Chronological Aging of the Yeast *Saccharomyces Cerevisiae* Is Linked to a Differentiation Program Converting Quiescent Cells into Non-Quiescent Cells

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ABSTRACT

Chronological Aging of the Yeast *Saccharomyces Cerevisiae* Is Linked to a Differentiation Program Converting Quiescent Cells into Non-Quiescent Cells

Rachel Clare Feldman

A diet known as caloric restriction is known to extend longevity of chronologically aging yeast. In studies described in this thesis, I elucidated how this longevity-extending dietary regimen influences differentiation of a population of chronologically aging yeast cells into subpopulations of quiescent and non-quiescent cells. My studies have identified two differentiation programs that define longevity of chronologically aging yeast. One of these differentiation programs progresses in yeast cultured under caloric restriction conditions, whereas another program functions in yeast not limited in calorie supply. My findings imply that each of the two differentiation programs defines longevity of chronologically aging yeast by linking cellular aging to cell cycle regulation, maintenance of a quiescent state, and entry into and progression through a non-quiescent state. I also investigated how lithocholic acid (a potent natural anti-aging compound), the pro-aging Ras family GTPase/cAMP/protein kinase A signaling pathway (one of the key regulators linking carbon source availability to cell growth and metabolism) and trehalose (a non-reducing disaccharide) regulate the two longevity-defining differentiation programs that I have discovered in studies described here. Based on my findings, here I propose a model of how lithocholic acid, the Ras family GTPase/cAMP/protein kinase A signaling pathway and trehalose define longevity of chronologically aging yeast by regulating various stages of the two differentiation programs linking cellular aging to cell cycle regulation, maintenance of a quiescent state, and entry into and progression through a non-quiescent state.

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List of Abbreviations

AMPK/TOR, the AMP-activated protein kinase/target of rapamycin signaling pathway; cAMP/PKA, the cAMP/protein kinase A signaling pathway; CFU, colony forming units; CLS, chronological lifespan; CR, caloric restriction; CW, Calcofluor White M2R; D, diauxic growth phase; DIC, differential interference contrast; DHR, dihydrorhodamine 123; DR, dietary restriction; IGF-1, the insulin/insulin-like growth factor 1 signaling pathway; L, logarithmic growth phase; LCA, lithocholic acid; NQ, non-quiescent; OD_{600}, optical density at 600 nm; PD, post-diauxic growth phase; PKA, protein kinase A; Q, quiescent; Ras, Ras family GTPase; ROS, reactive oxygen species; Sch9, serine-threonine protein kinase Sch9; ST, stationary growth phase; TOR, target of rapamycin; WT, wild-type strain; $\Delta\Psi_m$ mitochondrial membrane potential.

1 Introduction

1.1 The yeast *Saccharomyces cerevisiae* is a model organism for understanding mechanisms underlying cellular aging in multicellular eukaryotes

The budding yeast *S. cerevisiae* is a unicellular eukaryotic organism [1]. To elucidate mechanisms underlying complex biological processes taking place within individual yeast cells and their populations, many genetic, cell biological, biochemical, chemical biological, system biological and microfluidic methods of thorough molecular analysis have been developed [2 - 7]. It is also important to note that *S. cerevisiae* has relatively short replicative and chronological lifespans, both of which are easy to monitor under controllable laboratory conditions using well-established experimental approaches [8 - 17]. Due to such powerful combination of beneficial properties as a model organism for studying mechanisms of cellular aging, *S. cerevisiae* has been successfully used for the identification of many genes shown to modulate cellular aging, define organismal lifespan and influence organismal health-span not only in *S. cerevisiae* and other yeast species but also in various multicellular eukaryotic organisms [8 - 21]. Furthermore, using *S. cerevisiae* as a model organism for elucidating mechanisms of cellular aging, several nutrient- and energy-sensing signaling pathways have been discovered; these pathways appear to regulate an evolutionarily conserved set of cellular processes not only in *S. cerevisiae* and other yeasts but also in eukaryotes across phyla [8 - 17, 22 - 25]. Moreover, *S. cerevisiae* has been a model organism fruitfully employed for discovering several low molecular weight molecules that slow down aging and extend longevity; all these small molecules have been shown to decelerate the process of aging, extend health-span and delay the onset of age-related diseases in a broad variety of multicellular eukaryotic organisms [8 - 17, 26 - 32]. All these studies employing *S.*

cerevisiae as a model organism have provided comprehensive evidence that the main features of the aging process ; as well as the mechanisms through which cellular and organismal aging are influenced by certain genetic, dietary and pharmacological interventions targeting a distinct set of nutrient-and-energy-sensing signaling pathwayshave been conserved in the course of evolution [8 - 35].

1.2 The replicative mode of yeast aging

One aspect of the aging process in *S. cerevisiae* is that a mother cell can undergo a finite number of asymmetric divisions before becoming senescent because its mitotic cell cycle is arrested [9 - 13]. This aspect of the aging process reflects a so-called replicative mode of yeast aging [36 - 39]. Yeast replicative aging is defined by the maximum number of daughter cells that a mother cell can make by dividing asymmetrically before this mother cell undergoes cell cycle arrest (Figure 1.1) [37 - 39]. Commonly, to assess the replicative mode of yeast aging under controllable laboratory conditions, a budding daughter cell is removed from the mother cell with the help of a micromanipulator and the total number of asymmetric mitotic divisions this mother cell could make is recorded (Figure 1.1) [38, 39]; in recent years, several major modifications of such assay have been introduced and translated into a high-throughput format to allow systemic analyses of yeast replicative aging [4, 6, 7, 12]. It is commonly accepted that the replicative mode of yeast aging closely imitates aging of proliferating, mitotically active cells in a multicellular eukaryotic organism; the often cited examples of such cells are lymphocytes and various populations of stem cells [9, 10, 40].

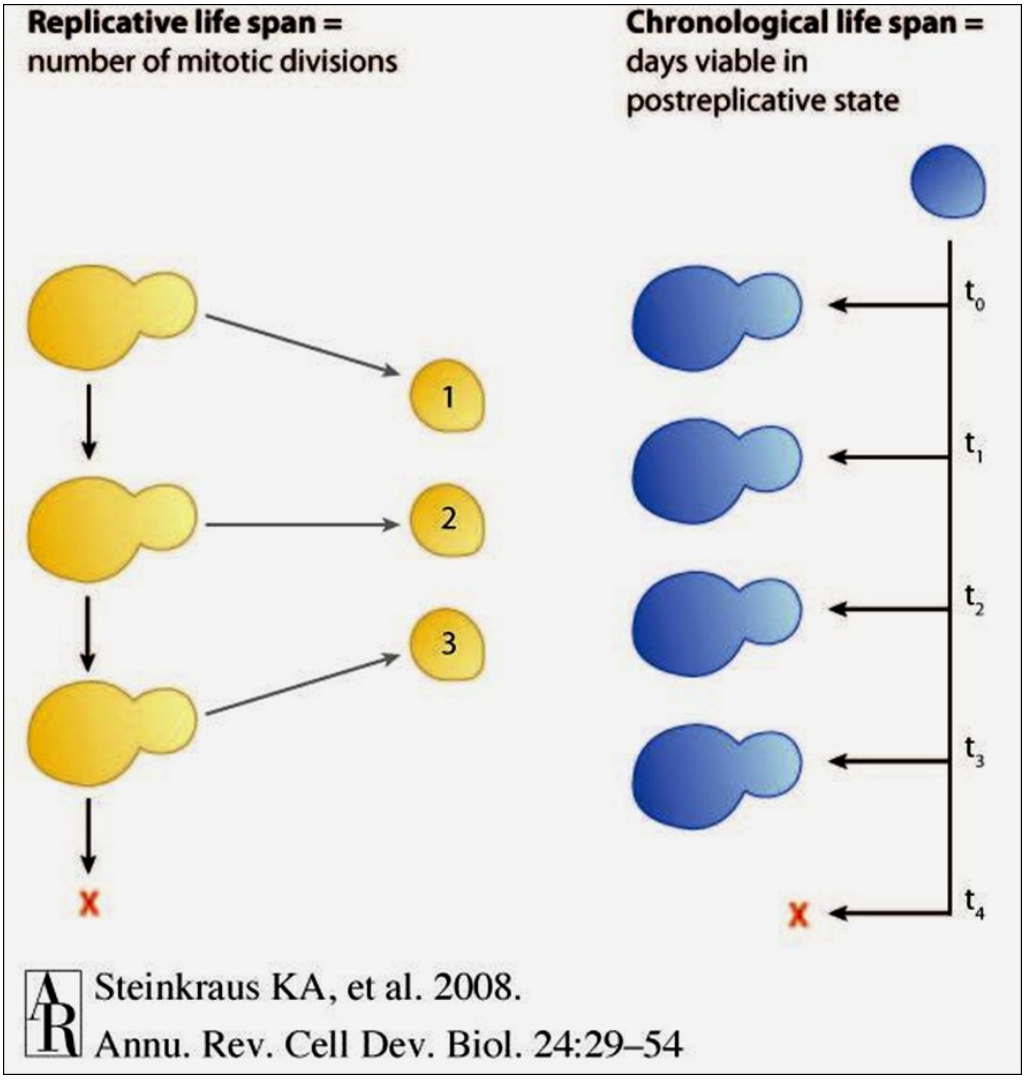

Figure 1.1. Two different ways to study aging of the yeast *Saccharomyces cerevisiae*. Replicative aging is studied by measuring the maximum number of daughter cells that a mother cell can produce before becoming senescent. Chronological aging is studied by measuring the length of time during which a cell remains viable after an arrest of its growth and division.

Growing evidence implies that the spatiotemporal dynamics of certain processes taking place within a mitotically competent mother cell undergoing replicative aging can define yeast replicative lifespan; this is because the spatiotemporal dynamics of such cellular processes can establish a rate with which the dividing mother cell progressively accumulates so-called ″aging factors″ [10, 12, 13, 17, 35, 41 - 46]. These longevity-defining cellular processes occur during

one of the three following stages of replicative aging; they are known as the ″early age″, ″intermediate age″ and ″late age″ stages [12]. Aging factors that progressively accumulating in a replicatively aging mother yeast cell include alkalinized and enlarged vacuoles, oxidatively damaged and aggregated cytosolic proteins, dysfunctional mitochondria that have a tendency to fragment and aggregate, extrachromosomal rDNA circles (ERC) that amass in the nucleus, the loss of heterozygosity at the nuclear rDNA locus, and reduced peroxisomal protein import [12, 13, 17, 18, 33, 35, 37, 45 - 57]. The spatiotemporal dynamics of cellular processes that can establish a rate with which the dividing mother cell progressively accumulates aging factors is controlled by two kinds of cell-autonomous mechanisms. One kind of these cell-autonomous mechanism modulates the rates with which different aging factors form and amass within the mother cell, whereas another kind of cell-autonomous mechanism regulates the rates with which the mother cell retains some aging factors to prevent their transmission into the daughter cell [12, 13, 17, 18, 33, 35, 41, 44, 58 - 75].

A mitotically competent mother cell undergoing replicative aging can also modulate longevities of other replicatively aging cells in a yeast population via a cell-non-autonomous mechanism involving intercellular communications. Indeed, it has been demonstrated that in populations of replicatively aging yeast maintained on solid surfaces under laboratory conditions, replicatively ″young″ mother cells limited in calorie supply produce and secrete small molecules that can extend longevities of other cells [76]. The chemical nature and mechanism of action of such transmissible longevity factors remain to be established [76].

1.3 The chronological mode of yeast aging

There is another aspect of the aging process in *S. cerevisiae*. Specifically, after a replicatively aged yeast cell undergoes cell cycle arrest to become senescent, this cell can remain viable only within a finite period of time [9, 10]. This aspect of the aging process reflects a so-called chronological mode of yeast aging [14, 15, 19, 20, 77]. Yeast chronological aging is defined by the length of time a yeast cell stays alive after becoming senescent (Figure 1.1) [9, 10, 77]. Usually, the viability of a chronologically aging yeast cell under controllable laboratory conditions is monitored by assessing its ability to form a colony on the surface of a solid nutrient-rich medium; under these conditions, a cell considered to be alive if it can proliferate to form a colony of cells (Figure 1.1) [10, 77, 78]. To enable systemic analyses of yeast chronological aging, several high-throughput modifications of such clonogenic assay have been developed [79 - 81]. It is thought that the chronological mode of yeast aging imitates aging of non-dividing, post-mitotic cells in a multicellular eukaryotic organism such as muscle and neural cells [9, 10, 19, 40].

Emergent evidence supports the view that the spatiotemporal dynamics of certain cellular processes defines the chronological lifespan of yeast cultured in liquid media under controllable laboratory conditions [9, 10, 14, 17, 18, 33, 82 - 98]. Among these longevity-defining processes are cell growth and division, certain metabolic pathways, the biogenesis of some organelles and interorganellar communications, the homeostasis of proteins and lipids, nucleic acid stability, cell stress response, and programmed cell death [9, 10, 14, 17, 84, 91 - 96]. It is believed that each of these longevity-defining processes progresses through a series of early-life ″checkpoints″ in logarithmic (L), diauxic (D) and post-diauxic (PD) growth phases, as well as through several late-life ″checkpoints″ in stationary (ST) growth phase [14, 15, 17, 92]. By monitoring the relative rates of some longevity-defining cellular processes and the intracellular concentrations

of some key metabolites at each of the early and late-life ″checkpoints″, a group of regulatory proteins respond to the age-related changes in such rates and concentrations by altering their intensity at various critical periods of yeast chronological lifespan [14, 15, 17]. Such ″sensory circuit″ allows to establish the pace of cellular aging, thus defining yeast chronological lifespan [14, 15, 17, 92].

Two groups of regulatory mechanisms underlie this longevity-defining sensory circuit. One group of these mechanisms operates in a cell-autonomous manner by modulating the longevity-defining cellular processes from inside of the cell [9, 10, 14, 17, 18, 33, 82 - 98]. Another group of mechanisms are cell-non-autonomous mechanisms of intercellular communication; these mechanisms involve certain low molecular weight transmissible longevity factors (such as ethanol, acetic acid and hydrogen sulfide) circulating within populations of yeast cells [9, 10, 14, 15, 17, 34, 83, 84, 99 - 104].

1.4 Relationship between the replicative and chronological modes of yeast aging

The experimental assays that are used to monitor the replicative mode of yeast aging are different and conducted independently from the experimental assays that assess the chronological mode of yeast aging (Figure 1.1) [9, 10, 37 - 39, 77, 78]. Until recently it was unclear how these two modes of yeast aging relate to each other. Recent studies have addressed several aspects of such relationship. It has been found that only some of the numerous monogenic knock-out mutations known to delay yeast replicative aging can also slow down the chronological mode of yeast aging [8 - 10, 100]. Moreover, while the addition of buffers that avert the acidification of liquid growth media and allow to maintain different values of alkaline pH has been shown to prolong longevity of chronologically aging yeast, the addition of such buffers does not alter longevity of replicatively aging yeast [104 - 106]. Thus, some of the

regulatory mechanisms underlying yeast replicative aging differ from those underlying the chronological mode of yeast aging, and vice versa [8 - 10, 100, 104 - 106].

However, it seems that there are regulatory mechanisms that are common to both the replicative and chronological modes of yeast aging. In fact, it has been shown that: 1) the progression of both modes of yeast aging can be slowed down by caloric restriction (CR), a nutritional intervention known to delay aging and extend longevity of eukaryotic organisms across phyla [8 - 10, 107], 2) the lifespans of both replicatively and chronologically aging yeast can be prolonged by mutations or chemical compounds that impair or inhibit either the pro-aging TOR/Sch9 (target of rapamycin/serine-threonine protein kinase Sch9) signaling pathway or the pro-aging Ras/cAMP/PKA (Ras family GTPase/cAMP/protein kinase A) signaling pathway [8 – 10, 100], and 3) the older a yeast cell becomes when it undergoes chronological aging in a liquid medium supplemented with excess of calories, the shorter a remaining replicative age of such cell becomes if it regains proliferation capability after being transferred to a fresh medium [104, 108 - 110]. Based on these observations, it has been proposed that the replicative and chronological modes of yeast aging may converge into a single aging process; it has been also suggested that the extent of such overlapping between the two modes of yeast aging may differ in different ecological and laboratory niches [15].

1.5 Aging yeast cells developing multicellular communities on solid surfaces

In the wild and under controllable laboratory conditions, individual yeast cells exist mainly within yeast populations. Thus, individual yeast cells progress through the aging process as part of organized multicellular communities. One example of such communities is a multicellular community which is called a colony; it is formed by yeast cells that are kept on solid surfaces

under laboratory conditions (Figure 1.2) [111, 112]. Recent findings provide evidence that the spatiotemporal dynamics of an intercellular flow of some low molecular weight metabolites within such colony of yeast cells generates a complex quorum-sensing system [113 - 116]. The relationships between individual cells and cell subpopulations within such quorum-sensing system are created, maintained and progressed because the cells and cell subpopulations establishing the system can generate, release and respond to these metabolites [113, 116]. The establishment of the intricate quorum-sensing system guides a multistep process of horizontal and vertical differentiation of a yeast colony; because of such differentiation, cells in different regions of the colony have differential patterns of growth, division, metabolism, gene expression, stress response and programmed death [113, 116, 117 - 119]. Some of these patterns prolong yeast longevity, whereas others shorten it; therefore, cells that are located in different areas of a colony differ in their longevities [116, 120, 121]. Under these circumstances, the metabolites within a colony that establish the quorum-sensing system guiding cell differentiation and aging act as low molecular weight transmissible longevity factors. The identities of some of these longevity factors, as well as the mechanisms by which they modulate aging and regulate longevity of yeast cells comprising a colony, have been established. They are outlined below.

The lifespan of a yeast colony is known to progress through two phases of reversible changes in extracellular pH (Figure 1.2) [114, 122, 123]. Several hours after an individual yeast cell is positioned on solid growth medium to form a microcolony or a suspension of yeast cells was placed on such medium to form a giant colony, the first alkali phase of fast cell growth starts; a characteristic feature of this phase is the release of volatile ammonia (Figure 1.2) [122, 123]. In microcolonies that are developed by cells of yeast natural strains, such release of volatile ammonia during the first alkali phase stimulates the dimorphic transition from the yeast to the

pseudomycelial form only in those cells that are located at the borders of microcolonies; the cells of adjacent microcolonies then extend towards each other to create a biofilm [116, 124, 125]. Unlike natural strains of yeast, laboratory yeast strains are unable to undergo dimorphic transition and microcolony expansion into a biofilm in response to the release of volatile ammonia during the first alkali phase within microcolonies [116, 124, 125].

When cell growth within a colony slows, the colony begins to acidify medium and enters the first acidic phase (Figure 1.2) [121, 123]. During this phase, cell population within the colony is not differentiated; it consists of non-dividing, chronologically aging yeast cells [113, 118, 120]. Late in the first acidic phase, the intracellular and extracellular concentrations of reactive oxygen species (ROS) within the colony increase; these ROS include both superoxide radicals and hydrogen peroxide [115 - 117].

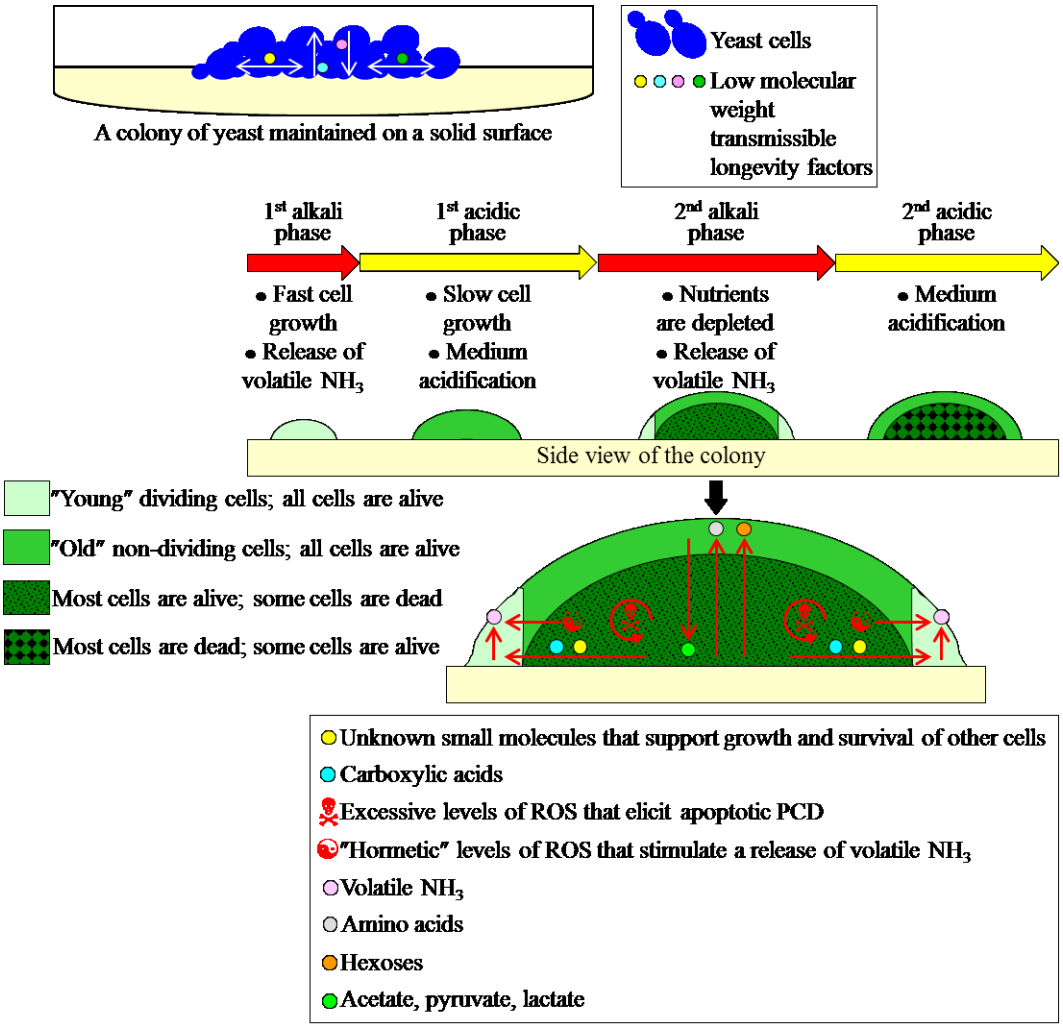

Figure 1.2. An intercellular flow of metabolites establishes a quorum sensing system that guides differentiation and aging of yeast organized into a cell colony on a solid surface. Side views of a yeast colony advancing through two reversible phases of volatile ammonia release and two phases of medium acidification are shown. The directions of an intercellular flow of some metabolites within a colony advancing through the second alkali phase are presented. Abbreviations: PCD, programmed cell death; ROS, reactive oxygen species.

Recent findings suggest that the relative concentrations of hydrogen peroxide in mitochondria and cytosol establish the pace of colony advancement from the first acidic phase to the second alkali phase; it has been shown that if hydrogen peroxide concentration in the cytosol rises above a certain threshold, such advancement is delayed [113, 117].

The second alkali phase and the second release of volatile ammonia take place when all

nutrient sources within the colony have been exhausted (Figure 1.2) [116, 119, 122]. During this phase, the cells in the peripheral colony areas undergo significant alterations in gene expression patterns; such alterations are consistent with the following largescale metabolic remodeling of these cells: 1) stimulation of such processes as ammonia secretion, cellular uptake of carboxylic acids and their transformation to oxaloacetate in the cytosol, peroxisomal β-oxidation of fatty acids into acetyl-CoA, mitochondrial uptake of oxaloacetate and acetyl-CoA, mitochondrial glyoxylate cycle which provides oxaloacetate for the synthesis of amino acids, mitochondrial synthesis of aspartate for making other amino acids, and amino acid degradation in vacuoles, as well as 2) weakening of such processes as mitochondrial oxidative phosphorylation, some reactions of the TCA cycle in mitochondria and cellular response to oxidative stress [117, 119, 123, 126]. Such significant alterations in gene expression and metabolism during the second alkali phase in the cells within the outer areas of the colony support their proliferation and long-term viability (Figure 1.2) [119, 123]. Unlike the cells in the peripheral colony areas, many cells in the center of the colony accumulate ROS and undergo programmed apoptotic death during the second alkali phase; the dying cells in the central colony region release certain metabolites (whose identity remains to be established) that are essential for growth and survival of other cells in the center of the colony as well as cells situated at the borders of the colony (Figure 1.2) [115, 116]. Unlike the population of cells in the peripheral colony areas, the population of cells in the center of the colony consists mainly of chronologically ″old″, non-dividing cells [116, 118, 127]. During the second alkali phase, these chronologically ″old″ cells in the center of the colony undergo differentiation both in horizontal and vertical directions, thus developing populations of upper (U) and lower (L) cells (Figure 1.2) [118, 127]. U cells in the upper colony region display decreased mitochondrial respiration, reduced concentrations of ROS, augmented autophagy, and

amplified levels of storage carbohydrates and lipids; these cells are resistant to various kinds of excessive stresses and are also long-lived (Figure 1.2) [118, 120]. L cells in the lower colony region have increased concentrations of ROS, diminished autophagy, and reduced concentrations of storage carbohydrates and lipids; unlike U cells, L cells are sensitive to various kinds of excessive stresses and are also short-lived (Figure 1.2) [118, 120].

Altogether, these findings imply the ability of yeast cells that are organized into a colony can generate, release and respond to some small molecules (including volatile ammonia, ROS, carboxylic acids, amino acids, hexoses and, possibly, other metabolites) that guide a multistep process of cell differentiation; consequently, yeast cells in different areas of the colony have different longevities (Figure 1.2).

1.6 Aging of yeast cells cultured in liquid media

Another example of individual yeast cells progressing through the aging process as multicellular communities is a population of yeast cells that are cultured under controllable laboratory conditions in liquid media. Usually these experiments are conducted by culturing yeast in the nutrient-rich YEPD medium initially containing 2% glucose as a sole carbon source [9, 10, 19, 77, 78, 80, 84, 92]. The full growth cycle of yeast cells cultured under such conditions starts with logarithmic (L; which is also called exponential) phase and then progresses through diauxic (D) and post-diauxic (PD) phases to stationary (ST) phase [19, 77, 78, 80, 84, 92]. A transition from L phase to D phase occurs when yeast cells exhaust glucose from the medium [19, 77, 78, 80, 84, 92]. During such transition, the cells undergo cell cycle arrest in the G_1 phase and enter a distinctive differentiation program [128 - 136]. This differentiation program gives rise to several cell populations which differ from each other in their morphologies, metabolic and gene

expression patterns, reproductive abilities, susceptibilities to various exogenous and endogenous stresses, and longevities (Figure 1.3) [128 - 136].

One of these cell populations is a population of so-called quiescent (Q) yeast cells. It has been shown that Q cells are predominantly daughter cells formed during L phase by asymmetric mitotic divisions of mother cells; the population of Q cells also includes some of the ″young″ mother cells that have asymmetrically divided not more than once (Figure 1.3) [128, 129, 131, 134]. All Q cells in the population are believed to be in a distinct non-proliferative state, which is called G_0 [128 - 136]. All Q cells also share a characteristic set of morphological, biochemical, physiological and reproductive traits, as outlined below. Gradient density centrifugation has revealed that Q cells have higher density than that of non-quiescent (NQ) cells, whereas phase-contrast microscopy of purified Q cells has shown that they are very refractive, equally sized, delimited by a dense cell wall and devoid of visible buds [128, 129, 131, 134, 135]. Furthermore, Q cells

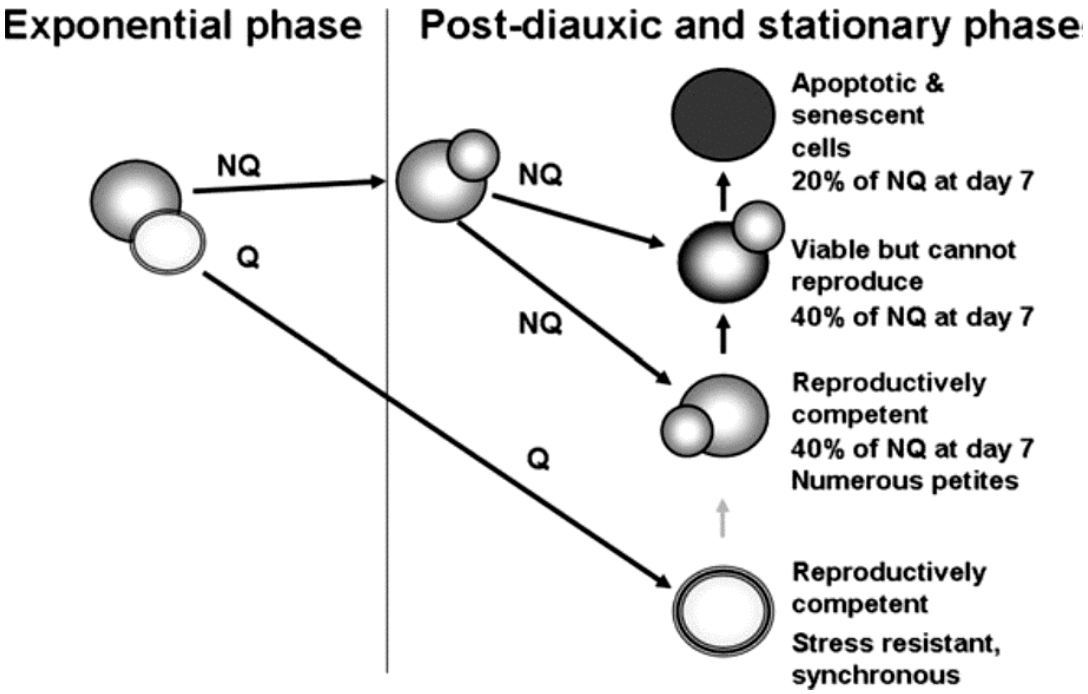

Figure 1.3. When a population of yeast cells cultured in a liquid nutrient-rich medium initially

containing 2% glucose undergoes transition from exponential phase to diauxic phase, it undergoes cell cycle arrest in the G_1 phase and then differentiates into quiescent (Q) cells and several populations of non-quiescent (NQ) cells; such differentiation is one of the key factors defining yeast longevity. From reference [134].

display high intracellular levels of such reserve carbohydrates as glycogen and trehalose, show high rate of such key trait of mitochondrial functionality as coupled respiration, and exhibit low concentrations of by-products of mitochondrial respiration such as ROS [128, 129, 131, 134, 135]. Moreover, Q cells are resistant to excessive thermal and oxidative stresses, and display high genomic stability [128, 129, 131, 134, 135]. Q cells are also viable (judging from their high metabolic activities assessed with the help of certain fluorescent reporter molecules) and capable of reproduction (judging from their abilities of synchronously re-enter the cell cycle and proliferate after being transferred to a fresh medium) [128, 129, 131, 132, 134, 135].

