Empirical Testing of the Evolutionary Theories of Aging Using Laboratory-Evolved Yeast Mutants with Extended Lifespan

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ABSTRACT

Empirical Testing of the Evolutionary Theories of Aging Using Laboratory-Evolved Yeast Mutants with Extended Lifespan

Alejandra Gomez-Perez, M.Sc.

We recently conducted a multistep selection of long-lived yeast mutants by a lasting exposure to lithocholic acid, a longevity-extending natural compound. Three selected long-lived mutant strains, called 3, 5 and 12, were able to maintain their extended lifespans following numerous passages in medium lacking lithocholic acid. In studies described in this thesis I demonstrated that the greatly extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant polygenic trait caused by mutations in more than two genes. To empirically validate evolutionary theories of programmed or non-programmed aging and age-related death, I investigated if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence early-life fitness when each of these mutants grows and ages alone, in the absence of a parental wild-type yeast strain that does not carry longevity-extending mutations. My studies revealed that these mutations (1) do not affect such key traits of early-life fitness as the exponential growth rate, efficacy of post-exponential growth and fecundity of yeast cells; and (2) enhance such key traits of early-life fitness as cell susceptibility to chronic exogenous stresses, mitochondria-controlled apoptosis triggered by a brief exposure to exogenous hydrogen peroxide, and lipoptotic form of death triggered by a short-term exposure to exogenous palmitoleic acid. These findings provide irrefutable proof of evolutionary theories of aging based on the concept of programmed aging and age-related death and invalidate evolutionary theories of non-programmed aging and age-

related death. I then examined if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence the relative fitness of each of these mutants in a direct competition assay with a parental wild-type strain. This assay mimics under various laboratory conditions the process of natural selection within a mixed population of yeast cells that (1) exhibit different longevity-defining genetic backgrounds; (2) differ in their lifespans if grow as a genetically homogenous cell population; and (3) compete for nutrients and other environmental resources. I found that in a population of mixed cells grown on 1% ethanol the dominant mutations that extend longevity of the three long-lived yeast mutants 3, 5 and 12 reduce the relative fitness of each of them in a direct competition assay with a parental wild-type strain. Based on these findings, I concluded that under laboratory conditions mimicking the process of natural selection within an ecosystem composed of yeast cells having different longevity-defining genetic backgrounds, each of the three long-lived mutants is forced out of the ecosystem by a parental wild-type strain exhibiting shorter lifespan. My findings imply that (1) yeast cells have evolved some mechanisms for limiting their lifespan upon reaching a certain chronological age; and (2) these mechanisms drive the evolution of yeast longevity towards maintaining a finite yeast lifespan within ecosystems. I hypothesize that these mechanisms may consist in the ability of a parental wild-type strain to secrete into an ecosystem certain compounds (small molecules and/or proteins) that slow down growth and/or kill long-lived yeast mutants within this ecosystem.

Acknowledgements

I am grateful to my supervisor, Dr. Vladimir Titorenko, for his guidance and support during the two years I spent in his laboratory. I would like to thank the members of my committee, Dr. Patrick Gulick and Dr. Reginald Storms, for their valuable suggestions during the course of my graduate research and studies. Many thanks go to all of my current and former lab-mates Adam Beach, Simon Bourque, Michelle Tali Burstein, Rachel Feldman, Alexander Goldberg, Olivia Koupaki, Pavlo Kyryakov, Anna Leonov, Vincent Richard and Sara Shafikhani for their friendship and support.

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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; DR, dietary restriction; IGF-1, the insulin/insulin-like growth factor 1 signaling pathway; LCA, lithocholic acid; NC, nitrocellulose; NPD, non-parental ditype tetrad; OD_{600}, optical density at 600 nm; PD, parental ditype tetrad; ROS, reactive oxygen species; ST, stationary growth phase; T, tetratype tetrad; WT, wild-type strain.

1 Introduction

1.1 Dysregulation of many processes within cells causes cellular and organismal aging

Aging of multicellular and unicellular eukaryotic organisms is due to impaired regulation of numerous processes within cells. These processes define the replicative and chronological age of a eukaryotic cell. They include cell cycle, cell growth, stress response, protein homeostasis, apoptotic and necrotic cell death, autophagy, proteasomal protein degradation, actin organization, signal transduction, nuclear DNA replication, chromatin maintenance, ribosome biogenesis and translation, lipid and carbohydrate metabolism, trehalose homeostasis, oxidative metabolism and protein synthesis in mitochondria, NAD^+ homeostasis, amino acid biosynthesis and degradation, and ammonium and amino acid uptake [1 - 6].

The spatiotemporal organization of all these cellular processes and their functional states are governed by an evolutionarily conserved signaling network. The network integrates the insulin/insulin-like growth factor 1 (IGF-1), AMP-activated protein kinase/target of rapamycin (AMPK/TOR) and cAMP/protein kinase A (cAMP/PKA) nutrient- and energy-sensing signaling pathways (Figure 1.1) [1, 3, 4, 6 - 8]. By sensing the intracellular and organismal nutrient and energy status, this longevity signaling network coordinates information flow along its convergent, divergent and multiply branched pathways to regulate cellular aging, influence age-related pathologies and define organismal lifespan [1, 6 - 8]. The network also responds to the age-related partial mitochondrial dysfunction and is modulated by mitochondrially produced reactive

oxygen species (ROS) [6, 8, 9 - 12].

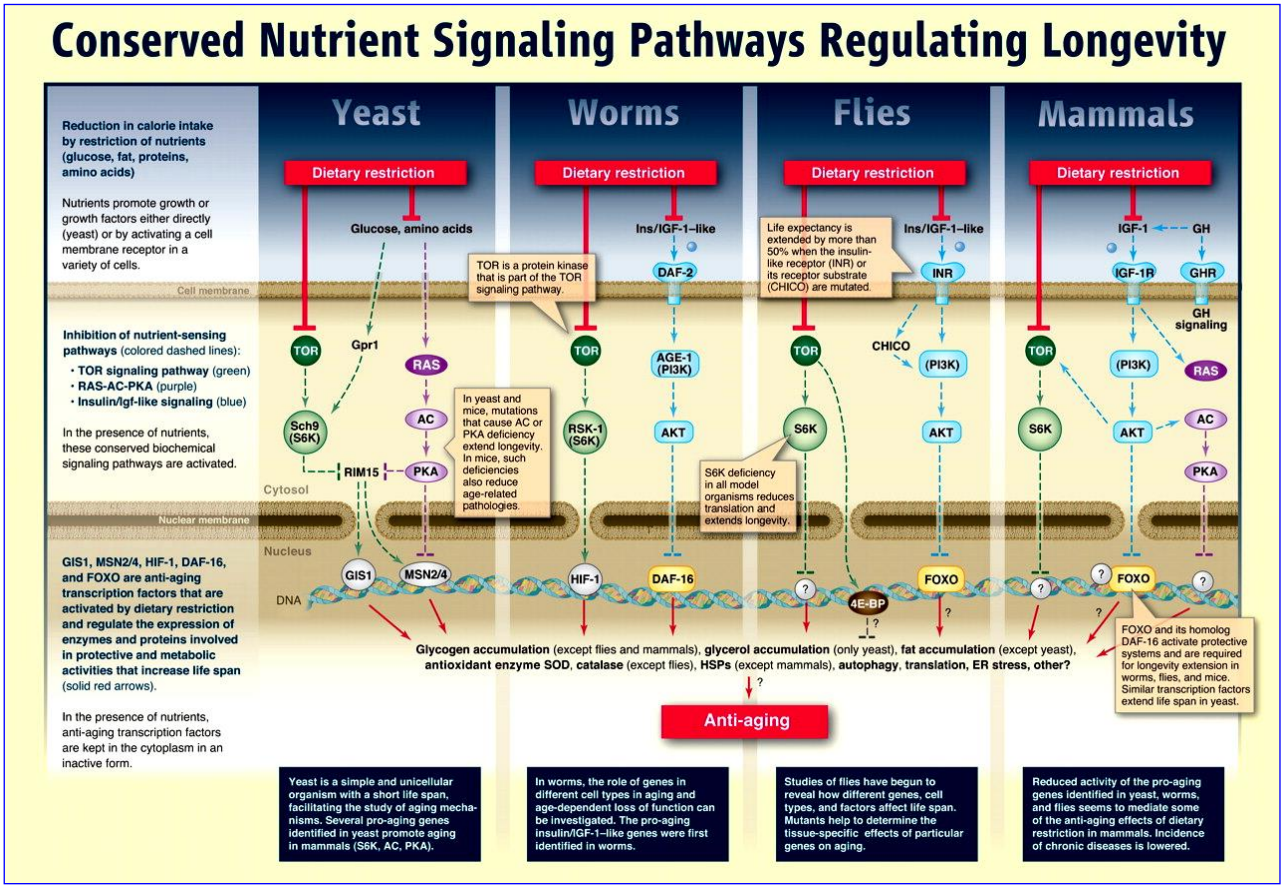


Figure 1.1. The spatiotemporal organization of all longevity-defining cellular processes and their functional states are governed by an evolutionarily conserved signaling network. The network integrates the insulin/insulin-like growth factor 1 (IGF-1), AMP-activated protein kinase/target of rapamycin (AMPK/TOR) and cAMP/protein kinase A (cAMP/PKA) nutrient- and energy-sensing signaling pathways. Reproduced from reference 3.

Certain dietary and pharmacological interventions slow down cellular aging, ameliorate aging-related pathology and extend organismal lifespan across phyla by

attenuating information flow along the pro-aging IGF-1, AMPK/TOR and cAMP/PKA pathways. These interventions include (1) caloric restriction (CR), a diet in which only calorie intake is reduced but the supply of amino acids, vitamins and other nutrients is not compromised; (2) dietary restriction (DR), in which the intake of nutrients (but not necessarily of calories) is reduced by limiting food supply without causing malnutrition; and (3) rapamycin, resveratrol, metformin, spermidine and several other small molecules [3, 13 - 25].

1.2 The yeast *Saccharomyces cerevisiae* is a valuable model for studying mechanisms of cellular and organismal aging

The budding yeast *S. cerevisiae*, a unicellular eukaryote with an annotated genome, is one of the most important model organisms used for unveiling molecular mechanisms of cellular and organismal aging in multicellular eukaryotes [3, 6, 22]. Due to the relatively short and easily monitored lifespan of this genetically and biochemically manipulable organism amendable to high-throughput screening procedures, studies of budding yeast have led to the identification of genes, signaling pathways, cellular processes and pharmacological interventions that subsequently have been implicated in defining longevity of invertebrate and mammalian organisms [3, 6, 22, 26 - 28]. A growing body of evidence supports the view that the key features and molecular mechanisms of cellular aging discovered in yeast have been evolutionarily conserved in invertebrates and mammals [3, 6, 22].

Yeast aging can be measured in two different ways. Replicative aging is defined by the maximum number of daughter cells that a mother cell can produce before senescence

and models aging of mitotically active mammalian cells [6, 27, 28]. Chronological aging is measured by the length of time a yeast cell remains viable in a nondividing state and models aging of postmitotic mammalian cells (*e.g.*, neurons) [6, 26, 27].

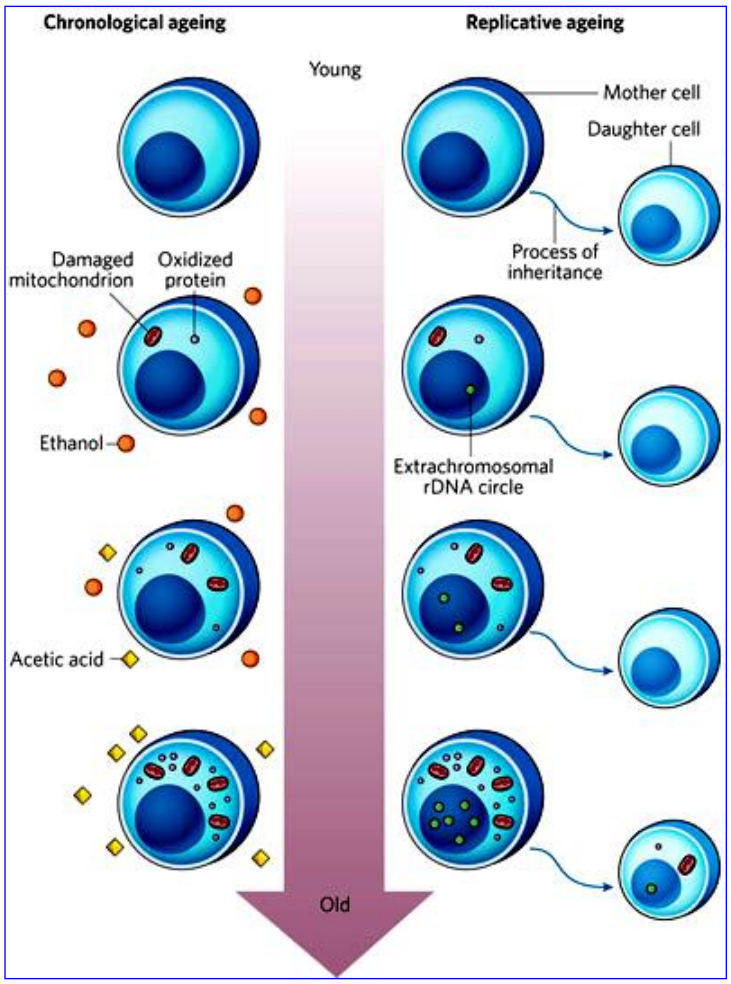


Figure 1.2. Yeast aging can be measured in two different ways. Replicative aging is defined by the maximum number of daughter cells that a mother cell can produce before senescence and models aging of mitotically active mammalian cells. Chronological aging is measured by the length of time a yeast cell remains viable in a nondividing state and models aging of postmitotic mammalian cells. Reproduced from reference 22.

1.3 A hypothesis on the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms within ecosystems

In a quest for small molecules that can increase yeast chronological lifespan, our laboratory used a custom-designed chemical genetic screen of extensive compound libraries to identify 24 previously unknown longevity-extending compounds [21]. One of them, a bile acid called lithocholic acid (LCA) (Figure 1.3), acts in synergy with a longevity-extending CR diet to enable a significant further extension of yeast lifespan [21]. Our recent studies revealed the molecular mechanisms underlying the ability of LCA to delay cellular aging in yeast by remodeling lipid metabolism and transport within mitochondria, the endoplasmic reticulum, lipid droplets and peroxisomes [2, 21, 29 - 32].

It has been shown that, unlike mammals, yeast do not synthesize LCA or any other bile acids [21, 33, 34]. Our laboratory therefore proposed that bile acids released into the environment by mammals may act as interspecies chemical signals extending longevity of yeast species and, perhaps, of other organisms that can (1) sense these mildly toxic molecules with detergent-like properties; and (2) respond to the resulting mild cellular damage by developing the most efficient stress protective mechanisms [35, 36]. We hypothesized that such mechanisms may provide effective protection of yeast and other organisms not only against cellular damage caused by bile acids but also against molecular and cellular damage accumulated with age [35, 36]. In our hypothesis, those species of the organisms within an ecosystem that that have been selected for the most effective (as compared to their counterparts of the same species) mechanisms providing protection against bile acids are expected to (1) live longer than their counterparts within the same species; and (2) evolve the most effective anti-aging mechanisms that are

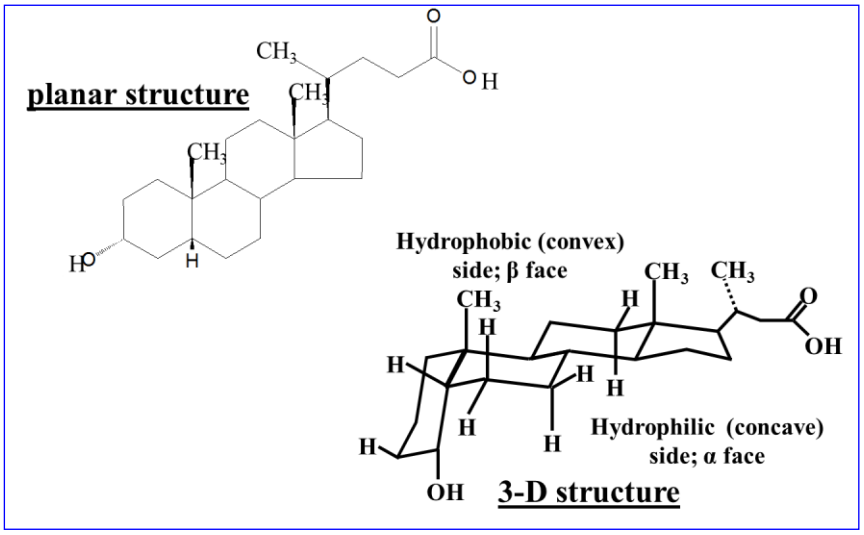


Figure 1.3. Planar and 3-D structures of a bile acid called lithocholic acid, a natural anti-aging compound that extends longevity of chronologically aging yeast.

sensitive to regulation by bile acids [35, 36]. Thus, our hypothesis posits that the ability of certain non-mammalian species within an ecosystem to sense bile acids produced by mammals and then to respond by undergoing certain longevity-extending changes to their physiology will increase their chances of survival - thereby creating selective force aimed at maintaining such ability and driving the evolution of their longevity regulation mechanisms [35, 36].

Our hypothesis on longevity regulation by bile acids within ecosystems may also explain the evolutionary origin of the lifespan-extending effect of another anti-aging natural compound, called rapamycin. This macrocyclic lactone synthesized by soil bacteria to inhibit growth of fungal competitors has been shown to extend longevity of yeast, fruit flies and mice by specifically inhibiting the nutrient-sensory protein kinase TOR, a master negative regulator of the pro-aging TOR signaling pathway [37 - 42].

Rapamycin exhibits a potent cytostatic effect by causing G1 cell cycle arrest and greatly delaying proliferative growth of organisms across phyla [37 - 42]. We therefore hypothesized that rapamycin released into the environment by soil bacteria not only suppresses growth of fungal competitors, but may also may create selective pressure for the evolution of yeast, fruit fly and mammalian species that can respond to rapamycin-induced growth retardation by developing certain mechanisms aimed at such remodeling of their anabolic and catabolic processes that would increase their chances of survival under conditions of slow growth [35, 36]. We proposed that some of these mechanisms delay aging by optimizing essential longevity-related processes and remain sensitive to modulation by rapamycin [35, 36]. In our hypothesis, the ability of yeast, fruit fly and mammalian species within an ecosystem to sense rapamycin produced by soil bacteria and then to respond by undergoing certain lifespan-extending changes to their metabolism and physiology could increase their chances of survival - thereby creating selective force aimed at maintaining such ability and driving the evolution of their longevity regulation mechanisms [35, 36].

Our hypothesis on longevity regulation by bile acids and rapamycin within ecosystems complements the “xenohormesis” hypothesis Howitz and Sinclair [43 - 45]. According to this hypothesis, in response to various hormetic environmental stresses - such as UV light, dehydration, infection, predation, cellular damage and nutrient deprivation - plants and other autotrophic organisms synthesize a group of secondary metabolites called xenohormetic phytochemicals [43 - 45]. Prior to being released into the environment, these secondary metabolites activate defense systems protecting the host autotrophic organisms against hormetic environmental stresses that caused their

synthesis [43 - 45]. After being released into the environment, these xenohormetic phytochemicals provide benefits to health and longevity of heterotrophic organisms within the ecosystem. It was proposed by Howitz and Sinclair that xenohormetic phytochemicals cause such life-extending and health-improving effects not by operating as mildly toxic hormetic molecules, but by activating the key enzymes of stress-response, anti-aging pathways known to govern longevity-related processes in heterotrophic organisms [43 - 45].

Recent studies revealed that some xenohormetic phytochemicals, such as resveratrol and caffeine, extend longevity of heterotrophic organisms by attenuating the adaptable TOR signaling pathway known to accelerate their aging [39, 46 - 51]. Because the TOR pathway also plays a pivotal role in promoting proliferative growth of all heterotrophic organisms, resveratrol and caffeine exhibit a cytostatic effect in these organisms [39, 46 - 51]. Based on these recent findings, we extended the xenohormesis hypothesis of Howitz and Sinclair by proposing that within each of the heterotrophic species composing an ecosystem there are organisms that (1) possess the most effective (as compared to their counterparts of the same species) mechanisms for sensing xenohormetic and cytostatic phytochemicals released into the environment by autotrophic species; and (2) can respond to these phytochemicals by activating the key enzymes of stress-response, anti-aging pathways and/or by attenuating the adaptable, pro-aging TOR signaling pathway - thereby undergoing life-extending changes to their metabolism and physiology [35, 36]. In our hypothesis, these heterotrophic organisms are expected to live longer than their counterparts within the same species [35, 36]. Thus, their ability to sense the longevity-extending xenohormetic and cytostatic phytochemicals released into

the environment by autotrophic species and to respond to these phytochemicals by undergoing certain life-extending metabolic and physiological changes is expected to (1) increase their chances of survival; (2) create selective forces aimed at maintaining such ability; and (3) drive the evolution of their longevity regulation mechanisms [35, 36].

In sum, our analysis of how several small molecules synthesized and released into the environment by one species of the organisms composing an ecosystem extend longevity of other species within this ecosystem suggested a unified hypothesis in which these interspecies chemical signals create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms (Figure 1.4) [35, 36]. In this unified hypothesis, after being released into the environment by one species of organisms capable of synthesizing such small molecules, they can activate anti-aging processes and/or inhibit pro-aging processes in other species within an ecosystem. Within each of these other species, there are organisms that possess the most effective (as compared to their counterparts of the same species) mechanisms for sensing the interspecies chemical signals and for responding to such signals by undergoing certain lifespan-extending changes to their metabolism and physiology; such lifespan-extending changes could be hormetic and/or cytostatic by their nature (Figure 1.4) [35, 36]. These organisms therefore are expected to live longer than their counterparts of the same species within the ecosystem. Thus, the ability of a species of the organisms composing an ecosystem to sense the longevity-modulating interspecies chemical signals released into the environment by other species within the ecosystem and to respond to these signals by undergoing certain life-extending metabolic and physiological changes is expected to increase its chances to survive, thereby creating selective force aimed at

maintaining such ability (Figure 1.4) [35, 36]. Our unified hypothesis implies that the evolution of longevity regulation mechanisms in each species of the organisms composing an ecosystem is driven by the ability of this species to undergo specific lifespan-extending metabolic or physiological changes in response to xenohormetic, hormetic or cytostatic chemical compounds that are released to the ecosystem by other species.

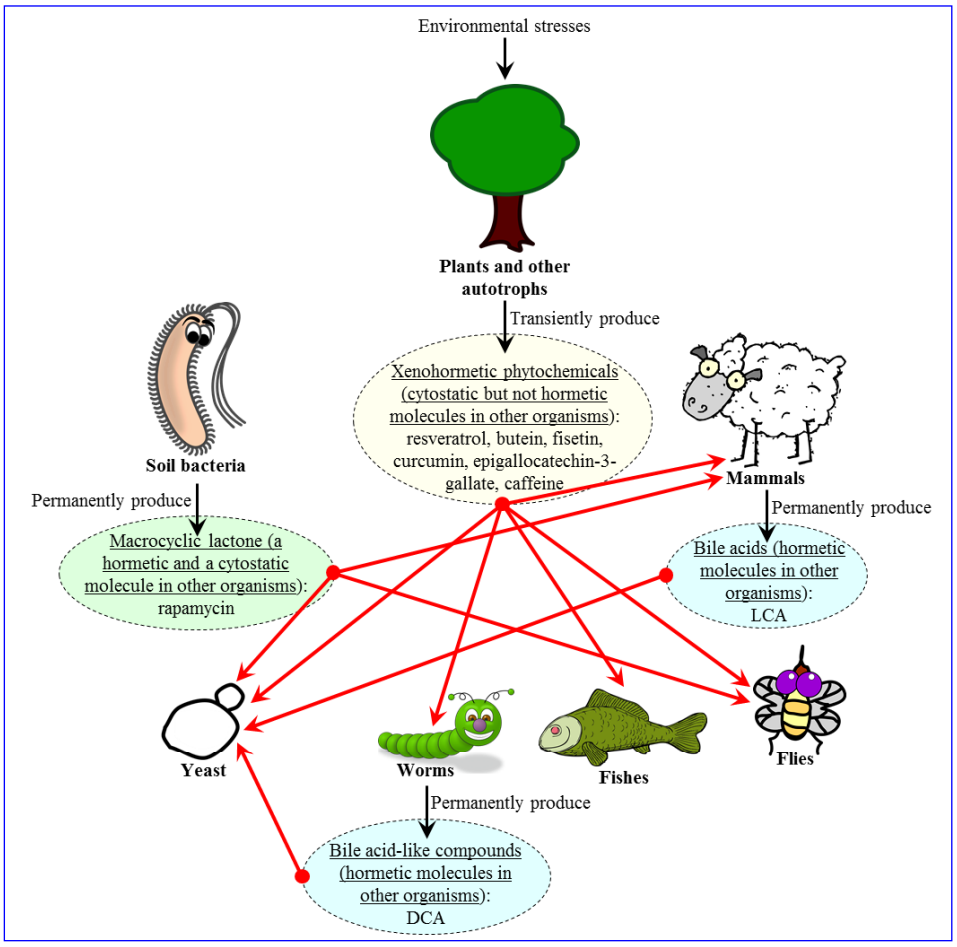


Figure 1.4. The xenohormetic, hormetic and cytostatic selective forces may drive the evolution of longevity regulation mechanisms within an ecosystem. We proposed that organisms from all domains of life within an ecosystem synthesize chemical compounds

that (1) are produced and then released into the environment permanently or only in response to deteriorating environmental conditions, increased population density of competitors and/or predators, or changes in food availability and its nutrient and/or caloric content; (2) are mildly toxic compounds that trigger a hormetic response in an organism that senses them or, alternatively, are not toxic for any organism within the ecosystem and do not cause a hormetic response; (3) are cytostatic compounds that attenuate the TOR-governed signaling network or, alternatively, do not modulate this growth-promoting network; and (4) extend longevity of organisms that can sense these compounds (red arrows), thereby increasing their chances of survival and creating selective force aimed at maintaining the ability of organisms composing the ecosystem to respond to these compounds by undergoing specific lifespan-extending changes to their physiology [35, 36]. In our hypothesis, the evolution of longevity regulation mechanisms in each group of the organisms composing an ecosystem is driven by the ability of this group of organisms to undergo specific lifespan-extending changes to their physiology in response to a compendium of "critical" chemical compounds that are permanently or transiently released to the ecosystem by other groups of organisms. Abbreviations: LCA, lithocholic acid; DCA, bile acid-like dafachronic acids. Reproduced from reference 35.

1.4 Empirical confirmation of a hypothesis on the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the ecosystemic level

To empirically verify our hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the

ecosystemic level, our laboratory carried out a 3-step selection of long-lived yeast mutants by a lasting exposure to LCA under laboratory conditions [52]; in collaboration with Michelle Tali Burstein and Anastasia Glebov, I carried out this selection as a Honours undergraduate student in the Titorenko laboratory.

For the first step of such selection, yeast cells from an overnight culture were inoculated into a complete growth medium (with or without LCA) containing 0.2% glucose to the initial cell titre of 1×10^5 cells/ml (Figure 1.5). After one week of the incubation in this medium and following approximately 45 cell generations, yeast entered into a non-proliferative state by reaching stationary (ST) growth phase at the cell titre of 2×10^8 cells/ml (Figure 1.5). Following their entry into a non-proliferative state, yeast cells were cultured for additional 5 weeks. By the end of this cultivation, only ~ 0.02% of cells in the medium without LCA remained viable. In the cell cultures that were supplemented with LCA, ~ 1% of cells remained viable – due to the ability of LCA to increase the chronological lifespan (CLS) of non-dividing (quiescent) yeast cells. Thus, the enrichment factor for long-lived mutants by the end of each selection step was 10^2. Five cultures were used to carry out the selection of long-lived yeast mutants: the first culture lacked LCA; the second culture contained 5 μM LCA added at the moment of cell inoculation; the third culture contained 50 μM LCA added at the moment of cell inoculation; the fourth culture contained 250 μM LCA added at the moment of cell inoculation; and the fifth culture was supplemented with 10 doses of 5 μM LCA each by adding this bile acid every 3 or 4 days. At the end of the first selection step, aliquots of each of the five cultures were plated onto plates with solid complete growth medium containing 2% glucose. After 2 days, each of the 10 randomly chosen colonies was

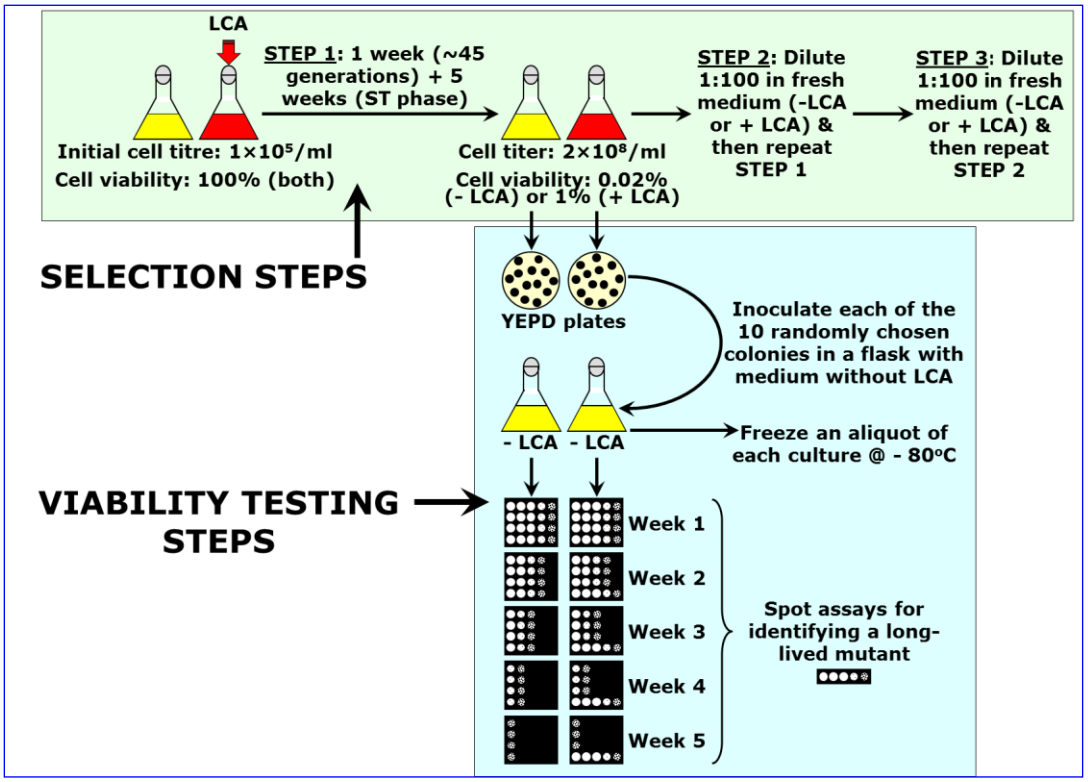


Figure 1.5. A 3-step process of the LCA-driven experimental evolution of longevity regulation mechanisms by conducting selection of long-lived yeast mutants through a lasting exposure to LCA. Reproduced from reference 52. See text for details.

inoculated into a liquid medium without LCA to carry out a viability testing step (Figure 1.5). Every week, an aliquot of each culture was used for a spot assay of cell viability to identify long-lived mutants (if any) induced due to a lasting exposure of yeast to LCA during the selection step (Figure 1.5). If such mutants were detected, an aliquot of their culture in medium lacking LCA was frozen at -80^{o}C.

To conduct the second selection step, an aliquot of the culture recovered at the end of the first step of selection was diluted 100 folds in a complete growth medium

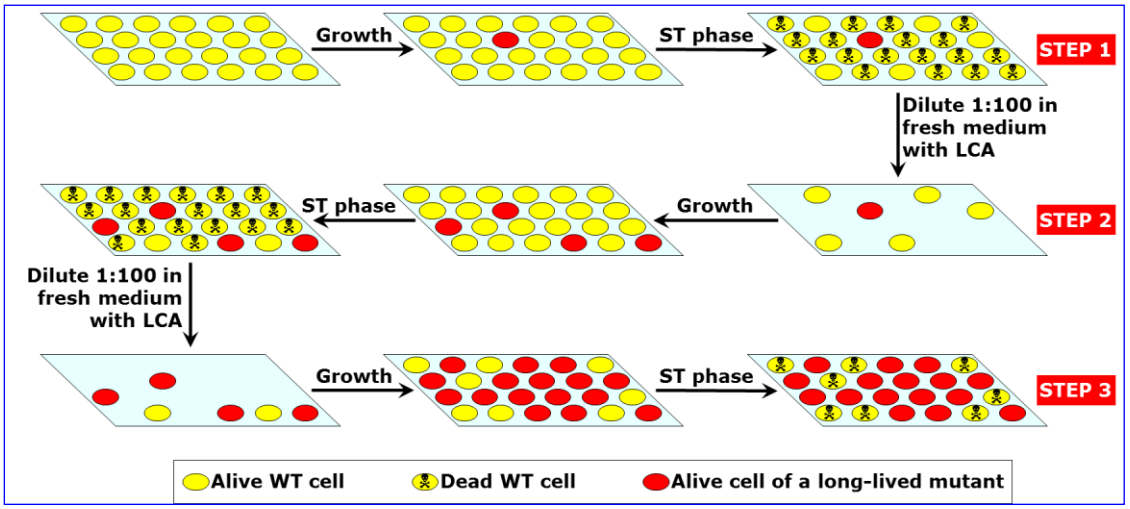


Figure 1.6. The fraction of long-lived mutants in a population of yeast is increased by the end of each of the 3 steps of the LCA-driven experimental evolution of longevity regulation mechanisms. Reproduced from reference 52. See text for details.

containing 0.2% glucose, with or without LCA; if added to a culture for the second selection step, LCA was present at the same final concentration as that in the medium used for the first step of selection (see above). From that point, the second selection step was carried out exactly as the first one and was followed by a spot-assay viability step described above (Figure 1.5). The second selection step was followed by the third selection step and then by a spot-assay viability step, both conducted as described above and depicted in Figure 1.5.

The 3-step selection of long-lived yeast mutants depicted in Figure 1.5 was expected to result in the increased fraction of long-lived mutants in a population of yeast exposed to LCA (Figure 1.6). Moreover, the fraction of such long-lived mutants in a population was anticipated to progressively increase at each of the consecutive selection

steps (Figure 1.6).

By carrying out this 3-step selection of long-lived yeast mutants under laboratory conditions, we found no such mutants at the end of the first selection step, 4 mutants at the end of the second selection step, and 16 mutants at the end of the third selection step [52]. We therefore concluded that a long-term exposure of wild-type (WT) yeast to LCA following the 3 selection steps did result in selection of yeast mutants that live longer in the absence of LCA than their ancestor [52].

The 3 steps of experimental evolution of long-lived yeast mutants by a prolonged exposure of WT yeast to LCA resulted in selection of 20 mutants that in a spot-assay lived longer than their ancestor in medium lacking LCA [52]. An aliquot of the culture of each of these long-lived yeast mutants was frozen at -80^{o}C immediately after being recovered during the second or third selection step. To test the ability of these species to maintain their greatly extended lifespans following the 1st passage in medium without LCA, each aliquot was thawed and then inoculated into a complete liquid medium lacking this bile acid and containing 0.2% glucose. Our comparative analysis of the CLS of WT strain and all of these selected long-lived yeast mutants revealed that mutants 3, 5 and 12 maintained their ability to live much longer than WT following the 1st passage (Figure 1.7) [52].

The selected long-lived yeast mutants 3, 5 and 12 underwent four more passages (each carried out as described for the 1st passage) in a complete liquid medium without LCA and then tested again for their ability to maintain greatly extended lifespans, now following five successive passages in medium without this bile acid. As we found, each of these three long-lived yeast mutants evolved under laboratory conditions maintained

its greatly extended lifespan following five passages in medium without LCA (Figure 1.8) [52].

