

Discovery of Plant Extracts That Delay Yeast Chronological Aging through Different Signaling
Pathways

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ABSTRACT

Discovery of Plant Extracts That Delay Yeast Chronological Aging through Different Signaling Pathways

Vicky Lutchman, M.Sc.

The yeast *Saccharomyces cerevisiae* is a beneficial organism for the discovery of genes, signaling pathways and chemical compounds that slow cellular and organismal aging in eukaryotes across phyla. As a first step towards uncovering novel aging-delaying chemical compounds, I conducted a screen of a library of plant extracts for those that can extend yeast chronological lifespan. My screen revealed six plant extracts that increase yeast chronological lifespan considerably more efficiently than any of the longevity-extending chemical compounds yet described. I show that each of the six plant extracts is a geroprotector which delays the onset and decreases the rate of yeast chronological aging by eliciting a hormetic stress response. Because the rate of aging in yeast is controlled by a network of integrated signaling pathways and protein kinases, I assessed how single-gene-deletion mutations eliminating each of these pathways and kinases affect the aging-delaying efficiencies of the six plant extracts. My findings imply that these extracts slow aging in the following ways: 1) plant extract 4 decreases the efficiency with which the pro-aging TORC1 pathway inhibits the anti-aging SNF1 pathway; 2) plant extract 5 mitigates two different branches of the pro-aging PKA pathway; 3) plant extract 6 coordinates processes that are not assimilated into the network of presently known signaling pathways/protein kinases; 4) plant extract 8 diminishes the inhibitory action of PKA on SNF1; 5) plant extract 12 intensifies the anti-aging protein kinase Rim15; and 6) plant extract 21 inhibits a form of the pro-aging protein kinase Sch9 that is activated by the pro-aging PKH1/2 pathway.

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Table of Contents

| | | |
|----------|---|--------------|
| 1 | Introduction | 1-25 |
| 1.1 | The budding yeast <i>Saccharomyces cerevisiae</i> as an advantageous model organism for unveiling mechanisms of cellular aging in multicellular eukaryotes | 1-2 |
| 1.2 | Replicative and chronological models of yeast aging | 3-13 |
| 1.3 | The rate of chronological aging in yeast is controlled by a network of integrated signalling pathways and protein kinases | 13-20 |
| 1.4 | Phytochemicals are secondary metabolites produced by plants and by bacteria and fungi that resides within the plants | 20- 22 |
| 1.5 | Phytochemicals extend lifespan in evolutionarily distant organisms by targeting an evolutionarily conserved set of longevity-defining cellular processes | 22- 24 |
| 1.6 | Phytochemicals can act as interspecies chemical signals that contribute to the evolution of longevity regulation mechanisms within natural ecosystems | 25-26 |
| 1.7 | The objectives of studies described in this thesis | 27 |
| 2 | Discovery of plant extracts that greatly delay yeast chronological aging | 28-55 |
| 2.1 | Abstract | 28 |
| 2.2 | Materials and Methods | 29-30 |
| 2.3 | Results | 31-48 |
| 2.3.1 | A screen for PEs that can extend longevity of chronologically aging | 31-45 |
| 2.3.2 | For each of the six lifespan-prolonging PEs, the longevity-extending efficacy under CR conditions is significantly lower than that under non-CR conditions | 46 |
| 2.3.3 | Each of the six longevity-extending PEs is a geroprotector which delays the onset and slows the progression of yeast chronological aging by eliciting a hormetic stress response | 47-48 |
| 2.4 | Discussion | 48-55 |

| | | |
|----------|---|---------------|
| 3 | Six plant extracts delay yeast chronological aging through different signaling pathways | 56-94 |
| 3.1 | Abstract | 56 |
| 3.2 | Materials and Methods | 57-58 |
| 3.3 | Results | 59-89 |
| 3.3.1 | The rationale of my experimental approach | 59-62 |
| 3.3.2 | PE4 delays chronological aging by attenuating the inhibitory effect of TORC1 on SNF1 | 62-68 |
| 3.3.3 | PE5 slows chronological aging by impeding two branches of the PKA pathway | 68-73 |
| 3.3.4 | PE6 delays chronological aging by coordinating processes that are not integrated into the network of longevity-defining signaling pathways/protein kinases | 74-77 |
| 3.3.5 | PE8 slows chronological aging by weakening the inhibitory effect of PKA on SNF1 | 78 |
| 3.3.6 | PE12 delays chronological aging by stimulating Rim15 | 79-86 |
| 3.3.7 | PE21 slows chronological aging by inhibiting a PKH1/2-sensitive form of Sch9 | 87-90 |
| 3.4 | Discussion | 91-94 |
| 4 | References | 95-125 |

List of Figures and Tables

| | | |
|------------|---|---|
| Figure 1.1 | Aging pathways are evolutionarily conserved throughout phyla | 2 |
| Figure 1.2 | Replicative vs. chronological assays most commonly used in experiments with yeast | 4 |
| Figure 1.3 | Damage accumulation in aging yeast for chronological vs. replicative lifespan assays | 6 |
| Figure 1.4 | The metabolism of carbon sources in the chronologically aging <i>S. cerevisiae</i> | 9 |

| | | |
|-------------|---|----|
| Figure 1.5 | Growth curve for yeast cultured in glucose-rich medium initially containing 2% glucose | 10 |
| Figure 1.6 | Model of quiescent and non-quiescent states in yeast | 12 |
| Figure 1.7 | A network of pro-aging and anti-aging pathways operating in chronologically aging yeast | 14 |
| Figure 2.1 | PE4, PE5, PE6 and PE8, but not PE1, PE2, PE3 or PE7, extend the CLS of WT yeast grown under non-CR conditions | 36 |
| Figure 2.2 | PE12, but not PE9, PE10, PE11, PE13, PE14, PE15 or PE16, extends the CLS of WT yeast grown under non-CR conditions | 37 |
| Figure 2.3 | PE21, but not PE17, PE18, PE19, PE20, PE22, PE24 or PE25, extends the CLS of WT yeast grown under non-CR conditions | 38 |
| Figure 2.4 | PE27, PE28, PE29, PE30, PE31, PE32, PE33 and PE34 do not extend the CLS of WT yeast grown under non-CR conditions | 39 |
| Figure 2.5 | PE35, PE36 and PE37 do not extend the CLS of WT yeast grown under non-CR conditions | 40 |
| Figure 2.6 | PE4, PE5, PE6, PE8, PE12 and PE21 extend the chronological lifespan (CLS) of yeast grown under non-caloric restriction (non-CR) conditions | 41 |
| Figure 2.7 | PE5 and PE21, but not PE4, PE6, PE8 or PE12, extend the CLS yeast grown under CR conditions | 42 |
| Figure 2.8 | The longevity-extending efficacy under non-CR conditions significantly exceeds that under CR conditions for each of the six lifespan-prolonging PEs | 43 |
| Figure 2.9 | PE4, PE5, PE6, PE8, PE12 and PE21 do not cause significant effects on growth of WT yeast under non-CR conditions | 44 |
| Figure 2.10 | PE4, PE5, PE6, PE8, PE12 and PE21 do not cause significant effects on growth of WT yeast under CR conditions | 45 |
| Figure 2.11 | Analysis of the Gompertz mortality function indicates that PE4, PE5, PE6, PE8, PE12 and PE21 significantly decrease the rate of chronological aging in yeast | 50 |