Unlike the homogeneous population of Q cells, the population of NQ cells recovered from ST phase includes three different types of cells. Some of NQ cells in the population are viable and reproductively proficient cells that contain respiration-deficient mitochondria, amass high concentrations of ROS and display reduced genomic stability; this type of NQ cells may derive from Q cells that undergo an aging-related differentiation (Figure 1.3) [128, 129, 131, 134]. Furthermore, some of NQ cells in the population are viable (judging from their high metabolic activities assessed using some fluorescent reporter molecules) but incapable of reproduction (i.e. they are unable to synchronously re-enter the cell cycle and proliferate after being transferred to a fresh medium); this type of NQ cells may originate from NQ cells that are both viable and reproductively proficient (Figure 1.3) [128, 129, 131, 134]. Moreover, some of NQ cells in the population are dying cells that exhibit several hallmark features of apoptotic and/or necrotic forms of programmed death; this type of NQ cells may derive from NQ cells that are viable but

incapable of reproduction (Figure 1.3) [128, 129, 131, 134].

Emergent evidence supports the view that the longevity-defining differentiation program giving rise to the population of Q cells and the three populations of NQ cells is under the stringent control of a distinct set of signaling pathways and protein kinases. These regulators of the differentiation program which defines yeast longevity by modulating the relative abundancies of Q and NQ cells include the following pathways and protein kinases: 1) the pro-aging TOR/Sch9 signaling pathway, which in yeast links nitrogen source availability and quality to cell proliferation and metabolism [133, 137 - 143], 2) the pro-aging Ras/cAMP/PKA signaling pathway, which in yeast is one of the key regulators linking carbon source availability to cell proliferation and metabolism [137 - 143], 3) the anti-aging plasma membrane sensor Mtl1, which in yeast not only participates in the cell wall integrity pathway but also attenuates both the TOR/Sch9 and Ras/cAMP/PKA signaling pathways during transition from L phase to D phase [137], 4) the anti-aging transcriptional repressor Xbp1, whose ability to suppress expression of many genes is essential for maintaining cell cycle arrest in the G_1 phase [136], 5) the AMP-activated serine/threonine protein kinase Snf1, which in yeast cells entering D phase regulates carbon metabolism by phosphorylating and altering activities of many transcription factors [139 - 143], and 6) the pro-aging cyclin-dependent kinase Pho85, which in yeast cells entering D phase is one of the global regulators of cell cycle progression and metabolism [139 - 143].

Altogether, these findings imply that when a population of yeast cells cultured in a liquid nutrient-rich medium initially containing 2% glucose enters D phase, it undergoes G_1 arrest and then differentiates into Q cells and several populations of NQ cells; such differentiation is one of the key factors defining yeast longevity.

1.7 The objectives of studies described in this thesis

Studies described in my thesis had the following two objectives.

The first objective of my studies was to investigate how caloric restriction (CR), a dietary intervention known to delay aging and extend longevity of yeast and various multicellular eukaryotes [8 - 10, 14, 15, 84, 107], influences differentiation of a population of chronologically aging yeast cells into subpopulations of Q and NQ cells. With the help of centrifugation in Percoll density gradient, I have separated ″heavy″ (high-density) Q cells from ″light″ (low-density) NQ cells that were recovered from chronologically aging yeast cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions). Using a combination of cell biological and biochemical approaches, I then examined various morphological, biochemical, physiological and reproductive properties of Q and NQ cells that were purified at different stages of the chronological aging process. These analyses have revealed that in chronologically aging yeast two differentiation programs link cellular aging to cell cycle regulation, maintenance and aging-related depletion of a niche of ″heavy″ (high-density) Q cells, conversion of ″heavy″ (high-density) Q cells to ″light″ (low-density) Q cells, and aging-related differentiation of ″light″ (low-density) Q cells into ″light″ (low-density) NQ cells; one of these differentiation programs operates in yeast cultured under CR conditions, whereas another program progresses in yeast grown under non-CR conditions. Based on my findings, I concluded that each of these two differentiation programs defines longevity of chronologically aging yeast by setting up the following: 1) a growth phase in which G_1 cell cycle arrest occurs, 2) the efficacy with which a niche of ″heavy″ (high-density) Q cells is maintained and depleted in an age-related manner, 3) the age-related rate with which ″heavy″ (high-density) Q cells are converted to ″light″ (low-density) Q cells, and 4) the age-related rate

with which ″light″ (low-density) Q cells differentiate into ″light″ (low-density) NQ cells.

The second objective of my studies was to investigate how each of these two differentiation programs defining longevity of chronologically aging yeast is regulated. To attain this objective, I have examined how several genetic and pharmacological interventions known to extend longevity of chronologically aging yeast by modulating certain signaling pathways and/or metabolic processes influence the following: 1) the efficacy with which a niche of ″heavy″ (high-density) Q cells is maintained and depleted in an age-related manner, 2) the age-related rate with which ″heavy″ (high-density) Q cells are converted to ″light″ (low-density) Q cells, and 3) the age-related rate with which ″light″ (low-density) Q cells differentiate into ″light″ (low-density) NQ cells.

1.8 Thesis outline

Chapter 1 of my thesis outlines the importance of using the budding yeast *S. cerevisiae* as a model organism for understanding mechanisms underlying cellular aging in multicellular eukaryotes. Furthermore, this Chapter describes molecular mechanisms underlying the replicative and chronological modes of yeast aging; it also discusses the relationships existing between the two modes of yeast aging. Moreover, Chapter 1 outlines cell-autonomous and cell-non-autonomous mechanisms underlying aging of yeast cells developing multicellular communities on solid surfaces or in liquid media. A section of this Chapter describing mechanisms regulating longevity of yeast cells progressing through the aging process as multicellular populations cultured in liquid media is currently in preparation as a manuscript of a review paper for submission to *Aging*. I expect this manuscript to be submitted for publication in October or November of 2015.

Chapter 2 of the thesis describes my data implying that there are two differentiation programs linking cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state. This Chapter outlines evidence that one of these two differentiation programs functions in yeast cells cultured under CR conditions, while another program acts in yeast cells maintained in a liquid culture under non-CR conditions. Chapter 2 also discusses my findings strongly suggesting that longevity of chronologically aging yeast cultured in a liquid medium critically depends on the relative rates of several key stages comprising each of the two differentiation programs. Findings described in Chapter 2 are presented in the manuscript of a paper that is currently in preparation for submission to *Microbial Cell*. I expect this manuscript to be submitted for publication in October or November of 2015.

Chapter 3 of the thesis presents my data on how lithocholic acid (LCA; a potent natural anti-aging compound), the pro-aging Ras/cAMP/PKA signaling pathway and trehalose metabolism define longevity of chronologically aging yeast by regulating various stages of each of the two differentiation programs linking cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state. Findings described in this Chapter are presented in the manuscript of a paper that is currently in preparation for submission to *Microbial Cell*. I expect this manuscript to be submitted for publication in October or November of 2015.

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 Two differentiation programs define longevity of chronologically aging yeast by linking cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state

2.1 Abstract

A diet known as caloric restriction (CR) has been shown to extend longevity of chronologically aging yeast [8 - 10, 14, 15, 84, 107]. In studies described in this Chapter of my thesis, I elucidated how the CR diet influences differentiation of a population of chronologically aging yeast cells into subpopulations of Q and NQ cells. My studies have identified two differentiation programs that define longevity of chronologically aging yeast. One of these differentiation programs progress in yeast cultured under CR conditions, while another program functions in yeast grown under non-CR conditions. My findings imply each of the two differentiation programs defines longevity of chronologically aging yeast by linking cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state.

2.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MAT⟨ his3⊗1 leu2⊗0 lys2⊗0 ura3⊗0*) from Thermo Scientific/Open Biosystems was grown in YEP medium (1% yeast extract, 2% peptone; both from Fisher Scientific; #BP1422-2 and #BP1420-2, respectively) initially containing 0.2% or 2% glucose (#D16-10; Fisher Scientific) as a carbon source. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1.

Chronological lifespan assay for cell cultures

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 per ml of culture using a hemacytometer (#0267110; Fisher Scientific). 10 ⌈l (1:10 to $1:10^3$) of cells that were serially diluted were applied to the hemacytometer, where each large square is calibrated to hold 0.1 ⌈l. The number of cells in 4 large square grids was 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 × dilution factor × 10 × 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 (1% yeast extract, 2% peptone, 2% glucose) plates in duplicate in order to count the number of viable cells per ml of each culture. 100 ⌈l of diluted culture was plated onto each plate. After 48-h incubation at 30^oC, 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 CFU × dilution factor × 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 × 100%. The % viability of cells in mid-logarithmic phase was set at 100% viability for that particular culture.

Separation of Q and NQ cells by centrifugation in Percoll density gradient

1 ml of 1.5 M NaCl (#S7653; Sigma) was placed into a 50-ml Falcon tube, and 8 ml of the Percoll solution (#P1644; Sigma) was added to this tube. The NaCl and Percoll solutions were then mixed by pipetting. To form two Percoll density gradients, 4 ml of the NaCl/Percoll mixture

was put into each of the two polyallomer tubes for an MLS-50 rotor for an Optima MAX ultracentrifuge (all from Beckman Coulter). The tubes were centrifuged at 25,000 × g (16,000 rpm) for 15 min at 4°C in an Optima MAX ultracentrifuge. A sample of yeast 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 per ml of culture using a hemacytometer (#0267110; Fisher Scientific). For each Percoll density gradient, 1×10^9 yeast cells were placed into a 15-ml Falcon tube and then pelleted by centrifugation at 5,000 rpm for 7 min at room temperature in a Centra CL2 clinical centrifuge (Thermo IEC). Pelleted cells were resuspended in 500 µl of 50 mM Tris/HCl buffer (pH 7.5), overlaid onto the preformed gradient and centrifuged at 2,300 × g (5,000 rpm) for 30 min at 25°C in an Optima MAX ultracentrifuge. The upper and lower fractions of cells were removed with a pipette, Percoll was removed by washing cells twice with 50 mM Tris/HCl buffer (pH 7.5) and cells were resuspended in 50 mM Tris/HCl buffer (pH 7.5) for subsequent assays.

Cell number measurement for Q and NQ cells separated by centrifugation in Percoll density gradient

An aliquot of the upper or lower fraction of cells recovered from the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) was diluted in order to determine the total number of cells per fraction using a hemacytometer (#0267110; Fisher Scientific). 10 ⌈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 ⌈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 × dilution factor × 10 × 1,000 = total number of cells per ml of fraction.

Reproductive (colony forming) capability assay for Q and NQ cells separated by centrifugation in Percoll density gradient

An aliquot of the upper or lower fraction of cells recovered from the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) was diluted in order to determine the total number of cells per fraction using a hemacytometer (#0267110; Fisher Scientific). 10 ⌈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 ⌈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 × dilution factor × 10 × 1,000 = total number of cells per ml of fraction. Serial dilutions ($1:10^2$ to $1:10^5$) of cells were plated onto YEPD (1% yeast extract, 2% peptone, 2% glucose) plates in duplicate in order to count the number of viable cells per ml of each cell fraction. 100 ⌈l of diluted culture was plated onto each plate. After 48-h incubation at 30^oC, the number of colonies per plate was counted. The number of colony forming units (CFU) equals to the number of reproductively capable cells in a sample. Therefore, the number of reproductively capable cells was calculated as follows: number of CFU × dilution factor × 10 = number of reproductively capable cells per ml. For each cell fraction assayed, % reproductive capability of the cells was calculated as follows: number of CFU per ml / total number of cells per ml × 100%.

Synchronous re-entry into mitosis assay for Q and NQ cells separated by centrifugation in Percoll density gradient

5×10^6 cells recovered in the upper or lower fraction of the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) were harvested by centrifugation for 1 min at 21,000 × g

at room temperature. Pelleted cells were washed twice with water and then inoculated into 50 ml of YEP medium (1% yeast extract, 2% peptone; both from Fisher Scientific; #BP1422-2 and #BP1420-2, respectively) initially containing 0.2% or 2% glucose (#D16-10; Fisher Scientific) as carbon source. Cells were cultured for 4 h 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 taken from a culture at a certain time-point and examined microscopically for the percentage of cells with new buds. At least 500 cells were examined per time point, and the budding percentage was calculated as follows: (number of cells with new buds per ml / total number of cells per ml) × 100%.

Viability (metabolic activity) assay for Q and NQ cells separated by centrifugation in Percoll density gradient

2×10^7 cells recovered in the upper or lower fraction of the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) were harvested by centrifugation for 1 min at 21,000 × g at room temperature. Pelleted cells were washed twice with water and then stained using a LIVE/DEAD yeast viability kit for measuring metabolic activity (#L7009; Invitrogen) following the manufacturer's instructions.

Oxygen consumption assay for Q and NQ cells separated by centrifugation in Percoll density gradient

From 2×10^7 cells to 1×10^8 cells recovered in the upper or lower fraction of the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) were used for continuous measurement of the rate of oxygen consumption. The measurement was conducted 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 YEP medium (1% yeast extract, 2% peptone; both from Fisher Scientific; #BP1422-2 and #BP1420-2, respectively) supplemented with 0.2% glucose was added to the electrode for approximately 5 min to obtain a baseline. Cells recovered in the upper or lower fraction of the Percoll gradient of a known titer were pelleted by centrifugation at 3,000 × g for 5 min. The resulting pellet was resuspended in YEP 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} × 10^9 cells.

Monitoring the mitochondrial membrane potential ($\Delta\Psi_m$) in Q and NQ cells separated by centrifugation in Percoll density gradient

From 2 × 10^7 cells to 1 × 10^8 cells recovered in the upper or lower fraction of the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) were used for monitoring $\Delta\Psi_m$ by incubation with Rhodamine 123 (R123). R123 (Invitrogen) staining for monitoring $\Delta\Psi_m$ 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 5-9 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.

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 Yeast cells cultured under CR conditions enter a differentiation program leading to formation of Q and NQ cell subpopulations when yeast culture reaches L growth phase

I first investigated how CR, a dietary intervention known to delay aging and extend longevity of yeast and various multicellular eukaryotes [8 - 10, 14, 15, 84, 107], influences differentiation of a population of chronologically aging yeast cells into subpopulations of Q and NQ cells. Q yeast cells can be separated from NQ yeast cells by centrifugation in Percoll density gradient [128, 133, 134]. I have adapted this protocol for separating Q cells from NQ cells that were recovered from chronologically aging yeast cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) [84] or 2% glucose (non-CR conditions) [84]. Current view suggests that yeast cells that reach ST growth phase under non-CR conditions (i.e. in medium initially containing 2% glucose) arrest their cell cycle at the checkpoint START A in late G_1 and enter a differentiation program leading to formation of two cell subpopulations, ″heavy″ (high-density) Q cells and ″light″ (low-density) NQ cells (Figure 2.1) [128, 133, 134].

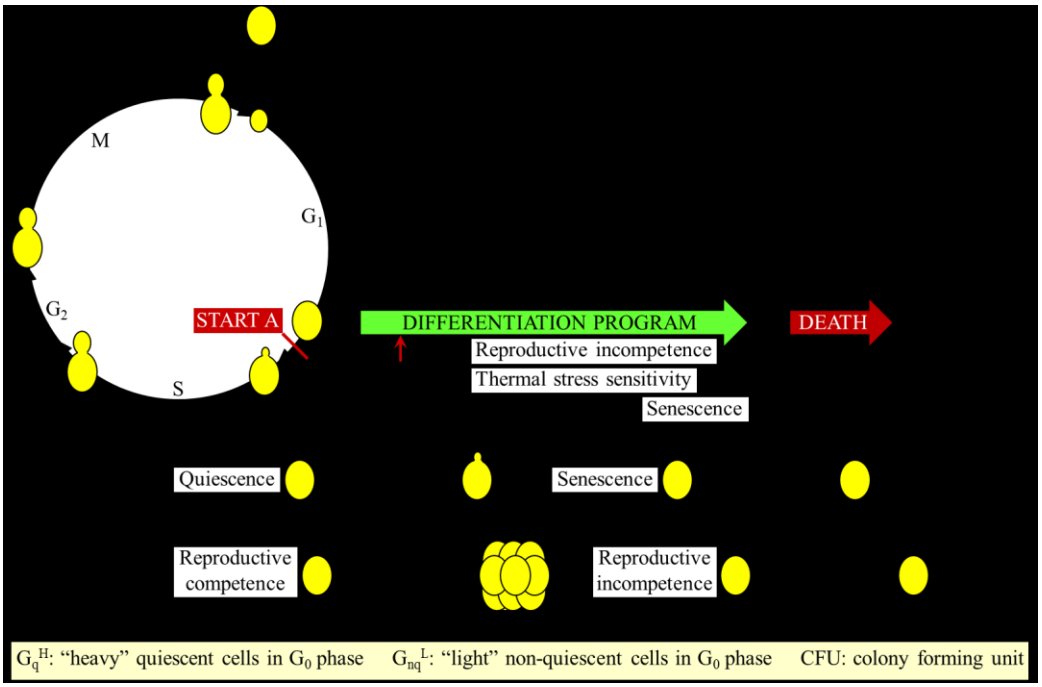

Figure 2.1. Current view on mechanisms that link cellular aging to cell cycle regulation, maintenance of a Q state, and entry into an NQ state. In this view, yeast cells that reach ST growth phase under non-CR conditions (i.e. in medium initially containing 2% glucose) arrest their cell cycle at the checkpoint START A in late G_1 and enter a differentiation program leading to formation of two cell subpopulations, ″heavy″ (high-density) Q cells and ″light″ (low-density) NQ cells. Based on references 128 and 134.

Using the centrifugation-based approach for separating such cell subpopulations, I found that yeast cells cultured under CR on 0.2% glucose enter differentiation program leading to formation of ″heavy″ (high-density) Q and ″light″ (low-density) NQ cell subpopulations when a yeast culture reaches L growth phase (Figures 2.2 and 2.3) - i.e. much earlier than yeast cells cultured under non-CR conditions on 2% glucose and known to differentiate only when they reach ST growth phase (Figures 2.2 and 2.3) [128, 133, 134].

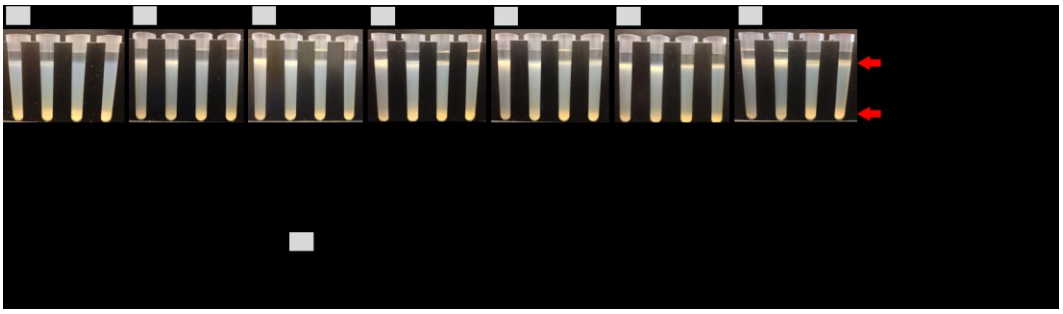

Figure 2.2. Yeast cells grown under CR on 0.2% glucose enter differentiation program leading to formation of ″heavy″ (high-density) Q and ″light″ (low-density) NQ cell subpopulations when a yeast culture reaches L growth phase. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″.

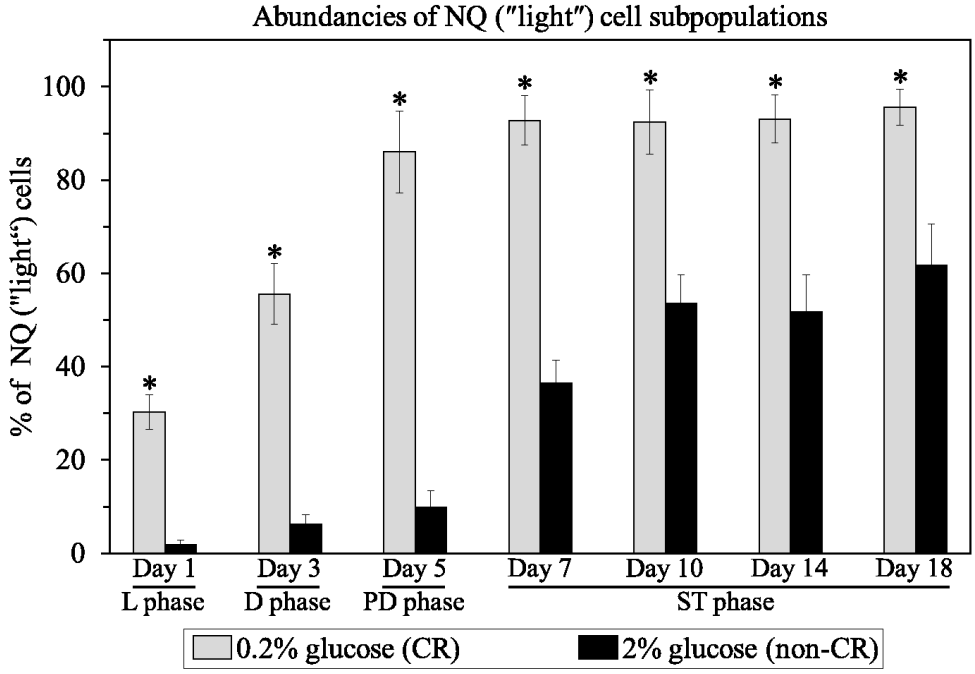

Figure 2.3. Yeast cells grown under CR on 0.2% glucose enter differentiation program leading to formation of ″heavy″ (high-density) Q and ″light″ (low-density) NQ cell subpopulations when a yeast culture reaches L growth phase. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 21 – 34); * $p < 0.05$.

2.3.2 Q cells differ from NQ cells in size and ability to form buds

I then used differential interference contrast (DIC) microscopy to compare sizes of Q and NQ cells recovered from different growth phases and subjected to centrifugation in Percoll density gradient. My DIC microscopical examination and subsequent morphometric analysis of Q and NQ cell subpopulations recovered following centrifugation in Percoll density gradient have revealed that throughout the entire chronological lifespan: 1) ″heavy″ Q cells cultured under CR conditions remain smaller than ″heavy″ Q cells cultured under non-CR conditions (Figures 2.4 and 2.5), 2) ″light″ NQ cells cultured under CR conditions have sizes similar to those of ″light″ NQ cells cultured under non-CR conditions (Figures 2.4 and 2.6), 3) ″heavy″ Q cells cultured either under CR conditions or under non-CR conditions remain unbudded (Figure 2.7), and 4) ″light″ NQ cells cultured either under CR conditions or under non-CR conditions represent a mixture of budded and unbudded cells (Figure 2.8).

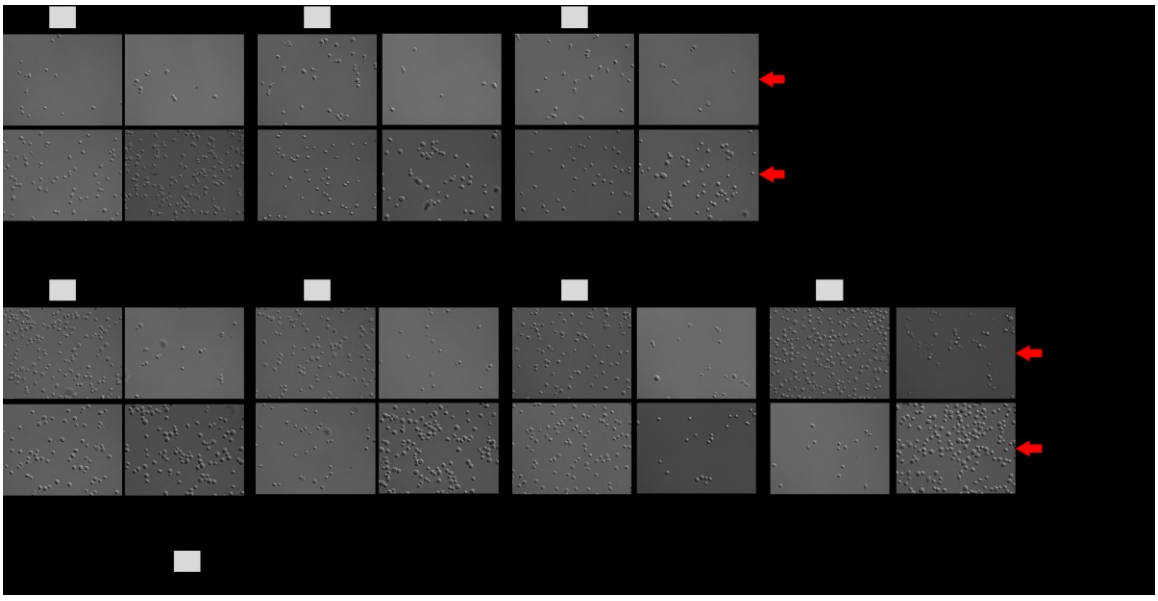

Figure 2.4. Throughout the entire chronological lifespan, ″heavy″ Q cells cultured under CR conditions remain smaller than ″heavy″ Q cells cultured under non-CR conditions. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll

density gradient as described in ″Materials and Methods″. ″Light″ and ″heavy″ cell subpopulations of NQ and Q cells, respectively, recovered following centrifugation in Percoll density gradient were subjected to differential interference contrast microscopical examination.

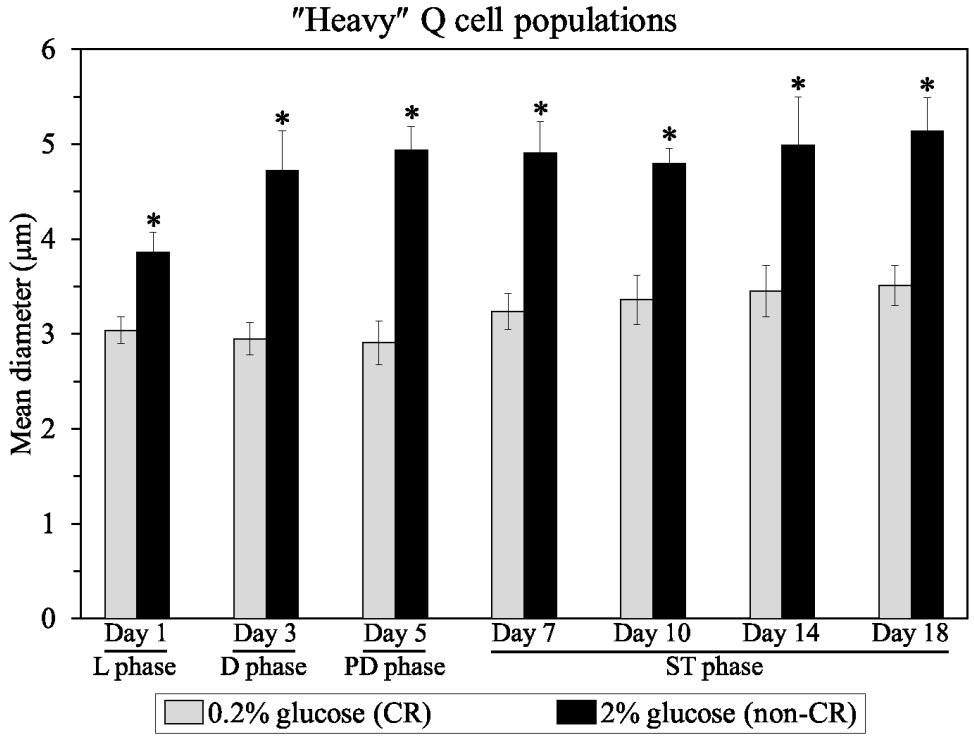

Figure 2.5. Throughout the entire chronological lifespan, ″heavy″ Q cells cultured under CR conditions remain smaller than ″heavy″ Q cells cultured under non-CR conditions. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. ″Light″ and ″heavy″ cell subpopulations of NQ and Q cells, respectively, recovered following centrifugation in Percoll density gradient were subjected to differential interference contrast microscopical examination. The mean diameter of ″heavy″ Q cells was calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 7 – 11); * $p < 0.05$.