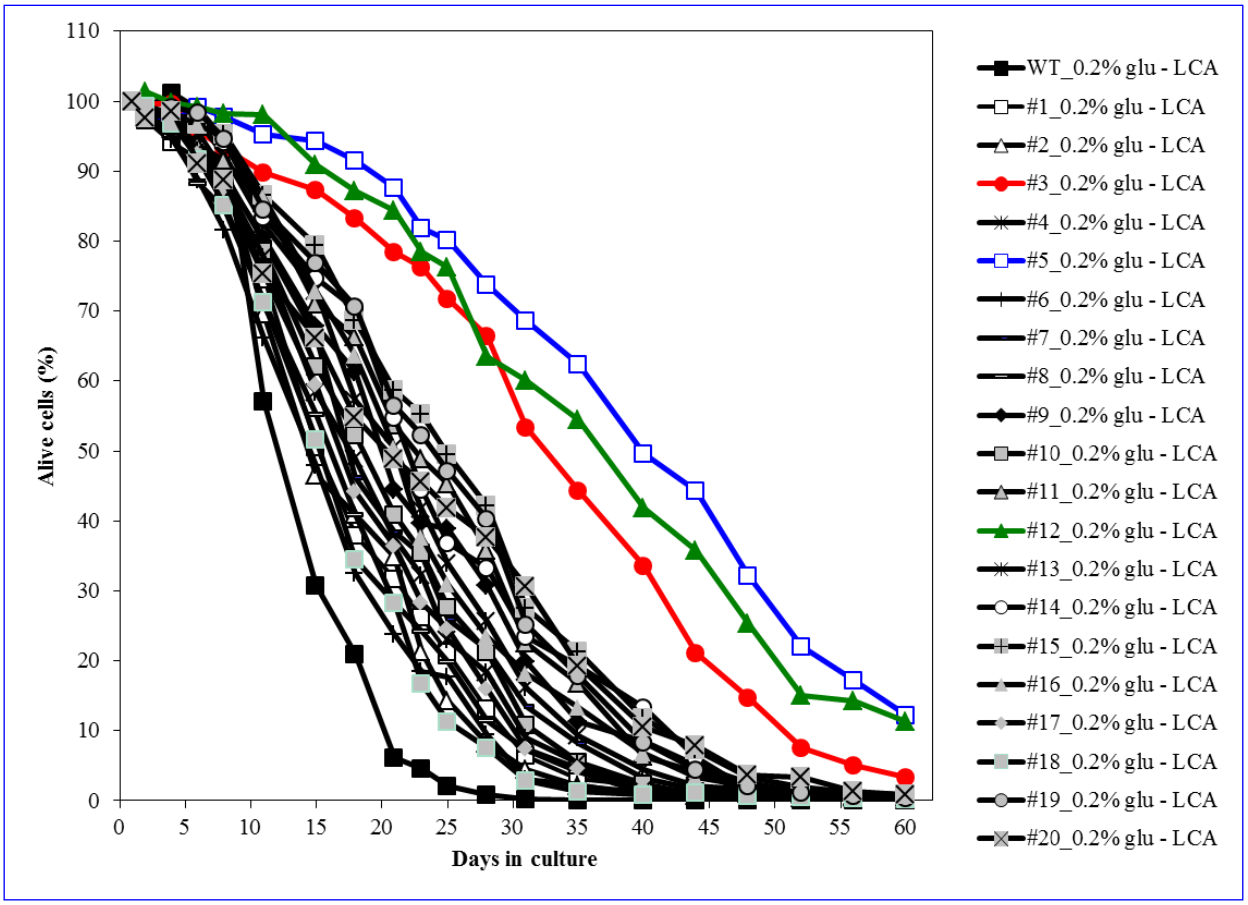


Figure 1.7. The selected long-lived yeast mutants 3, 5 and 12 maintain their ability to live much longer than WT following the 1st passage in medium lacking LCA. Survival of chronologically aging WT and of the yeast mutants 3, 5 and 12 cultured under CR conditions in a liquid complete medium initially containing 0.2% glucose without LCA. Reproduced from reference 52.

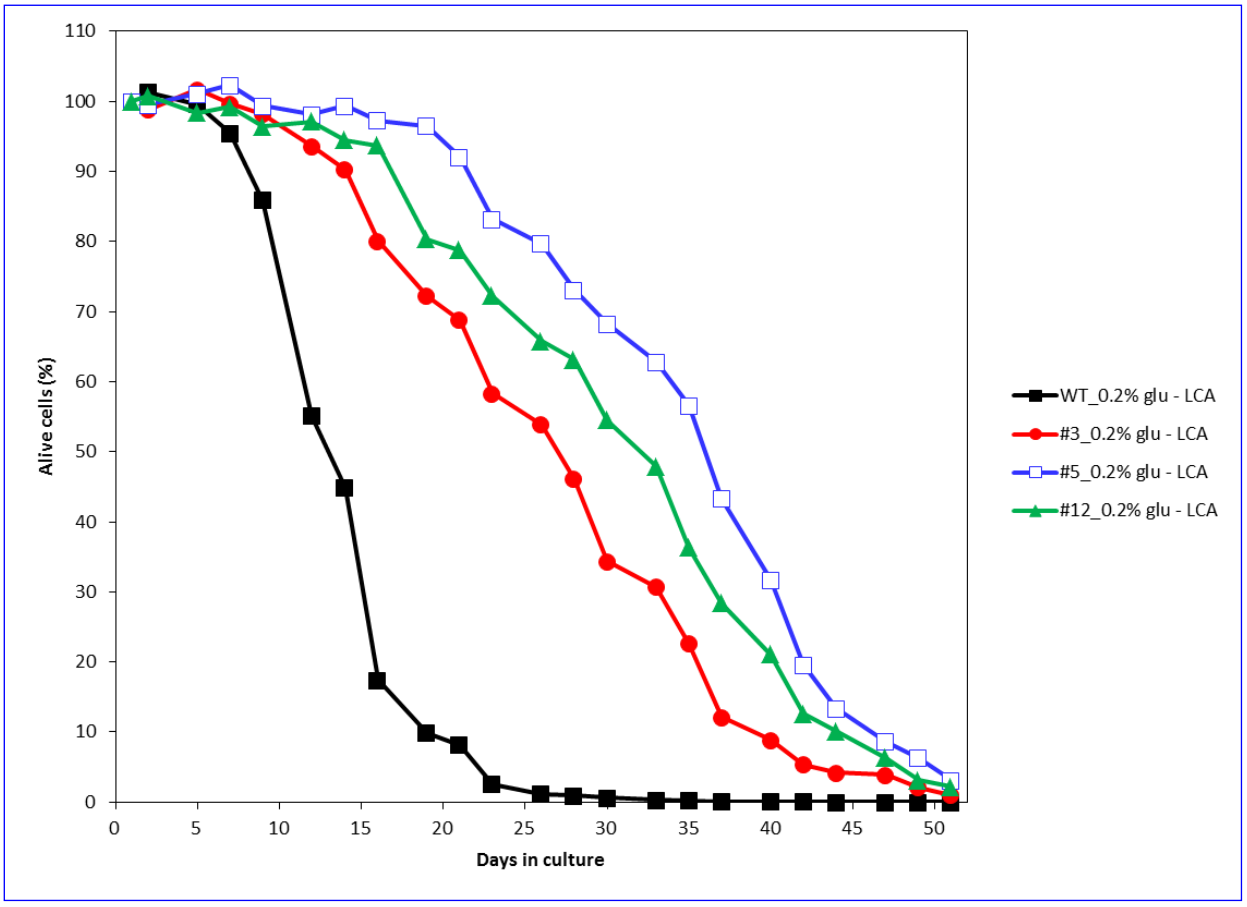


Figure 1.8. The selected long-lived yeast mutants 3, 5 and 12 maintain their ability to live much longer than WT following five successive passages in medium lacking LCA. Survival of chronologically aging WT and of the yeast mutants 3, 5 and 12 cultured under CR conditions in a liquid complete medium initially containing 0.2% glucose without LCA. Reproduced from reference 52.

1.5 The evolutionary theories of aging: the concepts of programmed and non-programmed aging

In theory, living organisms can avoid age-related death for a potentially unlimited period of time [53, 54]. This is because from the point of view of thermodynamics living

organisms are open self-organizing systems that can use exogenous energy to resist a progressive increase in entropy and the resulting molecular damage and disorder as they age [53, 54]. Yet, it is well known that organismal lifespan (1) has a limit that is unique to each species; and (2) varies drastically between different species (Figure 1.9) [55 - 58].

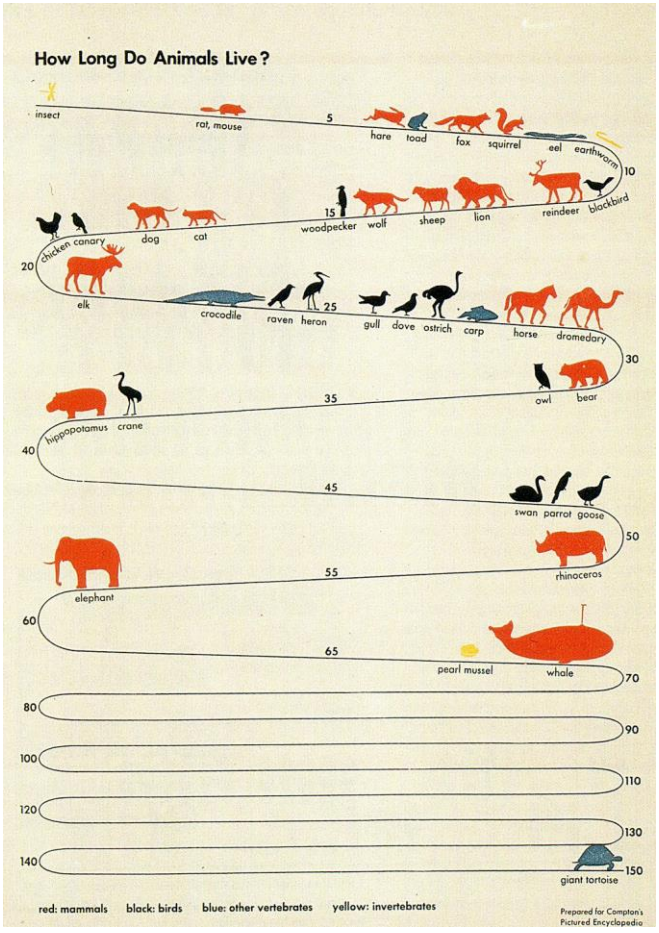


Figure 1.9. Average ages which certain animals may be expected to reach, based on reports of zoos and estimates of biologist. Data from Field Museum of Natural History. Prepared for Complon's Pictured Encyclopedia. Reproduced from http://www.woohome.com/photograph/how-long-do-animals-live.

Since late XIX century, numerous evolutionary theories of aging have been proposed in an attempt to resolve this paradox [53 - 57]. One group of these theories is based on the concept of programmed aging and age-related death; these programmed aging theories are trying to explain how the evolutionary force actively limits organismal lifespan at an age unique to each species [54, 57, 59 - 69]. Another group of evolutionary aging theories is based on the notion of non-programmed aging and age-related death; these non-programmed aging theories are attempting to rationalize how lack of the evolutionary force passively limits organismal lifespan at an age characteristic of each species [54, 56, 57, 59, 60, 69]. These two groups of evolutionary aging theories are discussed below.

The first evolutionary theory of aging, known as the theory of programmed death, was developed by August Weismann. According to this theory, natural selection resulted in preferential reproduction of those members of a particular species that are able to die when they reach a certain age, which is unique to this species [57, 69, 70]. By undergoing a "programmed" death at such species-specific age, older members of this species are eliminated from a competition with their younger counterparts for natural resources [57, 69, 70]. In the programmed death theory, the evolutionary advantage to having a limited lifespan at a species-specific age consists in providing a benefit to survival of a group of individuals by creating a disadvantage to those individuals within the group that has reached such an age [57, 69, 70].

Recent advances in our understanding of molecular mechanisms underlying cellular aging and organismal longevity marked a Renaissance period in developing evolutionary theories of aging that are based on the concept of programmed aging and age-related death [53, 54, 56, 57, 59 - 69]. These relatively recently developed

evolutionary theories of programmed aging and age-related death include (1) group selection theory [57, 59, 69]; (2) kin selection theory [57, 59, 69, 71]; (3) evolvability theory [57, 59, 69, 72]; (4) phenoptosis theory [61 - 63, 73]; and (5) altruistic aging theory [66 - 68, 74 - 77]. Akin to the theory of programmed death developed by August Weismann [57, 69, 70], all these contemporary evolutionary theories of aging are based on the premise that natural selection resulted in preferential reproduction of those members of various species that have evolved certain mechanisms for limiting their lifespans in a species-specific fashion and upon reaching a species-specific age [57, 59, 61- 63, 66, 69, 71 - 73].

It needs to be emphasized that recent studies of molecular mechanisms underlying cellular aging and organismal longevity provided a body of evidence favoring evolutionary theories of aging based on the concept of programmed aging and age-related death. In particular, it has been shown that cellular aging can be significantly delayed and organismal longevity can be substantially extended by certain genetic, dietary and pharmacological interventions that attenuate a limited set of pro-aging pathways and processes integrated into a pro-aging signaling network governing cellular aging; recent evidence supports the view that this pro-aging signaling network has evolved to limit lifespan in evolutionarily distant organisms in a species-specific fashion and upon reaching a certain species-specific age [1, 3, 4, 6 - 10, 13 - 25, 29, 37, 41 - 43, 46 - 51].

One of the key features of all evolutionary theories of aging based on the concept of programmed aging and age-related death is that various mutations delaying cellular aging and extending organismal longevity may or may not reduce early-life fitness; it was assumed that mutations affecting some of the modules of the pro-aging signaling network

could reduce early-life fitness, whereas mutations affecting other modules of this network could have no effect on it [66, 78 - 91]. Early-life fitness is known to include the following traits: (1) metabolic rate under various environmental conditions; (2) growth rate and, in yeast, the ability to utilize alternative carbon sources; (3) physical activity; (4) fecundity - *i.e.*, efficacies of mating and reproduction (including sporulation in yeast); (5) resistance to fluctuations in temperature, light, humidity and other environmental factors (such as osmolarity fluctuations in yeast); and (6) susceptibility to environmental toxins [66, 78 - 91]. Noteworthy, until now the effects of various genetic longevity-extending interventions on early-life fitness have been analyzed mainly under laboratory conditions under which long-lived mutants of evolutionarily distant species were growing, developing and aging alone, in the absence of "WT" individuals of the same species that do not carry longevity-extending mutations and thus do not have lifespan extended beyond a species-specific age [1, 3, 4, 6 - 10, 13 - 25, 29, 37, 41 - 43, 46 - 51]. However, these laboratory conditions do not mimic the process of natural selection within a mixed population of individuals that belong to the same species but exhibit different longevity-defining genetic backgrounds, have lifespans at a species-specific age and beyond it, and compete for nutrients and other environmental resources [91 - 97].

Unlike the evolutionary theories of aging that are based on the concept of programmed aging and age-related death, all evolutionary theories of non-programmed aging and age-related death posit that organismal lifespan is limited at an age characteristic of each species due to lack of the evolutionary force [54, 56, 57, 59, 60, 69]. These evolutionary theories of non-programmed aging and age-related death include (1) the mutation accumulation theory [57, 59, 69, 98, 99] and its modified version known

as the late-life mortality plateau theory [57, 59, 69, 100]; and (2) the antagonistic pleiotropy theory [57, 59, 69, 101] and its contemporary version called the disposable soma theory [57, 59, 69, 102 - 104].

Both, the mutation accumulation theory and the late-life mortality plateau theory, postulate that natural selection favours alleles (alternative forms) of a gene that are beneficial early in organismal life over alleles of the same gene that provide an advantage late in life of this organism [57, 59, 69, 98 - 100]. Thus, by eliminating gene alleles that are beneficial late in life, natural selection will diminish its power with age of an organism and will limit its lifespan at an age that is unique to each species [57, 59, 69, 98 - 100].

In contrast, the antagonistic pleiotropy theory and the disposable soma theory assume that alleles of certain genes that are beneficial in early life of an organism exhibit detrimental effects in its late life [57, 59, 69, 101 - 104]. Because different alleles of these genes display age-related antagonistic effects on several fitness-defining traits of an organism, these genes are called pleiotropic genes. According to both the antagonistic pleiotropy theory and the disposable soma theory, natural selection limits organismal lifespan at an age unique to each species by actively retaining only those alleles of pleiotropic genes that increase early-life fitness and thus reduce fitness at old age [57, 59, 69, 101 - 104].

It should be stressed that contemporary evolutionary theories of programmed aging and age-related death postulate that organisms of all species possess mechanisms that have been evolved to actively limit their lifespans at a species-specific age [57, 59, 61-63, 66, 69, 71 - 73]. In contrast, evolutionary theories of non-programmed aging and age-

related death assume that such mechanisms cannot exist, just because organismal lifespan is limited at a species-specific age passively - *i.e.*, due to lack of the evolutionary force [57, 59, 69, 98 - 104]. It was concluded therefore that the demonstrated ability of certain genetic, dietary and pharmacological interventions to significantly extend lifespan in evolutionarily distant species by targeting mechanisms that actively limit organismal lifespan at a species-specific age [1, 3, 4, 6 - 10, 13 - 25, 29, 37, 41 - 43, 46 - 51] provides irrefutable proof of evolutionary theories of aging based on the concept of programmed aging and age-related death and invalidates evolutionary theories of non-programmed aging and age-related death [57, 59 - 69]. However, in all these cases the ability of genetic, dietary and pharmacological interventions to significantly extend organismal lifespan has been revealed under laboratory conditions; as discussed above, these conditions do not mimic the process of natural selection within a genetically mixed population of individuals that belong to the same species, compete for environmental resources, and have lifespans at a species-specific age and beyond it [91 - 97]. But none of the evolutionary theories of non-programmed aging and age-related death assumes that in the absence of natural selection (*i.e.*, under laboratory conditions) longevity-extending mutant gene alleles reducing early-life fitness cannot exist; all these theories only proclaim that such mutant gene alleles will be eliminated from the gene pool of a species under the pressure of natural selection (*i.e.*, in the wild or under field-like laboratory conditions) [57, 59, 69, 98 - 104]. Furthermore, it seems impossible in the wild or under field-like laboratory conditions to impose any of the currently known longevity-extending dietary or pharmacological interventions (such as CR, DR or anti-aging natural compounds) only on some individuals of the same species; thus, it is unlikely that such

non-genetic interventions can be used for empirical validation of evolutionary theories of programmed or non-programmed aging and age-related death.

In conclusion, one could think that the initial and essential test for empirical validation of evolutionary theories of programmed or non-programmed aging and age-related death consists in evaluating how a longevity-extending genetic intervention influences early-life fitness of an organism under laboratory conditions; under such conditions (*i.e.*, in the absence of natural selection) long-lived mutants of evolutionarily distant species grow, develop and age alone, in the absence of "WT" individuals of the same species that do not carry longevity-extending mutations and thus do not have lifespan extended beyond a species-specific age. As discussed above, all evolutionary theories of non-programmed aging and age-related death posit that under such conditions any longevity-extending genetic intervention must reduce early-life fitness of an organism [57, 59, 69, 98 - 104]. In contrast, all contemporary evolutionary theories of programmed aging and age-related death assume that only those longevity-extending genetic interventions that affect critical for early-life fitness modules of the pro-aging signaling network could reduce such fitness, whereas the longevity-extending genetic interventions that affect other modules of such signaling network are unlikely to have an effect on organismal early-life fitness [66, 78 - 91].

1.6 The objectives of studies described in this thesis

Studies described in my thesis had the following three objectives.

The first objective of my studies was to test if the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions (1) is a recessive or a

dominant genetic trait; and (2) is due to a mutation in a single gene or is a polygenic genetic trait. To attain this objective, I used (1) the backcross mating of each of the three long-lived yeast mutants evolved under laboratory conditions with a WT yeast strain of opposite mating type to create a diploid strain; and (2) the subsequent tetrad analysis of meiotic segregants that originated from individual asci for each of the sporulated diploid strains. To define the genetic nature of the "extended longevity" trait for each of the three long-lived yeast mutants evolved under laboratory conditions, I measured the CLS of diploids and meiotic segregants derived from these monohybrid genetic analyses.

The second objective of my studies was to investigate if mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence early-life fitness when each of these mutants was incubated alone, in the absence of a WT yeast strain that does not carry longevity-extending mutations. To attain this objective, I analyzed the following key traits of early-life fitness: (1) exponential growth rate of yeast cells in media containing a fermentable carbon source (*i.e.*, glucose at the initial concentration of 0.2% [CR conditions] or 2% [non-CR conditions]) or a non-fermentable carbon source (*i.e.*, ethanol at the initial concentration of 1% or glycerol at the initial concentration of 3%); (2) the efficacy of post-exponential growth of yeast cells in media containing a fermentable carbon source (*i.e.*, glucose at the initial concentration of 0.2% [CR conditions] or 2% [non-CR conditions]) or a non-fermentable carbon source (*i.e.*, ethanol at the initial concentration of 1% or glycerol at the initial concentration of 3%); (3) fecundity of yeast cells, which was quantitatively assessed by monitoring the efficacies of their sexual reproduction by mating and sporulation following recovery of cells that were pre-grown in media containing glucose

at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1%; (4) cell susceptibility to chronic oxidative, thermal and osmotic stresses administered to yeast cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1%; (5) cell susceptibility to a mitochondria-controlled apoptotic form of death triggered by a brief exposure to exogenous hydrogen peroxide of yeast cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1%; and (6) cell susceptibility to a "lipoptotic" form of death triggered by a brief exposure to exogenous palmitoleic acid of yeast cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1% .

The third objective of my studies was to elucidate if mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence the relative fitness of each of them in a direct competition assay with the parental WT strain. The mutant and parental WT strains used in such competition assay were (1) pre-grown separately in media containing a fermentable carbon source (*i.e.*, glucose at the initial concentration of 0.2% [CR conditions] or 2% [non-CR conditions]) or a non-fermentable carbon source (*i.e.*, ethanol at the initial concentration of 1%); and then (2) mixed in equal numbers and co-cultured in media containing 0.2% glucose [CR conditions], 2% glucose [non-CR conditions] or 1% ethanol.

1.7 Thesis outline

Chapter 1 of my thesis outlines cellular processes that define organismal lifespan in eukaryotes, describes an evolutionarily conserved signaling network that governs the spatiotemporal organization of these processes, and characterizes dietary and pharmacological interventions that extend organismal lifespan across phyla by modulating the flow of information along this signaling network. The key features of the budding yeast *S. cerevisiae*, all of which make this unicellular eukaryote a beneficial model for uncovering mechanisms of cellular and organismal aging in multicellular eukaryotes, are also presented in Chapter 1. Furthermore, this Chapter outlines our hypothesis on the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms within ecosystems, and summarizes our recent empirical evidence in support of this hypothesis. Moreover, Chapter 1 discusses evolutionary theories of aging based on the concept of programmed aging and age-related death, and critically compares them to evolutionary aging theories assuming that aging and age-related death are non-programmed processes. Findings described in Chapter 1 are presented in the manuscript of a paper [Burstein MT, Glebov A, **Gomez-Perez A**, Kyryakov P, Akkari R, Asbah N, Titorenko VI. Empirical verification of a hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms within ecosystems] that is currently in preparation for submission to *Biology Open*. I expect this manuscript to be submitted for publication in late September or early October of 2013.

Chapter 2 of my thesis describes experiments aimed at testing if the extended longevity of each of the three long-lived yeast mutants that we selected by a lasting

exposure to LCA under laboratory conditions is a recessive or a dominant genetic trait and if it is a monogenic or polygenic genetic trait. As described in this Chapter, I first carried out the backcross mating of each of the three long-lived yeast mutants with a WT yeast strain of opposite mating type to create a diploid strain. I then conducted the tetrad analysis of meiotic segregants that originated from individual asci for each of the three sporulated diploid strains. As described in Chapter 2 of the thesis, my measurement of the CLS of the diploid strains and meiotic segregants derived from these monohybrid genetic analyses implies that the greatly extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant polygenic trait. Findings described in Chapter 2 are presented in the manuscript of a paper [**Gomez-Perez A**, Burstein MT, Kyryakov P, Bruno L, Meunier C, Akkari R, Asbah N, Titorenko VI. Empirical testing of the evolutionary theories of aging using laboratory-evolved yeast mutants with extended lifespan] that is currently in preparation for submission to *Evolution*. I expect this manuscript to be submitted for publication in late November or early December of 2013.

Chapter 3 of my thesis describes experiments aimed at investigating if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence early-life fitness if each of these mutants grows and ages alone, in the absence of a WT yeast strain that does not carry longevity-extending mutations. I carried out these experiments by analyzing the following key traits of early-life fitness: (1) exponential growth rate of yeast cells; (2) the efficacy of post-exponential growth of yeast cells; (3) fecundity of yeast cells, which was quantitatively assessed by monitoring the efficacies of their sexual reproduction by mating and

sporulation; (4) cell susceptibility to chronic oxidative, thermal and osmotic stresses; (5) cell susceptibility to a mitochondria-controlled apoptotic form of death triggered by a brief exposure to exogenous hydrogen peroxide; and (6) cell susceptibility to a "lipoptotic" form of death triggered by a brief exposure to exogenous palmitoleic acid. As described in Chapter 3 of the thesis, my analyses revealed that the dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions do not affect such key traits of early-life fitness as the exponential growth rate, efficacy of post-exponential growth and fecundity of yeast cells. Moreover, my studies described in Chapter 3 demonstrated that the dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance such key traits of early-life fitness as cell susceptibility to chronic exogenous stresses, mitochondria-controlled apoptosis triggered by a brief exposure to exogenous hydrogen peroxide, and lipoptotic form of death triggered by a short-term exposure to exogenous palmitoleic acid. Findings described in Chapter 3 are presented in the manuscript of a paper [**Gomez-Perez A**, Burstein MT, Kyryakov P, Bruno L, Meunier C, Akkari R, Asbah N, Titorenko VI. Empirical testing of the evolutionary theories of aging using laboratory-evolved yeast mutants with extended lifespan] that is currently in preparation for submission to *Evolution*. I expect this manuscript to be submitted for publication in late November or early December of 2013.

Chapter 4 of my thesis describes experiments aimed at elucidating if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence the relative fitness of each of them in a direct

competition assay with the parental WT strain. As described in Chapter 4 of the thesis, my analyses revealed that if each of these long-lived mutants grows together with the parental WT strain in medium containing ethanol, it is outcompeted within a "laboratory-like" ecosystem (*i.e.*, forced out of the ecosystem) by the parental WT strain. Findings described in Chapter 4 are presented in the manuscript of a paper [**Gomez-Perez A**, Burstein MT, Kyryakov P, Bruno L, Meunier C, Akkari R, Asbah N, Titorenko VI. Empirical testing of the evolutionary theories of aging using laboratory-evolved yeast mutants with extended lifespan] that is currently in preparation for submission to *Evolution*. I expect this manuscript to be submitted for publication in late November or early December of 2013.

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 The extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant polygenic trait

2.1 Abstract

To empirically validate our recently proposed hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms within ecosystems (Figure 1.4) [35, 36], we conducted a multistep selection of long-lived yeast mutants by a long-term exposure to LCA under laboratory conditions [52]. Three of the 20 selected mutant strains, called 3, 5 and 12, were able to maintain their extended lifespans following several passages in medium lacking LCA [52]. Experiments described in this chapter of my thesis were aimed at testing if the extended longevity of each of the three selected mutants is a recessive or a dominant genetic trait and if it is a monogenic or polygenic genetic trait. I first carried out the backcross mating of each of the three long-lived yeast mutants with a WT yeast strain of opposite mating type to create a diploid strain. I then conducted the tetrad analysis of meiotic segregants that originated from individual asci for each of the three sporulated diploid strains. As described in this chapter of the thesis, my measurement of the CLS of the diploid strains and meiotic segregants derived from these monohybrid genetic analyses implies that the greatly extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant polygenic trait.

2.2 Introduction

We recently analyzed how the xenohormetic phytochemicals resveratrol and caffeine, the hormetic mild stressors LCA and other bile acids, and the cytostatic macrocyclic lactone rapamycin extend longevity of some species of the organisms composing an ecosystem after being synthesized and released into the environment by other species of the organisms within this ecosystem [35, 36]. Based on our analysis, we proposed a hypothesis in which these interspecies chemical signals create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms (Figure 1.4) [35, 36]. A detailed description of this hypothesis can be found in Chapter 1 of my thesis. In brief, our hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms at the ecosystemic level posits that such evolution in each species of the organisms composing an ecosystem is driven by the ability of this species to undergo specific lifespan-extending metabolic or physiological changes in response to xenohormetic, hormetic or cytostatic chemical compounds released into the environment by other species of the organisms within this ecosystem. We sought to use an empirical approach to verify the proposed hypothesis. We therefore conducted a multistep selection of long-lived yeast mutants by a lasting exposure to LCA under laboratory conditions and selected 20 of such mutants [52]. Three of them, called 3, 5 and 12, were able to maintain their greatly extended lifespans following five passages in medium without LCA [52]. As a first step towards using these three long-lived yeast mutants evolved under laboratory conditions for empirical verification of various evolutionary theories of aging and age-related death, in experiments described in this chapter of my thesis I elucidated if the extended longevity of each of them is a recessive or a dominant genetic trait. I also

assessed if the extended longevity of each of these mutants is due to a single-gene mutation or is a polygenic genetic trait. My experimental approach consisted in using the backcross mating of each of the three long-lived yeast mutants evolved under laboratory conditions with a WT yeast strain of opposite mating type to create a diploid strain; I then compared the CLS of each of the resulting diploid strains to those of the parental haploid mutant and WT strains as well as to the CLS of diploids formed between two WT strains of opposite mating types (*i.e.*, **a** and ***α***). Moreover, I subjected meiotic segregants that originated from individual asci for each of the three sporulated diploids to a tetrad analysis by comparing the CLS of individual meiotic segregants to those of the parental mutant and WT strains.

2.3 Materials and Methods

Yeast strains

The haploid WT strains BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) of the yeast *S. cerevisiae* (both from Open Biosystems) as well as the long-lived mutant strains 3, 5 and 12 (each in the BY4742 genetic background) were used in this study.

Qualitative mating assay

A small patch of cells of the haploid WT strain BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was applied to the surface of a master YEPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) plate. 10^6 cells of mating type ***α*** (*i.e.*, the haploid WT strain BY4742 [*MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*] or the selected long-lived haploid mutant strains

3, 5 or 12) were spread on the surface of a separate crossing plate with YEPD medium. The master plate was replica plated onto a lawn of cells on each of the four crossing plates; different velvet was used for each crossing plate. The crossing plates were incubated overnight at 30^{o}C. Each of the four crossing plates was then replica plated onto a synthetic minimal YNB medium plate (0.67% Yeast Nitrogen Base without Amino Acids, 2% glucose, 2% agar) supplemented with 20 mg/l L-histidine, 30 mg/l L-leucine and 20 mg/l uracil. These plates were incubated overnight at 30^{o}C. A positive mating reaction between cells of the the haploid WT strain BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and cells of the haploid WT strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) or cells of each of the selected long-lived haploid mutant strains 3, 5 or 12 resulted in confluent growth of diploid cells on a YNB plate (supplemented with L-histidine, L-leucine and uracil) at the position of a patch of haploid BY4741 cells.

Qualitative sporulation assay

Cells of each of the four diploid strains recovered in the qualitative mating assay were spread on a separate SPO (0.1% yeast extract, 1% potassium acetate, 0.05% glucose, 2% agar) plate supplemented with 20 mg/l L-histidine, 30 mg/l L-leucine and 20 mg/l uracil. The plates were incubated at 30^{o}C for 5-6 days, until numerous asci were observed microscopically.

Dissection of asci, separation of ascospores and tetrad analysis

An inoculating loop was used to resuspend spores from the lawn on SPO plates in 50 µl of 0.2 mg/ml Zymolyase solution in sterile water. The suspension of spores was

incubated for 10 min at room temperature. 450 µl of sterile water was gently added to the suspension of spores. The mix was then incubated for 30-40 min at room temperature. An inoculating loop was used to spread an aliquot of digested asci onto a tetrad dissecting YEPDD (1% yeast extract, 2% peptone, 2% glucose, 4% agar) plate. Dissection of asci, separation of ascospores and tetrad analysis were performed according to established procedures [105].

A plating assay for the analysis of CLS

Cells were grown in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose as carbon source at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a flask volume/medium volume ratio of 5:1. A sample of cells was removed from each culture at various time points. A fraction of the cell sample was diluted in order to determine the total number of cells per ml of culture using a hemacytometer. 10 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 x 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 triplicate 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 colonies × 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.

2.4 Results

2.4.1 The extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant genetic trait

Using qualitative mating assay, I subjected each of the three long-lived yeast mutants (each of mating type ***α*** and in the BY4742 genetic background, *i.e.*, *MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) evolved under laboratory conditions to the backcross mating with the WT strain BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) of opposite mating type. I also used this assay to create a WT × WT diploid strain that was formed by mating between the haploid WT strains BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). I then compared the CLS of each, the WT × 3, WT × 5 and WT × 12 diploid strain, to those of the parental haploid mutant and WT strains as well as to the CLS of the WT × WT diploid strain; yeast cells were cultured under CR conditions in the nutrient-rich YP (1% yeast extract, 2% peptone) medium initially containing low (0.2%) concentration of glucose. I found that chronologically aging cells of the WT × 3, WT × 5 and WT × 12 diploid strains live (1) almost as long as chronologically aging cells of the parental haploid mutant strain; and (2) significantly longer than chronologically aging cells of the WT × WT diploid

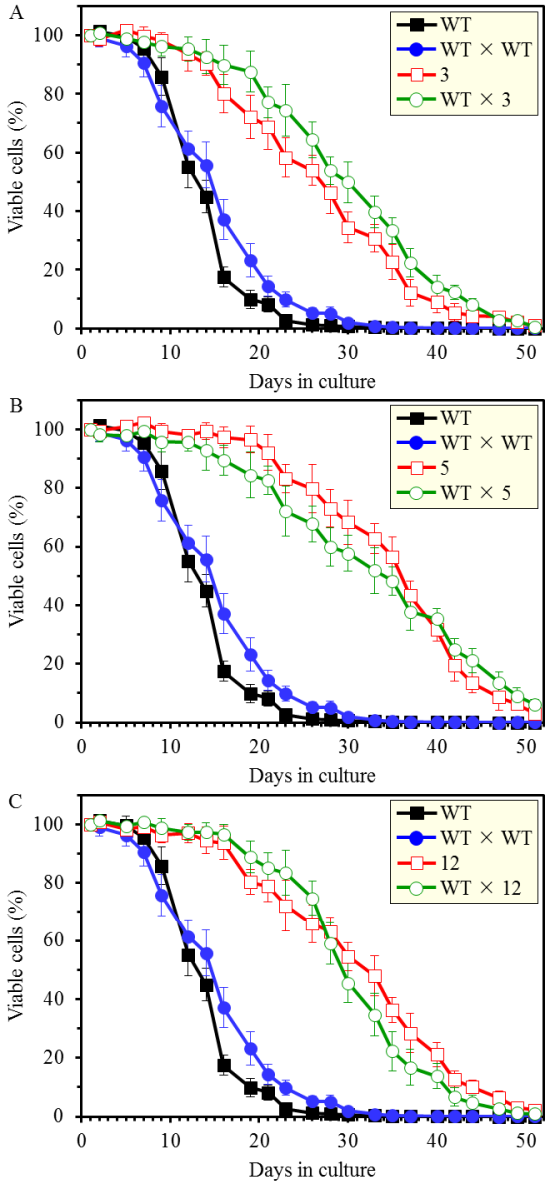


Figure 2.1. The extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant genetic trait. The parental haploid WT strain BY4742, the WT × WT diploid strain formed by mating between the haploid WT strains BY4741 and BY4742, the long-lived mutant strains 3, 5 and 12 (each in the BY4742 genetic background), and the WT × 3, WT × 5 and WT × 12 diploid strains were cultured in the nutrient-rich YP medium initially containing 0.2% glucose. Survival curves of chronologically aging cells are shown. Data are presented as means ± SEM (n = 5-7).

