328:321-326.

By defining the organismal and intracellular nutrient and energy status, nutrient intake plays an important role in modulating lifespan and influences age-related pathologies [55, 56]. Two dietary regimens are known to have the most profound longevity-extending effects across species and to improve overall health by delaying the onset of age-related diseases. They include: (1) caloric restriction (CR), a diet in which only calorie intake is reduced but the supply of amino acids, vitamins and other nutrients is not compromised [56 - 58]; and (2) dietary restriction (DR), in which the intake of nutrients (but not necessarily of calories) is reduced by limiting food supply without causing malnutrition [59 - 61]. In a "TOR-centric" view of longevity regulation, TOR alone governs the longevity-extending and health-improving effects of CR/DR by: (1) integrating the flow of information on the organismal and intracellular nutrient and energy status from the protein kinases AMPK, PKA, PKB/AKT (the insulin/IGF-1 pathway) and ERK1/2 (the PKA-inhibited Raf/MEK/ERK protein kinase cascade) as well as from the mitochondrial redox protein $P66^{Shc}$; (2) sensing the intracellular levels of amino acids in an AMPK-independent manner; and (3) operating as a control center which, based on the information it has gathered and processed, modulates many longevity-related processes in a sirtuin-independent fashion [62 - 64]. The inability of CR to increase the replicative lifespan (RLS) of yeast mutants lacking components of the TOR pathway [65] and the lack of the beneficial effect of DR on lifespan in worms with reduced TOR signaling [66, 67] support the proposed central role for TOR in orchestrating the longevity-extending effect of CR/DR in these two longevity paradigms.

Moreover, while the postulated by the TOR-centric model dispensability of sirtuins for the longevity benefit associated with DR has been confirmed in worms [67], the importance of the sirtuin Sir2p in mediating the longevity-extending effect of CR in replicatively aging yeast is debated [65, 68 - 70]. Noteworthy, while TOR is a central regulator of the longevity-extending effect of CR in replicatively aging yeast, the longevity benefit associated with CR in chronologically aging yeast is mediated by a signaling network that includes: (1) the TOR and cAMP/PKA pathways converged on Rim15p, which therefore acts as a nutritional integrator; and (2) some other, currently unknown pathways that are not centered on Rim15p [53]. Considering the likely convergence of the insulin/IGF-1, AMPK/TOR and cAMP/PKA signaling pathways into a network regulating longevity in worms, fruit flies and mammals (see above), it is feasible that - akin to TOR - the insulin/IGF-1 and cAMP/PKA pathways may contribute to the beneficial effect of CR/DR on their longevity. Although some findings in worms, fruit flies and mammals support the involvement of the insulin/IGF-1 pathway in the longevity benefit associated with CR/DR, other data imply that such benefit is independent of insulin/IGF-1 [51]. The role of cAMP/PKA signaling in the longevity-extending effect of CR/DR in these multicellular eukaryotes remains to be tested. Importantly, the recently reported in worms involvement of both independent and overlapping pathways in lifespan extension by different DR regimens [71] supports the notion that the longevity benefit associated with nutrient limitation is mediated by a signaling network that integrates several pathways.

Akin to CR and DR regimens, certain pharmacological interventions can extend longevity across phyla and improve health by beneficially influencing age-related

pathologies. Noteworthy, all of the currently known anti-aging compounds increase lifespan under non-CR or non-DR conditions. Under such conditions, these compounds have been shown to: (1) provide the longevity and health benefits associated with CR and DR, but without restricting caloric and nutrient intake; and (2) mimic numerous longevity-extending effects of CR and DR on gene expression pattern, metabolic and physiological processes, and stress response pathways. Therefore, the names "CR mimetics" and "DR mimetics" have been coined for them [72, 73].

Importantly, most CR mimetics and DR mimetics target signaling pathways that modulate longevity in response to the organismal and intracellular nutrient and energy status, including the insulin/IGF-1 and AMPK/TOR pathways as well as the sirtuin-governed protein deacetylation module of the longevity signaling network integrating these pathways [14]. Furthermore, such compounds as resveratrol, metformin and mianserin increase lifespan only under non-CR or non-DR conditions, but are unable to do so if the supply of calories or nutrients is limited [47, 74 - 77]. Hence, one could envision that most, if not all, longevity pathways are "adaptable" by nature, *i.e.*, that they modulate longevity only in response to certain changes in the extracellular and intracellular nutrient and energy status of an organism. However, Li^+ in worms and rapamycin in fruits flies extend lifespan even under DR conditions [78, 79]. It is likely therefore that some longevity pathways could be ″constitutive″ or ″housekeeping″ by nature, i.e., that they: (1) modulate longevity irrespective of the organismal and intracellular nutrient and energy status; and (2) do not overlap (or only partially overlap) with the adaptable longevity pathways that are under the stringent control of calorie and/or nutrient availability. The challenge is to identify such housekeeping longevity

pathways, perhaps by using a chemical screen for compounds that can extend longevity even under CR/DR conditions. Because under such conditions the adaptable pro-aging pathways are fully suppressed and the adaptable anti-aging pathways are fully activated, a compound that can increase lifespan is expected to target the housekeeping longevity pathways.

Noteworthy, two anti-aging compounds alter lipid levels in mammals and fruit flies under non-DR conditions. Indeed, resveratrol treatment reduces the levels of the neutral lipids triacylglycerols (TAG) and increases free fatty acid (FFA) levels in mouse adipocytes [80]. Furthermore, feeding rapamycin to fruit flies results in elevated TAG levels [79]. Although it remains to be seen if such effects of resveratrol and rapamycin on lipid levels play a casual role in their anti-aging action under non-DR conditions, it should be stressed that lipid metabolism has been shown to be involved in longevity regulation in yeast [24, 81], worms [82 - 85], fruit flies [83, 86] and mice [80, 83, 87 - 90]. We recently proposed a mechanism linking yeast longevity and lipid dynamics in the endoplasmic reticulum (ER), lipid droplets and peroxisomes. In this mechanism, a CR diet extends yeast chronological lifespan (CLS) by activating FFA oxidation in peroxisomes [24, 81, 91 - 93]. We expected that the identification of small molecules targeting this mechanism could yield novel anti-aging compounds. Such compounds can be used as research tools for defining the roles for different longevity pathways in modulating lipid metabolism and in integrating lipid dynamics with other longevity-related processes. Furthermore, the availability of such compounds would enable a quest for housekeeping longevity assurance pathways that do not overlap (or only partially overlap) with the adaptable TOR and cAMP/PKA pathways. Moreover, such compounds

would have a potential to be used as pharmaceutical agents for increasing lifespan and promoting healthy aging by delaying the onset of age-related diseases, regardless of an organism's dietary regimen.

We therefore conducted a high-throughput chemical genetic screen aimed at identifying small molecules that increase the CLS of yeast under CR conditions by targeting lipid metabolism and modulating housekeeping longevity assurance pathways. Our screen identified lithocholic acid (LCA), a bile acid, as one of such small molecules [14]. This thesis describes studies that led to the identification of some of the mechanisms through which LCA extends yeast chronological lifespan, drives the evolution of longevity regulation within ecosystems and suppresses mitochondrial deficiency known to cause a neurological disorder in humans.

1.2 Thesis outline and contributions of colleagues

Chapter 2 of this thesis outlines our evidence that the age-dependent dynamics of the mitochondrial tubular network regulates longevity of chronologically aging yeast by modulating age-related apoptosis. This mitochondria-controlled form of programmed apoptotic death is elicited by the efflux of the pro-apoptotic proteins cytochrome *c*, Aif1p and Nuc1p from mitochondria in reproductively mature yeast cells that enter stationary growth phase; furthermore, this form of age-related apoptotic death depends on the metacaspase Yca1p. Findings described in Chapter 2 provide evidence that the CR diet delays the fragmentation of the mitochondrial tubular network during early stationary phase. This, in turn, slows down the age-related exit of pro-apoptotic proteins from mitochondria, attenuates apoptotic cell death, and ultimately prolongs lifespan. Findings

described in Chapter 2 also revealed that LCA further increases the chronological lifespan of CR yeast by preventing mitochondrial fragmentation during late stationary phase, thus averting the age-related exit of pro-apoptotic proteins from mitochondria and inhibiting programmed apoptotic cell death.

Findings reported in Chapter 3 of this thesis imply that LCA extends longevity of chronologically aging yeast only if added at certain critical periods of their lifespan. Based on these findings, we propose a hypothesis of a biomolecular longevity network undergoing a stepwise progression through a series of checkpoints in chronologically aging yeast.

Findings reported in Chapter 4 of this thesis provide evidence that LCA enters yeast cells, accumulates mainly in the inner mitochondrial membrane, and elicits a remodeling of phospholipid synthesis and movement within both mitochondrial membranes. Such remodeling of mitochondrial phospholipid dynamics causes changes in mitochondrial membrane lipidome. These changes in the composition of membrane phospholipids alter mitochondrial abundance and morphology, thereby triggering changes in the age-related chronology of several longevity-defining redox processes confined to mitochondria.

In Chapter 5 of this thesis we propose a hypothesis in which LCA - as well as other interspecies chemical signals released into the environment - create xenohormetic, hormetic and cytostatic selective forces driving the ecosystemic evolution of longevity regulation mechanisms.

Findings described in Chapter 6 of this thesis suggest a previously unknown mechanism by which LCA suppresses mitochondrial deficiency causing the late-onset

Leigh syndrome, a severe neurological disorder in humans.

All findings described in Chapter 2 are presented in the manuscript of a paper that is currently in preparation for submission to *Aging Cell*. I expect this manuscript to be submitted for publication in October 2014. I carried out and supervised more than 70% of the work described in this manuscript and prepared its first draft. Dr. V. Titorenko provided intellectual leadership of this project and is currently editing the first draft of the manuscript.

All findings described in Chapter 3 have been published in *Cell Cycle* [Burstein MT, Kyryakov P, Beach A, Richard VR, Koupaki O, Gomez-Perez A, Leonov A, Levy S, Noohi F, Titorenko VI (2012). Lithocholic acid extends longevity of chronologically aging yeast only if added at certain critical periods of their lifespan. Cell Cycle 11:3443-3462]. I carried out and supervised more than 60% of the work described in this publication and prepared the first draft of the entire manuscript. Dr. V. Titorenko provided intellectual leadership of this project and edited the first draft of the manuscript.

All findings described in Chapter 4 have been published in *Redox Biol* [Burstein MT, Titorenko VI (2014). A mitochondrially targeted compound delays aging in yeast through a mechanism linking mitochondrial membrane lipid metabolism to mitochondrial redox biology. Redox Biol 2:305-307]. I prepared the first draft of the entire manuscript. Dr. V. Titorenko provided intellectual leadership of this project and edited the first draft of the manuscript.

All findings described in Chapter 5 have been published in *Dose Response* [Burstein MT, Beach A, Richard VR, Koupaki O, Gomez-Perez A, Goldberg AA, Kyryakov P, Bourque SD, Glebov A, Titorenko VI (2012). 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 1075-1082]. I prepared the first draft of the entire manuscript. Dr. V. Titorenko provided intellectual leadership of this project and edited the first draft of the manuscript.

All findings described in Chapter 6 are presented in the manuscript of a paper that is currently in preparation for submission to *eLife*. I expect this manuscript to be submitted for publication in September 2014. I carried out all of the work described in this manuscript and prepared its first draft. Dr. V. Titorenko provided intellectual leadership of this project and is currently editing the first draft of 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 Age-dependent dynamics of the mitochondrial tubular network regulates longevity of chronologically aging yeast by modulating a mitochondria-controlled form of programmed apoptotic death

2.1 Introduction

One of the early events of apoptosis in mammalian cells is the fragmentation of their interconnected tubular mitochondria [94 - 96]. Such fragmentation is known to be driven by the coordinated action of the mitochondrial fission and fusion machines, whose protein components control the efflux of cytochrome c and several other pro-apoptotic proteins from the mitochondrial intermembrane space [96]. The release of these pro-apoptotic proteins from mitochondria triggers caspase activation and nuclear DNA cleavage [97, 98]. Yeast cells also undergo apoptosis [99]. Their short-term exposure to hydrogen peroxide, acetic acid, hyperosmotic stress or α pheromone causes apoptotic cell death [100, 101] that has been linked to mitochondrial fragmentation, mitochondrial outer membrane permeabilization, and the release of several intermembrane space proteins from mitochondria [102 - 108]. The exit of the apoptosis inducing factor Aif1p and endonuclease G (Nuc1p) from yeast mitochondria and their subsequent import into the nucleus trigger such exogenously induced apoptosis by promoting DNA cleavage [104, 107] (Figure 2.1). Another intermembrane space protein that is released from yeast mitochondria during exogenously induced apoptosis is cytochrome *c* [102, 105, 109]. Although some data suggest that - akin to its essential role in triggering the apoptotic caspase cascade in mammalian cells [98] - cytochrome *c* in the cytosol of yeast cells

activates the metacaspase Yca1p [102, 105, 110 - 112], the involvement of cytosolic cytochrome *c* in Yca1p activation remains a controversial issue [109, 113].

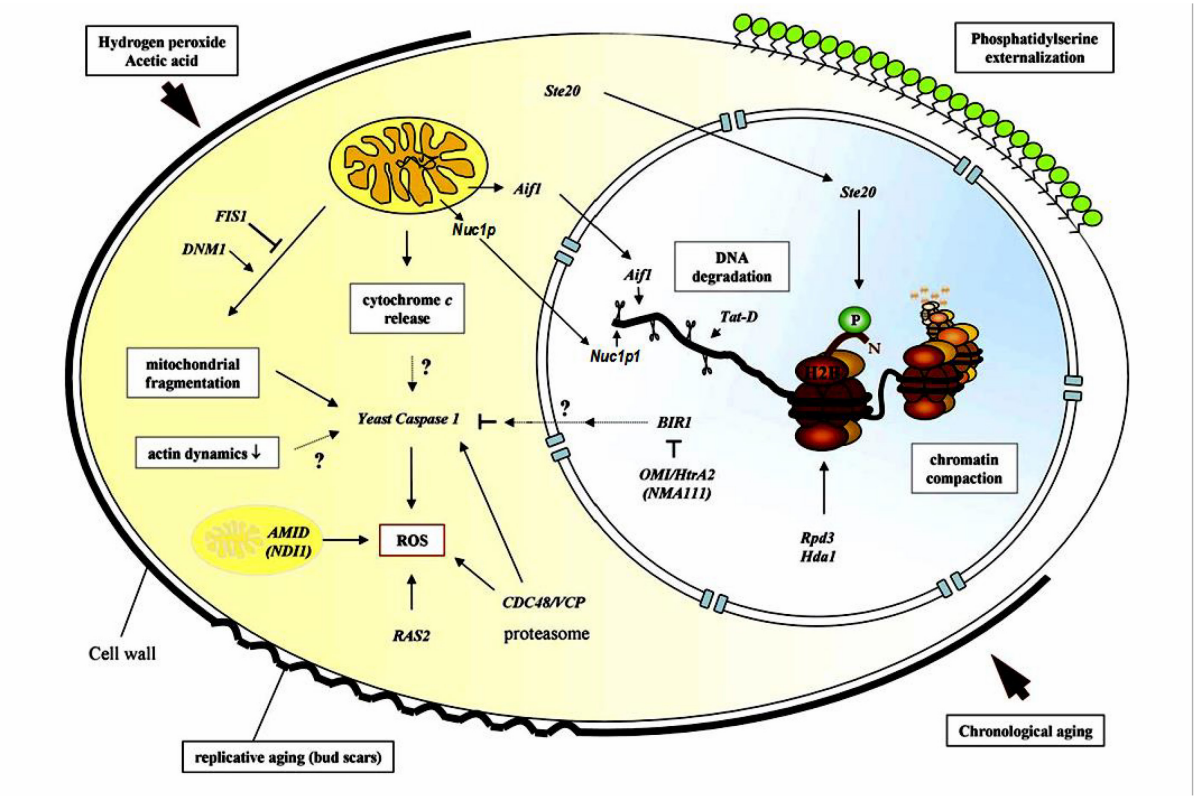

Figure 2.1. The basic molecular machinery of yeast apoptosis. From: Carmona-Gutierrez D, Madeo F (2006). Yeast unravels epigenetic apoptosis control: deadly chat within a histone tail. Mol Cell 2006 24:167-169.

Importantly, chronologically aging yeast die, in an Aif1p-, Nuc1p- and Yca1p-dependent fashion, exhibiting characteristic markers of apoptosis such as chromatin condensation, nuclear fragmentation, DNA cleavage, phosphatidylserine (PS) externalization, ROS production, and caspase activation [104, 107, 114 - 117]. Thus, the chronological aging of yeast is linked to an apoptosis-like programmed cell death [118 -

121]. This age-related apoptotic cell death is induced in response to presently unknown endogenous stimuli, somehow controlled by mitochondria (perhaps, through the release of pro-apoptotic proteins from the mitochondrial intermembrane space), and governed by yet-to-be-identified mechanisms. In studies described in this chapter of the thesis, we investigated these mechanisms.

2.2 Materials and Methods

Strains and media

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and mutant strains *fis1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 fis1Δ::kanMX4*), *dnm1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dnm1Δ::kanMX4*), *mdv1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mdv1Δ::kanMX4*), *caf4*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 caf4Δ::kanMX4*), *fzo1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 fzo1Δ::kanMX4*), *ugo1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ugo1Δ::kanMX4*), *mgm1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mgm1Δ::kanMX4*), *mdm30*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mdm30Δ::kanMX4*), *pcp1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pcp1Δ::kanMX4*), *aif1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 aif1Δ::kanMX4*), *nuc1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 nuc1Δ::kanMX4*), *yca1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yca1Δ::kanMX4*), *cyt1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 cyt1Δ::kanMX4*), *rip1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 rip1Δ::kanMX4*), *bcs1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 bcs1Δ::kanMX4*), *cox5A*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 cox5AΔ::kanMX4*), *cox8*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 cox8Δ::kanMX4*),

*pet100*Δ (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 pet100*Δ*::kanMX4*), *aco1*Δ (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 aco1*Δ*::kanMX4*), *kgd1*Δ (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 kgd1*Δ*::kanMX4*), *mdh1*Δ (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 mdh1*Δ*::kanMX4*), *sod1*Δ (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 sod1*Δ*::kanMX4*), *sod2*Δ (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 sod2*Δ*::kanMX4*), *ctt1*Δ (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 ctt1*Δ*::kanMX4*) and *cta1*Δ (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 cta1*Δ*::kanMX4*) were used in this study. Media components were as follows: (1) YEPD (0.2% glucose), 1% yeast extract, 2% peptone, 0.2% glucose; and (2) YEPD (2% glucose), 1% yeast extract, 2% peptone, 2% glucose.

A plating assay for the analysis of chronological lifespan

Cells were grown in YEPD (0.2% glucose) medium at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a flask volume/medium volume ratio of 5:1. A sample of cells was removed from each culture at various time points. A fraction of the cell sample was diluted in order to determine the total number of cells per ml of culture using a hemacytometer. 10 μ l of serial dilutions (1:10 to 1:10^{3}) of cells were applied to the hemacytometer, where each large square is calibrated to hold 0.1 μ l. The number of cells in 4 large squares was then counted and an average was taken in order to ensure greater accuracy. The concentration of cells was calculated as follows: number of cells per large square x dilution factor x 10 x 1,000 = total number of cells per ml of culture. A second fraction of the cell sample was diluted and serial dilutions (1:10^{2} to 1:10^{5}) of cells were plated onto YEPD (2% glucose) plates in triplicate in order to count the number of viable

cells per ml of each culture. 100 µ l of diluted culture was plated onto each plate. After a 48-h incubation at 30^{o}C, the number of colonies per plate was counted. The number of colony forming units (CFU) equals to the number of viable cells in a sample. Therefore, the number of viable cells was calculated as follows: number of colonies x dilution factor x 10 = number of viable cells per ml. For each culture assayed, % viability of the cells was calculated as follows: number of viable cells per ml / total number of cells per ml x 100%. The % viability of cells in mid-logarithmic phase was set at 100% viability for that particular culture. The life span curves for wild-type and some of the mutant strains were also validated using a LIVE/DEAD yeast viability kit (Invitrogen) following the manufacturer's instructions for stationary-phase cultures.

Monitoring the formation of reactive oxygen species (ROS)

Wild-type and mutant cells grown in YEPD (0.2% glucose) were tested microscopically for the production of ROS by incubation with dihydrorhodamine 123 (DHR). In the cell, this nonfluorescent compound can be oxidized to the fluorescent chromophore rhodamine 123 by ROS. Cells were also probed with a fluorescent counterstain Calcofluor White M2R (CW), which stains the yeast cell walls fluorescent blue. CW was added to each sample in order to label all cells for their proper visualization. DHR was stored in the dark at –20^{o}C as 50 µ l aliquots of a 1 mg/ml solution in ethanol. CW was stored in the dark at –20^{o}C as the 5 mM stock solution in anhydrous DMSO (dimethylsulfoxide). The concurrent staining of cells with DHR and CW was carried out as follows. The required amounts of the 50 µ l DHR aliquots (1 mg/ml) and of the 5 mM stock solution of CW were taken out of the freezer and warmed to room temperature. The solutions of DHR

and CW were then centrifuged at 21,000 x g for 5 min in order to clear them of any aggregates of fluorophores. For cell cultures with a titre of ~ 10^7 cells/ml, 100 µ l was taken out of the culture to be treated. If the cell titre was lower, proportionally larger volumes were used. 6 µ l of the 1 mg/ml DHR and 1 µ l of the 5 mM CW solutions were added to each 100 µ l aliquot of culture. After a 2-h incubation in the dark at room temperature, the samples were centrifuged at 21,000 x g for 5 min. Pellets were resuspended in 10 µ l of PBS buffer (20 mM KH_2PO_4/KOH, pH 7.5, and 150 mM NaCl). Each sample was then supplemented with 5 µ l of mounting medium, added to a microscope slide, covered with a coverslip, and sealed using nail polish. Once the slides were prepared, they were visualized under the Zeiss Axioplan fluorescence microscope mounted with a SPOT Insight 2 megapixel color mosaic digital camera. Several pictures of the cells on each slide were taken, with two pictures taken of each frame. One of the two pictures was of the cells seen through a rhodamine filter in order to detect cells dyed with DHR. The second picture was of the cells seen through a DAPI filter in order to visualize CW, and therefore all the cells present in the frame. For evaluating the percentage of DHR-positive cells, the UTHSCSA Image Tool (Version 3.0) software was used to calculate both the total number of cells and the number of stained cells. Fluorescence of individual DHR-positive cells in arbitrary units was determined by using the UTHSCSA Image Tool software (Version 3.0). In each of 3-5 independent experiments, the value of median fluorescence was calculated by analyzing at least 800-1000 cells that were collected at each time point. The median fluorescence values were plotted as a function of the number of days cells were cultured.

Monitoring the mitochondrial membrane potential ($\Delta\psi$)

Rhodamine 123 (R123) (Invitrogen) staining for monitoring the mitochondrial membrane potential ($\Delta\psi$) was performed according to established procedure (Invitrogen manual for the Yeast Mitochondrial Stain Sampler Kit). Images were collected with a Zeiss Axioplan fluorescence microscope (Zeiss) mounted with a SPOT Insight 2 megapixel color mosaic digital camera (Spot Diagnostic Instruments). For evaluating the percentage of R123-positive cells, the UTHSCSA Image Tool (Version 3.0) software was used to calculate both the total number of cells and the number of stained cells. Fluorescence of individual R123-positive cells in arbitrary units was determined by using the UTHSCSA Image Tool software (Version 3.0). In each of 3-6 independent experiments, the value of median fluorescence was calculated by analyzing at least 800-1000 cells that were collected at each time point. The median fluorescence values were plotted as a function of the number of days cells were cultured.

Immunofluorescence microscopy

Cell cultures were fixed in 3.7% formaldehyde for 45 min at room temperature. The cells were washed in solution B (100 mM KH_2PO_4/KOH pH 7.5, 1.2 M sorbitol), treated with Zymolyase 100T (MP Biomedicals, 1 µg Zymolyase 100T/1 mg cells) for 30 min at 30^{o}C and then processed as previously described [122]. Monoclonal antibody raised against porin (Invitrogen, 0.25 µg/µl in TBSB buffer [20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mg/ml BSA]) was used as a primary antibody. Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, 2 µg/µl in TBSB buffer) was used as a secondary antibody. The labeled samples were mounted in mounting solution (16.7 mM Tris/HCl pH 9.0, 1.7 mg/ml p-

phenylenediamine, 83% glycerol). Images were collected with a Zeiss Axioplan fluorescence microscope (Zeiss) mounted with a SPOT Insight 2 megapixel color mosaic digital camera (Spot Diagnostic Instruments).

Oxygen consumption assay

The rate of oxygen consumption by yeast cells recovered at various time points was measured continuously in a 2-ml stirred chamber using a custom-designed biological oxygen monitor (Science Technical Center of Concordia University) equipped with a Clark-type oxygen electrode. 1 ml of YEPD medium supplemented with 0.2% glucose was added to the electrode for approximately 5 minutes to obtain a baseline. Cultured cells of a known titre were spun down at 3,000 x g for 5 minutes. The resulting pellet was resuspended in YEPD medium supplemented with 0.2% glucose and then added to the electrode with the medium that was used to obtain a baseline. The resulting slope was used to calculate the rate of oxygen consumption in O_2% x min^{-1} x 10^9 cells.

Pharmacological manipulation of chronological lifespan

Chronological lifespan analysis was performed as described above in this section. The lithocholic (LCA) [#L6250] bile acid was from Sigma. The stock solution of LCA in DMSO was made on the day of adding this compound to cell cultures. LCA was added to growth medium 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).

Cell viability assay for monitoring the susceptibility of yeast to an apoptotic mode of cell death induced by hydrogen peroxide

A sample of cells was taken from a culture at a certain time-point. A fraction of the sample was diluted in order to determine the total number of cells using a hemacytometer. 2×10^7 cells were harvested by centrifugation for 1 min at 21,000 × g at room temperature and resuspended in 2 ml of YP medium containing 0.2% glucose as carbon source. Each cell suspension was divided into 2 equal aliquots. One aliquot was supplemented with hydrogen peroxide (#H325-500; Fisher Scientific) to the final concentration of 2.5 mM, whereas other aliquot remained untreated. Both aliquots were then incubated for 2 h at 30^{o}C on a Labquake rotator (#400110; Thermolyne/Barnstead International) set for 360^o rotation. Serial dilutions of cells were plated in duplicate onto plates containing YP medium with 2% glucose as carbon source. After 2 d of incubation at 30^{o}C, the number of colony forming units (CFU) per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each aliquot of cells exposed to hydrogen peroxide, the % of viable cells was calculated as follows: (number of viable cells per ml in the aliquot exposed to hydrogen peroxide/number of viable cells per ml in the control aliquot that was not exposed to hydrogen peroxide) × 100.

Statistical analysis

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

2.3 Results

2.3.1 Elucidating mechanisms that underlie characteristic changes in mitochondrial morphology and function taking place in chronologically aging yeast

We found that mitochondria in CR yeast entering the non-proliferative stationary (ST) phase exist as a tubular network, whereas in quiescent non-CR yeast this network is fragmented into individual mitochondria (Figure 2.2). It is known that the morphology of mitochondria depends on a balance between the processes of mitochondrial fission, fusion and tubulation [123 - 125] (Figure 2.3). Of note, we demonstrated that the *dnm1*Δ, *mdv1*Δ and *caf4*Δ mutations - all of which promote the formation of net-like mitochondria (Figure 2.4) by eliminating components of the mitochondrial fission machine [123] (Figure 2.5) - extend the lifespan of CR yeast (Figure 2.6). Conversely, the *fzo1*Δ, *ugo1*Δ, *mgm1*Δ, *mdm30*Δ and *pcp1*Δ mutations - all of which cause the accumulation of fragmented mitochondria (Figure 2.4) by eliminating components of the outer or inner membrane fusion machine [123] (Figure 2.7) - shorten the lifespan of yeast under CR (Figure 2.8). Furthermore, although the *fis1*Δ mutation eliminates an essential component of the mitochondrial fission machine (Figure 2.5), it impairs mitochondrial fission only in healthy, exponentially growing yeast cells [123, 124]. By contrast, *fis1*Δ does not abolish mitochondrial fission during exogenously induced apoptosis [126] and, akin to mutations that impair mitochondrial fusion, accelerates mitochondrial fragmentation in aging yeast under CR (Figure 2.4). Importantly, we found that the accumulation of fragmented mitochondria in the *fis1*Δ mutant coincides with its shortened chronological lifespan (Figure 2.9). Based on all these findings, we hypothesize that the ability of yeast cells that enter a non-proliferative state under CR conditions to maintain a network appearance of mitochondria is essential for the extension of their chronological lifespan by CR.

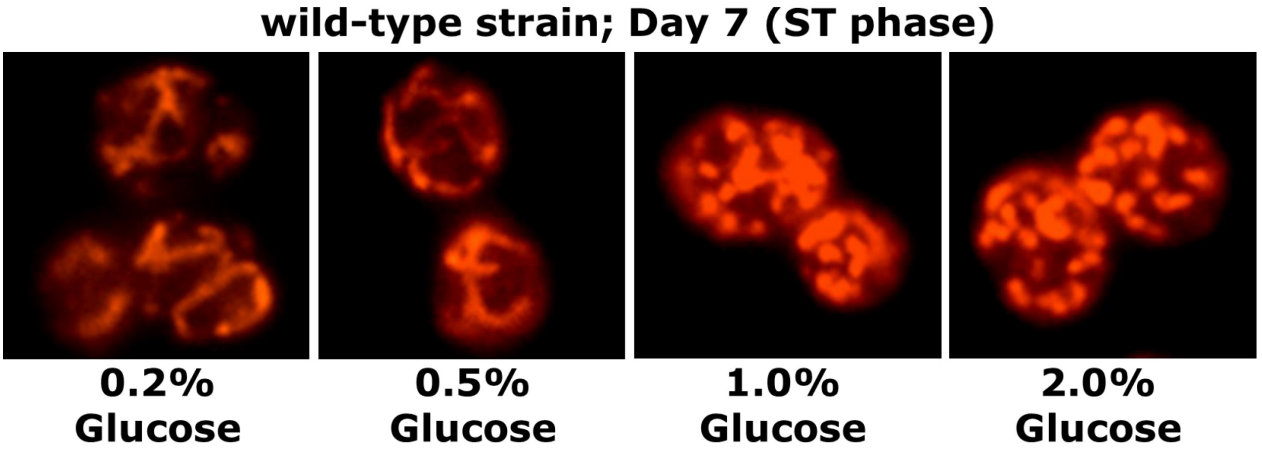

Figure 2.2. Mitochondria in yeast cells entering the non-proliferative stationary (ST) phase under CR conditions exist as a tubular network, whereas in quiescent yeast cells under non-CR conditions the network is fragmented into individual mitochondria. Mitochondria were visualized by indirect immunofluorescence microscopy using monoclonal anti-porin antibodies.

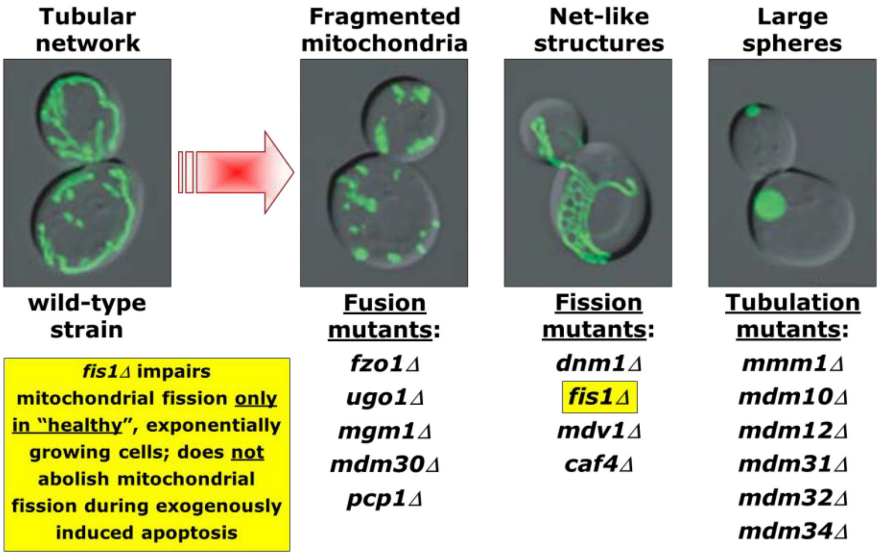

Figure 2.3. Morphology of mitochondria depends on a balance between the processes of mitochondrial fission, fusion and tubulation. From: Okamoto K, Shaw JM (2005). Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu Rev Genet 39:503-536.