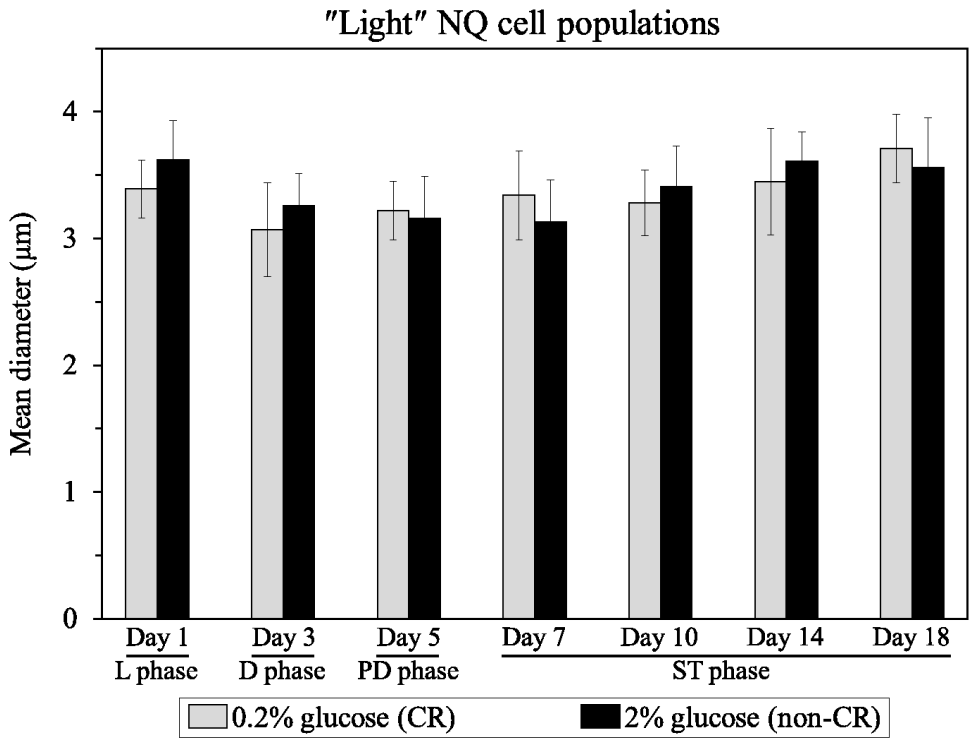

Figure 2.6. Throughout the entire chronological lifespan, ″light″ NQ cells cultured under CR conditions have sizes similar to those of ″light″ NQ cells cultured under non-CR conditions. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. ″Light″ and ″heavy″ cell subpopulations of NQ and Q cells, respectively, recovered following centrifugation in Percoll density gradient were subjected to differential interference contrast microscopical examination. The mean diameter of ″light″ NQ cells was calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 7 – 11).

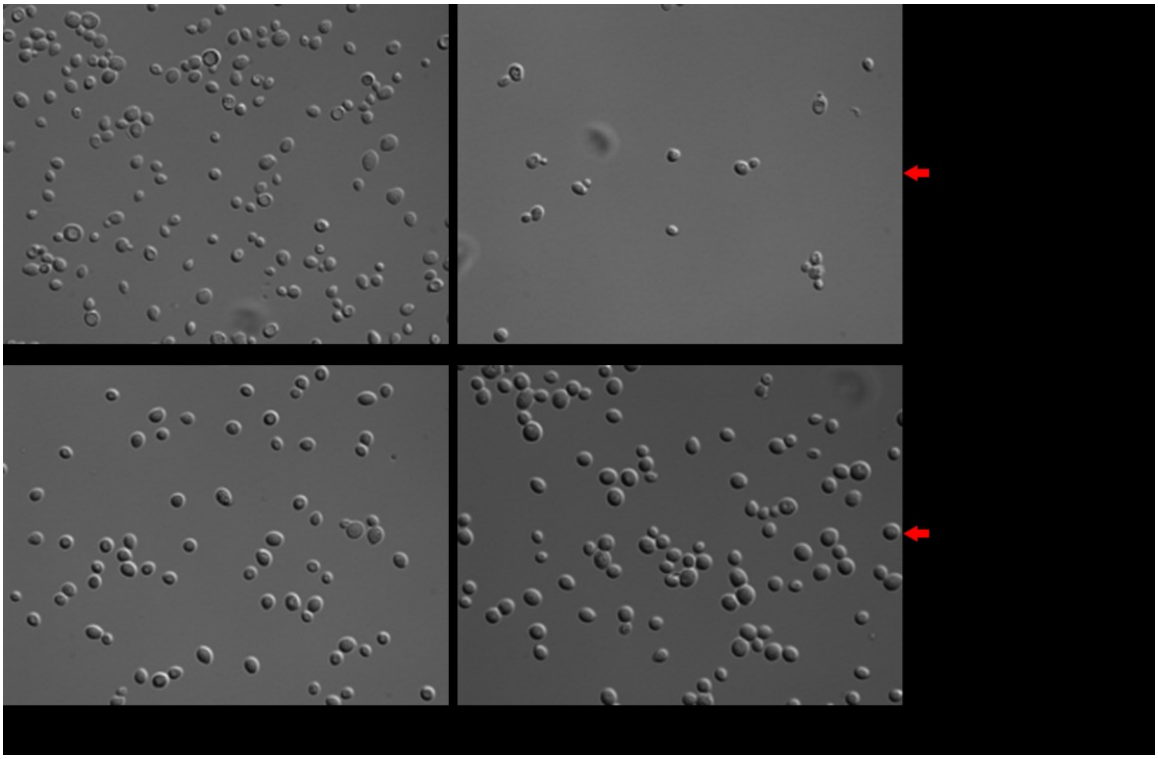

Figure 2.7. Throughout the entire chronological lifespan, ″heavy″ Q cells cultured either under CR conditions or under non-CR conditions remain unbudded. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. ″Light″ and ″heavy″ cell subpopulations of NQ and Q cells, respectively, recovered following centrifugation in Percoll density gradient were subjected to differential interference contrast microscopical examination.

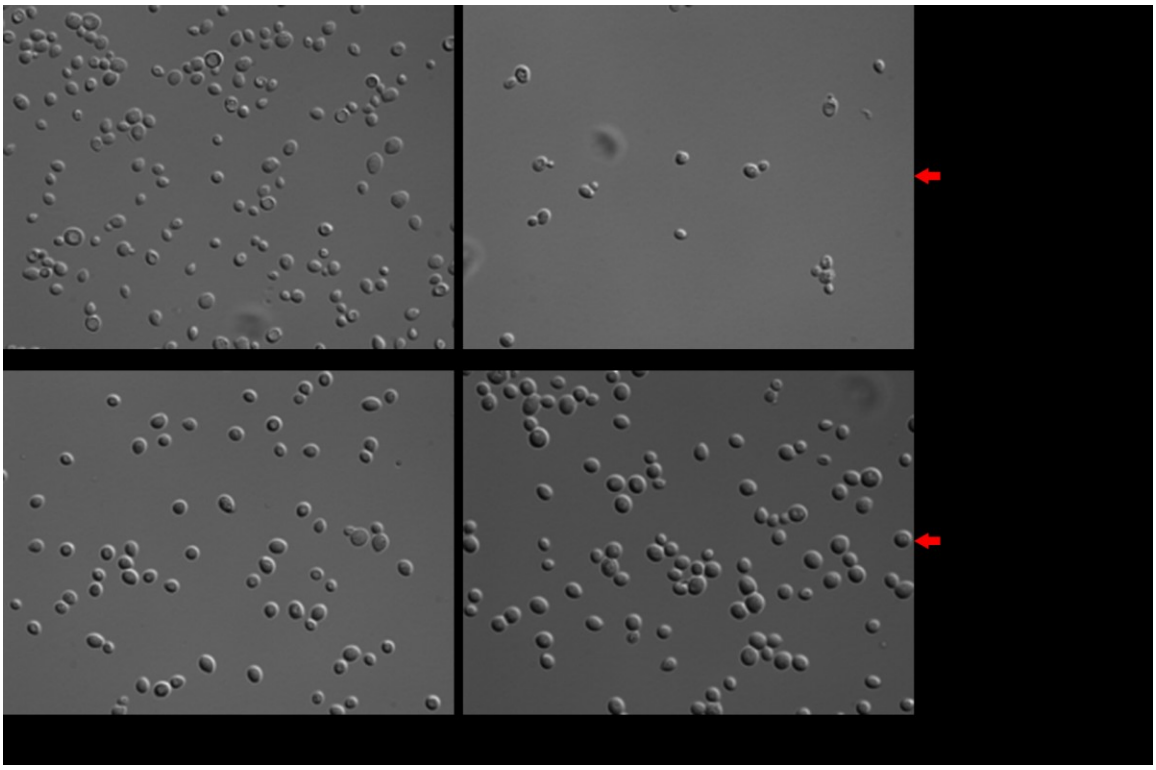

Figure 2.8. Throughout the entire chronological lifespan, ″light″ NQ cells cultured either under CR conditions or under non-CR conditions represent a mixture of budded and unbudded cells. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in "Materials and Methods". ″Light″ and ″heavy″ cell subpopulations of NQ and Q cells, respectively, recovered following centrifugation in Percoll density gradient were subjected to differential interference contrast microscopical examination.

2.3.3 Reproductive (colony-forming) capacities of Q and NQ cells

I then investigated how CR influences the reproductive (colony-forming) capacity of ″heavy″ Q cells and ″light″ NQ cells recovered from chronologically aging yeast cultures with the help of centrifugation in Percoll density gradient. This capacity was monitored by assessing the ability of recovered ″heavy″ Q cells and ″light″ NQ cells to form colonies on solid YEP medium after 2 days of incubation, as described in ″Materials and Methods″. I found that: 1) ″light″ NQ cells cultured under CR conditions maintain reproductive (colony-forming) capacity for a long time, whereas ″light″ NQ cells cultured under non-CR conditions exhibit a much more rapid decline in

reproductive competence (Figure 2.9), and 2) ″heavy″ Q cells cultured under CR conditions maintain reproductive (colony-forming) capacity for a long time, whereas ″heavy″ Q cells cultured under non-CR conditions exhibit an age-related gradual decline in reproductive competence (Figure 2.10).

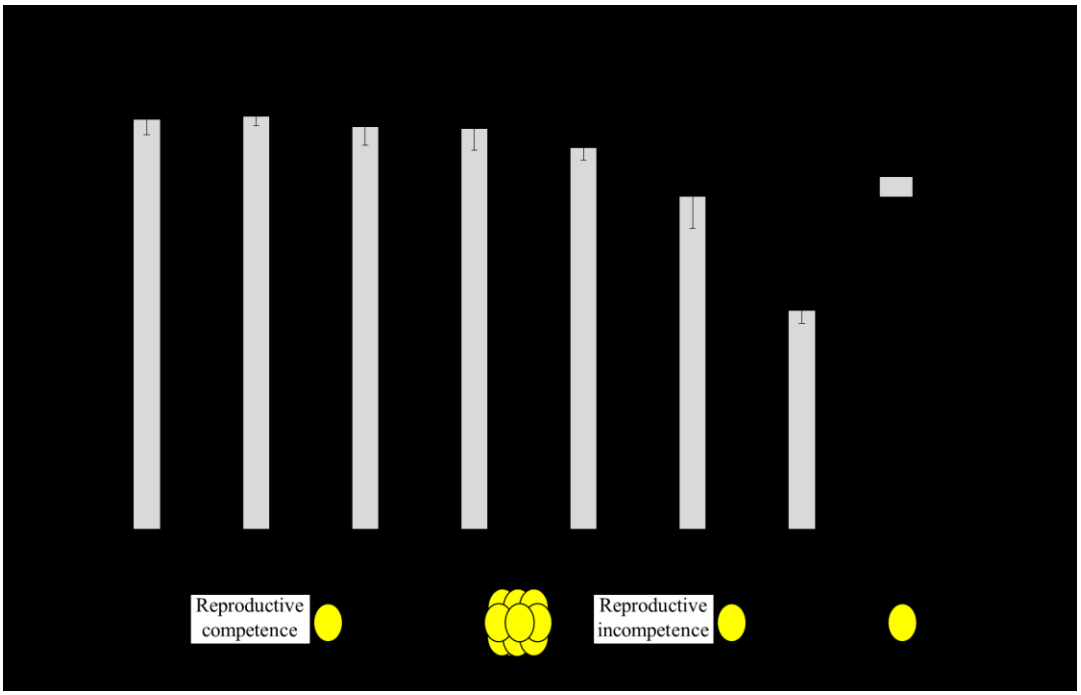

Figure 2.9. ″Light″ NQ cells cultured under CR conditions maintain reproductive (colony-forming) capacity for a long time, whereas ″light″ NQ cells cultured under non-CR conditions exhibit a much more rapid decline in reproductive competence. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered ″light″ NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 9 – 17); * $p < 0.05$.

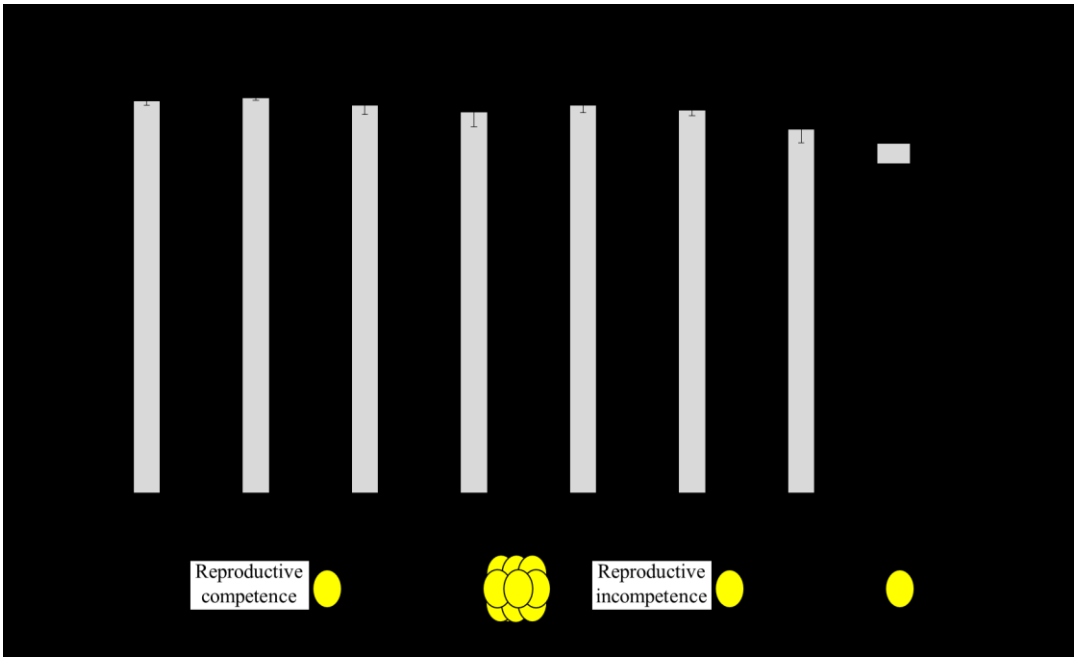

Figure 2.10. ″Heavy″ Q cells cultured under CR conditions maintain reproductive (colony-forming) capacity for a long time, whereas ″heavy″ Q cells cultured under non-CR conditions exhibit an age-related gradual decline in reproductive competence. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered ″heavy″ Q cells to form colonies on solid YPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 11 – 18); * $p < 0.05$.

2.3.4 Abilities of Q and NQ cells to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium

I then examined how CR impacts the abilities of ″light″ NQ cells and ″heavy″ Q cells to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and incubation for 1 to 4 h. I found that: 1) ″light″ NQ cells cultured under CR conditions

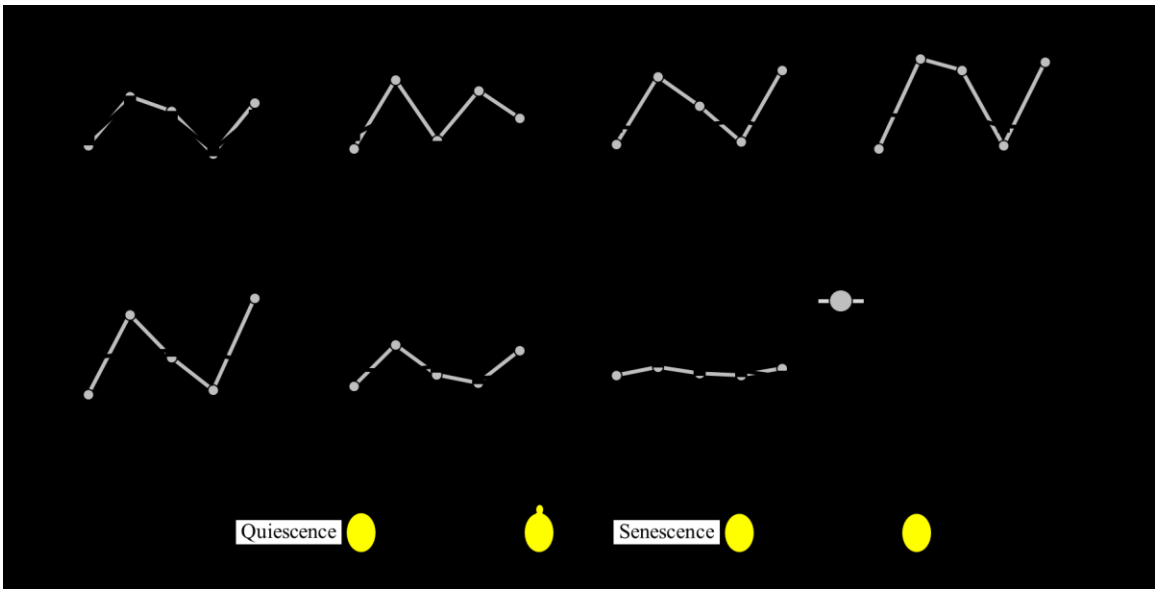

Figure 2.11. ″Light″ NQ cells cultured under CR conditions remain quiescent (i.e. maintain the ability to synchronously re-enter the mitotic cell cycle) for a long time, whereas ″light″ NQ cells cultured under non-CR conditions become non-quiescent (i.e. senescent) soon after being formed in the process of differentiation. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The ability of ″light″ NQ cells to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and incubation for 1 to 4 h was monitored as described in ″Materials and Methods″. Data are presented as means (n = 3 – 6).

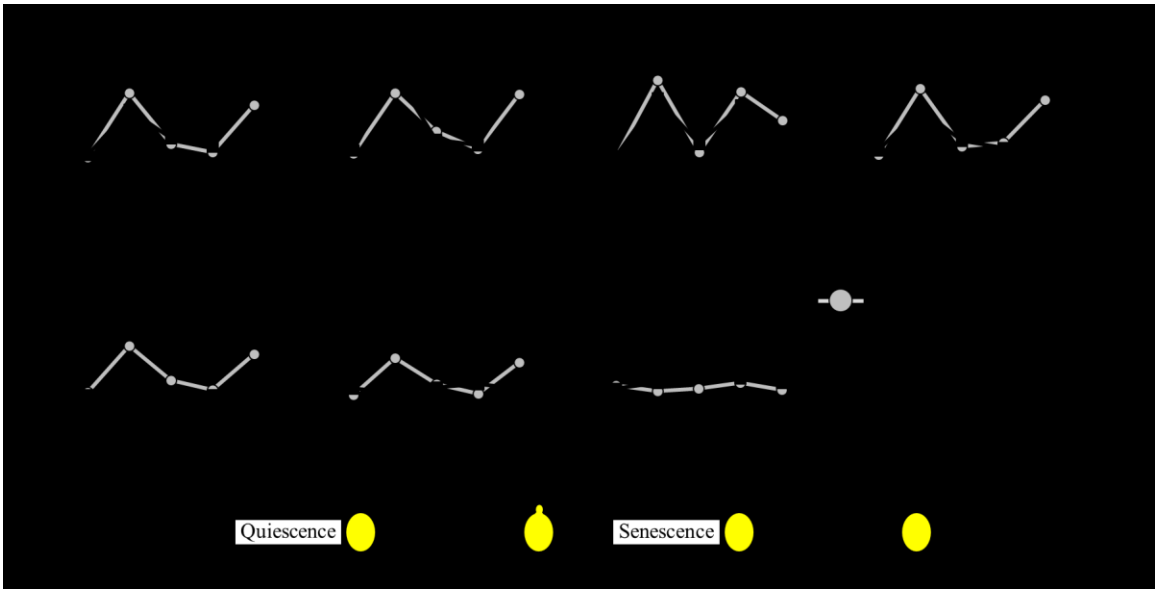

Figure 2.12. ″Heavy″ Q cells cultured under CR conditions remain quiescent (i.e. maintain the ability to synchronously re-enter the mitotic cell cycle) for a long time, whereas ″heavy″ Q cells cultured under non-CR conditions gradually become non-quiescent (i.e. senescent). Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The ability of ″heavy″ Q cells to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and incubation for 1 to 4 h was monitored as described in ″Materials and Methods″. Data are presented as means (n = 3 – 6).

remain quiescent (i.e. maintain the ability to synchronously re-enter the mitotic cell cycle) for a long time, whereas ″light″ NQ cells cultured under non-CR conditions become non-quiescent (i.e. senescent) soon after being formed in the process of differentiation (Figure 2.11), and 2) ″heavy″ Q cells cultured under CR conditions remain quiescent (i.e. maintain the ability to synchronously re-enter the mitotic cell cycle) for a long time, whereas ″heavy″ Q cells cultured under non-CR conditions gradually become non-quiescent (i.e. senescent) (Figure 2.12).

2.3.5 Abilities of Q and NQ cells to maintain viability measured as metabolic activity

I then used a LIVE/DEAD yeast viability kit to monitor the effect of CR on the abilities of ″light″ NQ and ″heavy″ Q cells to maintain viability through the process of chronological aging. In the LIVE/DEAD assay, viable (and, thus, metabolically active) yeast cells convert the yellow-green-fluorescent intracellular staining of FUN 1 into red-orange-fluorescent intra-vacuolar structures that are deposited in these organelles. My data imply that: 1) ″light″ NQ cells cultured under CR conditions maintain viability for a long time, whereas ″light″ NQ cells cultured under non-CR conditions exhibit a much more rapid decline in viability (Figure 2.13), and 2) ″heavy″ Q cells cultured under CR conditions maintain viability for a long time, whereas ″heavy″ Q cells

cultured under non-CR conditions exhibit an age-related gradual decline in viability (Figure 2.14).

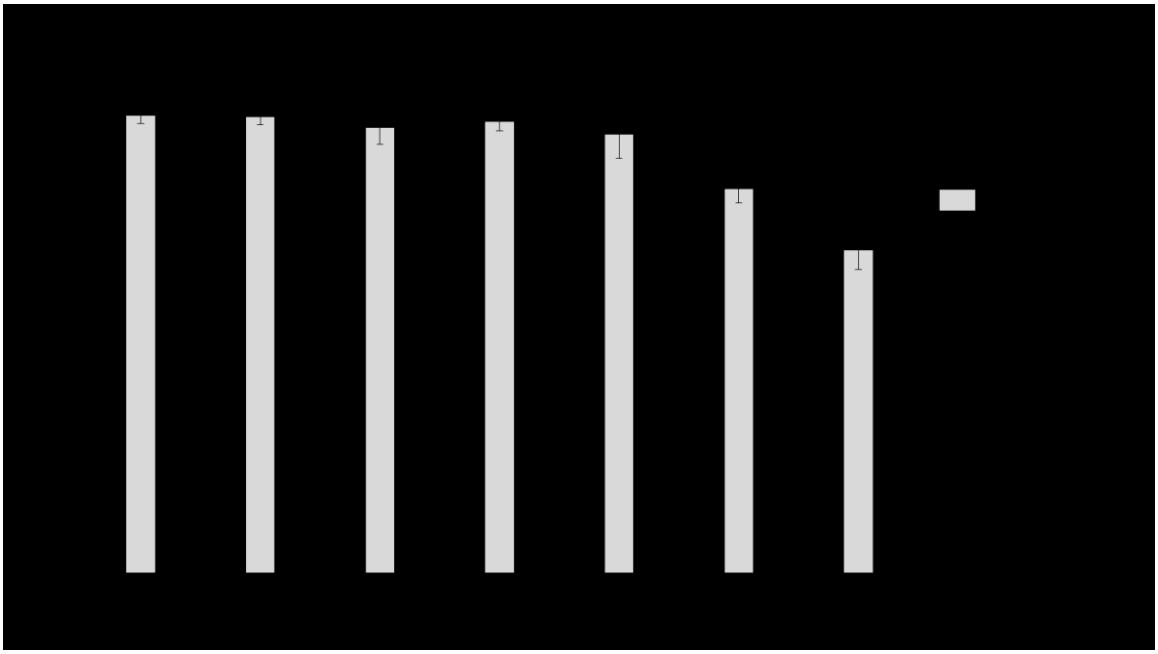

Figure 2.13. ″Light″ NQ cells cultured under CR conditions maintain viability for a long time, whereas ″light″ NQ cells cultured under non-CR conditions exhibit a much more rapid decline in viability. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. Viability of ″light″ NQ cells was measured using a LIVE/DEAD yeast viability kit. Viable cells convert the yellow-green-fluorescent intracellular staining of FUN 1 into red-orange-fluorescent intra-vacuolar structures. Data are presented as means ± SEM (n = 7 – 12); * $p < 0.05$.

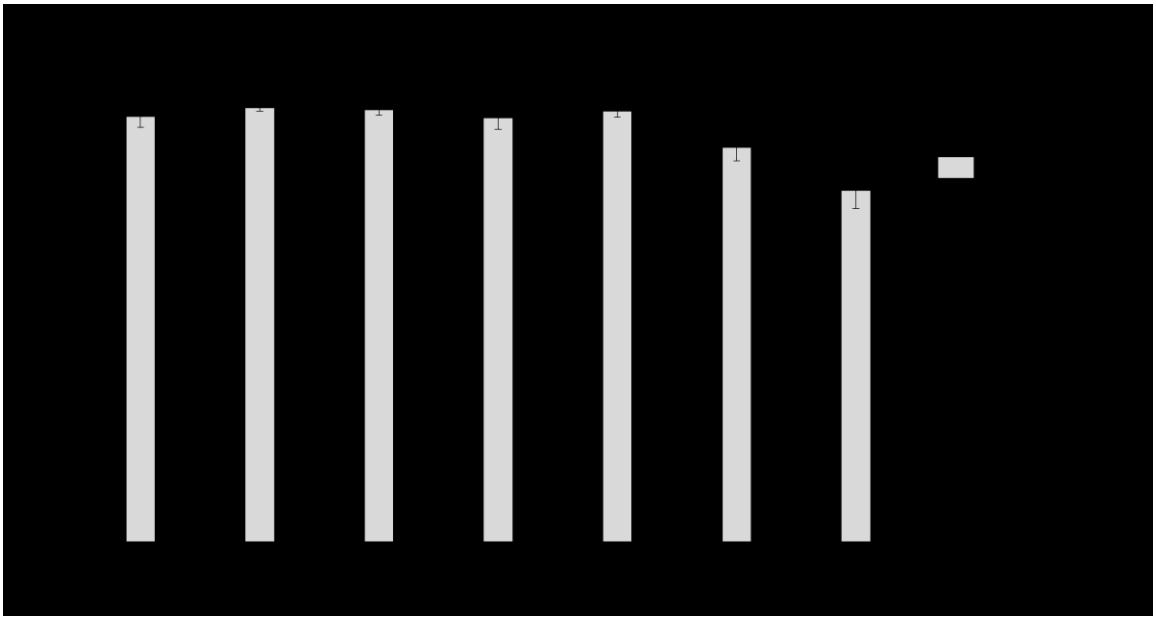

Figure 2.14. ″Heavy″ Q cells cultured under CR conditions maintain viability for a long time, whereas ″heavy″ Q cells cultured under non-CR conditions exhibit an age-related gradual decline in viability. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. Viability of ″heavy″ Q cells was measured using a LIVE/DEAD yeast viability kit. Viable cells convert the yellow-green-fluorescent intracellular staining of FUN 1 into red-orange-fluorescent intra-vacuolar structures. Data are presented as means ± SEM (n = 7 - 12); * $p < 0.05$.