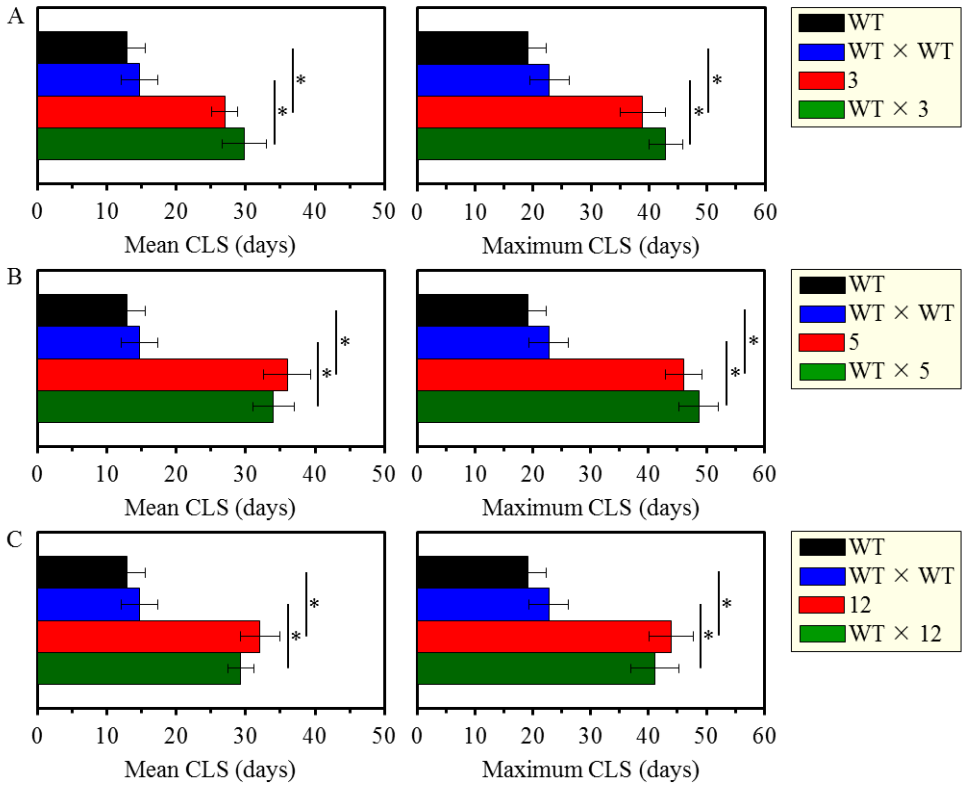


Figure 2.2. The extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant genetic trait. The parental haploid WT strain BY4742, the WT × WT diploid strain formed by mating between the haploid WT strains BY4741 and BY4742, the long-lived mutant strains 3, 5 and 12 (each in the BY4742 genetic background), and the WT × 3, WT × 5 and WT × 12 diploid strains were cultured in the nutrient-rich YP medium initially containing 0.2% glucose. The mean and maximum CLS are shown. Data originate from Figure 2.1 and are presented as means ± SEM (n = 5-7; *$p < 0.05$).

strain and the parental haploid WT strain (Figures 2.1A, 2.1B and 2.1C, respectively). Both the mean and maximum CLS of each, the WT × 3, WT × 5 and WT × 12 diploid strain, exceeded those of the WT × WT diploid strain to a similar degree as the mean and

maximum CLS of the parental haploid mutant strains exceeded those of the parental haploid WT strain (Figures 2.2A, 2.2B and 2.2C, respectively).

Based on these observations, I concluded that the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant genetic trait.

2.4.2 The extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a polygenic genetic trait

To elucidate if the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a monogenic or polygenic genetic trait, I first subjected the WT × 3, WT × 5 and WT × 12 diploid strains to sporulation. I then removed the outer cell wall surrounding the four ascospores within individual asci by treatment with Zymolyase, and used micromanipulation to separate these ascospores. For each of the three sporulated diploids, I conducted tetrad analysis of ascospores from six randomly chosen asci (the four ascospores recovered within each ascus) by comparing the CLS of individual meiotic segregants to those of the parental mutant and WT strains.

If the extended longevity of a long-lived yeast mutant evolved under laboratory conditions is a monogenic genetic trait, it was expected that (1) chronologically aging cells of two of the four ascospores within each of the six tetrads do not exhibit an extended CLS characteristic of the parental mutant strain and live almost as long as cells of the parental WT strain; and (2) chronologically aging cells of two other ascospores within each of the six tetrads display a prolonged CLS characteristic of the parental

mutant strain and live significantly longer than cells of the parental WT strain (Figure 2.3) [105].

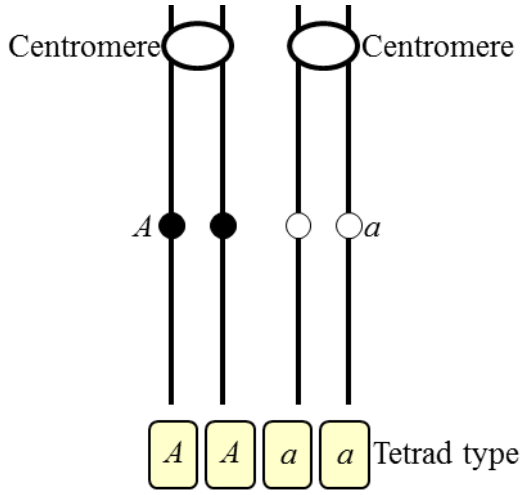
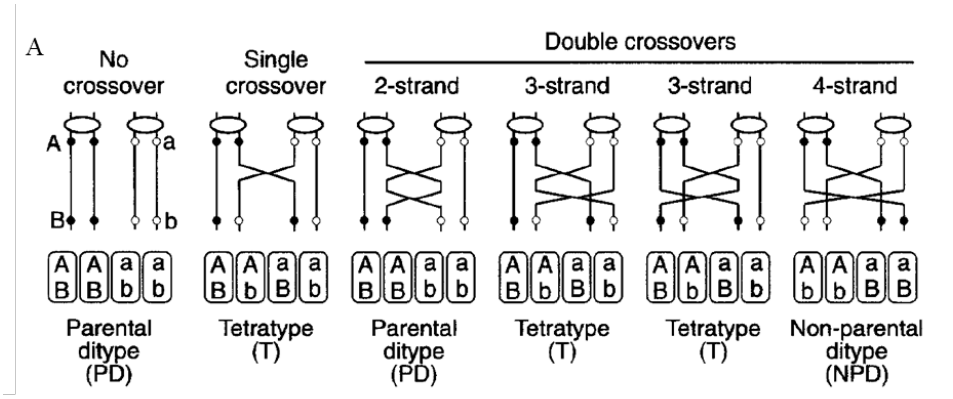


Figure 2.3. A single type of tetrads originated from an *A* × *a* hybrid which is heterozygous for a single gene. Two of the four ascospores within each tetrad exhibit a phenotype defined by the *A* allele of this gene, whereas two other ascospores within each tetrad display a phenotype defined by its *a* allele.

Of note, my findings imply that the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant genetic trait (see section 2.4.1). Thus, if the extended longevity of a long-lived yeast mutant evolved under laboratory conditions is due to mutations in two genes - *e.g.*, the dominant mutations A^d and B^d in the genes *A* and *B*, respectively – and if these two genes are widely separated on the same chromosome, or if these two genes are on different chromosomes and at least one of them is not centromere-linked, it was expected that (1) chronologically aging cells

of two of the four ascospores within one out of the six tetrads do not exhibit an extended CLS characteristic of the parental mutant strain and live almost as long as cells of the parental WT strain, whereas chronologically aging cells of two other ascospores within this tetrad display a prolonged CLS characteristic of the parental mutant strain and live significantly longer than cells of the parental WT strain (see a parental ditype [PD] ascus for randomly assorted genes in Figure 2.4); (2) chronologically aging cells of all four ascospores within one out of the six tetrads exhibit an extended CLS characteristic of the parental mutant strain (see a non-parental ditype [NPD] ascus for randomly assorted genes in Figure 2.4); and (3) chronologically aging cells of one of the four ascospores within four out of the six tetrads do not exhibit an extended CLS characteristic of the parental mutant strain and live almost as long as cells of the parental WT strain, whereas chronologically aging cells of three other ascospores within each of these tetrads display a prolonged CLS characteristic of the parental mutant strain and live significantly longer than cells of the parental WT strain (see a tetratype [T] ascus for randomly assorted genes in Figure 2.4) [105]. Furthermore, if the extended longevity of a long-lived yeast mutant evolved under laboratory conditions is due to mutations in two genes - *e.g.*, the dominant mutations A^d and B^d in the genes *A* and *B*, respectively – and if these two genes are tightly linked, it was expected that (1) chronologically aging cells of two of the four ascospores within three (or likely more) out of the six tetrads do not exhibit an extended CLS characteristic of the parental mutant strain and live almost as long as cells of the parental WT strain, whereas chronologically aging cells of two other ascospores within each of these tetrads display a prolonged CLS characteristic of the parental mutant strain and live significantly longer than cells of the parental WT strain (see a PD ascus for

linked to each other genes in Figure 2.4); and (2) chronologically aging cells of all four ascospores within three (or likely less) out of the six tetrads exhibit an extended CLS characteristic of the parental mutant strain (see a an NPD ascus for linked to each other genes in Figure 2.4) [105]. Moreover, if the extended longevity of a long-lived yeast mutant evolved under laboratory conditions is due to mutations in two genes - *e.g.*, the dominant mutations A^d and B^d in the genes *A* and *B*, respectively – and if these two genes are linked to their respective centromeres, it was expected that (1) chronologically aging cells of two of the four ascospores within one (or likely more) out of the six tetrads do not exhibit an extended CLS characteristic of the parental mutant strain and live almost as long as cells of the parental WT strain, whereas chronologically aging cells of two other ascospores within this tetrad display a prolonged CLS characteristic of the parental mutant strain and live significantly longer than cells of the parental WT strain (see a PD ascus for two genes linked to their respective centromeres in Figure 2.4); (2) chronologically aging cells of all four ascospores within one (or likely more) out of the six tetrads exhibit an extended CLS characteristic of the parental mutant strain (see an NPD ascus for two genes linked to their respective centromeres in Figure 2.4); and (3) chronologically aging cells of one of the four ascospores within four (or likely less) out of the six tetrads do not exhibit an extended CLS characteristic of the parental mutant strain and live almost as long as cells of the parental WT strain, whereas chronologically aging cells of three other ascospores within each of these tetrads display a prolonged CLS characteristic of the parental mutant strain and live significantly longer than cells of the parental WT strain (see a T ascus for two genes linked to their respective centromeres in Figure 2.4) [105].



B

	PD	NPD	T
	AB	*aB*	*AB*
	AB	*aB*	*Ab*
	ab	*Ab*	*ab*
	ab	*Ab*	*aB*
Random assortment	1 :	1 :	4
Linkage	>1 :	<1	
Centromere linkage	1 :	1 :	<4

Figure 2.4. (A) The three possible ratios of the parental ditype [PD], non-parental ditype [NPD] and tetratype [T] tetrads for an *AB* × *ab* hybrid which is heterozygous for two genes. These ratios depend on the distance between genes *A* and *B* as well as on the linkage of each of them to their respective centromeres. A different ratio of the PD, NPD and T tetrads for the *AB* × *ab* hybrid is expected in the following three situations: (1) the *A* and *B* genes are widely separated on the same chromosome, or are on different chromosomes and at least one of them is not centromere-linked; (2) the *A* and *B* genes are tightly linked; and (3) the *A* and *B* genes are linked to their respective centromeres. **(B) The PD, NPD and T types of tetrads for the *AB* × *ab* hybrid originate when there is no crossover, single crossover or double crossovers within an interval between the *A* and *B* genes.** Reproduced from reference 105.

I compared the CLS of individual meiotic segregants originated from the WT × 3, WT × 5 and WT × 12 diploid strains to the CLS of the parental mutant and WT strains. This comparative analysis revealed that: (1) chronologically aging cells of all four ascospores within each of the six tested tetrads exhibit an extended CLS characteristic of the parental mutant strain and live significantly longer than cells of the parental WT strain; and (2) this relationship between the CLS of individual meiotic segregants, the parental mutant strain and the parental WT strain is observed for each of the three diploid strains, namely WT × 3, WT × 5 and WT × 12 (Figures 2.5 to 2.10). Importantly, for each of these diploids the observed relationship between the CLS of individual meiotic segregants, the parental mutant strain and the parental WT strain differed from a relationship that was expected if the extended longevity of a long-lived yeast mutant evolved under laboratory conditions is due to a single-gene mutation or mutations in two genes (see above). Indeed, if the trait of extended longevity was monogenic, it was anticipated that two of the four ascospores within each of the six randomly chosen tetrads do not exhibit an extended CLS characteristic of the parental mutant strain and live almost as long as cells of the parental WT strain (see above). Furthermore, if the trait of extended longevity was caused by mutations in two genes, it was projected that one or two of the four ascospores within three, four or five out of the six randomly chosen tetrads do not exhibit an extended CLS characteristic of the parental mutant strain and live almost as long as cells of the parental WT strain (see above).

Based on all these observations, I concluded that the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a polygenic genetic trait caused by mutations in more than two genes.

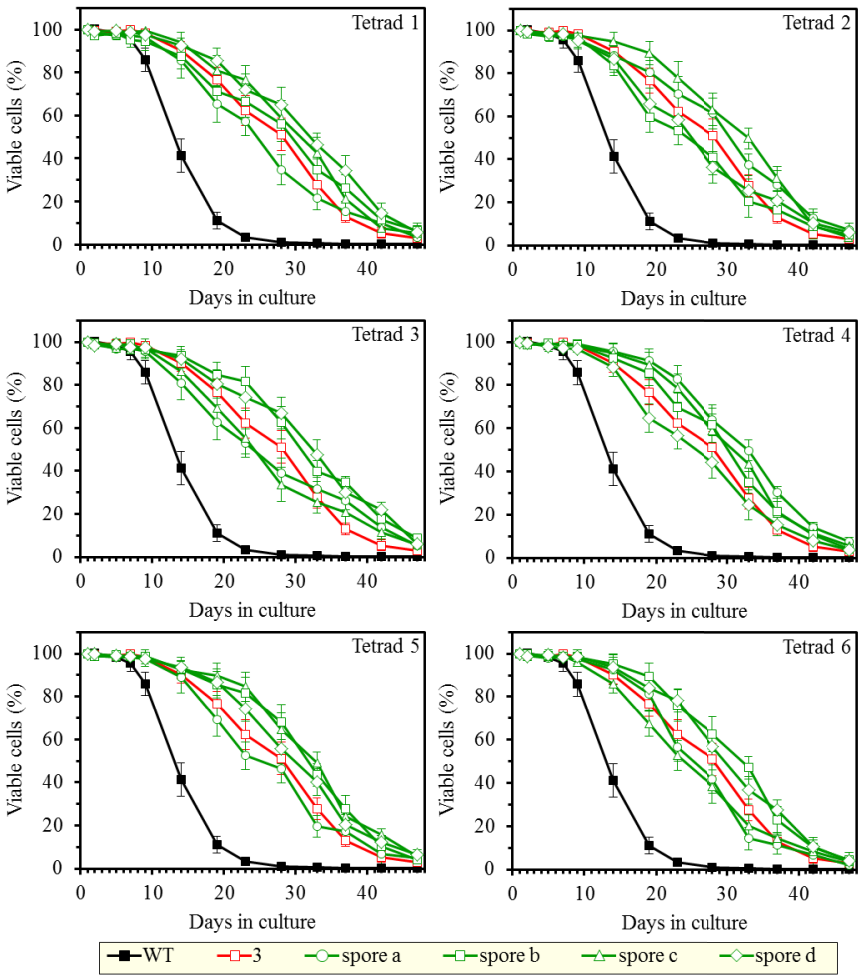


Figure 2.5. Chronologically aging cells of all four ascospores within each of the six randomly chosen tetrads that originated from the WT × 3 diploid exhibit an extended CLS characteristic of the parental mutant strain 3 and live significantly longer than cells of the parental WT strain. The parental WT strain BY4742, the long-lived parental mutant strain 3 (in the BY4742 genetic background) and the four ascospores recovered from each of the six randomly chosen tetrads were cultured in the nutrient-rich YP medium initially containing 0.2% glucose. Survival curves of chronologically aging cells are shown. Data are presented as means ± SEM (n = 5-7 for the parental WT and mutant strains; n = 2 for ascospores a, b, c and d).

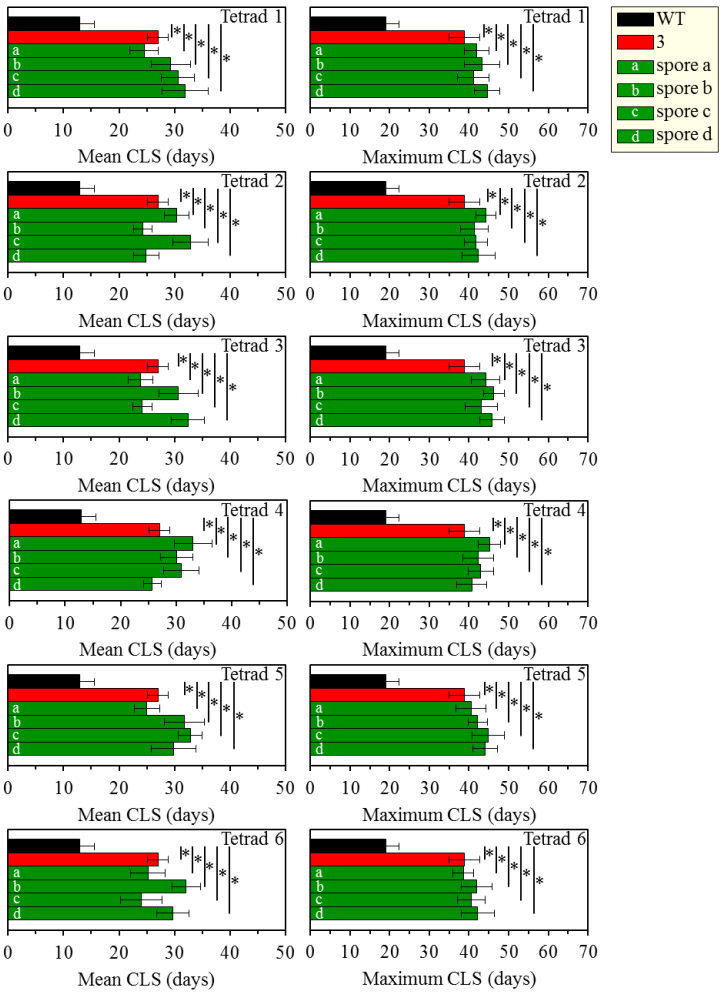


Figure 2.6. Chronologically aging cells of all four ascospores within each of the six randomly chosen tetrads that originated from the WT × 3 diploid exhibit an extended CLS characteristic of the parental mutant strain 3 and live significantly longer than cells of the parental WT strain. The parental WT strain BY4742, the long-lived parental mutant strain 3 (in the BY4742 genetic background) and the four ascospores recovered from each of the six randomly chosen tetrads were cultured in the nutrient-rich YP medium initially containing 0.2% glucose. The mean and maximum CLS are shown. Data originate from Figure 2.5 and are presented as means ± SEM (n = 5-7 for the parental WT and mutant strains; n = 2 for ascospores a, b, c and d; *$p < 0.05$).

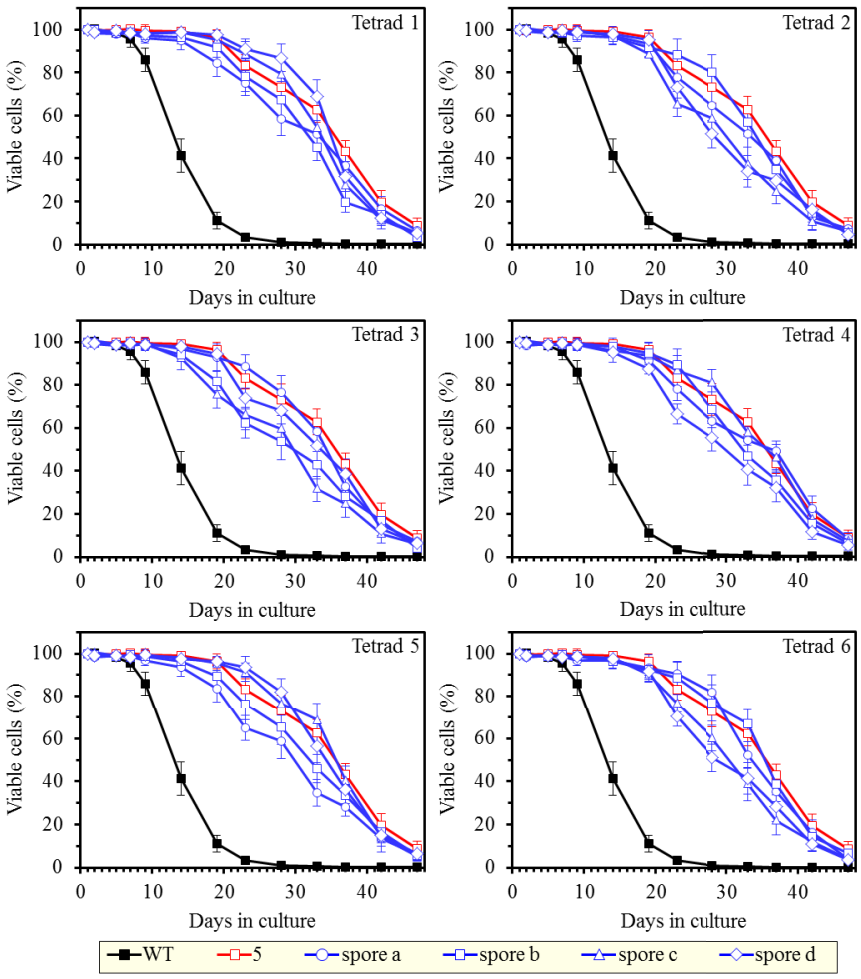


Figure 2.7. Chronologically aging cells of all four ascospores within each of the six randomly chosen tetrads that originated from the WT × 5 diploid exhibit an extended CLS characteristic of the parental mutant strain 5 and live significantly longer than cells of the parental WT strain. The parental WT strain BY4742, the long-lived parental mutant strain 5 (in the BY4742 genetic background) and the four ascospores recovered from each of the six randomly chosen tetrads were cultured in the nutrient-rich YP medium initially containing 0.2% glucose. Survival curves of chronologically aging cells are shown. Data are presented as means ± SEM (n = 5-7 for the parental WT and mutant strains; n = 2 for ascospores a, b, c and d).

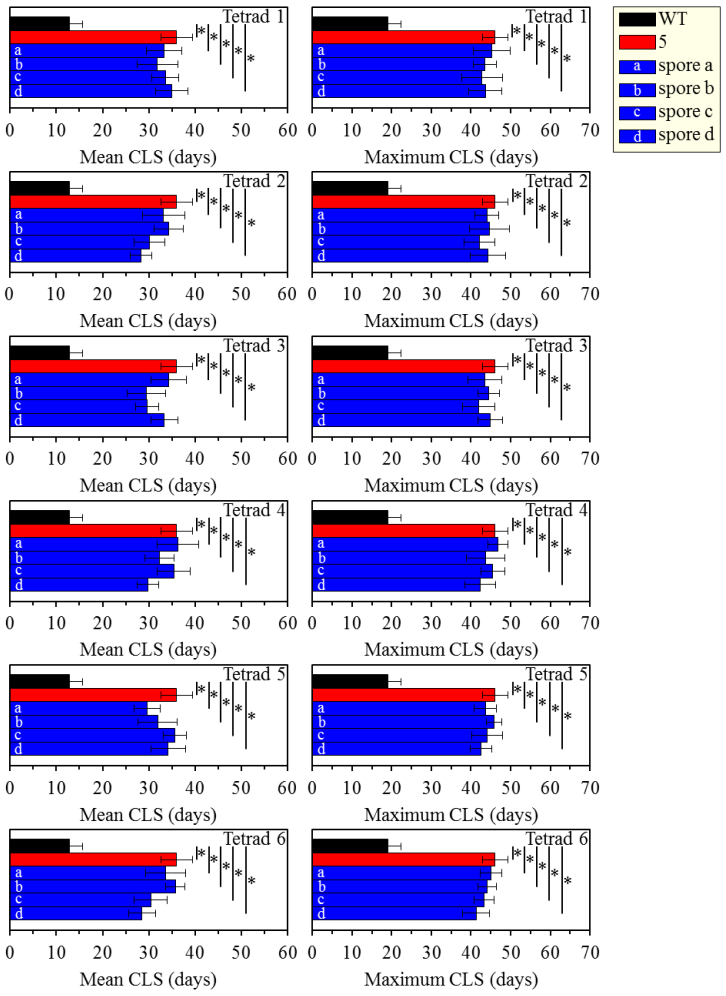


Figure 2.8. Chronologically aging cells of all four ascospores within each of the six randomly chosen tetrads that originated from the WT × 5 diploid exhibit an extended CLS characteristic of the parental mutant strain 5 and live significantly longer than cells of the parental WT strain. The parental WT strain BY4742, the long-lived parental mutant strain 5 (in the BY4742 genetic background) and the four ascospores recovered from each of the six randomly chosen tetrads were cultured in the nutrient-rich YP medium initially containing 0.2% glucose. The mean and maximum CLS are shown. Data originate from Figure 2.7 and are presented as means ± SEM (n = 5-7 for the parental WT and mutant strains; n = 2 for ascospores a, b, c and d; *$p < 0.05$).

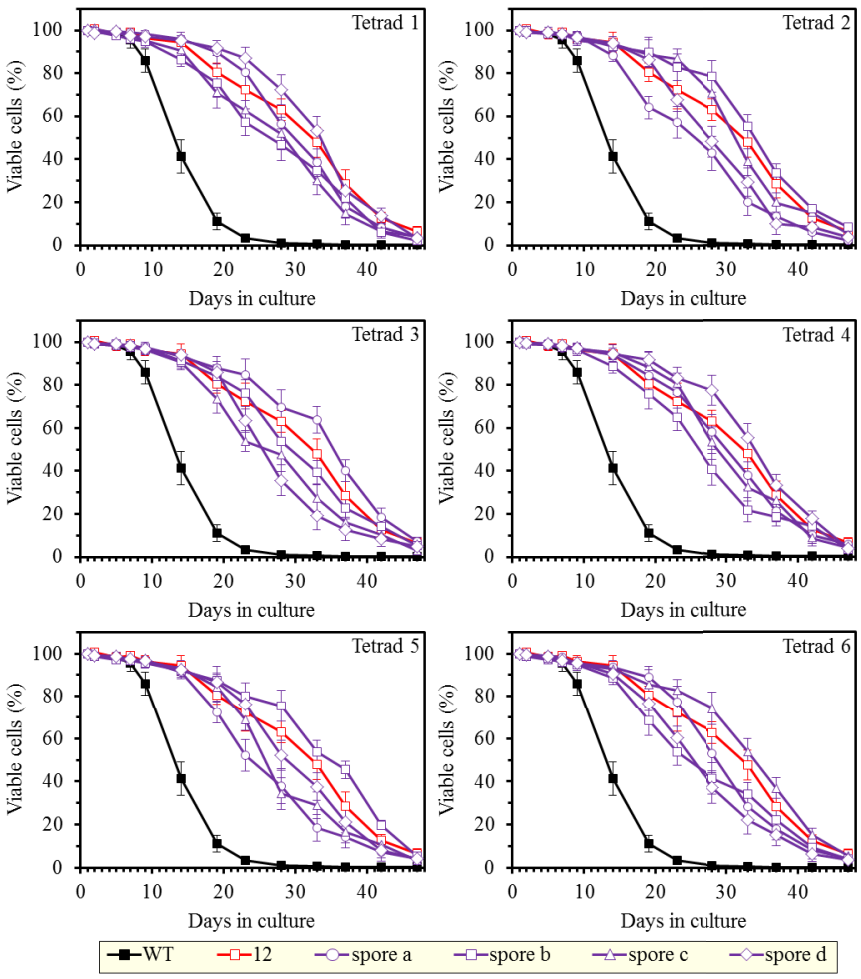


Figure 2.9. Chronologically aging cells of all four ascospores within each of the six randomly chosen tetrads that originated from the WT × 12 diploid exhibit an extended CLS characteristic of the parental mutant strain 12 and live significantly longer than cells of the parental WT strain. The parental WT strain BY4742, the long-lived parental mutant strain 12 (in the BY4742 genetic background) and the four ascospores recovered from each of the six randomly chosen tetrads were cultured in the nutrient-rich YP medium initially containing 0.2% glucose. Survival curves of chronologically aging cells are shown. Data are presented as means ± SEM (n = 5-7 for the parental WT and mutant strains; n = 2 for ascospores a, b, c and d).

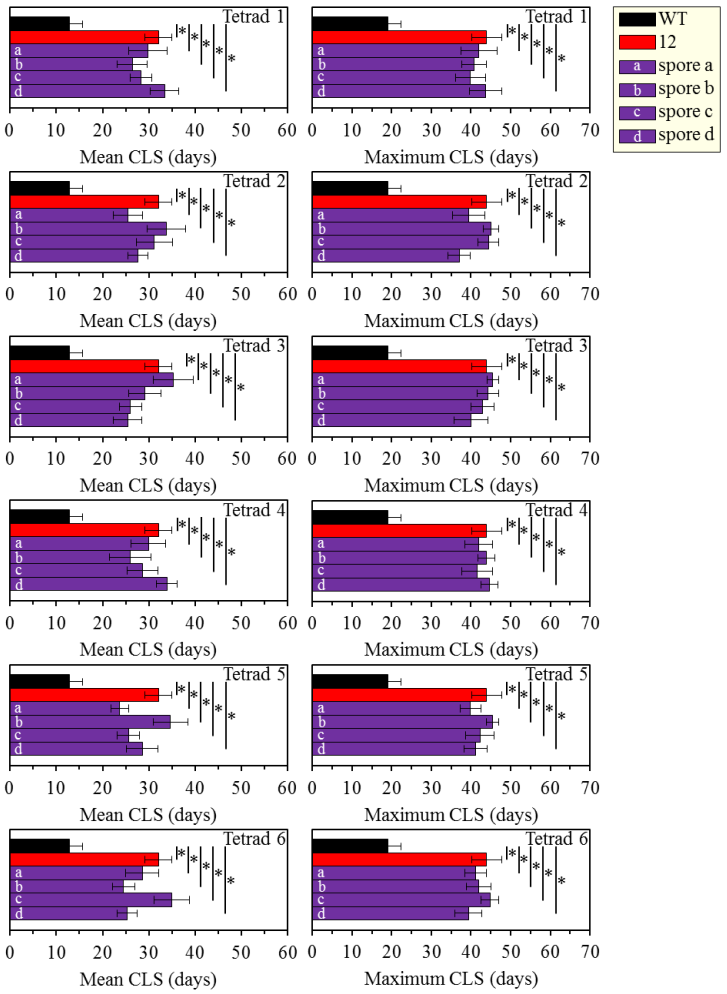


Figure 2.10. Chronologically aging cells of all four ascospores within each of the six randomly chosen tetrads that originated from the WT × 12 diploid exhibit an extended CLS characteristic of the parental mutant strain 12 and live significantly longer than cells of the parental WT strain. The parental WT strain BY4742, the long-lived parental mutant strain 12 (in the BY4742 genetic background) and the four ascospores recovered from each of the six randomly chosen tetrads were cultured in the nutrient-rich YP medium initially containing 0.2% glucose. The mean and maximum CLS are shown. Data originate from Figure 2.9 and are presented as means ± SEM (n = 5-7 for the parental WT and mutant strains; n = 2 for ascospores a, b, c and d; *$p < 0.05$).

2.5 Discussion

Findings described in this chapter of my thesis provide comprehensive evidence that the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions is a dominant polygenic trait caused by mutations in more than two genes.

The major challenge now is to get a greater insight into molecular and cellular mechanisms underlying the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions. To address this challenge, the following important questions need to be answered.

First, what genes are affected by dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants selected by a lasting exposure to LCA? To answer this question, the entire genome of each of these mutant strains needs to be sequenced and to be compared to the genome sequence of the parental WT strain. This will enable to establish, for the first time, the molecular landscape of the evolution of longevity under laboratory conditions.

Second, which molecular components of the two currently known signaling networks governing cellular aging in yeast (Figure 2.11) [3, 6, 21] are affected by dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions? One of these networks integrates the TOR and cAMP/PKA signaling pathways that are called “adaptable” because they regulate longevity only in response to certain changes in the extracellular and intracellular nutrient and energy status of a yeast cell (Figure 2.11) [1, 3, 6 - 8, 21]. Recent evidence supports the view that this pro-aging adaptable signaling network has evolved to limit lifespan in evolutionarily distant organisms in a species-specific fashion

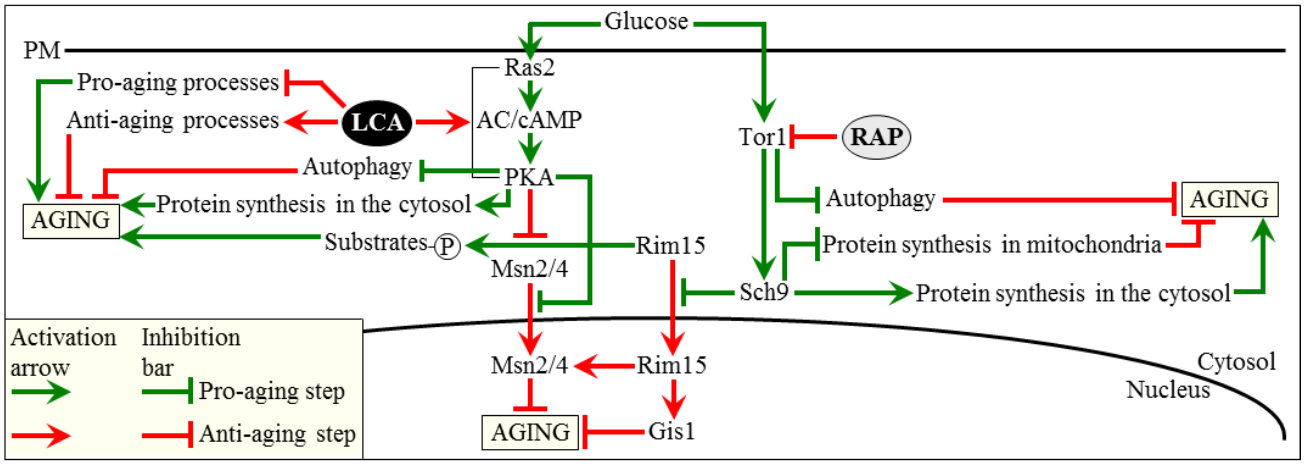


Figure 2.11. Two signaling networks govern cellular aging in yeast. One of these networks integrates the TOR and cAMP/PKA signaling pathways; these pathways are called "adaptable" because they regulate longevity only in response to certain changes in the extracellular and intracellular nutrient and energy status of a yeast cell. The TOR signaling pathway is modulated by rapamycin (RAP). The other network is modulated by LCA; it integrates longevity signaling pathways that are "constitutive" or "housekeeping" by nature because they regulate yeast longevity irrespective of the extracellular and intracellular nutrient and energy status of a cell and do not overlap with the adaptable longevity pathways. Activation arrows and inhibition bars denote pro-aging processes (displayed in green color) or anti-aging processes (displayed in red color). Reproduced from reference 21.

and upon reaching a certain species-specific age [1, 3, 4, 6 - 10, 13 - 25, 29, 37, 41 - 43, 46 - 51]. As we recently demonstrated, the other network integrates longevity signaling pathways that are "constitutive" or "housekeeping" by nature because they: (1) modulate yeast longevity irrespective of the extracellular and intracellular nutrient and energy

status of a cell; and (2) do not overlap (or only partially overlap) with the adaptable longevity pathways that are under the stringent control of calorie and/or nutrient availability (Figure 2.11) [21].

Importantly, it has been shown this housekeeping longevity signaling network in chronologically aging yeast is modulated by LCA (Figure 2.11) [21]. Because we selected each of the three long-lived yeast mutants by a long-term exposure of cells to LCA [52], I propose here that their extended longevity is caused by dominant mutations that affect the modulated by LCA housekeeping longevity signaling network. LCA is known to extend longevity of chronologically aging yeast by targeting two different mechanisms (Figure 2.11) [21]. One mechanism extends longevity regardless of the number of available calories. This mechanism involves the LCA-governed modulation of certain housekeeping longevity assurance pathways that do not overlap with the adaptable TOR and cAMP/PKA pathways (Figure 2.11); several recent papers from our laboratory identify a compendium of processes that compose LCA-targeted housekeeping longevity assurance pathways [2, 21, 29 - 32]. As a recent study from our laboratory has revealed, the other mechanism underlying the longevity-extending effect of LCA in chronologically aging yeast consists in LCA-driven unmasking of the previously unknown anti-aging potential of PKA, a key player in the adaptable cAMP/PKA pathway (Figure 2.11) [21]. Based on the findings of this study, it has been proposed that LCA uncovers the anti-aging potential of PKA by activating PKA-dependent phosphorylation of the cytosolic pool of a nutrient-sensory protein kinase Rim15p (Figure 2.11) [21]. It is well known that (1) the adaptable TOR and cAMP/PKA pathways converge on Rim15p to regulate yeast longevity in a calorie availability-dependent fashion; and (2) the nuclear

pool of Rim15p plays an anti-aging role by enabling a pro-longevity transcriptional program driven by transcription factors Msn2p, Msn4p and Gis1p (Figure 2.11) [21, 106, 107]. In a proposed by our laboratory mechanism underlying the LCA-driven unmasking of the previously unknown anti-aging potential of PKA (1) unlike its nuclear pool, the cytosolic pool of Rim15p has an essential pro-aging function in phosphorylating a distinct set of its cytosolic target proteins [108] some of which promote yeast aging only if phosphorylated; (2) under non-CR conditions LCA activates the PKA-dependent phosphorylation of Rim15p; and (3) because the phosphorylation of Rim15p inactivates its protein kinase activity [107], the dephosphorylation of pro-aging target proteins of Rim15p in the cytosol by phosphatases inhibits the ability of these target proteins to promote aging (Figure 2.11) [21].