| | | |
|-------------|--|----|
| Figure 3.1 | A logical framework for identifying signaling pathways and/or protein kinases controlled by the longevity-extending PE(x) and PE(y) | 62 |
| Figure 3.2 | PE4 extends yeast CLS by weakening the restraining action of TORC1 on SNF1 | 64 |
| Figure 3.3 | PE4 is unable to extend the chronological lifespans (CLS) of the <i>tor1Δ</i> and <i>snf1Δ</i> mutant strains, and exhibits additive CLS-extending effects with the <i>ras2Δ</i>, <i>sch9Δ</i> and <i>pkh2Δ</i> mutations | 65 |
| Figure 3.4 | Analysis of the Gompertz mortality function indicates that PE4 delays yeast chronological aging by attenuating the inhibitory effect of TORC1 on SNF1 | 66 |
| Figure 3.5 | PE5 extends yeast CLS by weakening two branches of the PKA signaling pathway. | 70 |
| Figure 3.6 | PE5 is unable to extend the chronological lifespan (CLS) of the <i>ras2Δ</i> mutant strain, exhibits an additive CLS-extending effect with the <i>sch9Δ</i> mutation, and increases yeast CLS in synergy with the <i>tor1Δ</i> and <i>pkh2Δ</i> mutations | 72 |
| Figure 3.7 | Analysis of the Gompertz mortality function indicates that PE5 delays yeast chronological aging by attenuating two branches of the PKA signaling pathway | 73 |
| Figure 3.8 | PE6 extends yeast CLS independently of presently known longevity-defining signaling pathways/protein kinases | 75 |
| Figure 3.9 | PE6 exhibits additive CLS-extending effects with the <i>rim15Δ</i>, <i>sch9Δ</i> and <i>atg1Δ</i> mutations; PE6 also increases yeast CLS in synergy with the <i>tor1Δ</i>, <i>ras2Δ</i>, <i>pkh2Δ</i> and <i>snf1Δ</i> mutations | 76 |
| Figure 3.10 | Analysis of the Gompertz mortality function indicates that PE6 delays yeast chronological aging independently of presently known longevity-defining signaling pathways/protein kinases | 77 |
| Figure 3.11 | PE8 extends yeast CLS by attenuating the inhibitory effect of PKA on SNF1 | 80 |
| Figure 3.12 | PE8 is unable to extend the chronological lifespans (CLS) of the <i>ras2Δ</i> and <i>snf1Δ</i> mutant strains, exhibits an additive CLS-extending | 81 |

| | | |
|-------------|---|-------|
| | effect with the <i>sch9Δ</i> mutation, and increases yeast CLS in synergy with the <i>tor1Δ</i> and <i>pkh2Δ</i> mutations | |
| Figure 3.13 | Analysis of the Gompertz mortality function indicates that PE8 delays yeast chronological aging by attenuating the inhibitory effect of PKA on SNF1 | 82 |
| Figure 3.14 | PE12 extends yeast CLS by stimulating Rim15 | 84 |
| Figure 3.15 | PE12 is unable to extend the chronological lifespan (CLS) of the <i>rim15Δ</i> mutant strain, exhibits additive CLS-extending effects with the <i>tor1Δ</i>, <i>ras2Δ</i> and <i>sch9Δ</i> mutations, and increases yeast CLS in synergy with the <i>pkh2Δ</i> mutation | 85 |
| Figure 3.16 | Analysis of the Gompertz mortality function indicates that PE12 delays yeast chronological aging by stimulating Rim15 | 86 |
| Figure 3.17 | PE21 extends yeast CLS by attenuating a PKH1/2-sensitive form of Sch9 | 88 |
| Figure 3.18 | PE21 extends the chronological lifespan (CLS) of the <i>sch9Δ</i> mutant strain significantly less efficiently than that of the wild-type (WT) strain, exhibits additive CLS-extending effects with the <i>tor1Δ</i> and <i>ras2Δ</i> mutations, and increases yeast CLS in synergy with the <i>pkh2Δ</i> mutation | 89 |
| Figure 3.19 | Analysis of the Gompertz mortality function indicates that PE21 delays yeast chronological aging by inhibiting a PKH1/2-sensitive form of Sch9 | 90 |
| Figure 3.20 | A model for how PE4, PE5, PE6, PE8, PE12 and PE21 delay yeast chronological aging via the longevity-defining network of signaling pathways/protein kinases | 92 |
| Table 2.1 | A list of plant extracts that have been used in this study | 32-33 |
| Table 2.2 | Properties of plant extracts that have been used in this study | 34-35 |
| Table 2.3 | Percent increase of lifespan by geroprotective PEs discovered in this study and by longevity-extending chemical compounds that have been previously identified | 52-55 |

| | | |
|-----------|--|-------|
| Table 3.1 | Single-gene-deletion mutations used in this study and their known effects on longevity-defining signaling pathways and longevity of chronologically aging yeast | 61 |
| Table 3.2 | <i>p</i> Values for pairs of survival curves of a yeast strain cultured with or without the indicated plant extract (PE) | 67 |
| Table 3.3 | <i>p</i> Values for pairs of survival curves of the wild-type (WT) and mutant strain, both cultured in the presence of the indicated PE | 67-68 |

List of Abbreviations

AMPK/TOR, the AMP-activated protein kinase/target of rapamycin signaling pathway; cAMP/PKA, the cAMP/protein kinase A signaling pathway; CFU, colony forming units; CL, cardiolipin; CLS, chronological lifespan; CR, caloric restriction; D, diauxic growth phase; DR, dietary restriction; ER, endoplasmic reticulum; L, logarithmic growth phase; LCA, lithocholic acid; LD, lipid droplet; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; non-CR, non-caloric restriction; OD₆₀₀, optical density at 600 nm; PD, post-diauxic growth phase; PKA, protein kinase A; RLS, replicative lifespan; ROS, reactive oxygen species; Sch9, serine-threonine protein kinase Sch9; ST, stationary growth phase; TAG, triacylglycerols; TCA cycle, tricarboxylic acid cycle; TORC1, target of rapamycin complex 1; UPR, unfolded protein response; WT, wild-type strain.

Introduction

1.1 The budding yeast *Saccharomyces cerevisiae* as an advantageous model organism for unveiling mechanisms of cellular aging in multicellular eukaryotes

The single-celled budding yeast, *Saccharomyces cerevisiae* is one of the most commonly utilized organisms in aging research. [1, 2] This is due to many factors, but one of the most underlying reasons is because the longevity-defining genes and signaling pathways discovered in yeast have been shown to define organismal longevity in eukaryotes across phyla. [3, 4] Other factors include the amenability of yeast to genetic, cell biological, comprehensive biochemical, system biological and many other forms of analyses. [1-6]. These factors pave way for using yeast for gaining fundamental mechanistic insights into longevity processes and their regulation not only in yeast, but also in eukaryotic organisms that are more evolutionarily distant. [5, 6]

S. cerevisiae also provides ease in experimentation due to the fact that its short lifespan can easily be quantified allowing rapid progress in comparison with other, more complex organisms. [5] This rapidity gives way for accelerated progress in defining molecular mechanisms defining longevity within the organism as well as identifies numerous factors underlying its longevity. [5] The preferable source of carbon and/or energy for this unicellular organism is glucose [14], permitting easy and efficient scientific adjustment for caloric restriction (CR) experimentation and also providing high cost efficiency.

S. cerevisiae is an advantageous unicellular model organism in that it unveils fundamental mechanisms and biological principles, which can easily be compared to that

of more complex cellular aging in multicellular eukaryotes, due to the evolutionary conserved effects on lifespan in multicellular organisms, depicted in **Figure 1.1**. [2, 6] Despite the fact that there are many differences as well as complexities concerning humans in relation to yeast, the study of aging in yeast provides many key insights amongst the aging pathways which is also illustrated in **Figure 1.1** [5, 6] Provided that the entire yeast genome has been well-characterized, it can be shown that a substantial portion of gene sequences have homologs compared to that of the mammalian sequences. [13,15] With this in mind, the topic of longevity pathways which concern human aging and disease, *S. cerevisiae* will continue to serve as an attractive model organism of study. [4]