2.3.6 Mitochondrial respiration, electrochemical potential and ROS in Q and NQ cells

An age-related progressive decline in mitochondrial function is known to be one of the characteristic features (and, perhaps, is one of the underlying reasons) of cellular and organismal aging in yeast and multicellular eukaryotes [82, 85, 89, 92, 96, 144 - 156]. One of the key longevity-defining traits of mitochondrial functionality is coupled respiration [82, 89, 92, 96, 144, 148, 150]. Therefore, I monitored how CR impact mitochondrial respiration in ″light″ NQ

cells and ″heavy″ Q cells. I found that: 1) ″light″

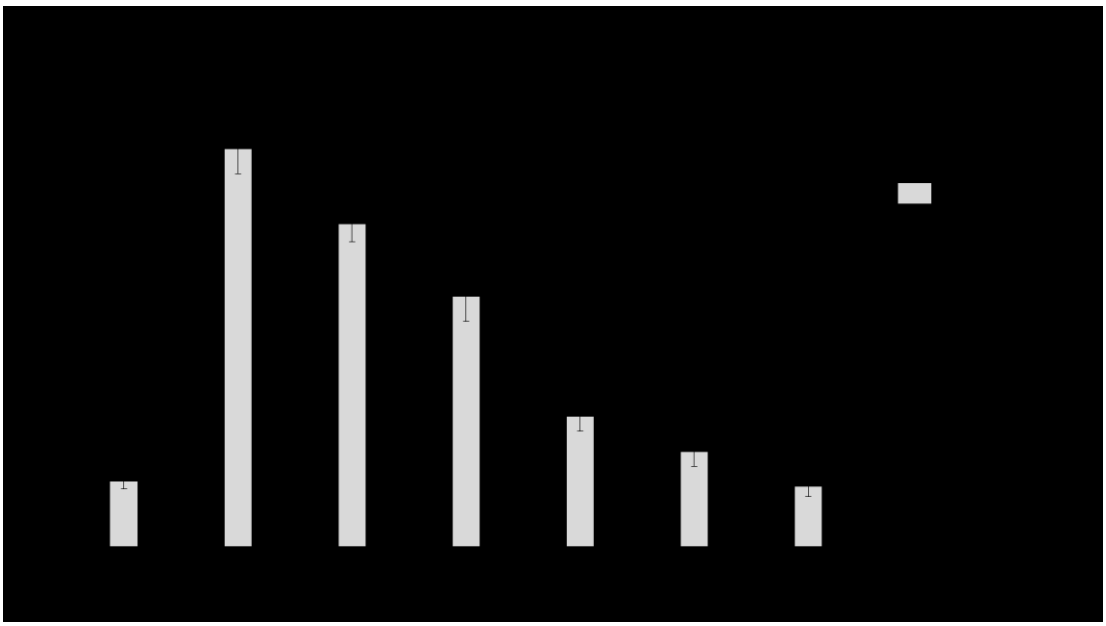

Figure 2.15. ″Light″ NQ cells cultured under CR conditions maintain a relatively high rate of mitochondrial respiration for a long period of time, whereas ″light″ NQ cells cultured under non-CR conditions exhibit a much lower rate of mitochondrial respiration and its rapid decline with age. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. Mitochondrial respiration of ″light″ NQ cells was measured as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 12 – 17); * $p < 0.05$.

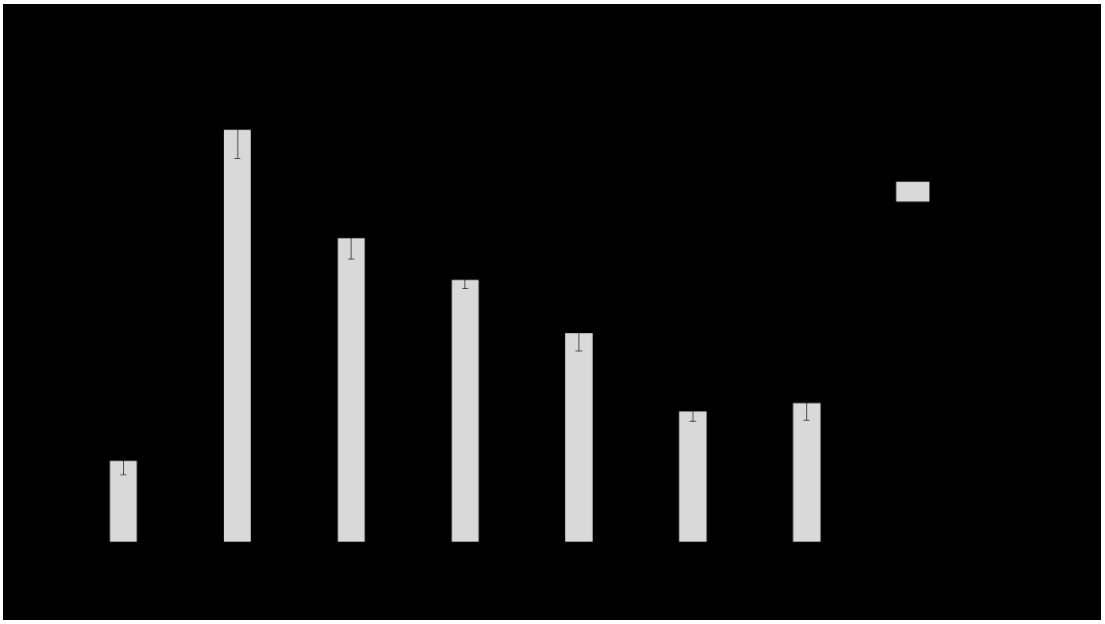

Figure 2.16. ″Heavy″ Q cells cultured under CR conditions maintain a very high rate of mitochondrial respiration for a long period of time, whereas ″heavy″ Q cells cultured under non-CR conditions exhibit a much lower rate of mitochondrial respiration during the same time period. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. Mitochondrial respiration of ″heavy″ Q cells was measured as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 12 – 17); * $p < 0.05$.

NQ cells cultured under CR conditions maintain a relatively high rate of mitochondrial respiration for a long time, whereas ″light″ NQ cells cultured under non-CR conditions exhibit a much lower rate of mitochondrial respiration and its rapid decline with age (Figure 2.15), and 2) ″heavy″ Q cells cultured under CR conditions sustain a very high rate of mitochondrial respiration for a long time, whereas ″heavy″ Q cells cultured under non-CR conditions exhibit a much lower rate of mitochondrial respiration for the same time interval (Figure 2.16).

I then assessed how CR influences the electrochemical potential across the inner mitochondrial membrane ($\Delta\Psi_m$) in ″light″ NQ cells and ″heavy″ Q cells. My data implies

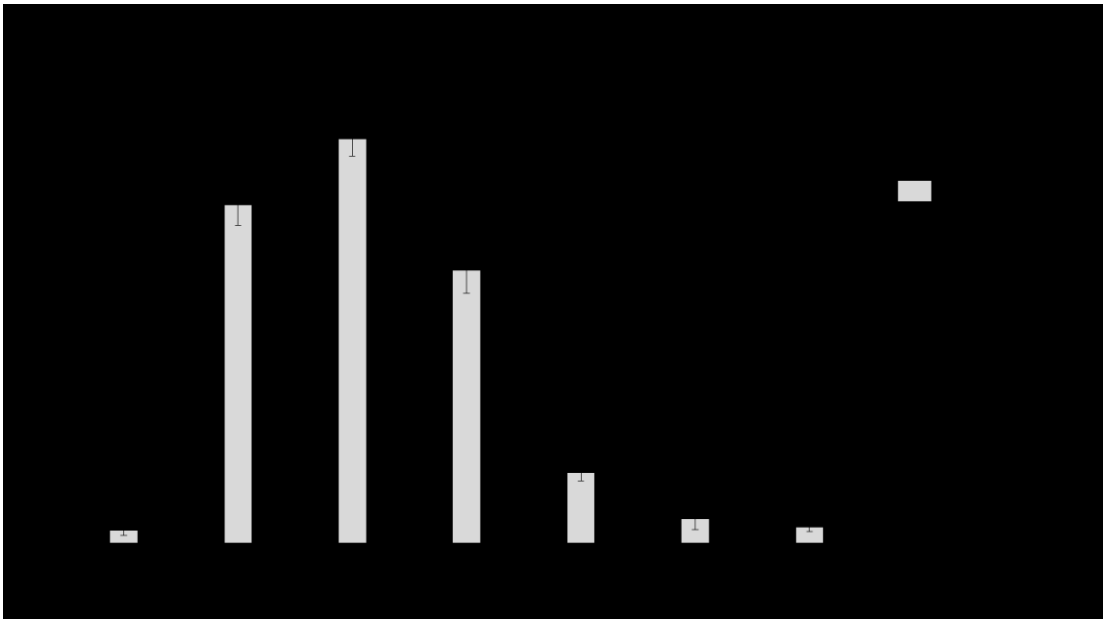

Figure 2.17. ″Light″ NQ cells cultured under CR conditions sustain a relatively high value of $\Delta\Psi_m$ for an extended period of time, whereas ″light″ NQ cells cultured under non-CR conditions display a significantly lower value of $\Delta\Psi_m$ and its fast drop with age. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. Mitochondrial electrochemical potential ($\Delta\Psi_m$) of ″light″ NQ cells was measured as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 9 – 13); * $p < 0.05$.

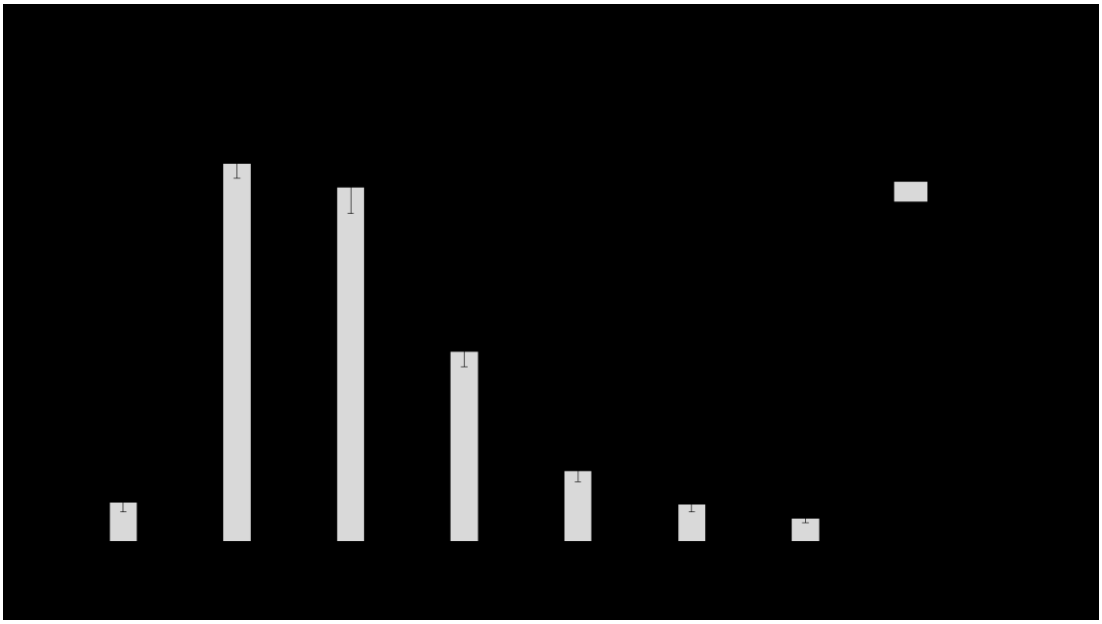

Figure 2.18. ″Heavy″ Q cells cultured under CR conditions sustain a very high value of $\Delta\Psi_m$ for an extended period of time, whereas ″heavy″ Q cells cultured under non-CR conditions exhibit a significantly lower value of $\Delta\Psi_m$ during the same time period. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) or 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. Mitochondrial electrochemical potential ($\Delta\Psi_m$) of ″heavy″ Q cells was measured as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 9 – 13); * $p < 0.05$.

that: 1) ″light″ NQ cells cultured under CR conditions sustain a high value of $\Delta\Psi_m$ for an extended period of time, whereas ″light″ NQ cells cultured under non-CR conditions display a significantly lower value of $\Delta\Psi_m$ and its fast drop with age (Figure 2.17), and 2) ″heavy″ Q cells cultured under CR conditions sustain a very high value of $\Delta\Psi_m$ for an extended period of time, whereas ″heavy″ Q cells cultured under non-CR conditions exhibit a significantly lower value of $\Delta\Psi_m$ during the same time period (Figure 2.18).

2.4 Discussion

Studies described in this chapter of my thesis have demonstrated that there are two differentiation programs that in chronologically aging yeast link cellular aging to cell cycle regulation, preservation of a Q state, and entry into and advancement through an NQ state. One of them functions in yeast cultured under CR conditions, whereas another program acts in yeast grown under non-CR conditions. My comparative analyses of morphological, biochemical, physiological and reproductive properties of Q and NQ cells, that were purified from yeast cultures progressing through different stages of the chronological aging process, suggest the following model for the two differentiation programs underlying the intricate age-related relationships existing between various subpopulations of Q and NQ cells (Figure 2.19).

In the model that I propose here, ″heavy″ Q cells in yeast cultured under CR or non-CR conditions represent a ″stem cell niche″ - i.e. a subpopulation of non-differentiated Q cells. I call these cells G_q^H; this is because they are arrested in the first ″gap″ phase of the cell cycle and represent Q cells of high density. As I demonstrated, almost all G_q^H cells constituting the stem cell niche are viable unbudded cells that exhibit high reproductive (colony-forming) capacity, able to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and have fully functional mitochondria (Figure 2.19). Of note, other students in the Titorenko laboratory have recently revealed that almost all G_q^H cells also display high concentrations of reserve carbohydrates such as trehalose and glycogen, exhibit low concentrations of ROS (which are formed mainly as by-products of mitochondrial respiration), do not accumulate oxidatively damaged proteins and lipids at high levels, are resistant to excessive oxidative and thermal stresses, have stable nuclear and mitochondria genomes (judging from the low frequencies of spontaneous point and deletion mutations in the nuclear and mitochondrial DNA), and display

reduced susceptibilities to mitochondria-controlled apoptotic and lipid-induced necrotic forms of programmed cell death. If a population of yeast cells is placed on a CR diet by culturing in the complete (nutrient-rich) medium initially containing 0.2% glucose, a significant portion of the non-differentiated G_q^H stem cells undergo cell cycle arrest when they reach late L growth phase. Judging from the very small size of G_q^H stem cells under CR conditions, their cell cycle is arrested at a previously unknown checkpoint in early G_1.

As the non-differentiated G_q^H stem cells under CR conditions age chronologically, some of them commit themselves to entry into the differentiation program 1 (Figure 2.19). Such commitment of G_q^H to differentiation is manifested in a significant reduction of their density, so that they are converted to a subpopulation of Q cells that I call G_q^L because of their low density. The progression of G_q^L cells through the differentiation program 1 causes a progressive decline in their viability, reproductive (colony-forming) capacity, ability to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and mitochondrial functionality (Figure 2.19). In addition (as other students in the Titorenko laboratory have recently demonstrated), such progression of G_q^L cells through the differentiation program 1 causes 1) a decline in the concentrations of trehalose and glycogen (such decline is, likely, responsible for the low density of G_q^L cells), 2) a gradual rise of intracellular ROS, 3) an increase in the levels of oxidatively damaged proteins and lipids, 4) a reduction in cell resistance to excessive oxidative and thermal stresses, 5) a decline in stabilities of nuclear and mitochondria genomes, 6) a rise in cell susceptibilities to mitochondria-controlled apoptotic and lipid-induced necrotic forms of programmed cell death. As studies described in this Chapter of my thesis revealed, at late stages of the differentiation program 1 G_q^L cells become NQ and then senescent (i.e. become unable to re-enter the mitotic cell cycle following cell transfer to a fresh medium) and, thus, undergo

conversion to a G_{nq}^{L} form (Figure 2.19). As other students in the Titorenko laboratory have recently shown, the subpopulation of aged G_{nq}^{L} cells is committed to undergoing an age-related apoptotic form of programmed cell death (Figure 2.19).

Furthermore, in the model that I propose here, when a yeast culture grown under CR conditions enters ST phase, most of non-differentiated G_{q}^{H} cells from the stem cell niche have committed themselves to entry into and progression through the differentiation program 1 and exist in a G_{nq}^{L} form (Figure 2.19). However, a minor portion of cells that reach ST phase still remains in a G_{q}^{H} form (Figure 2.19). Through the entire chronological lifespan, these non-differentiated stem cells progress through a maintenance program which I call ″maintenance program 1″. As studies described in this Chapter of my thesis have revealed, a progression of G_{q}^{H} stem cells through this maintenance program is manifested in a gradual deterioration in their viability, reproductive (colony-forming) capacity, ability to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and mitochondrial functionality (Figure 2.19). In addition (as other students in the Titorenko laboratory have recently revealed), such progression of G_{q}^{H} cells through the maintenance program 1 causes 1) an increase of intracellular ROS, 2) a rise in the levels of oxidatively damaged proteins and lipids, 3) a reduction in cell resistance to excessive oxidative and thermal stresses, 4) a deterioration in stabilities of nuclear and mitochondria genomes, and 5) an increase in cell susceptibilities to mitochondria-controlled apoptotic and lipid-induced necrotic forms of programmed cell death. As studies described in this Chapter of my thesis have demonstrated, at late stages of the maintenance program 1 G_{q}^{H} cells become NQ and then senescent (i.e. become unable to re-enter the mitotic cell cycle following cell transfer to a fresh medium) and, thus, undergo conversion to a G_{nq}^{H} form (Figure 2.19). As other students in the Titorenko laboratory have recently revealed, the subpopulation of

aged G_{nq}^{H} cells is committed to undergoing an age-related form of programmed cell death, either apoptotic or necrotic (Figure 2.19).

Moreover, the model I propose here also posits that, when a population of yeast cells is placed on a non-CR diet by culturing in the complete (nutrient-rich) medium

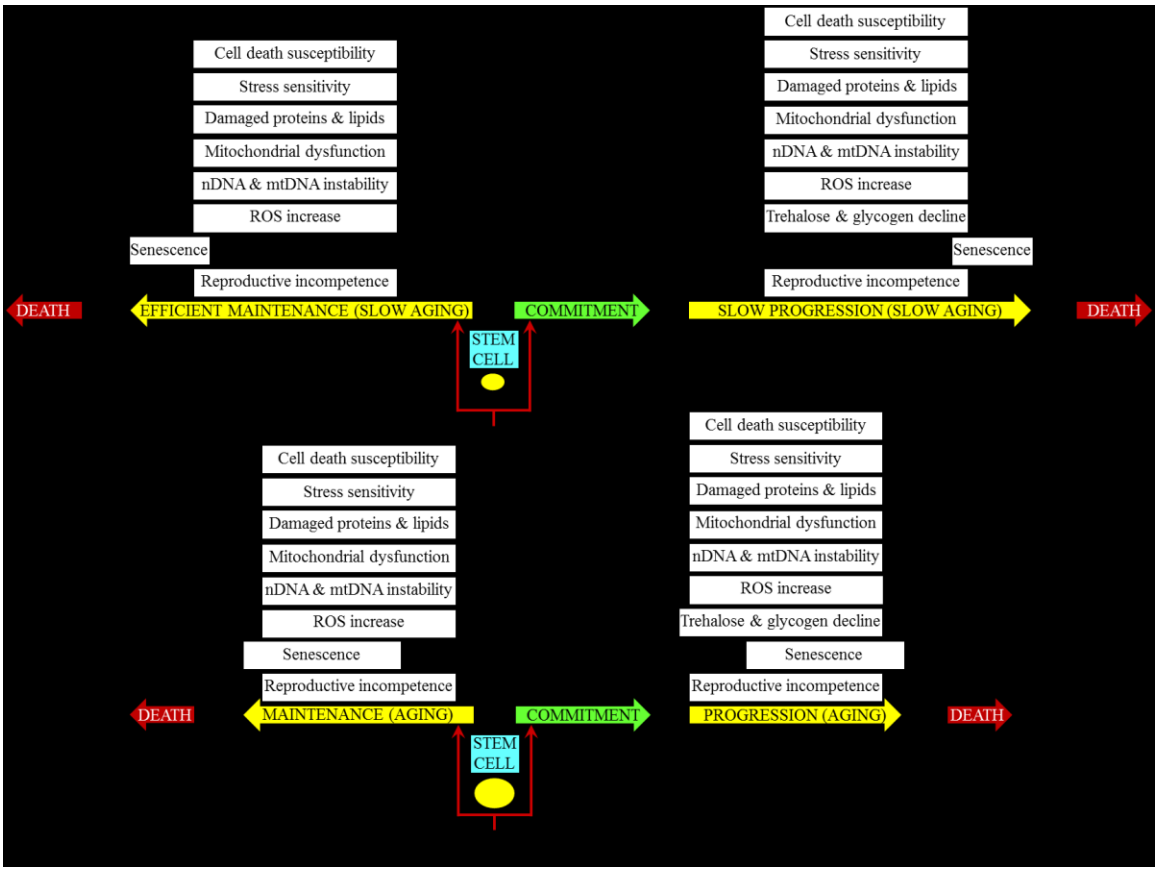

Figure 2.19. A model of two differentiation programs and two maintenance programs that in chronologically aging yeast link cellular aging to cell cycle regulation, preservation of a Q state, and entry into and advancement through an NQ state. The differentiation program 1 and the maintenance program 1 function in yeast cultured under CR conditions, whereas the differentiation program 2 and the maintenance program 2 operate in yeast grown under non-CR conditions. In this model, the two differentiation programs and the two maintenance program define longevity of chronologically aging yeast by setting up the following: 1) a growth phase in which G_1 cell cycle arrest occurs, 2) the efficacy with which a stem cell niche of heavy Q cells (G_q^{H}) is maintained and depleted by converting to heavy NQ cells (G_{nq}^{H}) in an age-related manner, 3) the age-related rate with which heavy Q cells progress through apoptotic and necrosis-like subroutines of programmed death, 4) the age-related rate with which heavy Q cells are converted to light Q cells (G_q^{L}), 5) the age-related rate with which light Q cells differentiate into light NQ cells (G_{nq}^{L}), and 6) the age-related rate with which light NQ cells advance through apoptotic and necrosis-like forms of programmed death. Abbreviations: CR, caloric restriction;

L, logarithmic; nDNA, nuclear DNA; NL, nutrient limitation; mDNA, mitochondrial DNA; ST, stationary.

initially containing 2% glucose, a significant portion of the G_q^H stem cells undergo cell cycle arrest only when they reach ST phase. Judging from the large size of G_q^H stem cells under non-CR conditions, their cell cycle is arrested at the checkpoint START A in late G_1 and is caused by nutrient limitation – likely because at that point cells are becoming deprived of nitrogen-containing compounds (Figure 2.19).

In my model, as G_q^H stem cells under non-CR conditions undergo chronological aging, some of them gradually become committed to entry into the differentiation program 2 (Figure 2.19). Such commitment of G_q^H stem cells to differentiation program 2 is reflected in a decrease of their density, so that they are converted to a subpopulation of G_q^L cells. As studies described in this Chapter of my thesis indicate, the progression of these G_q^L cells through the differentiation program 2 under non-CR conditions occurs faster than that of G_q^L cells through the differentiation program 1 under CR conditions; thus, the longevity-extending effect of CR is due, at least in part, to the decelerating effect of this dietary regimen on the differentiation of the subpopulation of G_q^L cells (Figure 2.19). Akin to the differentiation program 1 progressing under CR conditions, the differentiation program 2 under non-CR conditions reduces viability of G_q^L cells, decreases their reproductive (colony-forming) capacity and lowers their ability to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and mitochondrial functionality (Figure 2.19). Moreover, my findings imply that at late stages of the differentiation program 2 G_q^L cells rapidly become NQ and eventually senescent (i.e. they cannot re-enter anymore the mitotic cell cycle following cell transfer to a fresh medium) and, thus, undergo conversion to a G_{nq}^L form (Figure 2.19). According to data of other students in the

Titorenko laboratory, aged G_{nq}^{L} cells are committed to apoptotic programmed cell death (Figure 2.19).

According to my model, similar to G_q^H cells under CR conditions, some of the non-differentiated G_q^H stem cells under non-CR advance through a maintenance program which I call ″maintenance program 2″ (Figure 2.19). As my findings imply, an aging-related advancement of G_q^H cells through this maintenance program is manifested in a rapid decline in the viability of these stem cells, their reproductive (colony-forming) capacity, their ability to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and their mitochondrial functionality (Figure 2.19). At late stages of the maintenance program 2 G_q^H cells become NQ and ultimately senescent and, hence, undergo conversion to a G_{nq}^H form; some of these NQ cells undergo an apoptotic form of programmed death, whereas others are committed to a necrosis-like subroutine of programmed death (Figure 2.19).

Altogether, my findings imply that the differentiation program 1 and the maintenance program 1 (both operating in yeast cultured under CR conditions), as well as the differentiation program 2 and the maintenance program 2 (both of which act in yeast grown under non-CR conditions), define longevity of chronologically aging yeast by setting up the following: 1) a growth phase in which G_1 cell cycle arrest occurs, 2) the efficacy with which a stem cell niche of heavy Q cells is maintained and depleted in an age-related manner, 3) the age-related rate with which heavy Q cells progress through apoptotic and necrosis-like subroutines of programmed death, 4) the age-related rate with which heavy Q cells are converted to light Q cells, 5) the age-related rate with which light Q cells differentiate into light NQ cells, and 6) the age-related rate with which light NQ cells advance through apoptotic and necrosis-like forms of programmed death.

3 Regulation of longevity-defining differentiation programs that link cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state

3.1 Abstract

Lithocholic acid (LCA), a natural bile acid, has been shown to extend the chronological lifespan of yeast under both CR and non-CR conditions [16, 27, 88, 90]. Furthermore, the lifespan of chronologically aging yeast can also be extended by mutations that impair the pro-aging Ras/cAMP/PKA signaling pathway; this pathway is one of the key regulators linking carbon source availability to cell growth and metabolism [8 – 10, 100, 137 - 143]. Moreover, the intracellular concentrations of trehalose, a non-reducing disaccharide, prior to entry of a yeast culture into a non-proliferative state and following such entry play essential and differing roles in defining longevity of chronologically aging yeast; this is because trehalose is involved in modulating protein folding, misfolding, unfolding, refolding, oxidative damage, solubility and aggregation throughout lifespan [91]. In studies described in this Chapter of my thesis, I investigated how LCA, the Ras/cAMP/PKA signaling pathway and trehalose influence the two longevity-defining differentiation programs that I have discovered in studies described in Chapter 2 of my thesis. Here I provide evidence that LCA, the Ras/cAMP/PKA signaling pathway and trehalose define longevity of chronologically aging yeast by regulating various stages of the two differentiation programs linking cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state.

3.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), as well as the single-gene-deletion mutant strains *ras2Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ras2Δ::kanMX4*) and *tps1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tps1Δ::kanMX4*) in the BY4742 genetic background were all from Thermo Scientific/Open Biosystems. Yeast strains were grown in YEP medium (1% yeast extract, 2% peptone; both from Fisher Scientific; #BP1422-2 and #BP1420-2, respectively) initially containing 0.2% or 2% glucose (#D16-10; Fisher Scientific) as carbon source. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1.

Chronological lifespan assay for cell cultures

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 per ml of culture using a hemacytometer (#0267110; Fisher Scientific). 10 μl 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 μl. 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 × dilution factor × 10 × 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 (1% yeast extract, 2% peptone, 2% glucose) plates in duplicate in order to count the number of viable cells per ml of each culture. 100 μl of diluted culture was plated onto each plate. After 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 CFU × dilution factor × 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 × 100%. The % viability of cells in mid-logarithmic phase was set at 100% viability for that particular culture.

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 water was made on the day of adding this compound to cell cultures. LCA was added to growth medium in water at the final concentration of 50 μM immediately following cell inoculation into the medium.