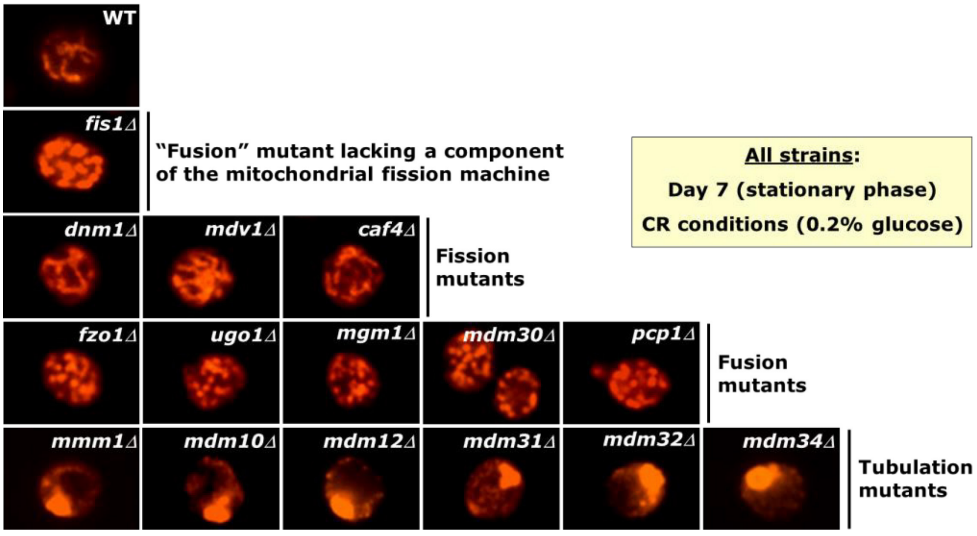

Figure 2.4. Morphology of mitochondria in wild-type cells and in mutant cells lacking individual components of the mitochondrial fission, fusion or tubulation machine. Mitochondria were visualized by indirect immunofluorescence microscopy using monoclonal anti-porin primary antibodies.

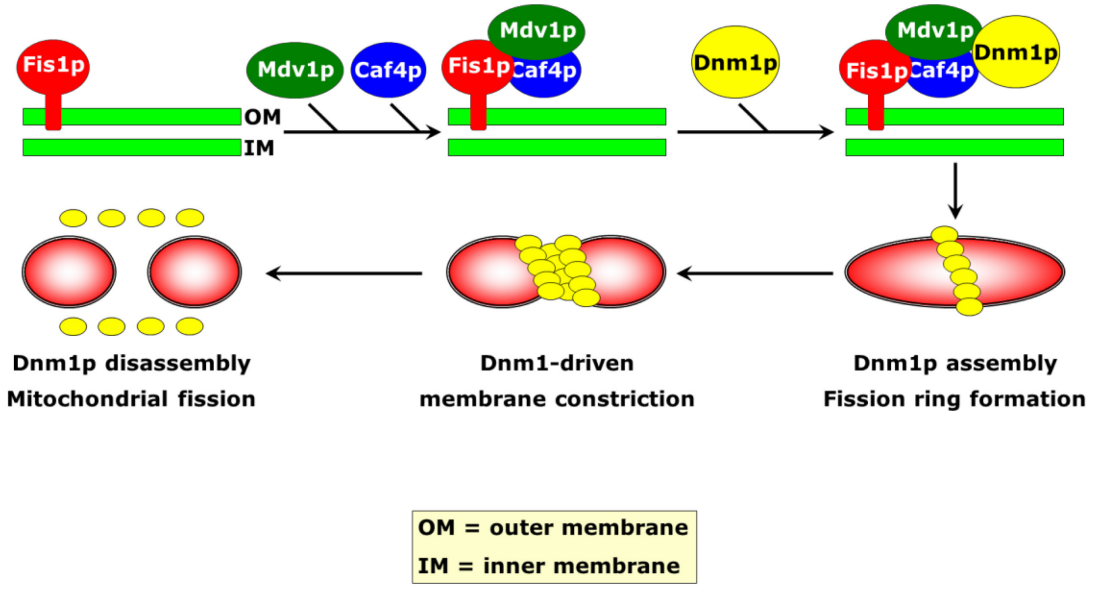

Figure 2.5. The spatiotemporal dynamics of proteins that compose the mitochondrial fission machine in yeast.

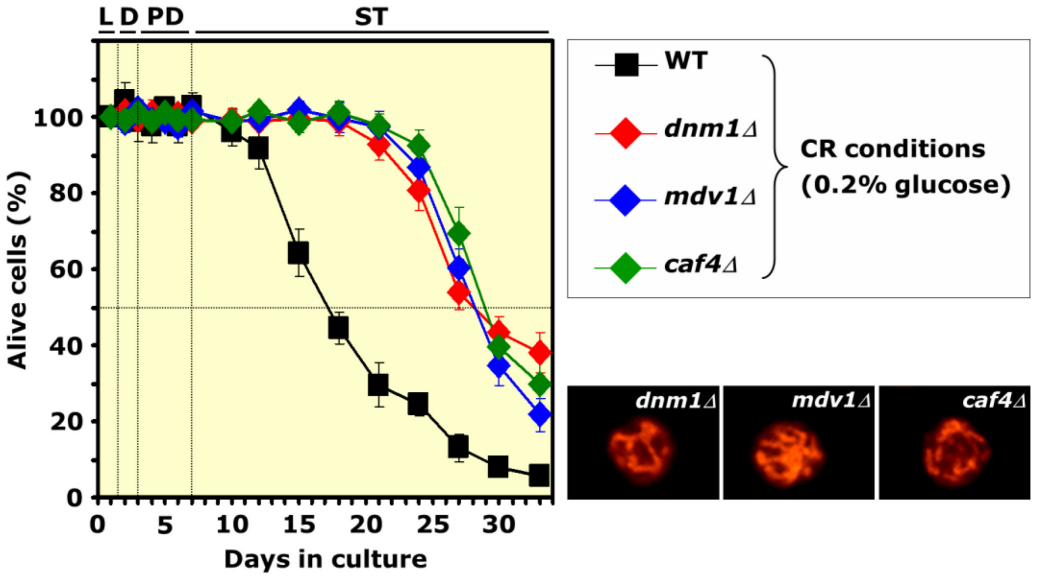

Figure 2.6. The *dnm1*Δ, *mdv1*Δ and *caf4*Δ mutations – all of which promote the formation of net-like mitochondria by eliminating components of the mitochondrial fission machine – extend yeast chronological lifespan under CR conditions.

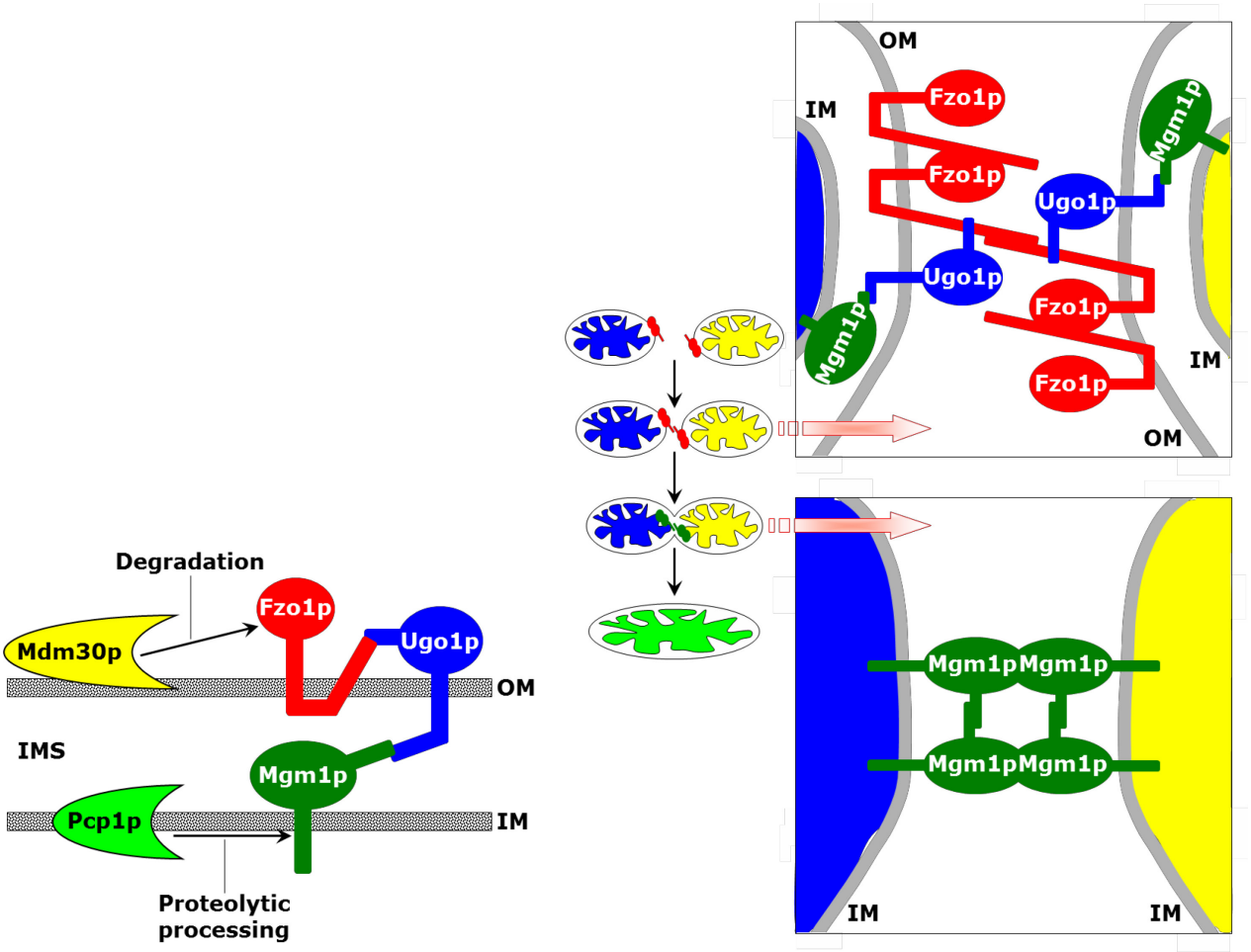

Figure 2.7. Two mitochondrial fusion machines serve the sequential fusion of the outer mitochondrial membranes (OM) and the inner mitochondrial membranes (IM). Localization, topology, interaction, processing and degradation of proteins that compose two machines serving the sequential fusion of the OM and the IM. Tethering of the opposing OM precedes fusion of their lipid bilayers. The opposing IM then tether and, subsequently, fuse.

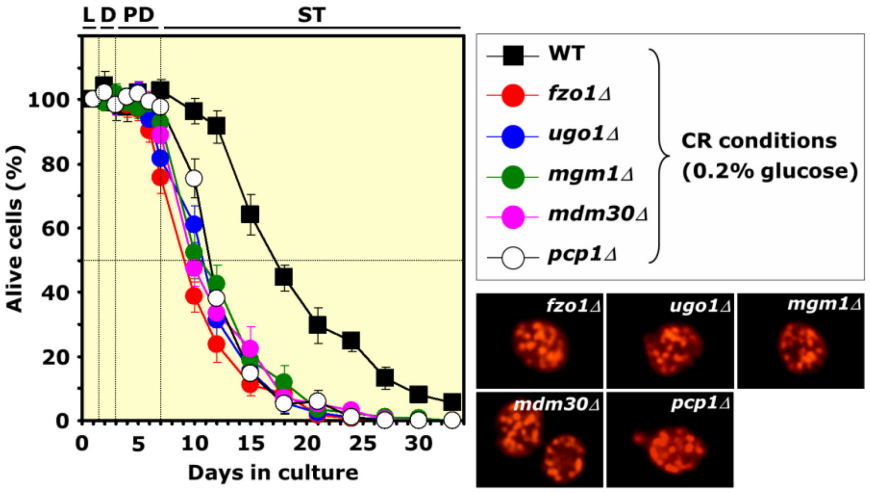

Figure 2.8. The *fzo1*Δ, *ugo1*Δ, *mgm1*Δ, *mdm30*Δ and *pcp1*Δ mutations – all of which cause the accumulation of fragmented mitochondria by eliminating components of the outer or inner mitochondrial membrane fusion machine – shorten yeast chronological lifespan under CR conditions.

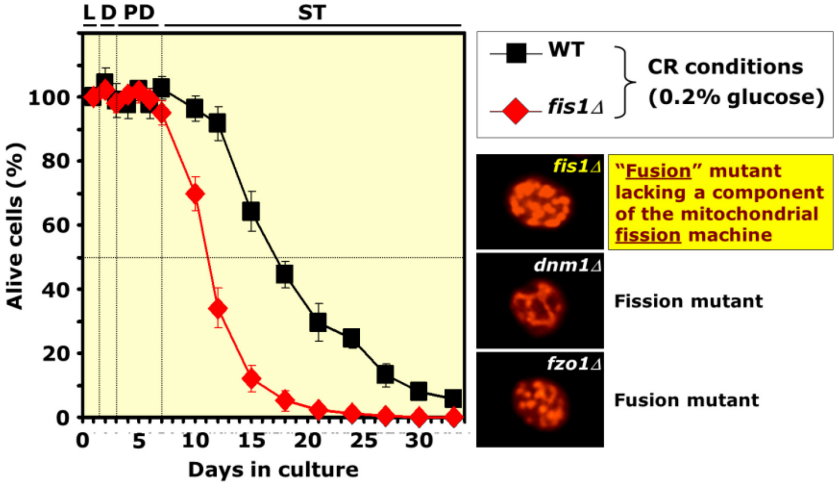

Figure 2.9. Akin to mutations that impair mitochondrial fusion, *fis1*□accelerates mitochondrial fragmentation in yeast cells grown under CR conditions and shortens their chronological lifespan.

We found that the tubular mitochondrial network undergoes fragmentation and is eventually converted to large spheres even in wild-type cells grown under CR conditions, particularly when these cells progress further into ST phase (Figure 2.10). Importantly, the potent anti-aging compound LCA - a bile acid which extends the chronological lifespan of wild-type strain under CR conditions [14] - prevents such fragmentation of the mitochondrial network and averts its conversion to large spheres (Figure 2.11). We demonstrated that LCA is unable to extend the chronological lifespans of the *fzo1*Δ and *ugo1*Δ mutant strains grown under CR conditions (Figures 2.12 and 2.14). By eliminating essential interacting components of the outer membrane fusion machine (Figure 2.7) [124, 127], the *fzo1*Δ and *ugo1*Δ mutations are known to cause the accumulation of fragmented mitochondria (Figure 2.4). The interaction between Fzo1p and Ugo1p has been shown to be essential for mitochondrial outer membrane fusion [123, 128]. It seems that the outer membrane GTPase Fzo1p initiates such fusion by forming an oligomeric complex in *cis* and in *trans*, thereby tethering outer membranes of two opposing mitochondria and bringing them into a docked state [124] (Figure 2.7). In addition to being essential for tethering of the mitochondrial outer membranes, both Fzo1p and Ugo1p are known to function, by a yet-to-be-identified mechanism, in merging of their lipid bilayers [127, 129].

Importantly, we revealed that, although LCA does not extend the chronological lifespans of *fzo1*Δ and *ugo1*Δ, it increases the lifespans of chronologically aging mutants lacking other components of the outer (*i.e.*, Mdm30p) or inner (*i.e.*, Mgm1p and Pcp1p) membrane fusion machines (Figures 2.13 and 2.14). Thus, it is conceivable that LCA extends longevity of wild-type yeast by targeting Fzo1p and Ugo1p in the outer

membranes of fusion partners (Figure 2.15). We hypothesize that, by promoting the ability of Fzo1p and Ugo1p to tether and fuse the opposing mitochondrial outer membranes in wild-type cells, LCA prevents fragmentation of the mitochondrial network during late ST phase (Figure 2.11) due to its ability to shift a balance between the opposing processes of mitochondrial fission and fusion towards fusion. Hence, it is plausible that LCA extends the chronological lifespan of wild-type yeast under CR conditions in part by preserving the tubular mitochondrial network late in ST phase.

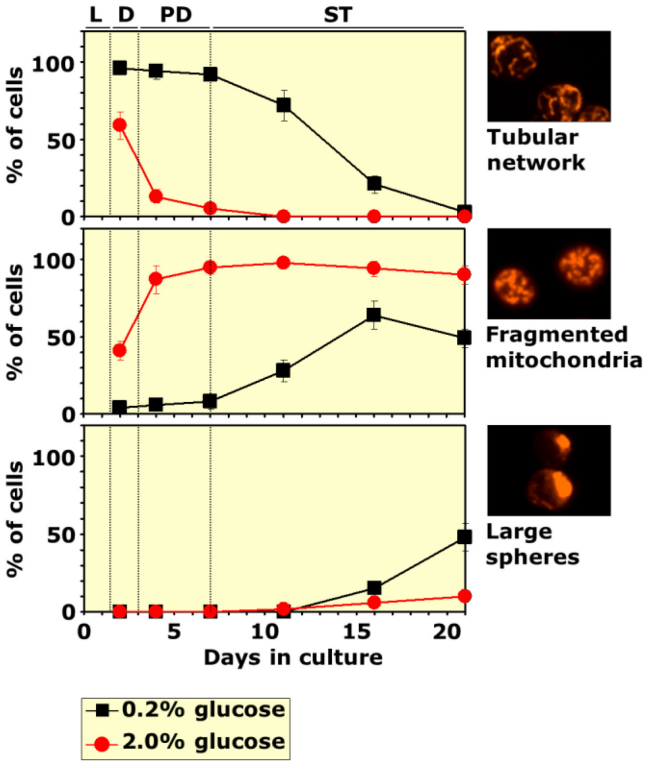

Figure 2.10. In chronologically aging wild-type cells that enter a non-proliferative state, CR diet delays the fragmentation of the tubular mitochondrial network. During late ST phase, CR diet promotes the conversion of fragmented mitochondria to large spheres. Mitochondria were visualized by indirect immunofluorescence microscopy using monoclonal anti-porin primary antibodies. The percentage of cells exhibiting the network

of long mitochondrial tubules, fragmented mitochondria or large spherical mitochondria was calculated. At least 600 cells were used for quantification of mitochondrial morphology at each time point. Data are presented as mean ± SEM (n = 3). Cells were cultured in medium containing 0.2% or 2.0% glucose.

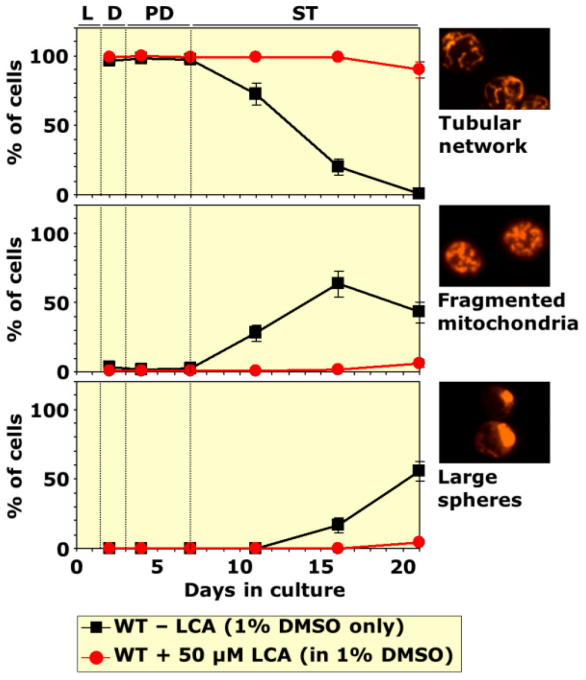

Figure 2.11. In chronologically aging wild-type cells grown under CR conditions, LCA prevents both the fragmentation of the tubular mitochondrial network and the accumulation of large spheres. Mitochondria were visualized by indirect immunofluorescence microscopy using monoclonal anti-porin primary antibodies. The percentage of cells exhibiting the network of long mitochondrial tubules, fragmented mitochondria or large spherical mitochondria was calculated. At least 600 cells were used for quantification of mitochondrial morphology at each time point. Data are presented as mean ± SEM (n = 3). Cells were cultured in medium containing 0.2% glucose.

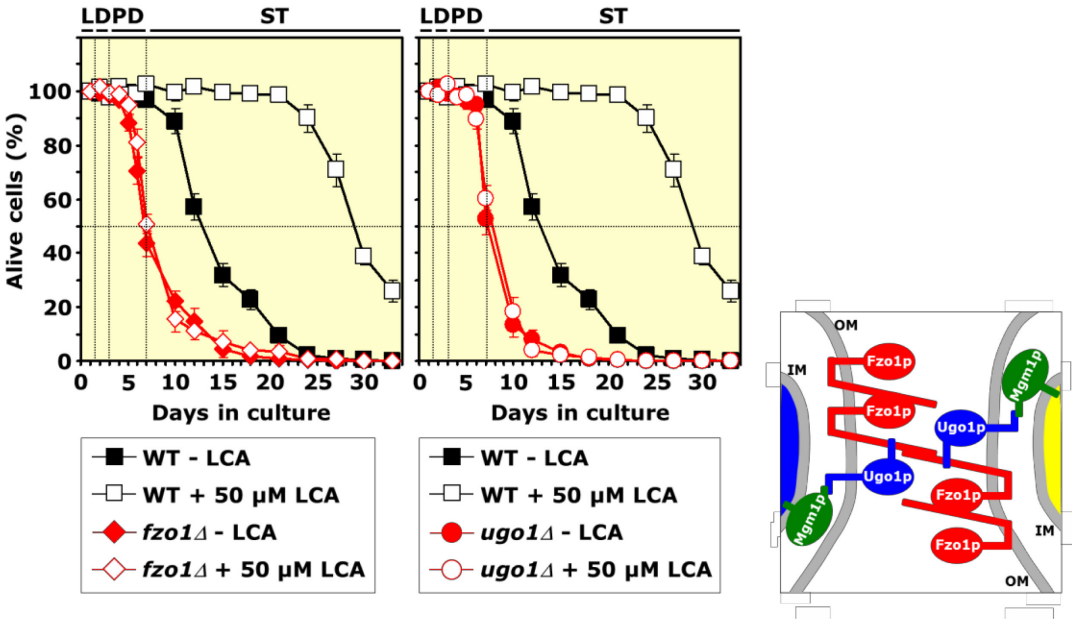

Figure 2.12. LCA does not extend the chronological lifespans of the *fzo1*Δ and *ugo1*Δ mutants that under CR conditions accumulate fragmented mitochondria.

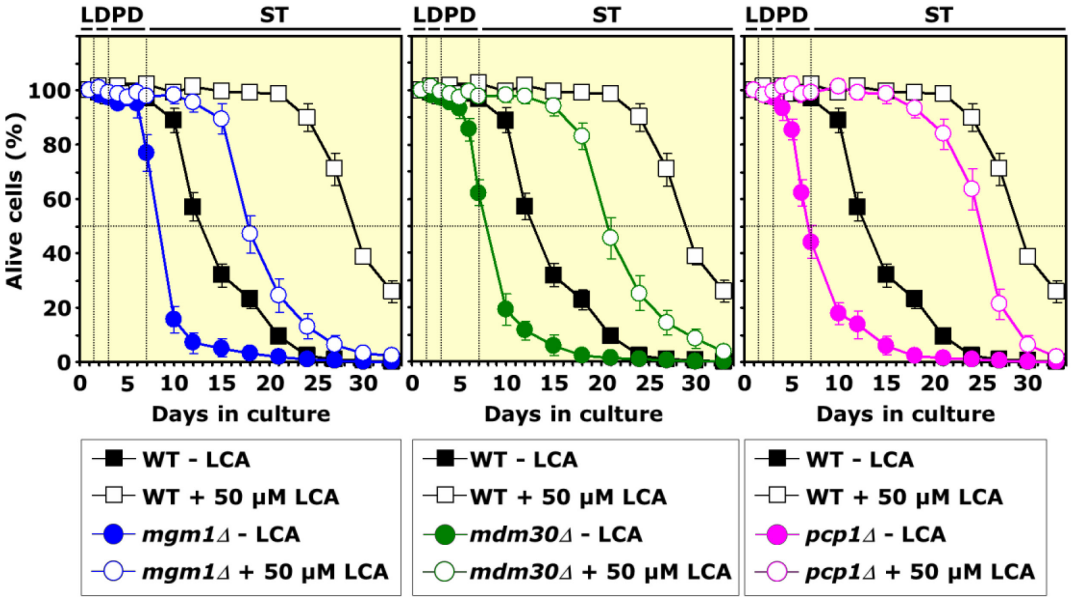

Figure 2.13. Although LCA does not extend the chronological lifespans of the *fzo1*Δ and *ugo1*Δ mutants, it increases the chronological lifespans of mutants lacking other

components of the outer (*i.e.*, Mdm30p) or inner (*i.e.*, Mgm1p and Pcp1p) membrane fusion machines.

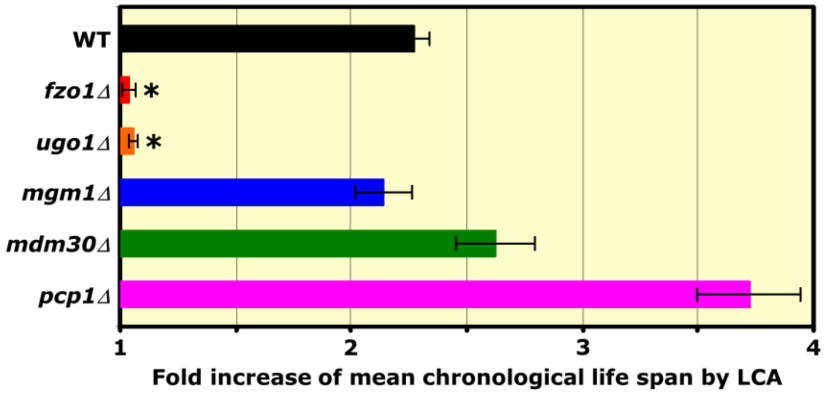

Figure 2.14. Although LCA does not extend the mean chronological lifespans of the *fzo1*Δ and *ugo1*Δ mutants, it increases the mean chronological lifespans of mutants lacking other components of the outer (*i.e.*, Mdm30p) or inner (*i.e.*, Mgm1p and Pcp1p) membrane fusion machines.

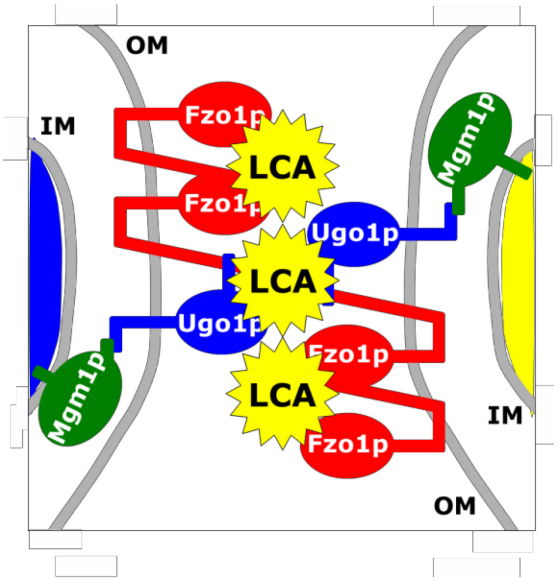

Figure 2.15. Hypothesis: LCA extends longevity of wild-type yeast by targeting Fzo1p and Ugo1p in the outer membranes of fusion partners.

2.3.2. Discovering mechanisms that underlie the role of mitochondria in longevity regulation

One of the objectives of studies described in this thesis was to establish the mechanism that links age-related changes in mitochondrial morphology to an apoptosis-like cell death of aging yeast. Based on findings described in previous section, we hypothesized that: (1) the observed fragmentation of the mitochondrial network in chronologically aging yeast cells that enter ST phase (Figures 2.4, 2.10 and 2.11) could trigger apoptosis; and (2) this age-related, mitochondria-controlled apoptosis could play an essential role in regulating longevity. In support of this hypothesis, we found that chronologically aging non-CR yeast cells die exhibiting characteristic markers of apoptosis (Figure 2.16) such as nuclear fragmentation (Figure 2.17), PS translocation from the inner to the outer leaflet of the plasma membrane (Figure 2.18), cleavage of chromosomal DNA (Figure 2.19), mitochondrial fragmentation (Figure 2.20) and the efflux of cytochrome *c* from mitochondria (Figures 2.21 and 2.22). We demonstrated that a CR diet - which slows down the fragmentation of the mitochondrial tubular network early in ST phase - delays such age-related apoptosis (Figures 2.17 to 2.22). Moreover, we also revealed that the anti-aging compound LCA - which under CR conditions completely abolishes mitochondrial fragmentation - prevents age-related apoptosis even in CR yeast that have reached late ST phase (Figures 2.17 to 2.22).

It should be emphasized that, as we recently demonstrated, CR also significantly reduces the susceptibility of yeast to apoptosis induced in response to a short-term exposure to exogenous hydrogen peroxide or acetic acid, and LCA almost completely abolishes such apoptosis under CR conditions [14, 24]. Of note, the hydrogen peroxide-

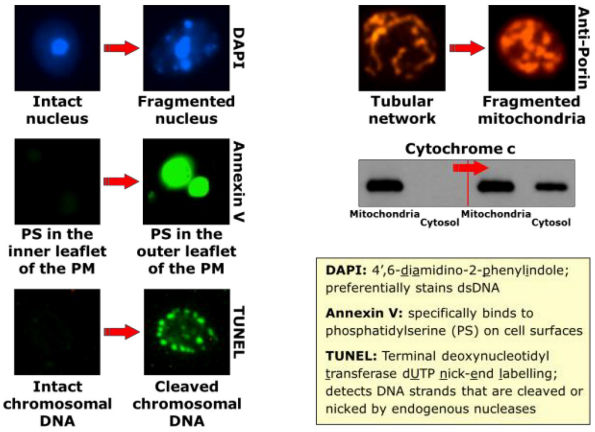

Figure 2.16. Hallmark events of apoptotic cell death.

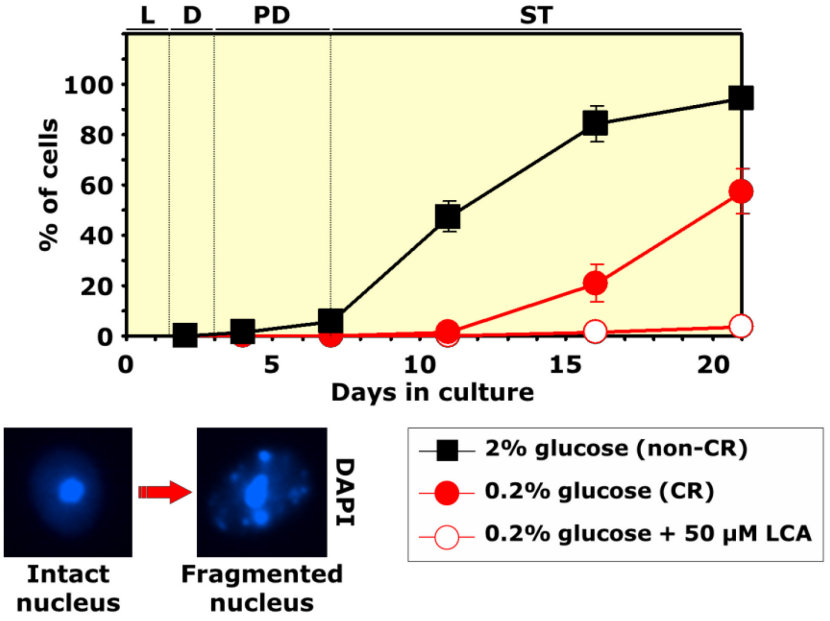

Figure 2.17. CR delays and LCA almost completely abolishes nuclear fragmentation, a hallmark event of apoptosis.

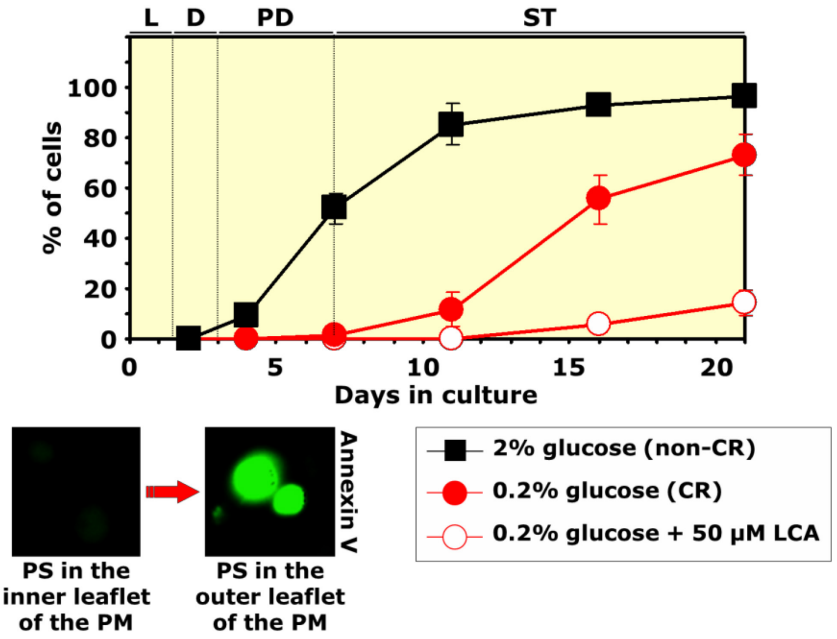

Figure 2.18. CR delays and LCA almost completely abolishes externalization of PS, a hallmark event of apoptosis.