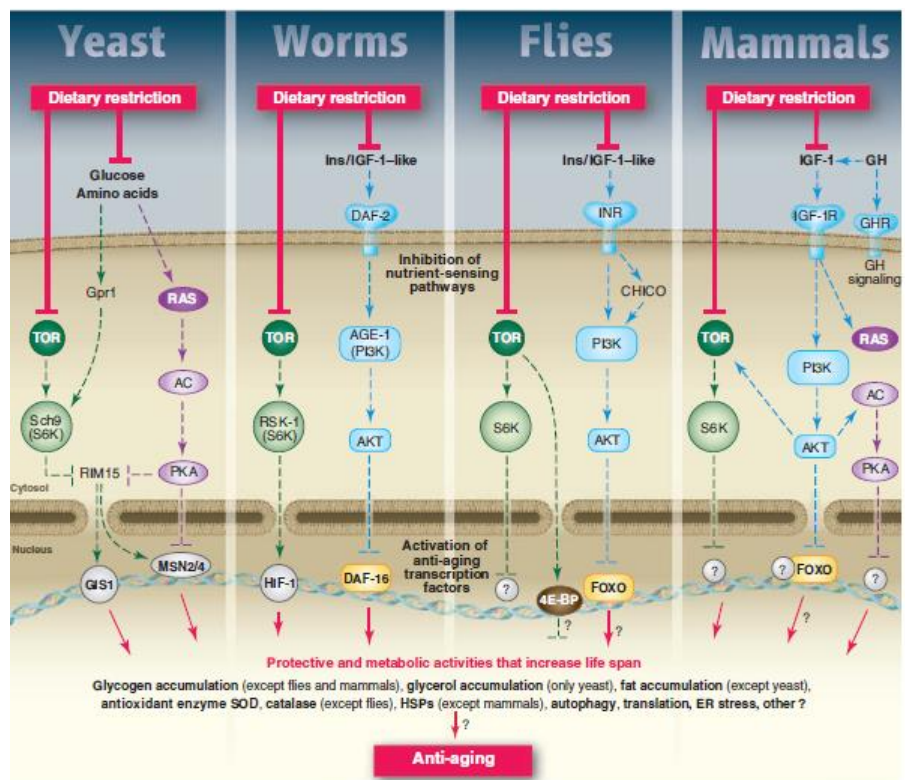


Figure 1.1. Aging pathways are evolutionarily conserved throughout phyla. Aging pathways exhibit many similar features in the unicellular organism, *S. cerevisiae*, and in more complex multicellular organisms. [6]

1.2 Replicative and chronological models of yeast aging

One of the main aspects in research pertaining to longevity is to establish the fundamental concepts which contribute to the nature of aging as well as its associated diseases. [5] This is where the different specificity concerning the lifespan of *S. cerevisiae* and how it is differentiated come to hand. In budding yeast, there are two main approaches in which yeast can be studied in accordance to its lifespan; the replicative lifespan approach and the chronological lifespan approach.

The replicative lifespan (RLS) is defined as the study of the number of asymmetric mitotic divisions a mother cell can undergo preceding cell cycle arrest, as illustrated in **Figure 1.2**. [1] The chronological lifespan is defined as the study of the viability of a cell over time after cell cycle arrest. The viability of a cell is categorized as the ability of re-entry into the cell-cycle in response to the addition of new essential nutrients, which is also illustrated in **Figure 1.2**. [1] Studies of yeast longevity through replicative and chronological lifespan assays under controllable laboratory settings have advanced our understanding of longevity regulation; these studies have also revealed longevity-defining cellular processes and mechanisms within a single cell in eukaryotic organisms across phyla. [2]

The replicative lifespan (RLS):

S. cerevisiae reproduces through the means of asymmetric cell division. The concept of RLS in *S. cerevisiae* is thought to emulate the longevity of dividing, mitotically active cells in multicellular eukaryotic organisms, which highlights the importance of RLS assays. [2]

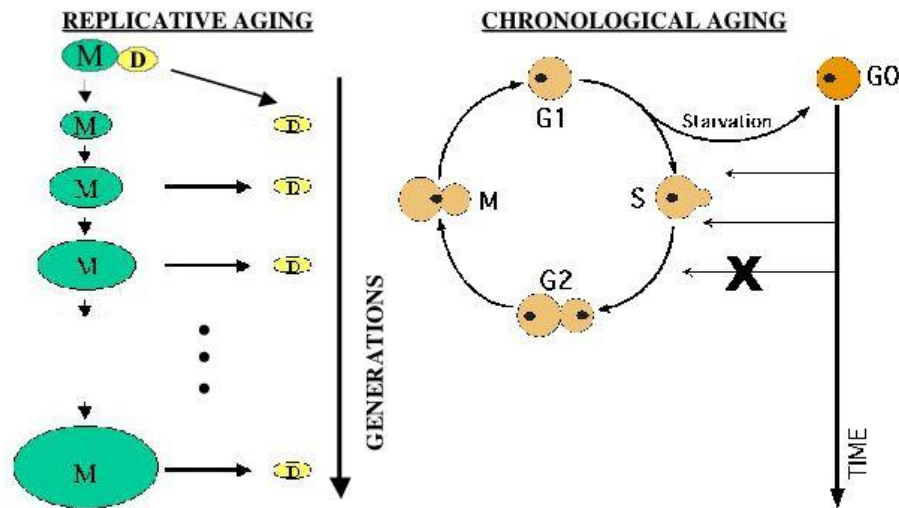


Figure 1.2. Replicative vs. chronological assays most commonly used in experiments with yeast. Replicative aging in yeast is measured over generations as the number of daughter cells produced by a mother cell. Chronological aging in yeast is assessed as viability of the cells over time after cell cycle arrest; cell viability in this assay is defined as the ability of the cell to proliferate if transferred to a nutrient-rich medium. [9]

RLS research focuses on this asymmetric cell division through the use of a standard tetrad microscope equipped with a micromanipulator. [2, 4] Within laboratory condition, budding progeny of the mother cell is removed with using this micromanipulator and the cumulative number of asymmetric mitotic divisions is counted in order to assess how many of these divisions a single mother cell can undergo. [2] Recent studies stray from this micromanipulator method, and rather involve selectively eradicating daughter cells or microfluidic flow chambers. Although these newly introduced high-throughput methods are still being perfected, the micromanipulator method still remains the standard method for RLS assays. [4]

The illustration depicted in **Figure 1.3** simplifies the differences between the processes and longevity effects in that of replicative lifespan compared to that of

chronological lifespan, as well as the damage accumulated over time within each assay.

The mother cell within the RLS assay undergoes longevity-defined cellular processes.

Figure 1.3 portrays the processes of aging for RLS which take place in three defined and consecutive stages; the early, intermediate and late age stages, respectively. These cellular processes are also confounded to various cellular compartments, where longevity is coordinated and co-regulated. Over time, the mother cell progressively accumulates age-related factors known as “senescence factors” which in essence define yeast RLS. [1]

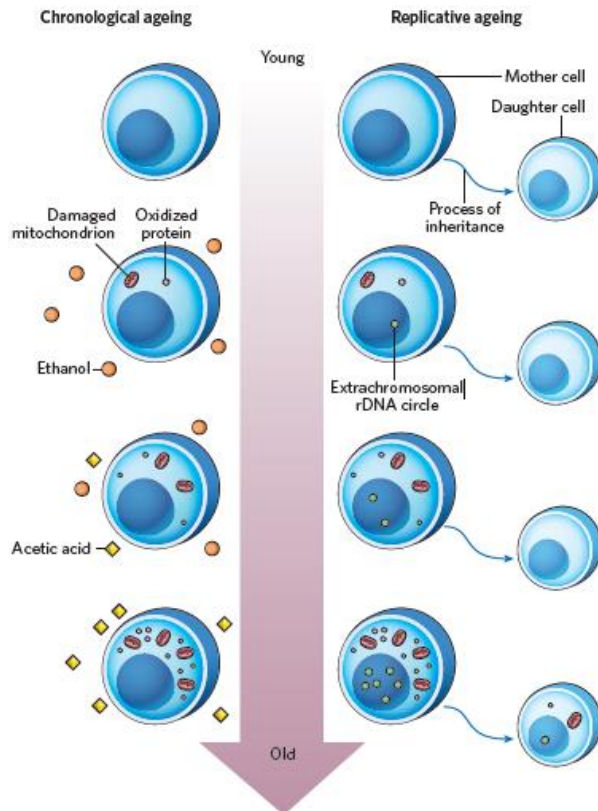


Figure 1.3. Damage accumulation in aging yeast for chronological vs. replicative lifespan assays. For CLS, age-related damage accumulates in non-dividing cells, whereas for RLs, the damage is inherited by the asymmetrically mitotically dividing daughter cells. In chronologically aging yeast, ethanol accumulates within the medium, which later converts to acetic acid and stimulates senescence. Within the chronologically aging cell, on the other hand, damage accumulation is built up from oxidized proteins as well as impaired mitochondria, which also contributes to cellular senescence. In replicatively aging yeast, cellular senescence is obtained through oxidized proteins and impaired mitochondria (alike CLS), along with nuclear extrachromosomal rDNA circles. Over time, the daughter cells can inherit an ample amount of accumulated damage which can cause premature aging due to the very aged mother cells. [5]