Separation of Q and NQ cells by centrifugation in Percoll density gradient

1 ml of 1.5 M NaCl (#S7653; Sigma) was placed into a 50-ml Falcon tube, and 8 ml of the Percoll solution (#P1644; Sigma) was added to this tube. The NaCl and Percoll solutions were then mixed by pipetting. To form two Percoll density gradients, 4 ml of the NaCl/Percoll mixture was put into each of the two polyallomer tubes for an MLS-50 rotor for an Optima MAX ultracentrifuge (all from Beckman Coulter). The tubes were centrifuged at 25,000 × g (16,000 rpm) for 15 min at 4^{o}C in an Optima MAX ultracentrifuge. A sample of yeast 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 per ml of culture using a hemacytometer (#0267110; Fisher Scientific). For each Percoll density gradient, 1×10^9 yeast cells were placed into a 15-ml Falcon tube and then pelleted by centrifugation at 5,000 rpm for 7 min at room temperature in a Centra CL2

clinical centrifuge (Thermo IEC). Pelleted cells were resuspended in 500 µl of 50 mM Tris/HCl buffer (pH 7.5), overlaid onto the preformed gradient and centrifuged at 2,300 × g (5,000 rpm) for 30 min at 25^{o}C in an Optima MAX ultracentrifuge. The upper and lower fractions of cells were removed with a pipette, Percoll was removed by washing cells twice with 50 mM Tris/HCl buffer (pH 7.5) and cells were resuspended in 50 mM Tris/HCl buffer (pH 7.5) for subsequent assays.

Cell number measurement for Q and NQ cells separated by centrifugation in Percoll density gradient

An aliquot of the upper or lower fraction of cells recovered from the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) was diluted in order to determine the total number of cells per fraction using a hemacytometer (#0267110; Fisher Scientific). 10 µl 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 µl. 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 × dilution factor × 10 × 1,000 = total number of cells per ml of fraction.

Reproductive (colony-forming) capability assay for Q and NQ cells separated by centrifugation in Percoll density gradient

An aliquot of the upper or lower fraction of cells recovered from the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) was diluted in order to determine the total number of cells per fraction using a hemacytometer (#0267110; Fisher Scientific). 10 µl 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 μl. 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 × dilution factor × 10 × 1,000 = total number of cells per ml of fraction. Serial dilutions ($1:10^2$ to $1:10^5$) of cells were plated onto YEPD (1% yeast extract, 2% peptone, 2% glucose) plates in duplicate in order to count the number of viable cells per ml of each cell fraction. 100 μl of diluted culture was plated onto each plate. After 48-h incubation at 30°C, the number of colonies per plate was counted. The number of colony forming units (CFU) equals to the number of reproductively capable cells in a sample. Therefore, the number of reproductively capable cells was calculated as follows: number of CFU × dilution factor × 10 = number of reproductively capable cells per ml. For each cell fraction assayed, % reproductive capability of the cells was calculated as follows: number of CFU per ml / total number of cells per ml × 100%.

Synchronous re-entry into mitosis assay for Q and NQ cells separated by centrifugation in Percoll density gradient

5×10^6 cells recovered in the upper or lower fraction of the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) were harvested by centrifugation for 1 min at 21,000 × g at room temperature. Pelleted cells were washed twice with water and then inoculated into 50 ml of YEP medium (1% yeast extract, 2% peptone; both from Fisher Scientific; #BP1422-2 and #BP1420-2, respectively) initially containing 0.2% or 2% glucose (#D16-10; Fisher Scientific) as carbon source. Cells were cultured for 4 h at 30°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 taken from a culture at a certain time-point and examined microscopically for the percentage of cells with new buds. At least 500 cells were examined per time point, and the budding percentage was

calculated as follows: (number of cells with new buds per ml / total number of cells per ml) × 100%.

Oxygen consumption assay for Q and NQ cells separated by centrifugation in Percoll density gradient

From 2×10^7 cells to 1×10^8 cells recovered in the upper or lower fraction of the Percoll gradient and washed twice with 50 mM Tris/HCl buffer (pH 7.5) were used for continuous measurement of the rate of oxygen consumption. The measurement was conducted 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 YEP medium (1% yeast extract, 2% peptone; both from Fisher Scientific; #BP1422-2 and #BP1420-2, respectively) supplemented with 0.2% glucose was added to the electrode for approximately 5 min to obtain a baseline. Cells recovered in the upper or lower fraction of the Percoll gradient of a known titer were pelleted by centrifugation at 3,000 × g for 5 min. The resulting pellet was resuspended in YEP 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 $min^{-1} \times 10^9$ cells.

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 extends yeast longevity by improving maintenance of the G_q^H stem cell niches as well as by delaying their entry into and progression through the differentiation programs linking cell cycle regulation to cellular aging

Using centrifugation of chronologically aging yeast cultures in Percoll density gradient to separate light NQ cells from heavy Q cells, I first investigated how LCA, a potent natural anti-aging compound [16, 27, 88, 90], impacts age-related changes in the relative abundancies of these two cell subpopulations. My studies described in Chapter 2 of this thesis have revealed that the relative abundance of light NQ cells is directly proportional to the efficacy with which cells of the G_q^H stem cell niche (all of which are heavy cells) are committed to a differentiation program leading to their conversion to G_q^L cells (all of which are light cells) (Figure 2.19). I found that in yeast cultured under CR on 0.2% glucose, LCA significantly slows down the age-related increase in the relative abundance of light NQ cells (Figure 3.1); thus, LCA considerably delays the entry into the differentiation program 1 leading to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19). I also discovered that in yeast cultured under non-CR conditions on 2% glucose, LCA considerably delays the age-related rise in the relative abundance of light NQ cells (Figure 3.2); I therefore concluded that LCA significantly slows down the entry into the differentiation program 2 known to lead to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19).

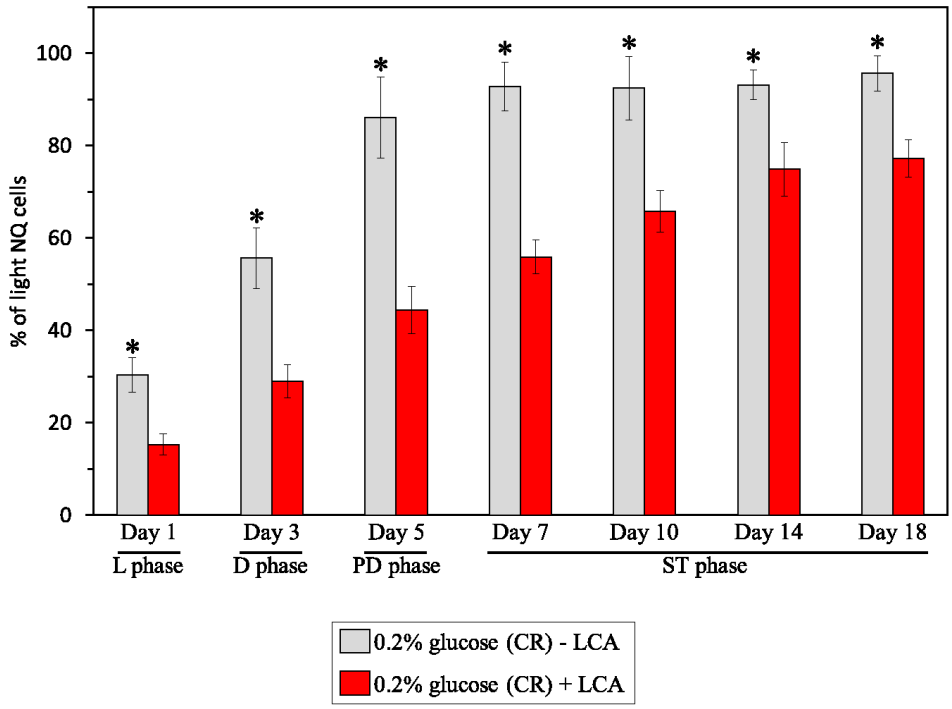

Figure 3.1. In yeast cultures under CR on 0.2% glucose, LCA significantly slows down the age-related increase in the relative abundance of light NQ cells. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 9 – 14); * $p < 0.05$.

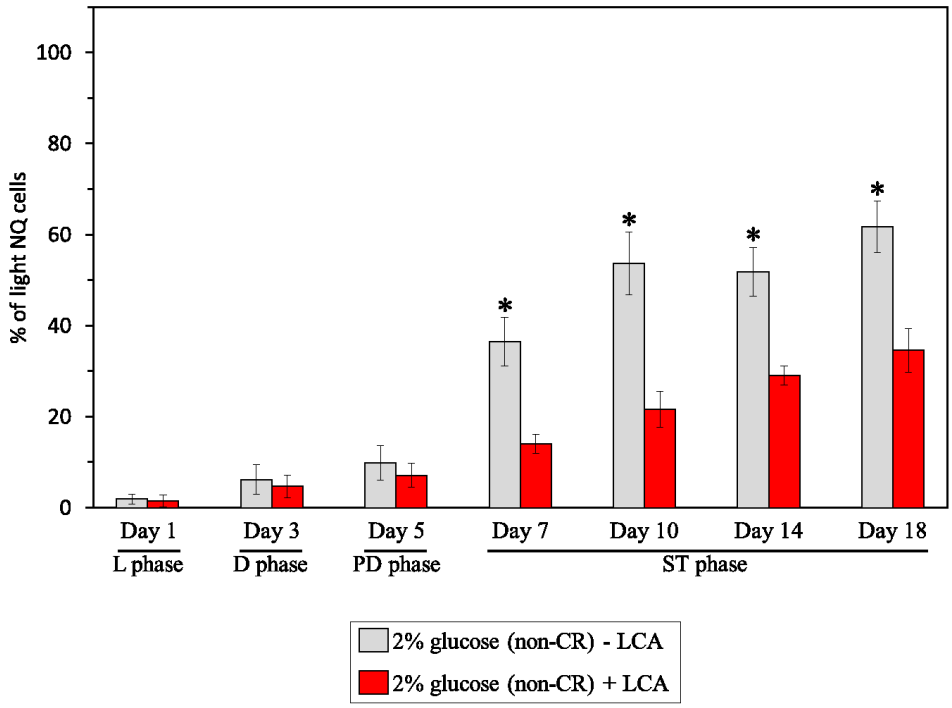

Figure 3.2. In yeast cultures under non-CR conditions on 2% glucose, LCA considerably delays the age-related rise in the relative abundance of light NQ cells. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 9 – 14); * $p < 0.05$.

I then investigated how LCA influences the reproductive (colony-forming) capacity of light NQ and heavy Q cells that were recovered from chronologically aging yeast cultures using centrifugation in Percoll density gradient. This capacity was monitored by assessing the ability of purified light NQ and heavy Q cells to form colonies on solid YEP medium after 2 days of incubation, as described in ″Materials and Methods″. For light NQ cells, the reproductive (colony-forming) capacity is reversibly proportional to the rate with which G_q^L cells progress through the differentiation programs 1 or 2 (for yeast cultured under CR or non-CR conditions, respectively); both these programs underlie the conversion of G_q^L cells to G_{nq}^L cells and, thus,

accelerate yeast chronological aging (Figure 2.19). For heavy Q cells, the reproductive (colony-forming) capacity is directly proportional to the efficacy with which the G_q^H stem cell niche is maintained via the maintenance programs 1 or 2 (for yeast cultured under CR or non-CR conditions, respectively); both these programs underlie the conversion of G_q^H cells to G_{nq}^H cells and, thus, accelerate yeast chronological aging (Figure 2.19). I found that LCA increases the reproductive (colony-forming) capacity of light NQ cells cultured under CR or non-CR conditions (Figures 3.3 and 3.4, respectively); thus, LCA slows down yeast chronological aging in part because it delays the conversion of G_q^L cells to G_{nq}^L cells. Furthermore, I discovered that LCA increases the reproductive (colony-forming) capacity of heavy Q cells cultured under CR conditions (Figure 3.5); therefore, LCA delays yeast chronological aging in part because it decelerates the conversion of G_q^H stem cells to G_{nq}^H cells through the maintenance program 1. However, LCA did not alter the reproductive (colony-forming) capacity of heavy Q cells cultured under non-CR conditions (Figure 3.6); therefore, LCA does not influence the conversion of G_q^H stem cells to G_{nq}^H cells via the maintenance program 2.

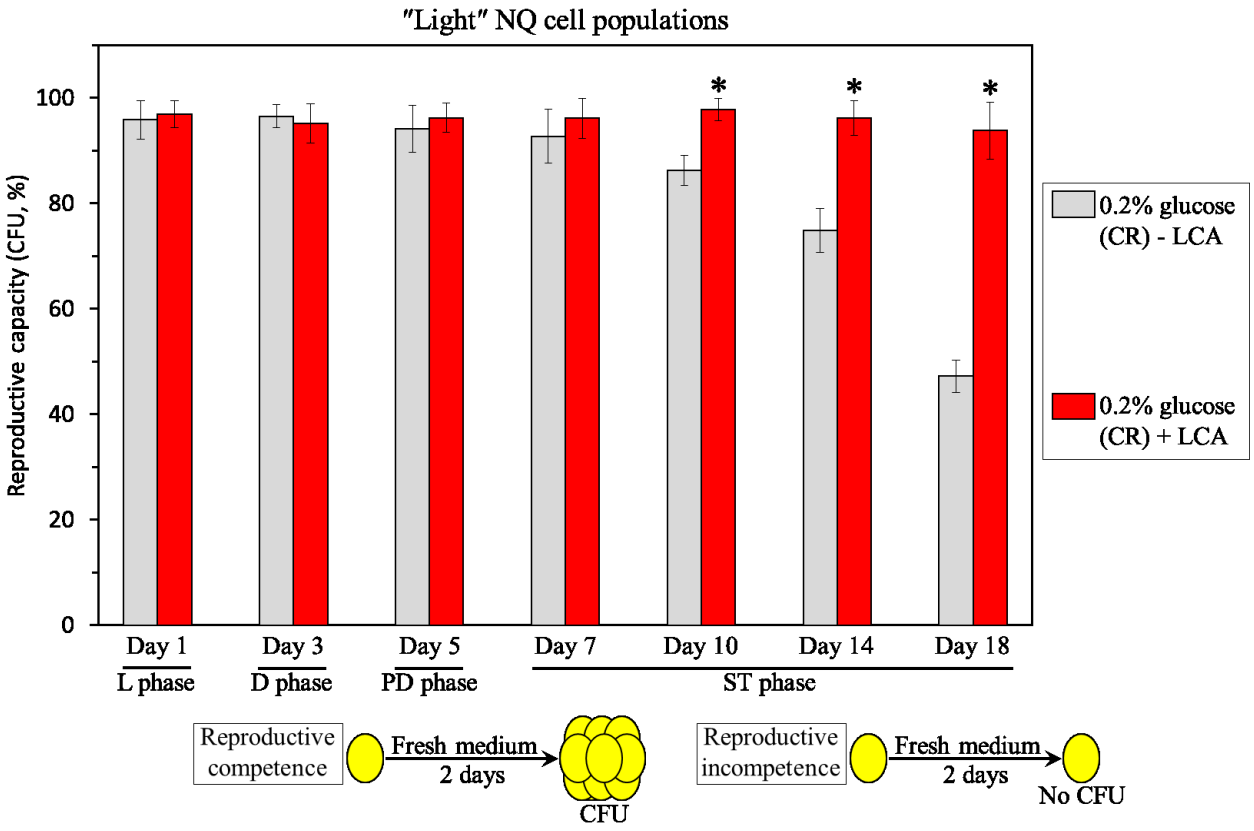

Figure 3.3. LCA increases the reproductive (colony-forming) capacity of light NQ cells cultures under CR conditions. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in "Materials and Methods". The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered light NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in "Materials and Methods". Data are presented as means ± SEM (n = 6 – 11); * $p < 0.05$.

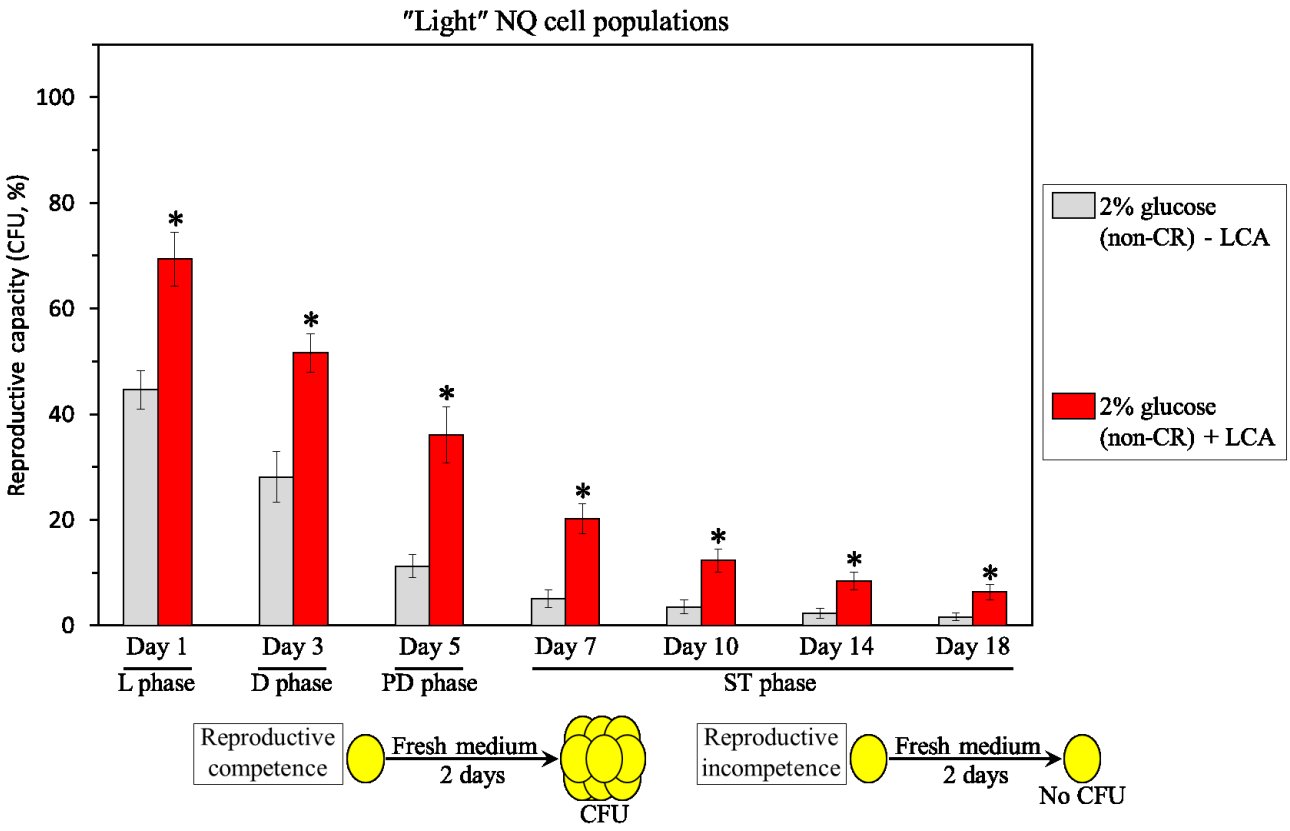

Figure 3.4. LCA increases the reproductive (colony-forming) capacity of light NQ cells cultures under non-CR conditions. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in "Materials and Methods". The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered light NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in "Materials and Methods". Data are presented as means ± SEM (n = 6 – 11); * $p < 0.05$.

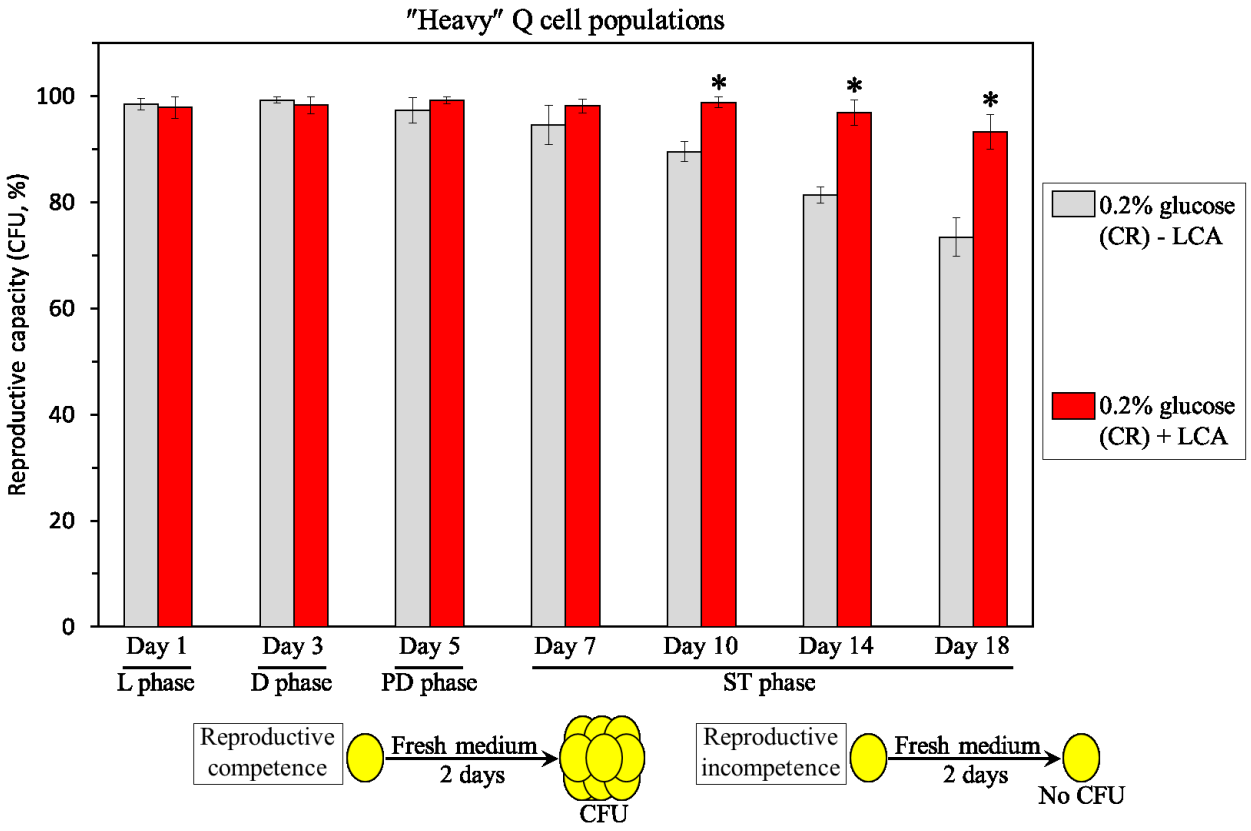

Figure 3.5. LCA increases the reproductive (colony-forming) capacity of heavy Q cells under CR conditions. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in "Materials and Methods". The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered heavy Q cells to form colonies on solid YEPD medium after 2 days of incubation, as described in "Materials and Methods". Data are presented as means ± SEM (n = 6 – 11); * $p < 0.05$.

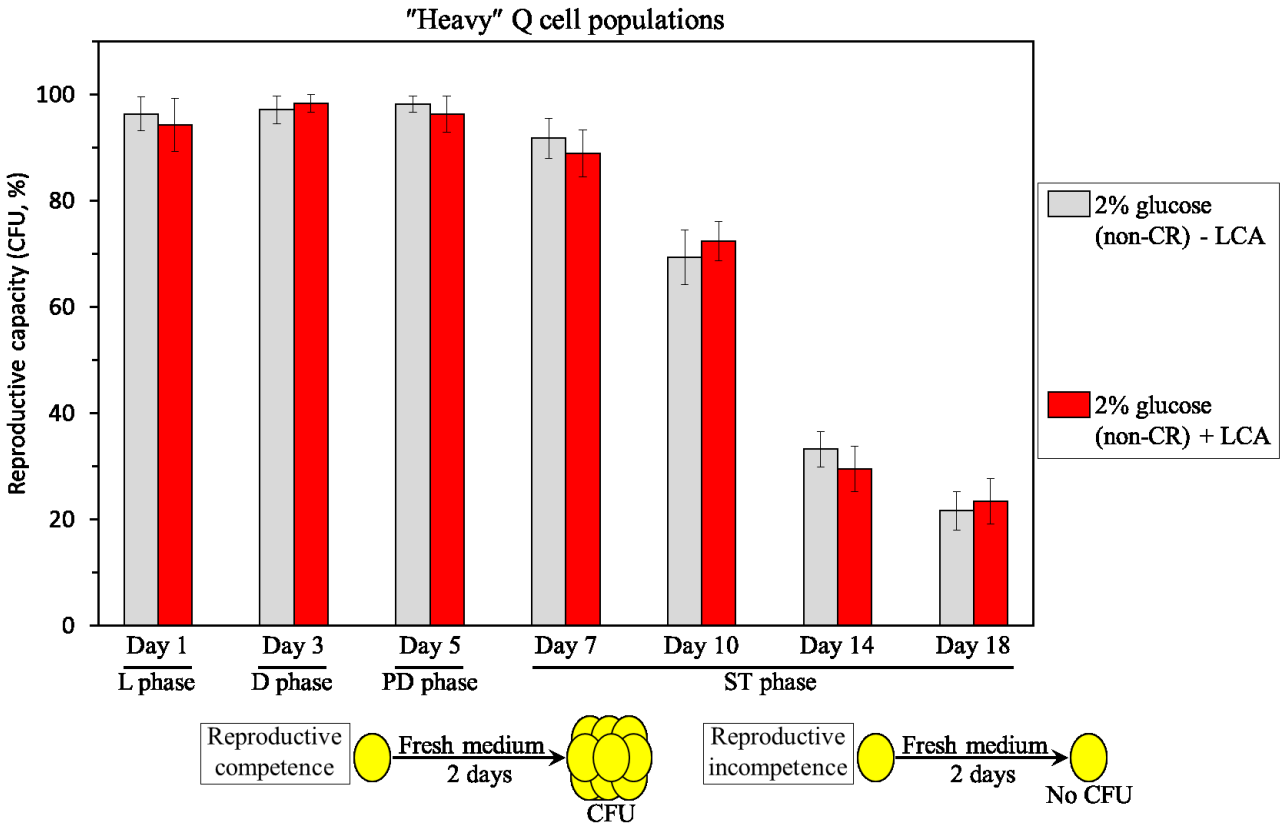

Figure 3.6. LCA does not alter the reproductive (colony-forming) capacity of heavy Q cells under non-CR conditions. Yeast cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered heavy Q cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 6 – 11); * $p < 0.05$.

I then examined how LCA impacts the abilities of light NQ and heavy Q cells under CR conditions to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and incubation for 1 to 4 h. For light NQ cells cultured under CR conditions, such ability is reversibly proportional to the rate with which G_q^L cells progress through the differentiation program 1; as I demonstrated in Chapter 2, this program causes the conversion of G_q^L cells to G_{nq}^L cells and, thus, accelerates yeast chronological aging (Figure 2.19). For heavy Q cells

cultured under CR conditions, the ability of cells to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium is directly proportional to the efficacy with which the G_q^H stem cell niche is maintained via the maintenance program 1; this program underlies the conversion of G_q^H cells to G_{nq}^H cells and, thus, accelerates yeast chronological aging (Figure 2.19).

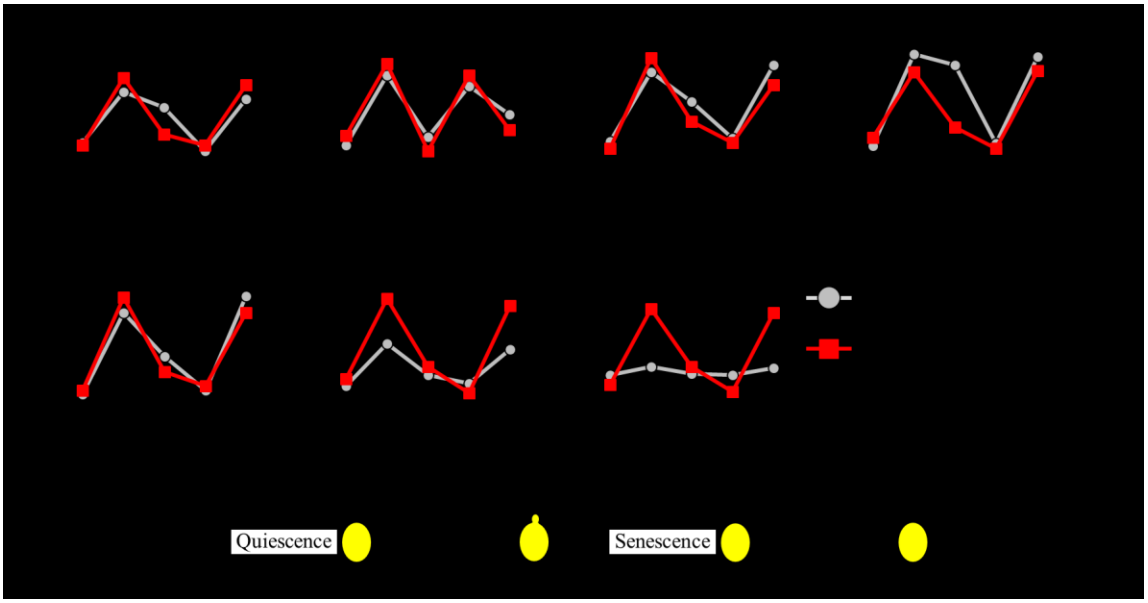

Figure 3.7. LCA extends the period of time during which light NQ cells under CR maintain the ability to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium. Yeast cells were cultured with or without LCA in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The ability of light NQ cells to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and incubation for 1 to 4 h was monitored as described in ″Materials and Methods″. Data are presented as means (n = 2 – 4).