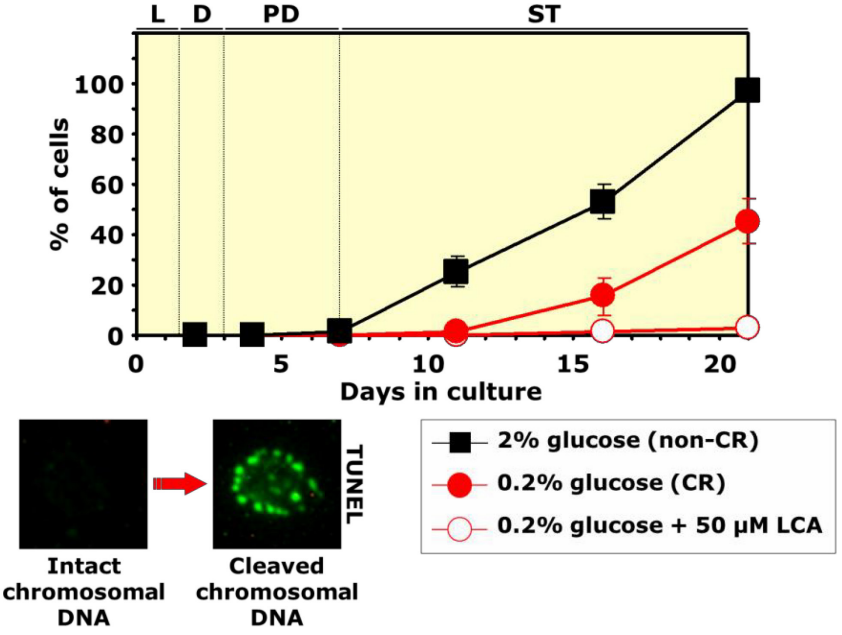

Figure 2.19. CR delays and LCA almost completely abolishes cleavage of chromosomal DNA, a hallmark event of apoptosis.

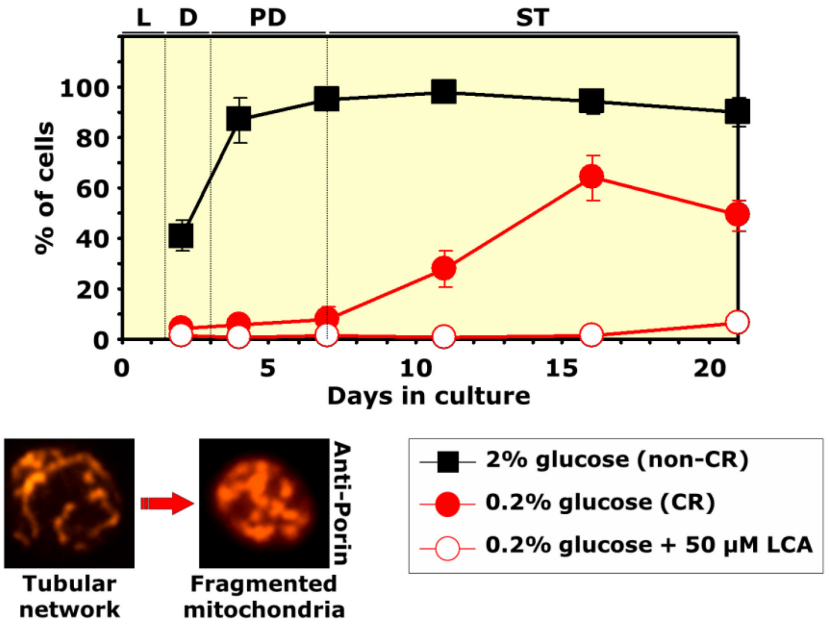

Figure 2.20. CR delays and LCA almost completely abolishes mitochondrial fragmentation, a hallmark event of apoptosis.

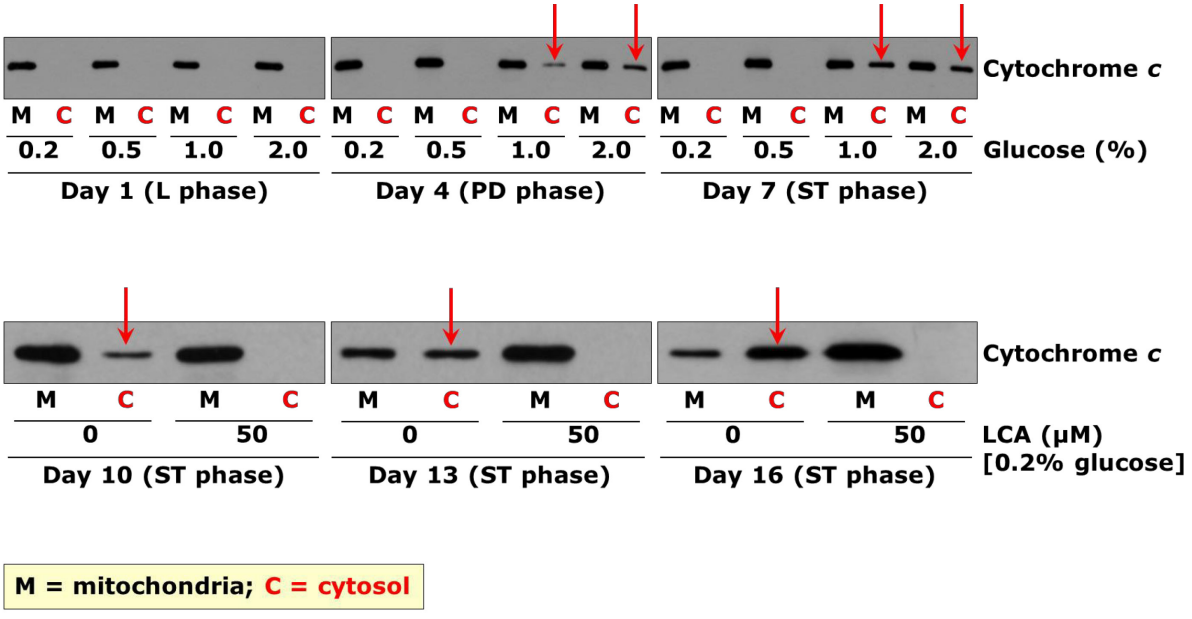

Figure 2.21. CR delays and LCA almost completely abolishes the efflux of cytochrome c from mitochondria, a hallmark event of apoptosis.

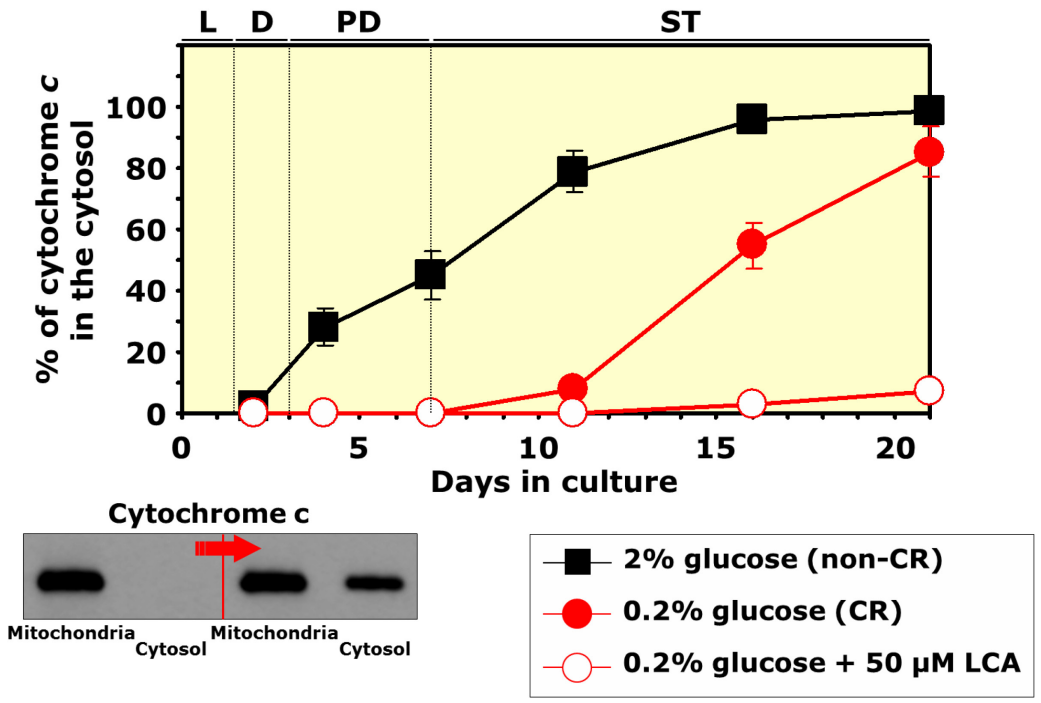

Figure 2.22. CR delays and LCA almost completely abolishes the efflux of cytochrome c from mitochondria, a hallmark event of apoptosis.

and acetic acid-induced forms of apoptosis have been linked to mitochondrial fragmentation, mitochondrial outer membrane permeabilization, and the release of several intermembrane space proteins from mitochondria [102, 104 - 109, 126]. Importantly, we found that the age-related apoptotic cell death of CR yeast during late ST phase depends on the metacaspase Yca1p as well as on such mitochondrial intermembrane space proteins as cytochrome *c*, Aif1p and Nuc1p. In fact, lack of Yca1p, both isoforms (*i.e.*, Cyc1p and Cyc7p) of cytochrome *c*, Aif1p or Nuc1p extends longevity of chronologically aging yeast even under CR conditions (Figure 2.23). Furthermore, the pro-aging activities of at least some of these proteins could be reduced

by LCA, which under CR conditions increases the chronological lifespans of mutants that lack only one of them but maintain the others (Figures 2.24 to 2.27). Moreover, we found that LCA increases the chronological lifespans of mutants that lack Yca1p, both isoforms (*i.e.*, Cyc1p and Cyc7p) of cytochrome *c*, Aif1p or Nuc1p to a significantly lesser degree than it does for wild-type strain (Figures 2.24 to 2.27). We therefore hypothesize that each of these pro-apoptotic proteins plays a dual role in regulating the beneficial effect of CR and LCA on yeast longevity. Specifically, while Yca1p, both isoforms (*i.e.*, Cyc1p and Cyc7p) of cytochrome *c*, Aif1p and Nuc1p weaken (by a yet-to-be-established mechanism) the lifespan-expending effect of CR, these pro-apoptotic proteins somehow enhance the ability of LCA to extend yeast longevity under CR conditions (Figure 2.28).

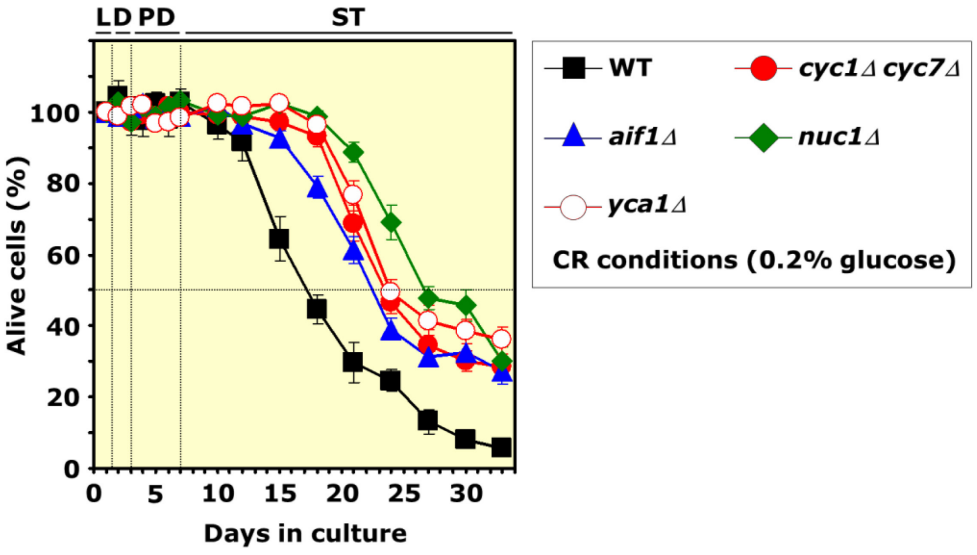

Figure 2.23. Age-related apoptosis of CR yeast during late ST phase depends on the metacaspase Yca1p and mitochondrial intermembrane space proteins cytochrome c (a pro-apoptotic protein), Aif1p (an apoptosis inducing factor) and Nuc1p (a pro-apoptotic endonuclease G).

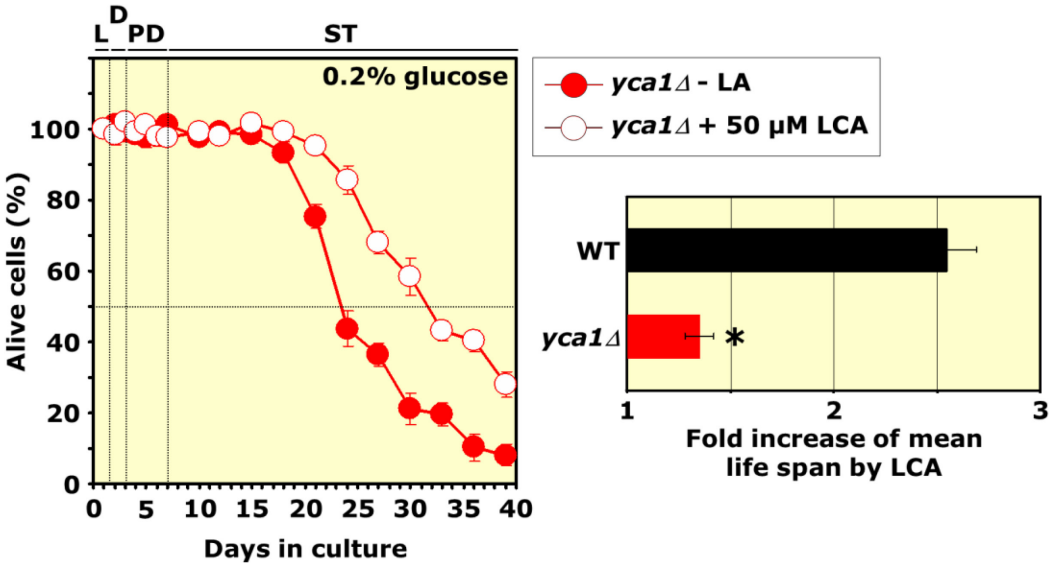

Figure 2.24. LCA increases the chronological lifespan of a mutant that lacks the metacaspase Yca1p, but to a significantly lesser degree than it does for WT strain. Thus, paradoxically, Yca1p plays an essential role in promoting the anti-aging effect of LCA.

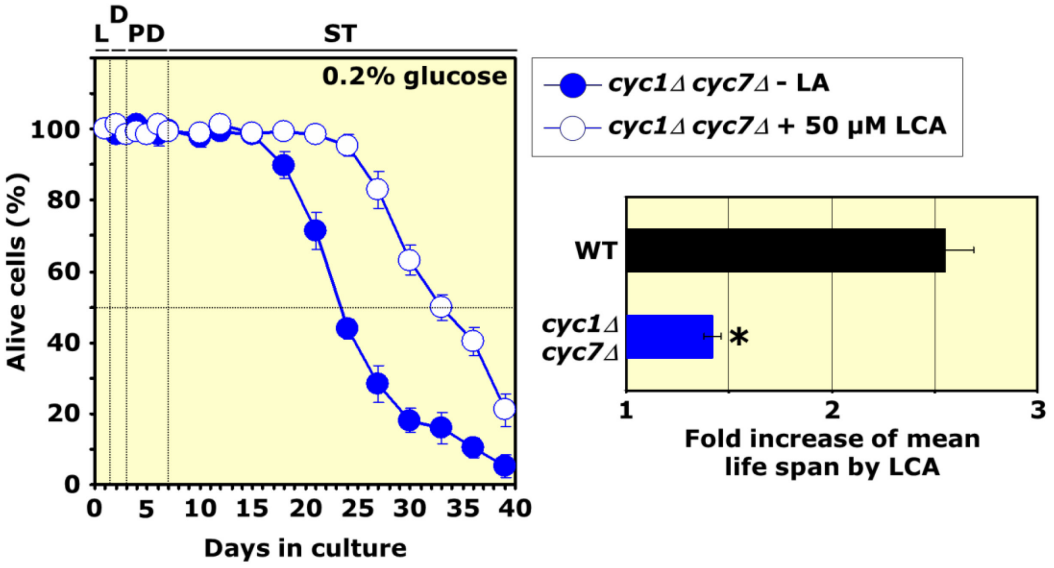

Figure 2.25. LCA increases the chronological lifespan of a mutant that lacks the Cyc1p and Cyc7p isoforms of cytochrome *c*, but to a significantly lesser degree than it does for wild-type strain. Thus, paradoxically, cytochrome *c* plays an essential role in promoting the anti-aging effect of LCA.

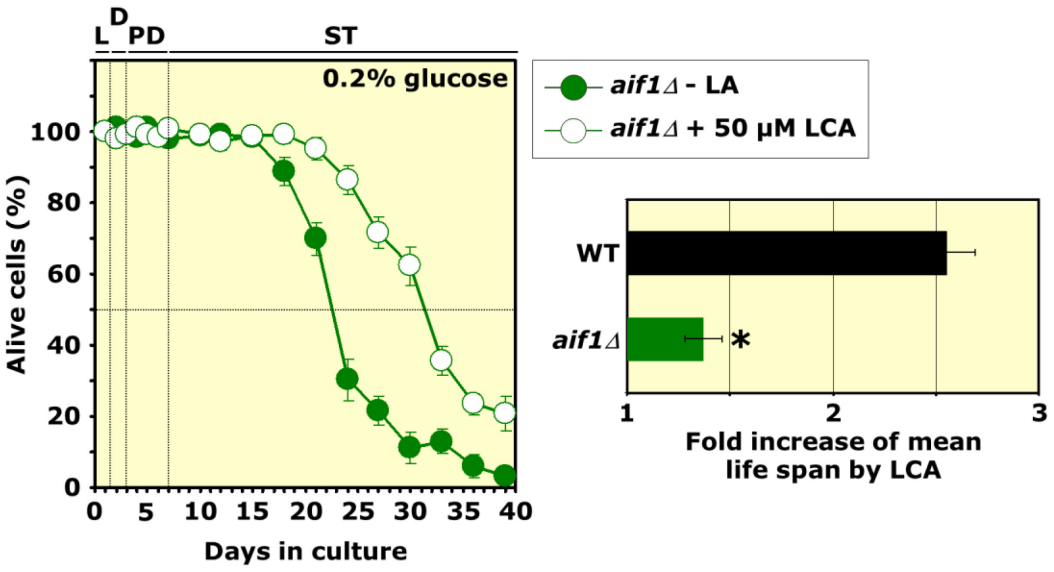

Figure 2.26. LCA increases the chronological lifespan of a mutant strain that lacks the apoptosis inducing factor Aif1p, but to a significantly lesser degree than it does for wild-type strain. Thus, paradoxically, Aif1p plays an essential role in promoting the anti-aging effect of LCA.

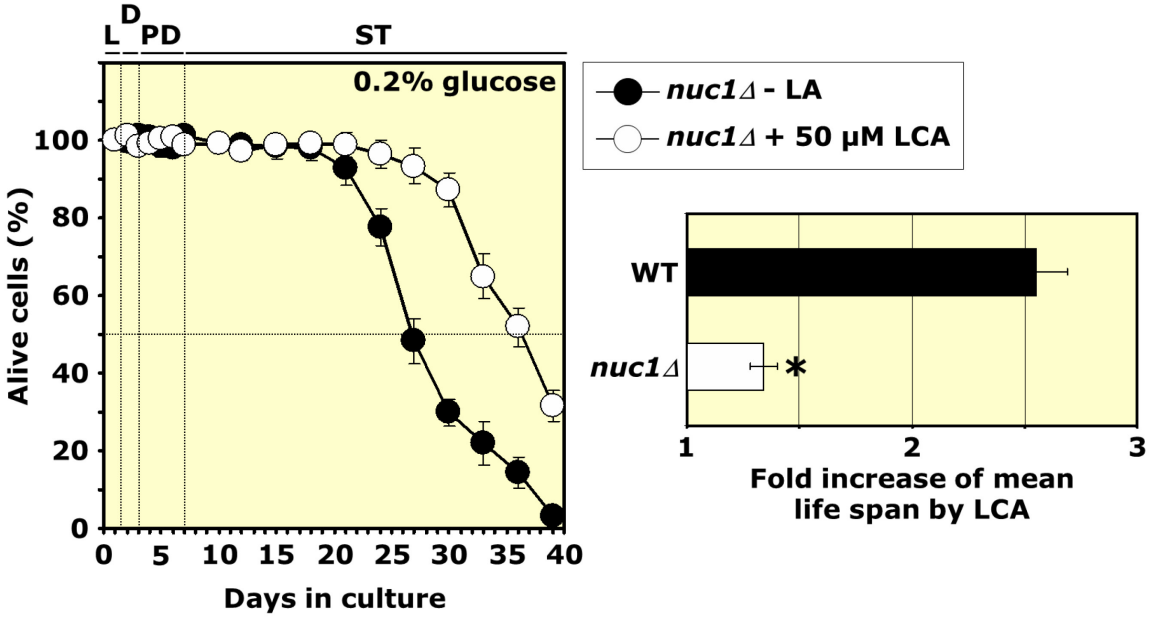

Figure 2.27. LCA increases the chronological lifespan of a mutant that lacks the pro-apoptotic endonuclease Nuc1p, but to a significantly lesser degree than it does for wild-

type strain. Thus, paradoxically, Nuc1p plays an essential role in promoting the anti-aging effect of LCA.

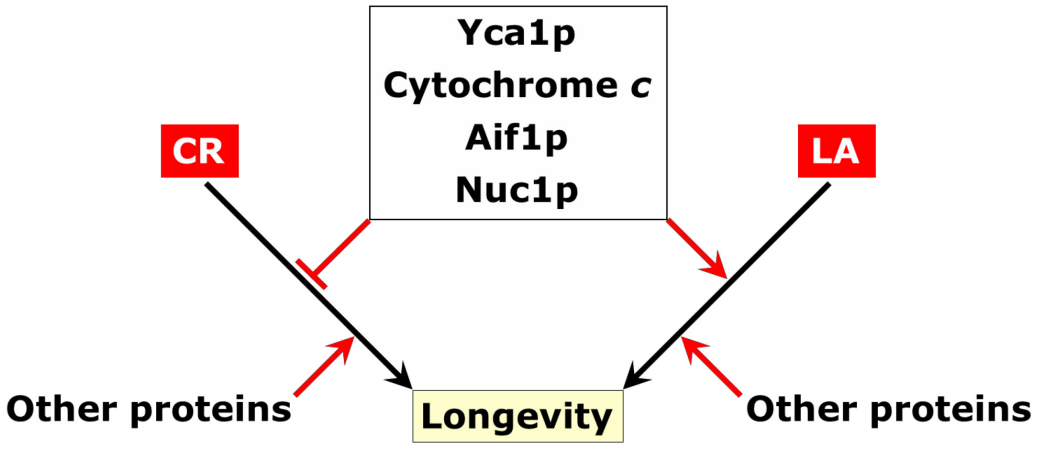

Figure 2.28. Dual roles for the pro-apoptotic proteins Yca1p, cytochrome c, Aif1p and Nuc1p in regulating the beneficial effects of CR and LCA on yeast longevity.

It needs to be emphasized that, as we found, the ability of CR to delay mitochondrial fragmentation in early ST phase and attenuate both age-related and exogenously induced apoptosis coincides with its ability to slow down the release of cytochrome *c* from mitochondria into the cytosol (Figures 2.21 and 2.22). Moreover, we demonstrated that LCA - which under CR conditions impedes mitochondrial fragmentation late in ST phase and prevents both age-related and exogenously induced apoptosis - completely abolishes such efflux of cytochrome *c* from mitochondria (Figures 2.21 and 2.22). Based on these findings, we hypothesize that the age-dependent dynamics of the mitochondrial tubular network regulates longevity by modulating age-related

apoptosis. This mitochondria-controlled apoptosis: (1) is triggered by the efflux of the pro-apoptotic proteins cytochrome *c*, Aif1p and Nuc1p from mitochondria in aging cells; and (2) depends on the metacaspase Yca1p. In our hypothesis, CR delays the fragmentation of the mitochondrial tubular network during early ST phase. This, in turn, slows down the age-related exit of pro-apoptotic proteins from mitochondria, attenuates apoptotic cell death, and ultimately prolongs lifespan. Moreover, we hypothesize that LCA further increases the lifespan of CR yeast by preventing mitochondrial fragmentation during late ST phase, thereby averting the age-related exit of pro-apoptotic proteins from mitochondria and inhibiting apoptosis.

As we found, LCA extends yeast longevity in part by promoting the ability of Fzo1p and Ugo1p to fuse the opposing mitochondrial outer membranes, thereby preventing fragmentation of the mitochondrial network (see above in this chapter). It should be stressed that, by maintaining the connectivity of the mitochondrial network, mitochondrial fusion is known to play a key role in cell and organismal life and death [130 - 132]. Defects in mitochondrial fusion in humans are known to manifest primarily as neurodegenerative diseases [132]. In fact, mutations affecting mitochondrial fusion proteins Mfn2 (a mammalian homolog of yeast Fzo1p) and OPA1 (a mammalian homolog of yeast Mgm1p) have been shown to lead to peripheral neuropathy Charcot-Marie-Tooth type 2A and dominant optic atrophy 1, respectively [130 - 132]. One of the primary phenotypes associated with the impairment of mitochondrial fusion in patients suffering from these diseases is loss of mitochondrial respiratory function, which is an important factor contributing to their etiology [130 - 132]. It is therefore important to mention that recent unpublished findings of other students in the Titorenko laboratory

revealed that under CR conditions LCA is unable to extend the lifespans of mutants lacking components of the complexes III, IV and V of the mitochondrial electron transport chain (ETC) or mitochondrial chaperones required for the assembly of these complexes (Beach et al., manuscript in preparation). Thus, the ability of LCA to delay aging relies on the intact mitochondrial respiratory chain. Moreover, in studies described in this chapter of the thesis we found that LCA alters the age-dependent dynamics of several mitochondrial activities in CR yeast, including oxygen consumption, mitochondrial membrane potential ($\Delta\psi$) and ROS generation. In particular, in CR yeast entering D phase, LCA decreases the amplitude of the spike in all these mitochondrial activities (Figures 2.29 to 2.31). Furthermore, during PD and ST phases, LCA prevents a sharp decline of all these activities, maintaining them at the levels they reached by the end of PD phase (Figures 2.29 to 2.31).

Interestingly, by analyzing yeast mutants lacking redundant components of the mitochondrial tricarboxylic acid (TCA) cycle, other students in the Titorenko laboratory recently revealed that the effects of LCA on lifespan and mitochondrial functions are very similar to those seen in CR yeast carrying mutations eliminating the Idh1p or Idh2p subunits of isocitrate dehydrogenase (Beach et al., manuscript in preparation). One could reason that mitochondria lacking a single redundant component of the TCA cycle (such as Idh1p or Idh2p) would still be able to produce NADH and $FADH_2$, the two donors of electrons for the ETC, but in reduced amounts. This, in turn, would reduce mitochondrial ROS generated as by-products of electron flow through the ETC. As other students in the Titorenko laboratory demonstrated, akin to LCA (Figures 2.29 to 2.31), the *idh1*Δ and *idh2*Δ mutations extend the chronological lifespan of CR yeast and alter - in an LCA-like

fashion - the age-dependent dynamics of oxygen consumption, $\Delta\psi$ and ROS generation. Specifically, it was revealed that, in CR yeast entering D phase, both mutations decrease the amplitude of the spike in all of these mitochondrial activities (Beach et al., manuscript in preparation). During PD and ST phases, both mutations were shown to prevent a sharp decline of all these activities. In addition, it was found that, during ST phase, *idh1*Δ and *idh2*Δ avert the loss of cytochrome c oxidase (CCO) and succinate dehydrogenase (SDH) activities (Beach et al., manuscript in preparation), both of which are known to represent the major mitochondrial targets of oxidative damage by ROS [133, 134]. Furthermore, it was demonstrated by other students in the Titorenko laboratory that both mutations protect aconitase (ACO), an enzyme of the TCA cycle, from age-related inactivation (Beach et al., manuscript in preparation) known to be caused by the oxidation-dependent loss of one iron from its Fe/S cluster [135]. Moreover, using mass spectrometry-based proteomics, other students in the Titorenko laboratory revealed that *idh1*Δ and *idh2*Δ increase the abundance of cytosolic and mitochondrial anti-stress chaperones, ROS-decomposing proteins, and proteins that protect mtDNA from oxidative damage (Beach et al., manuscript in preparation). Consistent with the elevated levels of these proteins, both mutations were found to enhance heat-shock and oxidative stress resistance of aging CR yeast. Importantly, it was shown that, akin to *idh1*Δ and *idh2*Δ, LCA protects yeast from chronic oxidative stress under CR conditions (Beach et al., manuscript in preparation).

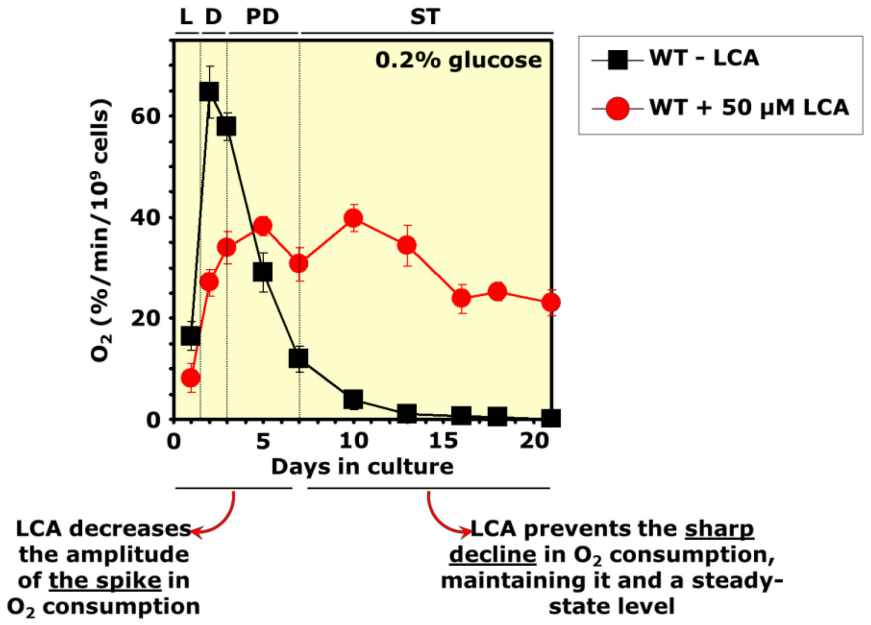

Figure 2.29. LCA alters the age-dependent dynamics of cellular respiration by modulating mitochondrial O_2 consumption.

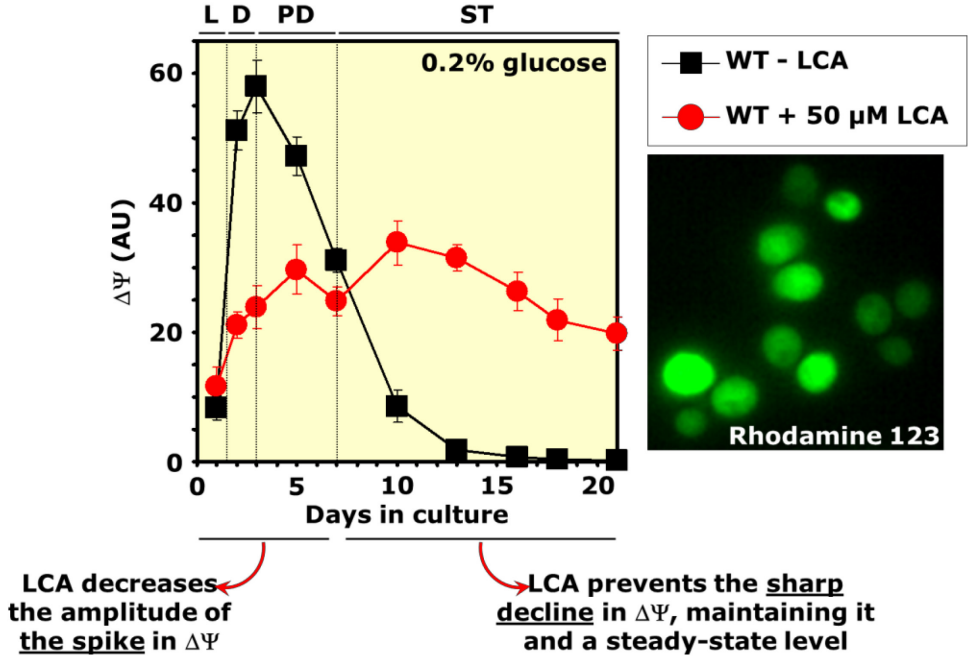

Figure 2.30. LCA alters the age-dependent dynamics of mitochondrial membrane potential ($\Delta\psi$).