One of the distinct forms of molecular damage within RLS, which is specific to the mother cell, is the accumulation of extrachromosomal ribosomal DNA (rDNA) circles, known as ERCs (**Figure 1.3**). These ERCs result from the homologous recombination within the rDNA consequentially forming a self-replicating circular DNA molecule which is kept in the nucleus of the mother cell. This ERC is therefore passed on to the daughter cell through asymmetric mitotic cell division. [5, 16] Proteins such as sirtuins,

specifically histone deacetylase Sir2, influence the formation of ERCs and are therefore a very important factor when observing RLS of yeast. Sirtuins are a group of NAD⁺-dependent protein deacetylases found to be of major importance in ample studies. [5, 16] Much remains unclear and is yet to be discovered concerning Sir2 in its role of replicative senescence, but some studies revealed that its overexpression increases RLS whereas its deletion decrease the RLS. [5]

The chronological lifespan (CLS):

In contrast to yeast RLS, in chronological lifespan (CLS) of yeast, the age-related damage accumulated over time is observed within non-dividing cells, where a threshold is met and the cells can no longer re-enter the cell cycle (**Figure 1.3**). The CLS paradigm is studied due to the fact that it serves as straightforward model for cellular longevity as well as mimics the aging of post-mitotic, non-dividing cells in that of a more complex eukaryotic organism. [2] The CLS is assessed within the controlled environment of the laboratory within a liquid media where its distinct cellular processes are evaluated before cellular cessation. These distinct cellular processes comprise of: cellular growth and division, cellular stress response and death, organelle biogenesis, interorganellar communication, cellular metabolism, and macromolecular homeostasis. [1]

Within laboratory conditions, the CLS assay is achieved through the use of clonogenic assay. This clonogenic assay quantifies the percentage of yeast cell viability at different time points during growth in a liquid medium of 2% glucose, followed by assessing its ability to form colonies on fresh solid nutrient-rich media. [2, 5] In general, the yeast cells are grown in a liquid medium with 2% glucose as the only initial carbon

source. As the yeast cells consume the glucose through fermentation, ethanol is the main product of such fermentation and it accumulates in the extracellular environment. Once the glucose reserve is completely depleted, the cells begin metabolize the ethanol within the extracellular environment through mitochondrial respiration, as depicted in **Figure 1.4**.

Studies suggest that through the process of chronological aging, the mitochondrial production of reactive oxygen species (ROS) causes oxidative damage to proteins, lipids and DNA within the mitochondria. Concomitantly with the age-related ROS accumulation in chronologically aging yeast, cell resistance to oxidative stress has been shown to decrease. [5] One of the key factors pertaining to this oxidative stress is the accumulation of acetic acid. As the chronologically aging yeast persists to use ethanol as its secondary carbon source for mitochondrial respiration, acetic acid (as well as some other organic acids) are leached out into the extracellular environment. Studies have shown that the presence of acetic acid limits the lifespan in chronologically aging yeast due to the fact that acetic acid (along with other acidic organic by-products) acidifies the growth medium and thereby shortens longevity of chronologically aging yeast. [5]

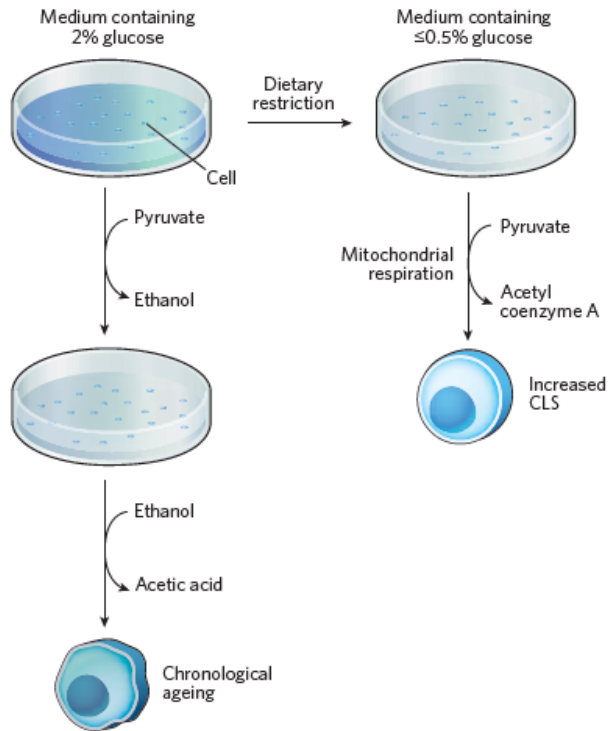


Figure 1.4. The metabolism of carbon sources in the chronologically aging *S. cerevisiae*. Under non-caloric restriction conditions on 2% glucose, the yeast cells metabolize this carbon source into ethanol. As glucose is depleted over time, ethanol is then metabolized through mitochondrial respiration, with acetic acid as the final product secreted into the extracellular environment. Under caloric restriction conditions on 0.5% glucose, glucose is metabolized not through fermentation but through mitochondrial respiration, thus extending chronological lifespan and increasing resistance to oxidative stress. [5].

Other factors that define the longevity of chronologically aging yeast are so-called “biomolecular networks”. [1] These biomolecular networks operate at several distinct checkpoints during the CLS of *S. cerevisiae*; the entry into and progression through each of these checkpoints is regulated by a limited number of protein regulators. The protein regulators act synergistically to coordinate the pace of the aging processes, thus defining yeast CLS. [1]

The CLS checkpoints occur in several consecutive growth phases, which are shown in **Figure 1.5**. The checkpoints have been identified in the logarithmic/exponential

(so-called early-life checkpoints), diauxic, post-diauxic and stationary (so-called late-life checkpoints) growth phases. [1] As previously mentioned, cells that are inoculated into a glucose-rich medium with 2% glucose, progress quickly through the exponential/logarithmic phase. When 50% of glucose supplies are consumed, glycogen synthesis begins. The diauxic shift phase is defined as a phase in which glucose has been fully depleted, cells reduce their growth rate and then enter the post-diauxic phase; during this post-diauxic phase, yeast cells undergo slow respiratory growth using the glucose fermentation products ethanol and acetate as carbon sources (**Figure 1.4**). [12]

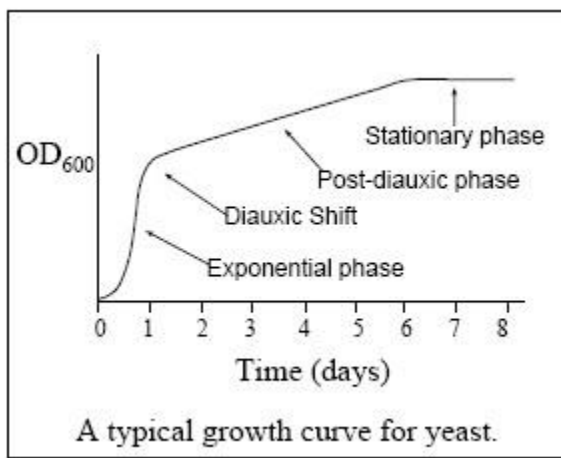


Figure 1.5. Growth curve for yeast cultured in glucose-rich medium initially containing 2% glucose. This curve illustrates the different phases through which chronologically aging yeast progress. A limited number of proteins and cellular processes orchestrates the progression of chronologically aging yeast through each of these phases. [11]

Each checkpoint the yeast cell undergoes within its lifespan is monitored by several “master regulator proteins” which regulate the intercellular concentrations of various metabolic pathways as well as the rates in which they proceed, therefore regulating the interorganellar communication. [1] These master regulator proteins

modulate the concentrations of some key metabolites and the rates of their interorganellar flow. Because of this, these master regulator proteins define the efficiency of such longevity-defining cellular processes as cellular metabolism, cellular division, organelle biogenesis, interorganellar communication, stress response, macromolecular homeostasis and cellular senescence. [1] Although each of these processes is a program for maintaining the overall survival of yeast, none of them is a program for defining aging. Rather, a dynamic equilibrium between these various processes is a key factor which defines longevity of chronologically aging yeast. [1]

Another main feature to the study of chronologically aging yeast is its ability to enter into a characteristic state of cell cycle arrest known as quiescence. Such entry into a state of quiescence has been shown to delay yeast aging because it allows yeast to adapt to different nutrient stresses and, thus, to delay aging inflicted by environmental stresses. The aging of yeast that enter the quiescent state is a valuable model for understanding aging of post-mitotic mammalian cells; this is because this state of quiescence is a state in which cells undergo complex changes in their metabolism and physiology.