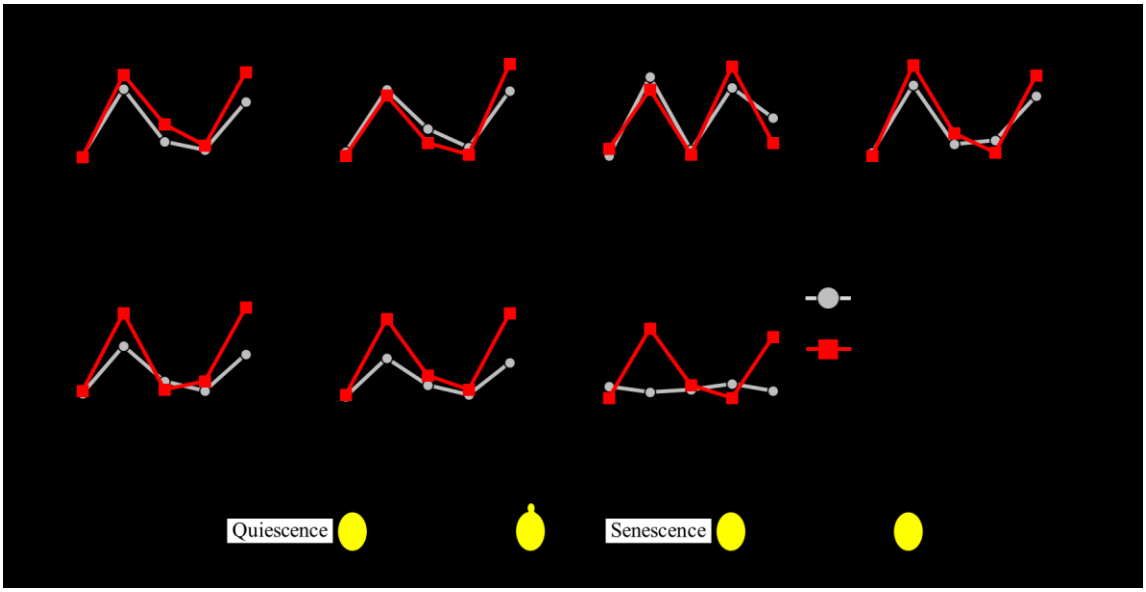

Figure 3.8. LCA extends the period of time during which heavy Q cells under CR maintain the ability to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium. Yeast cells were cultured with or without LCA in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The ability of heavy Q cells to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium and incubation for 1 to 4 h was monitored as described in ″Materials and Methods″. Data are presented as means (n = 2 – 4).

I found that LCA extends the period of time during which light NQ cells under CR sustain the ability to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium (Figure 3.7); this finding provides an additional support for my conclusion (see above) that LCA slows down yeast chronological aging in part because it delays the conversion of G_q^L cells to G_{nq}^L cells. I also found that LCA extends the period of time during which heavy Q cells under CR maintain the ability to synchronously re-enter the mitotic cell cycle after cell transfer to a fresh medium (Figure 3.8); this finding further supports my conclusion (see above) that LCA delays yeast chronological aging in part because it slows down the conversion of G_q^H stem cells to G_{nq}^H cells through the maintenance program 1.

I also investigated how LCA influences mitochondrial respiration, one of the key longevity-defining traits [82, 89, 92, 96, 144, 148, 150], in light NQ and heavy Q cells cultured under CR or non-CR conditions. I found that, although LCA reduces mitochondrial respiration early in life of light NQ cells, it extends the period of time during these cells maintain high rate of mitochondrial respiration after they enter the differentiation program 1 or 2 (for yeast cultured under CR or non-CR conditions, respectively) (Figures 3.9 and 3.10); both these programs underlie the conversion of G_q^L cells to G_{nq}^L cells and, hence, accelerate yeast chronological aging (Figure 2.19). Because my data provide evidence that LCA slows down yeast chronological aging in part because it delays the conversion of G_q^L cells to G_{nq}^L cells (see above), it is plausible that such delaying effect of LCA on the G_q^L-to-G_{nq}^L conversion is due to its ability to support the maintenance of functional mitochondria for a longer period of time. Moreover, I found that, although LCA reduces mitochondrial respiration early in life of heavy Q cells, it extends the period of time during which these cells maintain high rate of mitochondrial respiration during late stages of their preservation as a stem cell niche for the maintenance program 1 or 2 (for yeast cultured under CR or non-CR conditions, respectively) (Figures 3.11 and 3.12); both these programs underlie the conversion of G_q^H cells to G_{nq}^H cells and, thus, accelerate yeast chronological aging (Figure 2.19). Because my data provide evidence that LCA decelerate yeast chronological aging in part because it delays the conversion of G_q^H cells to G_{nq}^H cells (see above), it is conceivable that such delaying effect of LCA on the G_q^H-to-G_{nq}^H conversion is caused by its ability to sustain functional mitochondria for a longer period of time.

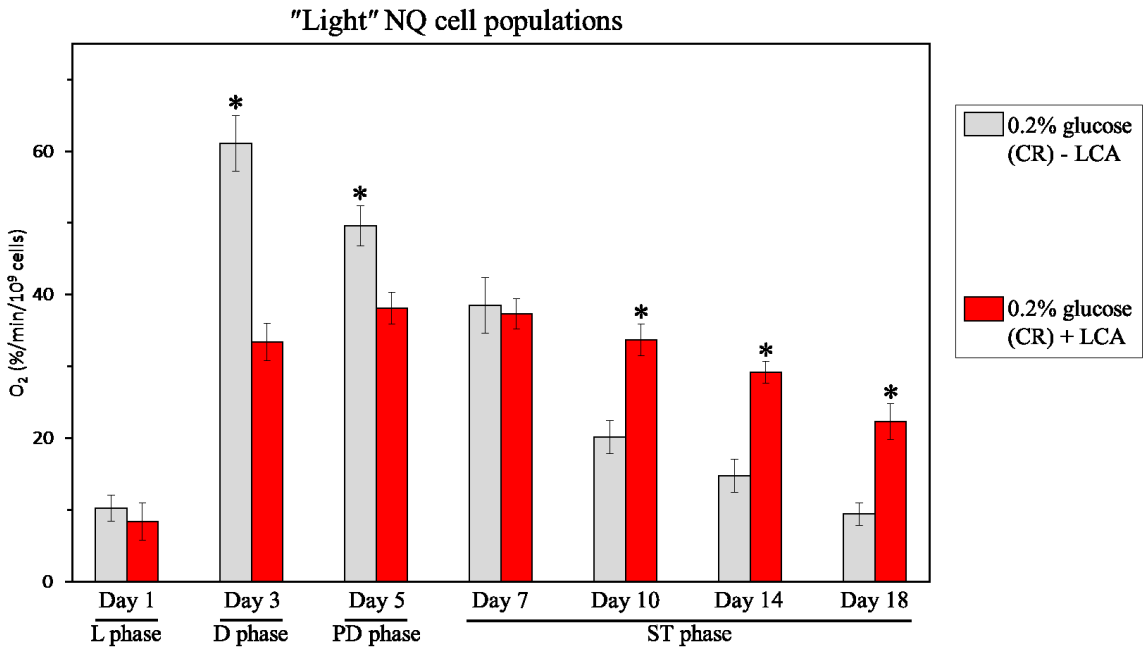

Figure 3.9. Although LCA reduces mitochondrial respiration early in life of light NQ cells cultured under CR conditions, it extends the period of time during which these cells maintain high rate of mitochondrial respiration after they enter the differentiation program 1. Yeast cells were cultured with or without LCA in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in "Materials and Methods". Mitochondrial respiration of light NQ cells was measured as described in "Materials and Methods". Data are presented as means ± SEM (n = 5 – 8); * $p < 0.05$.

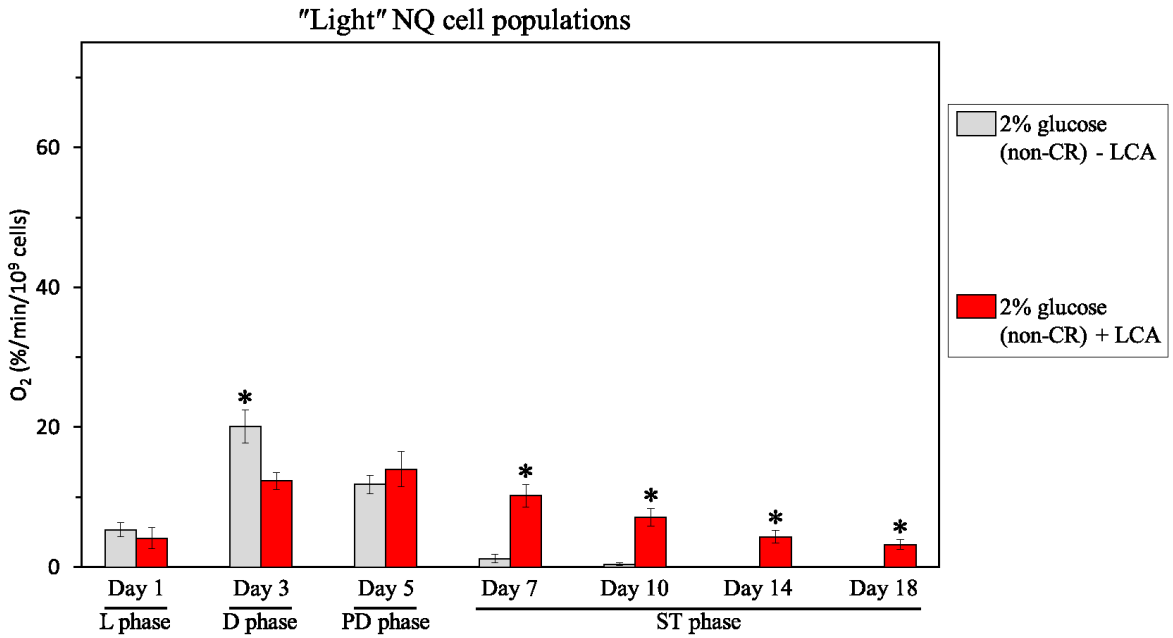

Figure 3.10. Although LCA reduces mitochondrial respiration early in life of light NQ cells cultured under non-CR conditions, it extends the period of time during which these cells maintain high rate of mitochondrial respiration after they enter the differentiation program 2. Yeast cells were cultured with or without LCA in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in "Materials and Methods". Mitochondrial respiration of light NQ cells was measured as described in "Materials and Methods". Data are presented as means ± SEM (n = 5 – 8); * $p < 0.05$.

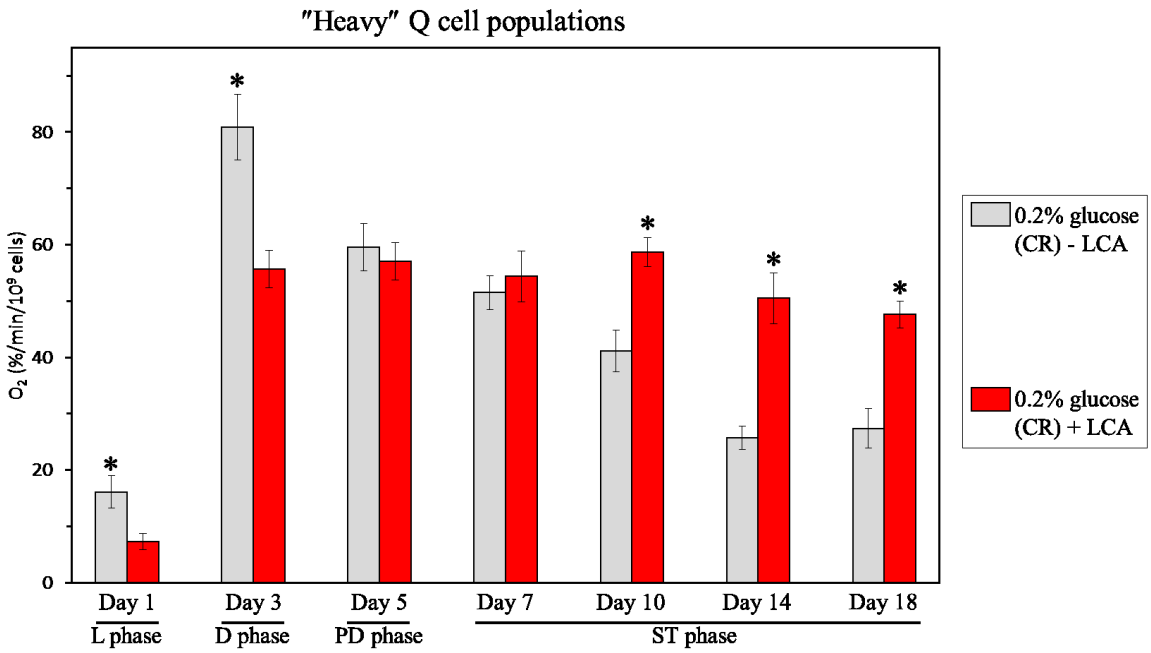

Figure 3.11. Although LCA reduces mitochondrial respiration early in life of heavy Q cells cultured under CR conditions, it extends the period of time during which these cells maintain high rate of mitochondrial respiration during late stages of their preservation as a stem cell niche for the maintenance program 1. Yeast cells were cultured with or without LCA in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. Mitochondrial respiration of heavy Q cells was measured as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 5 – 8); * $p < 0.05$.

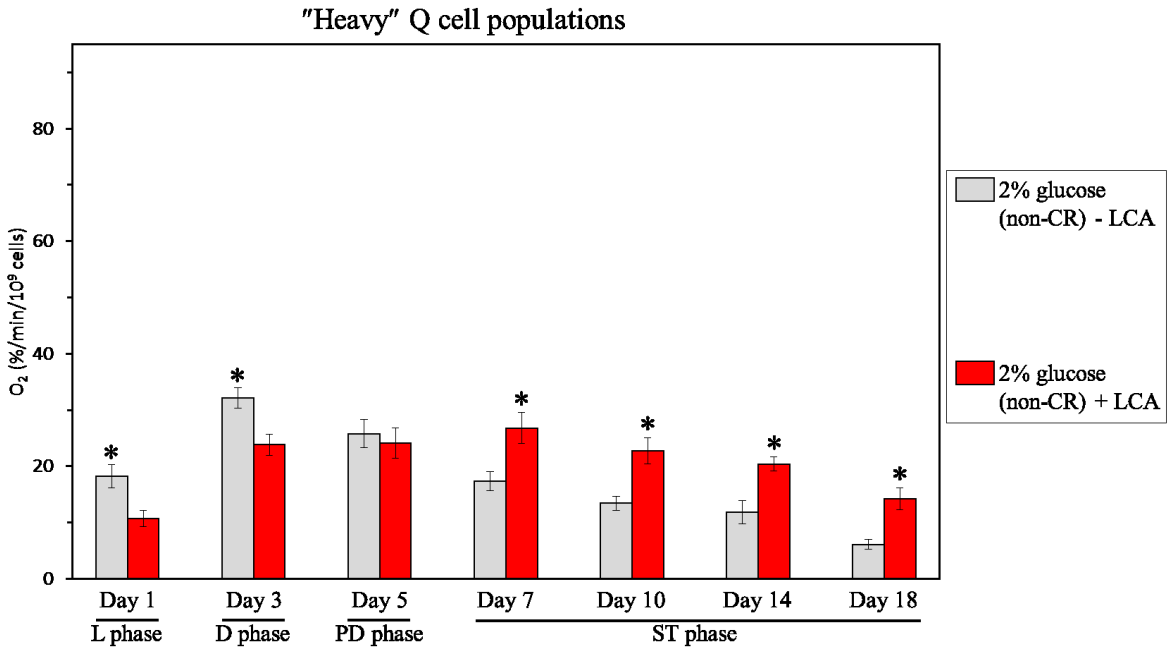

Figure 3.12. Although LCA reduces mitochondrial respiration early in life of heavy Q cells cultured under non-CR conditions, it extends the period of time during which these cells maintain high rate of mitochondrial respiration during late stages of their preservation as a stem cell niche for the maintenance program 2. Yeast cells were cultured with or without LCA in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. Mitochondrial respiration of heavy Q cells was measured as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 5 – 8); * $p < 0.05$.

3.3.2 The Ras/cAMP/PKA signaling pathway operates as a pro-aging pathway by weakening maintenance of niches of stem cells and also exhibits an anti-aging potential by delaying their entry into the differentiation programs linking cell cycle regulation to cellular aging

The Ras/cAMP/PKA signaling pathway is considered as one of the key pro-aging pathways that accelerates yeast chronological aging; this pathway is also one of the key regulators linking carbon source availability to cell growth and metabolism [8 – 10, 100, 137 - 143]. Lack of the small G-protein Ras2, an activator of adenylate cyclase, is known to attenuate the

Ras/cAMP/PKA signaling pathway because in the absence of Ras2 the activity of PKA is greatly reduced [8 – 10, 19, 77]. I found that the single-gene-deletion mutation *ras2Δ*, which eliminates Ras2, extends longevity of chronologically aging yeast cultured either under CR or non-CR conditions in the absence of LCA (Figure 3.13) - as it was expected because the Ras/cAMP/PKA signaling pathway is considered as one of the key pro-aging pathways [8 – 10, 100, 137 - 143]. However, a recent study has revealed that the single-gene-deletion mutation *ras2Δ* also significantly reduces the longevity-extending efficacy of LCA in chronologically aging yeast cultured either under CR or under non-CR conditions (Figure 3.14) [27] – as if the Ras/cAMP/PKA signaling pathway may also have an anti-aging potential because it is required for the maximal longevity-extending effect of LCA. Therefore, I have decided to investigate how this signaling pathway regulates the two longevity-defining differentiation programs that link cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state; I discovered these differentiation programs in studies that are described in Chapter 2 of my thesis. I first examined how the *ras2Δ* mutation influences age-related changes in the relative abundancies of subpopulations of light NQ cells and heavy Q cells. My studies described in Chapter 2 of this thesis have revealed

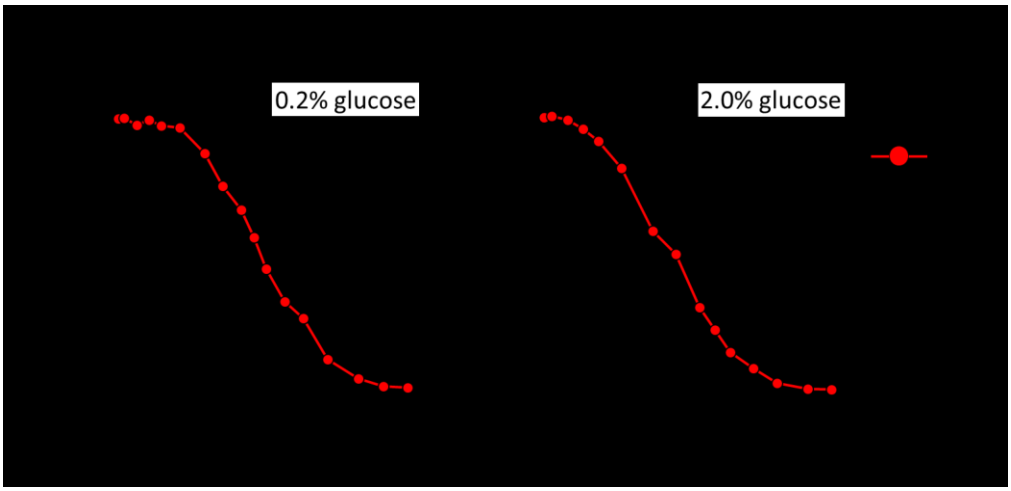

Figure 3.13. The single-gene-deletion mutation *ras2Δ*, which eliminates Ras2, extends longevity of chronologically aging yeast cultured in the absence of LCA under CR conditions on 0.2% glucose (A) or in the absence of LCA under non-CR conditions on 2% glucose (B). Wild-type (WT) and *ras2Δ* mutant cells were cultured without LCA in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (A; CR conditions) or 2% glucose (B; non-CR conditions). Survival curves of chronologically aging yeast are shown; data are presented as means ± SEM (n = 11-14).

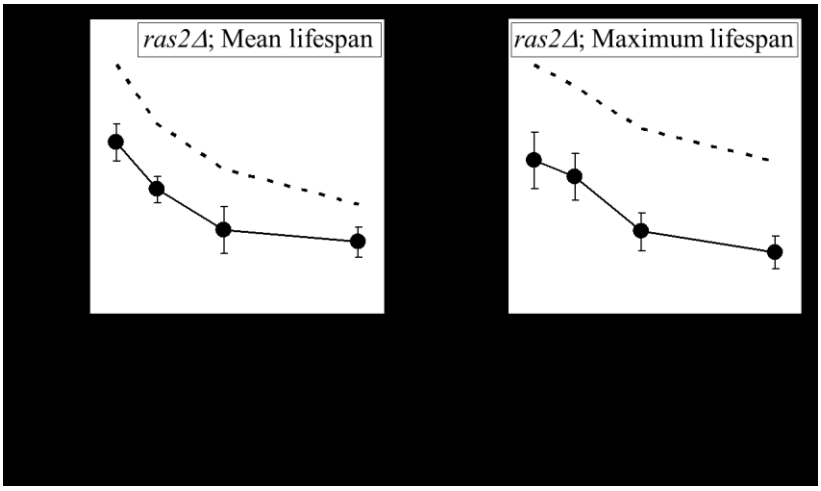

Figure 3.14. The single-gene-deletion mutation *ras2Δ* significantly decreases the longevity-extending efficacy of LCA in chronologically aging yeast cultured either under CR or under non-CR conditions. From reference [27].

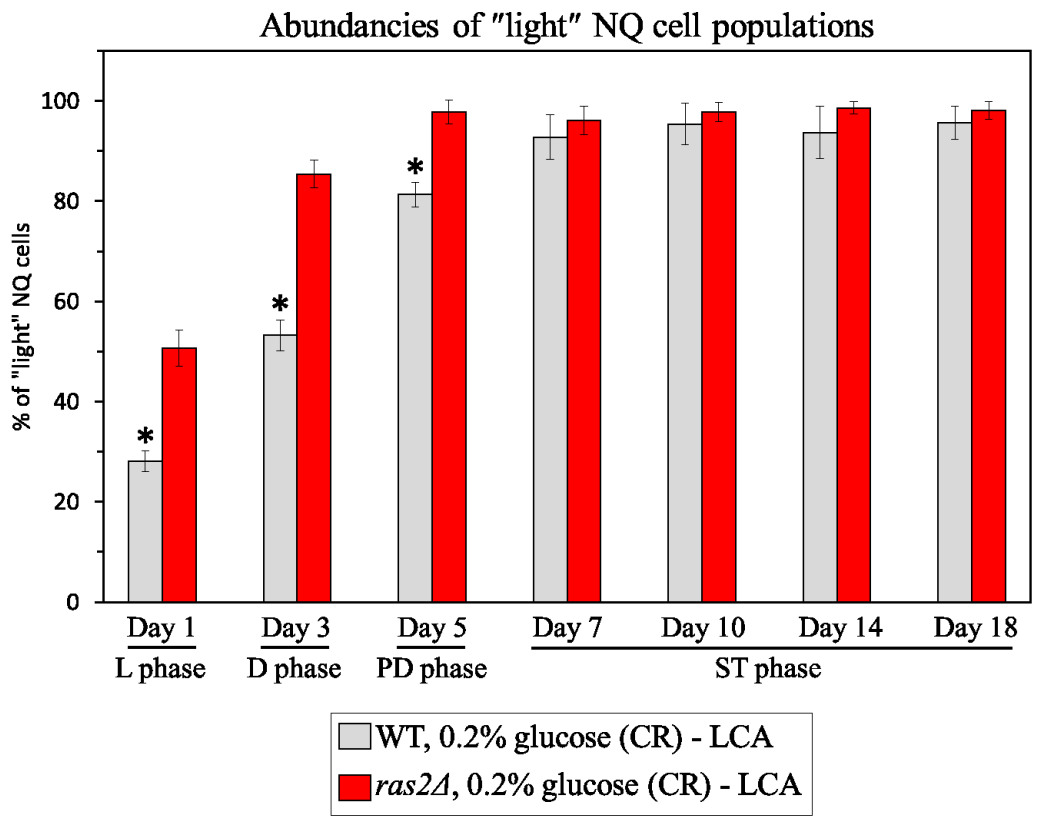

Figure 3.15. In yeast cultures under CR on 0.2% glucose, lack of Ras2 accelerates the age-related increase in the relative abundance of light NQ cells. Wild-type (WT) and *ras2Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 4 – 6); * $p < 0.05$.

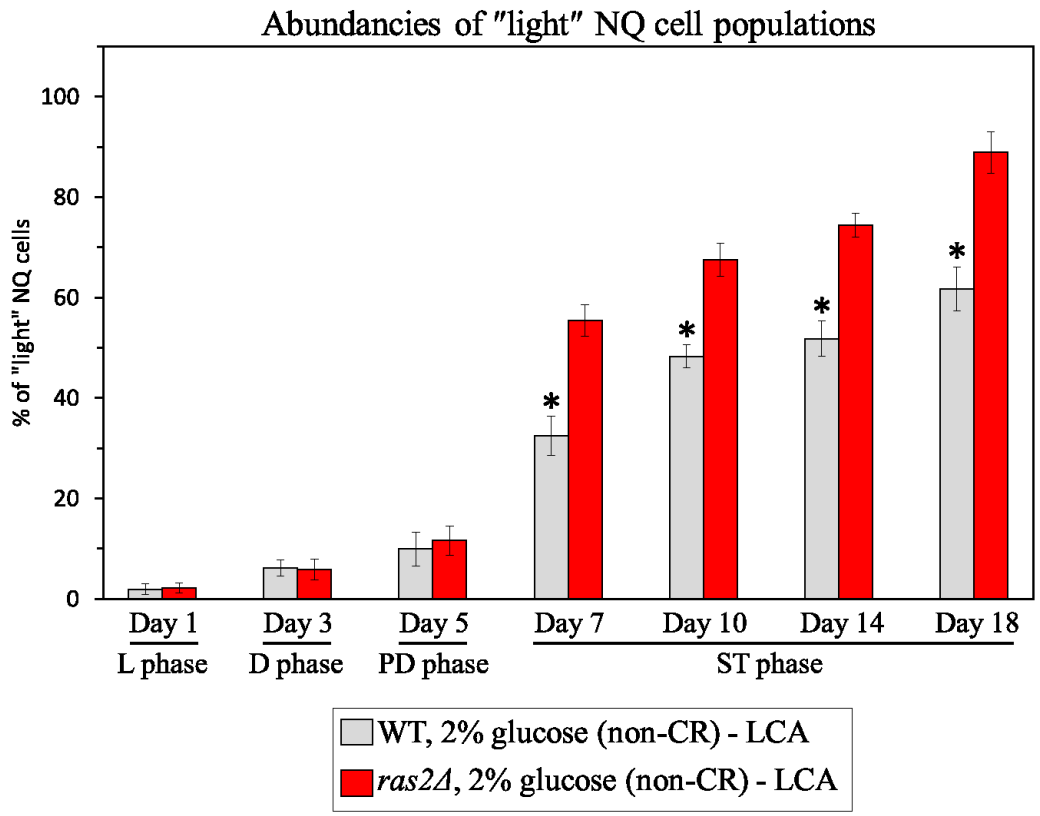

Figure 3.16. In yeast cultured under non-CR conditions on 2% glucose, lack of Ras2 speeds up the age-related increase in the relative abundance of light NQ cells. Wild-type (WT) and *ras2Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 4 – 6); * $p < 0.05$.

that the relative abundance of light NQ cells is directly proportional to the efficacy with which cells of the G_q^H stem cell niche (all of which are heavy cells) are committed to a differentiation program leading to their conversion to G_q^L cells (all of which are light cells) (Figure 2.19). I found that in yeast cultures under CR on 0.2% glucose, lack of Ras2 accelerates the age-related increase in the relative abundance of light NQ cells (Figure 3.15); I therefore concluded that in wild-type yeast cells Ras2 delays the entry into the differentiation program 1 leading to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19). I also discovered that in yeast cultured

under non-CR conditions on 2% glucose, lack of Ras2 speeds up the age-related increase in the relative abundance of light NQ cells (Figure 3.16); hence, in wild-type yeast cells Ras2 slows down the entry into the differentiation program 2 known to lead to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19).

I then investigated how the *ras2Δ* mutation may impact the efficacy with which LCA alters the relative abundancies of subpopulations of light NQ cells and heavy Q cells in an age-related manner. I found that: 1) in yeast cultures under CR on 0.2% glucose, lack of Ras2 abolishes the ability of LCA to slow down the age-related increase in the relative abundance of light NQ cells (Figure 3.17); thus, in the absence of Ras2, LCA does not exhibit its ability to delay the entry into the differentiation program 1 which leads to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19); and 2) in yeast cultured under non-CR conditions on 2% glucose, lack of Ras2 obliterates the ability of LCA to delay the age-related rise in the relative abundance of light NQ cells (Figure 3.18); therefore, the absence of Ras2 eliminates the ability of LCA to slow down the entry into the differentiation program 2 known to lead to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19).