All these considerations provide a valuable conceptual framework for the near future studies in our laboratory aimed at elucidating which molecular components of the two described above signaling networks governing cellular aging (Figure 2.11) are affected by dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions.

3 Dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions do not affect several key traits of early-life fitness and enhance other such traits if each of these mutants grows and ages in the absence of a parental WT yeast strain

3.1 Abstract

The objective of experiments described in this chapter was to perform the initial and essential test for empirical validation of evolutionary theories of programmed or non-programmed aging and age-related death. To attain this objective, I investigated if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence early-life fitness when each of these mutants grows and ages alone, in the absence of a parental WT yeast strain that does not carry longevity-extending mutations. As described in this chapter, my studies revealed that these mutations do not affect such key traits of early-life fitness as the exponential growth rate, efficacy of post-exponential growth and fecundity of yeast cells. Moreover, my studies demonstrated that the dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance such key traits of early-life fitness as cell susceptibility to chronic exogenous stresses, mitochondria-controlled apoptosis triggered by a brief exposure to exogenous hydrogen peroxide, and lipoptotic form of death triggered by a short-term exposure to exogenous palmitoleic acid. Taken together, these findings provide irrefutable proof of evolutionary theories of aging based on the concept of programmed

aging and age-related death and invalidate evolutionary theories of non-programmed aging and age-related death.

3.2 Introduction

As detailed in Chapter 1 of my thesis, the initial and essential test for empirical validation of evolutionary theories of programmed or non-programmed aging and age-related death consists in evaluating how a longevity-extending genetic intervention influences early-life fitness of an organism under laboratory conditions; under such conditions (*i.e.*, in the absence of natural selection) long-lived mutants of evolutionarily distant species grow, develop and age alone, in the absence of "WT" individuals of the same species that do not carry longevity-extending mutations and thus do not have lifespan extended beyond a species-specific age. It needs to be emphasized that all evolutionary theories of non-programmed aging and age-related death posit that under such conditions any longevity-extending genetic intervention must reduce early-life fitness of an organism [57, 59, 69, 98 - 104]. In contrast, all contemporary evolutionary theories of programmed aging and age-related death assume that only those longevity-extending genetic interventions that affect critical for early-life fitness modules of the pro-aging signaling network could reduce such fitness, whereas the longevity-extending genetic interventions that affect other modules of such signaling network are unlikely to have an effect on organismal early-life fitness [66, 78 - 91]. I sought to perform the initial and essential test for empirical validation of these two groups of theories by investigating if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence early-life fitness when each of these mutants was

incubated alone, in the absence of a parental WT yeast strain that does not carry longevity-extending mutations. To attain this objective, I analyzed the following key traits of early-life fitness: (1) exponential growth rate of yeast cells in media containing a fermentable carbon source (*i.e.*, glucose at the initial concentration of 0.2% [CR conditions] or 2% [non-CR conditions]) or a non-fermentable carbon source (*i.e.*, ethanol at the initial concentration of 1% or glycerol at the initial concentration of 3%); (2) the efficacy of post-exponential growth of yeast cells in media containing a fermentable carbon source (*i.e.*, glucose at the initial concentration of 0.2% [CR conditions] or 2% [non-CR conditions]) or a non-fermentable carbon source (*i.e.*, ethanol at the initial concentration of 1% or glycerol at the initial concentration of 3%); (3) fecundity of yeast cells, which was quantitatively assessed by monitoring the efficacies of their sexual reproduction by mating and sporulation following recovery of cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1%; (4) cell susceptibility to chronic oxidative, thermal and osmotic stresses administered to yeast cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1%; (5) cell susceptibility to a mitochondria-controlled apoptotic form of death triggered by a brief exposure to exogenous hydrogen peroxide of yeast cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1%; and (6) cell susceptibility to a "lipoptotic" form of death triggered by a brief exposure to exogenous palmitoleic acid of yeast cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1% .

3.3 Materials and Methods

Yeast strains and growth conditions

The haploid WT strains BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) of the yeast *S. cerevisiae*, the long-lived mutant strains 3, 5 and 12 (each in the BY4742 genetic background), as well as the single-gene-deletion mutant strains *rpp2BΔ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 rpp2BΔ::kanMX4*) and *dbp3Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dbp3Δ::kanMX4*) (each in the BY4742 genetic background) were used in this study. All strains were from Open Biosystems. Cells were grown in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose (a fermentable carbon source; CR conditions), 2% glucose (a fermentable carbon source; non-CR conditions), 1% ethanol (a non-fermentable carbon source) or 3% glycerol (a non-fermentable 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.

Quantitative mating assay

Cultures of mating type *MAT**a*** (*i.e.*, the haploid WT strain BY4741 [*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*]) and mating type ***α*** (*i.e.*, the haploid WT strain BY4742 [*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*] or the selected long-lived haploid mutant strains 3, 5 or 12) were grown separately to mid-logarithmic phase in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose (a fermentable carbon source; CR conditions) or 1% ethanol (a non-fermentable carbon source). Equal numbers (5×10^{6}) of cells of each mating type were mixed and then collected on a 0.45-μm pore, 25-mm diameter nitrocellulose (NC) filter. The filters were placed on the surface of a YEPD (1%

yeast extract, 2% peptone, 2% glucose, 2% agar) plate and incubated at 30°C for 5 hours. The filters were then transferred to Eppendorf tubes and resuspended in 1 ml of a synthetic minimal YNB medium plate (0.67% Yeast Nitrogen Base without Amino Acids, 2% glucose). The suspensions were used for serial 10-fold dilutions. 100-µl aliquots of each dilution were spread on (1) a synthetic minimal YNB medium plate (0.67% Yeast Nitrogen Base without Amino Acids, 2% glucose, 2% agar) without supplements; and (1) a synthetic minimal YNB medium plate supplemented with 20 mg/l L-histidine, 30 mg/l L-leucine and 20 mg/l uracil. These plates were incubated at 30°C for 2 days. The numbers of diploid cells (N_d) were counted on synthetic minimal YNB medium plates without supplements, whereas the total numbers of cells (N_t) were counted on synthetic minimal YNB medium plates supplemented with 20 mg/l L-histidine, 30 mg/l L-leucine and 20 mg/l uracil. The efficiency of mating was calculated as the number of colonies of *MAT**a***/*MAT**α*** diploids (N_d) divided by the sum of *MAT**a***/*MAT**α*** diploids plus haploid colonies (N_t). Crosses between two WT strains of opposite mating types (*i.e.*, the haploid strain BY4741 [*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*]) and the haploid strain BY4742 [*MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*]) were used as controls. All tests were carried out in triplicate in three independent experiments.

Quantitative sporulation assay

A small patch of cells of the haploid WT strain BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was applied to the surface of a master YEPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) plate. 10^6 cells of mating type ***α*** (*i.e.*, the haploid WT strain BY4742 [*MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*] or the selected long-lived haploid mutant strains

3, 5 or 12) were spread on the surface of a separate crossing plate with YEPD medium. The master plate was replica plated onto a lawn of cells on each of the four crossing plates; different velvet was used for each crossing plate. The crossing plates were incubated overnight at 30^{o}C. Each of the four crossing plates was then replica plated onto a synthetic minimal YNB medium plate (0.67% Yeast Nitrogen Base without Amino Acids, 2% glucose, 2% agar) supplemented with 20 mg/l L-histidine, 30 mg/l L-leucine and 20 mg/l uracil. These plates were incubated overnight at 30^{o}C. A positive mating reaction between cells of the the haploid WT strain BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and cells of the haploid WT strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) or cells of each of the selected long-lived haploid mutant strains 3, 5 or 12 resulted in confluent growth of diploid cells on a YNB plate (supplemented with L-histidine, L-leucine and uracil) at the position of a patch of haploid BY4741 cells. To measure sporulation efficiency, cells of each of the four recovered diploid strains were first grown to mid-logarithmic phase in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose (a fermentable carbon source; CR conditions) or 1% ethanol (a non-fermentable carbon source). The cell cycle of these cells was then synchronized by growing them in YPA medium (1% yeast extract, 2% peptone, 2% potassium acetate) from a starting optical density at 600 nm (OD_{600}) of 0.2 to final OD_{600} of 1.0; cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 10:1. 2×10^{7} of cells from this synchronized culture were then incubated in liquid SPO (0.1% yeast extract, 1% potassium acetate, 0.05% glucose) medium supplemented with 20 mg/l L-histidine, 30 mg/l L-leucine and 20 mg/l uracil at 30^{o}C for the duration of experiment. At various time

points, aliquots of cells were examined for sporulation efficiency by DIC microscopy with an Olympus BX microscope with a × 100 oil immersion objective. Sporulation efficiency was measured as the percentage of tetrads and dyads produced by a strain, relative to the total number of cells. All tests were carried out in triplicate in three independent experiments.

Plating assays for the analysis of cell susceptibility to various chronic stresses

For the analysis of hydrogen peroxide (oxidative stress) resistance, serial dilutions ($1:10^0$ to $1:10^5$) of WT and mutant cells removed from each culture at various time-points were spotted onto two sets of plates. One set of plates contained YP medium with 2% glucose alone, whereas the other set contained YP medium with 2% glucose supplemented with 5 mM hydrogen peroxide. Pictures were taken after a 3-day incubation at 30°C. For the analysis of thermal stress resistance, serial dilutions ($1:10^0$ to $1:10^5$) of WT and mutant cells removed from each culture at various time-points were spotted onto two sets of plates containing YP medium with 2% glucose. One set of plates was incubated at 30°C. The other set of plates was initially incubated at 55°C for 30 min, and was then transferred to 30°C. Pictures were taken after a 3-day incubation at 30°C. For the analysis of osmotic stress resistance, serial dilutions ($1:10^0$ to $1:10^5$) of WT and mutant cells removed from each culture at various time-points were spotted onto two sets of plates. One set of plates contained YP medium with 2% glucose alone, whereas the other set contained YP medium with 2% glucose supplemented with 1 M sorbitol. Pictures were taken after a 3-day incubation at 30°C.

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

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

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

A sample of cells was taken from a culture at a certain time-point. A fraction of the sample was diluted in order to determine the total number of cells using a hemacytometer. 2×10^7 cells were harvested by centrifugation for 1 min at 21,000 × g at

room temperature and resuspended in 2 ml of YP medium containing 0.2% glucose as carbon source. Each cell suspension was divided into 2 equal aliquots. One aliquot was supplemented with palmitoleic acid (#P9417; Sigma) from a 50 mM stock solution (in 10% chloroform, 45% hexane and 45% ethanol); the final concentration of palmitoleic acid was 0.15 mM (in 0.03% chloroform, 0.135% hexane and 0.135% ethanol). Other aliquot was supplemented with chloroform, hexane and ethanol added to the final concentrations of 0.03%, 0.135% and 0.135%, respectively. Both aliquots were then incubated for 2 h at 30^{o}C on a Labquake rotator set for 360^{o} rotation. Serial dilutions of cells were plated in duplicate onto plates containing YP medium with 2% glucose as carbon source. After 2 d of incubation at 30^{o}C, the number of CFU per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each aliquot of cells exposed to palmitoleic acid, the % of viable cells was calculated as follows: (number of viable cells per ml in the aliquot exposed to palmitoleic acid/number of viable cells per ml in the control aliquot that was not exposed to palmitoleic acid) × 100.

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.

3.4 Results

3.4.1 Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions do not affect such key traits of early-life fitness as the exponential growth rate, efficacy of post-exponential growth and fecundity of yeast cells

To perform the initial and essential test for empirical validation of evolutionary theories of programmed or non-programmed aging and age-related death, I elucidated if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence early-life fitness when each of these mutants grows and ages alone, in the absence of a parental WT yeast strain that does not carry longevity-extending mutations. I first tested if the long-lived mutant strains 3, 5 and/or 12 exhibit altered exponential growth rate and/or efficacy of post-exponential growth in media containing (1) a fermentable carbon source - *i.e.*, glucose at the initial concentration of 0.2% [CR conditions] or 2% [non-CR conditions]; and 2) a non-fermentable carbon source - i.e., ethanol at the initial concentration of 1% or glycerol at the initial concentration of 3%. In these experiments, I used the single-gene-deletion mutant strains *rpp2BΔ* and *dbp3Δ* as control strains known to exhibit an extended replicative lifespan and reduced growth rate on 2% glucose; *rpp2BΔ* lacks a gene encoding ribosomal protein P2 beta, whereas *dbp3Δ* lacks a gene encoding a DEAD-box family protein involved in ribosomal biogenesis [109].

By monitoring the OD_{600} of cell cultures recovered at different growth phases as a measure of cell growth, I found that the long-lived mutant strains 3, 5 and 12: (1) do not

differ from the parental WT strain BY4742 in exponential growth rate and post-exponential growth efficacy in medium containing 0.2% glucose (a fermentable carbon

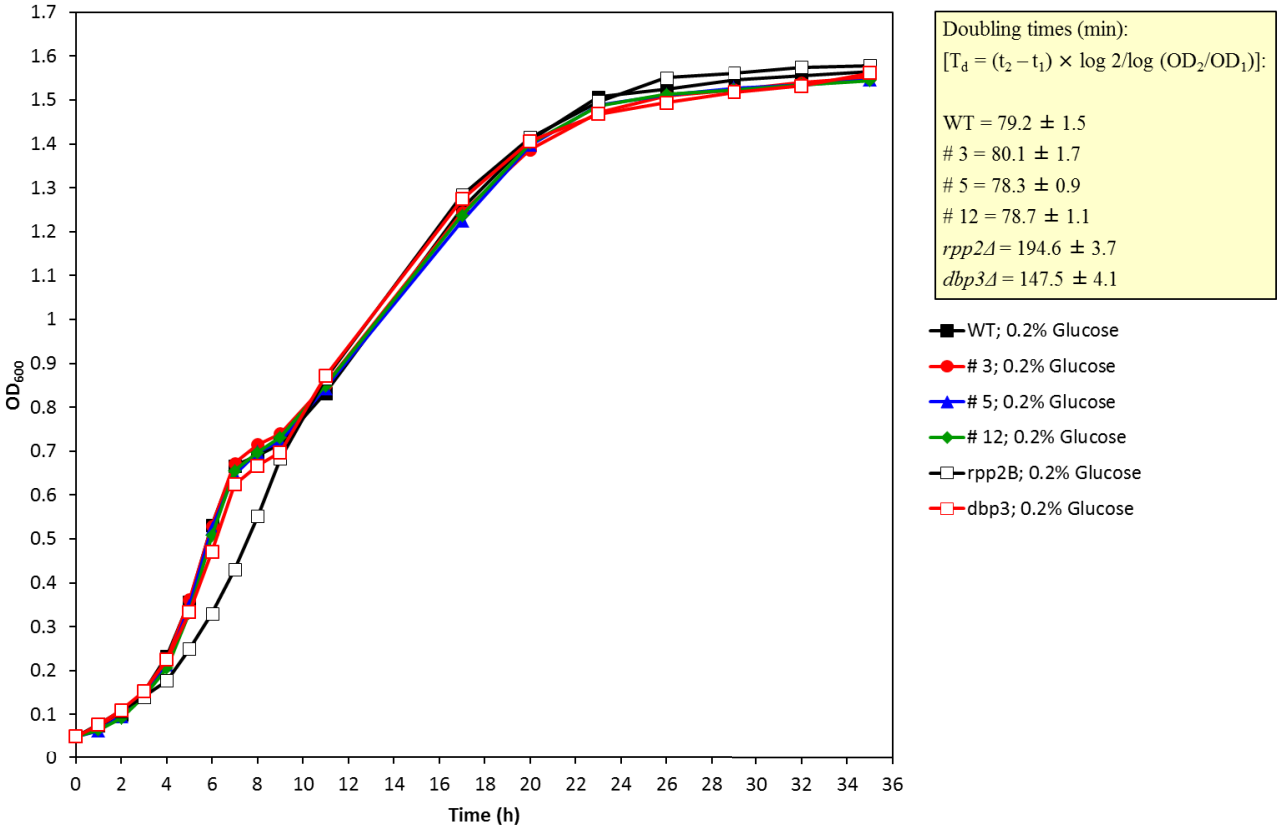


Figure 3.1. The long-lived mutant strains 3, 5 and 12 do not differ from the parental WT strain BY4742 in exponential growth rate and post-exponential growth efficacy in medium initially containing 0.2% glucose (a fermentable carbon source; CR conditions). For each strain, a doubling time (min) was calculated as $T_d = (t_2 - t_1) \times \log 2/\log (OD_2/OD_1)$.

source; CR conditions) (Figure 3.1); (2) do not differ from the parental WT strain BY4742 in exponential growth rate and post-exponential growth efficacy in medium containing 2% glucose (a non-fermentable carbon source; non-CR conditions) (Figure

3.2); (3) do not differ from the parental WT strain BY4742 in exponential growth rate and post-exponential growth efficacy in medium containing 1% ethanol (a non-fermentable carbon source) (Figure 3.3); and (4) do not differ from the parental WT strain BY4742 in exponential growth rate and post-exponential growth efficacy in medium containing 3% glycerol (a non-fermentable carbon source) (Figure 3.4).

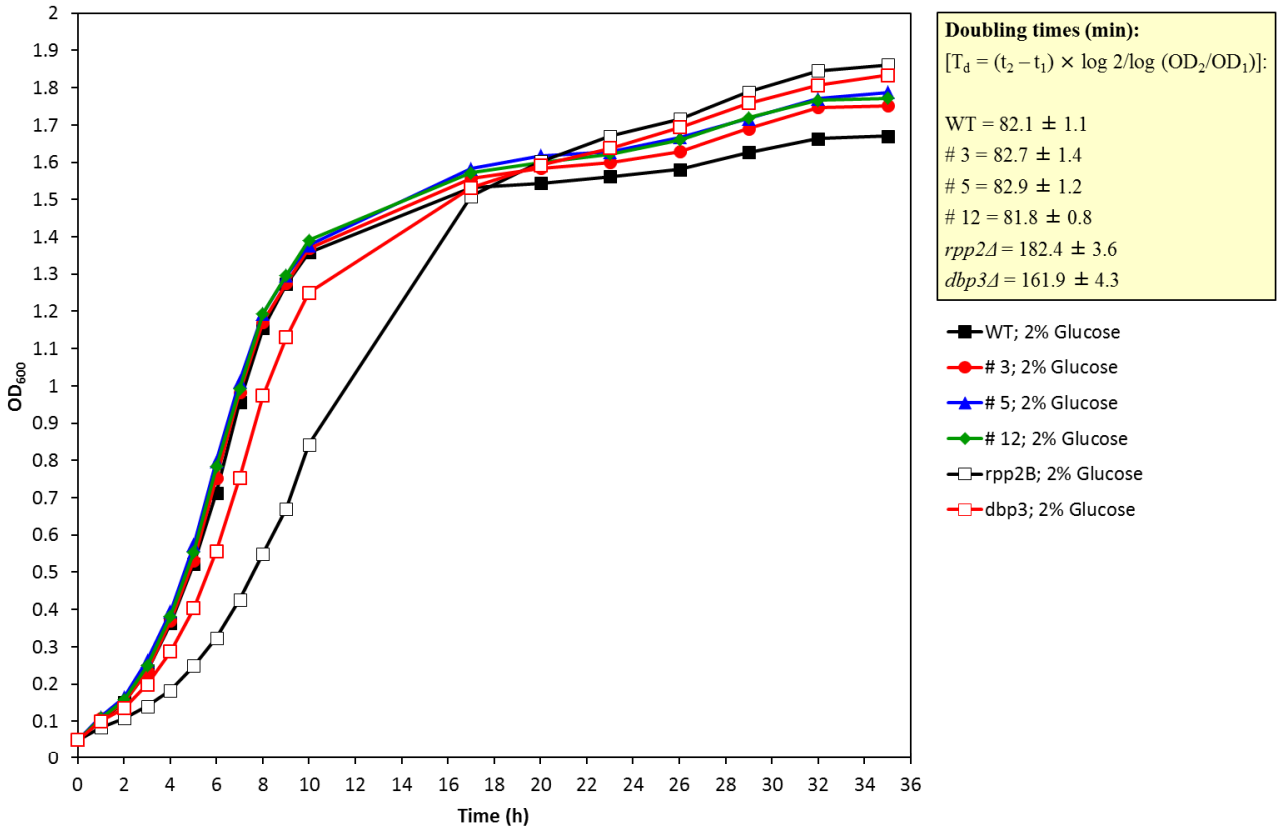


Figure 3.2. The long-lived mutant strains 3, 5 and 12 do not differ from the parental WT strain BY4742 in exponential growth rate and post-exponential growth efficacy in medium initially containing 2% glucose (a fermentable carbon source; non-CR conditions). For each strain, a doubling time (min) was calculated as $T_d = (t_2 - t_1) \times \log 2/\log (OD_2/OD_1)$.

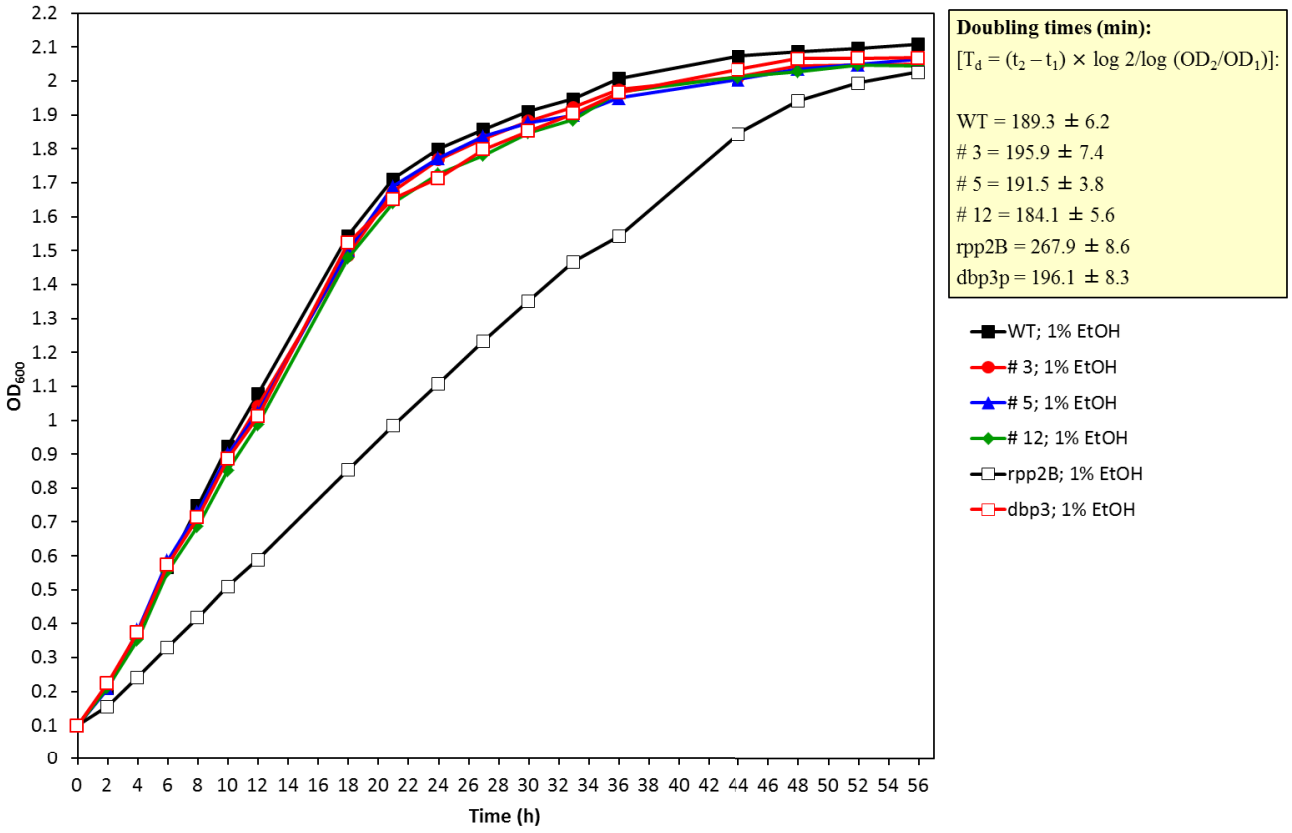


Figure 3.3. The long-lived mutant strains 3, 5 and 12 do not differ from the parental WT strain BY4742 in exponential growth rate and post-exponential growth efficacy in medium initially containing 1% ethanol (a non-fermentable carbon source). For each strain, a doubling time (min) was calculated as $T_d = (t_2 - t_1) \times \log 2/\log (OD_2/OD_1)$.

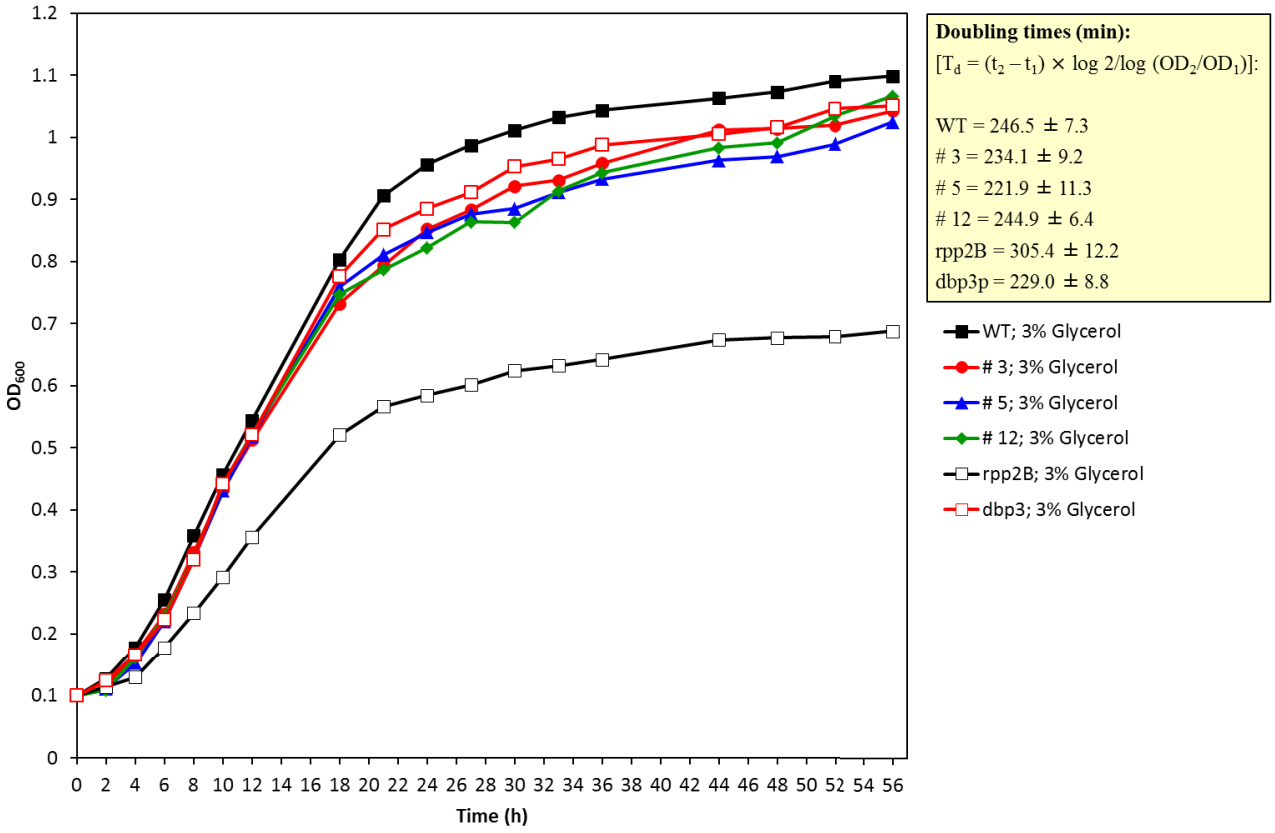


Figure 3.4. The long-lived mutant strains 3, 5 and 12 do not differ from the parental WT strain BY4742 in exponential growth rate and post-exponential growth efficacy in medium initially containing 3% glycerol (a non-fermentable carbon source). For each strain, a doubling time (min) was calculated as $T_d = (t_2 - t_1) \times \log 2/\log (OD_2/OD_1)$.

I then tested if the long-lived mutant strains 3, 5 and/or 12 exhibit altered efficacy of their sexual reproduction by mating, one of the measures of fecundity. In these experiments, yeast cells of mating type *MAT**a*** (*i.e.*, the haploid WT strain BY4741 [*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*]) and mating type ***α*** (*i.e.*, the haploid WT strain BY4742 [*MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*] or the selected long-lived haploid mutant

strains 3, 5 or 12) were pre-grown separately to mid-logarithmic phase in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose (a fermentable carbon source; CR conditions) or 1% ethanol (a non-fermentable carbon source). The efficiency of mating was measured as described in the "Materials and methods" section; it was calculated as the number of colonies of *MAT**a***/*MAT**α*** diploids divided by the sum of *MAT**a***/*MAT**α*** diploids plus haploid colonies. Crosses between two WT strains of opposite mating types (*i.e.*, the haploid strain BY4741 [*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*]) and the haploid strain BY4742 [*MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*]) were used as controls. I found that (1) the long-lived mutant strains 3, 5 and 12 do not differ from the parental WT strain BY4742 in efficacy of their sexual reproduction by mating if pre-grown in medium initially containing 0.2% glucose (a fermentable carbon source; CR conditions) (Figure 3.5); and (2) the long-lived mutant strains 3, 5 and 12 do not differ from the parental WT strain BY4742 in efficacy of their sexual reproduction by mating if pre-grown in medium initially containing 1% ethanol (a non-fermentable carbon source) (Figure 3.5).

I then assessed if the long-lived mutant strains 3, 5 and/or 12 exhibit altered efficacy of their sexual reproduction by sporulation, another measure of fecundity. In these experiments, each of the four diploid strains formed between cells of the haploid WT strain BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and cells of the haploid WT strain BY4742 (*MAT**α** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) or cells of each of the selected long-lived haploid mutant strains 3, 5 or 12 were pre-grown to mid-logarithmic phase in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose (a fermentable carbon source; CR conditions) or 1% ethanol (a non-fermentable carbon

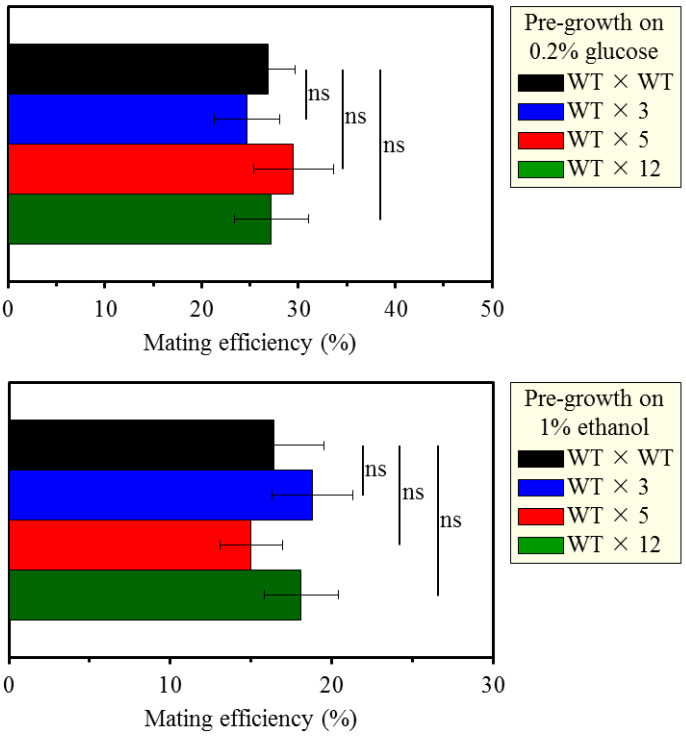


Figure 3.5. Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions do not alter the efficacy of sexual reproduction by mating, one of the measures of fecundity and a key trait of early-life fitness. Yeast cells of mating type *MAT**a*** (*i.e.*, the haploid WT strain BY4741) and mating type ***α*** (*i.e.*, the haploid WT strain BY4742 or the selected long-lived haploid mutant strains 3, 5 or 12) were pre-grown separately to mid-logarithmic phase in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose (a fermentable carbon source; CR conditions) or 1% ethanol (a non-fermentable carbon source). The efficiency of mating was measured as described in the "Materials and methods" section; it was calculated as the number of colonies of *MAT**a***/*MAT**α*** diploids divided by the sum of *MAT**a***/*MAT**α*** diploids plus haploid colonies. Data are presented as means ± SEM (n = 3; *ns*, not significant difference).

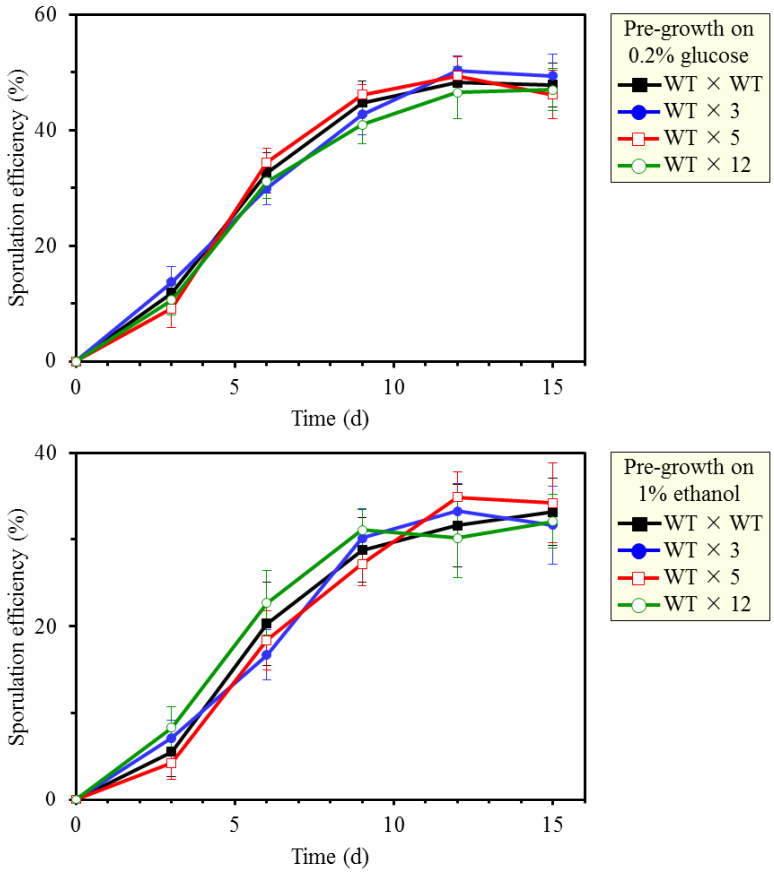


Figure 3.6. Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions do not alter the efficacy of sexual reproduction by sporulation, one of the measures of fecundity and a key trait of early-life fitness. Each of the four diploid strains formed between cells of the haploid WT strain BY4741 (*MAT****a***) and cells of the haploid WT strain BY4742 (*MAT****α***) or cells of each of the selected long-lived haploid mutant strains 3, 5 or 12 were pre-grown to mid-logarithmic phase in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose (a fermentable carbon source; CR conditions) or 1% ethanol (a non-fermentable carbon). The efficiency of sporulation of each of the four diploid strains was measured at various time points since the beginning of a sporulation assay as described in

the “Materials and methods” section; it was calculated as the percentage of tetrads and dyads produced by a diploid strain, relative to the total number of cells. Data are presented as means ± SEM (n = 3).

source). The efficiency of sporulation of each of the four diploid strains was measured at various time points since the beginning of a sporulation assay as described in the “Materials and methods” section; it was calculated as the percentage of tetrads and dyads produced by a diploid strain, relative to the total number of cells. I found that (1) the long-lived mutant strains 3, 5 and 12 do not differ from the parental WT strain BY4742 in efficacy of their sexual reproduction by sporulation when cells of the hybrid each of them formed with the haploid WT strain BY4741 of opposite mating type were pre-grown in medium initially containing 0.2% glucose (a fermentable carbon source; CR conditions) (Figure 3.6); and (2) the long-lived mutant strains 3, 5 and 12 do not differ from the parental WT strain BY4742 in efficacy of their sexual reproduction by sporulation when cells of the hybrid each of them formed with the haploid WT strain BY4741 of opposite mating type were pre-grown in medium initially containing 1% ethanol (a non-fermentable carbon source) (Figure 3.6).