As discussed previously, yeast CLS is mainly monitored using cells grown in a glucose-rich liquid medium, progressing through the stationary phase of starvation and ultimately dying because of the excessive endogenous stress. Because yeast cells progressing through the stationary phase undergo starvation, these cells exit cell cycle in the G1 phase and enter a non-dividing phase in which cells undergo characteristic morphological, biochemical and physiological changes to adapt to the environmental stress (**Figure 1.6**). [12] This non-dividing phase of the cell cycle is known as the G0 phase and is often called “cellular quiescence”. When nutrients become available, these

quiescent cells may re-enter the cell cycle to proliferate once again. Under starvation conditions, not all cells exit the cell cycle and enter the quiescent state; some cells enter a different state to become nonquiescent cells. Due to the starvation conditions, most cells eventually die and are therefore broken down; this releases some nutrient components that can then be reused by cells that are still alive to maintain their survival.

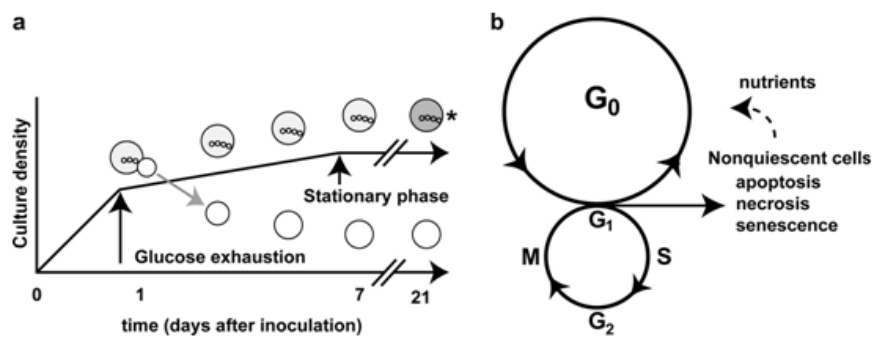


Figure 1.6. Model of quiescent and non-quiescent states in yeast. After glucose depletion, during the diauxic shift, cells can enter the G₀ quiescent state. Cells in this quiescent state can re-enter the cell cycle if and when nutrients are replenished. In the quiescent state, cells undergo different programs of adaptation in order to survive under starvation conditions. Cells which do not enter the quiescent modification state (nonquiescent) continue within the mitotic cell cycle, where most of which become senescent, apoptotic or necrotic. These cells are eventually released as nutrients and replenish those cells that have entered the G₀ quiescent state. The few nonquiescent cells may retain the ability to reproduce when nutrients become available once again. [8]

Quiescent cells were once thought to be a dormant state for the cells, but have recently been found to progress through a distinctive adaptive phases of cellular programming; such programming enables cells to live longer and to withstand environmental stresses. [12] These quiescent programs overcome environmental challenges through the configuration of various signaling pathways as well as regulating pathway-specific effectors which in essence modulate these signaling pathways. [12] The

role of signaling pathways and yeast longevity is of much importance due to their individual anti- or pro-aging characteristics.

1.3 The rate of chronological aging in yeast is controlled by a network of integrated signalling pathways and protein kinases

Certain signaling pathways and protein kinases converge into a network that controls the rate of chronological aging in yeast, as shown in **Figure 1.7**. In chronologically aging yeast, this network integrates the following: 1) the pro-aging TORC1 (target of rapamycin complex 1) pathway; 2) the pro-aging PKA (protein kinase A) pathway; 3) the pro-aging PKH1/2 (Pkb-activating kinase homolog) pathway; 4) the anti-aging SNF1 (sucrose non-fermenting) pathway; 5) the anti-aging ATG (autophagy) pathway; 6) the pro-aging protein kinase Sch9, which is stimulated by the TORC1 and PKH1/2 pathways; and 7) the anti-aging protein kinase Rim15, which is inhibited by the TORC1, PKA and PKH1/2 pathways. This network of signaling pathways and protein kinases coordinates certain longevity-defining cellular processes, including stress responses, protein synthesis in the cytosol and mitochondria, maintenance of nuclear and mitochondrial genomes, autophagy, mitochondrial respiration, peroxisome biogenesis, gluconeogenesis, lipid metabolism, glyoxylate cycle, glycogen synthesis and degradation, and the synthesis of amino acids and fatty acids.

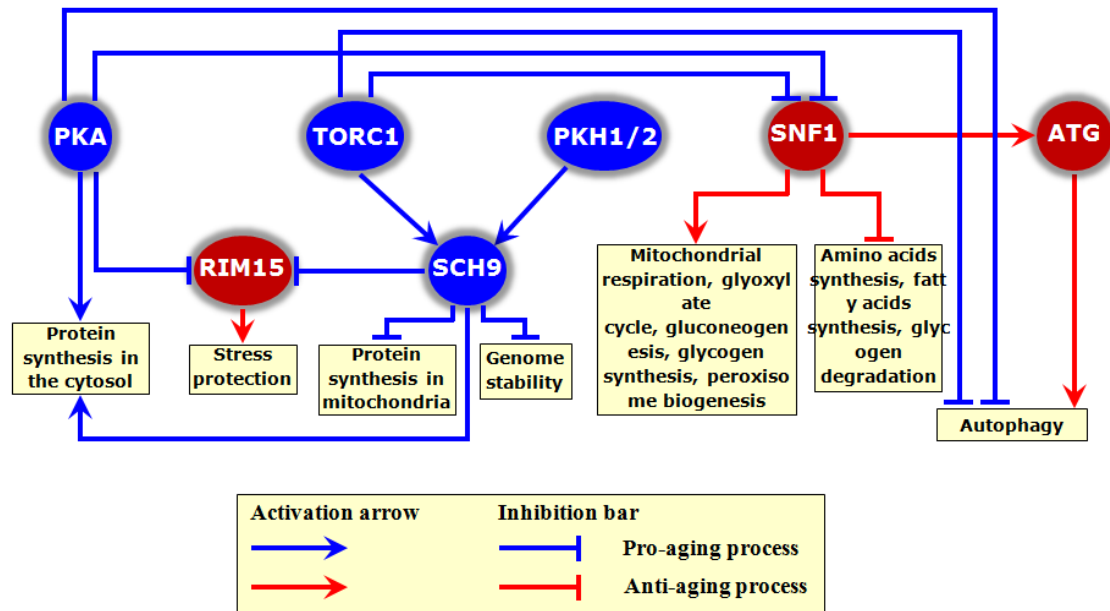


Figure 1.7. A network of pro-aging and anti-aging pathways operating in chronologically aging yeast. This signaling network controls the relative rates of a distinct set of cellular processes.

The pro-aging TORC1 (target of rapamycin complex 1) pathway:

The *TOR* gene encodes an evolutionarily conserved master regulator of longevity in all eukaryotic organisms. This master regulator protein is called TOR (target of rapamycin). While higher eukaryotes possess only one TOR protein assembled with certain interacting proteins into the TORC1 complex, the yeast *S. cerevisiae* has two TOR proteins (which known as Tor1p and Tor2p) interacting with a partially overlapping set of proteins. [14] In *S. cerevisiae*, an interaction of Tor1p and Tor2p with three other proteins results in the assembly of the TORC1 complex. [14] An association between Tor2p and five other proteins forms the TORC2 complex. [14] The TORC2 complex regulates cell polarity and the actin cytoskeleton. [14] The TORC1 regulates cell growth, the transition from the logarithmic growth to the diauxic and the stationary growth phases, and the progression through the stationary phase. Because of these essential roles

in cell progression through different growth phases, TORC1 is a main focus of many studies pertaining to aging. [14] The TORC1 responds to nutrient availability, being especially sensitive to the availability of nitrogen sources. [14] The TORC1 pathway has been found to be inhibited by rapamycin (unlike the TORC2 pathway) and by nitrogen starvation. [12, 14] The inhibition of TORC1 decreases protein synthesis, delays the onset of autophagy and prevents the entry into the G0 (quiescent) state of the cell cycle. [12, 14] The inhibition of the TORC1 pathway by rapamycin or nitrogen starvation activates several stress-responsive transcriptional factors, thereby causing the reprogramming of gene expression and extending longevity in yeast. [14] There are two main protein targets of TORC1, namely Tap42p (regulator of protein phosphatase 2A) and Sch9p. These two proteins regulate most of the cellular processes known to be under control of the TORC1 signaling pathway. [14]