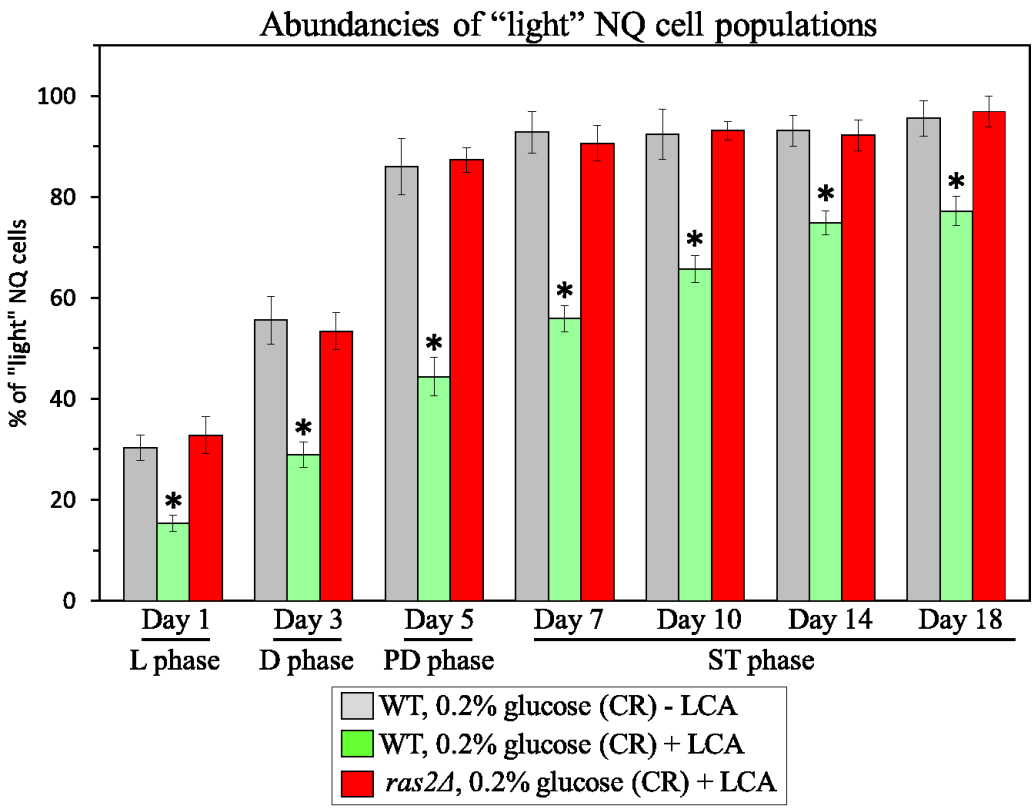

Figure 3.17. In yeast cultures under CR on 0.2% glucose, lack of Ras2 abolishes the ability of LCA to slow down the age-related increase in the relative abundance of light NQ cells. Wild-type (WT) and *ras2Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6); * $p < 0.05$.

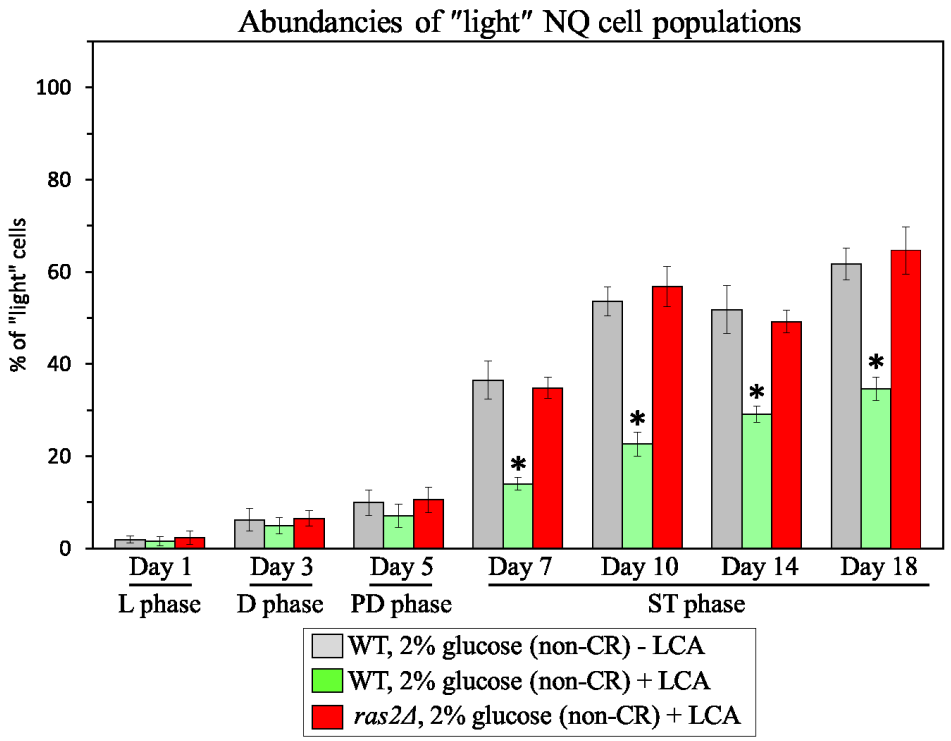

Figure 3.18. In yeast cultured under non-CR conditions on 2% glucose, lack of Ras2 obliterates the ability of LCA to delay the age-related rise in the relative abundance of light NQ cells. Wild-type (WT) and *ras2Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) with or without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6); * $p < 0.05$.

I also examined how the *ras2Δ* mutation impacts the reproductive (colony-forming) capacity of light NQ and heavy Q cells that were purified from chronologically aging yeast cultures with the help of centrifugation in Percoll density gradient. As described in ″Materials and Methods″, this capacity was monitored by assessing the ability of purified light NQ and heavy Q cells to form colonies on solid YEP medium after 2 days of incubation. As I have established in studies described in Chapter 2, for light NQ cells, the reproductive (colony-forming) capacity is reversibly proportional to the rate with which G_q^L cells progress through the

differentiation program 1 or 2 (for yeast cultured under CR or non-CR conditions, respectively); both these programs allow the conversion of G_q^L cells to G_{nq}^L cells and, thus, speed up yeast chronological aging (Figure 2.19). As I have established in studies described in Chapter 2, for heavy Q cells, the reproductive (colony-forming) capacity is directly proportional to the efficacy with which the G_q^H stem cell niche is sustained via the maintenance programs 1 or 2 (for yeast cultured under CR or non-CR conditions, respectively); both these programs supports the conversion of G_q^H cells to G_{nq}^H cells and, thus, promote yeast chronological aging (Figure 2.19). I found that lack of Ras2 does not alter the reproductive (colony-forming) capacity of light NQ cells cultured under CR or non-CR conditions without LCA (Figures 3.19 and 3.20, respectively); thus, in wild-type yeast cells Ras2 is not required for the conversion of G_q^L cells to G_{nq}^L cells via the differentiation program 1 or 2 (for yeast cultured under CR or non-CR conditions, respectively). However, lack of Ras2 increased the reproductive (colony-forming) capacity of heavy Q cells cultured under CR or non-CR conditions without LCA (Figures 3.21 and 3.22, respectively); I therefore concluded that in wild-type yeast cells cultured under CR or non-CR conditions (without LCA) Ras2 accelerates yeast chronological aging in part because it promotes the conversion of G_q^H stem cells to G_{nq}^H cells through the maintenance program 1 or 2, respectively.

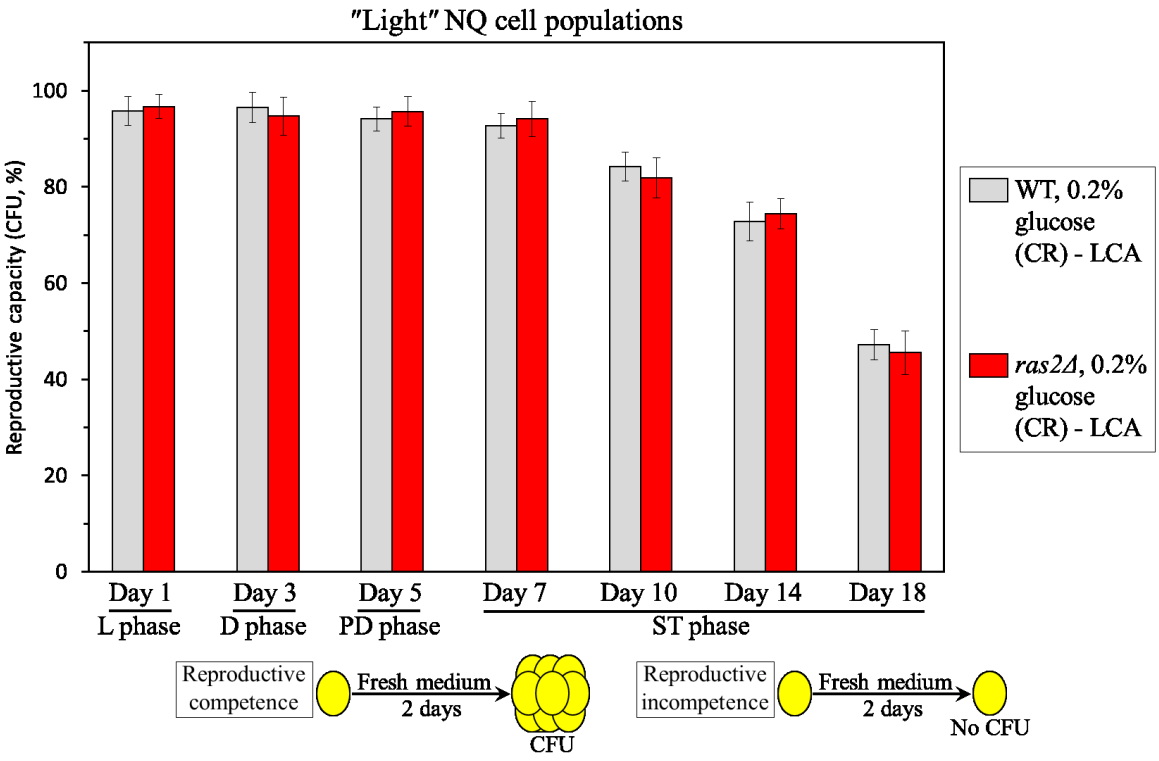

Figure 3.19. Lack of Ras2 does not alter the reproductive (colony-forming) capacity of light NQ cells cultured under CR conditions without LCA. Wild-type (WT) and *ras2Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in "Materials and Methods". The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered light NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in "Materials and Methods". Data are presented as means ± SEM (n = 3 – 6).

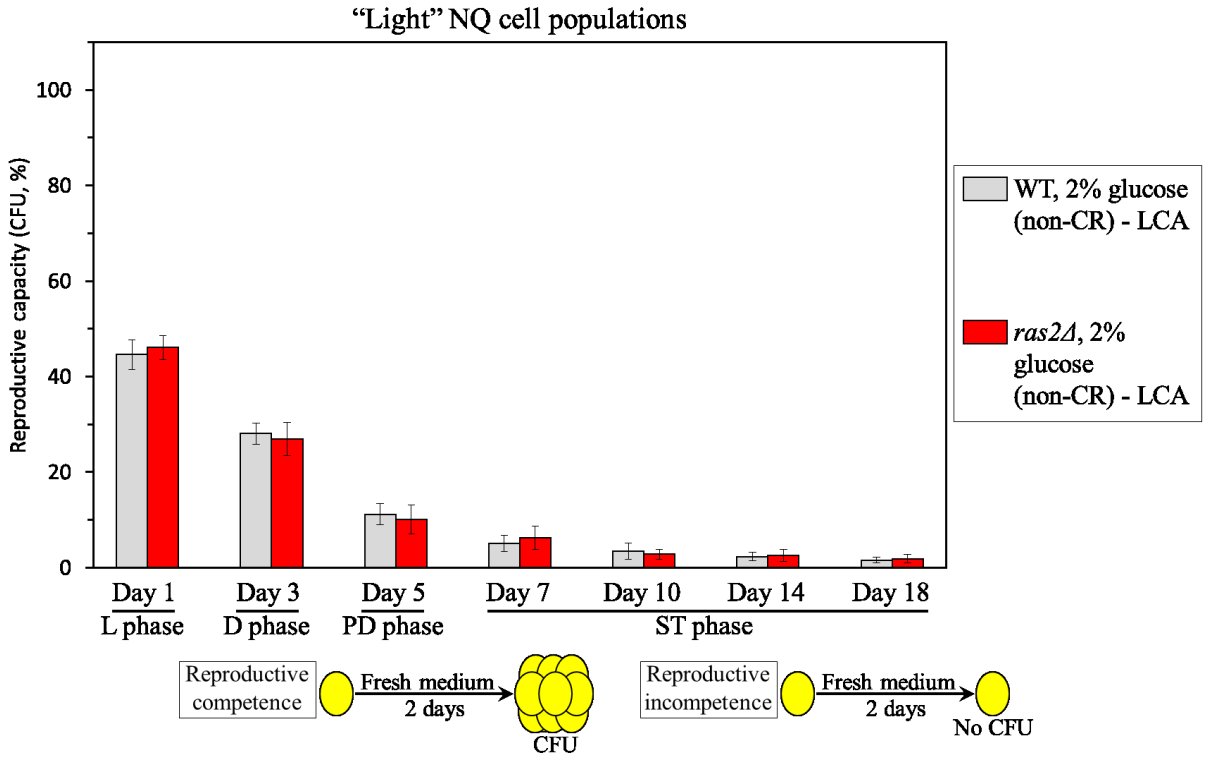

Figure 3.20. Lack of Ras2 does not alter the reproductive (colony-forming) capacity of light NQ cells cultured under non-CR conditions without LCA. Wild-type (WT) and *ras2Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered light NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6).

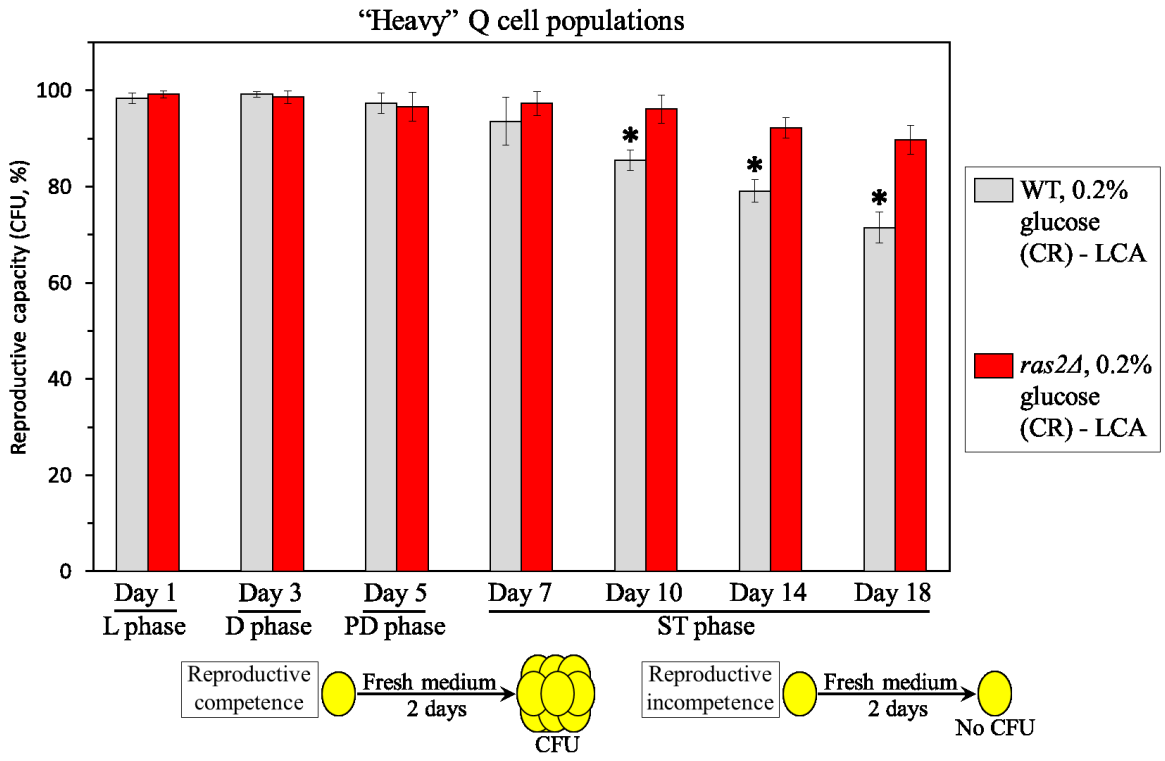

Figure 3.21. Lack of Ras2 increases the reproductive (colony-forming) capacity of heavy Q cells cultured under CR conditions without LCA. Wild-type (WT) and *ras2Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered light NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6); * $p < 0.05$.

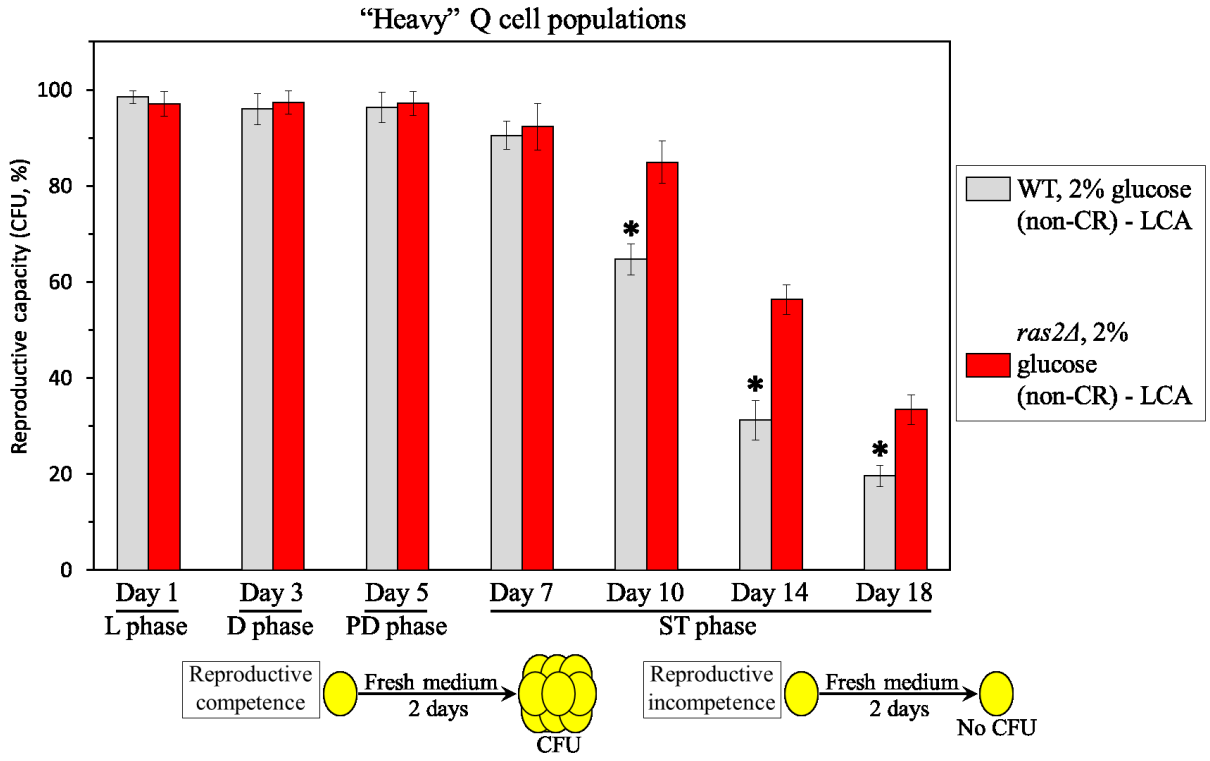

Figure 3.22. Lack of Ras2 increases the reproductive (colony-forming) capacity of heavy Q cells cultured under non-CR conditions without LCA. Wild-type (WT) and *ras2Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered light NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6); * $p < 0.05$.

3.3.3 Trehalose extends yeast longevity by improving maintenance of the G_q^H stem cell niches as well as by delaying their entry into and progression through the differentiation programs linking cell cycle regulation to cellular aging

Trehalose is a non-reducing disaccharide which has been long considered only as a reserve carbohydrate [1, 157]. However, recent studies in yeast revealed that the intracellular concentrations of trehalose prior to cell entry into a non-proliferative state and following such entry play essential and differing roles in defining longevity of chronologically aging yeast; this

is because trehalose is involved in modulating protein folding, misfolding, unfolding, refolding, oxidative damage, solubility and aggregation throughout lifespan (Figure 3.23) [14, 91]. The single-gene-deletion mutation *tps1Δ*, which in yeast eliminates one of the catalytic subunits of the trehalose synthase complex

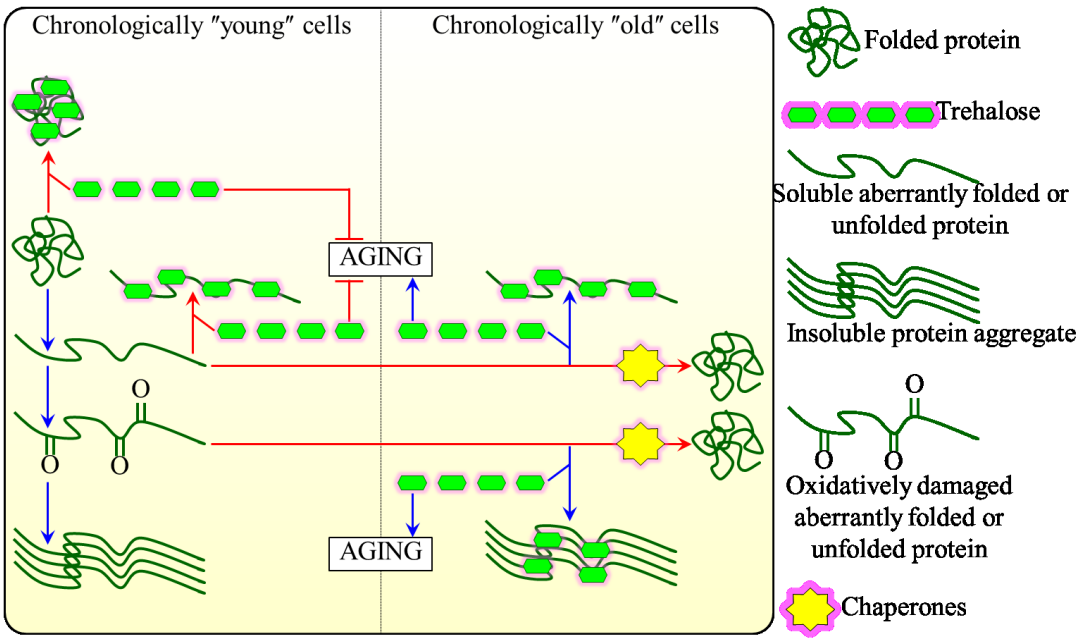

Figure 3.23. Molecular mechanisms through which trehalose regulates the process of cellular aging in yeast by modulating protein folding, misfolding, unfolding, refolding, oxidative damage, solubility and aggregation in chronologically ″young″ and ″old″ yeast cells. Modified from references [14] and [91].

(Figure 3.24), has been shown to decrease intracellular trehalose concentration and shorten chronological lifespan [91]. Therefore, I have decided to examine how trehalose

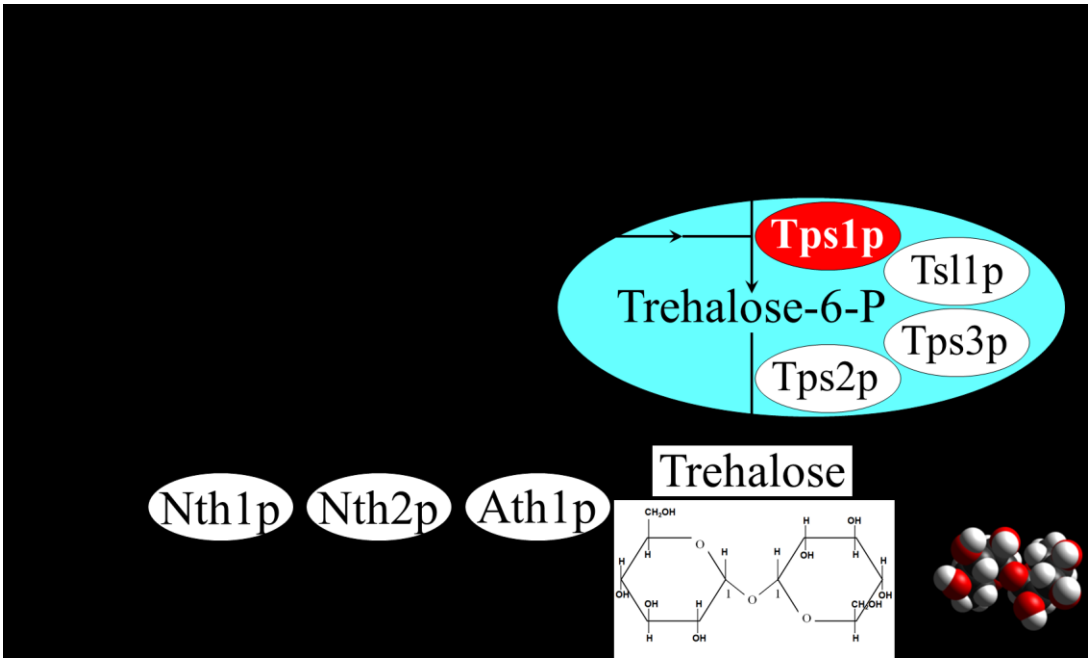

Figure 3.24. Outline of metabolic pathways for the biosynthesis and degradation of the reserve carbohydrate trehalose, a non-reducing disaccharide and osmolyte. Tps1 is one of the catalytic subunits of the trehalose synthase complex. Modified from reference [91].

regulates the two longevity-defining differentiation programs that link cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state; I discovered these differentiation programs in studies that are presented in Chapter 2 of my thesis. I first examined how the *tps1Δ* mutation influences age-related changes in the relative abundancies of subpopulations of light NQ cells and heavy Q cells. I found that in yeast cultures under CR on 0.2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose does not alter the extent of the age-related rise in the relative abundance of light NQ cells (Figure 3.25); thus, trehalose in wild-type yeast cells is not essential for the entry into the differentiation program 1 leading to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19). However, in yeast cultures under non-CR conditions on 2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose accelerates the age-related increase in the relative abundance of light NQ cells (Figure 3.26); I

therefore concluded that trehalose in wild-type yeast cells delays the entry into the differentiation program 2 leading to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19).

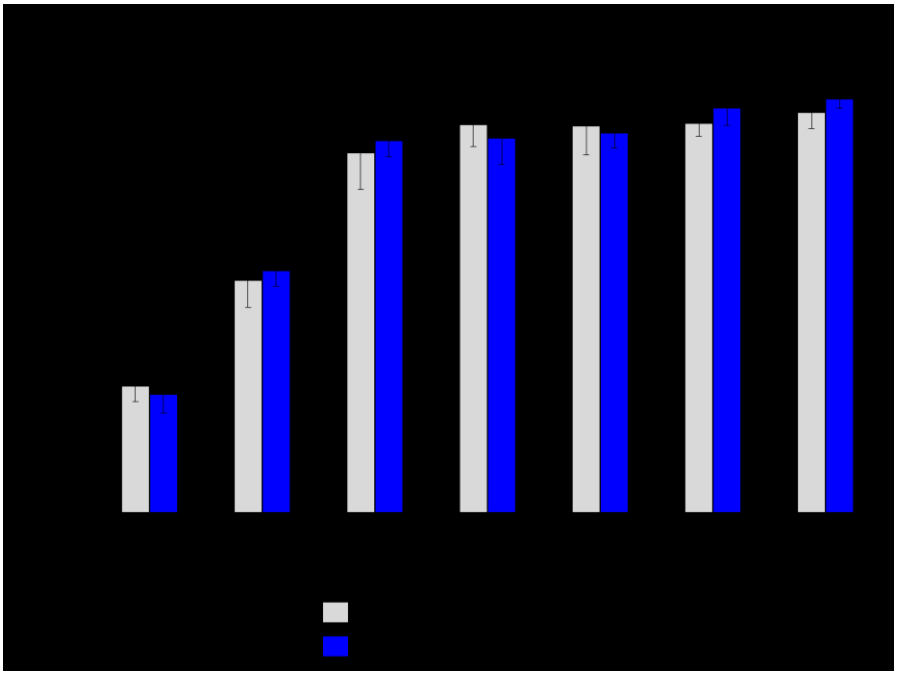

Figure 3.25. In yeast cultures under CR on 0.2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose does not alter the extent of the age-related rise in the relative abundance of light NQ cells. Wild-type (WT) and *tps1Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 - 4).