Taken together, findings described in this section of my thesis imply that the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions do not affect such key traits of early-life fitness as the exponential growth rate, efficacy of post-exponential growth and fecundity of yeast cells. This trend was seen for mutant cells cultured in medium initially containing either

0.2% glucose (a fermentable carbon source; CR conditions) or 1% ethanol (a non-fermentable carbon).

3.4.2 Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance such key traits of early-life fitness as cell susceptibility to chronic exogenous stresses, mitochondria-controlled apoptosis and lipoptotic death

I also investigated if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence other essential aspects of early-life fitness when mutant cells grow and age alone, in the absence of a parental WT yeast strain. I found that in each of the three mutant strains these mutations enhance the following key traits of early-life fitness: (1) cell susceptibility to chronic oxidative, thermal and osmotic stresses administered to yeast cells that were pre-grown in medium containing glucose at the initial concentration of 0.2% [CR conditions] or ethanol at the initial concentration of 1% (Figure 3.7); (2) cell susceptibility to a mitochondria-controlled apoptotic form of death triggered by a brief exposure to exogenous hydrogen peroxide [21] of yeast cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] (Figure 3.8) or ethanol at the initial concentration of 1% (Figure 3.9); and (3) cell susceptibility to a "lipoptotic" form of death triggered by a brief exposure to exogenous palmitoleic acid [21] of yeast cells that were pre-grown in media containing glucose at the initial concentration of 0.2% [CR conditions] (Figure 3.10) or ethanol at the initial concentration of 1% (Figure 3.11).

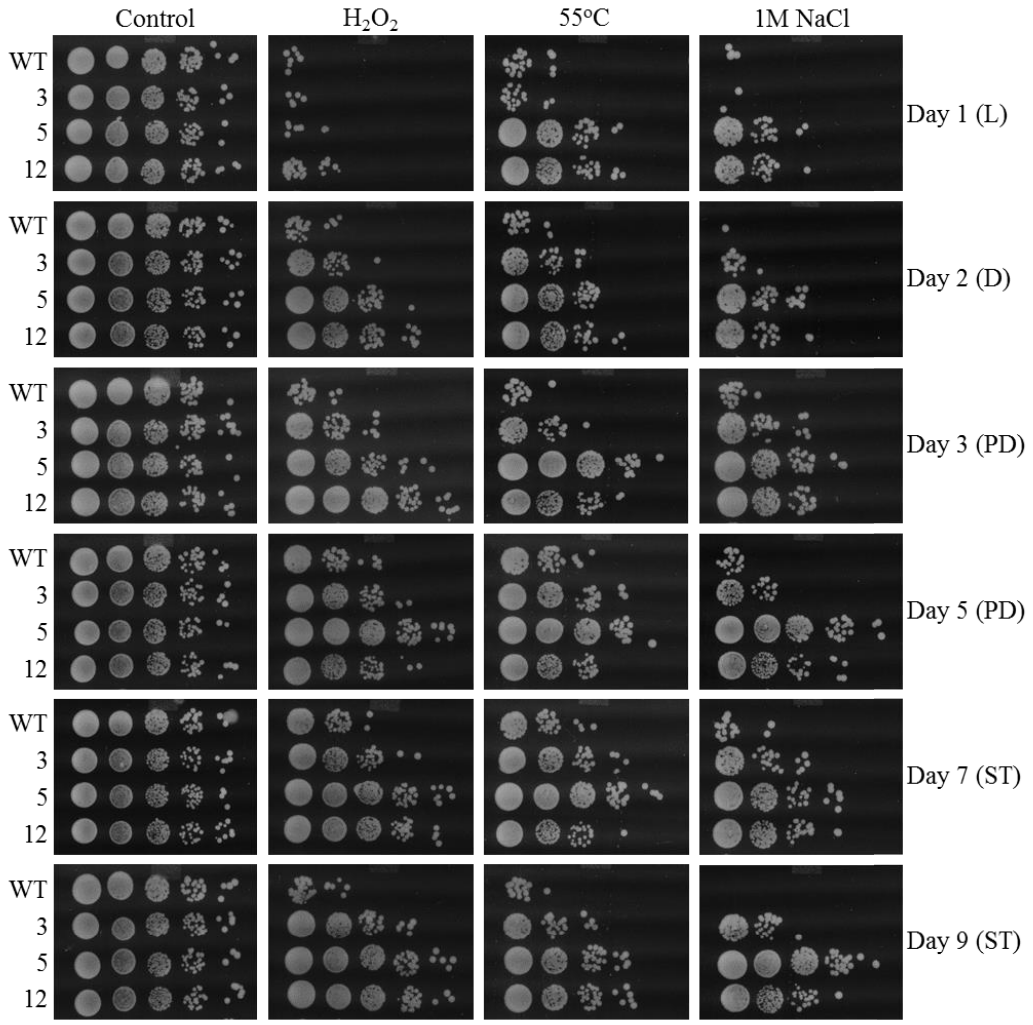


Figure 3.7. Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance the resistance of yeast to chronic oxidative, thermal and osmotic stresses. Yeast cells were grown in YP medium initially containing 0.2% glucose. Cell aliquots were recovered from various growth phases. The resistance of yeast to chronic oxidative, thermal and osmotic stresses was monitored as described in the "Materials and methods" section. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

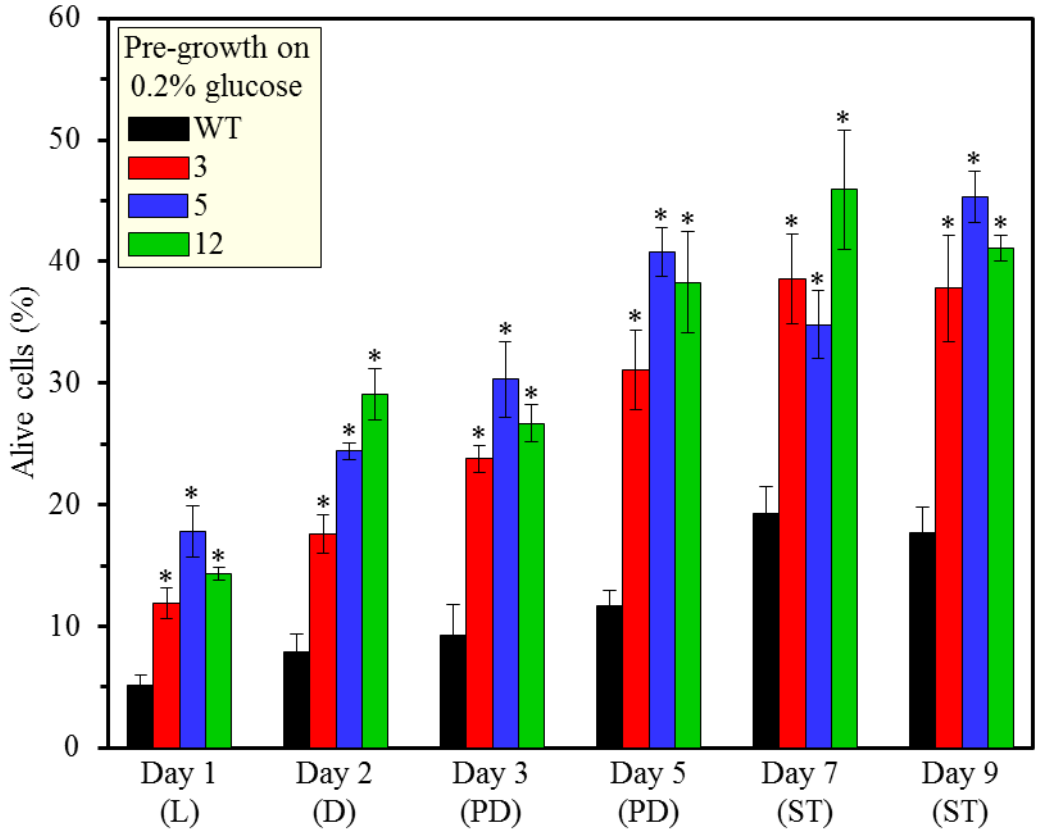


Figure 3.8. Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance the resistance of yeast to mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide. Yeast cells were grown in YP medium initially containing 0.2% glucose. Cell aliquots were recovered from various growth phases and then treated for 2 h with 2.5 mM hydrogen peroxide to induce mitochondria-controlled apoptosis as described in reference [21] and in the "Materials and methods" section of this thesis. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase. Data originate are presented as means ± SEM (n = 3; *p < 0.05).

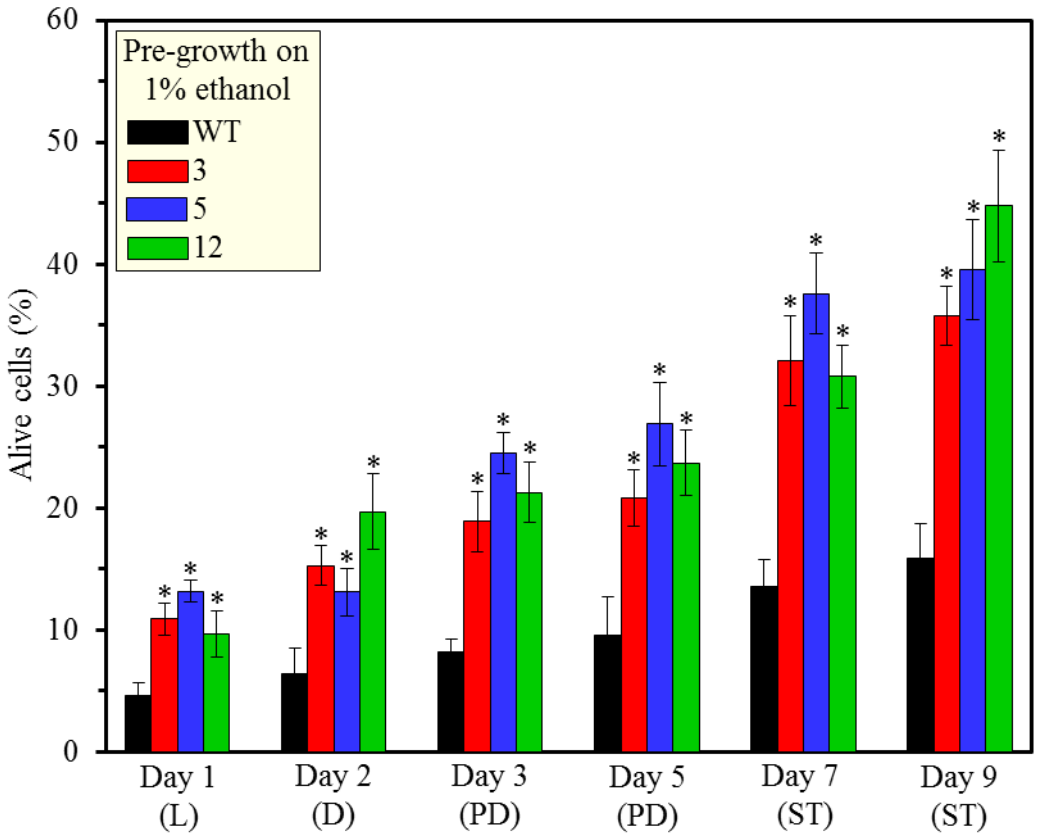


Figure 3.9. Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance the resistance of yeast to mitochondria-controlled apoptotic cell death triggered by exogenously added hydrogen peroxide. Yeast cells were grown in YP medium initially containing 1% ethanol. Cell aliquots were recovered from various growth phases and then treated for 2 h with 2.5 mM hydrogen peroxide to induce mitochondria-controlled apoptosis as described in reference [21] and in the "Materials and methods" section of this thesis. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase. Data originate are presented as means ± SEM ($n = 3$; $*p < 0.05$).

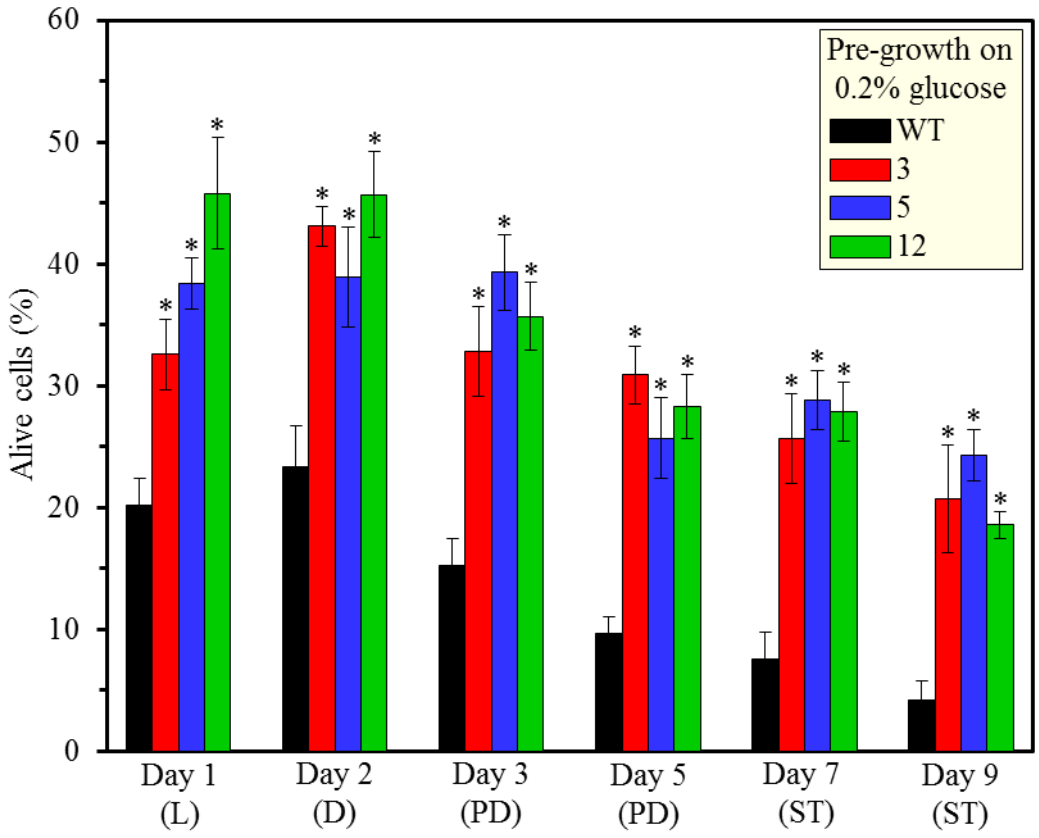


Figure 3.10. Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance the resistance of yeast to lipoptotic cell death induced by exogenously added palmitoleic acid. Yeast cells were grown in YP medium initially containing 0.2% glucose. Cell aliquots were recovered from various growth phases and then Cell aliquots were recovered from various growth phases and then exposed for 2 h to 0.2 mM palmitoleic acid to induce a lipoptotic mode of cell death as described in reference [21] and in the "Materials and methods" section of this thesis. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase. Data originate are presented as means ± SEM (n = 3; *p < 0.05).

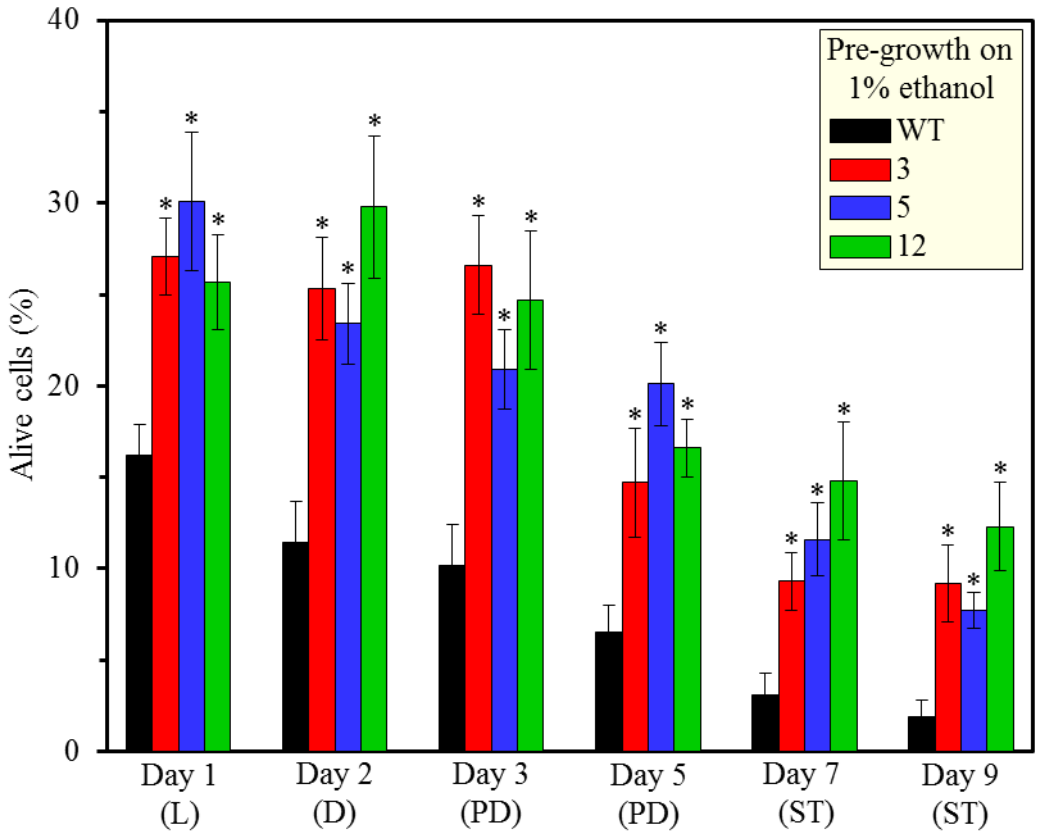


Figure 3.11. Dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance the resistance of yeast to lipoptotic cell death induced by exogenously added palmitoleic acid. Yeast cells were grown in YP medium initially containing 1% ethanol. Cell aliquots were recovered from various growth phases and then Cell aliquots were recovered from various growth phases and then exposed for 2 h to 0.2 mM palmitoleic acid to induce a lipoptotic mode of cell death as described in reference [21] and in the "Materials and methods" section of this thesis. Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase. Data originate are presented as means ± SEM (n = 3; *$p < 0.05$).

Taken together, findings described in this section of my thesis imply that the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions enhance such key traits of early-life fitness as cell susceptibility to chronic exogenous stresses, mitochondria-controlled apoptosis and lipoptotic death. This trend was seen for mutant cells cultured in medium initially containing either 0.2% glucose (a fermentable carbon source; CR conditions) or 1% ethanol (a non-fermentable carbon).

3.5 Discussion

Data presented in this chapter of my thesis demonstrate that the dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions do not affect several key traits of early-life fitness and enhance other such traits if each of these mutants grows and ages in the absence of a parental WT yeast strain. This observation provides the first irrefutable proof of evolutionary theories of aging based on the concept of programmed aging and age-related death and invalidate evolutionary theories of non-programmed aging and age-related death. Indeed, as detailed in Chapter 1 of my thesis, all evolutionary theories of non-programmed aging and age-related death predict that any longevity-extending mutation or mutations must reduce early-life fitness of an organism if it grows, develops and ages alone, in the absence of "WT" individuals of the same species that do not carry longevity-extending mutations and thus do not have lifespan extended beyond a species-specific age [57, 59, 69, 98 - 104]. In contrast, as described in details in Chapter 1 of my thesis, all evolutionary theories of programmed aging and age-related death postulate that only

those longevity-extending mutations that affect critical for early-life fitness modules of the pro-aging signaling network could reduce such fitness, whereas the longevity-extending mutations that affect other modules of such signaling network are unlikely to impact organismal early-life fitness [66, 78 - 91]. It should be stressed that, as studies described in this Chapter of my thesis have revealed for the first time, some longevity-extending mutations in yeast can actually enhance several key traits of early-life fitness if a long-lived mutant strain grows and ages in the absence of a parental WT yeast strain (*i.e.*, in the absence of natural selection).

4 Dominant mutations responsible for the extended longevity of each of the three long-lived yeast mutants evolved under laboratory conditions reduce the relative fitness of each of these mutants in a direct competition assay with a parental WT yeast strain grown in medium with 1% ethanol

4.1 Abstract

The objective of experiments described in this chapter was to investigate if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence the relative fitness of each of these mutants in a direct competition assay with the parental WT strain BY4742. The mutant and parental WT strains used in such competition assay were (1) pre-grown separately in media containing a fermentable carbon source (*i.e.*, glucose at the initial concentration of 0.2% [CR conditions] or 2% [non-CR conditions]) or a non-fermentable carbon source (*i.e.*, ethanol at the initial concentration of 1%); and then (2) mixed in equal numbers and co-cultured in media containing 0.2% glucose [CR conditions], 2% glucose [non-CR conditions] or 1% ethanol. This direct competition assay mimics the process of natural selection within a mixed population of individuals that belong to the same species but exhibit different longevity-defining genetic backgrounds, have lifespans at a species-specific age and beyond it, and compete for nutrients and other environmental resources. As described in this chapter, I found that in a population of mixed cells grown on 1% ethanol the dominant mutations that extend longevity of the three long-lived yeast mutants 3, 5 and 12 reduce the relative fitness of each of them in a direct competition assay with the parental WT strain BY4742. Thus, under some laboratory conditions

mimicking the process of natural selection within an ecosystem composed of yeast cells having different longevity-defining genetic backgrounds, each of the three long-lived mutants is forced out of the ecosystem by a parental WT strain exhibiting shorter lifespan. I therefore concluded that yeast cells have evolved some mechanisms for limiting their lifespan upon reaching a certain chronological age. These mechanisms drive the evolution of yeast longevity towards maintaining a finite yeast lifespan within ecosystems.

4.2 Introduction

As detailed in Chapter 1 of my thesis, the evolutionary theories of programmed aging and age-related death posit that the evolutionary force actively limits organismal lifespan at an age unique to each species [53, 54, 56, 57, 59 - 69]. All these theories of aging are based on the premise that natural selection resulted in preferential reproduction of those members of various species that have evolved certain mechanisms for limiting their lifespans in a species-specific fashion and upon reaching a species-specific age [57, 59, 61- 63, 66, 69, 71 - 73]. The availability of the three long-lived yeast mutants that we selected under laboratory conditions enables empirical validation of these theories by investigating if the dominant mutations that extend their longevity influence the relative fitness of each of these mutants in a direct competition assay with the parental WT strain BY4742. To attain this objective, in studies described in this chapter of my thesis I developed such direct competition assay and used it to mimic under various laboratory conditions the process of natural selection within a mixed population of yeast cells that (1) exhibit different longevity-defining genetic backgrounds; (2) differ in their lifespans

if grow as a genetically homogenous cell population; and (3) compete for nutrients and other environmental resources.

4.3 Materials and Methods

Quantifying the relative fitness of a long-lived mutant strain in a direct competition assay with a parental WT strain

The WT strains BY4739 (*MATα leu2Δ0 lys2Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), the single-gene-deletion mutant strain *dbp3Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dbp3Δ::kanMX4*) in the BY4742 genetic background (all from Open Biosystems), and the long-lived mutant strains 3, 5 and 12 (all three in the BY4742 genetic background) were grown separately in YP medium (1% yeast extract, 2% peptone) initially containing 0.2% glucose, 2% glucose or 1% ethanol as carbon source until mid-exponential phase. Cells were cultured at 30^{o}C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1. The single-gene-deletion mutant strain *dbp3Δ* lacks a gene encoding a DEAD-box family protein involved in ribosomal biogenesis [109]. *dbp3Δ* was used as a control mutant strain because it is known to exhibit (1) an extended replicative lifespan (as compared to the parental WT strain BY4742) [109]; (2) a reduced growth rate on 0.2% glucose (see Chapter 3 of this thesis), 2% glucose [109] and 1% ethanol (see Chapter 3 of this thesis); and (3) a reduced relative fitness when is co-cultured with a parental WT strain in medium initially containing 2% glucose [109]. 2.5×10^6 cells of the WT strain BY4739 (*MATα leu2Δ0 lys2Δ0 ura3Δ0*) were mixed with the same number of cells of the BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), *dbp3Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*

dbp3Δ::kanMX4), 3, 5 or 12 strain in 50 ml of YP medium initially containing 0.2% glucose, 2% glucose or 1% ethanol as carbon source. After culturing the cell mixture at 30^{o}C for 7 days, an aliquot of cell suspension was diluted and plated on a solid YP medium supplemented with 2% glucose. Following 2 days of incubation at 30^{o}C, colonies on each plate were replicated onto 2 plates with solid YNB (Yeast Nitrogen Base) medium without amino acids supplemented with 2% glucose; one of these plates contained leucine, lysine, uracil and histidine [hereafter called a "His^{+}" plate], whereas the other plate contained leucine, lysine and uracil [hereafter called a "His^{-}" plate]. After 2 days of incubation at 30^{o}C, the number of CFU on "His^{+}" and "His^{-}" plates was counted. The relative fitness of each His^{+} strain (relative to the His^{-} strain BY4739 [*MATα leu2Δ0 lys2Δ0 ura3Δ0*]) was calculated as $\log_2 [(CFU^{x}{}_{mutant}/CFU^{x}{}_{WT}/(CFU^{0}{}_{mutant}/CFU^{0}{}_{WT})]$, where: CFU^{x} is the colony count at the end of week x, whereas CFU^{0} is the colony count at initial inoculation of a mixed culture.

4.4 Results

4.4.1 Development and validation of a quantitative assay for assessing the relative fitness of a long-lived mutant strain competing for nutrients and other environmental resources with a parental WT strain

To investigate if the dominant mutations that extend longevity of each of the three long-lived yeast mutants evolved under laboratory conditions influence the relative fitness of each of these mutants when it competes for nutrients and other environmental resources with a parental WT strain, I developed a direct competition assay. In this assay (Figure 4.1), the WT strains BY4739 (*MATα leu2Δ0 lys2Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1*

leu2Δ0 lys2Δ0 ura3Δ0), the single-gene-deletion mutant strain *dbp3Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dbp3Δ::kanMX4*) in the BY4742 genetic background, and the long-lived mutant strains 3, 5 and 12 (all three in the BY4742 genetic background) were grown separately in liquid YP medium initially containing 0.2% glucose, 2% glucose or 1% ethanol as carbon source until mid-exponential phase. The single-gene-deletion mutant strain *dbp3Δ* lacks a gene encoding a DEAD-box family protein involved in ribosomal biogenesis [109]; *dbp3Δ* was used as a control mutant strain because it is known to exhibit (1) an extended replicative lifespan (as compared to the parental WT strain BY4742) [109]; (2) a reduced growth rate on 0.2% glucose (see Chapter 3 of this thesis), 2% glucose [109] and 1% ethanol (see Chapter 3 of this thesis); and (3) a reduced relative fitness when is co-cultured with a parental WT strain in medium initially containing 2% glucose [109]. Cells of the WT strain BY4739 were mixed with the same number of cells of the BY4742, *dbp3Δ*, 3, 5 or 12 strain in YP medium initially containing 0.2% glucose, 2% glucose or 1% ethanol as carbon source. After culturing the cell mixture for 7 days, an aliquot of cell suspension was diluted and plated on a solid YP medium supplemented with 2% glucose. Following 2 days of incubation, colonies on each plate were replicated onto plates with a synthetic minimal YNB medium without amino acids supplemented with 2% glucose; one of these plates contained leucine, lysine, uracil and histidine (it was called a "His^+" plate), whereas the other plate contained leucine, lysine and uracil (it was called a "His^-" plate). After 2 days of incubation at 30^oC, the number of CFU on "His^+" and "His^-" plates was counted. The relative fitness of each His^+ strain (*i.e.*, the BY4742, *dbp3Δ*, 3, 5 or 12 strain) in a direct competition with the His^- strain BY4739 was calculated as $\log_2 [(CFU^x_{mutant}/CFU^x_{WT}/(CFU^0_{mutant}/CFU^0$

$_{WT}$)], where: CFU^x is the colony count at the end of week x, whereas CFU^0 is the colony count at initial inoculation of a mixed culture (Figure 4.1).

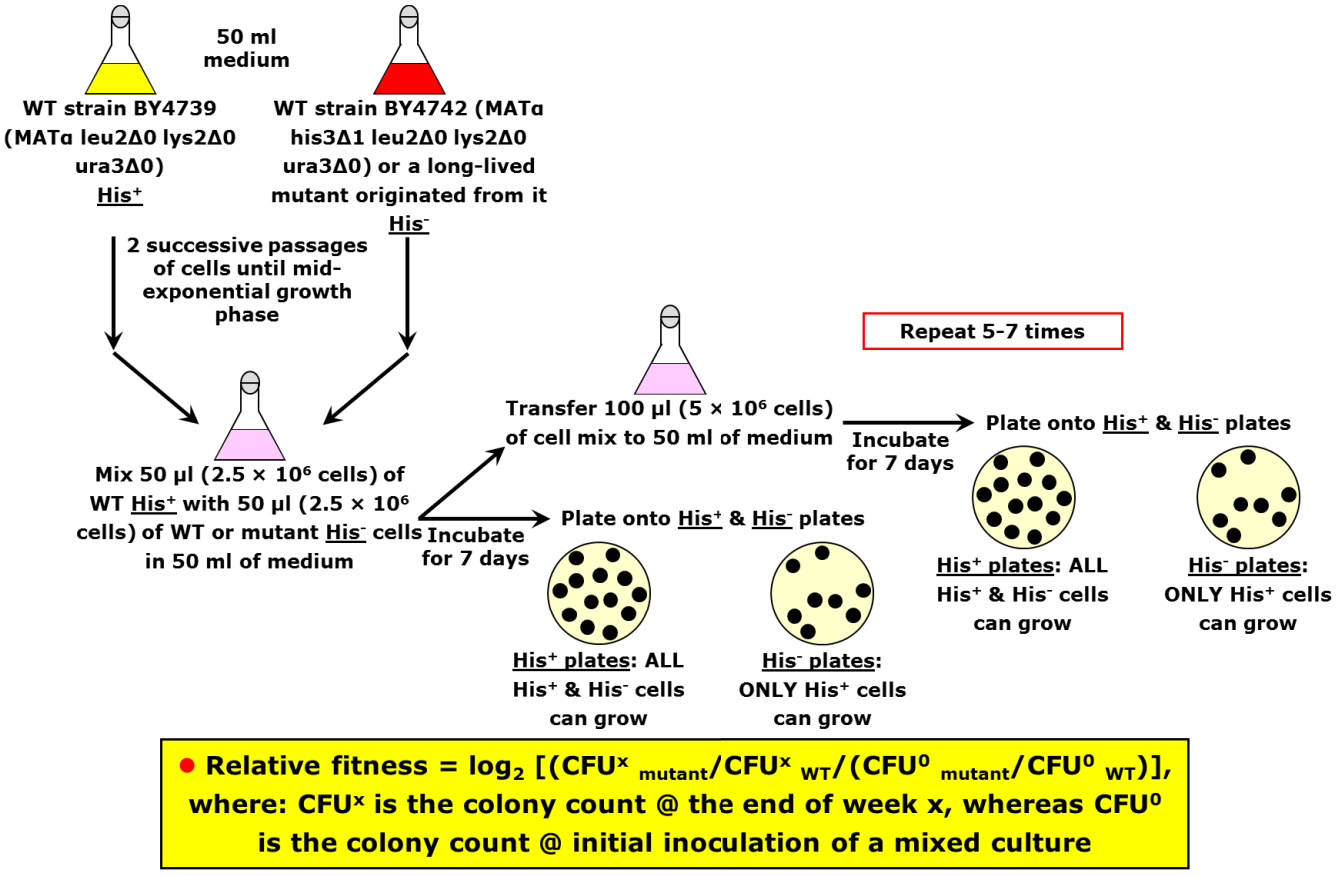


Figure 4.1. Quantifying the relative fitness of a long-lived mutant strain in a direct competition assay with a parental WT strain.

To validate this assay, I used it to compare the fitness of the WT strains BY4739 (His^+) and BY4742 (His^-) in a control experiment. I found that even after six consecutive 7-days incubations together the parental (to the long-lived mutant strains 3, 5 and 12) WT strain BY4742 (His^-) exhibits similar relative fitness in a direct competition assay with

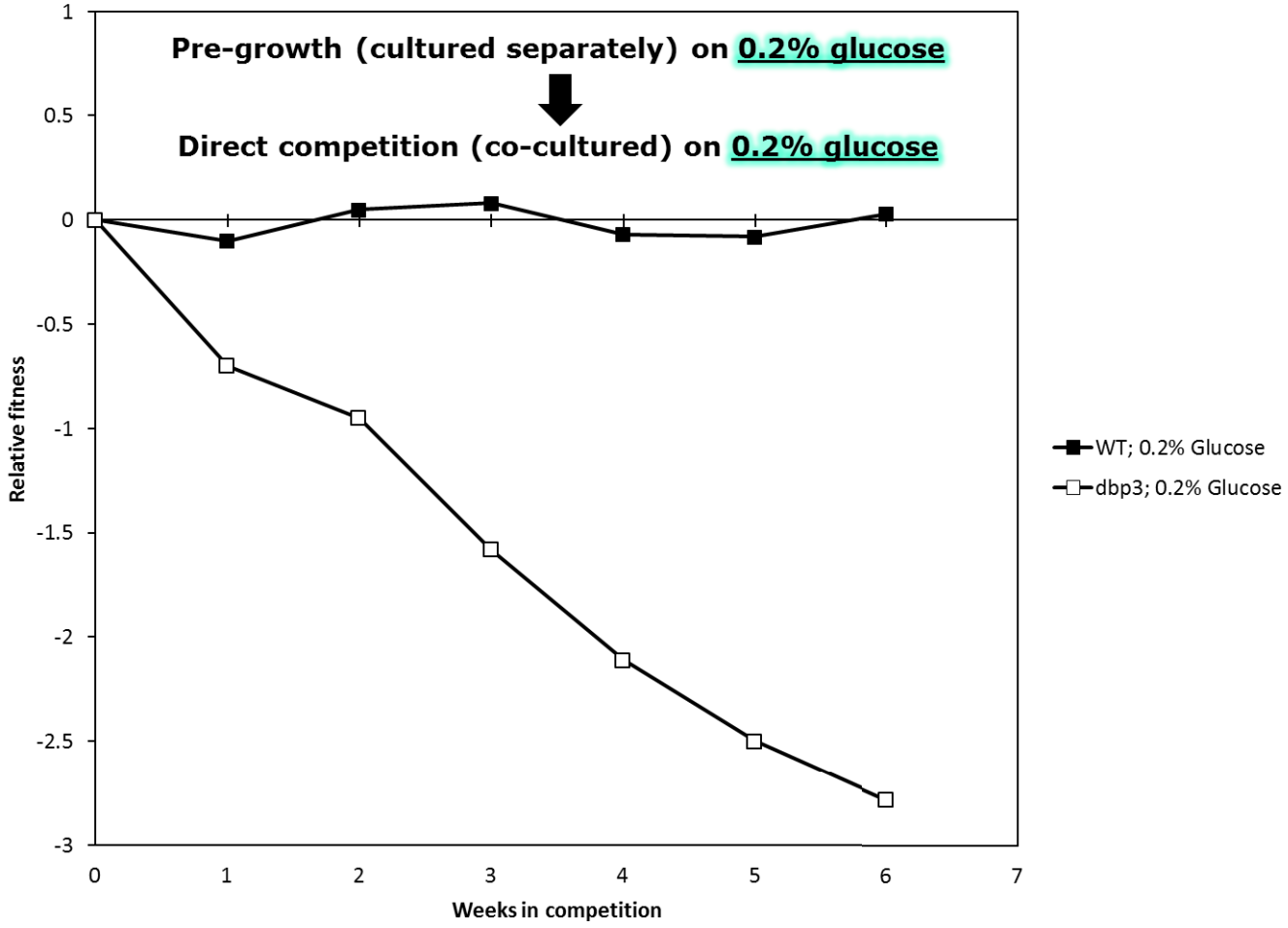


Figure 4.2. The parental (to the long-lived mutant strains 3, 5 and 12) wild-type strain BY4742 exhibits similar relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to BY4742) co-cultured in medium containing 0.2% glucose.