The pro-aging protein kinase Sch9, which is stimulated by the TORC1 and PKH1/2 pathways:

Sch9 is one of the key regulators of cell cycle initiation, thus contributing to cell size control. [14] Sch9 also regulates ribosome assembly. [14] Therefore, a deletion of the *SCH9* gene makes yeast cells very small. Sch9 is dually regulated by the TORC1 and the PKH1/2 signaling pathways. Such dual regulation of Sch9 enables the efficient control of yeast growth and survival in response to intracellular and external cues. [18] The inhibition of TORC1 by way rapamycin dephosphorylates and inactivates Sch9. [14] This inactivation of Sch9 activates the nuclear translocation of the protein kinase Rim15p, one of the key regulators of G0 entry. [14] As mentioned previously, the entry into the G0

state of cellular quiescence results in the reprogramming of gene expression in yeast; such reprogramming increases stress resistance and extends longevity. It has also been shown that lack of Sch9 or TORC1 decreases the concentrations of ethanol and acetic acid in growth medium as well as increases the efflux of glycerol from the yeast cell. [6] These changes in the concentrations of ethanol, acetic acid and glycerol extend yeast chronological lifespan via mechanisms similar to those responsible for lifespan extension by caloric restriction. [6]

The pro-aging PKH1/2 (Pkb-activating kinase homolog) pathway:

In addition to being regulated by TORC1, Sch9 is under control of the PKH1/2 signaling pathway. While TORC1 phosphorylates the serine and threonine residues at the C-terminus of Sch9, the nutrient-sensing protein kinases Pkh1 and Pkh2 phosphorylate the threonine 570 (T570) residue at the activation loop of the kinase domain of Sch9. [18] Sch9 is fully active only if phosphorylated in these various positions by TORC1 and PKH1/2. [18] Both, Pkh1 and Pkh2, are known to be activated by sphingolipids. [18, 19] Sphingolipids are signaling molecules implicated in the regulation of cellular growth and migration, senescence, inflammation, differentiation, and apoptotic cell death. [18] Sphingolipid synthesis is known to be initiated by an enzyme inhibited by myriocin. [20] The inhibition of sphingolipid synthesis by myriocin has been shown to extend yeast chronological lifespan because it weakens the PKH1/2-dependent phosphorylation and activation of Sch9. [19]

The anti-aging protein kinase Rim15, which is inhibited by the TORC1, PKA and PKH1/2 pathways:

As previously mentioned, the inactivation of Sch9 allows the nuclear translocation of Rim15p. Rim15p is an anti-aging protein kinase required for the entry into G0. [14] Therefore, activation of Rim15p under conditions of mild intracellular or extracellular stress promotes the entry into the state of cellular quiescence and activates expression of numerous stress resistance genes, thus extending yeast CLS. [14] *RIM15* gene deletion impacts key cellular processes in the stationary growth phase. [14] Specifically, lack of Rim15p prevents cell cycle arrest in the G1 phase, impairs the entry into G0 and averts transcription of stress-inducible genes; all these processes are critical for assuring yeast longevity. [14] Deletion of the *RIM15* gene also decreases thermotolerance, and decelerates the accumulation of trehalose and glycogen. [14]

Rim15p is inhibited by phosphorylation exerted by the PKA signaling pathway. [14] Its nucleocytoplasmic distribution is controlled by the Sch9 branch of the TORC1 signaling pathway and by the nutrient-sensitive Pho80p-Pho85p signaling pathway. [14]

The nutrient-sensitive Pho80p-Pho85p signaling pathway phosphorylates Rim15p at the threonine 1075 residue in the Bmh2p-binding site of Rim15p; such phosphorylation of Rim15p impairs its nuclear import and retains it in the cytoplasm. [14] Sch9 also keeps Rim15p in the cytoplasm, by phosphorylating the threonine 1075 residue in its Bmh2p-binding site; upon the inhibition of TORC1, this site of Rim15p gets dephosphorylated, thus enabling Rim15p to enter the nucleus and to activate transcription of many stress-response genes. [14]

The pro-aging PKA (protein kinase A) pathway:

Like the TORC1 pathway, the pro-aging PKA signaling pathway orchestrates key longevity-defining processes in chronologically aging yeast and in all other eukaryotes. PKA inhibits the anti-aging signaling pathways RIM15 and SNF1; it also inhibits autophagy and plays a crucial role in progression through the cell cycle. [12, 14]

PKA is a heterotetramer compiled of two regulatory (encoded by the *BCY1* gene) and two catalytic subunits (encoded by three closely related genes, *TPK1*, *TPK2* and *TPK3*). [14] Cyclic AMP (cAMP) must bind to the Bcy1p (inhibitory subunit) in order for the Bcy1p to dissociate from the catalytic subunit and therefore allow Tpk1p, Tpk2p and Tpk3p (the catalytic subunit) to be activated. [14] Other protein components have been found to modulate the PKA signaling pathway in response to certain environmental cues; these upstream regulators of the PKA pathway include the transcriptional complex Ccr4p-Not, Srb9p and subunits of the Mediator complex of RNA polymerase II. [14]

The PKA pathway inhibits stress response and some metabolic pathways in yeast cells entering the diauxic growth phase. [12] PKA has been found to play a major inhibitory role in the transition from the exponential to the diauxic and then to the stationary phases of growth. [14] Increased activity of PKA has been shown to shorten yeast CLS by attenuating stress response in yeast cells entering the stationary growth phase. [14] Decreased activity of PKA activity, on the other hand, is known to extend yeast CLS by enhancing stress response. [14]

The anti-aging SNF1 (sucrose non-fermenting) pathway:

SNF1, like RIM15, is an anti-aging signaling pathway operating in yeast. SNF1 is known to regulate transcription of almost 400 genes. [12] Yeast SNF1 is an ortholog of the mammalian AMP-activated protein kinase (AMPK); it controls transcription of numerous genes by inhibiting transcriptional repressors, directly modulating the core transcriptional machinery and stimulating transcriptional activators. [12, 14] SNF1 has also been shown to play key roles in other processes, including chromatin modification, autophagy, translation and activation of some metabolic pathways. [12] SNF1 complex is composed of multiple subunits; they include the regulatory Snf1p and Snf4p subunits, Gal83p, Sip1p and Sip2p. [14] Snf1p is autoinhibited when glucose is available to yeast cells. [14] When glucose is completely consumed, the regulatory subunit Snf4p attenuates such autoinhibition, thus activating Snf1p. [14] Since the SNF1 pathway is inhibited when glucose is available to yeast, cells lacking SNF1 activity are unable to grow in the presence of carbon sources other than glucose. Therefore, these yeast cells die soon after the diauxic shift and exhibit shortened CLS. [14]

The anti-aging ATG (autophagy) pathway:

The ATG signaling pathway is an anti-aging pathway which is involved in autophagy. Autophagy is the process of delivering various cytoplasmic substrates through different routes to the lysosome (the vacuole in yeast) for degradation. [17] Several types of autophagy are known, such as macroautophagy, microautophagy and chaperone-mediated autophagy. [17] Once the cytoplasmic substrates (including individual proteins, protein aggregates and organelles) are broken down within the lysosome, the degradation

products are released by permeases and recycled within the cytosol. [17] The process of autophagy is extremely important when nutrients are scarce. Under such stressful conditions, yeast cells enter the G0 phase of cell quiescence. When nutrients become readily available, these cells can re-enter the cell cycle once again for cellular division. [17] The bioenergetic demands of the cells under stressful conditions of nutrient limitation can be fulfilled with the help of autophagy, which allows to renew the energy-rich compounds so high in demand under such conditions. [17] This implies that autophagy is not a destructive process leading to cell death, but rather a cytoprotective process of longevity assurance and lifespan maintenance. [17]

While the pro-aging TORC1 pathway regulates not only autophagy but also many other cellular processes, the ATG pathway controls only autophagy. [17] A body of evidence indicates that the ATG pathway in chronologically aging yeast undergoes age-related attenuation. [17] The mechanism underlying such attenuation of this anti-aging pathway remains to be established.