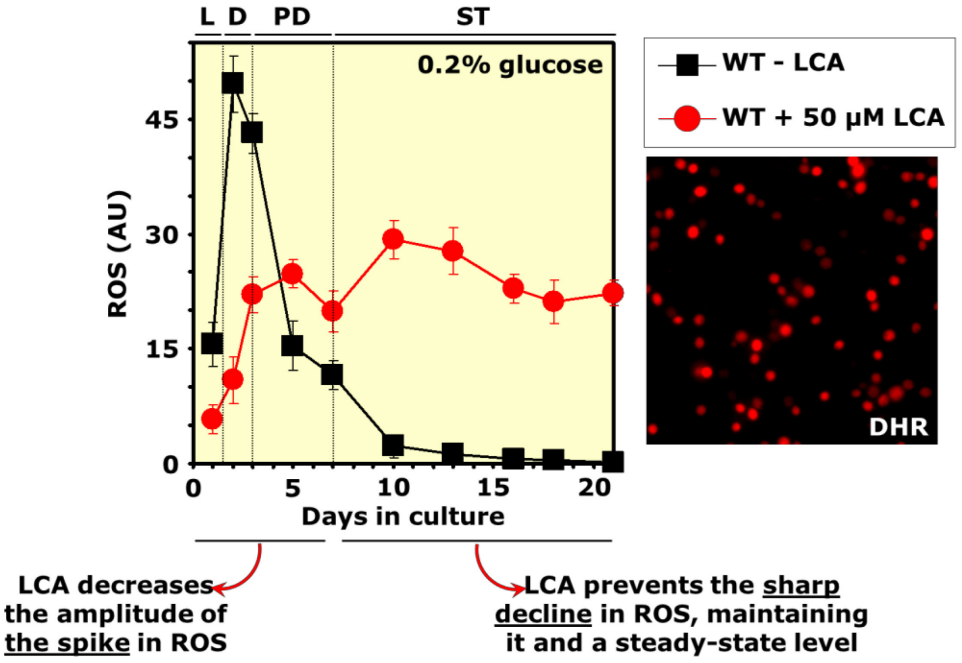

Figure 2.31. LCA alters the age-dependent dynamics of ROS generation in mitochondria.

2.4 Discussion

Based on our observation that LCA alters the age-dependent dynamics of several mitochondrial activities in CR yeast, including oxygen consumption, mitochondrial membrane potential ($\Delta\psi$) and ROS generation and considering data of other students in the Titorenko laboratory on the effect of *idh1*Δ and *idh2*Δ on longevity and mitochondrial functions, we hypothesize that age-related, mitochondria-controlled apoptosis is not the only mechanism by which mitochondria regulate longevity. The other mechanism is based on the LCA-dependent ability of mitochondria in aging yeast to maintain ROS homeostasis. In particular, we propose that ROS, which are mostly generated as by-products of mitochondrial respiration [136, 137], play a dual role in regulating longevity (Figure 2.32). First, if yeast mitochondria are unable (due to a dietary regimen or unavailability of LCA) to maintain ROS concentration below a critically high level, ROS

promote aging by oxidatively damaging certain mitochondrial proteins (such as CCO, SDH and ACO) (Figure 2.32). Second, if yeast mitochondria can (due to a dietary regimen or availability of LCA) maintain ROS concentration at an "optimal" level, ROS delay aging (Figure 2.32). This "optimal" level of ROS is insufficient to damage cellular macromolecules but can activate certain signaling networks [133, 136 - 142] that extend lifespan by increasing the abundance or activity of stress-protecting and other anti-aging proteins. The term "mitohormesis" has been coined for such anti-aging role of mitochondrially produced ROS [143]; the term "hormesis" refers to a beneficial defence response of an organism to a low-intensity biological stress [144, 145].

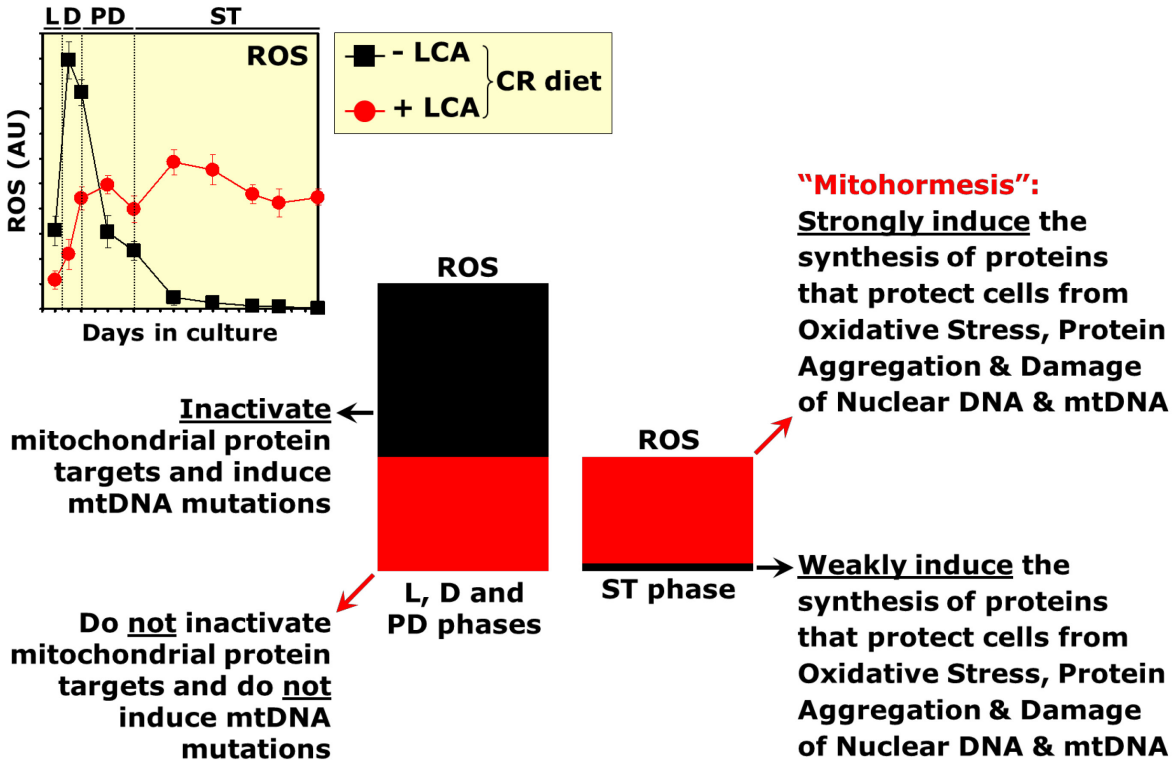

Figure 2.32. LCA extends longevity by reducing the damaging effect of ROS and amplifying their "hormetic" effect.

3 LCA extends longevity of chronologically aging yeast only if added at certain critical periods of their lifespan

3.1 Introduction

One way to look at the complexity of the aging process, in which a limited number of evolutionarily conserved nutrient- and energy-sensing signaling pathways (hereafter often called "master regulators") orchestrate a plethora of cellular processes in space and time [1, 50, 146, 147], is to consider each of these numerous processes as a functional module integrated with other modules into a biomolecular network [148 - 151]. In this conceptual framework (1) a synergistic action of individual modules comprising the network could define longevity by establishing the rate of cellular and organismal aging; and (2) 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 [24, 148 - 152]. Initially developed for theoretical modeling of the complexity of the senescence process in mammalian organisms [153], a network biology approach has been intensively used in recent years for reconstructing biomolecular networks of longevity and aging on the cellular and organismal levels. Numerous models of such networks have been proposed based on system biological and computational analyses of protein-protein interactions, age-related changes in gene expression or longevity-defining metabolic alterations [24, 147, 154 - 162]. Here we extend the network theory of aging by proposing that a biomolecular longevity network could progress through a series of checkpoints. At each of these checkpoints, a distinct set of master regulators senses the functional states of critical

modules comprising the network. Based on this information and considering the input of some environmental cues (such as caloric and dietary intake, environmental stresses, endocrine factors, etc.), master regulators modulate certain processes within monitored modules in order to limit the age-related accumulation of molecular and cellular damage. The resulting changes in the dynamics of individual modules comprising the network and in its general configuration are critically important for defining longevity by establishing the rate of cellular and organismal aging.

A confirmation of this concept for a stepwise development of a longevity network configuration at 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*. It seems that this organism employs 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 [163, 164]. 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 [163, 164]. 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 [165, 166] 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 [167]. 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) [163, 168]. Noteworthy, the magnitude of the effect of this second regulatory system on lifespan declines with age and becomes insignificant after several days of adulthood [163]. The third regulatory system influences the lifespan of *C. elegans* in a diet-restriction-specific fashion by operating exclusively during adulthood [169]. 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* [169]. 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 [163, 167 - 169].

It seems that some general aspects of the proposed here hypothesis of the longevity control system progressing through a series of checkpoints could be applicable to laboratory mice and rats. In fact, 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 [170 - 172]. 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 conceivable 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.

According to the proposed here hypothesis for a stepwise development of a longevity network configuration at a series of checkpoints, a pharmacological anti-aging intervention may modulate the key longevity-defining cellular processes that are monitored at a particular checkpoint by a master regulator of the longevity control system. Several anti-aging compounds are currently known for their ability to extend longevity across phyla and improve health by beneficially influencing age-related pathologies [1, 2, 14, 173]. Only one of these anti-aging compounds, a macrocyclic

lactone rapamycin synthesized by soil bacteria to inhibit growth of fungal competitors within an ecosystem [174], has been examined for its effects on lifespan and healthspan if implemented at different ages of an organism. If fed to genetically heterogeneous mice beginning at 270 days or 600 days of age, this specific inhibitor of the nutrient-sensory protein kinase mTORC1 (mammalian target of rapamycin complex 1) has been shown to be equally efficient in extending lifespan and beneficially influencing age-related pathologies [173, 175 - 178]. Our recent studies provided evidence that (1) LCA, a bile acid, extends longevity of chronologically aging yeast if added to growth medium at the time of cell inoculation [14]; and (2) longevity in chronologically aging yeast is programmed by the level of metabolic capacity and organelle organization that they 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 [24]. We therefore sought to investigate how LCA influences longevity and a compendium of longevity-defining cellular processes in chronologically aging yeast if added to growth medium at different periods of lifespan.

3.2 Materials and Methods

Strain and media

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MAT* α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) was used in this study. Media components were as follows: (1) YEPD (0.2% glucose), 1% yeast extract, 2% peptone, 0.2% glucose; and (2) YEPD (2% glucose), 1% yeast extract, 2% peptone, 2% glucose.

A plating assay for the analysis of chronological lifespan

Cells were grown in YEPD (0.2% glucose) medium at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a flask volume/medium volume ratio of 5:1. A sample of cells was removed from each culture at various time points. A fraction of the cell sample was diluted in order to determine the total number of cells per ml of culture using a hemacytometer. 10 µ l of serial dilutions (1:10 to 1:10^{3}) of cells were applied to the hemacytometer, where each large square is calibrated to hold 0.1 µ l. The number of cells in 4 large squares was then counted and an average was taken in order to ensure greater accuracy. The concentration of cells was calculated as follows: number of cells per large square x dilution factor x 10 x 1,000 = total number of cells per ml of culture. A second fraction of the cell sample was diluted and serial dilutions (1:10^{2} to 1:10^{5}) of cells were plated onto YEPD (2% glucose) plates in triplicate in order to count the number of viable cells per ml of each culture. 100 µ l of diluted culture was plated onto each plate. After a 48-h incubation at 30^{o}C, the number of colonies per plate was counted. The number of colony forming units (CFU) equals to the number of viable cells in a sample. Therefore, the number of viable cells was calculated as follows: number of colonies x dilution factor x 10 = number of viable cells per ml. For each culture assayed, % viability of the cells was calculated as follows: number of viable cells per ml / total number of cells per ml x 100%. The % viability of cells in mid-logarithmic phase was set at 100% viability for that particular culture. The life span curves for wild-type and some of the mutant strains were also validated using a LIVE/DEAD yeast viability kit (Invitrogen) following the manufacturer's instructions for stationary-phase cultures.

Plating assays for the analysis of resistance to various stresses

For the analysis of hydrogen peroxide resistance, serial dilutions ($1:10^0$ to $1:10^5$) of wild-type and mutant cells removed from mid-logarithmic phase (day 1) and from diauxic phase (days 2 and 3) in YEPD (0.2% glucose) were spotted onto two sets of plates. One set of plates contained YEPD (2% glucose) medium alone, whereas the other set contained YEPD (2% glucose) medium supplemented with 5 mM hydrogen peroxide. Pictures were taken after a 3-day incubation at 30°C.

For the analysis of oxidative stress resistance, serial dilutions ($1:10^0$ to $1:10^5$) of wild-type and mutant cells removed from mid-logarithmic phase (day 1) and from diauxic phase (days 2 and 3) in YEPD (0.2% glucose) were spotted onto two sets of plates. One set of plates contained YEPD (2% glucose) medium alone, whereas the other set contained YEPD (2% glucose) medium supplemented with 2.5 mM of the superoxide/hydrogen peroxide-generating agent paraquat. Pictures were taken after a 3-day incubation at 30°C.

For the analysis of heat-shock resistance, serial dilutions ($1:10^0$ to $1:10^5$) of wild-type and mutant cells removed from mid-logarithmic phase (day 1) and from diauxic phase (days 2 and 3) in YEPD (0.2% glucose) were spotted onto two sets of YEPD (2% glucose) plates. One set of plates was incubated at 30°C. The other set of plates was initially incubated at 55°C for 30 min, and was then transferred to 30°C. Pictures were taken after a 3-day incubation at 30°C.

For the analysis of salt stress resistance, serial dilutions ($1:10^0$ to $1:10^5$) of wild-type and mutant cells removed from mid-logarithmic phase (day 1) and from diauxic

phase (days 2 and 3) in YEPD (0.2% glucose) were spotted onto two sets of plates. One set of plates contained YEPD (2% glucose) medium alone, whereas the other set contained YEPD (2% glucose) medium supplemented with 0.5 M NaCl. Pictures were taken after a 3-day incubation at 30^{o}C. For the analysis of osmotic stress resistance, serial dilutions (1:10^{0} to 1:10^{5}) of wild-type and mutant cells removed from mid-logarithmic phase (day 1) and from diauxic phase (days 2 and 3) in YEPD (0.2% glucose) were spotted onto two sets of plates. One set of plates contained YEPD (2% glucose) medium alone, whereas the other set contained YEPD (2% glucose) medium supplemented with 1 M sorbitol. Pictures were taken after a 3-day incubation at 30^{o}C.

Pharmacological manipulation of chronological lifespan

Chronological lifespan analysis was performed as described above in this section. The lithocholic (LCA) [#L6250] bile acid was from Sigma. The stock solution of LCA in DMSO was made on the day of adding this compound to cell cultures. LCA was added to growth medium at the final concentration of 50 μM immediately following cell inoculation into the medium or on days 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in the medium, as indicated. 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).

Cell viability assay for monitoring the susceptibility of yeast to an apoptotic mode of cell death induced by hydrogen peroxide

A sample of cells was taken from a culture at a certain time-point. A fraction of the sample was diluted in order to determine the total number of cells using a hemacytometer. 2×10^7 cells were harvested by centrifugation for 1 min at 21,000 × g at room temperature and resuspended in 2 ml of YP medium containing 0.2% glucose as carbon source. Each cell suspension was divided into 2 equal aliquots. One aliquot was supplemented with hydrogen peroxide (#H325-500; Fisher Scientific) to the final concentration of 2.5 mM, whereas other aliquot remained untreated. Both aliquots were then incubated for 2 h at 30^{o}C on a Labquake rotator (#400110; Thermolyne/Barnstead International) set for 360^o rotation. Serial dilutions of cells were plated in duplicate onto plates containing YP medium with 2% glucose as carbon source. After 2 d of incubation at 30^{o}C, the number of colony forming units (CFU) per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each aliquot of cells exposed to hydrogen peroxide, the % of viable cells was calculated as follows: (number of viable cells per ml in the aliquot exposed to hydrogen peroxide/number of viable cells per ml in the control aliquot that was not exposed to hydrogen peroxide) × 100.

Cell viability assay for monitoring the susceptibility of yeast to a necrotic mode of cell death induced by palmitoleic acid

A sample of cells was taken from a culture at a certain time-point. A fraction of the sample was diluted in order to determine the total number of cells using a hemacytometer. 2×10^7 cells were harvested by centrifugation for 1 min at 21,000 × g at room temperature and resuspended in 2 ml of YP medium containing 0.2% glucose as carbon source. Each cell suspension was divided into 2 equal aliquots. One aliquot was

supplemented with palmitoleic acid (#P9417; Sigma) from a 50 mM stock solution (in 10% chloroform, 45% hexane and 45% ethanol) [#650498, #248878 and #34852, respectively; all from Sigma]; the final concentration of palmitoleic acid was 0.15 mM (in 0.03% chloroform, 0.135% hexane and 0.135% ethanol). Other aliquot was supplemented with chloroform, hexane and ethanol added to the final concentrations of 0.03%, 0.135% and 0.135%, respectively. Both aliquots were then incubated for 2 h at 30^{o}C on a Labquake rotator set for 360^{o} rotation. Serial dilutions of cells were plated in duplicate onto plates containing YP medium with 2% glucose as carbon source. After 2 d of incubation at 30^{o}C, the number of colony forming units (CFU) per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each aliquot of cells exposed to palmitoleic acid, the % of viable cells was calculated as follows: (number of viable cells per ml in the aliquot exposed to palmitoleic acid/number of viable cells per ml in the control aliquot that was not exposed to palmitoleic acid) × 100.

Measurement of the frequency of nuclear mutations

The frequency of spontaneous point mutations in the *CAN1* gene of nuclear DNA was evaluated by measuring the frequency of mutations that caused resistance to the antibiotic canavanine.[10] A sample of cells was removed from each culture at various time-points. Cells were plated in triplicate onto YNB (0.67% Yeast Nitrogen Base without amino acids [#DF0919153; Fisher Scientific]) plates containing 2% glucose and supplemented with L-canavanine (50 mg/L), histidine, leucine, lysine and uracil (#C1625, #H8125, #L8912, #L5751 and #U0750, respectively; all from Sigma). In addition, serial dilutions

of each sample were plated in triplicate onto YP plates containing 2% glucose for measuring the number of viable cells. The number of CFU was counted after 4 d of incubation at 30°C. For each culture, the frequency of mutations that caused resistance to canavanine was calculated as follows: number of CFU per ml on YNB plates containing 2% glucose, L-canavanine (50 mg/L), histidine, leucine, lysine and uracil/number of CFU per ml on YP plates containing 2% glucose.

Measurement of the frequency of mitochondrial mutations affecting mitochondrial components

The frequency of spontaneous single-gene (*mit*$^-$ and *syn*$^-$) and deletion (*rho*$^-$ and *rho*^{o}) mutations in mtDNA affecting essential mitochondrial components was evaluated by measuring the fraction of respiratory-competent (*rho*$^+$) yeast cells remaining in their aging population. *rho*$^+$ cells maintained intact their mtDNA and their nuclear genes encoding essential mitochondrial components. Therefore, *rho*$^+$ cells were able to grow on glycerol, a non-fermentable carbon source. In contrast, mutant cells deficient in mitochondrial respiration were unable to grow on glycerol. These mutant cells carried mutations in mtDNA (including single-gene *mit*$^-$ and *syn*$^-$ mutations or large deletions *rho*$^-$) or completely lacked this DNA (*rho*^{o} mutants).[10] Serial dilutions of cell samples removed from different phases of growth were plated in triplicate onto YP plates containing either 2% glucose or 3% glycerol (#BP229-4; Fisher Scientific) as carbon source. Plates were incubated at 30°C. The number of CFU on YP plates containing 2% glucose was counted after 2 d of incubation, whereas the number of CFU on YP plates containing 3% glycerol was counted after 6 d of incubation. For each culture, the

percentage of respiratory-deficient (*mit*$^-$, *syn*$^-$, *rho*$^-$, *rho*^{o} and *pet*$^-$) cells was calculated as follows: 100 - [(number of CFU per ml on YP plates containing 3% glycerol/number of CFU per ml on YP plates containing 2% glucose) × 100].

The frequency of spontaneous point mutations in the *rib2* and *rib3* loci of mtDNA was evaluated by measuring the frequency of mtDNA mutations that caused resistance to the antibiotic erythromycin.[10] These mutations impair only mtDNA.[10] A sample of cells was removed from each culture at various time-points. Cells were plated in triplicate onto YP plates containing 3% glycerol and erythromycin (1 mg/ml) [#227330050; Acros Organics]. In addition, serial dilutions of each sample were plated in triplicate onto YP plates containing 3% glycerol as carbon source for measuring the number of respiratory-competent (*rho*$^+$) cells. The number of CFU was counted after 6 d of incubation at 30^{o}C. For each culture, the frequency of mutations that caused resistance to erythromycin was calculated as follows: number of CFU per ml on YP plates containing 3% glycerol and erythromycin/number of CFU per ml on YP plates containing 3% glycerol.

Statistical analysis

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

3.3 Results

3.3.1 LCA increases the chronological lifespan of yeast cultured under CR or non-CR conditions only if added at certain critical periods

We sought to examine if the addition of LCA to chronologically aging yeast at different periods of lifespan has an effect on the longevity-extending efficacy of this bile acid. Yeast cells were cultured in YP medium under CR conditions on 0.2% glucose or under non-CR conditions on 2% glucose, and LCA was added to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. LCA was used at the final concentration of 50 μM, at which this natural anti-aging compound has been shown to exhibit the greatest beneficial effect on longevity of chronologically aging yeast under both CR and non-CR conditions [14].

We found that in yeast cultured under CR conditions on 0.2% glucose, there are two critical periods when the addition of LCA to growth medium can increase both their mean and maximum chronological lifespans (Figures 3.1 and 3.3). One of these two critical periods, which we term period 1, includes L (logarithmic) and D (diauxic) growth phases. The other critical period, which is called period 3, exists in early ST (stationary) phase. In contrast, LCA did not cause a significant extension of the mean or maximum chronological lifespan of CR yeast if it was added at period 2 or 4 that exists in PD (post-diauxic) or late ST phase, respectively (Figures 3.1 and 3.3).

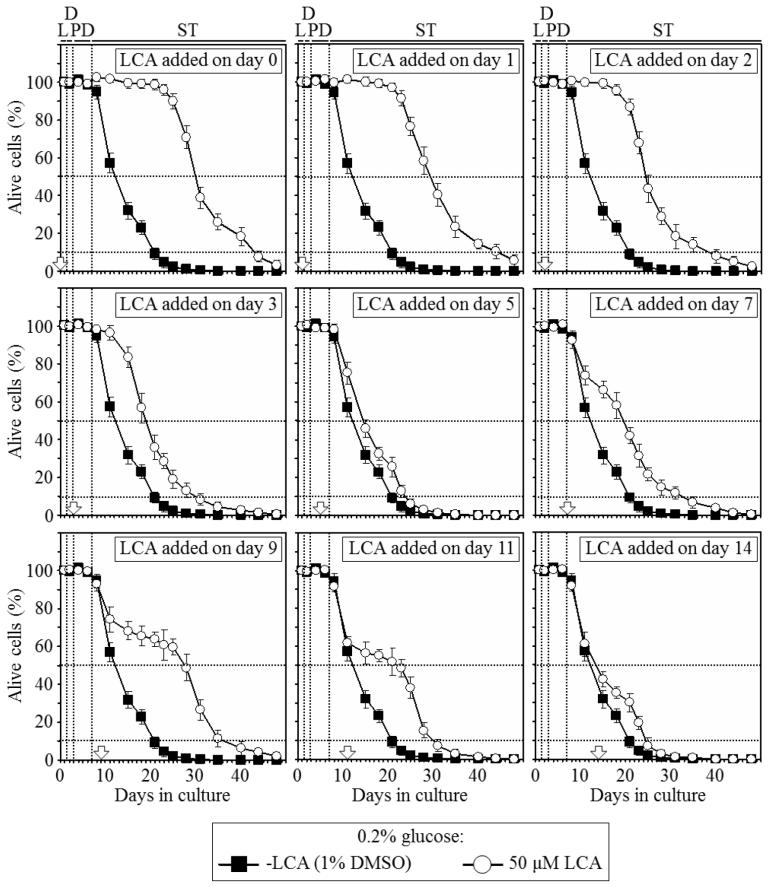

Figure 3.1. In yeast cultured under CR conditions on 0.2% glucose, there are two critical periods when the addition of LCA to growth medium can extend longevity. Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). Chronological lifespan analysis was performed as described in "Materials and Methods". Data are presented as mean ± SEM (n = 8-12). *Abbreviations*: diauxic (D), logarithmic (L), post-diauxic (PD) or stationary (ST) phase.

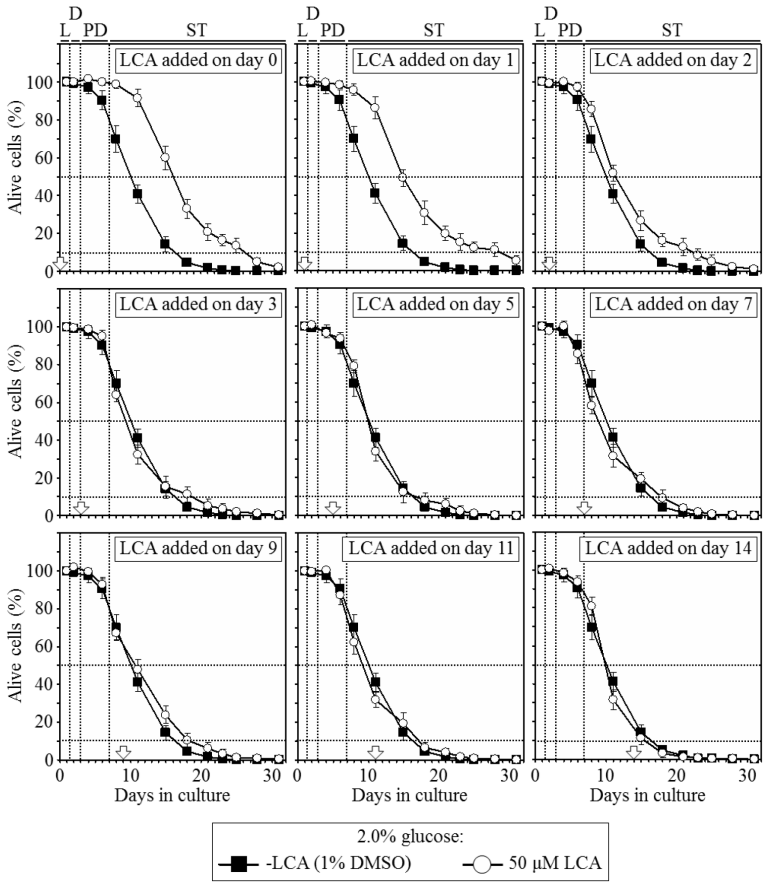

Figure 3.2. In yeast cultured under non-CR conditions on 2% glucose, there is only one critical period when the addition of LCA to growth medium can extend longevity. Wild-type yeast cells were cultured in YP medium initially containing 2% glucose, and LCA was added at the final concentration of 50 µM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). Chronological lifespan analysis was performed as described in "Materials and Methods". Data are presented as mean ± SEM (n = 6-7).

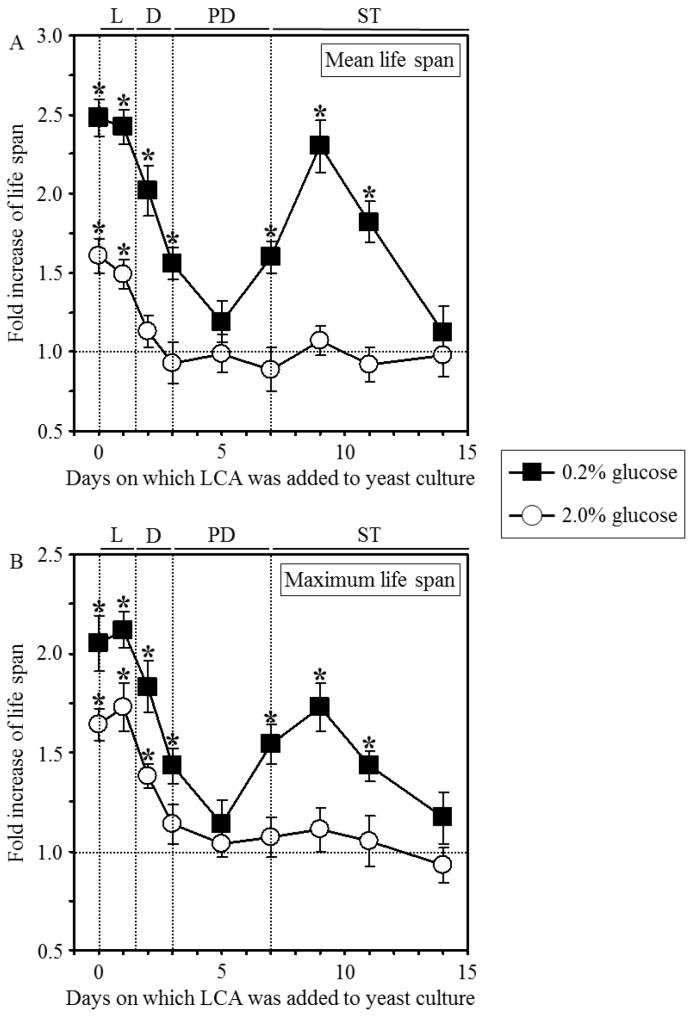

Figure 3.3. In yeast cultured under CR conditions on 0.2% glucose, there are two critical periods when the addition of LCA to growth medium can extend longevity. In yeast cultivated under non-CR conditions on 2% glucose, there is only one such a period. The mean (A) and maximum (B) chronological lifespans of different wild-type yeast cultures are shown. Wild-type yeast cells were cultured under CR or non-CR conditions and LCA was added as described in the legends for Fig. 1 and 2, respectively. Chronological lifespan analysis was performed as described in "Materials and Methods". Data are presented as mean ± SEM (n = 8-12 for (A); n = 6-7 for (B)); *$p < 0.05$ (relative to the mean or maximum chronological lifespan of yeast not exposed to LCA).

We also found that in yeast cultivated under non-CR conditions on 2% glucose, there is only one critical period, during L and D phases, when the addition of LCA to growth medium can increase both their mean and maximum chronological lifespans (Figures 3.2 and 3.3). However, if LCA was added to non-CR yeast at any time-point after this critical period ended, it did not significantly alter their mean or maximum chronological lifespan (Figures 3.2 and 3.3). Thus, unlike a substantial beneficial effect of LCA on yeast longevity seen if it was added in early ST phase under CR conditions, this bile acid was unable to extend longevity of chronologically aging yeast under non-CR conditions if added in the same phase.