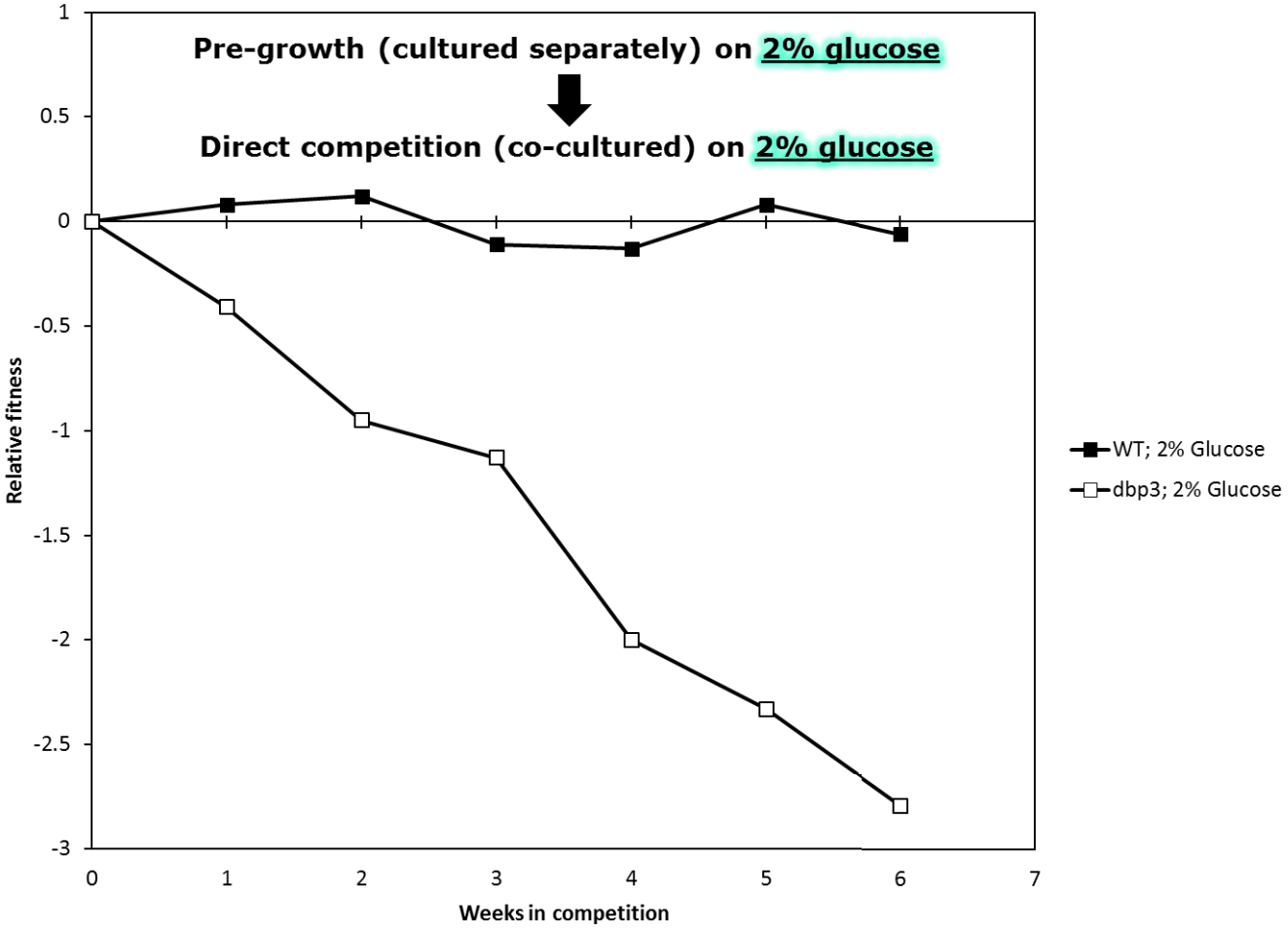


Figure 4.3. The parental (to the long-lived mutant strains 3, 5 and 12) wild-type strain BY4742 exhibits similar relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to BY4742) co-cultured in medium containing 2% glucose.

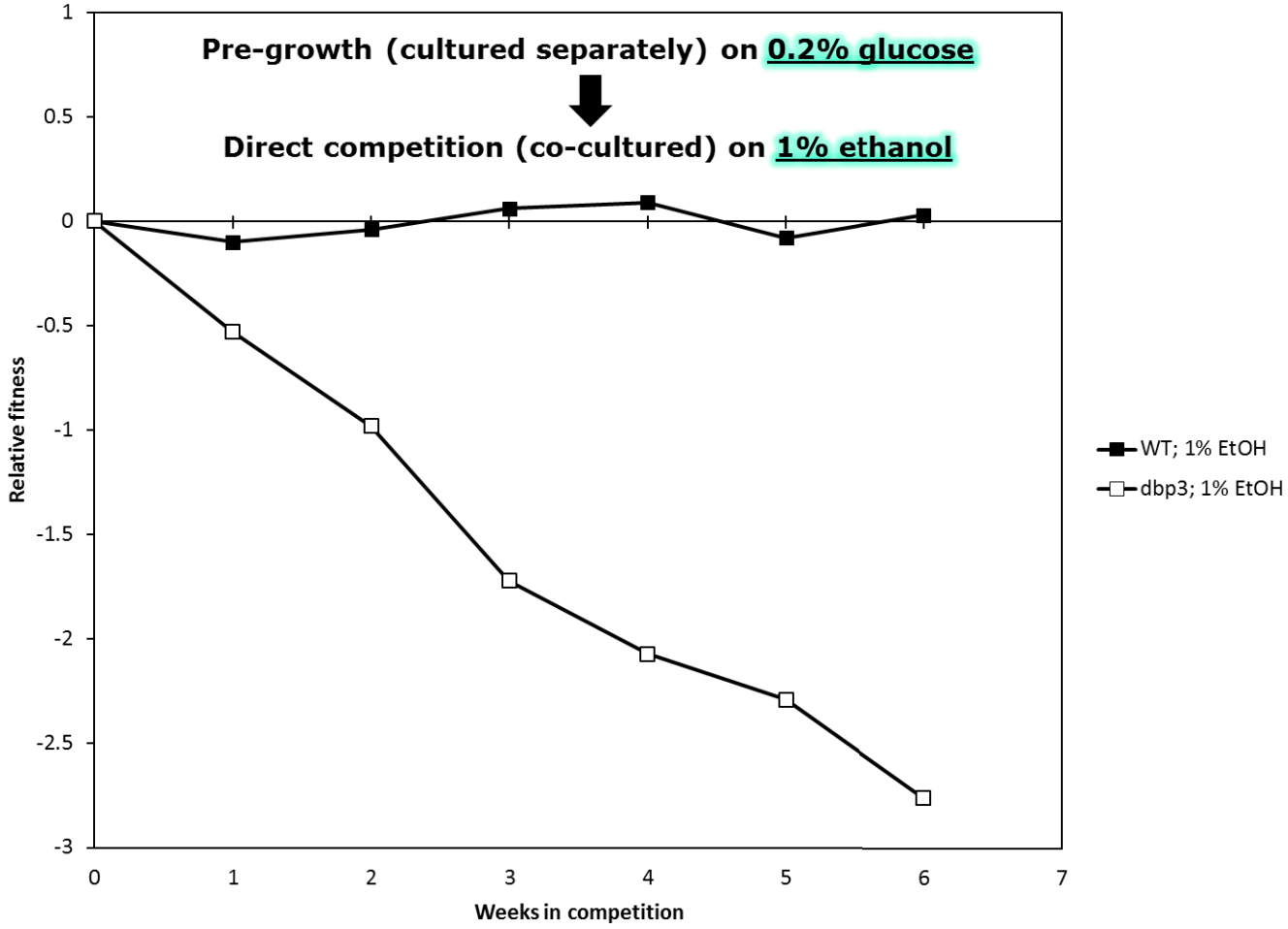


Figure 4.4. The parental (to the long-lived mutant strains 3, 5 and 12) wild-type strain BY4742 exhibits similar relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to BY4742) co-cultured in medium containing 1% ethanol.

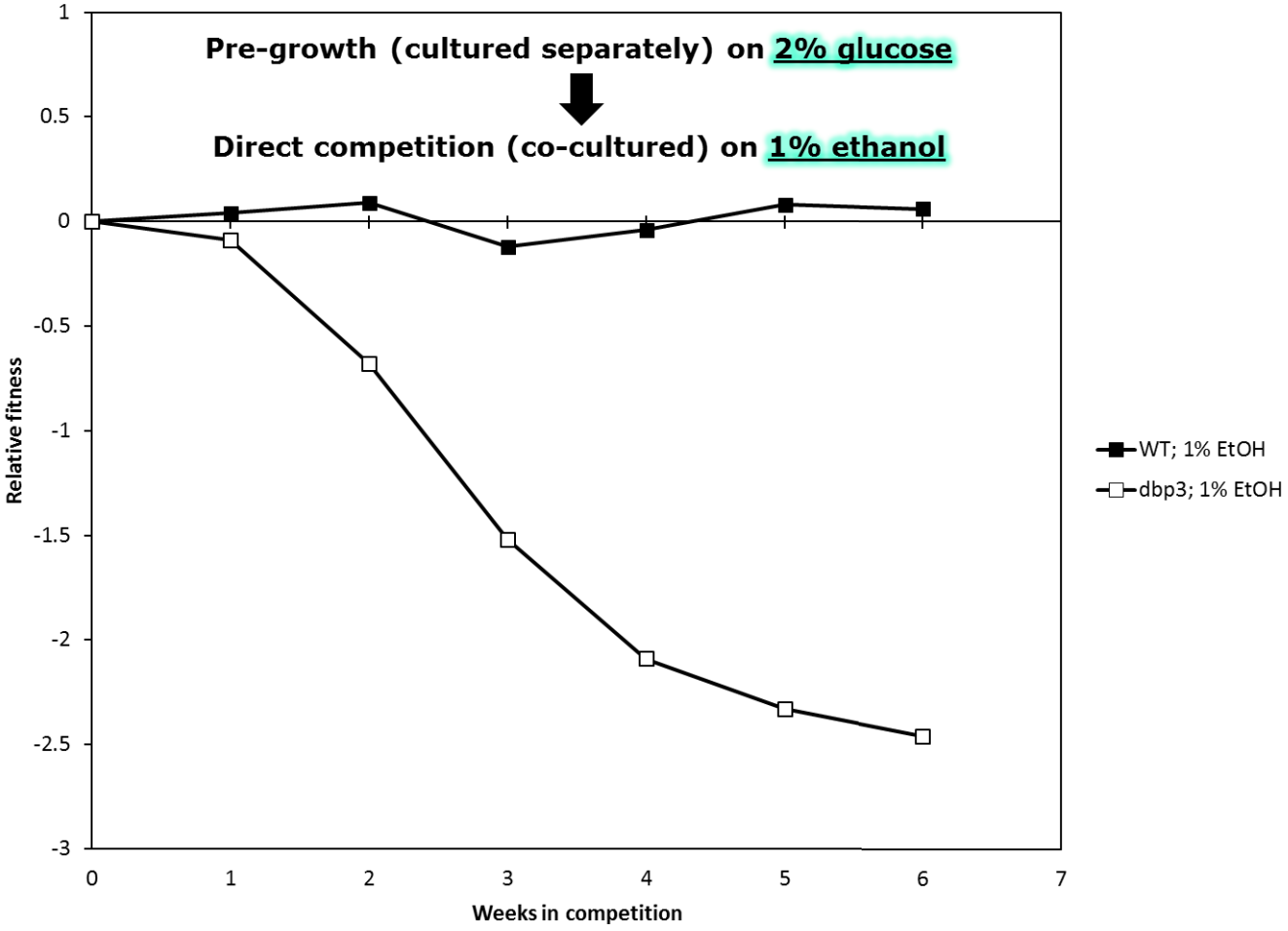


Figure 4.5. The parental (to the long-lived mutant strains 3, 5 and 12) wild-type strain BY4742 exhibits similar relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to BY4742) co-cultured in medium containing 1% ethanol.

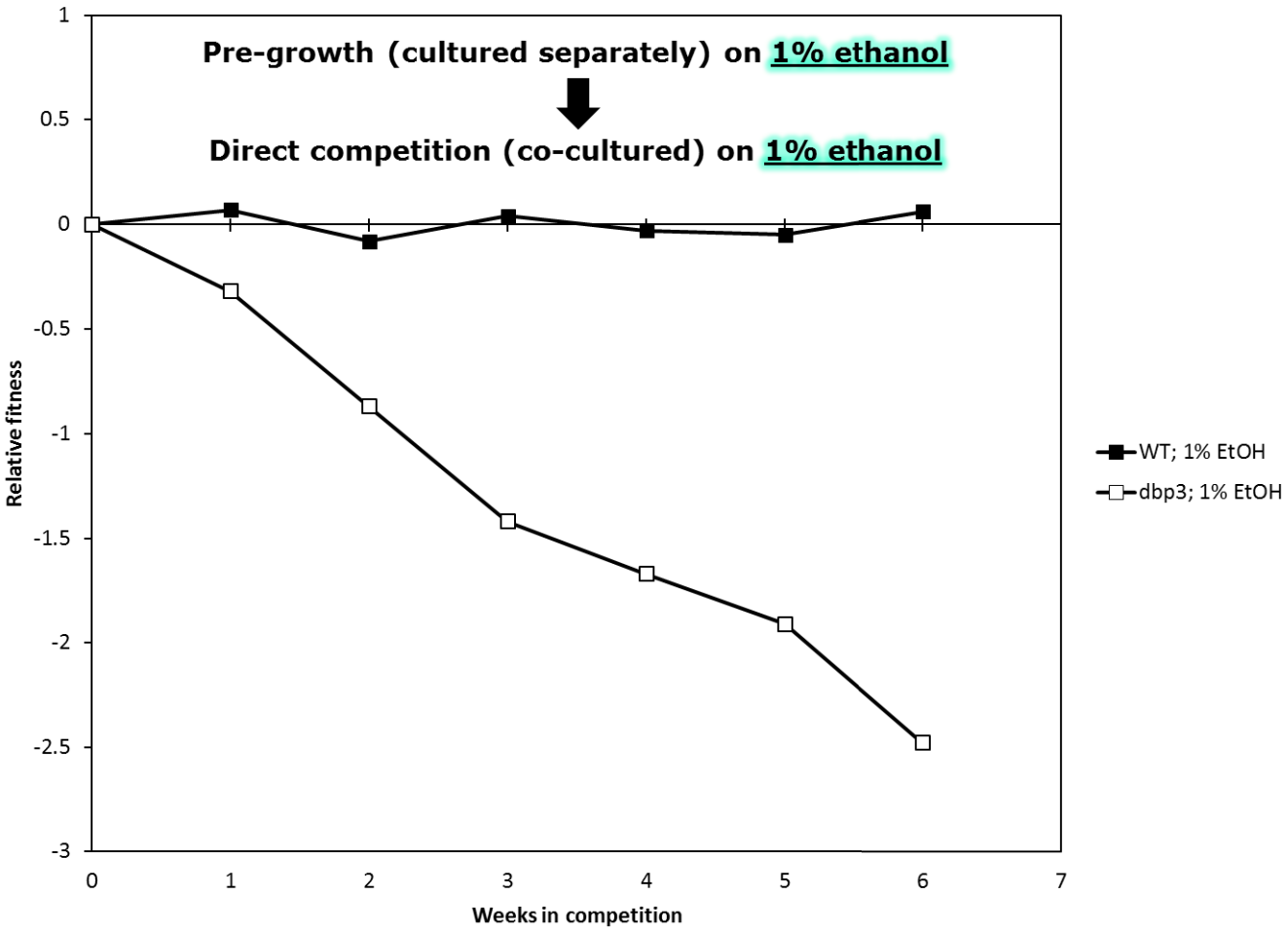


Figure 4.6. The parental (to the long-lived mutant strains 3, 5 and 12) wild-type strain BY4742 exhibits similar relative fitness in a direct competition assay with the WT strain BY4739 (His$^+$, but otherwise isogenic to BY4742) co-cultured in medium containing 1% ethanol.

the WT strain BY4739 (His$^+$, but otherwise isogenic to BY4742) that was co-cultured in YP medium containing: (1) 0.2% glucose, following cell transfer from 0.2% glucose (Figure 4.2); (2) 2% glucose, following cell transfer from 2% glucose (Figure 4.3); (3) 1% ethanol, following cell transfer from 0.2% glucose (Figure 4.4); (4) 1% ethanol,

following cell transfer from 2% glucose (Figure 4.5); or (5) 1% ethanol, following cell transfer from 1% ethanol (Figure 4.6). Based on these findings, I concluded that the developed direct competition assay outlined in Figure 4.1 accurately reproduces the expected equal fitness of the two WT strains used, *i.e*. BY4739 (His^+) and BY4742 (His^-, but otherwise isogenic to BY4739). Moreover, this assay also accurately reproduces the expected (see reference [109] and Chapter 3 of this thesis) reduced fitness of the mutant strain *dbp3Δ* (which is isogenic to the WT strain BY4742) in a direct competition assay with the parental WT strain BY4739 (His^+, but otherwise isogenic to BY4742) (Figures 4.2 - 4.6).

4.4.2 Dominant mutations responsible for the extended longevity of the yeast mutants 3, 5 and 12 reduce the relative fitness of each of these long-lived mutants in a direct competition assay with a parental WT yeast strain grown in medium with 1% ethanol

I first used the developed direct competition assay to quantify the fitness of the long-lived mutant strain 3 relative to the parental WT strain BY4742; cells of these two strains were first cultured separately in medium containing different concentrations of glucose or ethanol, then mixed as described in the "Materials and Methods" section, and underwent six consecutive 7-days incubations together. I found that the dominant mutations that extend longevity of the long-lived mutant strain 3 (which was His^- in the BY4742 genetic background) do not reduce its relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742 and the long-lived mutant strain 3) co-cultured in media containing: (1) 0.2% glucose, following

cell transfer from 0.2% glucose (Figure 4.7); and (2) 2% glucose, following cell transfer from 2% glucose (Figure 4.8). In contrast, the dominant mutations that extend longevity of the long-lived mutant strain 3 (which was His^- in the BY4742 genetic background) reduce its relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742 and the long-lived mutant strain 3) co-cultured in media containing: (1) 1% ethanol, following cell transfer from 0.2% glucose (Figure 4.9); (2) 1% ethanol, following cell transfer from 2% glucose (Figure 4.10); or (3) 1% ethanol, following cell transfer from 1% ethanol (Figure 4.11). It needs to be emphasized that, as a comparison of the data presented in Figures 4.9, 4.10 and 4.11 demonstrate, the conditions of pre-culturing of the long-lived mutant strain 3 do not influence the extent of its reduced relative fitness during the subsequent co-culturing with a parental WT strain in medium containing 1% ethanol (Figure 4.12). I therefore concluded that the long-lived mutant strain 3 does not keep a "memory" of conditions under which it has been grown prior to being mixed with a parental WT strain in medium containing 1% ethanol for a fitness competition.

I then used the developed direct competition assay to quantify the fitness of the long-lived mutant strain 5 relative to the parental WT strain BY4742; cells of these two strains were first cultured separately in medium containing different concentrations of glucose or ethanol, then mixed as described in the "Materials and Methods" section, and underwent six consecutive 7-days incubations together. I found that the dominant mutations that extend longevity of the long-lived mutant strain 5 (which was His^- in the BY4742 genetic background) do not reduce its relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to the parental WT strain

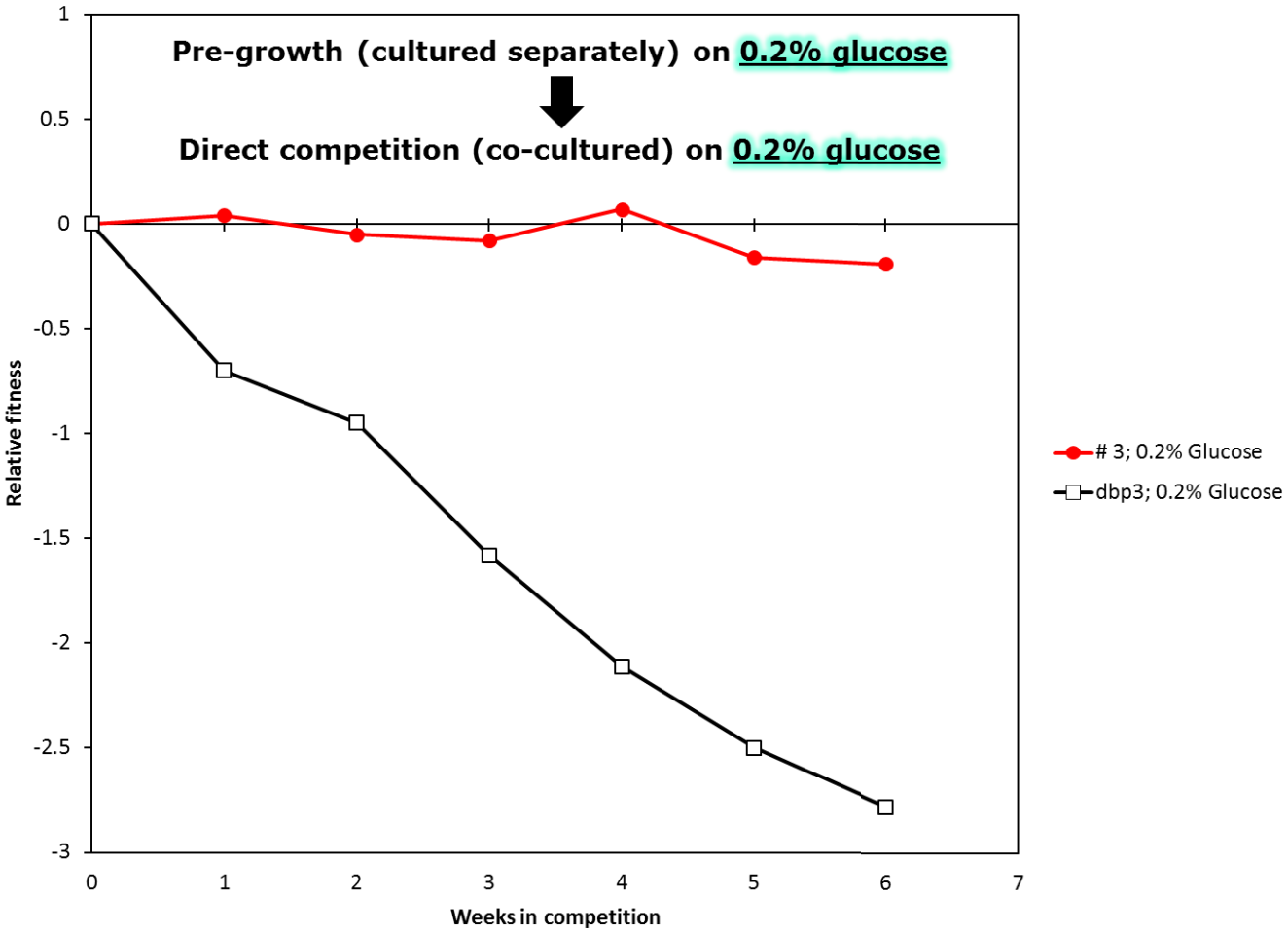


Figure 4.7. Mutations that extend longevity of the long-lived mutant strain 3 (His^-) do not reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 0.2% glucose.

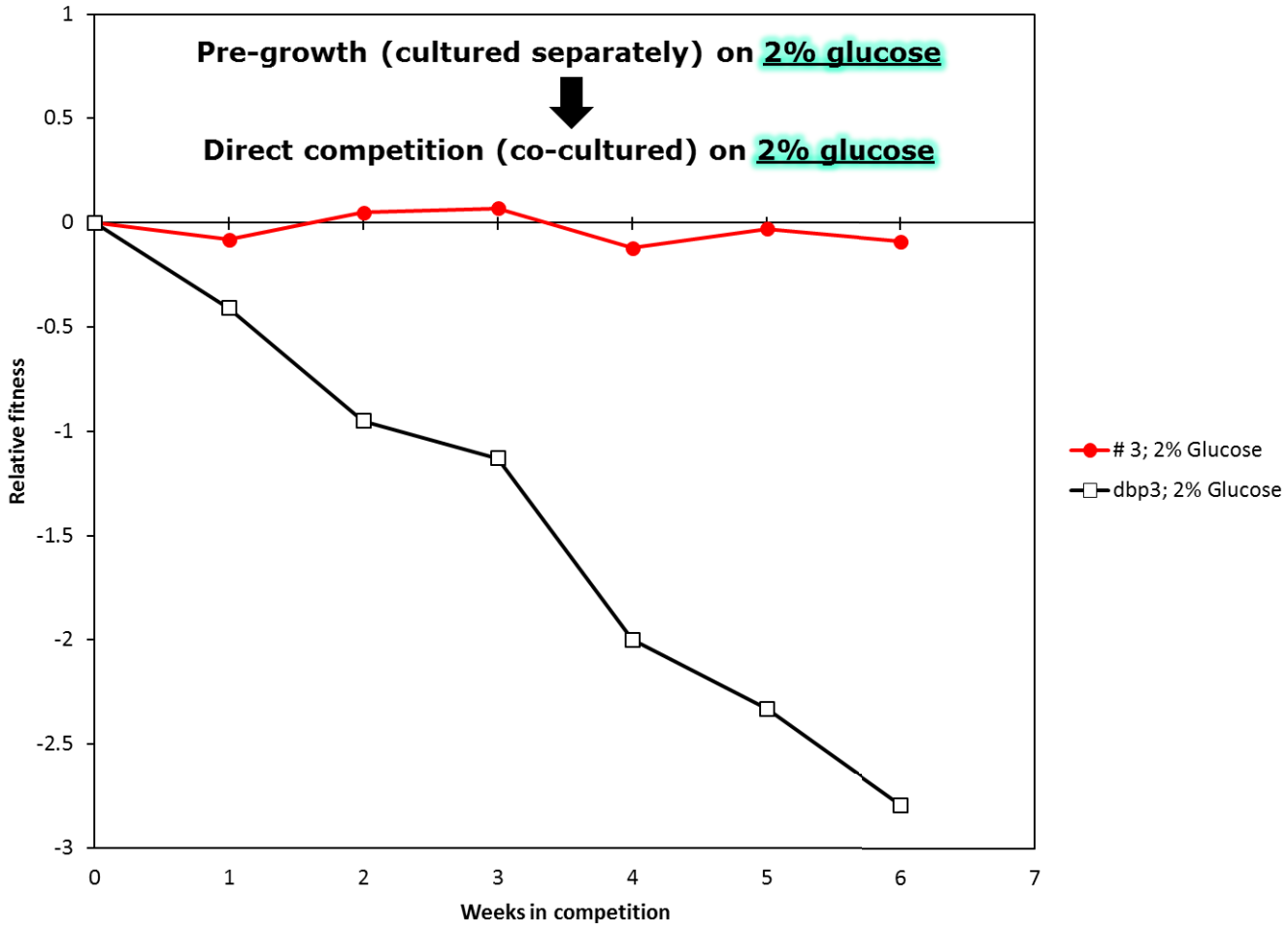


Figure 4.8. Mutations that extend longevity of the long-lived mutant strain 3 (His^-) do not reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 2% glucose.

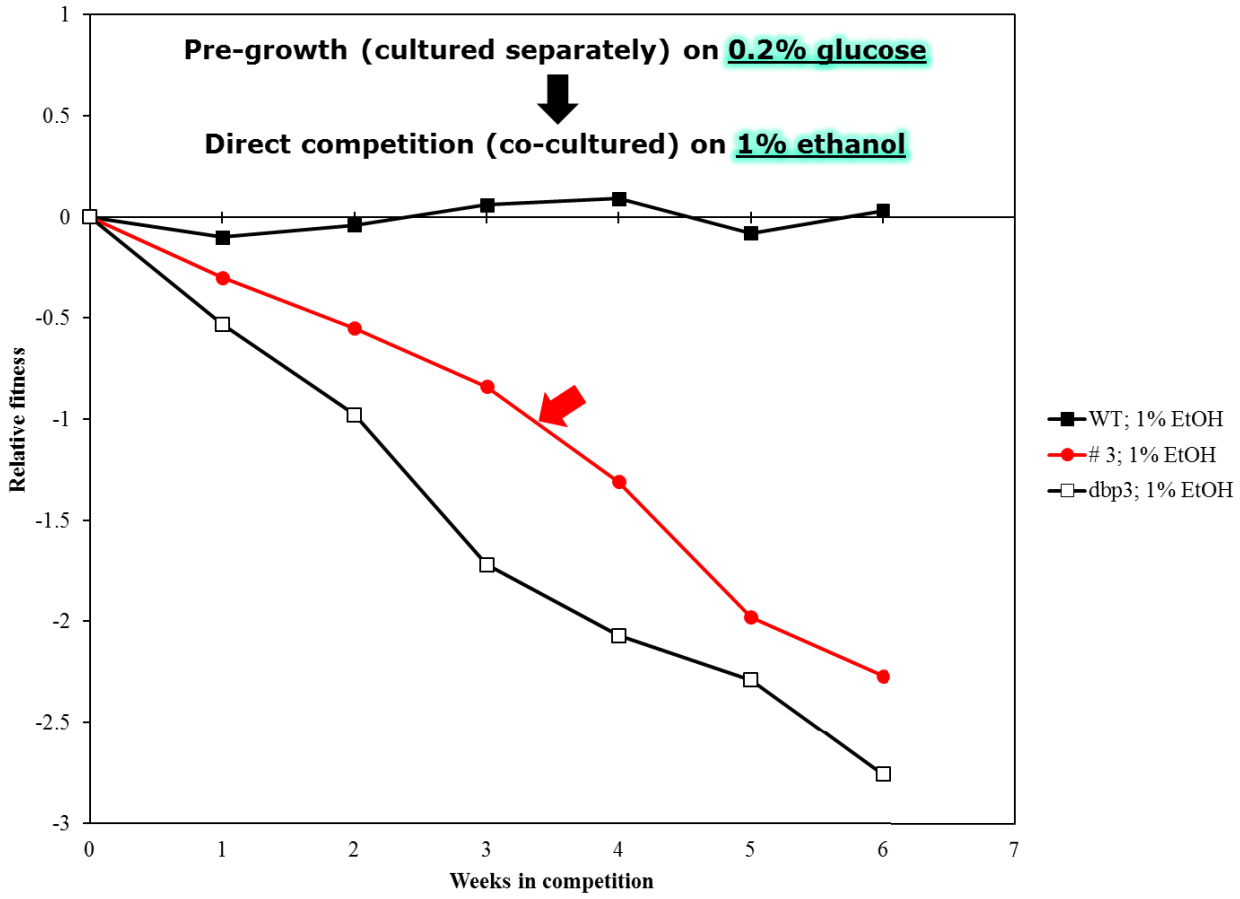


Figure 4.9. Mutations that extend longevity of the long-lived mutant strain 3 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

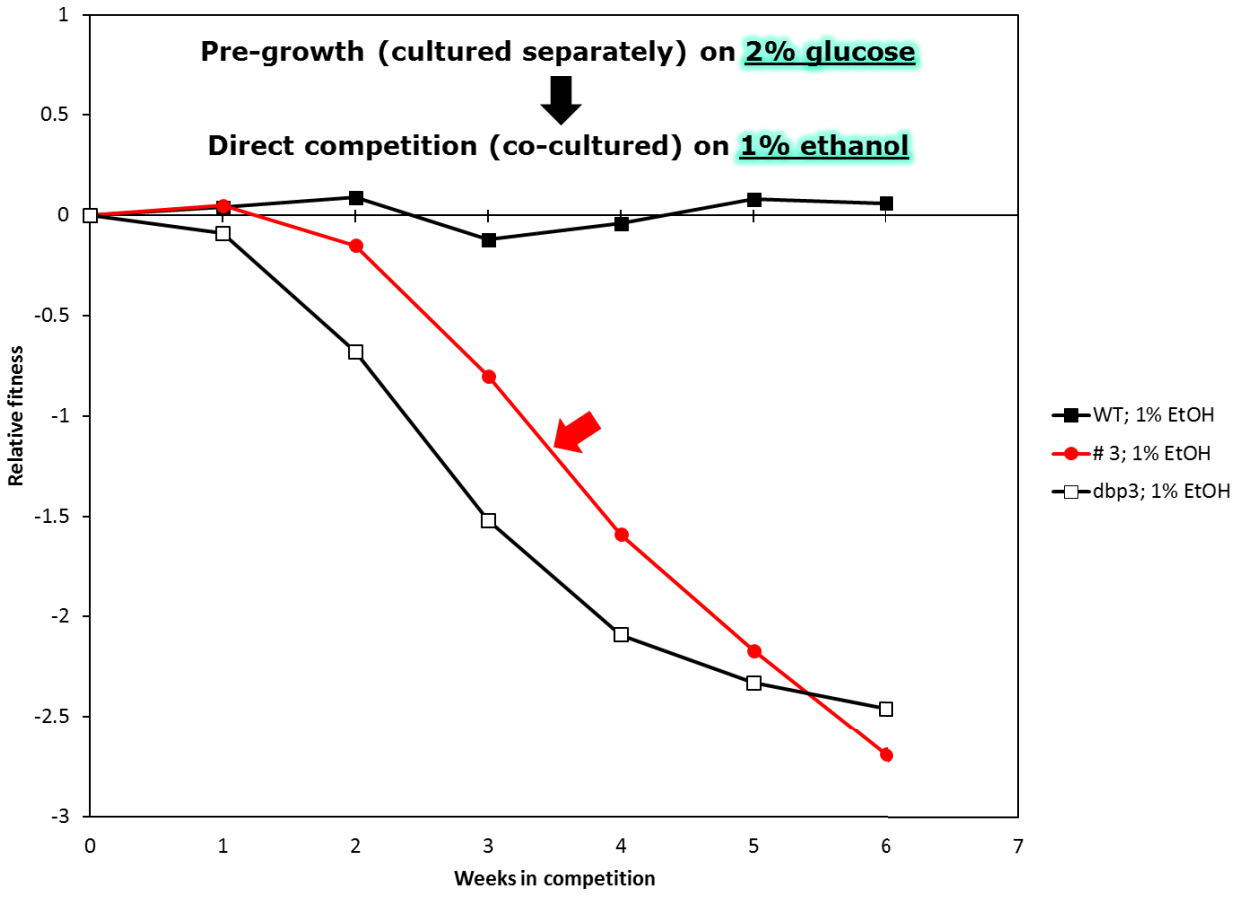


Figure 4.10. Mutations that extend longevity of the long-lived mutant strain 3 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

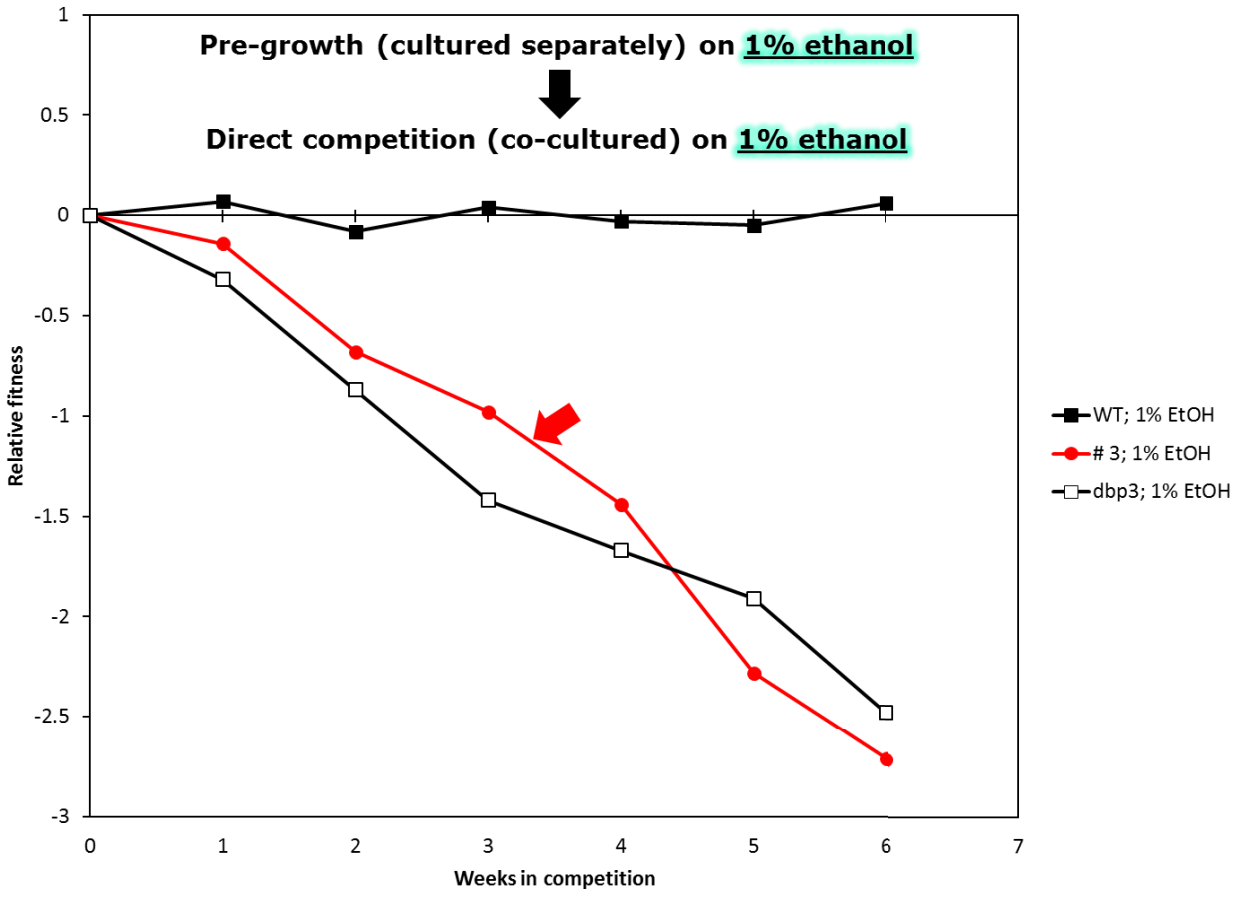


Figure 4.11. Mutations that extend longevity of the long-lived mutant strain 3 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

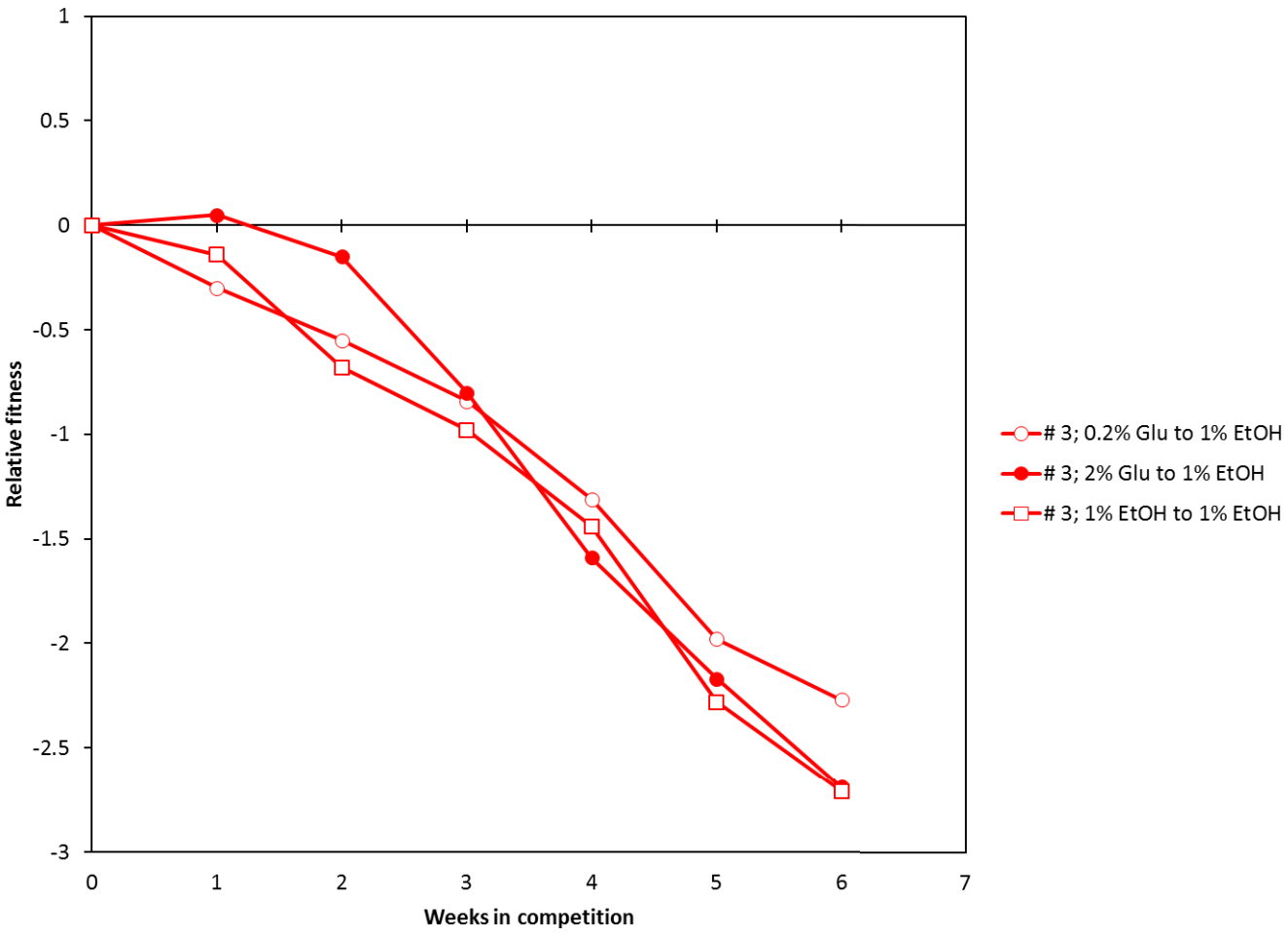


Figure 4.12. The conditions of pre-culturing of the long-lived mutant strain 3 do not influence the extent of its reduced relative fitness during the subsequent co-culturing with a parental WT strain in medium containing 1% ethanol.