1.4 Phytochemicals are secondary metabolites produced by plants and by bacteria and fungi that resides within the plants

Phytochemicals are secondary metabolites with diverse structures. [7] They are generated by plants as well as by non-pathogenic endosymbiont microorganisms residing within that very plant. [7] The secondary metabolites are of much importance to plants, due to the fact that they help the plants to survive. [7] Phytochemicals are of much importance to my research project as well, due to the fact that the extracts delaying yeast

aging and used in my studies were derived from whole plants and, thus, contained a mix of phytochemicals.

Phytochemicals aid plant survival by protecting the plant from environmental stresses, herbivorous organisms (by way of attracting predators for such herbivorous organisms), microbial infections and environmental pollutants. [7] Phytochemicals are also known to attract pollinator organisms and other symbiotes required for plant survival. [7] Phytochemicals have been shown to extend longevity in heterotrophic organisms across phyla through mechanisms that have been conserved in the course of evolution. [7]

The structurally diverse phytochemicals are divided into eleven different major classes which include the following: phenolic compounds, terpenes, betalains, polysulfides, organosulfur compounds, indole compounds, select protease inhibitors, oxalic and anacardic organic acids, modified purines, quinones, and polyamines. [7]

Phytochemicals are believed to be an acquired evolutionary adaptation to increase the survival of the stationary autotrophic organisms within the many diverse ecological niches, and to overcome the many different environmental stresses and/or other survival obstacles. [7, 21] Due to the fact that phytochemicals are evolutionarily adaptive to the specific stresses within that environment, these secondary metabolites are unique to the specific species and/or genera. [7, 21] Thus, phytochemicals do not interfere with the primary metabolic requirements for the organism, but rather allow the organism to overcome local survival challenges. [21] Moreover, phytochemicals are produced in so-called “secondary biochemical pathways” which are believed to be evolved to enable plant survival through multiple fashions. [7]

The necessity of phytochemicals can be highlighted through the energy expenditure for their synthesis, where the energy invested is far much in excess compared to that required to synthesize primary metabolites. [21] The survival skills benefited to the host plant upon the phytochemical synthesis include the following: survival through forms of environmental stresses (such as thermal stresses, extreme pH, UV damage, osmotic stresses, water deficiency and nutrient deficiency), protection against invading species (such as insects, competitor plant species and herbivorous animals), defence against infections (including viral, fungal and bacterial ones), protection against environmental pollutants, attraction of natural predators for invading species, and attraction of symbiotes and pollinators. [7] Furthermore, phytochemicals are produced not only by the plant itself, but also by the non-pathogenic endosymbiont microorganisms residing within the host plant; this promotes plant survival under conditions in which the plant is normally defenceless (that is, in plants exposed to invading species, pathogenic infections etc.). [7, 21]

1.5 Phytochemicals extend lifespan in evolutionarily distant organisms by targeting an evolutionarily conserved set of longevity-defining cellular processes

Phytochemicals not only provide added benefits to the survival of plants. A body of evidence indicates that these secondary metabolites of plant origin can enhance survival and/or extend longevity of various heterotrophic organisms through the targeting of an evolutionarily conserved set of longevity-defining cellular processes. [7] As mentioned above, phytochemicals are very diverse in nature and may belong to an assortment of chemical classes such as quinones, indole compounds etc.

Due to the natural structural diversity of phytochemicals, evolutionarily distant heterotrophic organisms use the benefits of phytochemicals to prolong their lifespans. Such heterotrophic organisms include *S. cerevisiae*, fruit flies (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*), fission yeast (*Schizosaccharomyces pombe*), mosquitos, honey bees (*Apis mellifera*), short-lived fish (*Nothobranchius furzeri*), laboratory mice and rats, multiple lines of cultured human fibroblasts, and human peripheral blood mononuclear cells. [7] Many studies support the notion that phytochemicals extend longevity in numerous heterotrophic organisms by mechanisms which have been conserved throughout the course of evolution. [7] These mechanisms target a distinct set of evolutionarily conserved nutrient-sensing signaling pathways.

Some of these pathways acting in *S. cerevisiae* have been mentioned above; they include the TOR and ATG pathways. These evolutionarily conserved pathways shown to be targeted by longevity-extending phytochemicals include the following: the TOR pathway, the IIS pathway, the sirtuins-governed protein deacetylation module of the longevity signaling network integrating the IIS and TOR pathways, the non-selective autophagy pathway for degradation of various cellular organelles and macromolecules, and the OSR-1/UNC-43 (CaMKII)/SEK-1 (p38 MAPK) stress-responsive signaling pathway. [7]

Furthermore, phytochemicals can delay aging and extend longevity by postponing the onset of a distinct set of cellular processes referred to as “cellular and molecular hallmarks of aging”. [7] Specifically, it has been shown that phytochemicals can slow the development of the following seven common traits of aging: loss of proteostasis,

genomic instability, epigenetic alterations, mitochondrial dysfunction, deregulated nutrient sensing, altered intercellular communication and cellular senescence. [7]

Many phytochemicals which have been shown to prolong longevity in evolutionarily distant organisms by targeting certain signaling pathways and cellular proteins. In brief, such signaling pathways and proteins include the following: sirtuins, (Sir1 in *S. cerevisiae*, SIR-2.1 in the nematode *C. elegans*, Sir2 in the fruit fly *D. melanogaster* and SIRT1 in mice), TORC1 (in the yeast *S. cerevisiae* and *Sch. pombe*), Sch9 and Gcn2 (in *S. cerevisiae*), Sod1 and Sod2 (in *S. cerevisiae*), and non-selective autophagy pathways (in *S. cerevisiae*, *C. elegans* and *D. melanogaster*). [7]

In the yeast *S. cerevisiae*, different longevity-extending phytochemicals have been shown to cause the following changes to various cellular processes: 1) caffeine enhances transcription of genes encoding heat-shock proteins and molecular chaperones; 2) cryptotanshinone reduces the intracellular concentrations of ROS; 3) phloridzin causes a decline in ROS, increases cell resistance to oxidative stress, and activates transcription of the *SOD1*, *SOD2* and *SIR2* genes; 4) quercetin decreases ROS as well as lowers cell susceptibility to oxidative stress, glutathione oxidation, lipid peroxidation and protein carbonylation; 5) resveratrol reduces the frequency of rDNA recombination; and 6) spermidine activates transcription of autophagy-related genes, delays the onset of age-related necrotic cell death, promotes histone H3 deacetylation and decreases activities of histone acetyltransferases. [7] Moreover, in another yeast species, *Sch. pombe*, caffeine has been shown to inhibit phosphorylation of ribosomal S6 protein, decelerate protein synthesis, alter transcription of numerous nuclear genes, cause cell-cycle arrest in G2 and slow down growth. [7]

1.6 Phytochemicals can act as interspecies chemical signals that may contribute to the evolution of longevity regulation mechanisms within natural ecosystems

One hypothesis in regards to phytochemicals and longevity regulation is that plants synthesize these secondary metabolites in response to hermetic environmental stresses such as dehydration, heat exposure, nutrient deprivation etc. This hypothesis is known as the xenohormesis hypothesis. [7] Hormesis is a term used to define a distinct type of response of cells and organisms to different doses of stress. In hormetic response, low doses of stress promote growth and survival, whereas high doses of the same stress impair growth and survival. [7]

The xenohormesis hypothesis posits that an exposure to low doses of stress allows the cell or organism to activate mechanisms that can protect this cell or organism from high doses of the same kind of stress. [7] According to this hypothesis, phytochemicals released into the environment by plants can create a moderate stress in the heterotrophic organisms within the ecosystem. [7] Therefore, phytochemicals can operate as hormetic stress agents that enable adaptation and survival of these heterotrophic organisms if they are exposed to the higher doses of the same stress. [7] Thus, phytochemicals can act as “chemically encoded information” about environmental changes and conditions within a particular ecosystem. [7] After being released into this ecosystem by plants, these chemical signals can extend longevity of heterotrophic organisms inhabiting the ecosystem by modulating some evolutionary conserved of survival. [7]

The xenohormesis hypothesis suggests that the ability for heterotrophic organisms to change their physiology and metabolism in response to mild environmental stresses created by phytochemicals can increase their chances of survival. [7] This enables

evolutionary selection of mechanisms for maintaining such survival capabilities and, thus, for extending longevity of these heterotrophic organisms. [7] In summary, the xenohormetic hypothesis states that phytochemicals can function as xenohormetic stress signals promoting the survival of heterotrophic organisms within ecosystem and driving the evolution of longevity regulation mechanisms. [7]

1.7 The objectives of studies described in this thesis

Research in the Titorenko laboratory is aimed at using *S. cerevisiae* as a model organism to discover chemical compounds that can slow aging and delay the onset of age-related diseases in evolutionarily distant eukaryotic organisms. Some of such geroprotective compounds have been previously revealed in natural products extracted from certain plants. As a first step towards uncovering novel aging-delaying chemical compounds of plant origin, my first objective was to conduct a screen for plant extracts that can extend yeast chronological lifespan. My screen revealed six plant extracts that increase yeast chronological lifespan considerably more efficiently than any of the longevity-extending chemical compounds yet described. Because the rate of aging in yeast is controlled by a network of integrated signaling pathways and protein kinases, my second objective was to assess how single-gene-deletion mutations eliminating each of these pathways and kinases affect the aging-delaying efficiencies of the six plant extracts.