3.3.2 There are several ways through which LCA could differentially influence longevity if added to CR yeast at different periods of their lifespan

Aging of multicellular and unicellular eukaryotic organisms affects numerous longevity-defining processes within cells [1, 2, 49, 50, 146, 147]. It is conceivable therefore that the observed ability of LCA to extend longevity of chronologically aging yeast under CR conditions only if added at period 1 or 3 of their lifespan (Figures 3.1 and 3.3) could be due to its differential and, perhaps, enduring effects on certain longevity-extending and longevity-shortening processes controlled at different lifespan periods. One could envisage several ways through which LCA can differentially influence these longevity-defining processes following its addition at different periods of yeast chronological lifespan (Figure 3.4). For example, LCA could activate a longevity-extending process (or several processes) controlled at certain checkpoints that may exist in L/D and early ST growth phases (periods 1 and 3, respectively), without influencing

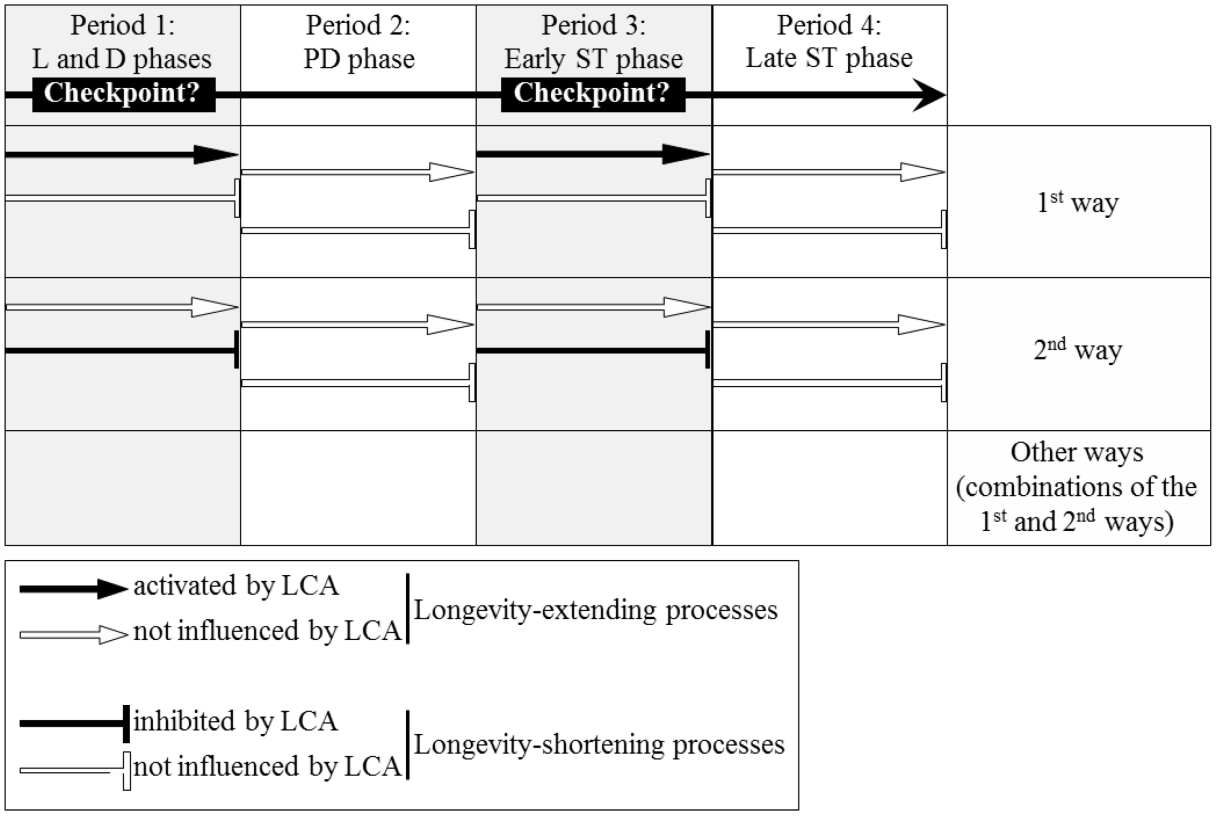

Figure 3.4. There are several ways through which LCA could differentially influence some longevity-extending and longevity-shortening processes following its addition at different periods of yeast chronological lifespan. LCA could control these longevity-defining processes at certain checkpoints that may exist in L/D and early ST phases (periods 1 and 3, respectively). PD and late ST phases constitute periods 2 and 4, respectively.

longevity-shortening processes at these envisioned lifespan checkpoints or having an effect on any longevity-defining processes during periods 2 and 4 in PD and late ST phases (Figure 3.4; see the 1st way). Alternatively, LCA could inhibit a longevity-shortening process (or several processes) controlled at certain checkpoints that may exist in L/D and early ST phases (periods 1 and 3, respectively), without influencing

longevity-extending processes at these envisioned lifespan checkpoints or having an effect on any longevity-defining processes during periods 2 and 4 in PD and late ST phases (Figure 3.4; see the 2nd way). Moreover, the observed ability of LCA to extend longevity of chronologically aging yeast under CR conditions only if added at periods 1 and 3 of their lifespan could be also due to various combinations of the 1st and the 2nd ways outlined above (Figure 3.4; see other ways). We therefore sought to examine how the addition of LCA at different periods of chronological lifespan in yeast cultured under CR conditions influences longevity-extending and longevity-shortening processes controlled during each of these periods.

3.3.3 LCA makes yeast cells resistant to mitochondria-controlled apoptotic death, a longevity-shortening process, only if added at period 1, 2 or 3 of their chronological lifespan

A short-term exposure of yeast to hydrogen peroxide, acetic acid, hyperosmotic stress, α pheromone or several other exogenous agents causes apoptotic cell death that has been linked to mitochondrial fragmentation, mitochondrial outer membrane permeabilization and the release of several intermembrane space proteins from mitochondria [103, 105, 109, 179]. The exit of Aif1p (apoptosis inducing factor 1) and Nuc1p (endonuclease G) from yeast mitochondria and their subsequent import into the nucleus trigger such exogenously induced apoptosis by promoting DNA cleavage [104, 107]. Another intermembrane space protein that is released from yeast mitochondria during exogenously induced apoptosis is cytochrome *c* [102, 105, 109]. Akin to its essential role in triggering the apoptotic caspase cascade in mammalian cells [98],

cytochrome *c* in the cytosol of yeast cells activates the metacaspase Yca1p [105, 111, 112, 180]. Importantly, chronologically aging yeast cells die, in an Aif1p-, Nuc1p- and Yca1p-dependent fashion, exhibiting characteristic markers of apoptosis such as chromatin condensation, nuclear fragmentation, DNA cleavage, PS externalization, ROS production and caspase activation [104, 107, 114 - 117]. Thus, the chronological aging of yeast is linked to an apoptosis-like, mitochondria-controlled programmed cell death [118 - 121, 181]. It should be emphasized that mutations eliminating pro-apoptotic proteins as well as such potent anti-aging interventions as a CR diet and LCA (1) extend longevity of chronologically aging yeast; (2) delay age-related apoptotic death controlled by mitochondria; and (3) reduce the susceptibility of yeast to cell death triggered by a short-term exposure to exogenous hydrogen peroxide and known to be caused by mitochondria-controlled apoptosis [14, 24, 99, 104, 107, 109, 114 - 121, 126, 181]. Taken together, these findings strongly support the notion that mitochondria-controlled apoptotic death plays an essential role in regulating longevity of chronologically aging yeast. This form of longevity-defining cell death can be triggered by a brief exposure of yeast to exogenous hydrogen peroxide [14, 24, 99, 109, 126]. We therefore examined how the addition of LCA at different periods of chronological lifespan influences a longevity-shortening process of mitochondria-controlled apoptotic cell death in yeast cultured under CR on 0.2% glucose. We monitored the susceptibility of yeast to cell death triggered by a short-term (for 2 h) exposure to exogenous hydrogen peroxide known to cause mitochondria-controlled apoptosis. We found that if added to growth medium on day 0, 1, 2, 3, 5, 7, 9 or 11, LCA reduces the susceptibility of CR yeast to apoptosis induced by a brief exposure to exogenous hydrogen peroxide following LCA

addition; this effect of LCA persists through the rest of the lifespan (Figure 3.5). In contrast, if added to growth medium on day 14, LCA does not alter the susceptibility of CR yeast to this form of apoptotic cell death for the remainder of the lifespan (Figure 3.5). Thus, LCA makes yeast cells resistant to mitochondria-controlled apoptotic death, a longevity-shortening process, only if added at period 1, 2 or 3 of chronological lifespan. This effect of LCA persists long after each of these lifespan periods ended.

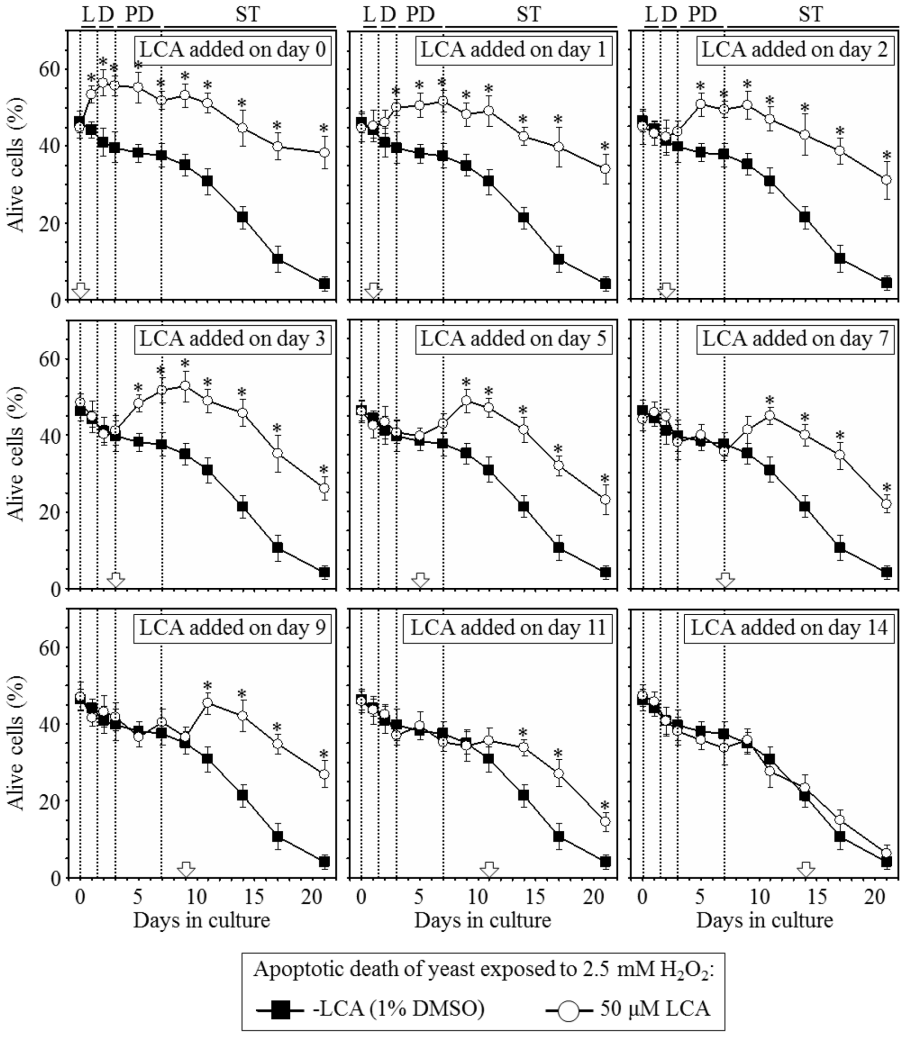

Figure 3.5. LCA reduces the susceptibility of yeast cells to mitochondria-controlled apoptotic death, a longevity-shortening process, only if added at period 1, 2 or 3 of chronological lifespan and for the rest of the lifespan. Wild-type yeast cells were cultured

in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). Cell viability assay for monitoring the susceptibility of yeast to an apoptotic mode of cell death induced by a 2-h exposure to exogenous hydrogen peroxide was performed as described in "Materials and Methods". Data are presented as mean ± SEM (n = 3 - 5); *p < 0.05 (relative to the % of alive cells in yeast cultures not exposed to LCA).

3.3.4 LCA differentially influences the susceptibility of chronologically aging yeast to palmitoleic acid-induced necrotic cell death, a longevity-shortening process, if added at different periods of their lifespan

In our recently proposed model for a mechanism linking yeast longevity and lipid dynamics in the endoplasmic reticulum, lipid bodies and peroxisomes, a remodeling of lipid metabolism in chronologically aging non-CR yeast shortens their lifespan by causing premature death in part due to necrotic cell death triggered by the accumulation of free fatty acids [14, 24, 81, 91, 182]. Importantly, both CR and LCA not only extend longevity of chronologically aging yeast but also reduce their susceptibility to a form of necrotic cell death triggered by a short-term exposure to exogenous palmitoleic fatty acid [14, 24, 81, 91, 182]. These findings imply that palmitoleic acid-induced necrotic cell death plays an essential role in regulating longevity of chronologically aging yeast. We therefore assessed how the addition of LCA at different periods of chronological lifespan

influences a longevity-shortening process of necrotic cell death in yeast cultured under CR on 0.2% glucose. We examined the susceptibility of yeast to cell death triggered by a short-term (for 2 h) exposure to exogenous palmitoleic acid. We found that if added to growth medium on day 0, 1 or 2, LCA reduces the susceptibility of CR yeast to necrosis induced by a brief exposure to palmitoleic acid following LCA addition; this effect of LCA persists through the remainder of the lifespan (Figure 3.6). In contrast, LCA either does not influence (if added on day 3, 9, 11 or 14) or increases for the rest of the lifespan (if added on day 5 or 7) the susceptibility of CR yeast to this form of necrotic cell death (Figure 3.6).

Thus, LCA makes yeast cells resistant to palmitoleic acid-induced necrotic death, a longevity-shortening process, only if added at period 1 that includes L and D growth phases; this effect of LCA persists long after the end of period 1. It is conceivable that the observed inability of LCA to extend yeast longevity if added at period 2 of chronological lifespan (Figures 3.1 and 3.3) could be due to the observed stimulating lifelong effect of LCA on a palmitoleic acid-induced form of necrotic cell death if added at this period in PD phase (Figure 3.6). Furthermore, despite LCA extends longevity of chronologically aging yeast if added at period 3, it does not affect their susceptibility to necrotic cell death if added at this period in early ST phase (Figure 3.6). Moreover, the observed lack of an effect of LCA on yeast longevity if added at period 4 of chronological lifespan (Figures 3.1, 3.3 and 3.4) coincides with its inability to alter cell susceptibility to necrotic cell death if added at this period in late ST phase (Figure 3.6).

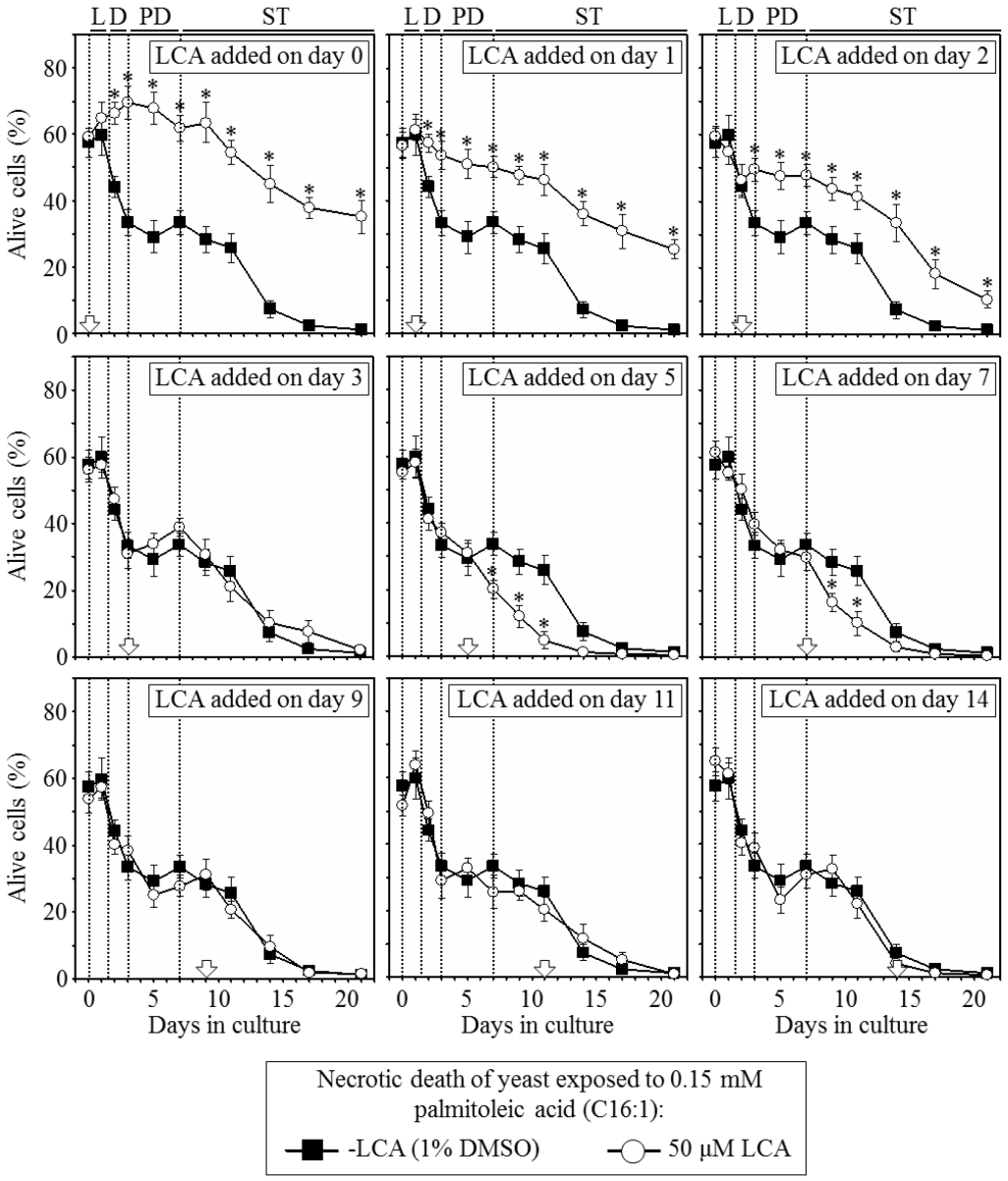

Figure 3.6. LCA differentially influences the susceptibility of chronologically aging yeast to palmitoleic acid-induced necrotic cell death, a longevity-shortening process, if added at different periods of lifespan. Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 µM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control

cultures supplemented with compound vehicle) was 1% (v/v). Cell viability assay for monitoring the susceptibility of yeast to a necrotic mode of cell death induced by a 2-h exposure to exogenous palmitoleic acid was performed as described in "Materials and Methods". Data are presented as mean ± SEM (n = 4); *$p < 0.05$ (relative to the % of alive cells in yeast cultures not exposed to LCA).

3.3.5 LCA differentially influences a longevity-extending process of the maintenance of nuclear and mitochondrial genomes if added at different periods of yeast chronological lifespan

A body of evidence supports the view that the maintenance of nDNA (nuclear DNA) and mtDNA (mitochondrial DNA) integrity is an essential longevity-extending process in evolutionarily distant organisms, including yeast [14, 24, 34, 49, 183, 184]. We therefore examined how the addition of LCA to yeast cultured under CR on 0.2% glucose at different periods of chronological lifespan influences (1) the frequency of spontaneous point mutations in the *CAN1* gene of nDNA; (2) the frequencies of spontaneous single-gene (*mit*$^-$ and *syn*$^-$) and deletion (*rho*$^-$ and *rho*^{o}) mutations in mtDNA, all causing a deficiency in mitochondrial respiration and impairing growth on glycerol; and (3) the frequencies of spontaneous point mutations in the *rib2* and *rib3* loci of mtDNA.

We found that if added to growth medium on day 0, 1 or 2, LCA reduces the frequency of spontaneous point mutations in the *CAN1* gene of nDNA for the rest of the lifespan (Figure 3.7). In contrast, LCA either does not influence (if added on day 3, 9, 11 or 14) or increases for the remainder of the lifespan (if added on day 5 or 7) the frequency

of these spontaneous point mutations in nDNA (Figure 3.7). Thus, LCA stimulates the maintenance of nDNA integrity, an essential longevity-extending process, only if added at period 1 that includes L and D growth phases; this effect of LCA persists long after the end of period 1. Our findings also suggest that the observed lifelong inhibitory effect of LCA on the maintenance of nDNA integrity if it is added at period 2 of yeast chronological lifespan (Figure 3.7) could in part be responsible for the inability of LCA to extend yeast longevity if added at this period in PD phase (Figures 3.1, 3.3 and 3.4). Furthermore, despite LCA extends longevity of chronologically aging yeast if added at period 3, it does not influence the maintenance of nDNA integrity if added at this period during early ST phase (Figure 3.7). Moreover, the observed lack of an effect of LCA on yeast longevity if added at period 4 of chronological lifespan (Figures 3.1, 3.3 and 3.4) coincides with the inability of this compound to alter the efficacy of the maintenance of nDNA integrity if added at this period in late ST phase (Figure 3.7).

We also found that if added to growth medium on day 0, 1, 2, 3, 5, 7, 9 or 11, LCA reduces for the rest of the lifespan (1) the frequencies of spontaneous single-gene (*mit*$^-$ and *syn*$^-$) and deletion (*rho*$^-$ and *rho*^{o}) mutations in mtDNA, all causing a deficiency in mitochondrial respiration and impairing growth on glycerol (Figure 3.8); and (2) the frequencies of spontaneous point mutations in the *rib2* and *rib3* loci of mtDNA (Figure 3.9). In contrast, if added to growth medium on day 14, LCA does not affect the frequencies of these spontaneous mutations in mtDNA (Figures 3.8 and 3.9). Thus, LCA stimulates the maintenance of mtDNA integrity, an essential longevity-extending process, only if added at period 1, 2 or 3 that exists in L/D, PD or early ST phase (respectively); this effect of LCA persists long after the end of each of these lifespan periods. Moreover,

the observed lack of an effect of LCA on yeast longevity if added at period 4 of chronological lifespan (Figures 3.1, 3.3 and 3.4) coincides with the inability of this compound to alter the efficacy of the maintenance of mtDNA integrity if added at this period in late ST phase (Figures 3.8 and 3.9).

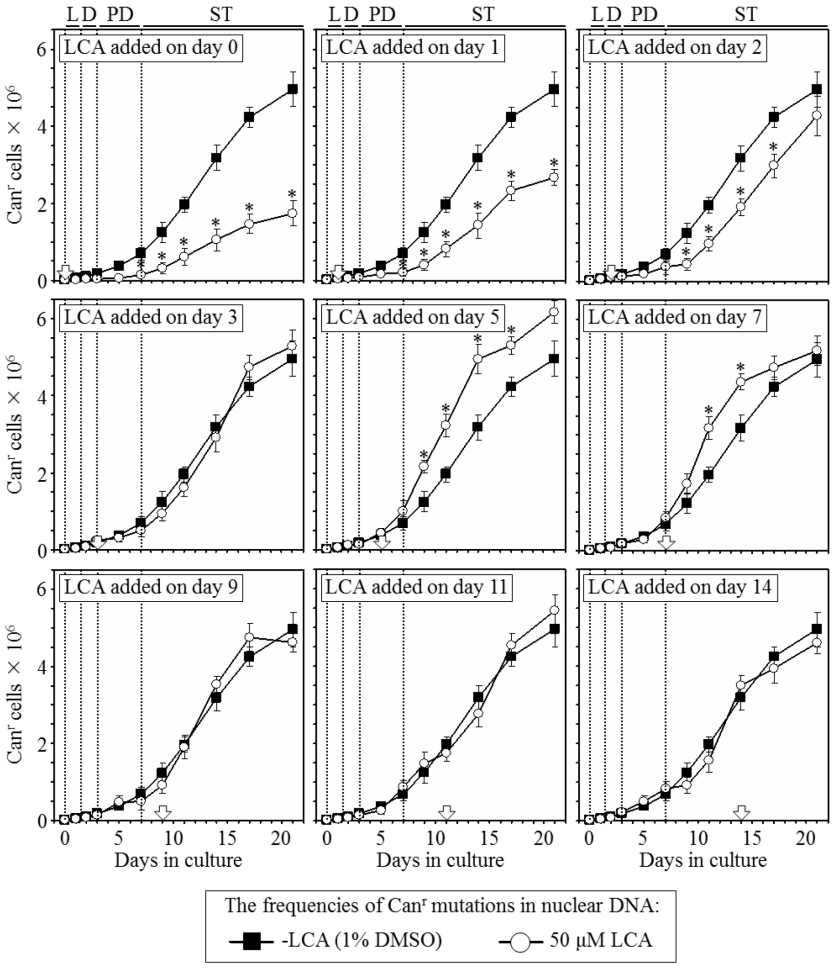

Figure 3.7. LCA differentially influences the maintenance of nDNA integrity, an essential longevity-extending process, if added at different periods of yeast lifespan. Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures

supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). The frequency of spontaneous point mutations in the *CAN1* gene of nDNA was measured as described in "Materials and Methods". Data are presented as mean ± SEM (n = 3); *p < 0.05 (relative to the frequency of spontaneous point mutations in the *CAN1* gene of nDNA in yeast cultures not exposed to LCA).

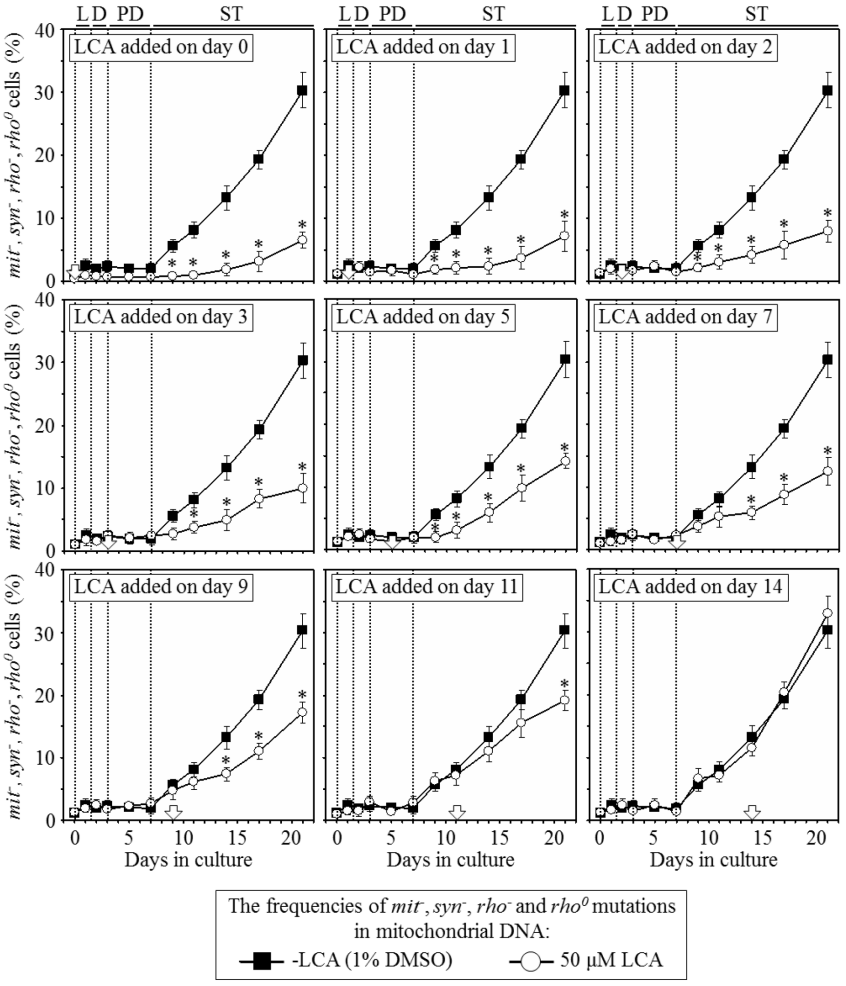

Figure 3.8. LCA differentially influences the maintenance of mtDNA integrity, an essential longevity-extending process, if added at different periods of yeast lifespan. Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14

of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). The frequencies of spontaneous single-gene (*mit*$^-$ and *syn*$^-$) and deletion (*rho*$^-$ and *rho*^{o}) mutations in mtDNA, all causing a deficiency in mitochondrial respiration and impairing growth on glycerol, were measured as described in "Materials and Methods". Data are presented as mean ± SEM (n = 5-6); *p < 0.05 (relative to the frequencies of spontaneous *mit*$^-$, *syn*$^-$, *rho*$^-$ and *rho*^{o} mutations in mtDNA in yeast cultures not exposed to LCA).

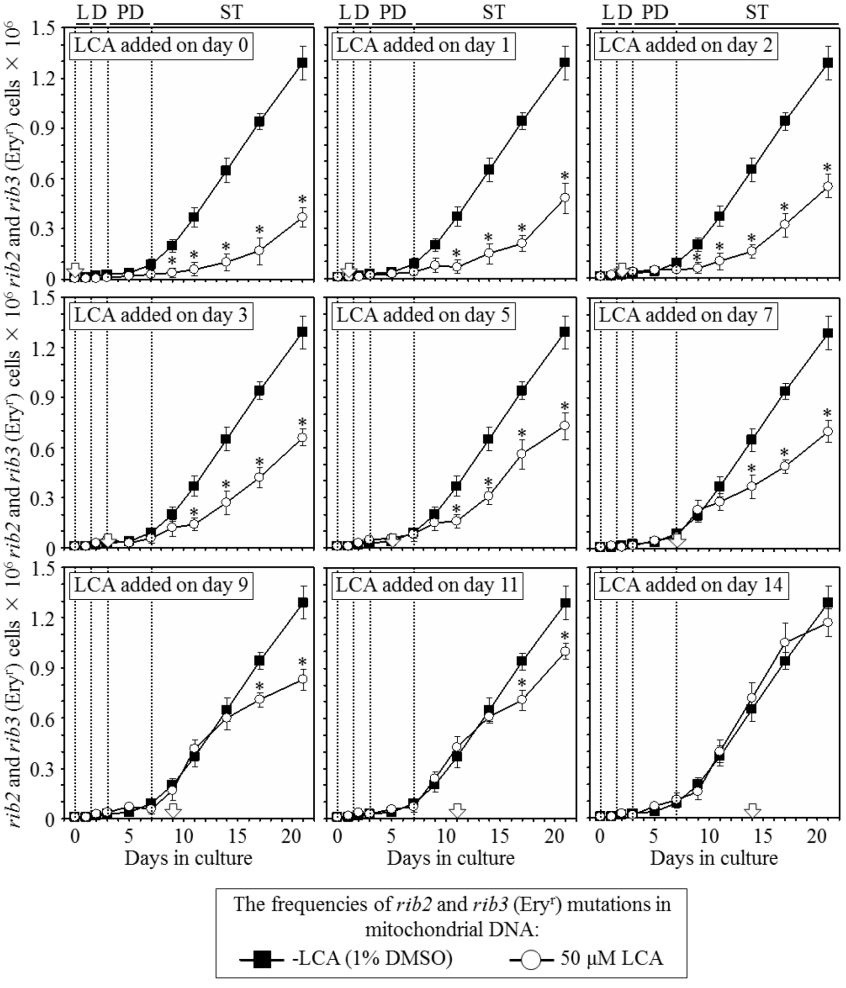

Figure 3.9. LCA differentially influences the maintenance of mtDNA integrity, an essential longevity-extending process, if added at different periods of yeast lifespan.

Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). The frequencies of spontaneous point mutations in the *rib2* and *rib3* loci of mtDNA were measured as described in "Materials and Methods". Data are presented as mean ± SEM (n = 3-4); *$p < 0.05$ (relative to the frequencies of spontaneous *rib2 and rib3* mutations in mtDNA in yeast cultures not exposed to LCA).

3.3.6 LCA differentially influences a longevity-extending process of the development of resistance to chronic stresses if added at different periods of yeast chronological lifespan

It is well established that the development of resistance to chronic (long-term) oxidative, thermal and osmotic stresses is an essential longevity-extending process in evolutionarily distant organisms, including yeast [1 - 3, 14, 24, 49, 53, 136, 137, 142, 185]. We therefore examined how the addition of LCA to yeast cultured under CR on 0.2% glucose at different periods of chronological lifespan influences their resistance to each of these chronic stresses. We found that if added to growth medium on day 0, 1, 2 or 3, LCA increases for the remainder of the lifespan the resistance of yeast to chronic oxidative and thermal stresses, but does not alter cell susceptibility to chronic osmotic stress (Figures 3.10 to 3.16). Moreover, LCA increases the resistance of yeast to all three chronic stresses if added to growth medium on day 7, 9 or 11; this effect of LCA persists

long after its addition (Figures 3.10 to 3.16). In contrast, LCA does not alter cell susceptibility to any of these chronic stresses if added on day 5 or 14 (Figures 3.10 to 3.16). Thus, in chronologically aging yeast LCA stimulates a longevity-extending process of the development of lifelong resistance to chronic oxidative and thermal stresses if added at period 1 that exists in L and D phases. If added at period 3 (which includes early ST phase), LCA not only stimulates the development of resistance to chronic oxidative and thermal stresses for the rest of the lifespan but also enhances a longevity-extending process of developing enduring resistance to chronic osmotic stress. If added at any of these two periods, LCA can extend longevity (Figures 3.1, 3.3 and 3.4). Noteworthy, the observed lack of an effect of LCA on yeast longevity if added at period 2 or period 4 of chronological lifespan (Figures 3.1, 3.3 and 3.4) coincides with the inability of this compound to alter cell susceptibility to any of these stresses at these two periods in PD and late ST phases, respectively.

3.4 Discussion

In sum, this study provides evidence that LCA, a bile acid, can extend longevity of CR yeast only if added at period 1 (which includes L and D growth phases) or period 3 (which exists in early ST phase) of chronological lifespan (Figure 49). In contrast, LCA is unable to extend the mean or maximum chronological lifespan of yeast cultured under CR conditions if added at period 2 or 4 in PD or late ST phase, respectively (Figure 49). We also found that longevity of yeast cultivated under non-CR conditions can be extended by LCA only if it is added at period 1, but not following its addition at period 2, 3 or 4.