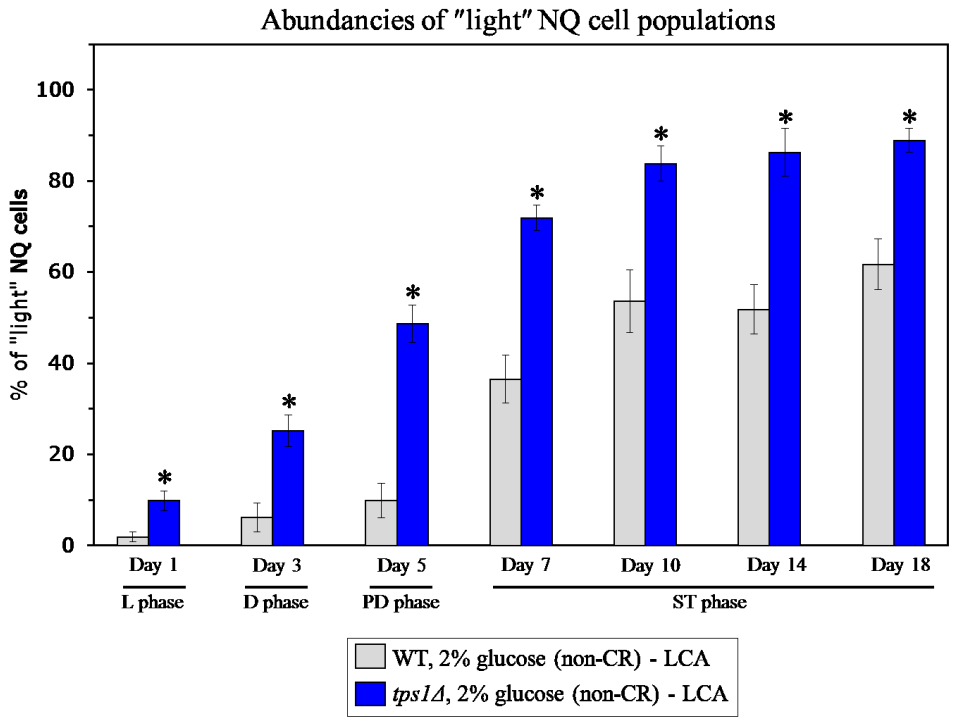

Figure 3.26. In yeast cultures under non-CR conditions on 2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose accelerates the age-related increase in the relative abundance of light NQ cells. Wild-type (WT) and *tps1Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 4); * $p < 0.05$.

I then examined how the *tps1Δ* mutation may impact the efficacy with which LCA alters the relative abundancies of subpopulations of light NQ cells and heavy Q cells in an age-related manner. I found that in yeast cultures under CR on 0.2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose does not alter the extent to which LCA decelerates the age-related increase in the relative abundance of light NQ cells (Figure 3.27); thus, trehalose in wild-type yeast cells does not modulate the ability of LCA to delay the entry into the differentiation program 1 which leads to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19). However, in yeast cultured under non-CR

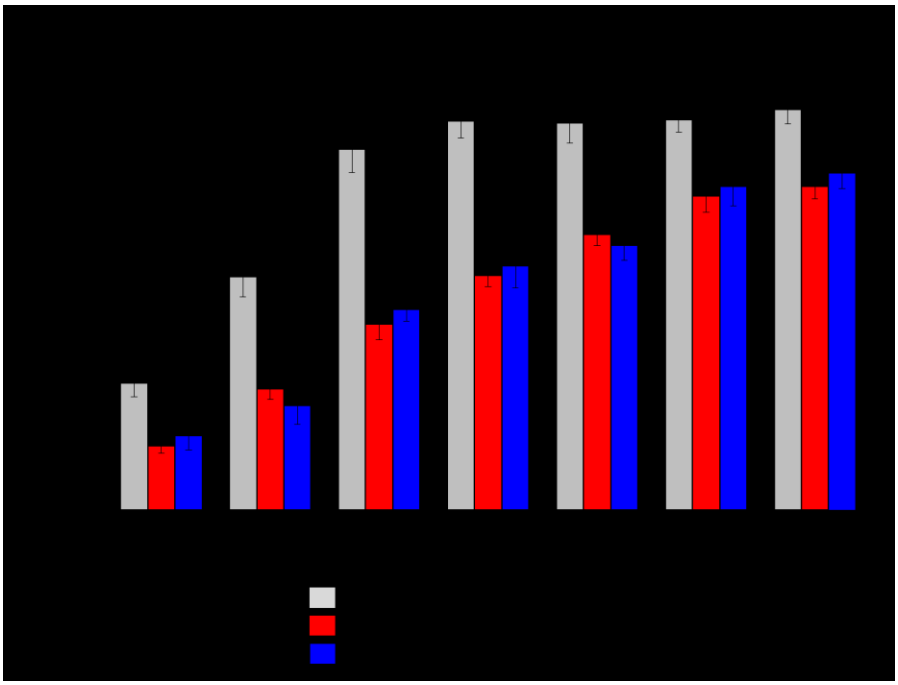

Figure 3.27. In yeast cultures under CR on 0.2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose does not alter the extent to which LCA decelerates the age-related increase in the relative abundance of light NQ cells. Wild-type (WT) and *tps1Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium with or without LCA initially containing 0.2% glucose (CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 4); * $p < 0.05$.

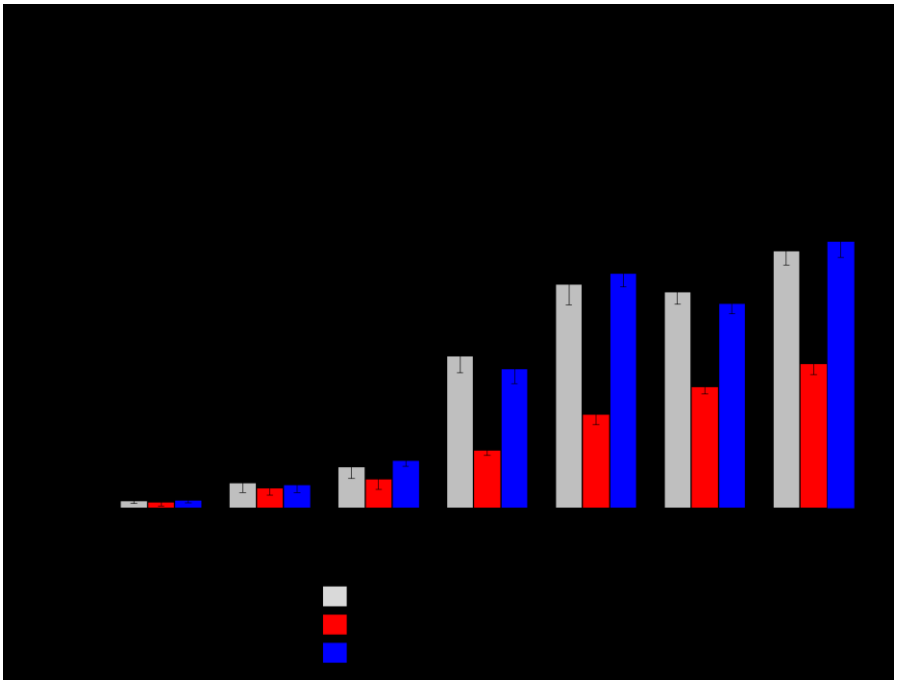

Figure 3.28. In yeast cultured under non-CR conditions on 2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose abolishes the ability of LCA to delay the age-related rise in the relative abundance of light NQ cells. Wild-type (WT) and *tps1Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium with or without LCA initially containing 2% glucose (non-CR conditions), recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The number and percentage of cells recovered in ″heavy″ Q and ″light″ NQ cell subpopulations were calculated as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 4); * $p < 0.05$.

conditions on 2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose abolishes the ability of LCA to delay the age-related rise in the relative abundance of light NQ cells (Figure 3.28); I therefore concluded that a significant reduction in the intracellular trehalose eliminates the ability of LCA to delay the entry into the differentiation program 2 leading to the conversion of G_q^H stem cells to G_q^L cells (Figure 2.19).

I also assessed how the *tps1Δ* mutation may influence the reproductive (colony-forming) capacity of light NQ and heavy Q cells that were purified from chronologically aging yeast

cultures with the help of centrifugation in Percoll density gradient. As described in ″Materials and Methods″, this capacity was monitored by assessing the ability of purified light NQ and heavy Q cells to form colonies on solid YEP medium after 2 days of incubation. I found that the *tps1Δ*-dependent decrease of intracellular trehalose reduces the reproductive (colony-forming) capacity of light NQ cells cultured under CR or non-CR conditions without LCA (Figures 3.29 and 3.30, respectively); I therefore concluded that trehalose in wild-type yeast cells slows down the conversion of G_q^L cells to G_{nq}^L cells via the differentiation program 1 or 2 (for yeast cultured under CR or non-CR conditions, respectively). Moreover, the *tps1Δ*-dependent decrease of intracellular trehalose also reduced the reproductive (colony-forming) capacity of heavy Q cells cultured under CR or non-CR conditions without LCA (Figures 3.31 and 3.32, respectively); thus, trehalose in wild-type yeast cells delays the conversion of G_q^H stem cells to G_{nq}^H cells through the maintenance program 1 or 2, respectively.

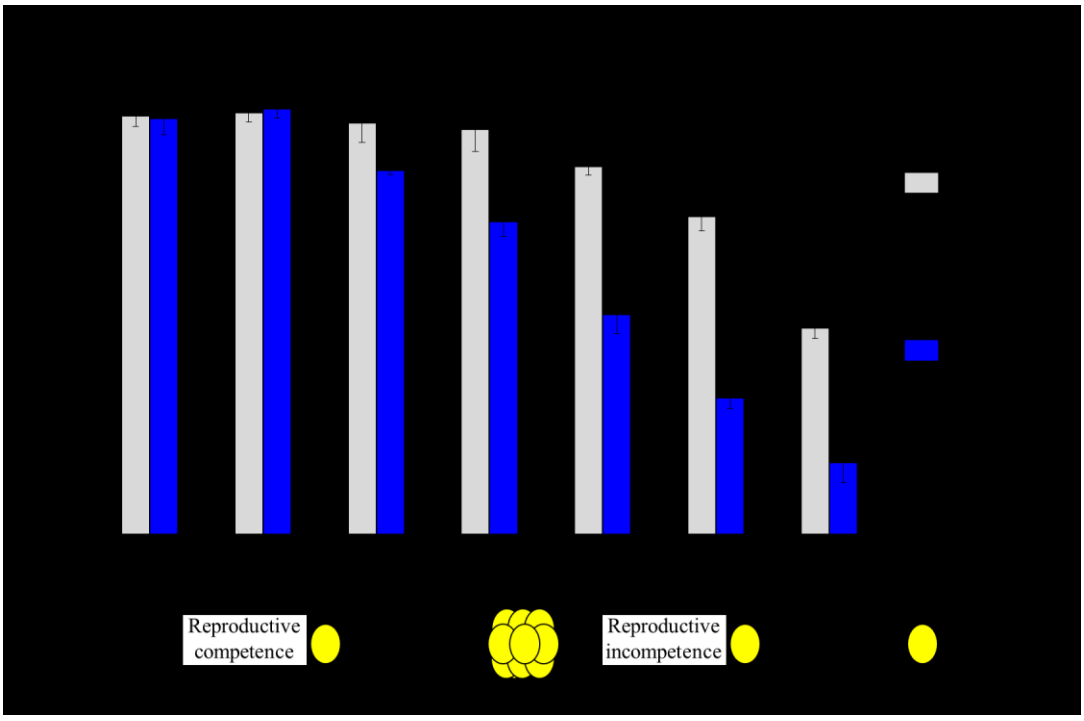

Figure 3.29. In yeast cultures under CR on 0.2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose reduces the reproductive (colony-forming) capacity of light NQ cells.

Wild-type (WT) and *tps1Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered light NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6); * $p < 0.05$.

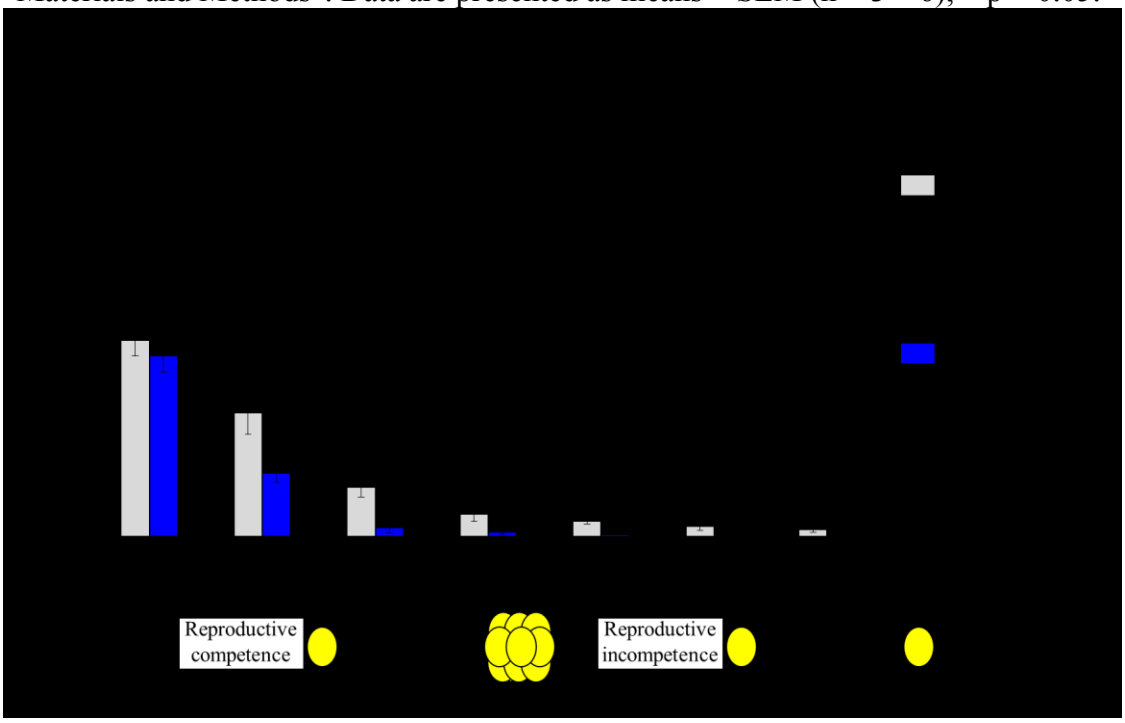

Figure 3.30. In yeast cultures under non-CR conditions on 2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose reduces the reproductive (colony-forming) capacity of light NQ cells. Wild-type (WT) and *tps1Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered light NQ cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6); * $p < 0.05$.

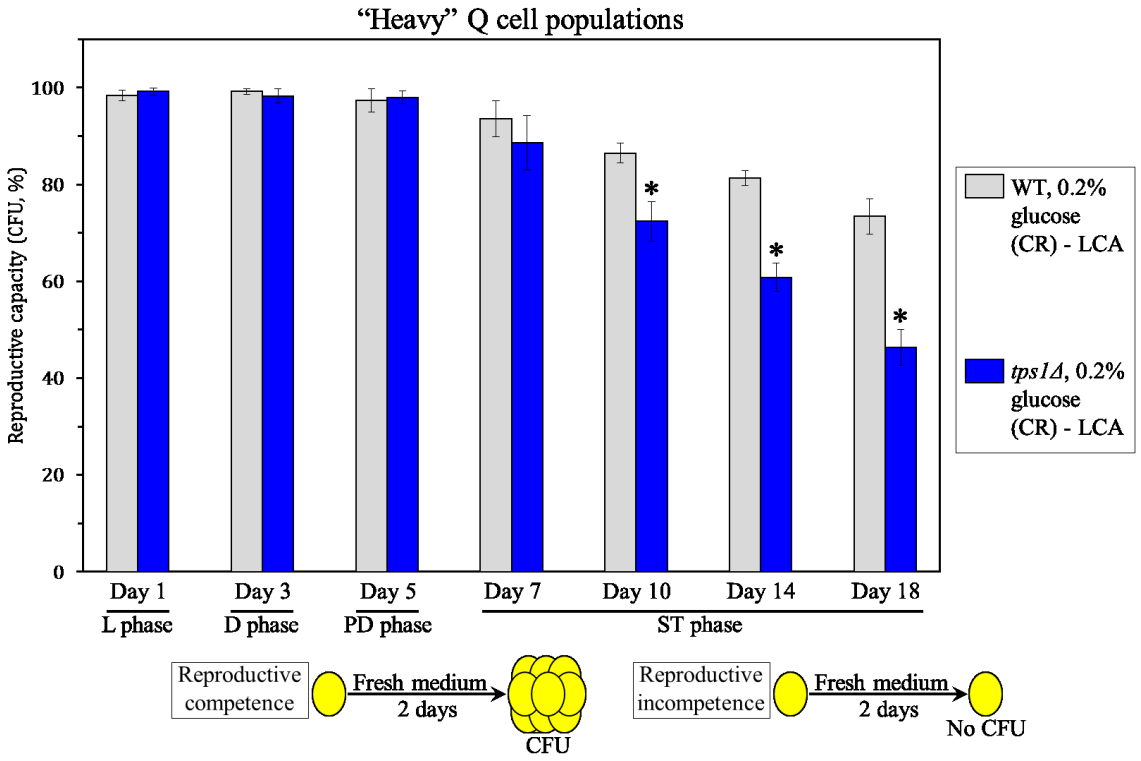

Figure 3.31. In yeast cultures under CR on 0.2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose reduces the reproductive (colony-forming) capacity of heavy Q cells. Wild-type (WT) and *tps1Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 0.2% glucose (CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered heavy Q cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6); * $p < 0.05$.

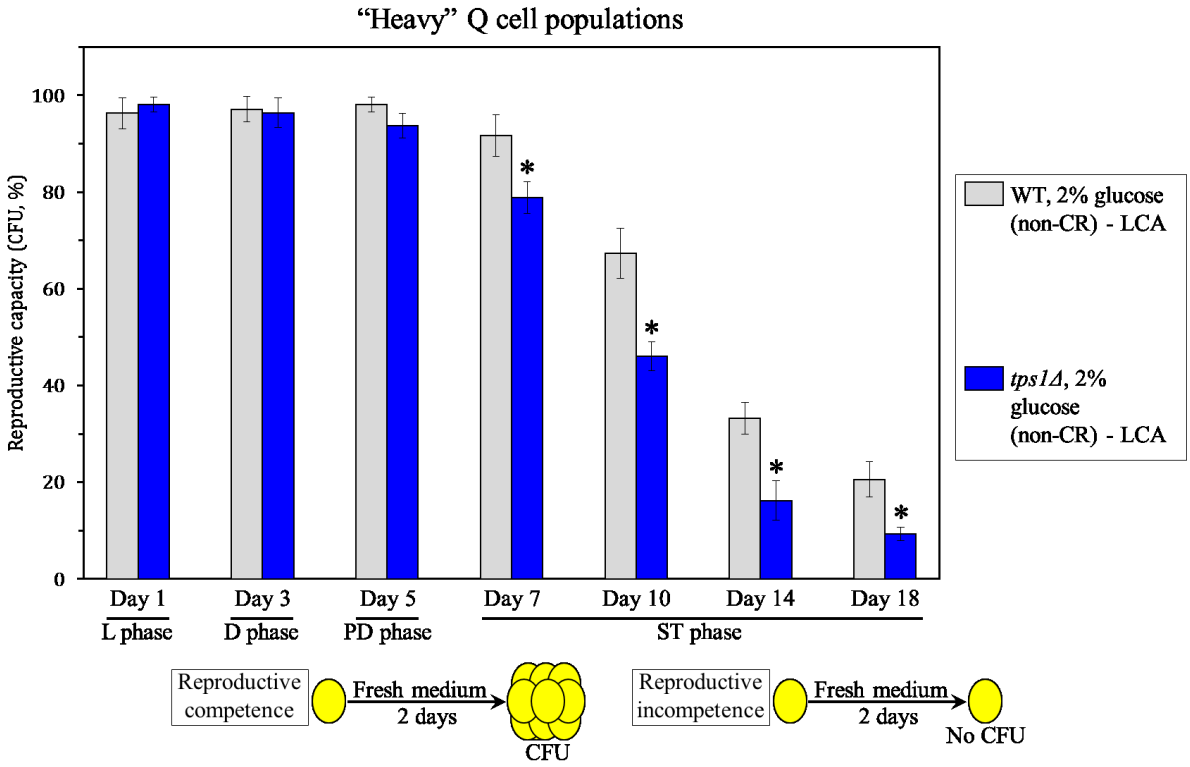

Figure 3.32. In yeast cultures under non-CR conditions on 2% glucose, the *tps1Δ*-dependent decrease of intracellular trehalose reduces the reproductive (colony-forming) capacity of heavy Q cells. Wild-type (WT) and *tps1Δ* mutant cells were cultured in the complete (nutrient-rich) YEP medium initially containing 2% glucose (non-CR conditions) without LCA, recovered from logarithmic (L), diauxic (D), post-diauxic (PD) or stationary (ST) growth phase and subjected to centrifugation in Percoll density gradient as described in ″Materials and Methods″. The reproductive (colony-forming) capacity was monitored by assessing the ability of recovered heavy Q cells to form colonies on solid YEPD medium after 2 days of incubation, as described in ″Materials and Methods″. Data are presented as means ± SEM (n = 3 – 6); * $p < 0.05$.

3.4 Discussion

Studies described in this chapter of my thesis suggest the following model of how LCA, the Ras/cAMP/PKA signaling pathway and trehalose define longevity of chronologically aging yeast by regulating various stages of the two differentiation programs linking cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state (Figure 3.33).

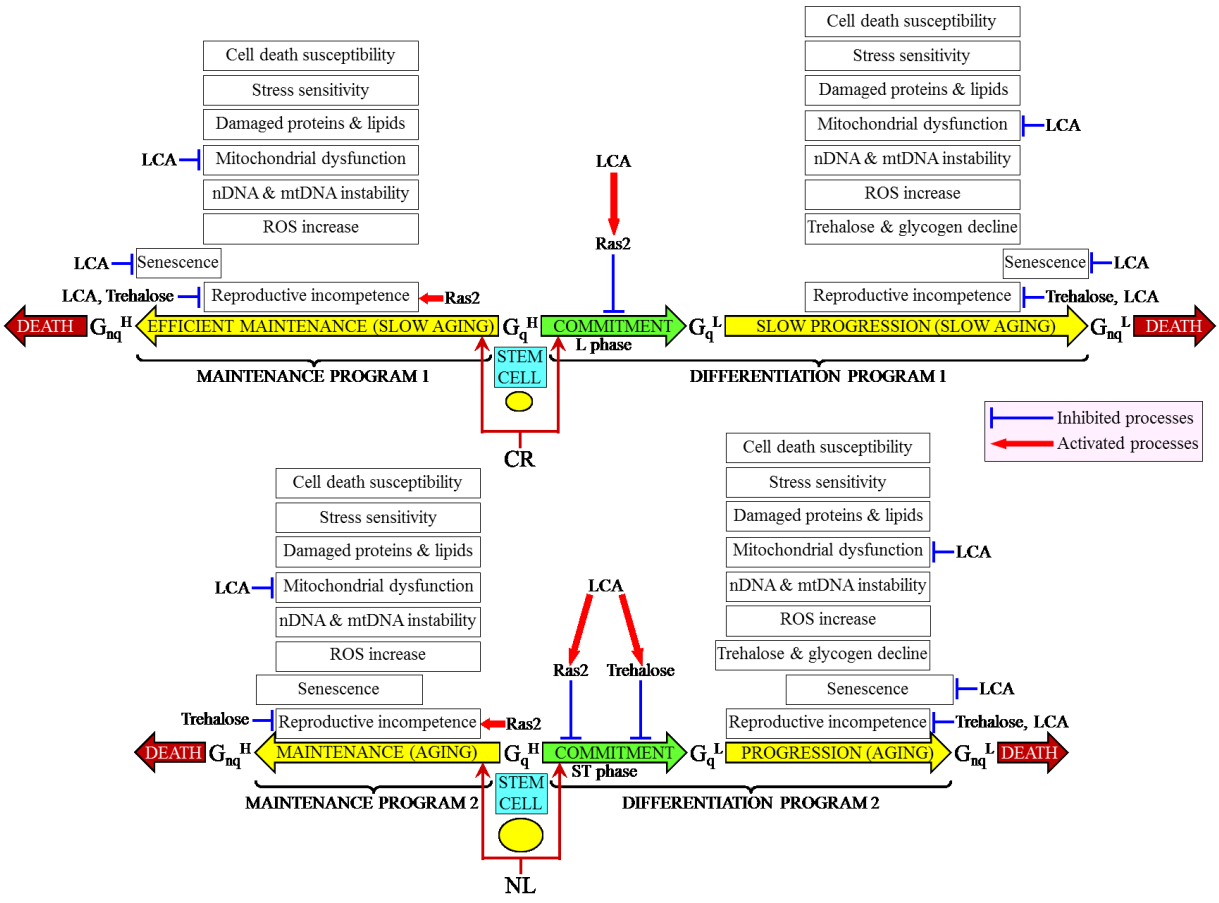

Figure 3.33. A model of how LCA, the Ras/cAMP/PKA signaling pathway and trehalose define longevity of chronologically aging yeast by regulating various stages of the two differentiation programs linking cellular aging to cell cycle regulation, maintenance of a Q state, and entry into and progression through an NQ state. The differentiation program 1 and the maintenance program 1 function in yeast cultured under CR conditions, whereas the differentiation program 2 and the maintenance program 2 operate in yeast grown under non-CR conditions. See text for more details. Abbreviations: CR, caloric restriction; G_q^H, a stem cell niche of heavy Q cells; G_{nq}^H, heavy NQ cells; G_q^L, light Q cells; G_{nq}^L, light NQ cells; L, logarithmic; nDNA, nuclear DNA; NL, nutrient limitation; mDNA, mitochondrial DNA; ST, stationary.

In the model that I propose here, in chronologically aging yeast limited in calorie supply the Ras/cAMP/PKA signaling pathway exhibits an anti-aging potential by delaying the commitment of the G_q^H stem cell niche to the differentiation program 1 leading to the conversion of these stem cells to G_q^L cells (Figure 3.33). Such previously unknown anti-aging potential of the Ras/cAMP/PKA signaling pathway can be significantly enhanced by LCA (Figure 3.33); a

mechanism by which LCA stimulates the ability the Ras/cAMP/PKA signaling pathway to delay the commitment of the G_q^H stem cell niche to a differentiation program 1 remains to be established. In yeast cultures under CR conditions, LCA also delays chronological aging by slowing down the progression of G_q^L cells through the differentiation program 1; this program underlies the conversion of G_q^L cells to G_{nq}^L cells (Figure 3.33). Such inhibitory effect of LCA on the progression of CR yeast through the differentiation program 1 is likely due to, at least in part, the ability of LCA to delay an age-related loss of mitochondrial functionality in yeast cells that have been committed to the differentiation program 1 (Figure 3.33). In addition, LCA also delays chronological aging of CR yeast by improving maintenance of the G_q^H stem cell niche via the maintenance program 1; such longevity-extending effect of LCA is due to its ability to slow down the conversion of the G_q^H stem cell niche to G_{nq}^H cells, in part because LCA can delay an age-related loss of mitochondrial functionality in yeast cells that are progressing through the maintenance program 1 (Figure 3.33). Furthermore, trehalose further delays the process of chronological aging in CR yeast not only by slowing down the progression of G_q^L cells through the differentiation program 1 but also by improving maintenance of the G_q^H stem cell niche via the maintenance program 1 (Figure 3.33). Moreover, in addition to the anti-aging potential the Ras/cAMP/PKA signaling pathway in CR yeast (see above), this pathway also displays a pro-aging effect by accelerating the progression of the G_q^H stem cell niche through the maintenance program 1 (Figure 3.33).

The model I propose here also posits that in chronologically aging yeast cultured under non-CR conditions: 1) the anti-aging potential of the Ras/cAMP/PKA signaling pathway consists in its ability to delay the commitment of the G_q^H stem cell niche to the differentiation program 2 leading to the conversion of these stem cells to G_q^L cells, 2) such anti-aging potential of the

Ras/cAMP/PKA signaling pathway can be significantly enhanced by LCA, 3) LCA also substantially amplify the ability of trehalose to slow down the commitment of the G_q^H stem cell niche to the differentiation program 2 leading to the conversion of these stem cells to G_q^L cells, 4) LCA and trehalose further delay the process of chronological aging in yeast cultures under non-CR conditions not only by slowing down the progression of G_q^L cells through the differentiation program 2 but also by improving maintenance of the G_q^H stem cell niche via the maintenance program 2, 5) such longevity-extending effects of LCA are likely due to its ability to delay an age-related loss of mitochondrial functionality in yeast cells that are progressing through the differentiation program 2 and through the maintenance program 2, and 6) the Ras/cAMP/PKA signaling pathway also has a pro-aging effect by accelerating the progression of the G_q^H stem cell niche through the maintenance program 2 (Figure 3.33).

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