BY4742 and the long-lived mutant strain 5) co-cultured in media containing: (1) 0.2% glucose, following cell transfer from 0.2% glucose (Figure 4.13); and (2) 2% glucose, following cell transfer from 2% glucose (Figure 4.14). In contrast, the dominant mutations that extend longevity of the long-lived mutant strain 5 (which was His^- in the

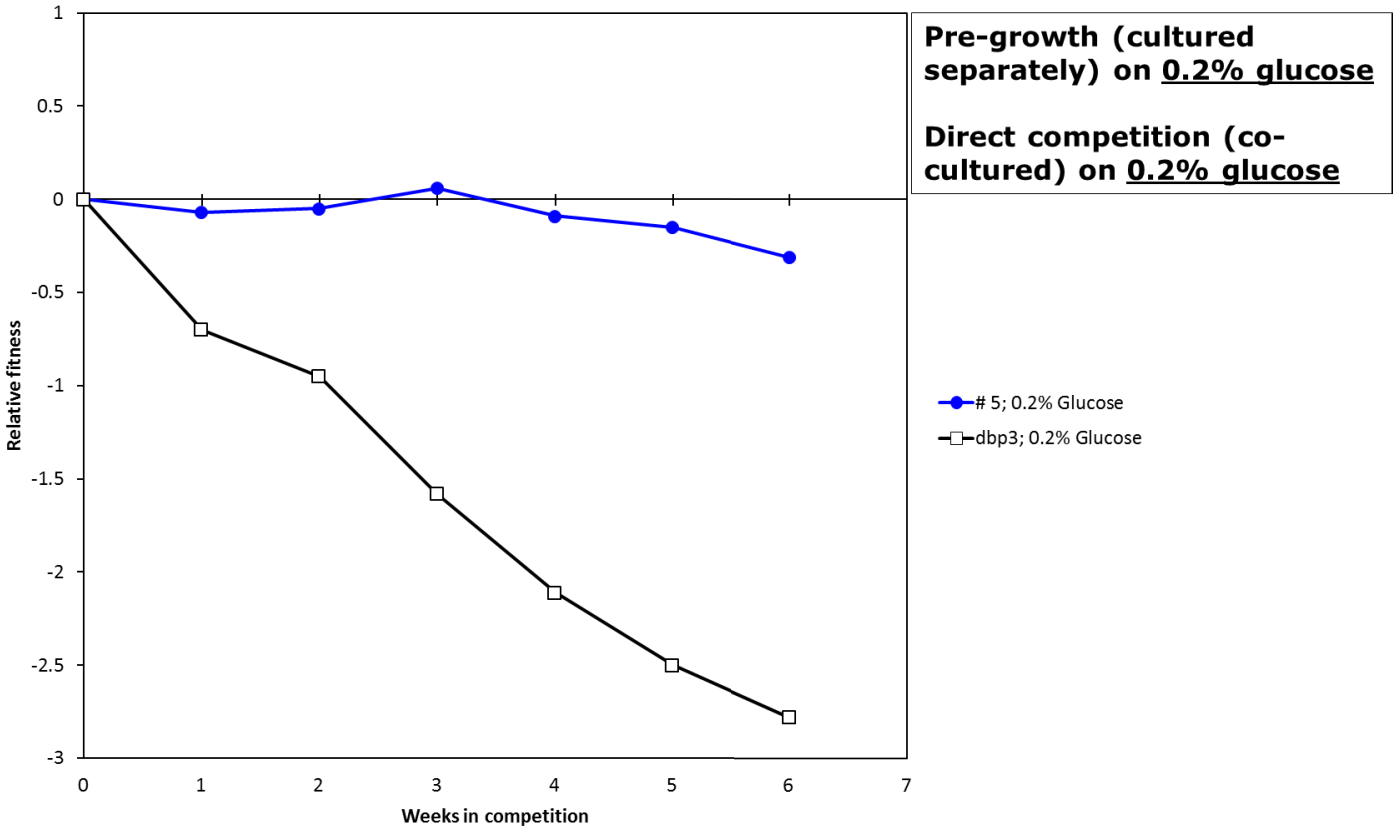


Figure 4.13. Mutations that extend longevity of the long-lived mutant strain 5 (His^-) do not reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 0.2% glucose.

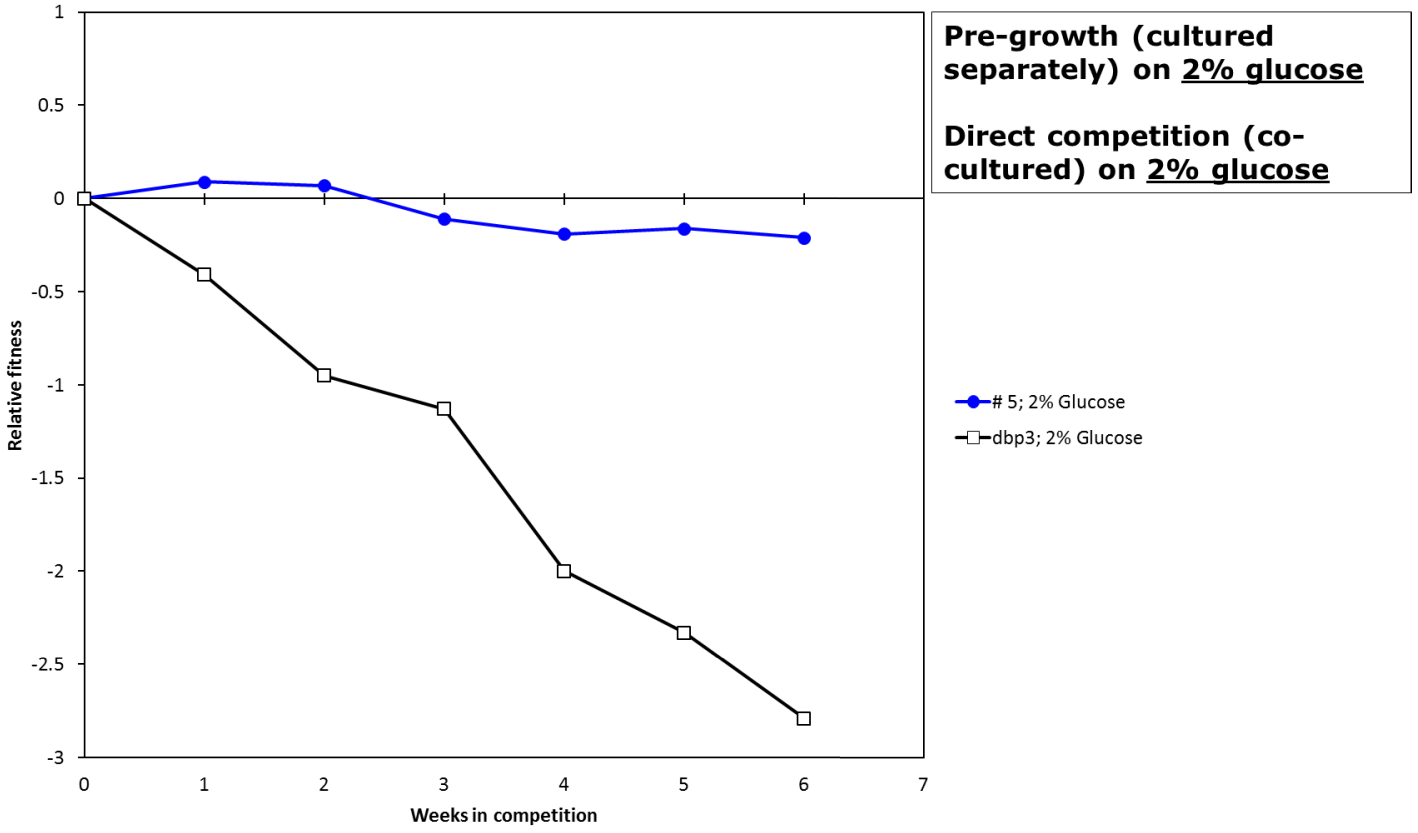


Figure 4.14. Mutations that extend longevity of the long-lived mutant strain 5 (His^-) do not reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 2% glucose.

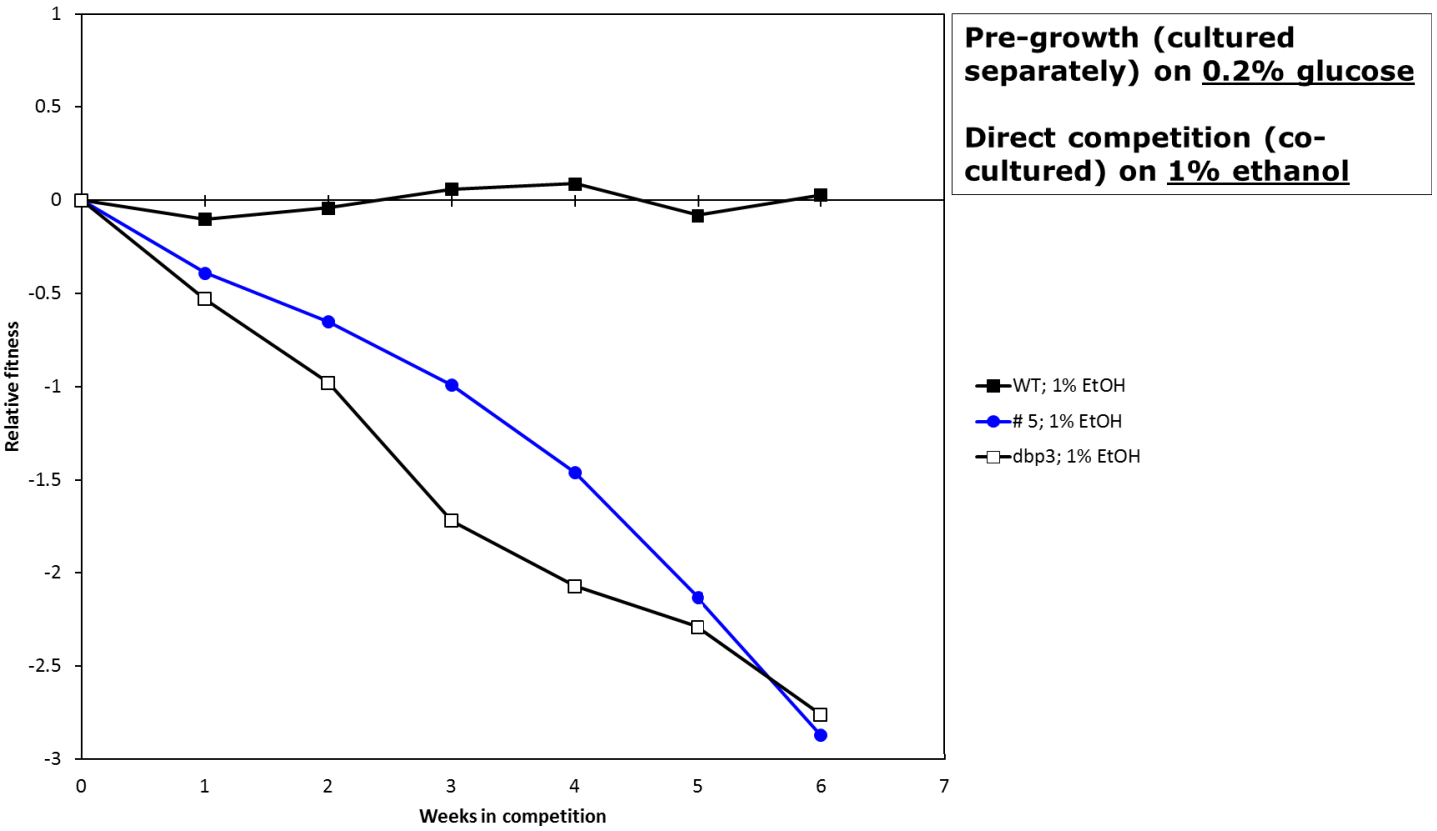


Figure 4.15. Mutations that extend longevity of the long-lived mutant strain 5 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

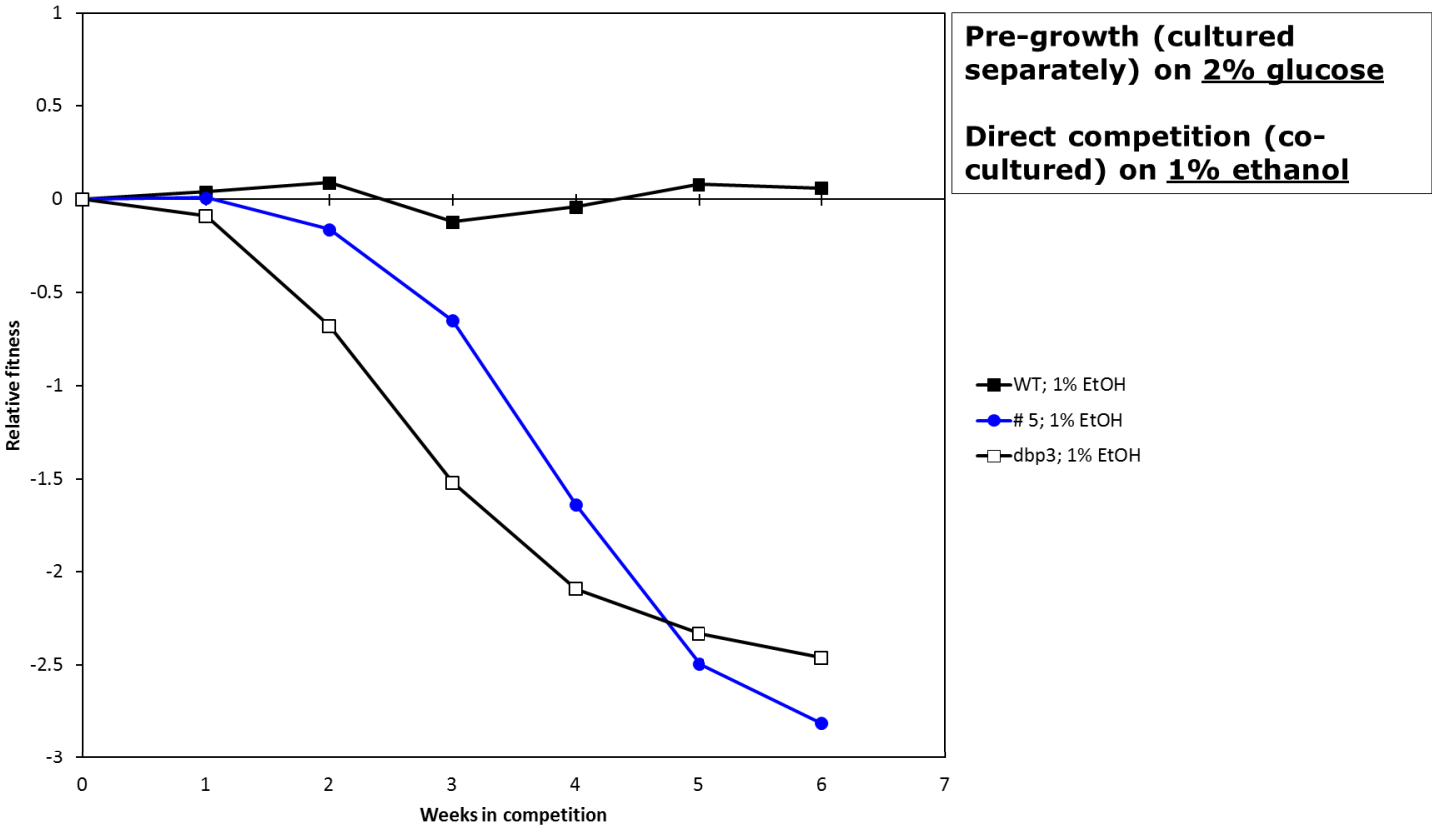


Figure 4.16. Mutations that extend longevity of the long-lived mutant strain 5 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

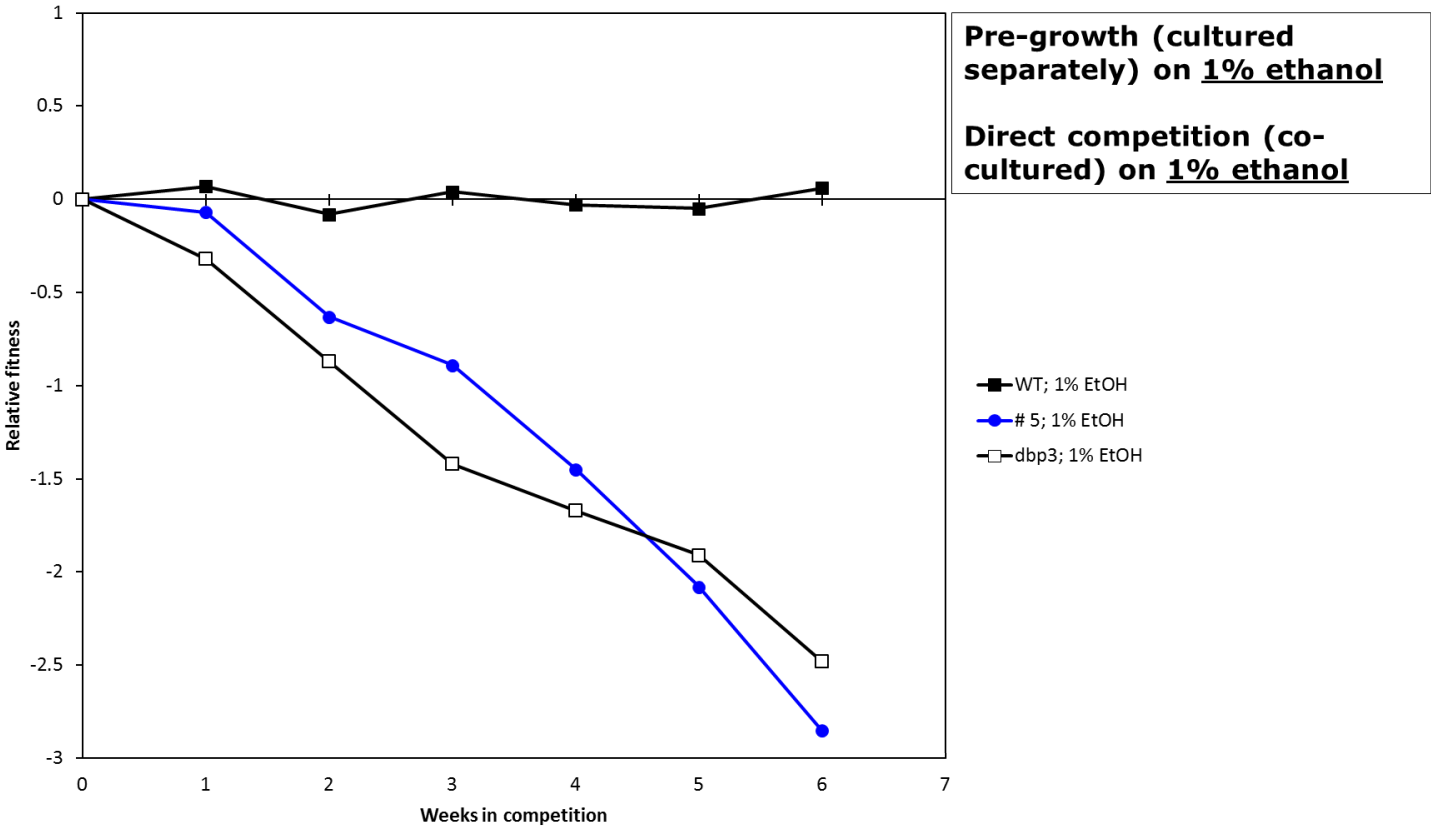


Figure 4.17. Mutations that extend longevity of the long-lived mutant strain 5 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

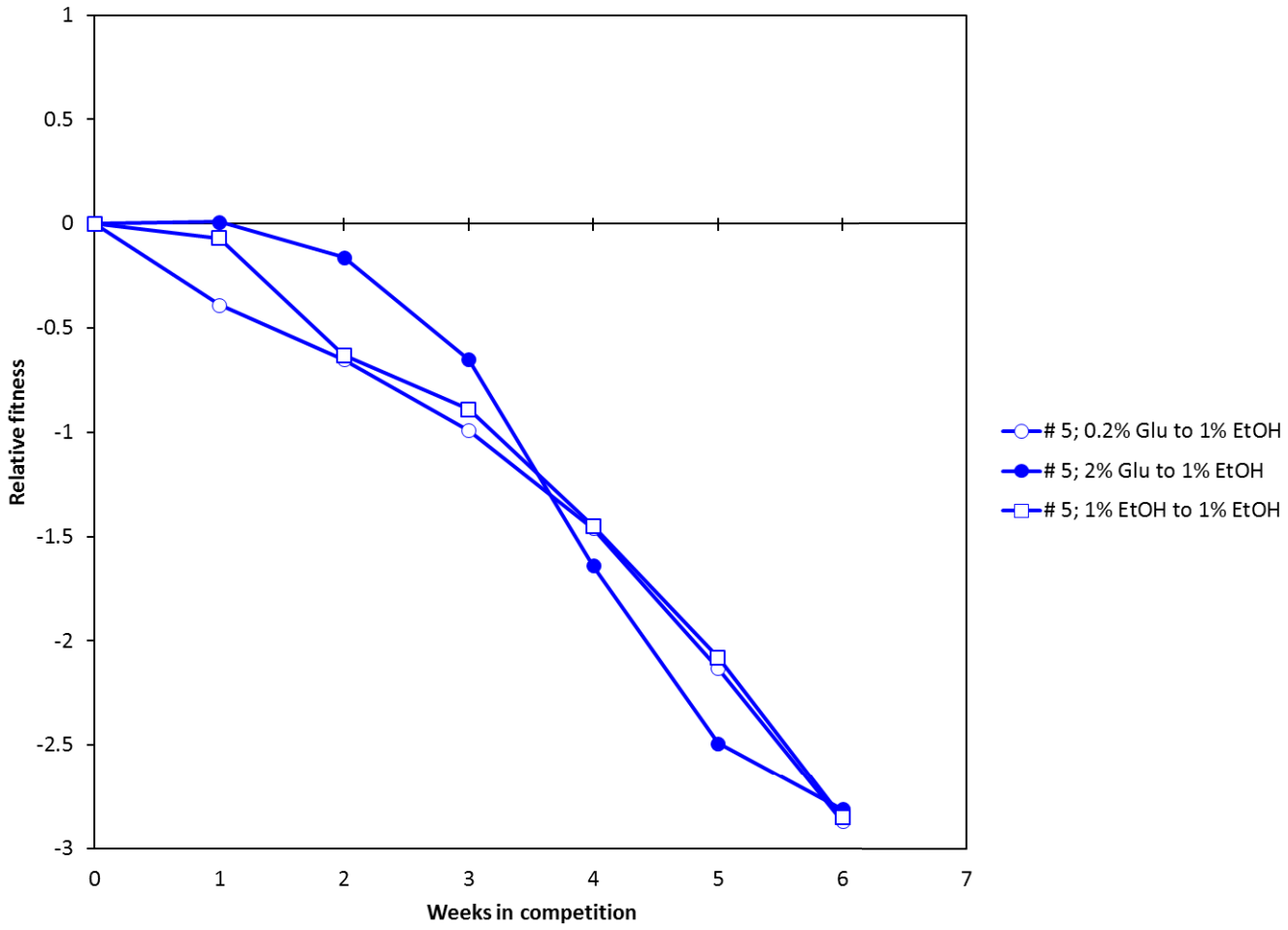


Figure 4.18. The conditions of pre-culturing of the long-lived mutant strain 5 do not influence the extent of its reduced relative fitness during the subsequent co-culturing with a parental WT strain in medium containing 1% ethanol.

BY4742 genetic background) reduce its relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742 and the long-lived mutant strain 5) co-cultured in media containing: (1) 1% ethanol, following cell transfer from 0.2% glucose (Figure 4.15); (2) 1% ethanol, following cell

transfer from 2% glucose (Figure 4.16); or (3) 1% ethanol, following cell transfer from 1% ethanol (Figure 4.17). It needs to be emphasized that, as a comparison of the data presented in Figures 4.15, 4.16 and 4.17 demonstrate, the conditions of pre-culturing of the long-lived mutant strain 5 do not influence the extent of its reduced relative fitness during the subsequent co-culturing with a parental WT strain in medium containing 1% ethanol (Figure 4.18). I therefore concluded that the long-lived mutant strain 5 does not keep a "memory" of conditions under which it has been grown prior to being mixed with a parental WT strain in medium containing 1% ethanol for a fitness competition.

I then used the developed direct competition assay to quantify the fitness of the long-lived mutant strain 12 relative to the parental WT strain BY4742; cells of these two strains were first cultured separately in medium containing different concentrations of glucose or ethanol, then mixed as described in the "Materials and Methods" section, and underwent six consecutive 7-days incubations together. I found that the dominant mutations that extend longevity of the long-lived mutant strain 12 (which was His^- in the BY4742 genetic background) do not reduce its relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742 and the long-lived mutant strain 12) co-cultured in media containing: (1) 0.2% glucose, following cell transfer from 0.2% glucose (Figure 4.19); and (2) 2% glucose, following cell transfer from 2% glucose (Figure 4.20). In contrast, the dominant mutations that extend longevity of the long-lived mutant strain 12 (which was His^- in the BY4742 genetic background) reduce its relative fitness in a direct competition assay with the WT strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742 and the long-lived mutant strain 12) co-cultured in media containing: (1) 1% ethanol,

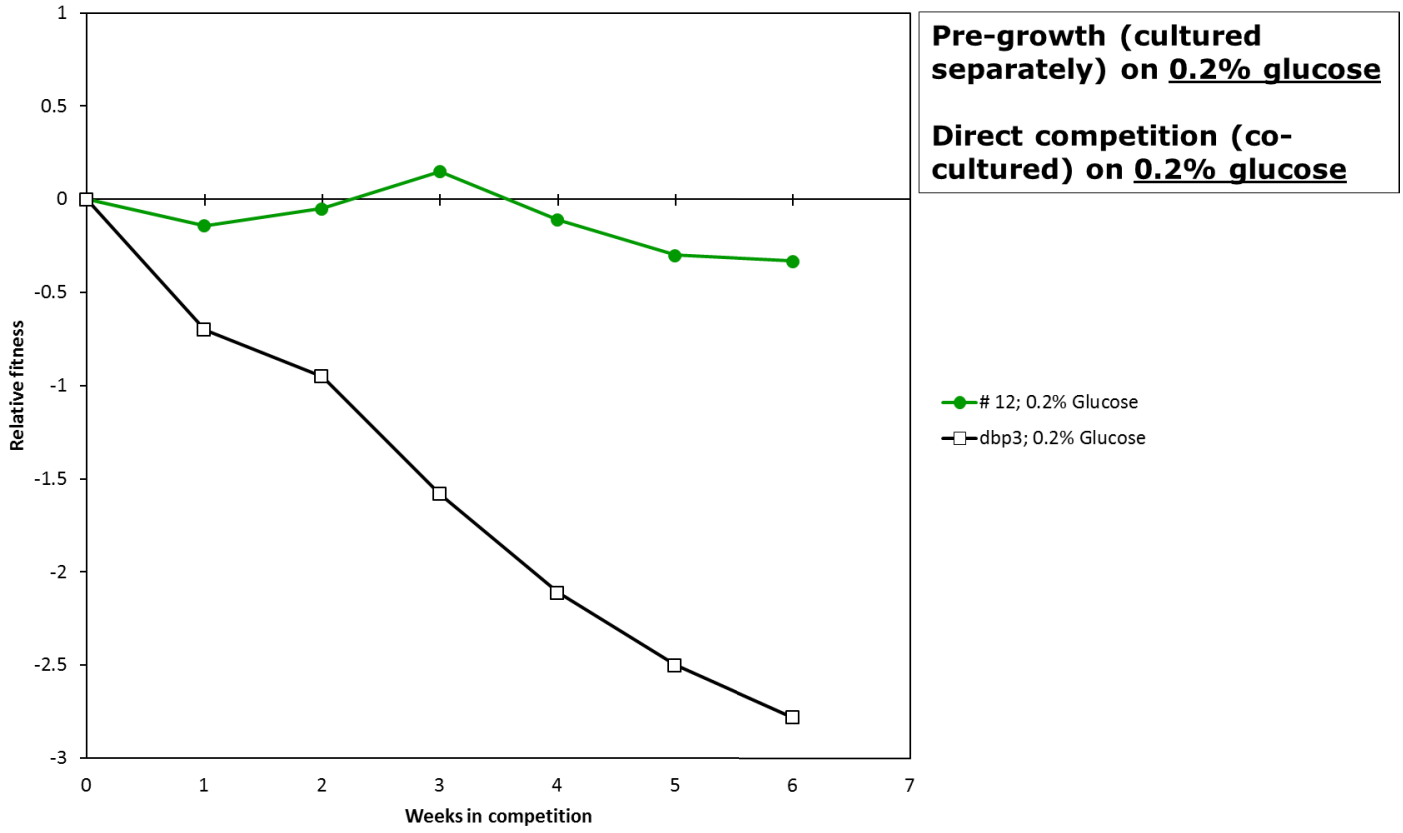


Figure 4.19. Mutations that extend longevity of the long-lived mutant strain 12 (His^-) do not reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 0.2% glucose.

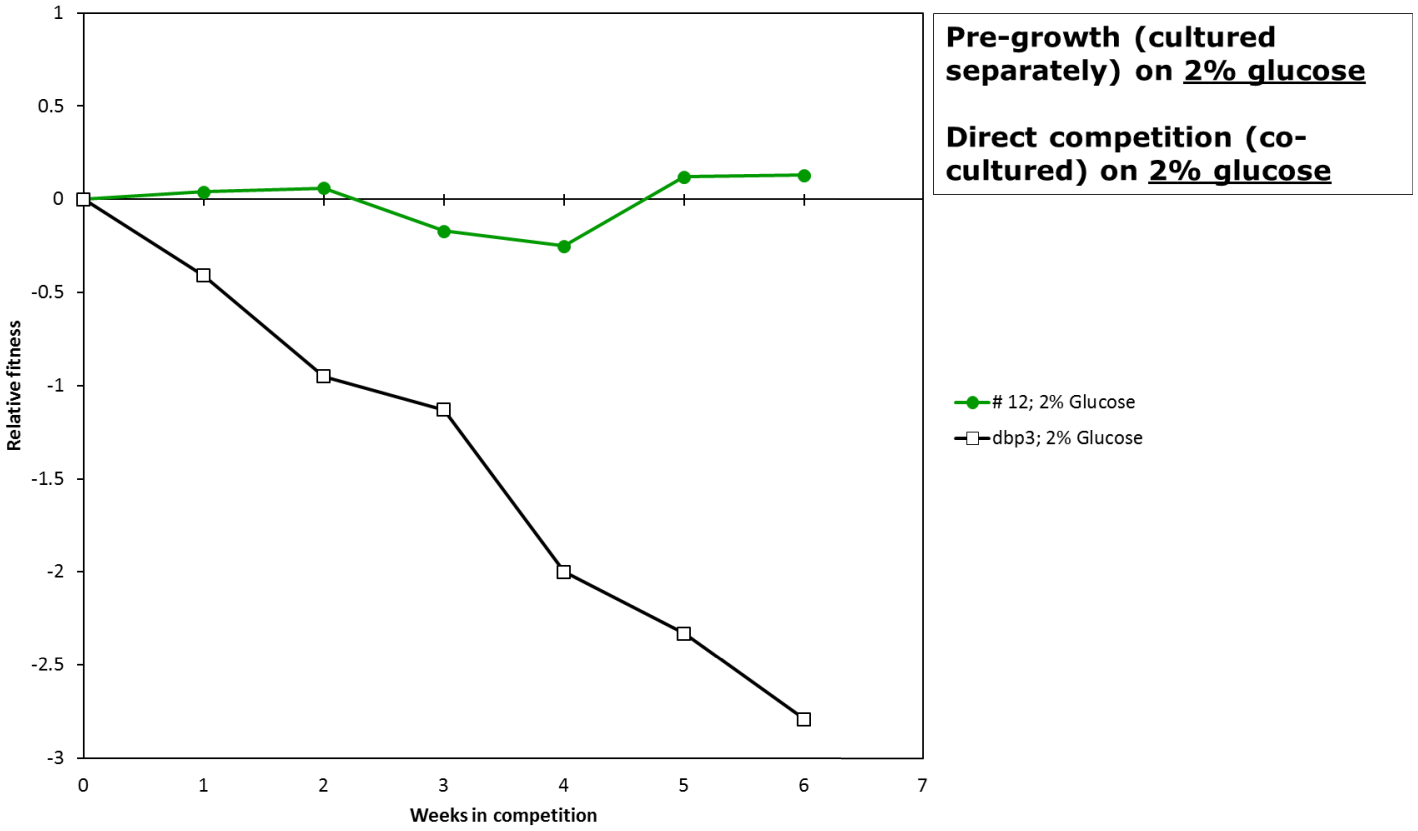


Figure 4.20. Mutations that extend longevity of the long-lived mutant strain 12 (His^-) do not reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 2% glucose.