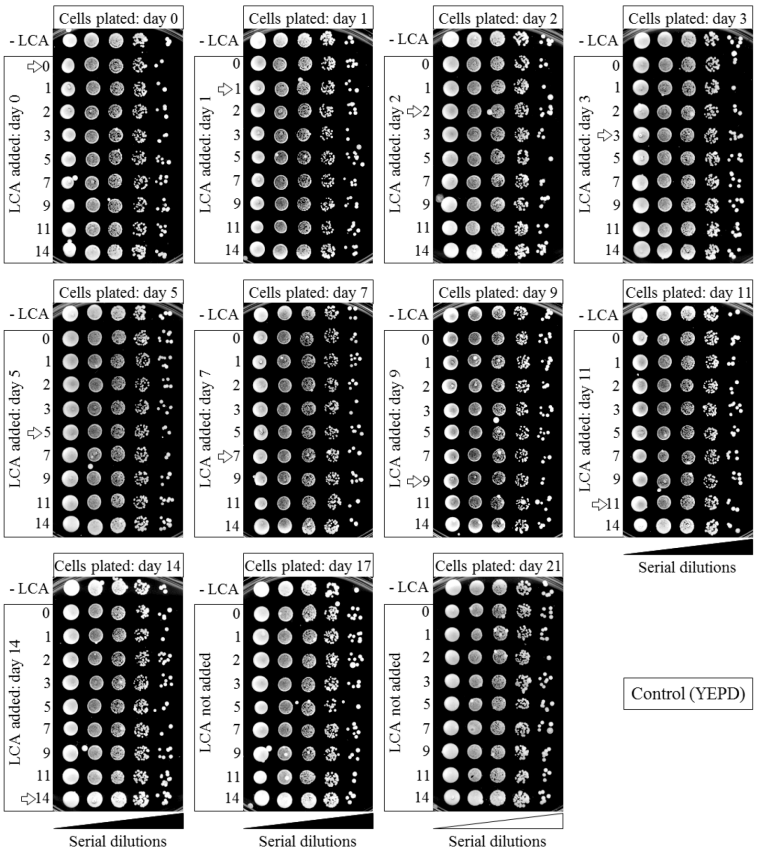

Figure 3.10. Effect of LCA added at different periods of yeast chronological lifespan on cell growth under non-stressful conditions. Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. Spot assays were performed as described in "Materials and Methods". Serial ten-fold dilutions of cells were spotted on plates with solid YP medium containing 2% glucose as carbon source. All pictures were taken after a 3-day incubation at 30^{o}C.

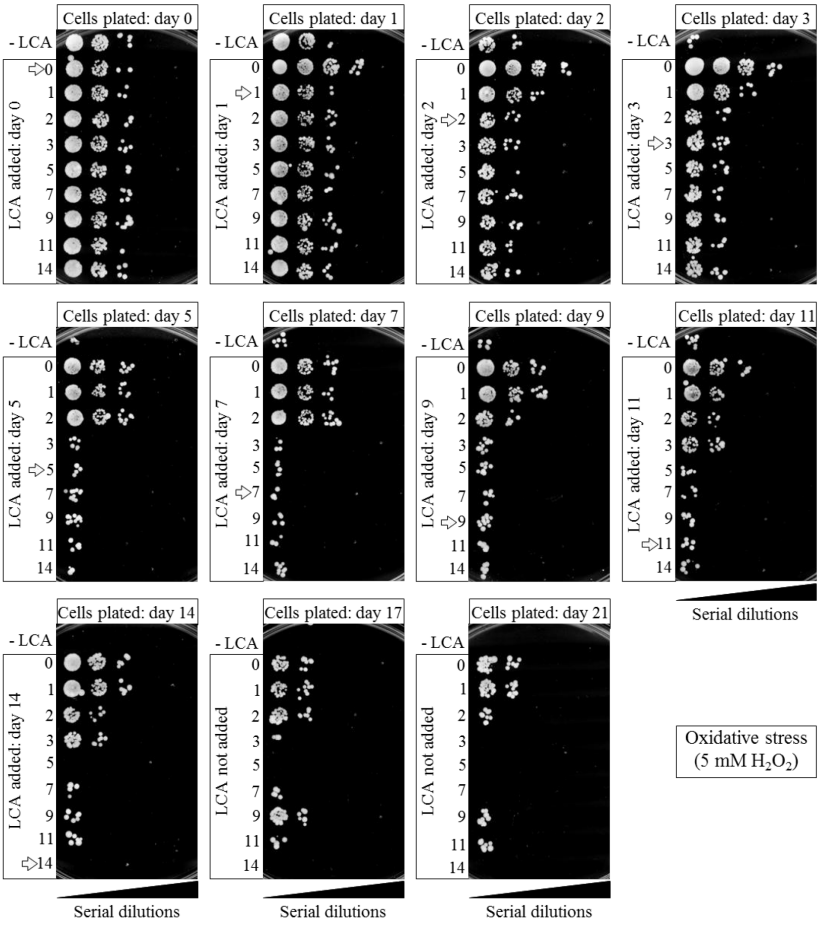

Figure 3.11. Effect of LCA added at different periods of chronological lifespan on the ability of yeast to resist chronic oxidative stress. Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). Spot assays for monitoring oxidative stress resistance were performed as described in "Materials and Methods". Serial ten-fold dilutions of cells were spotted on plates with

solid YP medium containing 2% glucose as carbon source and 5 mM hydrogen peroxide. All pictures were taken after a 3-day incubation at 30^{o}C.

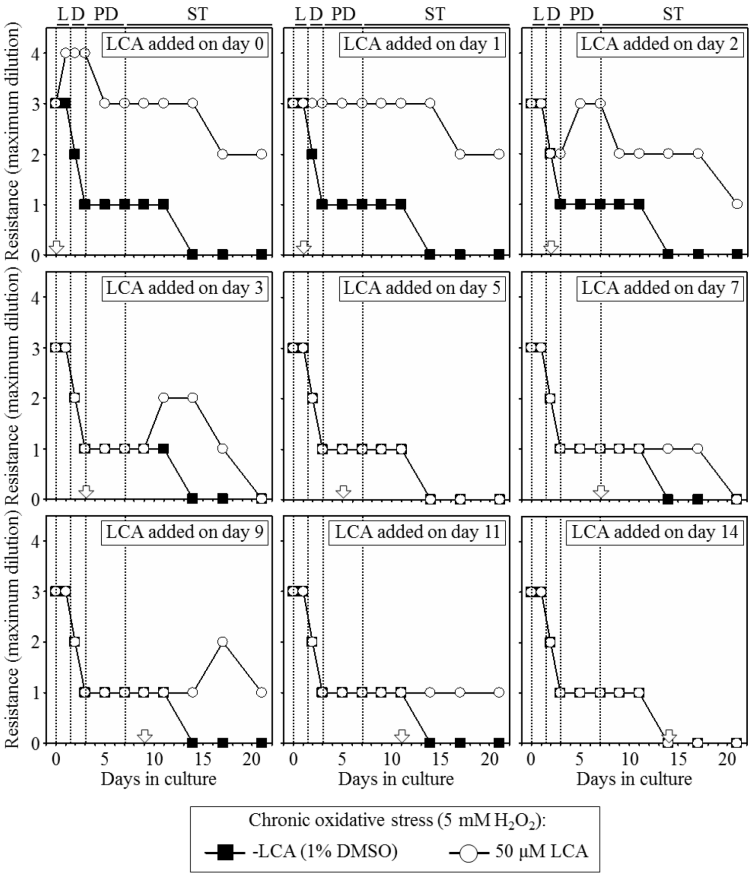

Figure 3.12. LCA differentially influences a longevity-extending process of the development of resistance to chronic oxidative stress if added at different periods of yeast chronological lifespan. A graphic presentation of the results of spot assays for monitoring oxidative stress resistance, which is shown in Figure 3.11.

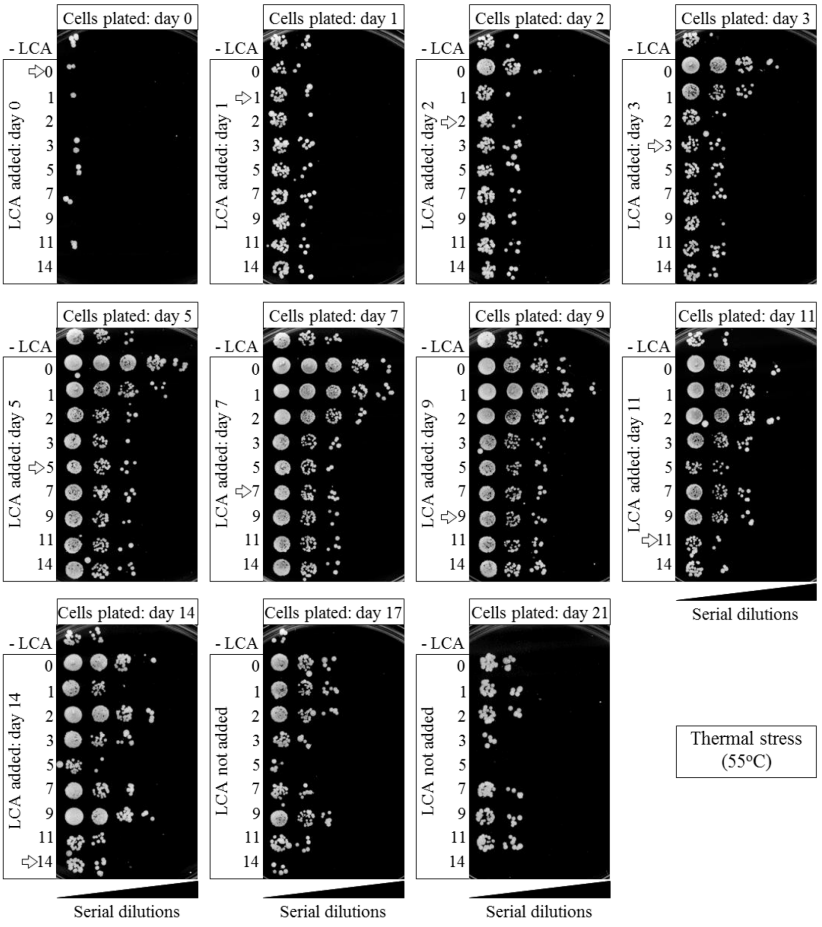

Figure 3.13. Effect of LCA added at different periods of chronological lifespan on the ability of yeast to resist chronic thermal stress. Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). Spot assays for monitoring thermal stress resistance were performed as described in "Materials and Methods". Serial ten-fold dilutions of cells were spotted on plates with solid YP medium containing 2% glucose as carbon source. Plates were initially incubated at 55^{o}C

for 30 min, and were then transferred to 30°C. All pictures were taken after a 3-day incubation at 30°C.

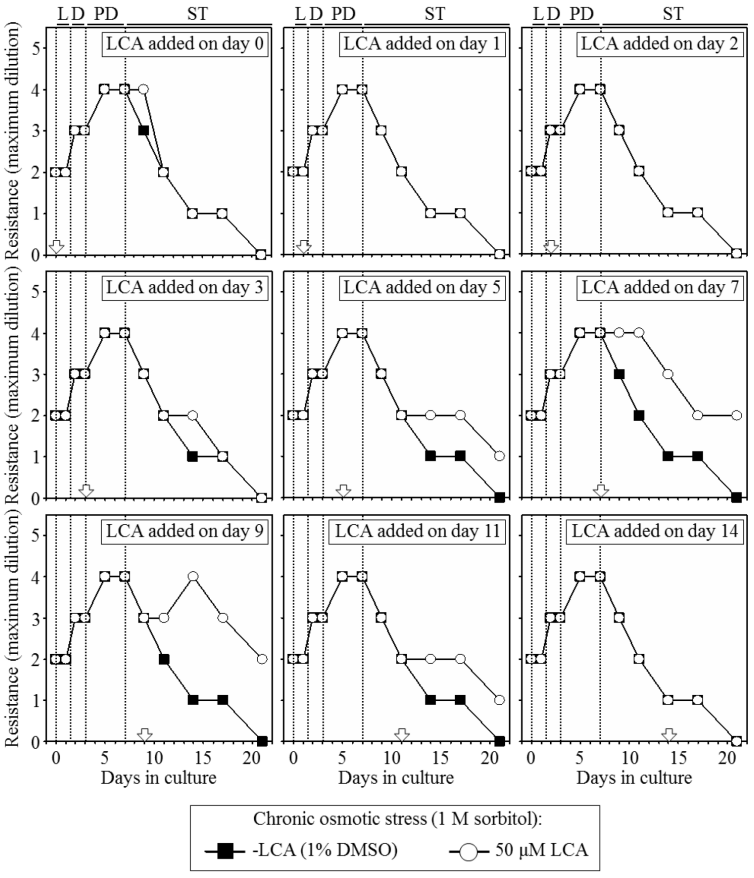

Figure 3.14. LCA differentially influences a longevity-extending process of the development of resistance to chronic thermal stress if added at different periods of yeast chronological lifespan. A graphic presentation of the results of spot assays for monitoring thermal stress resistance, which is shown in Figure 3.13.

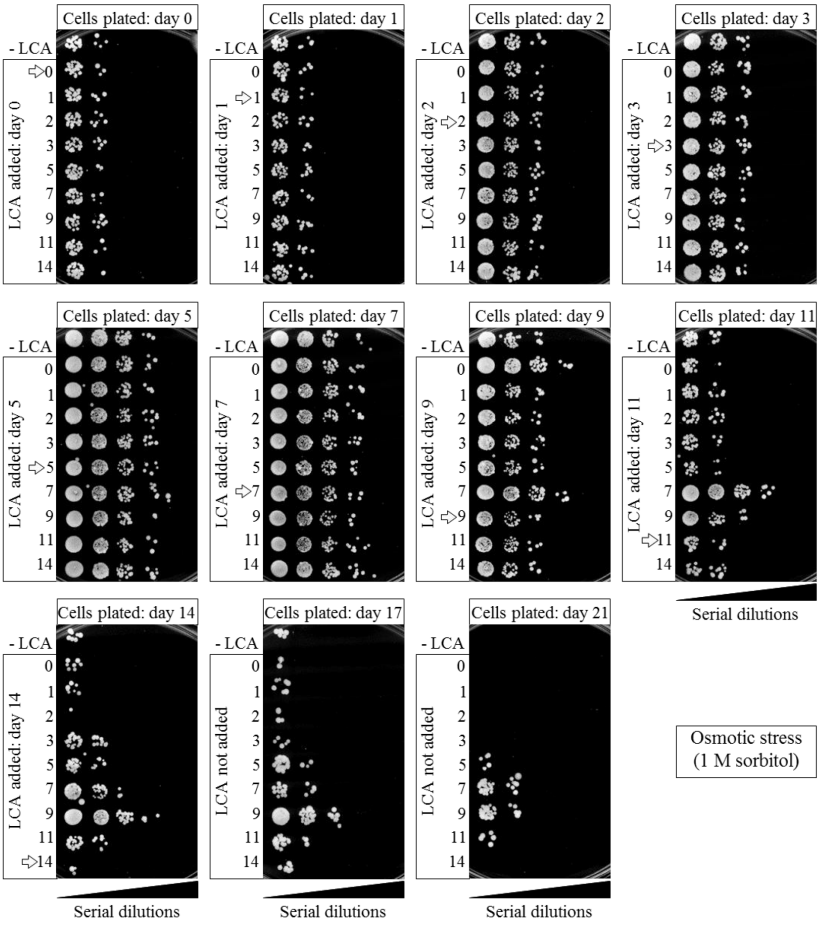

Figure 3.15. Effect of LCA added at different periods of chronological lifespan on the ability of yeast to resist chronic osmotic stress. Wild-type yeast cells were cultured in YP medium initially containing 0.2% glucose, and LCA was added at the final concentration of 50 μM to a cell culture immediately following cell inoculation into the medium (on day 0) or on day 1, 2, 3, 5, 7, 9, 11 or 14 of cell culturing in this medium. The final concentration of DMSO in yeast cultures supplemented with LCA (and in the corresponding control cultures supplemented with compound vehicle) was 1% (v/v). Spot assays for monitoring osmotic stress resistance were performed as described in "Materials and Methods". Serial ten-fold dilutions of cells were spotted on plates with

solid YP medium containing 2% glucose as carbon source and 1 M sorbitol. All pictures were taken after a 3-day incubation at 30^{o}C.

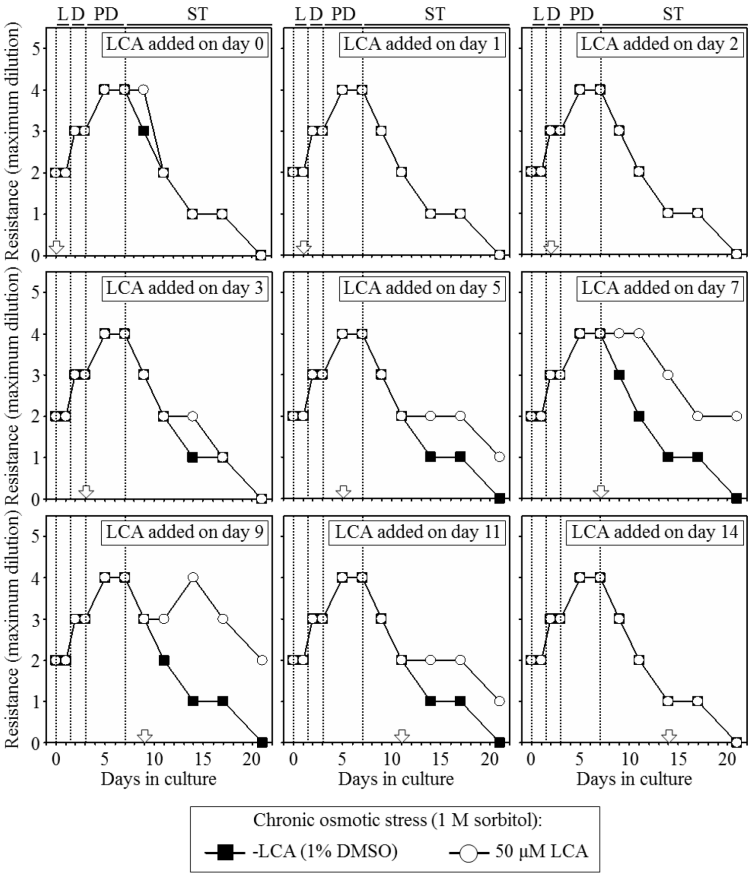

Figure 3.16. LCA differentially influences a longevity-extending process of the development of resistance to chronic osmotic stress if added at different periods of yeast chronological lifespan. A graphic presentation of the results of spot assays for monitoring osmotic stress resistance, which is shown in Figure 3.15.

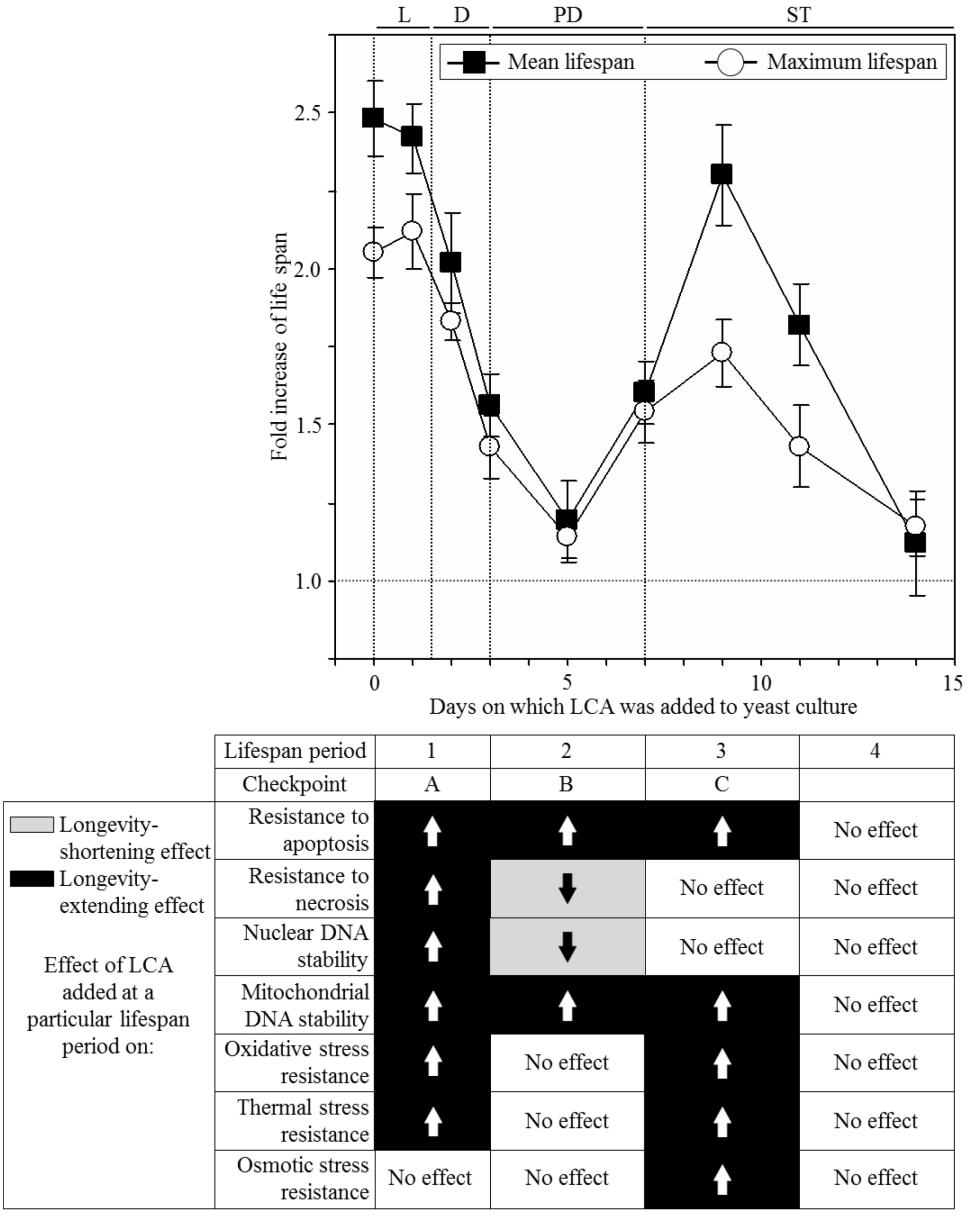

Figure 3.17. A mechanism that may link the ability of LCA to extend longevity of CR yeast only if added at period 1 or 3 of chronological lifespan to the differential effects of this compound on certain longevity-extending and longevity-shortening processes controlled at different lifespan periods. LCA may control these longevity-defining processes at the checkpoints A, B and C that exist in L/D (period 1), PD (period 2) and early ST (period 3) phases, respectively. See text for details.

Our analysis of how the addition of LCA to CR yeast at different periods of chronological lifespan influences several longevity-extending and longevity-shortening processes suggests a mechanism that may link the ability of LCA to increase the lifespan of CR yeast only if added at period 1 or 3 to its differential effects on a monitored in this study set of longevity-defining processes controlled at different lifespan periods (Figure 3.17). In this mechanism, LCA added to CR yeast at period 1 increases chronological lifespan because (1) its addition at this period has a beneficial effect on six longevity-defining processes; (2) the longevity-extending effect of added at this period LCA on each of these processes persists through the entire lifespan, long after this critical lifespan period ended; and (3) the addition of LCA at this period does not cause a lifespan-shortening effect on any of the longevity-defining processes monitored in this study (Figure 3.17). Furthermore, although the addition of LCA at period 2 imposes a lifelong beneficial effect on two longevity-defining cellular process (*i.e.*, it enhances cell resistance to mitochondria-controlled apoptotic death and stimulates mitochondrial genome maintenance), the observed inability of the compound to extend longevity if added at this period of lifespan could be due to the two enduring longevity-shortening effects that LCA exhibits following its addition at period 2 (Figure 3.17). These detrimental to longevity effects of LCA added at period 2 include a lifelong reduction of cell resistance to palmitoleic acid-induced necrotic death and a long-term decline of the efficacy with which the integrity of nuclear genome is maintained (Figure 3.17). In the mechanism that we propose here, LCA added to CR yeast at period 3 increases chronological lifespan because (1) its addition at this period has a beneficial effect on five

longevity-defining processes for the rest of the lifespan; and (2) the addition of LCA at this period does not cause a lifespan-shortening effect on any of the longevity-defining processes that we monitored (Figure 3.17). Moreover, the observed lack of an effect of LCA on a monitored set of longevity-defining processes following its addition at period 4 may satisfactorily explain the inability of LCA to extend longevity if added at this lifespan period (Figure 3.17).

Our model for a mechanism that may link the ability of LCA to increase the lifespan of CR yeast only if added at period 1 or 3 to its differential effects on several longevity-defining processes also foresees that LCA controls these processes at the checkpoints A, B and C that exist in L/D (period 1), PD (period 2) and early ST (period 3) phases, respectively (Figure 3.17). It is conceivable that at each of these checkpoints LCA modulates the key set of longevity-defining cellular processes (modules) that comprise a biomolecular longevity network and are monitored by certain master regulators of the longevity control system. Based on the information gathered and processed by the master regulators, they modulate certain longevity-defining processes within monitored modules of the longevity network in order to limit the age-related accumulation of molecular and cellular damage. The resulting changes in the dynamics of individual modules constituting the network and in its general configuration are critically important for establishing the rate of cellular aging and, thus, define longevity.

As outlined below, recent studies support the validity of the proposed here hypothesis on a stepwise progression of a biomolecular longevity network through a series of checkpoints, at each of which (1) some genetic, dietary and pharmacological anti-aging interventions modulate certain longevity-defining processes within modules

comprising the longevity network; and (2) some checkpoint-specific master regulators monitor and govern the functional states of these critical network modules.

Studies in chronologically aging yeast revealed that, by promoting coupled respiration in mitochondria, elevating the mitochondrial membrane potential and increasing mitochondrial ROS production during L phase, a CR diet and some pharmacological interventions extend longevity by causing changes in several longevity-defining processes during the subsequent D, PD and ST 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 [14, 24, 186 - 189]. It seems that in chronologically aging yeast TORC1 operates as one of the predicted by our hypothesis checkpoint-specific master regulators that at a checkpoint in L phase can monitor and govern a functional state of mitochondria [186 - 189]. 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 [24, 190]. At one of these lifespan checkpoints in PD 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 [190]. At another lifespan checkpoint in ST 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 [190].

Moreover, as it has been mentioned in the "Introduction" section, studies in the nematode *C. elegans* provided evidence that UBL-5/DVE-1, DAF-16 and PHA-4 operate as the checkpoint-specific master regulators of longevity by governing progression through the three consecutive checkpoints operating during the L3/L4 larval stage of development, early adulthood and late adulthood, respectively [163 - 169].

Taken together, these findings support the view 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. Other data supporting this view have been comprehensively discussed elsewhere [24, 62, 64, 115, 174, 190 - 195].

The major challenge now is to get a greater insight into mechanisms that in chronologically aging yeast underlie (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.

4 A mitochondrially targeted compound delays aging in yeast through a mechanism linking mitochondrial membrane lipid metabolism to mitochondrial redox biology

4.1 Introduction

A body of evidence supports the notion that mitochondria regulate cellular aging in evolutionarily distant eukaryotic organisms [20, 196, 197]. These organelles compartmentalize various redox processes known to be essential for establishing the rate of cellular and organismal aging. Such longevity-defining redox processes in mitochondria include the coupling of electron transport to ATP synthesis, modulation of mitochondrial membrane potential, maintenance of cellular homeostasis of reactive oxygen species (ROS), and formation and release of certain metabolites and macromolecules that can set off a pro- or anti-aging cellular pattern [18, 20, 188, 198 - 202]. Several small molecules have been shown to delay cellular and organismal aging in eukaryotes across phyla by modulating some of the mitochondria-confined redox processes. After being sorted to the mitochondrial matrix, the inner mitochondrial membrane (IMM) or the outer mitochondrial membrane (OMM), these anti-aging pharmaceuticals act as rechargeable antioxidants that attenuate oxidative damage to membrane proteins and/or phospholipids [203 - 209].

4.2 Results and Discussion

Our recent study revealed a previously unknown mechanism of delaying cellular aging by a mitochondrially targeted natural compound which specifically impacts

mitochondrial redox biology [210]. The name of this compound is lithocholic acid (LCA), a bile acid which we identified in a high-throughput chemical genetic screen for small molecules extending longevity of chronologically aging yeast [14]. In the mechanism that we discovered, an exogenously added LCA enters yeast cells and accumulates mostly in the IMM; a minor portion of this bile acid is also confined to the OMM (Figure 4.1). The accumulated in both mitochondrial membranes pools of LCA elicit an age-related remodeling of phospholipid synthesis and movement within the IMM and OMM (Figure 4.1). Such specific remodeling of mitochondrial phospholipid
dynamics progresses with the chronological age of a yeast cell and ultimately causes significant changes in mitochondrial membrane lipidome. These changes include: (1) a decrease in the relative levels of phosphatidylethanolamine (PE), cardiolipin (CL) and monolysocardiolipin (MLCL) within mitochondrial membranes; and (2) an increase in the relative levels of phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylglycerol (PG) within mitochondrial membranes (Figure 4.1). In turn, the LCA-driven changes in the composition of mitochondrial membrane phospholipids cause: (1) a substantial enlargement of mitochondria; (2) a significant decrease in mitochondrial number; (3) a reduction in the fraction of mitochondria with cristae extending from the IMM; and (4) a build-up within the mitochondrial matrix of abundant cristae disconnected from the IMM (Figure 4.1). These elicited by LCA major alterations in mitochondrial abundance and morphology in turn cause specific changes in the age-related chronology of such longevity-defining redox processes confined to mitochondria as respiration, the maintenance of

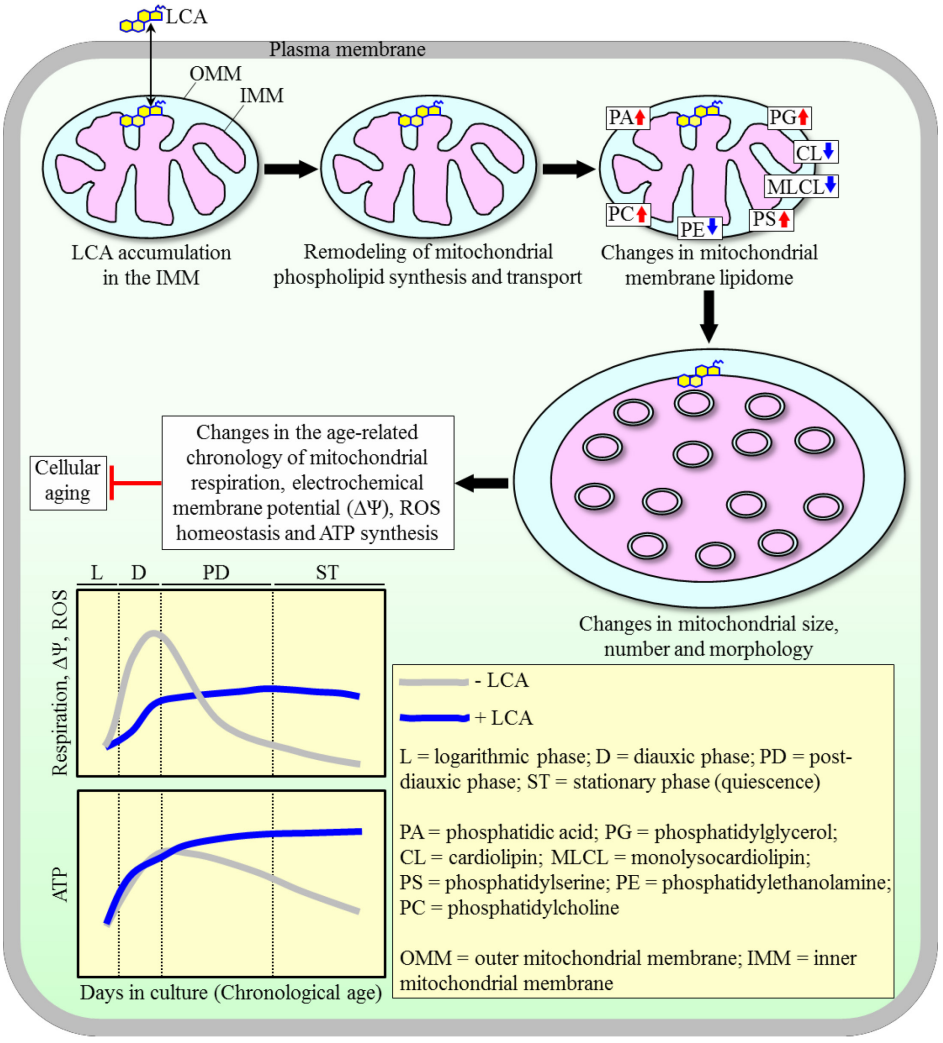

Figure 4.1. A mechanism of delaying aging in yeast by a mitochondrially targeted compound which impacts mitochondrial redox biology. Lithocholic bile acid (LCA) enters yeast cells, accumulates mainly in the inner mitochondrial membrane, and elicits a remodeling of phospholipid synthesis and movement within both mitochondrial membranes. Such remodeling of mitochondrial phospholipid dynamics causes changes in mitochondrial membrane lipidome. These changes in the composition of membrane phospholipids alter mitochondrial abundance and morphology, thereby triggering changes in the age-related chronology of several longevity-defining redox processes confined to mitochondria. For additional details, see text.

mitochondrial membrane potential, the preservation of cellular homeostasis of mitochondrially produced ROS, and the coupling of electron transport to ATP synthesis (Figure 4.1). The elevated efficiencies of mitochondrial respiration, membrane potential maintenance and ATP synthesis observed in chronologically "old", quiescent yeast cultured with LCA (as compared to those in age-matched cells cultured without LCA) are known to delay aging of yeast cells by increasing their long-term viability [14, 24, 186, 187, 189, 210]. Furthermore, the raised sub-lethal concentration of mitochondria-generated intracellular ROS detected in this yeast (Figure 4.1) has been demonstrated to delay cellular aging because it stimulates a signaling network promoting the long-term stress resistance of yeast cells [14, 24, 186, 188, 199, 200, 210]. Importantly, the longevity-extending pattern of mitochondrial redox processes characteristic of chronologically "old", quiescent yeast cultured with LCA (Figure 4.1) is likely due to the lowered below a toxic threshold intracellular concentration of ROS seen in chronologically "young", non-quiescent yeast cultured in the presence of this bile acid [14, 210]. Indeed, a reduced ROS level in chronologically "young" yeast cells exposed to certain anti-aging interventions has been shown to lessen the extent of oxidative damage to mitochondrial macromolecules in these cells, thereby allowing them to maintain the functionality of macromolecules involved in mitochondrial respiration, membrane potential maintenance and ATP synthesis when these cells become chronologically "old" [14, 24, 189, 210]. Ultimately, the elicited by LCA changes in the age-related chronology of these mitochondrial redox processes extend longevity of chronologically aging yeast.

5 Interspecies chemical signals released into the environment may create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms

5.1 Introduction

Aging of multicellular and unicellular eukaryotic organisms is a highly complex biological phenomenon, which affects numerous processes within cells [1, 49, 211, 212]. These cellular processes include cell cycle, cell growth, stress response, protein folding, apoptosis, autophagy, proteasomal protein degradation, actin organization, signal transduction, nuclear DNA replication, chromatin assembly and maintenance, ribosome biogenesis and translation, lipid and carbohydrate metabolism, oxidative metabolism in mitochondria, NAD^+ homeostasis, amino acid biosynthesis and degradation, and ammonium and amino acid uptake [2, 50, 51, 213, 214]. Across phyla, such plethora of longevity-defining processes is governed by a nutrient signaling network integrating the AMP-activated protein kinase/target of rapamycin (AMPK/TOR), cAMP/protein kinase A (cAMP/PKA) and insulin/insulin-like growth factor 1 (IGF-1) pathways, as well as a sirtuin-governed protein deacetylation module [1, 50, 51, 146, 215, 216]. Because this evolutionarily conserved signaling network regulates longevity only in response to certain changes in the organismal and intracellular nutrient and energy status, it is "adaptable" by its nature [14]. By altering the organismal and intracellular nutrient and energy status, caloric restriction and dietary restriction extend longevity and improve health across species by modulating the adaptable longevity network [1, 55 - 57, 59, 217]. Unlike signaling pathways and sirtuin-governed protein deacetylation module integrated

into the adaptable longevity network, some longevity-defining pathways are "constitutive" or "housekeeping" by their nature as they regulate longevity irrespective of the organismal and intracellular nutrient status [14].

It should be stressed that both adaptable and housekeeping longevity pathways are the targets of longevity-extending and health-improving small molecules that are produced and then released into the environment by organisms from all domains of life (*i.e.*, bacteria, fungi, plants and animals) within an ecosystem. We therefore propose a hypothesis in which interspecies chemical signals released into the environment create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms.

5.2 Discussion

5.2.1 Plants and other autotrophs release into the environment xenohormetic and cytostatic interspecies chemical signals that extend longevity of other organisms within an ecosystem

According to the "xenohormesis" hypothesis of Howitz and Sinclair, in response to various hormetic environmental stresses - such as UV light, dehydration, infection, predation, cellular damage and nutrient deprivation - plants and other autotrophic organisms synthesize a group of secondary metabolites called xenohormetic phytochemicals [47, 48, 218]. Prior to being released into the environment, these secondary metabolites activate defense systems protecting the host autotrophic organisms against hormetic environmental stresses that caused their synthesis [48, 218]. After being released into the environment, these xenohormetic phytochemicals provide benefits to

health and longevity of heterotrophic organisms within the ecosystem. It was proposed that xenohormetic phytochemicals cause such life-extending and health-improving effects not by operating as mildly toxic hormetic molecules, but by activating the key enzymes of stress-response, anti-aging pathways known to govern longevity-related processes in heterotrophic organisms [47, 48, 218]. Recent studies revealed that some xenohormetic phytochemicals, such as resveratrol and caffeine, extend longevity of heterotrophic organisms by attenuating the adaptable TOR signaling pathway known to accelerate their aging [64, 219 - 222]. Because the TOR pathway also plays a pivotal role in promoting proliferative growth of all heterotrophic organisms, resveratrol and caffeine exhibit a cytostatic effect in these organisms [64, 174, 195, 222].

By extending the xenohormesis hypothesis of Howitz and Sinclair, we propose that within each of the heterotrophic species composing an ecosystem there are organisms that (1) possess the most effective (as compared to their counterparts of the same species) mechanisms for sensing xenohormetic and cytostatic phytochemicals released into the environment by autotrophic species; and (2) can respond to these phytochemicals by activating the key enzymes of stress-response, anti-aging pathways and/or by attenuating the adaptable, pro-aging TOR signaling pathway - thereby undergoing life-extending changes to their metabolism and physiology. These heterotrophic organisms are expected to live longer then their counterparts within the same species. Thus, their ability to sense the longevity-extending xenohormetic and cytostatic phytochemicals released into the environment by autotrophic species and to respond to these phytochemicals by undergoing certain life-extending metabolic and physiological changes will: (1) increase their chances of survival; (2) create selective forces aimed at maintaining such ability;

and (3) drive the evolution of their longevity regulation mechanisms.

5.2.2 Mammals release into the environment bile acids, hormetic interspecies chemical signals that extend longevity of yeast and perhaps of other organisms within an ecosystem

In mammals, bile acids operate not only as trophic factors for the enteric epithelium and detergents for the emulsification and absorption of dietary lipids, but also as signaling molecules regulating lipid, glucose and energy homeostasis and activating detoxification of xenobiotics [223 - 228]. Bile acids have been shown to cause numerous health-improving metabolic effects in mammals and to protect them from xenobiotic toxins [223 - 228]. Therefore, it was proposed that by promoting chemical hormesis in mammals, bile acids – mildly toxic molecules with detergent-like properties – may extend their longevity by acting as endobiotic regulators of aging [185, 229 - 231]. Importantly, our recent study identified lithocholic acid, a bile acid, as an anti-aging compound that extends yeast longevity by activating a compendium of anti-aging processes and attenuating a distinct set of pro-aging processes [14]. Unlike mammals, yeast do not synthesize bile acids [14, 227]. We therefore propose that bile acids released into the environment by mammals may act as interspecies chemical signals extending longevity of yeast species and, perhaps, of other organisms that can: (1) sense these mildly toxic molecules with detergent-like properties; and (2) respond to the resulting mild cellular damage by developing the most efficient stress protective mechanisms. We hypothesize that such mechanisms may provide effective protection of yeast and other organisms not only against cellular damage caused by bile acids but also against

molecular and cellular damage accumulated with age. Thus, those species of the organisms within an ecosystem that that have been selected for the most effective (as compared to their counterparts of the same species) mechanisms providing protection against bile acids are expected to (1) live longer than their counterparts within the same species; and (2) evolve the most effective anti-aging mechanisms that are sensitive to regulation by bile acids. Thus, the ability of certain non-mammalian species within an ecosystem to sense bile acids produced by mammals and then to respond by undergoing certain longevity-extending changes to their physiology will increase their chances of survival - thereby creating selective force aimed at maintaining such ability and driving the evolution of their longevity regulation mechanisms.

5.2.3 Soil bacteria release into the environment rapamycin, a cytostatic interspecies chemical signal that extends longevity of other organisms within an ecosystem

The adaptable TOR signaling pathway can be attenuated not only by resveratrol and spermidine - the two longevity-extending xenohormetic and cytostatic phytochemicals released into the environment by autotrophic species – but also by rapamycin [174, 234, 235]. Rapamycin - a macrocyclic lactone synthesized by soil bacteria to inhibit growth of fungal competitors - extends longevity of yeast, fruit flies and mice by specifically inhibiting the nutrient-sensory protein kinase TOR, a master negative regulator of the pro-aging TOR signaling pathway [79, 173, 174, 235, 236]. Rapamycin exhibits a potent cytostatic effect by causing G1 cell cycle arrest and greatly delaying proliferative growth of organisms across phyla [174, 234 - 236]. We therefore

hypothesize that rapamycin released into the environment by soil bacteria not only suppresses growth of fungal competitors, but may also may create selective pressure for the evolution of yeast, fruit fly and mammalian species that can respond to rapamycin-induced growth retardation by developing certain mechanisms aimed at such remodeling of their anabolic and catabolic processes that would increase their chances of survival under conditions of slow growth. We propose that some of these mechanisms delay aging by optimizing essential longevity-related processes and remain sensitive to modulation by rapamycin. Therefore, the ability of yeast, fruit fly and mammalian species within an ecosystem to sense rapamycin produced by soil bacteria and then to respond by undergoing certain life-extending changes to their metabolism and physiology will increase their chances of survival - thereby creating selective force aimed at maintaining such ability and driving the evolution of their longevity regulation mechanisms.

5.2.4 A hypothesis of the xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms

Our analysis of how several small molecules synthesized and released into the environment by one species of the organisms composing an ecosystem extend longevity of other species within this ecosystem suggests a hypothesis in which these interspecies chemical signals create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms. In our hypothesis, after being released into the environment by one species of organisms capable of synthesizing such small molecules, they can activate anti-aging processes and/or inhibit pro-aging processes in other species within an ecosystem. Within each of these other species, there

are organisms that possess the most effective (as compared to their counterparts of the same species) mechanisms for sensing the interspecies chemical signals and for responding to such signals by undergoing certain life-extending changes to their metabolism and physiology; such life-extending changes could be hormetic and/or cytostatic by their nature. These organisms therefore are expected to live longer than their counterparts of the same species within the ecosystem. Thus, the ability of a species of the organisms composing an ecosystem to sense the longevity-modulating interspecies chemical signals released into the environment by other species within the ecosystem and to respond to these signals by undergoing certain life-extending metabolic and physiological changes is expected to increase its chances to survive, thereby creating selective force aimed at maintaining such ability. Our hypothesis implies that the evolution of longevity regulation mechanisms in each species of the organisms composing an ecosystem is driven by the ability of this species to undergo specific life-extending metabolic or physiological changes in response to hormetic or cytostatic chemical compounds that are released to the ecosystem by other species.

6 LCA suppresses mitochondrial deficiency known to cause a neurological disorder in humans

6.1 Introduction

Mitochondrial encephalomyopathies in humans are caused by cytochrome c oxidase (COX; respiratory complex IV) deficiency [237, 238]. Two major causes of COX deficiency in humans (and in the budding yeast *Saccharomyces cerevisiae*) are: (1) defects in mitochondrial translation of the mitochondrial DNA (mtDNA)-encoded Cox1p, Cox2p and Cox3p subunits of COX; and (2) defects in the assembly of COX within the mitochondrial inner membrane from the three subunits encoded by mtDNA and many subunits encoded by nuclear genes (Figures 6.1 and 6.2) [237 - 239]. Both these causes of COX deficiency in humans (and yeast) are due to mutations in nuclear genes. Several human translation and assembly factors for COX that have been implicated in mitochondrial encephalomyopathies have been shown to have yeast orthologues, including: (1) yeast Hah1p is an orthologue of human TACO1 implicated in the late-onset Leigh syndrome [238, 240]; (2) yeast Pet309p is an orthologue of human LRPPRC implicated in the French-Canadian variant of Leigh syndrome [238, 241, 242]; (3) yeast Shy1p is an orthologue of human SURF1 implicated in the Leigh syndrome [238, 243, 244]; and (4) yeast Sco1p is an orthologue of human SCO1 implicated in a mitochondrial encephalomyopathy called severe COX deficiency [238, 245, 246] (Figure 6.3).

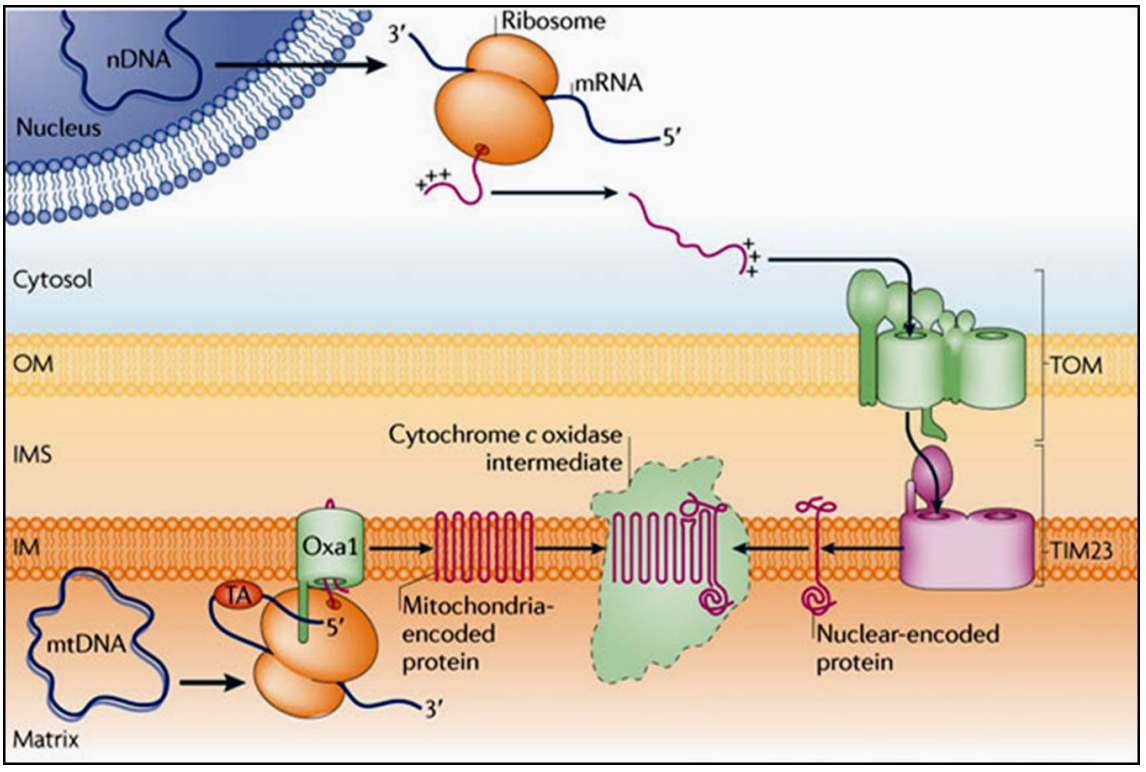

Figure 6.1. A stepwise assembly of the COX complex from three mitochondrial DNA (mtDNA)-encoded subunits (Cox1p, -2p and -3p) and eight nuclear DNA (nDNA)-encoded ones in the inner membrane of yeast mitochondria. From: Reference 238.

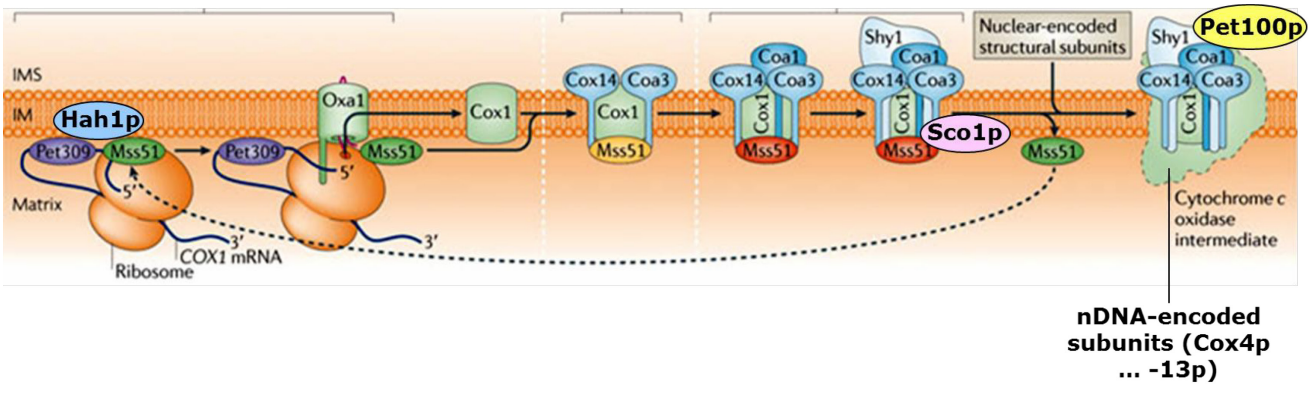

Figure 6.2. A model for the translation of Cox1p and assembly of the entire COX complex in the mitochondrial inner membrane of yeast. From: Reference 238; with modifications.

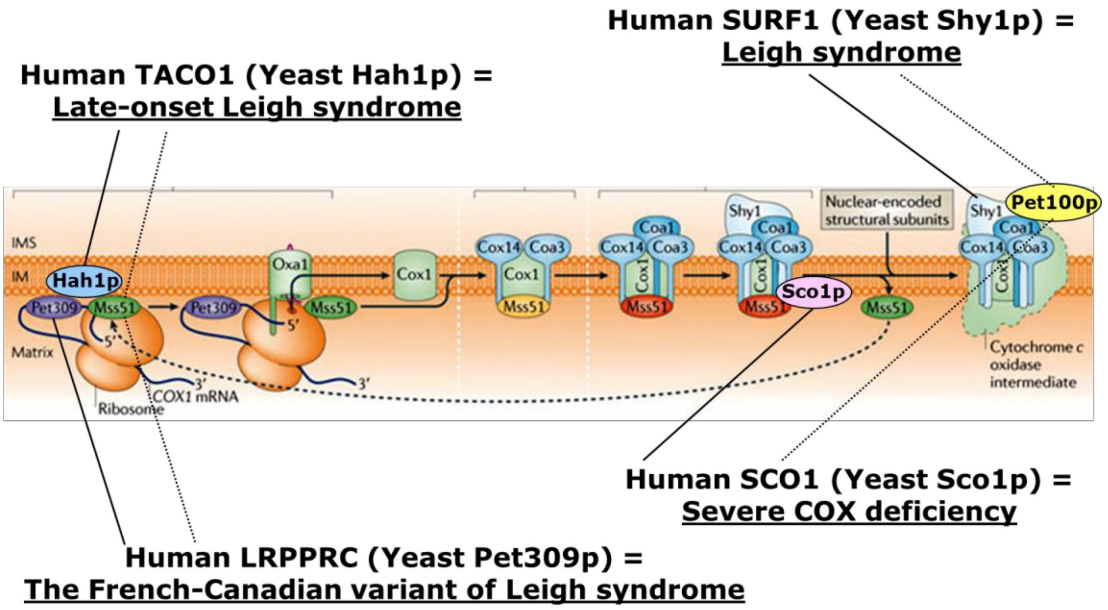

Figure 6.3. Several human translation and assembly factors for COX that have been implicated in mitochondrial encephalomyopathies have yeast orthologues. From: Reference 238; with modifications. See text for details.

6.2 Materials and Methods

Cell lines

Primary cell lines were established from subject skin fibroblasts. The subject and control cell lines were immortalized by transduction with a retroviral vector expressing the HPV-16 E7 gene plus a retroviral vector expressing the catalytic component of human telomerase (htert). The fibroblasts and HEK 293 line were grown at 37 °C in an atmosphere of 5% CO2 in high-glucose DMEM supplemented with 10% FBS.

Electrophoresis and immunobloting

Blue-Native polyacrylamide gradient electrophoresis (BN-PAGE) was used to separate samples in the first dimension on 6-15% polyacrylamide gradient gel. Mitoplasts were prepared from fibroblasts by treatment with 0.8 mg of digitonin/mg of protein and solubilized with 1% lauryl maltoside; 20 µg of the solubilized proteins were used for electrophoresis. Individual structural subunits of complexes I, II, III, IV and V were detected by immunoblot analysis using commercially available monoclonal antibodies (Molecular Probes).

RNA blot analysis

RNA from subject and control fibroblasts was isolated using the RNeasy Kit (Qiagen). 10 µg of total RNA were separated on a denaturing MOPS/formaldehyde agarose gel and transferred to a nylon membrane. 300- to 500-bp-long PCR products of individual mitochondrial genes were labeled with [α-^{32}P]-dCTP (GE Healthcare) using the MegaPrime DNA labeling kit (GE Healthcare). Hybridization was conducted according to the manufacturer's manual using ExpressHyb Hybridization Solution (Clontech) and the radioactive signal was detected using the Phosphoimager system.

TACO1 antibody production

A polyclonal antibody against two peptides (Ac-IKGPKDVERSRIFSKLC-amide and Ac-LEFIPNSKVQLAEPDLEQAAC-amide) from the human TACO1 protein was prepared by 21st Century Biochemicals (Marlboro, Massachusetts). Crude serum and affinity purified antibodies were tested on cell lines overexpressing TACO1 protein and

detected a band of approximately 28 kDa. The C-terminal affinity-purified antibody was used for further experiments.

Mitochondrial isolation

Fibroblasts were resuspended in ice-cold 250 mM sucrose/10 mM Tris-HCl/1 mM EDTA (pH 7.4) and homogenized with 10 passes through a pre-chilled, zero clearance homogenizer (Kimble/Kontes). Samples were centrifuged twice for 10 min at 600 x g to obtain a postnuclear supernatant. Mitochondria were pelleted by centrifugation for 10 min at 10 000 x g, and washed once in the same buffer, before being subjected SDS-PAGE.

Yeast strains and media

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and mutant strains *pet100*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pet100Δ::kanMX4*), *hah1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hah1Δ::kanMX4*), *pet309*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pet309Δ::kanMX4*), *mss51*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mss51Δ::kanMX4*), *sco1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 sco1Δ::kanMX4*), *shy1*Δ (*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 shy1Δ::kanMX4*), were used in this study. Media components were as follows: (1) YEPD (0.2% glucose), 1% yeast extract, 2% peptone, 0.2% glucose; and (2) YEPD (2% glucose), 1% yeast extract, 2% peptone, 2% glucose.

A plating assay for the analysis of chronological lifespan

Cells were grown in YEPD (0.2% glucose) medium at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a flask volume/medium volume ratio of 5:1. A sample of cells was removed from each culture at various time points. A fraction of the cell sample was diluted in order to determine the total number of cells per ml of culture using a hemacytometer. 10 µ l of serial dilutions (1:10 to 1:10^{3}) of cells were applied to the hemacytometer, where each large square is calibrated to hold 0.1 µ l. The number of cells in 4 large squares was then counted and an average was taken in order to ensure greater accuracy. The concentration of cells was calculated as follows: number of cells per large square x dilution factor x 10 x 1,000 = total number of cells per ml of culture. A second fraction of the cell sample was diluted and serial dilutions (1:10^{2} to 1:10^{5}) of cells were plated onto YEPD (2% glucose) plates in triplicate in order to count the number of viable cells per ml of each culture. 100 µ l of diluted culture was plated onto each plate. After a 48-h incubation at 30^{o}C, the number of colonies per plate was counted. The number of colony forming units (CFU) equals to the number of viable cells in a sample. Therefore, the number of viable cells was calculated as follows: number of colonies x dilution factor x 10 = number of viable cells per ml. For each culture assayed, % viability of the cells was calculated as follows: number of viable cells per ml / total number of cells per ml x 100%. The % viability of cells in mid-logarithmic phase was set at 100% viability for that particular culture. The life span curves for wild-type and some of the mutant strains were also validated using a LIVE/DEAD yeast viability kit (Invitrogen) following the manufacturer's instructions for stationary-phase cultures.

Pharmacological manipulation of chronological lifespan

Chronological lifespan analysis was performed as described above in this section. The lithocholic (LCA) [#L6250] bile acid was from Sigma. The stock solution of LCA in DMSO was made on the day of adding this compound to cell cultures. LCA was added to growth medium 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).

Statistical analysis

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

6.3 Results and Discussion

6.3.1 LCA can extend longevity of yeast mutants lacking some orthologues of human genes implicated in mitochondrial encephalomyopathies

Currently, there are no small molecules known to rescue or alleviate any of the human mitochondrial encephalomyopathies on the organismal or cellular (*i.e.*, in cultured human cells) level. Our recent unpublished findings revealed that LCA, a potent longevity-extending natural compound that delays cellular aging in yeast [14], stimulates mitochondrial protein translation and elevates the levels of mitochondrial protein

assembly factors in yeast cells. Therefore, we sought to investigate if LCA can extend longevity of yeast mutants lacking orthologues of human genes implicated in mitochondrial encephalomyopathies. We first tested how LCA influences the chronological lifespan of the *hah1Δ* mutant grown under CR conditions on 0.2% glucose. This mutant is considered as a yeast model of the human late-onset Leigh syndrome, a human neurological disorder that is caused by a recessive mutation in *TACO1* gene (Figure 6.3) [238, 240]. We found that under CR conditions LCA extends both the mean and maximal lifespans of the *hah1Δ* mutant to a greater degree than that of wild-type (WT) strain (Figure 6.4).

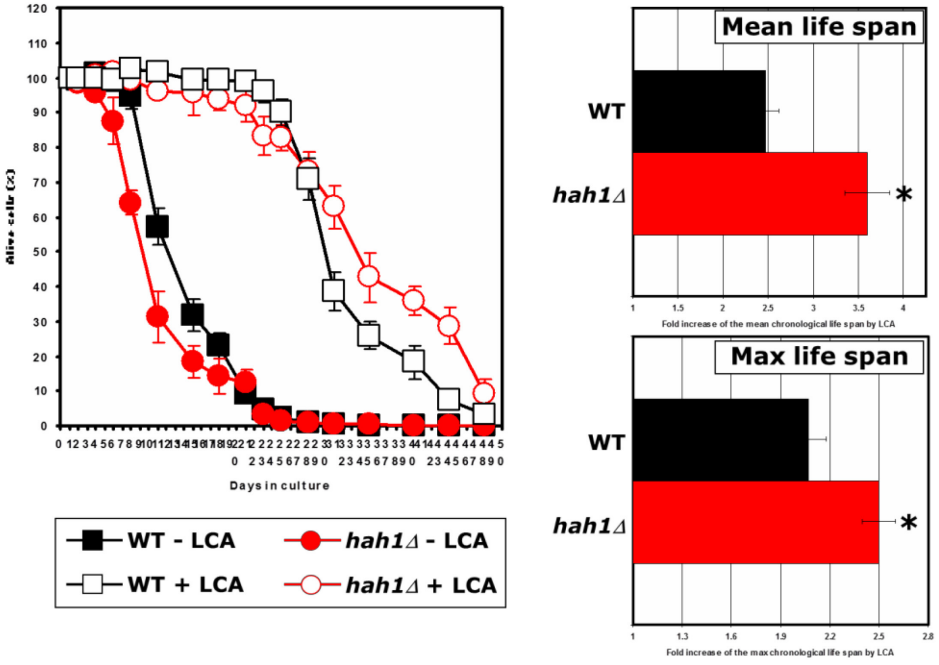

Figure 6.4. LCA extends longevity of the *hah1*Δ mutant (a yeast model of the human late-onset Leigh syndrome) to a greater degree than that of WT strain. Yeast cells were grown in YEPD medium containing 0.2% glucose as a carbon source, with or without LCA. The final concentration of LCA was 50 μM.

The *pet309*Δ mutant of yeast has been shown to be a model of the human French-Canadian variant of Leigh syndrome (Figure 6.3) [238, 241, 242]. We found that LCA does not extend longevity of this mutant under CR conditions (Figure 6.5).

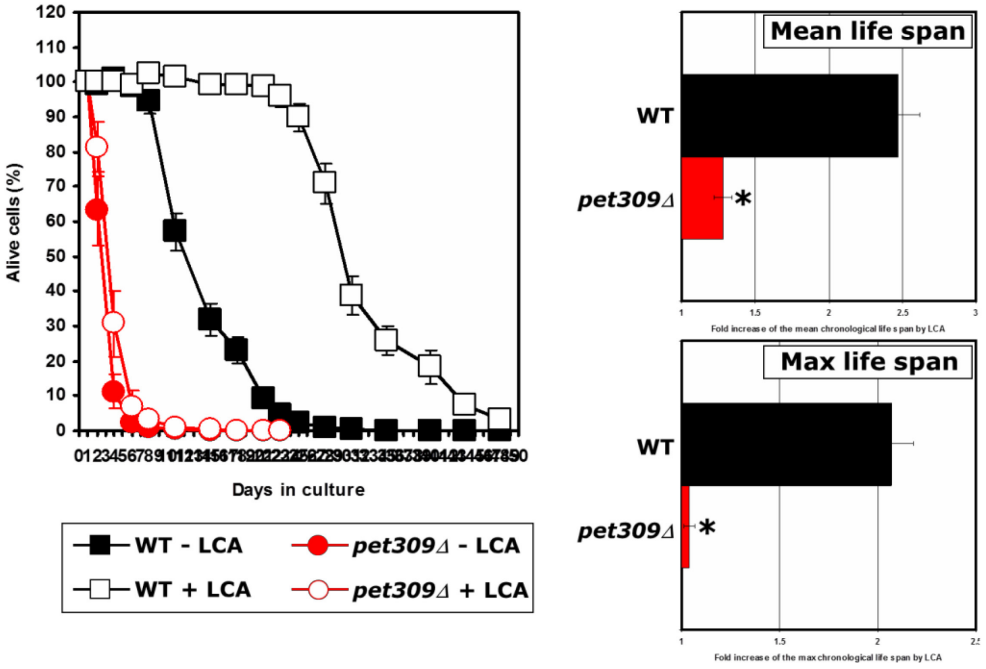

Figure 6.5. LCA does not extend longevity of the *pet309Δ* mutant, a yeast model of the human French-Canadian variant of Leigh syndrome. Yeast cells were grown in YEPD medium containing 0.2% glucose as a carbon source, with or without LCA. The final concentration of LCA was 50 μM.

We also found that under CR conditions LCA does not extend longevity of the *mss51*Δ mutant, which is impaired in the same step of COX assembly as yeast models of the Leigh syndrome and its French-Canadian variant (Figures 6.3 and 6.6).

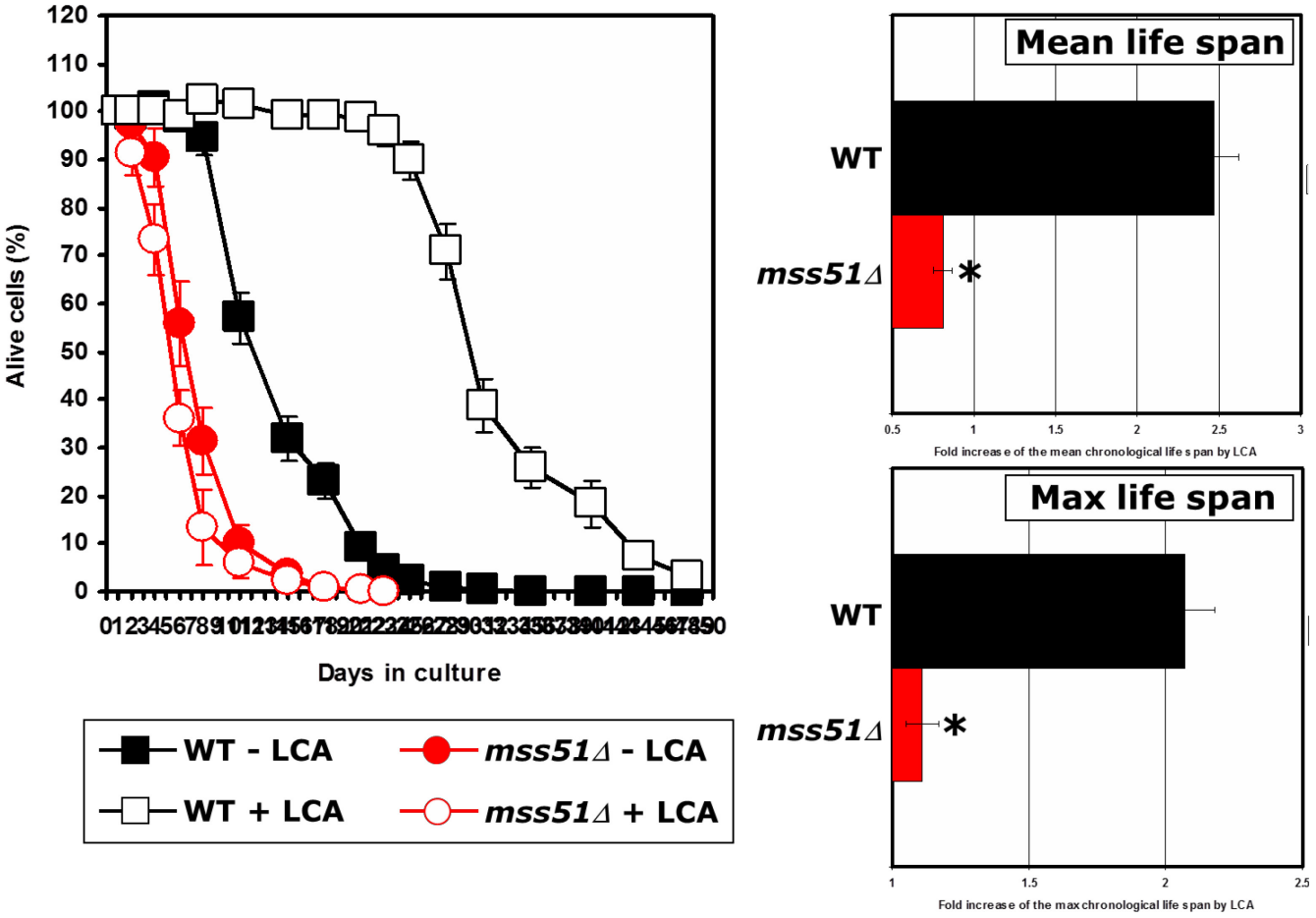

Figure 6.6. LCA does not extend longevity of the *mss51Δ* mutant, a yeast model of the Leigh syndrome and its French-Canadian variant. Yeast cells were grown in YEPD medium containing 0.2% glucose as a carbon source, with or without LCA. The final concentration of LCA was 50 μM.