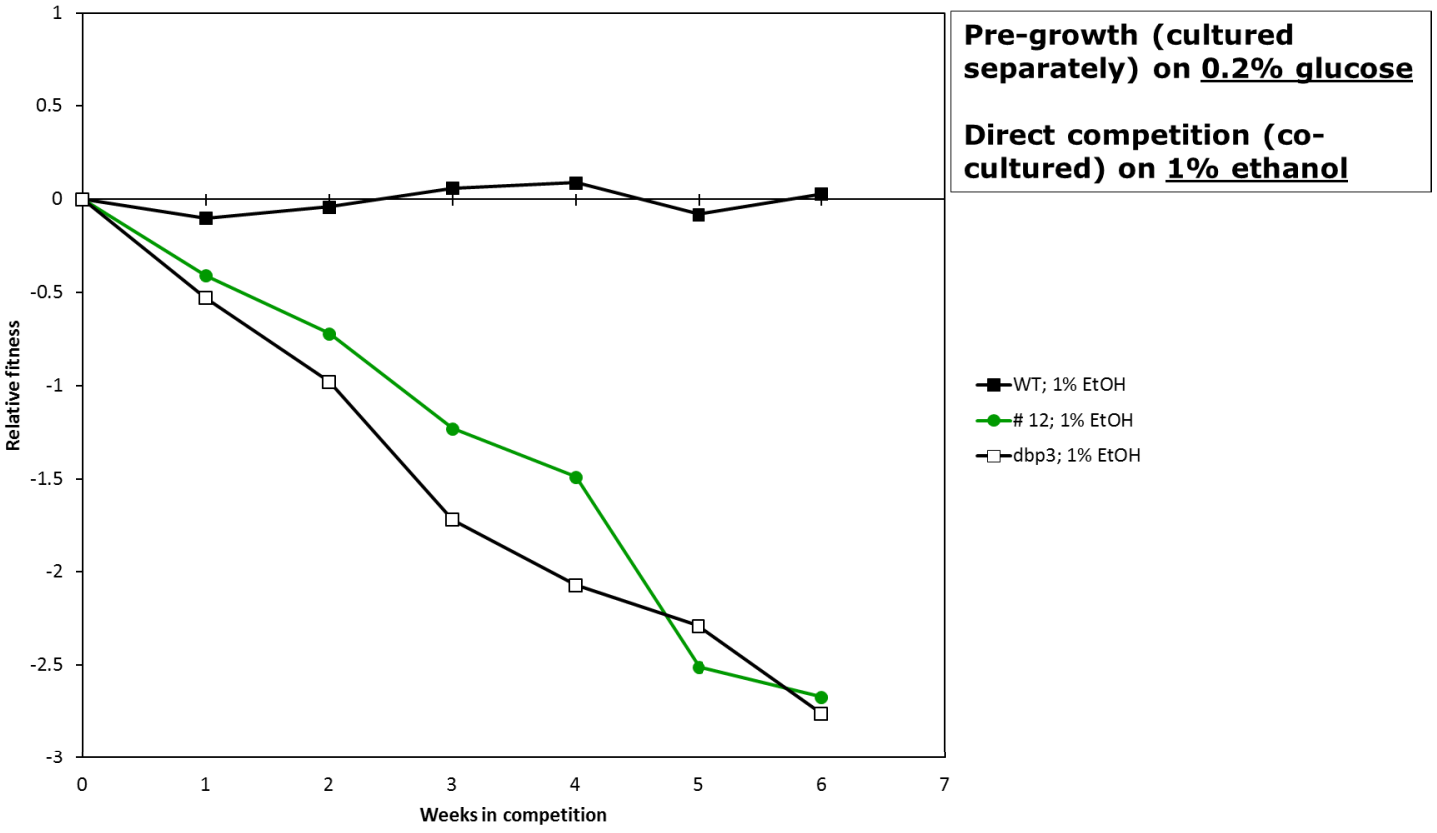


Figure 4.21. Mutations that extend longevity of the long-lived mutant strain 12 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

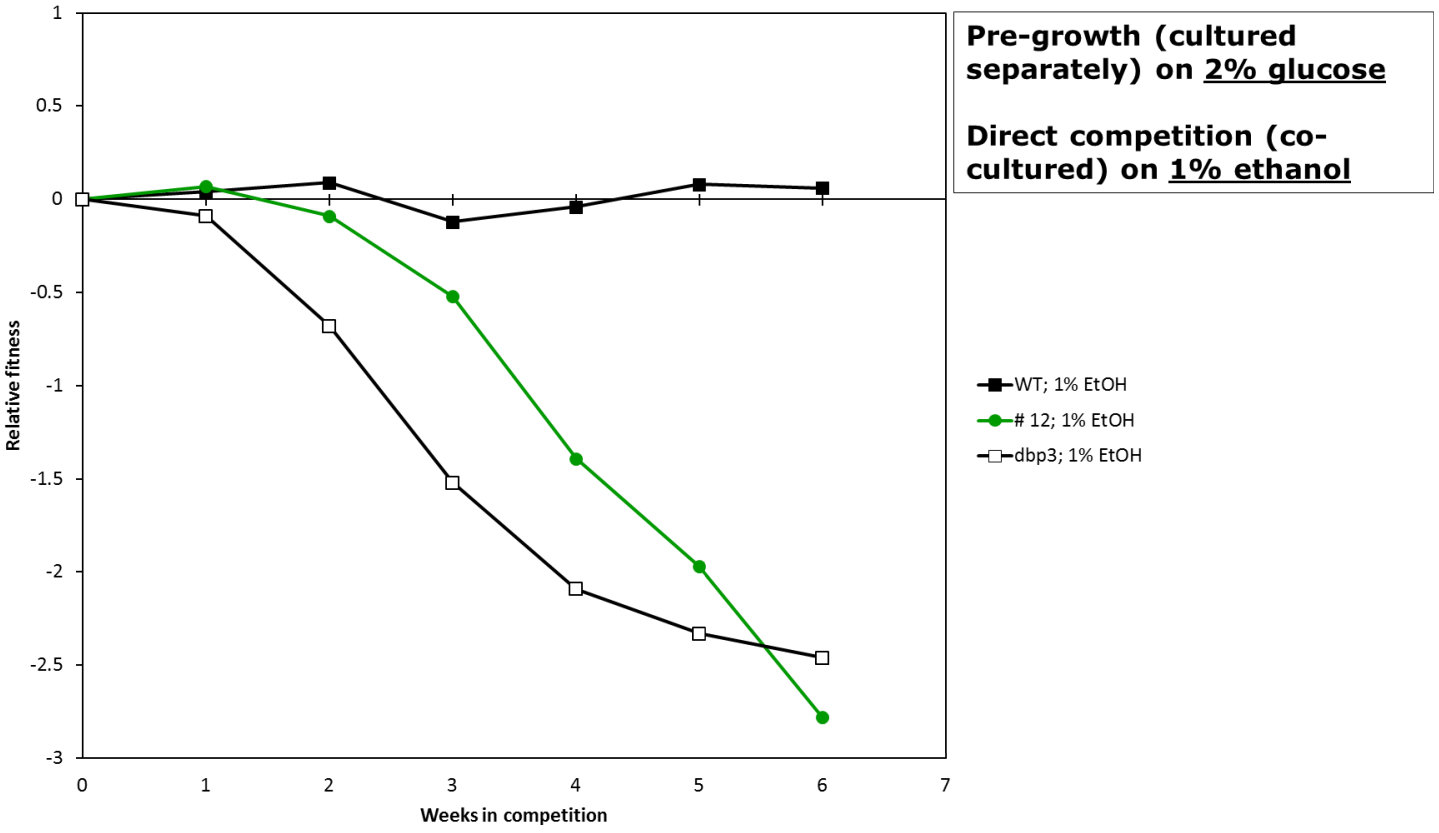


Figure 4.22. Mutations that extend longevity of the long-lived mutant strain 12 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

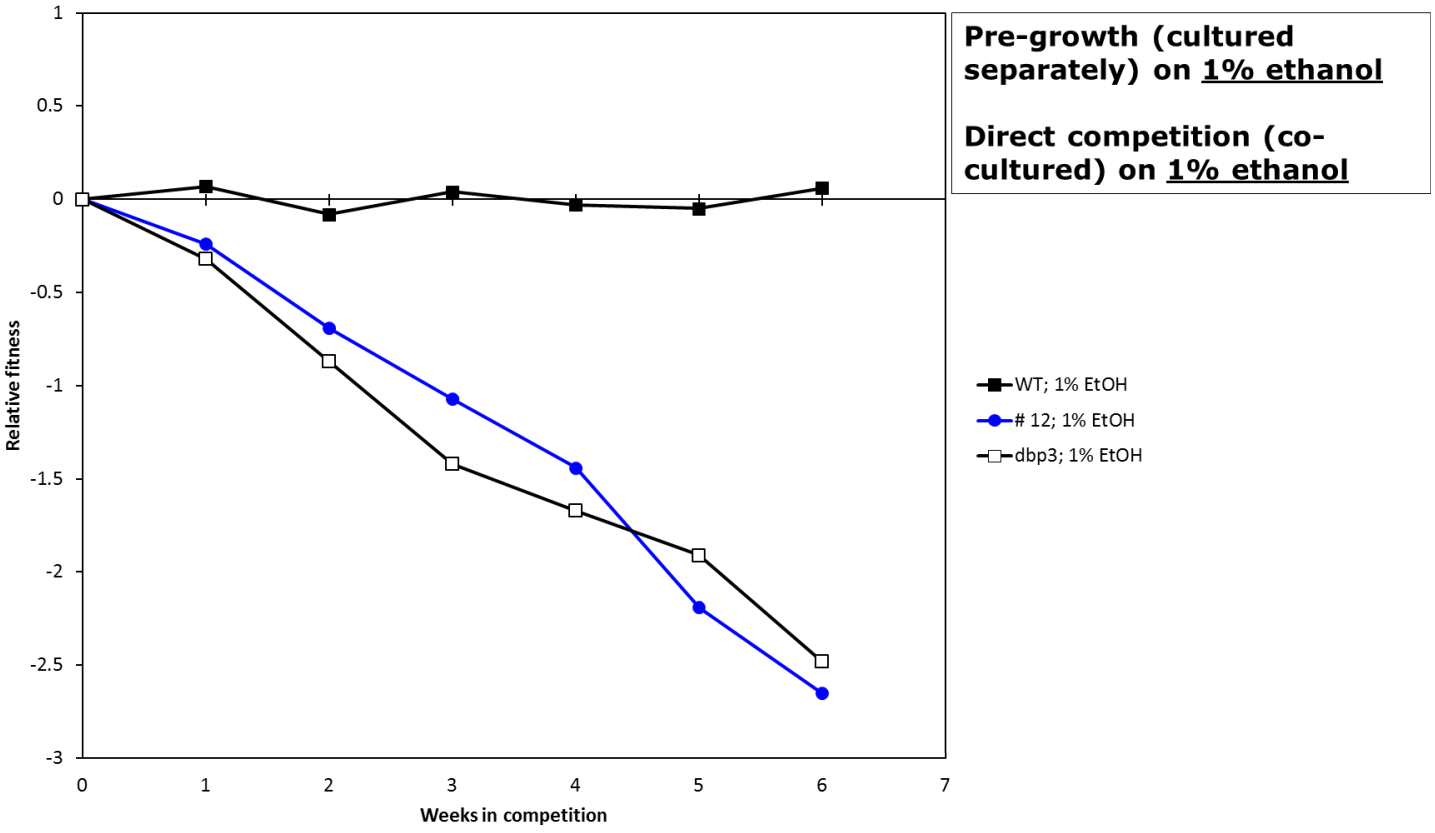


Figure 4.23. Mutations that extend longevity of the long-lived mutant strain 12 (His^-) reduce its relative fitness in a direct competition assay with the wild-type strain BY4739 (His^+, but otherwise isogenic to the parental WT strain BY4742) co-cultured in medium containing 1% ethanol.

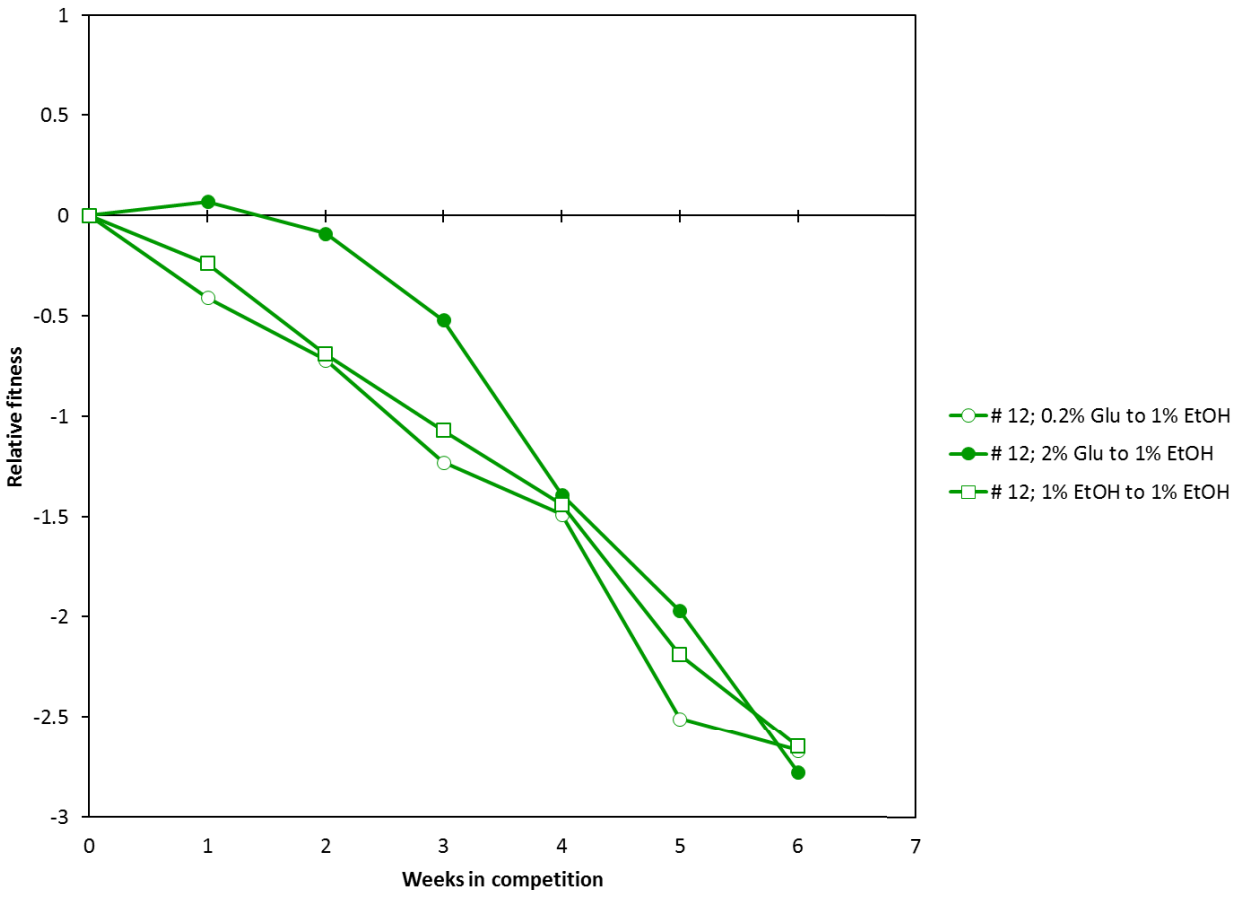


Figure 4.24. The conditions of pre-culturing of the long-lived mutant strain 12 do not influence the extent of its reduced relative fitness during the subsequent co-culturing with a parental WT strain in medium containing 1% ethanol.

following cell transfer from 0.2% glucose (Figure 4.21); (2) 1% ethanol, following cell transfer from 2% glucose (Figure 4.22); or (3) 1% ethanol, following cell transfer from 1% ethanol (Figure 4.23). It needs to be emphasized that, as a comparison of the data presented in Figures 4.21, 4.22 and 4.23 demonstrate, the conditions of pre-culturing of the long-lived mutant strain 12 do not influence the extent of its reduced relative fitness during the subsequent co-culturing with a parental WT strain in medium containing 1%

ethanol (Figure 4.24). I therefore concluded that the long-lived mutant strain 12 does not keep a "memory" of conditions under which it has been grown prior to being mixed with a parental WT strain in medium containing 1% ethanol for a fitness competition.

4.5 Discussion

In studies described in this chapter of my thesis I developed and validated a direct competition assay which under various laboratory conditions mimics the process of natural selection within a mixed population of yeast cells that (1) exhibit different longevity-defining genetic backgrounds; (2) differ in their lifespans if grow as a genetically homogenous cell population; and (3) compete for nutrients and other environmental resources. Using this assay, I found that in a population of mixed cells grown on 1% ethanol the dominant mutations that extend longevity of the three long-lived yeast mutants 3, 5 and 12 reduce the relative fitness of each of them in a direct competition with the parental WT strain BY4742. Based on these findings, I concluded that under laboratory conditions mimicking the process of natural selection within an ecosystem composed of yeast cells having different longevity-defining genetic backgrounds, each of the three long-lived mutants is forced out of the ecosystem by a parental WT strain exhibiting shorter lifespan. My findings imply that yeast cells have evolved some mechanisms for limiting their lifespan upon reaching a certain chronological age. It is conceivable that these mechanisms drive the evolution of yeast longevity towards maintaining a finite yeast lifespan within ecosystems. I propose here that these mechanisms may consist in the ability of the parental WT strain to secrete into growth medium certain compounds (small molecules and/or proteins) that slow down

growth and/or kill long-lived yeast mutants. The challenge for the near future is to identify these compounds responsible for the maintenance a finite yeast lifespan within ecosystems.

5 References

1. Greer EL, Brunet A. Signaling networks in aging. J Cell Sci 2008; 121:407-412.

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

3. Fontana L, Partridge L, Longo VD. Extending healthy life span - from yeast to humans. Science 2010; 328:321-326.

4. Kenyon CJ. The genetics of ageing. Nature 2010; 464:504-512.

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

6. Longo VD, Shadel GS, Kaeberlein M, Kennedy B. Replicative and chronological aging in *Saccharomyces cerevisiae*. Cell Metab 2012; 16:18-31.

7. Laplante M, Sabatini DM. mTOR signaling at a glance. J Cell Sci 2009; 122:3589-3594.

8. Narasimhan SD, Yen K, Tissenbaum HA. Converging pathways in lifespan regulation. Curr Biol 2009; 19:R657-R666.

9. Giorgio M, Trinei M, Migliaccio E, Pelicci PG. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? Nat Rev Mol Cell Biol 2007; 8:722-728.

10. Finley LW, Haigis MC. The coordination of nuclear and mitochondrial communication during aging and calorie restriction. Ageing Res Rev 2009; 8:173-188.

11. Ristow M, Zarse K. How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). Exp Gerontol 2010; 45:410-418.

12. Hekimi S, Lapointe J, Wen Y. Taking a "good" look at free radicals in the aging process. Trends Cell Biol 2011; 21:569-576.

13. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the *in vivo* evidence. Nat Rev Drug Discov 2006; 5:493-506.

14. Mair W, Dillin A. Aging and survival: the genetics of life span extension by dietary restriction. Annu Rev Biochem 2008; 77:727-754.

15. Wanke V, Cameroni E, Uotila A, Piccolis M, Urban J, Loewith R, De Virgilio C. Caffeine extends yeast lifespan by targeting TORC1. Mol Microbiol 2008; 69:277-285.

16. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, Allison DB, Cruzen C, Simmons HA, Kemnitz JW, Weindruch R. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 2009; 325:201-204.

17. Eisenberg T, Knauer H, Schauer A, Büttner S, Ruckenstuhl C, Carmona-Gutierrez D, Ring J, Schroeder S, Magnes C, Antonacci L, Fussi H, Deszcz L, Hartl R, Schraml E, Criollo A, Megalou E, Weiskopf D, Laun P, Heeren G, Breitenbach M, Grubeck-Loebenstein B, Herker E, Fahrenkrog B, Fröhlich KU, Sinner F, Tavernarakis N, Minois N, Kroemer G, Madeo F. Induction of autophagy by spermidine promotes longevity. Nat Cell Biol 2009; 11:1305-1314.

18. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson, JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 2009; 460:392-395.

19. Skulachev VP, Anisimov VN, Antonenko YN, Bakeeva LE, Chernyak BV, Erichev VP, Filenko OF, Kalinina NI, Kapelko VI, Kolosova NG, Kopnin BP, Korshunova GA, Lichinitser MR, Obukhova LA, Pasyukova EG, Pisarenko OI, Roginsky VA, Ruuge EK, Senin II, Severina II, Skulachev MV, Spivak IM, Tashlitsky VN, Tkachuk VA,

Vyssokikh MY, Yaguzhinsky LS, Zorov DB. An attempt to prevent senescence: a mitochondrial approach. Biochim Biophys Acta 2009; 1787:437-461.

20. Anderson RM, Weindruch R. Metabolic reprogramming, caloric restriction and aging. Trends Endocrinol Metab 2010; 21:134-141.

21. Goldberg AA, Richard VR, Kyryakov P, Bourque SD, Beach A, Burstein MT, Glebov A, Koupaki O, Boukh-Viner T, Gregg C, Juneau M, English AM, Thomas DY, Titorenko VI. Chemical genetic screen identifies lithocholic acid as an anti-aging compound that extends yeast chronological life span in a TOR-independent manner, by modulating housekeeping longevity assurance processes. Aging 2010; 2:393-414.

22. Kaeberlein M. Lessons on longevity from budding yeast. Nature 2010; 464:513-519.

23. Anisimov VN, Berstein LM, Popovich IG, Zabezhinski MA, Egormin PA, Piskunova TS, Semenchenko AV, Tyndyk ML, Yurova MN, Kovalenko IG, Poroshina TE. If started early in life, metformin treatment increases life span and postpones tumors in female SHR mice. Aging 2011a; 3:148-157.

24. Gems D, Partridge L. Genetics of longevity in model organisms: Debates and paradigm shifts. Annu Rev Physiol 2013; 75:621-644.

25. Smoliga JM, Vang O, Baur JA. Challenges of translating basic research into

therapeutics: resveratrol as an example. J Gerontol A Biol Sci Med Sci 2012; 67:158-167.

26. Fabrizio P, Longo VD. The chronological life span of *Saccharomyces cerevisiae*. Methods Mol Biol 2007; 371:89-95.

27. Lin SJ, Sinclair D. Molecular mechanisms of aging: insights from budding yeast. In: Molecular Biology of Aging (Guarente, L.P., Partridge, L. and Wallace, D.C., eds.) 2008; pp. 483-516; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

28. Steinkraus KA, Kaeberlein M, Kennedy BK. Replicative aging in yeast: the means to the end. Annu Rev Cell Dev Biol 2008; 24:29-54.

29. Beach A, Titorenko VI. In search of housekeeping pathways that regulate longevity. Cell Cycle 2011; 10:3042-3044.

30. Burstein MT, Kyryakov P, Beach A, Richard VR, Koupaki O, Gomez-Perez A, Leonov A, Levy S, Noohi F, Titorenko VI. Lithocholic acid extends longevity of chronologically aging yeast only if added at certain critical periods of their lifespan. Cell Cycle 2012; 11:3443-3462.

31. Richard VR, Leonov A, Beach A, Burstein MT, Koupaki O, Gomez-Perez A, Levy S, Pluska L, Mattie S, Rafesh R, Iouk T, Sheibani S, Greenwood M, Vali H, Titorenko VI. Macromitophagy is a longevity assurance process that in chronologically aging

yeast limited in calorie supply sustains functional mitochondria and maintains cellular lipid homeostasis. Aging 2013; 5:234-269.

32. Beach A, Richard VR, Leonov A, Burstein MT, Bourque SD, Koupaki O, Juneau M, Feldman R, Iouk T, Titorenko VI. Mitochondrial membrane lipidome defines yeast longevity. Aging 2013; 5:551-574.

33. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev 2009; 89:147-191.

34. Monte MJ, Marin JJ, Antelo A, and Vazquez-Tato J. Bile acids: chemistry, physiology, and pathophysiology. World J Gastroenterol 2009; 15:804-816.

35. Goldberg AA, Kyryakov P, Bourque SD, Titorenko VI. Xenohormetic, hormetic and cytostatic selective forces driving longevity at the ecosystemic level. Aging 2010; 2:461-470.

36. Burstein MT, Beach A, Richard VR, Koupaki O, Gomez-Perez A, Goldberg AA, Kyryakov P, Bourque SD, Glebov A, Titorenko VI. Interspecies chemical signals released into the environment may create xenohormetic, hormetic and cytostatic selective forces that drive the ecosystemic evolution of longevity regulation mechanisms. Dose Response 2012; 10:75-82.

37. Powers RW 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. Genes Dev 2006; 20:174-184.

38. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 206; 124:471-484.

39. Blagosklonny MV, Hall MN. Growth and aging: a common molecular mechanism. Aging 2009; 1:357-362.

40. Hands SL, Proud CG, Wyttenbach A. mTOR's role in ageing: protein synthesis or autophagy? Aging 2009; 1:586-597.

41. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 2009; 460:392-395.

42. Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L. Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. Cell Metab 2010; 11:35-46.

43. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE,

Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. 2003. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature 2003; 425:191-196.

44. Lamming DW, Wood JG, Sinclair DA. Small molecules that regulate lifespan: evidence for xenohormesis. Mol Microbiol 2004; 53:1003-1009.

45. Howitz KT, Sinclair DA. Xenohormesis: sensing the chemical cues of other species. Cell 2008; 133:387-391.

46. Dasgupta B, Milbrandt J. Resveratrol stimulates AMP kinase activity in neurons. Proc Natl Acad Sci USA 2007; 104:7217-7222.

47. Wanke V, Cameroni E, Uotila A, Piccolis M, Urban J, Loewith R, De Virgilio C. Caffeine extends yeast lifespan by targeting TORC1. Mol Microbiol 2008; 69:277-285.

48. Armour SM, Baur JA, Hsieh SN, Land-Bracha A, Thomas SM, Sinclair DA. Inhibition of mammalian S6 kinase by resveratrol suppresses autophagy. Aging 2009; 1:515-528.

49. Blagosklonny MV. Inhibition of S6K by resveratrol: in search of the purpose. Aging 2009; 1:511-514.

50. Demidenko ZN, Blagosklonny MV. At concentrations that inhibit mTOR, resveratrol suppresses cellular senescence. Cell Cycle 2009; 8:1901-1904.

51. Blagosklonny MV. Rapamycin and quasi-programmed aging: Four years later. Cell Cycle 2010; 9:1859-1862.

52. Burstein MT, Glebov A, Gomez-Perez A, Kyryakov P, Akkari R, Asbah N, Titorenko VI. Empirical verification of a hypothesis of the xenohormetic, hormetic and cytostatic selective forces driving the evolution of longevity regulation mechanisms within ecosystems. Biol Open 2013; in preparation.

53. Mitteldorf J. Aging is not a process of wear and tear. Rejuvenation Res 2010; 13:322-326.

54. Trindade LS, Aigaki T, Peixoto AA, Balduino A, Mânica da Cruz IB, Heddle JG. A novel classification system for evolutionary aging theories. Front Genet 2013; 4:25.

55. Kirkwood TB. Comparative life spans of species: why do species have the life spans they do? Am J Clin Nutr 1992; 55:1191S-1195S.

56. Ljubuncic P, Reznick AZ. The evolutionary theories of aging revisited - a mini-review. Gerontology 2009; 55:205-216.

57. Goldsmith T. Aging by Design. Azinet Press, Annapolis, 2011; ISBN 0-9788709-3-X.

58. Life span 2013. Encyclopædia Britannica Online. Retrieved 02 August, 2013, from http://www.britannica.com/EBchecked/topic/340297/life-span.

59. Goldsmith TC. On the programmed/non-programmed aging controversy. Biochemistry 2012; 77:729-732.

60. Mitteldorf JJ. Adaptive aging in the context of evolutionary theory. Biochemistry 2012; 77:716-725.

61. Skulachev VP. Phenoptosis: programmed death of an organism. Biochemistry 1999; 64:1418-1426.

62. Skulachev VP. Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms. Mol Aspects Med 1999; 20:139-184.

63. Skulachev VP. The programmed death phenomena, aging, and the Samurai law of biology. Exp Gerontol 2001; 36:995-1024.

64. Skulachev VP. Programmed death phenomena: from organelle to organism. Ann NY Acad Sci 2002; 959:214-237.

65. Skulachev VP. Programmed death in yeast as adaptation? FEBS Lett 2002; 528:23-26.

66. Longo VD, Mitteldorf J, Skulachev VP. Programmed and altruistic ageing. Nat Rev Genet 2005; 6:866-872.

67. Skulachev VP, Longo VD. Aging as a mitochondria-mediated atavistic program: can aging be switched off? Ann NY Acad Sci 2005; 1057:145-164.

68. Severin FF, Meer MV, Smirnova EA, Knorre DA, Skulachev VP. Natural causes of programmed death of yeast *Saccharomyces cerevisiae*. Biochim Biophys Acta 2008; 1783:1350-1353.

69. Goldsmith T. The Evolution of Aging 3rd ed. Azinet Press, Annapolis, 2013; ISBN: 0978870905.

70. Weismann A. Über die Dauer des Lebens. Fisher, Jena, 1882.

71. Libertini G. An adaptive theory of increasing mortality with increasing chronological age in populations in the wild. J Theor Biol 1988; 132:145-162.

72. Goldsmith TC. Aging, evolvability, and the individual benefit requirement; medical

implications of aging theory controversies. J Theor Biol 2008; 252:764-768.

73. Skulachev VP. Aging is a specific biological function rather than the result of a disorder in complex living systems: biochemical evidence in support of Weismann's hypothesis. Biochemistry 1997; 62:1191-1195.

74. Fabrizio P, Battistella L, Vardavas R, Gattazzo C, Liou LL, Diaspro A, Dossen JW, Gralla EB, Longo VD. Superoxide is a mediator of an altruistic aging program in *Saccharomyces cerevisiae*. J Cell Biol 2004; 166:1055-1067.

75. Herker E, Jungwirth H, Lehmann KA, Maldener C, Frohlich KU, Wissing S, Buttner S, Fehr M, Sigrist S, Madeo F. Chronological aging leads to apoptosis in yeast. J Cell Biol 2004; 164:501-507.

76. Vachova L, Palkova Z. Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. J Cell Biol 2005; 169:711-717.

77. Büttner S, Eisenberg T, Herker E, Carmona-Gutierrez D, Kroemer G, Madeo F. Why yeast cells can undergo apoptosis: death in times of peace, love, and war. J Cell Biol 2006; 175:521-525.

78. Parsons PA. Inherited stress resistance and longevity: a stress theory of ageing. Heredity 1995; 75:216-221.

79. Marden JH, Rogina B, Montooth KL, Helfand SL. Conditional tradeoffs between aging and organismal performance of Indy long-lived mutant flies. Proc Natl Acad Sci USA 2003; 100:3369-3373.

80. Partridge L, Gems D. Beyond the evolutionary theory of ageing, from functional genomics to evo-gero. Trends Ecol Evol 2006; 21:334-340.

81. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A *C. elegans* mutant that lives twice as long as wild type. Nature 1993; 366:461-464.

82. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, et al. Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science 2001; 292:104-106.

83. Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS. A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science 2001; 292:107-110.

84. Brown-Borg HM, Borg KE, Meliska CJ, Bartke A. Dwarf mice and the ageing process. Nature 1996; 384:33.

85. Partridge L, Prowse N, Pignatelli P. Another set of responses and correlated

responses to selection on age at reproduction in *Drosophila melanogaster*. Proc Biol Sci 1999; 266:255-261.

86. Buck S, Vettraino J, Force AG, Arking R. Extended longevity in Drosophila is consistently associated with a decrease in developmental viability. J Gerontol A Biol Sci Med Sci 2000; 55:292-301.

87. Zwaan BJ, Bijlsma R, Hoekstra RF. Direct selection on lifespan in *Drosophila melanogaster*. Evolution 1995; 49:649-659.

88. Stearns SC, Ackermann M, Doebeli M, Kaiser M. Experimental evolution of aging, growth and reproduction in fruit flies. Proc Natl Acad Sci USA 2000; 97:3309-3313.

89. Giannakou ME, Goss M, Jacobson J, Vinti G, Leevers SJ, Partridge L. Dynamics of the action of dFOXO on adult mortality in Drosophila. Aging Cell 2007; 6:429-438.

90. Liu X, Jiang N, Hughes B, Bigras E, Shoubridge E, Hekimi S. Evolutionary conservation of the *clk-1*-dependent mechanism of longevity: loss of *mclk1* increases cellular fitness and lifespan in mice. Genes Dev 2005; 19:2424-2434.

91. Delaney JR, Murakami CJ, Olsen B, Kennedy BK, Kaeberlein M. Quantitative evidence for early life fitness defects from 32 longevity-associated alleles in yeast. Cell Cycle 2011; 10:156-165.

92. Walker DW, McColl G, Jenkins NL, Harris J, Lithgow GJ. Evolution of lifespan in *C. elegans*. Nature 2000; 405:296-297.

93. McColl G, Jenkins NL, Walker DW, Lithgow GJ. Testing evolutionary theories of aging. Ann NY Acad Sci 2000; 908:319-320.

94. Marden JH, Rogina B, Montooth KL, Helfand SL. Conditional tradeoffs between aging and organismal performance of Indy long-lived mutant flies. Proc Natl Acad Sci USA 2003; 100:3369-3373.

95. Jenkins NL, McColl G, Lithgow GJ. Fitness cost of extended lifespan in *Caenorhabditis elegans*. Proc Biol Sci 2004; 271:2523-2526.

96. Anderson JL, Reynolds RM, Morran LT, Tolman-Thompson J, Phillips PC. Experimental evolution reveals antagonistic pleiotropy in reproductive timing but not life span in *Caenorhabditis elegans*. J Gerontol A Biol Sci Med Sci 2011; 66:1300-1308.

97. Qian W, Ma D, Xiao C, Wang Z, Zhang J. The genomic landscape and evolutionary resolution of antagonistic pleiotropy in yeast. Cell Rep 2012; 2:1399-1410.

98. Medawar PB. An Unsolved Problem of Biology. London, HK Lewis, 1952.

99. Charlesworth B. Fisher, Medawar, Hamilton and the evolution of aging. Genetics 2000; 156:927-931.

100. Charlesworth B: Patterns of age-specific means and genetic variances of mortality rates predicted by the mutation-accumulation theory of ageing. J Theor Biol 2001; 210:47-65.

101. Williams GC: Pleiotropy, natural selection, and the evolution of senescence. Evolution 1957; 11:398-411.

102. Kirkwood TB. Evolution of ageing. Nature 1977; 270:301-304.

103. Kirkwood TB, Holliday R. The evolution of ageing and longevity. Proc R Soc Lond B Biol Sci 1979; 205:531-546.

104. Kirkwood TB, Austad SN. Why do we age? Nature 2000; 408:233-238.

105. Sherman F. Getting started with yeast. Methods Enzymol 2002; 350:3-41.

106. Wei M, Fabrizio P, Hu J, Ge H, Cheng C, Li L, Longo VD. Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS Genet 2008; 4:e13.

107. Smets B, Ghillebert R, De Snijder P, Binda M, Swinnen E, De Virgilio C, Winderickx J. Life in the midst of scarcity: adaptations to nutrient availability in Saccharomyces cerevisiae. Curr Genet 2010; 56:1-32.

108. Ptacek J, Devgan G, Michaud G, Zhu H, Zhu X, Fasolo J, Guo H, Jona G, Breitkreutz A, Sopko R, McCartney RR, Schmidt MC, Rachidi N et al. Global analysis of protein phosphorylation in yeast. Nature 2005; 438:679-684.

109. Delaney JR, Murakami CJ, Olsen B, Kennedy BK, and Kaeberlein M. Quantitative evidence for early life fitness defects from 32 longevity-associated alleles in yeast. Cell Cycle (2011) 10:156-165.