The *sco1*Δ mutant of yeast has been shown to be a model of one of the mitochondrial encephalomyopathies called severe COX deficiency [238, 245, 246]. As we found, under CR conditions LCA extends both the mean and maximal chronological lifespans of the *sco1*Δ mutant to a greater degree than that of WT strain (Figure 6.7).

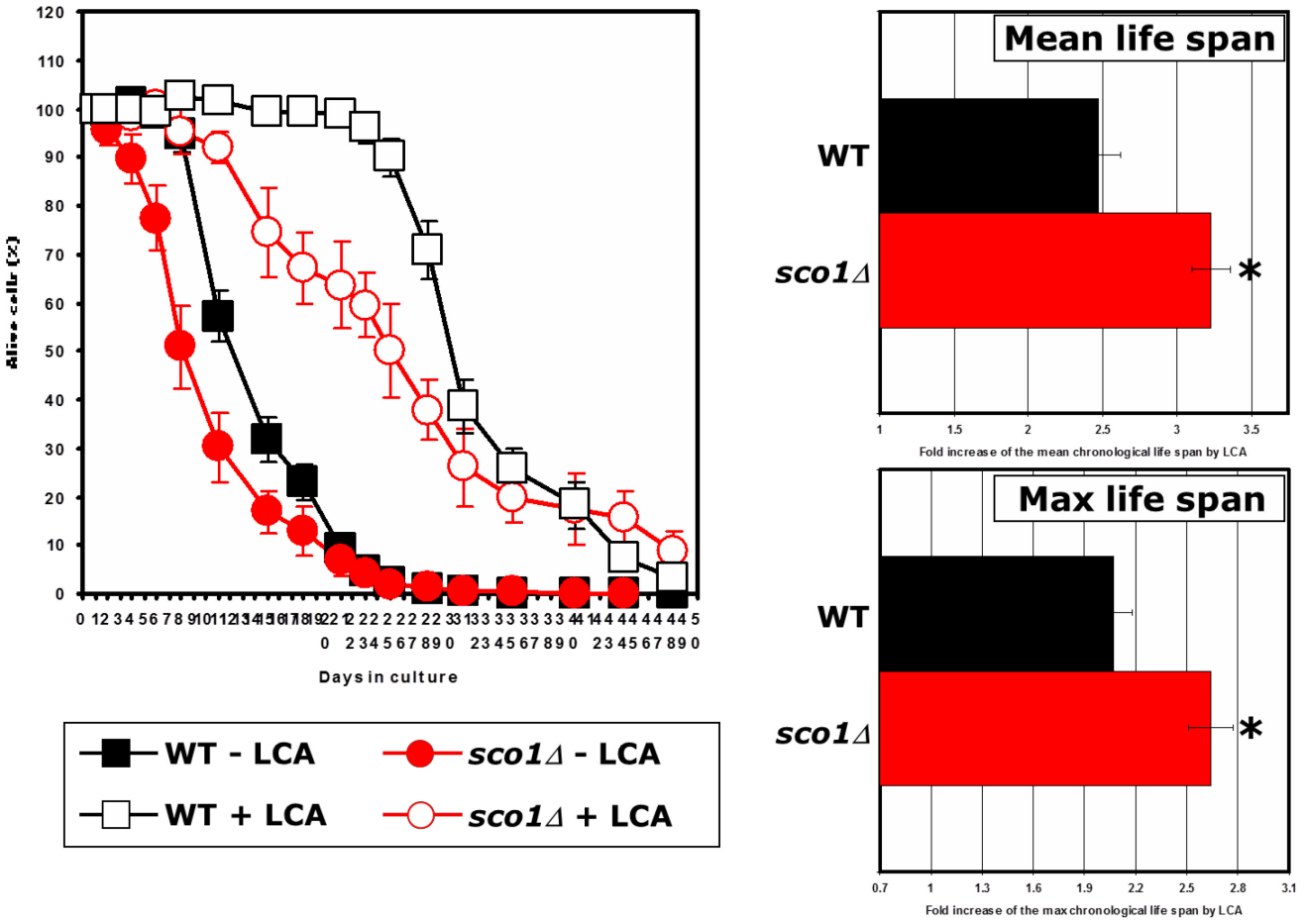

Figure 6.7. Under CR conditions LCA extends both the mean and maximal chronological lifespans of the *sco1Δ* mutant to a greater degree than that of WT strain. Yeast cells were grown in YEPD medium containing 0.2% glucose as a carbon source, with or without LCA. The final concentration of LCA was 50 μM.

Our evaluation of the effect of LCA on the chronological lifespan of the *shy1*Δ mutant, a yeast model of the Leigh syndrome [238, 243, 244], revealed that LCA does not extend its longevity (Figure 6.8).

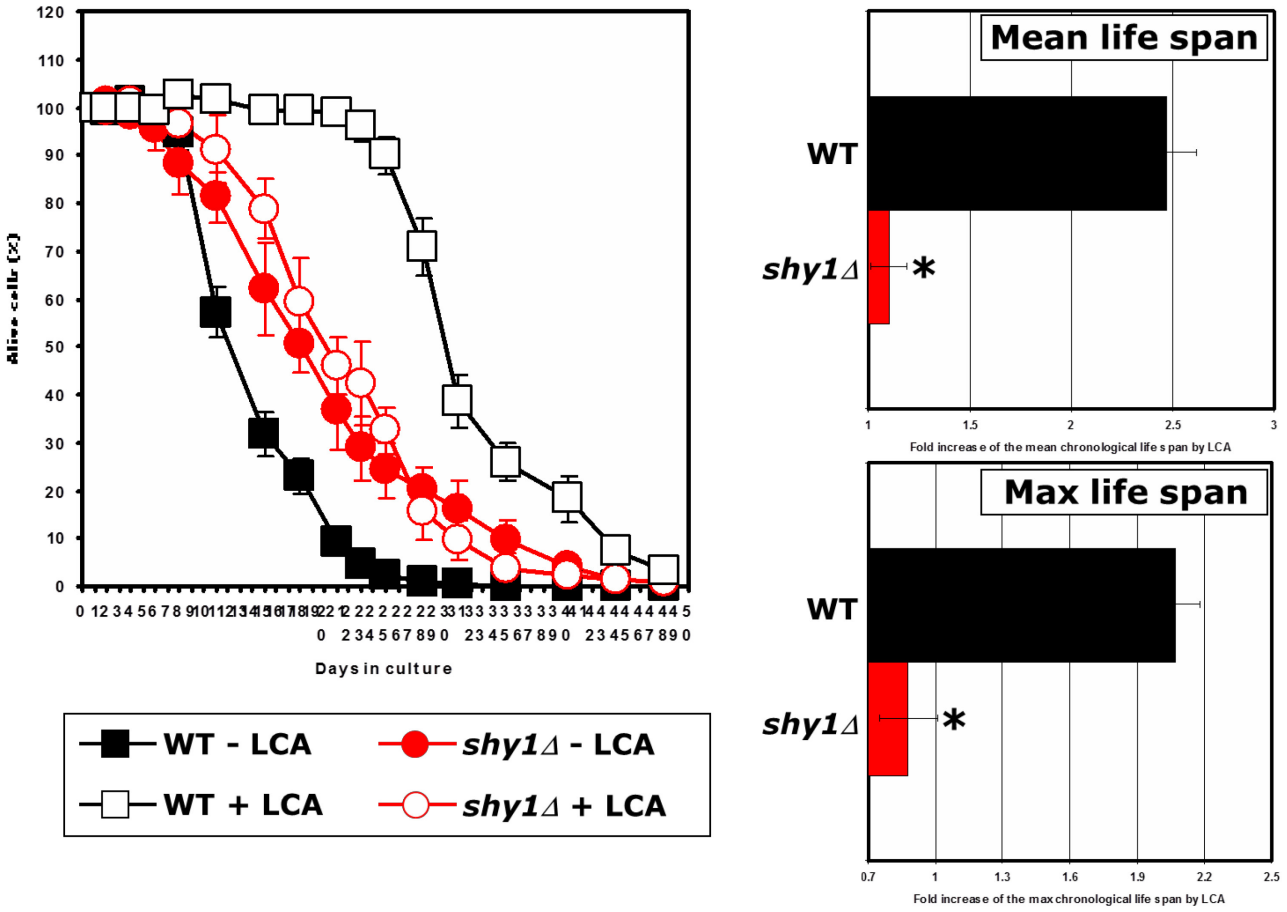

Figure 6.8. Under CR conditions LCA does not extend longevity of the *shy1Δ* mutant, a yeast model of the Leigh syndrome. Yeast cells were grown in YEPD medium containing 0.2% glucose as a carbon source, with or without LCA. The final concentration of LCA was 50 μM.

We then assessed how LCA influences longevity of the *pet100*Δ mutant of yeast, which is impaired in the same step of COX assembly as yeast models of the Leigh syndrome and severe COX deficiency [238]. We found that LCA does not extend longevity of the *pet100*Δ mutant (Figure 6.9).

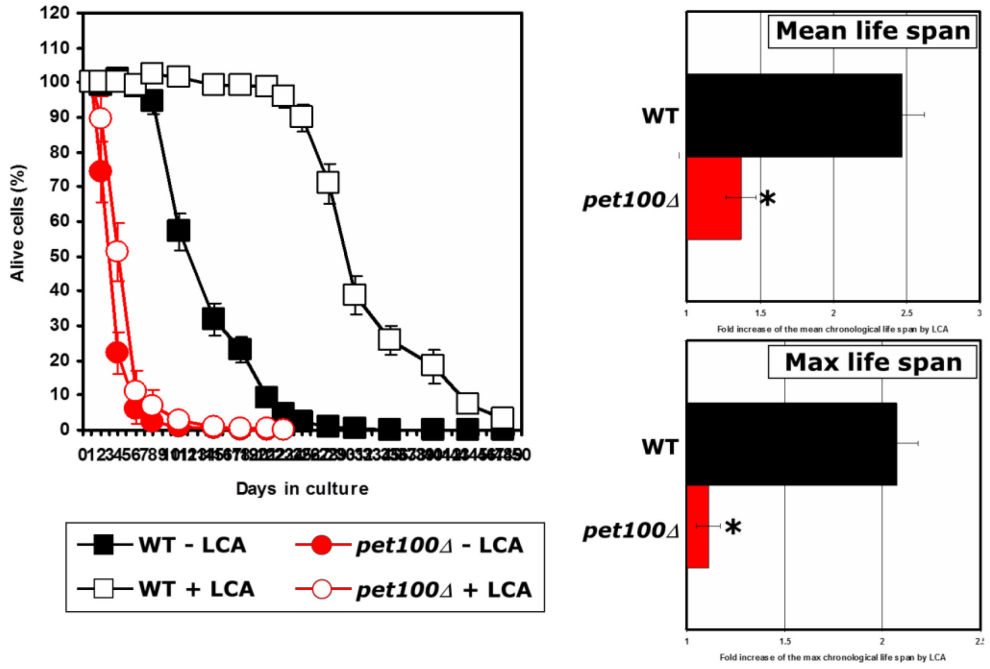

Figure 6.9. Under CR conditions LCA does not extend longevity of the *pet100*Δ mutant, which is impaired in the same step of COX assembly as yeast models of the Leigh syndrome and severe COX deficiency. Yeast cells were grown in YEPD medium containing 0.2% glucose as a carbon source, with or without LCA. The final concentration of LCA was 50 μM.

6.3.2 LCA can suppress mitochondrial COX deficiency causing Leigh syndrome, a human neurological disorder caused by a mutation in *TACO1* gene

Because our findings revealed that LCA extends both the mean and maximal chronological lifespans of the *hah1*Δ mutant (a yeast model of Leigh syndrome, a human neurological disorder caused by a recessive mutation in *TACO1* gene; Figures 6.3 and 6.4) to a greater degree than those of WT strain, we investigated if in cultured human fibroblasts of patients with this disorder LCA can suppress mitochondrial COX deficiency causing it. Human TACO1 protein is known to be encoded by nuclear DNA

and to operate as a translational activator of the mtDNA-encoded COX I subunit (Figure 6.10) [238, 240]. Leigh syndrome is caused by a recessive mutation in *TACO1* gene; this mutation is a one-base-pair insertion at position 472 (472insC) resulting in a frameshift and the creation of a premature stop codon (Figure 6.11) [240]. It has been demonstrated that this mutation in the *TACO1* gene significantly reduces: (1) the steady-state level of TACO1 mRNA (due to its nonsense-mediated decay); (2) the rate of COX I synthesis; (3) the steady-state level of COX I; and (4) the steady-state levels of COX II, COX III and the entire COX complex (Figure 6.12) [240].

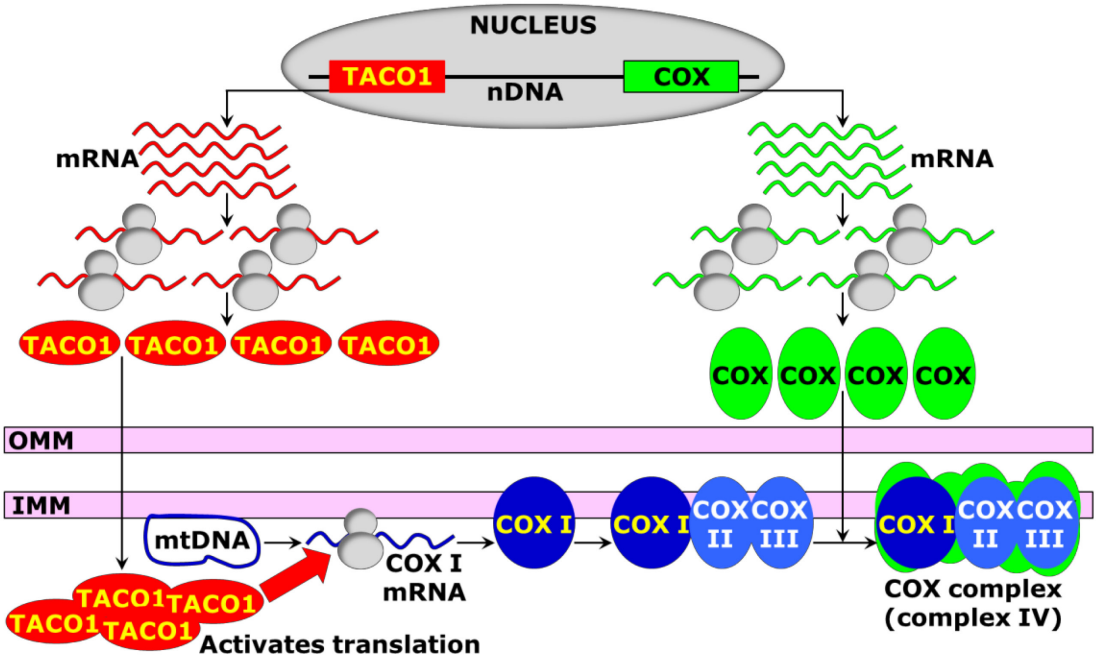

Figure 6.10. Human TACO1 protein is a translational activator of the mtDNA-encoded COX I subunit.

Figure 6.11. Leigh syndrome is caused by a recessive mutation in *TACO1* gene; this mutation is a one-base-pair insertion at position 472 (472insC) resulting in a frameshift and the creation of a premature stop codon.

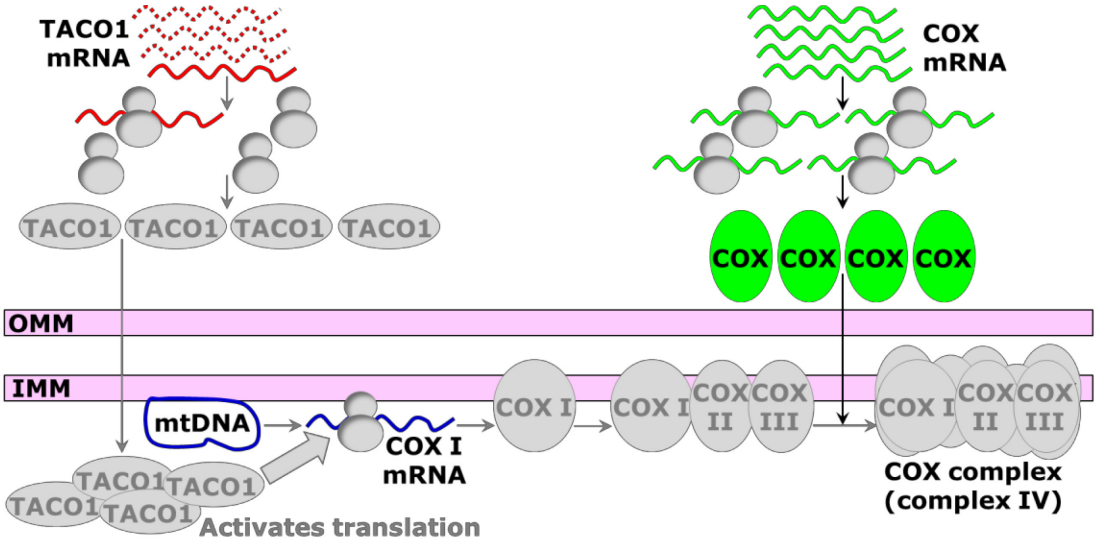

Figure 6.12. *TACO1* mutations reduces the steady-state level of TACO1 mRNA, rate of COX I synthesis, steady-state level of COX I, and steady-state levels of COX II, COX III and of the entire COX complex.

Using immunoblot analysis of purified mitochondria, we found that LCA greatly elevates the steady-state levels of the COX I, COX II and COX III subunits of the COX complex in mitochondria of a TACO1 subject (Figures 6.13 to 6.20). Our immunoblot analysis of purified mitochondria also revealed that significantly increases the steady-

state level of TACO1 protein in mitochondria of this TACO1 subject (Figures 6.21 and 6.22). It should be stressed that anti-TACO1 antibody is known to recognize the *C* terminus of TACO1 protein [240]. We therefore concluded that LCA promotes synthesis of the full-length TACO1 protein. Based on these observations, we hypothesize that in TACO1 mutant cells, LCA restores the assembly of the mitochondrial COX complex by increasing the steady-state level of TACO1 protein, perhaps by any of the following mechanisms: (1) stabilizing mutant TACO1 mRNA by decelerating its nonsense-mediated decay; and/or (2) enabling ribosomal readthrough of premature but not normal termination codons [247 – 250].

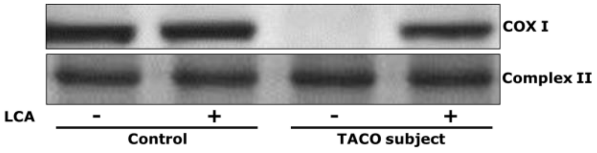

Figure 6.13. LCA greatly elevates the steady-state level of the COX I subunit of the COX protein complex. Immunoblot analysis of COX I steady-state level in purified mitochondria (the complex II 70-kDa subunit was used as a loading control).

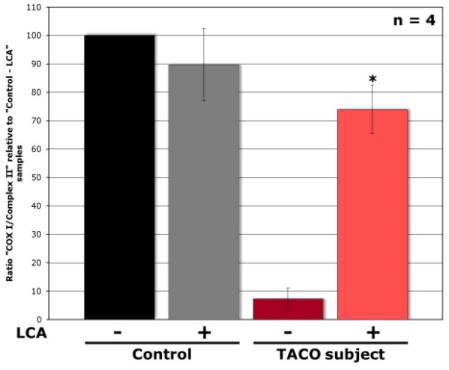

Figure 6.14. LCA greatly elevates the steady-state level of the COX I subunit of the COX protein complex. Immunoblot analysis of COX I steady-state level in purified mitochondria (the complex II 70-kDa subunit was used as a loading control; n = 4).

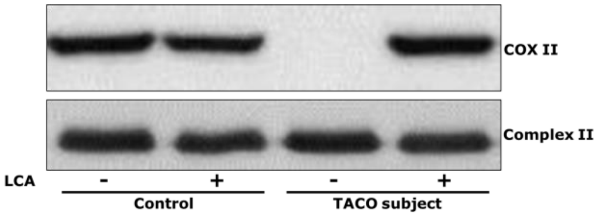

Figure 6.15. LCA greatly elevates the steady-state level of the COX II subunit of the COX protein complex. Immunoblot analysis of COX II steady-state level in purified mitochondria (the complex II 70-kDa subunit was used as a loading control).

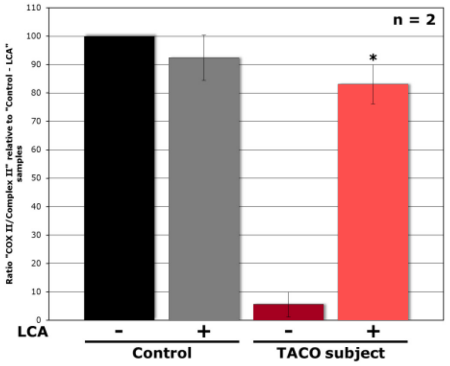

Figure 6.16. LCA greatly elevates the steady-state level of the COX II subunit of the COX protein complex. Immunoblot analysis of COX II steady-state level in purified mitochondria (the complex II 70-kDa subunit was used as a loading control; n = 2).

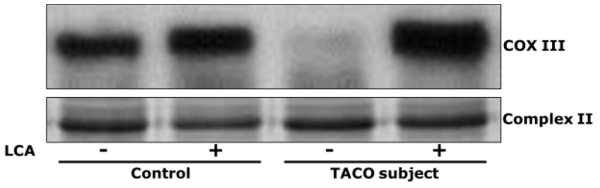

Figure 6.17. LCA greatly elevates the steady-state level of the COX III subunit of the COX protein complex. Immunoblot analysis of COX III steady-state level in purified mitochondria (the complex II 70-kDa subunit was used as a loading control).

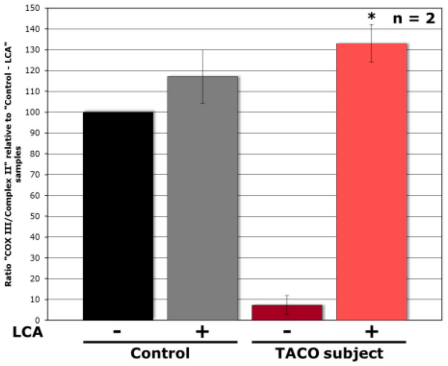

Figure 6.18. LCA greatly elevates the steady-state level of the COX III subunit of the COX protein complex. Immunoblot analysis of COX III steady-state level in purified mitochondria (the complex II 70-kDa subunit was used as a loading control; n = 2).

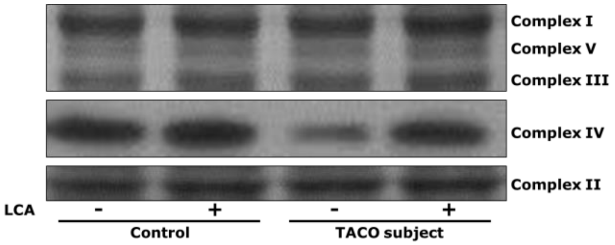

Figure 6.19. LCA increases the steady-state level of the entire COX protein complex (the respiratory complex IV). BN-PAGE of purified mitochondria followed by immunoblot analysis of complexes I to V. 10 µg protein of purified mitochondria were loaded per lane.

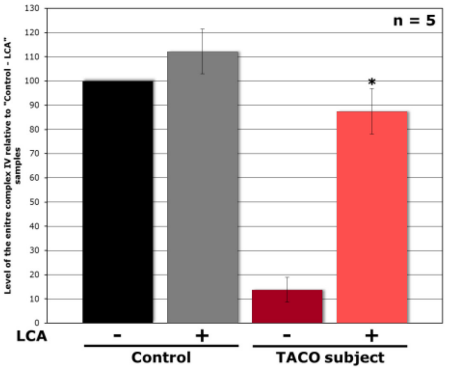

Figure 6.20. LCA increases the steady-state level of the entire COX protein complex (the

respiratory complex IV). BN-PAGE of purified mitochondria followed by immunoblot analysis of complexes I to V; n = 5.

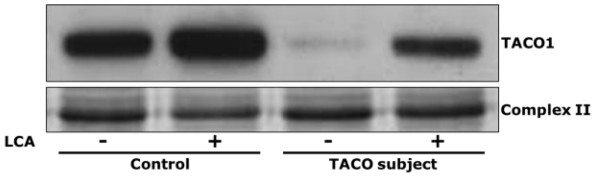

Figure 6.21. LCA increases the steady-state level of TACO1 protein. Immunoblot analysis of TACO1 steady-state level in purified mitochondria using an antibody that recognizes its C terminus (the complex II 70-kDa subunit was used as a loading control).

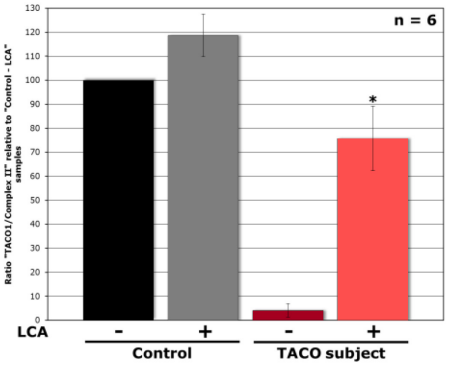

Figure 6.22. LCA increases the steady-state level of TACO1 protein. Immunoblot analysis of TACO1 steady-state level in purified mitochondria using an antibody that recognizes its C terminus (the complex II 70-kDa subunit was used as a loading control; n = 6).

7 Suggestions for future work

We demonstrated that LCA can suppress mitochondrial COX deficiency causing Leigh syndrome, a human neurological disorder caused by a recessive mutation in *TACO1* gene. Our findings provided evidence that LCA promotes synthesis of the full-length TACO1 protein. Based on these observations, we put forward a hypothesis that in TACO1 mutant cells LCA restores the assembly of the mitochondrial COX complex by increasing the steady-state level of TACO1 protein, perhaps by any of the following mechanisms: (1) stabilizing mutant TACO1 mRNA by decelerating its nonsense-mediated decay; and/or (2) enabling ribosomal readthrough of premature but not normal termination codons [247 - 250]. A challenge now is to get a greater insight into these mechanisms. To address this challenge, many important questions need to be answered. Does LCA enter cultured human fibroblasts or does it activate a signaling pathway elevating the steady-state level of TACO1 protein? Does LCA increase the level of TACO1 mRNA in TACO1 mutant cells? Will gentamycin and/or PTC124 (the two known drugs enabling ribosomal readthrough of premature stop codons) be able to restore COX assembly in TACO1 mutant cells by increasing the steady-state level of TACO1 protein? If so, will various combinations of LCA, gentamycin and/or PTC124 be more efficient in restoring COX assembly in TACO1 mutant cells than any of these compounds alone? Will siRNA directed against UPF1 and/or UPF2 (the two proteins that in mammalian cells are known to function in destabilizing mRNAs carrying premature stop codons) be able to restore COX assembly in TACO1 mutant cells by increasing the steady-state level of TACO1 protein?

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9 List of my publications and invited presentations

Publications:

1. Goldberg AA, Bourque SD, Kyryakov P, Gregg C, Boukh-Viner T, Beach A, **Burstein MT**, Machkalyan G, Richard V, Rampersad S, Cyr D, Milijevic S, Titorenko VI (2009). Effect of calorie restriction on the metabolic history of chronologically aging yeast. Exp Gerontol 44:555-571.

2. Goldberg AA, Bourque SD, Kyryakov P, Boukh-Viner T, Gregg C, Beach A, **Burstein MT**, Machkalyan G, Richard V, Rampersad S, Titorenko VI (2009). A novel function of lipid droplets in regulating longevity. Biochem Soc Trans 37:1050-1055.

3. Goldberg AA, Richard VR, Kyryakov P, Bourque SD, Beach A, **Burstein MT**, Glebov A, Koupaki O, Boukh-Viner T, Gregg C, Juneau M, English AM, Thomas DY, Titorenko VI (2010). 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 2:393-414.

4. **Burstein MT**, Beach A, Richard VR, Koupaki O, Gomez-Perez A, Goldberg AA, Kyryakov P, Bourque SD, Glebov A, Titorenko VI (2012). 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 10:75-82.

5. Kyryakov P, Beach A, Richard VR, **Burstein MT**, Leonov A, Levy S, Titorenko VI (2012).
Caloric restriction extends yeast chronological lifespan by altering a pattern of age-related changes in trehalose concentration. Front Physiol 3:256.

6. **Burstein MT**, Kyryakov P, Beach A, Richard VR, Koupaki O, Gomez-Perez A, Leonov A, Levy S, Noohi F, Titorenko VI. Lithocholic acid extends longevity of chronologically aging yeast only if added at certain critical periods of their lifespan. Cell Cycle 11:3443-3462.

7. Beach A, **Burstein MT**, Richard VR, Leonov A, Levy S, Titorenko VI (2012). Integration of peroxisomes into an endomembrane system that governs cellular aging. Front Physiol 3:283.

8. Richard VR, Leonov A, Beach A, **Burstein MT**, Koupaki O, Gomez-Perez A, Levy S, Pluska L, Mattie S, Rafesh R, Iouk T, Sheibani S, Greenwood M, Vali H, Titorenko VI (2013). Macromitophagy is a longevity assurance process that in chronologically aging yeast limited in calorie supply sustains functional mitochondria and maintains cellular lipid homeostasis. Aging 5:234-269.

9. Beach A, Richard VR, Leonov A, **Burstein MT**, Bourque SD, Koupaki O, Juneau M,

Feldman R, Iouk T, Titorenko VI (2013). Mitochondrial membrane lipidome defines yeast longevity. Aging 5:551-574.

10. **Burstein MT**, Titorenko VI (2014). A mitochondrially targeted compound delays aging in yeast through a mechanism linking mitochondrial membrane lipid metabolism to mitochondrial redox biology. Redox Biol 2:305-307.

Invited presentations:

1. The 2009 Northeast Regional Yeast Meeting, Cornell University, New York, USA; June 2009. Title of the talk: "By increasing the level of cardiolipin in the mitochondrial membrane, a novel anti-aging drug modulates longevity- and disease-related processes in mitochondria".

2. The Montreal Yeast Meeting; April 2010. Title of the talk: "A novel anti-aging compound modulates longevity- and disease-related processes in mitochondria".

3. The 2010 Northeast Regional Yeast Meeting, the State University of New York – Buffalo, New York, USA; May 2010. Title of the talk: "By altering the composition of mitochondrial membrane lipids, a novel anti-aging compound modulates many longevity- and disease-related processes in yeast mitochondria".

4. The 13th Annual Chemistry and Biochemistry Graduate Research Conference, Concordia University; November 2010. Title of the talk: "A novel anti-aging compound modulates longevity- and disease-related processes in mitochondria".

5. The 10th International Conference “Dose-Response: Implications for Toxicity, Medicine, and Risk Assessment”, Amherst, Massachusetts, USA; April 2011. Title of the talk: "Xenohormetic, hormetic, and cytostatic selective forces drive the evolution of longevity regulation mechanisms within ecosystems: A hypothesis and its empirical verification".

6. The Northeast Regional Yeast Meeting in Rochester, New York, USA; October 2011. Title of the talk: "Using the experimental evolution of long-lived yeast species for testing evolutionary theories of aging".

7. The 14th Annual Chemistry and Biochemistry Graduate Research Conference, Concordia University; November 2011. Title of the talk: "Using the experimental evolution of long-lived yeast species for testing evolutionary theories of aging".