2 Discovery of plant extracts that greatly delay yeast chronological aging

2.1 Abstract

As a first step towards uncovering novel aging-delaying chemical compounds of plant origin, I conducted a screen for plant extracts (PEs) that can extend yeast chronological lifespan (CLS). My screen revealed six PEs that increase yeast CLS considerably more efficiently than any of the longevity-extending chemical compounds yet described. These novel lifespan-extending plant extracts include the following: 1) 0.5% PE4 from *Cimicifuga racemosa*; 2) 0.5% PE5 from *Valeriana officinalis L.*; 3) 1.0% PE6 from *Passiflora incarnata L.*; 4) 0.3% PE8 from *Ginkgo biloba*; 5) 0.1% PE12 from *Apium graveolens L.*; and 6) 0.1% PE21 from *Salix alba*. One of these extracts, 0.1% PE21 from *Salix alba*, is the most potent longevity-extending pharmacological intervention yet described. I demonstrate that, for each of the six lifespan-prolonging PEs, the longevity-extending efficacy under caloric restriction (CR) conditions is significantly lower than that under non-CR conditions. I conclude that each of these PEs could mimic the longevity-extending effect of CR. I provide evidence that each of the six PEs is a geroprotector which delays the onset and decreases the rate of yeast chronological aging by eliciting a hormetic stress response.

2.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0*) from Thermo Scientific/Open Biosystems was grown in a synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% or 0.5% glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil. Cells were cultured at 30°C with rotational shaking at 200 rpm in Erlenmeyer flasks at a “flask volume/medium volume” ratio of 5:1.

CLS assay

A sample of cells was taken from a culture at a certain day following cell inoculation and PE addition into the medium. A fraction of the sample was diluted in order to determine the total number of cells using a hemacytometer. Another fraction of the cell sample was diluted and serial dilutions of cells were plated in duplicate onto YEP (1% yeast extract, 2% peptone) plates containing 2% glucose as carbon source. After 2 d of incubation at 30°C, the number of colony forming units (CFU) per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each culture, the percentage of viable cells was calculated as follows: (number of viable cells per ml/total number of cells per ml) \times 100. The percentage of viable cells in mid-logarithmic growth phase was set at 100%.

A screen for PEs that can extend yeast CLS

CLS analysis in the presence of various PEs was performed as described above. A 20% stock solution of each PE in ethanol was made on the day of adding this PE to cell cultures. For each PE, the stock solution was added to growth medium with 2% glucose immediately following cell inoculation into the medium. The final concentration of each PE in the medium was 0.02%, 0.04%, 0.06%, 0.08%, 0.1%, 0.3%, 0.5% or 1.0%.

Gompertz mortality function analysis

The age-specific mortality rate (q_x) [22, 24, 25], Gompertz slope or mortality rate coefficient (α) [23, 24, 25], and mortality rate doubling time (MRDT) [24, 26] were calculated as previously described. The value of q_x was calculated as the number of cells that lost viability (i.e. are unable to form a colony on the surface of a solid nutrient-rich medium) during each time interval divided by the number of viable (i.e. clonogenic) cells at the end of the interval. The natural logarithms of the values of q_x for each time interval were plotted against time. The value of α was calculated as the slope of the Gompertz mortality line, whereas the value of MRDT was calculated as $\ln 2/\alpha$.

Statistical analysis

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean \pm SEM. The p values for comparing the means of two groups (using an unpaired two-tailed t test) and survival curves (using a two-tailed t test) were calculated with the help of the GraphPad Prism statistics software.

2.3 Results

2.3.1 A screen for PEs that can extend longevity of chronologically aging yeast

I screened a library of PEs for extracts that can increase yeast CLS. This library includes 35 different PEs of known origin and properties (**Table 2.1** and **Table 2.2**, respectively). To perform the screen for lifespan-extending PEs, I used a robust assay for measuring yeast CLS. This assay was similar to the one described previously [27], but the wild-type strain BY4742 was cultured in the synthetic minimal YNB medium initially containing 2% glucose (instead of the nutrient-rich YEP medium supplemented with 0.5% glucose). Yeast cells cultured on 2% glucose are not limited in calorie supply; these cells age chronologically under so-called non-caloric restriction (non-CR) conditions that accelerate aging in different yeast genetic backgrounds, including BY4742 [2, 4, 5].

In my screen for longevity-extending PEs, each PE from the library was added to growth medium at the time of cell inoculation at a final concentration in the 0.02% to 1.0% range. Some PEs from the library did not alter the mean and maximum CLS of yeast under non-CR conditions at any concentration examined; among these PEs were PE9, PE13, PE16, PE22, PE28 and PE36 (**Figure 2.2 – Figure 2.5**). Many PEs from the library shortened the mean and/or maximum CLS of yeast under non-CR conditions at final concentrations ranging from 0.08% to 1.0%; among these PEs were PE1 - PE3, PE7, PE10, PE11, PE14, PE15, PE17 - PE20, PE24, PE25, PE27, PE29 - PE35 and PE37 (**Figure 2.1 – Figure 2.5**). 6 of the 35 PEs from the library significantly increased both the mean and maximum CLS of yeast under non-CR conditions if added at final concentrations ranging from 0.04% to 1.0% (**Figure 2.1 – Figure 2.3**). A group of these

longevity-extending PEs included the following extracts: 1) 0.5% PE4 from *Cimicifuga racemosa* (Figure 2.1, Figure 2.6A, Figure 2.8A, Figure 2.8B); 2) 0.5% PE5 from *Valeriana officinalis L.* (Figure 2.1, Figure 2.6B, Figure 2.8A, Figure 2.8B); 3) 1.0% PE6 from *Passiflora incarnata L.* (Figure 2.1, Figure 2.6C, Figure 2.8A, Figure 2.8B); 4) 0.3% PE8 from *Ginkgo biloba* (Figure 2.1, Figure 2.6D, Figure 2.8A, Figure 2.8B); 5) 0.1% PE12 from *Apium graveolens L.* (Figure 2.2, Figure 2.6E, Figure 2.8A, Figure 2.8B); and 6) 0.1% PE21 from *Salix alba* (Figure 2.3, Figure 2.6F, Figure 2.8A, Figure 2.8B). None of the six lifespan-prolonging PEs affected growth rates in logarithmic (L) and post-diauxic (PD) phases or impacted the maximum cell density in stationary (ST) phase of yeast cultures under non-CR conditions on 2% glucose (Figure 2.9). Thus, the observed lifespan extension by each of these PEs is unlikely to be caused by its ability to decrease growth rate or to make yeast more resistant to toxic substances accumulated during culturing in the synthetic minimal YNB medium.

Table 2.1. A list of plant extracts that have been used in this study.

| Abbreviated name | Botanical name | Plant part used | Commercial source |
|------------------|------------------------------------|------------------|--------------------|
| PE1 | <i>Echinacea purpurea</i> | Whole plant | Idunn Technologies |
| PE2 | <i>Astragalus membranaceous</i> | Root | Idunn Technologies |
| PE3 | <i>Rhodiola rosea L.</i> | Root | Idunn Technologies |
| PE4 | <i>Cimicifuga racemosa</i> | Root and rhizome | Idunn Technologies |
| PE5 | <i>Valeriana officinalis L.</i> | Root | Idunn Technologies |
| PE6 | <i>Passiflora incarnate L.</i> | Whole plant | Idunn Technologies |
| PE7 | <i>Polygonum cuspidatum</i> | Root and rhizome | Idunn Technologies |
| PE8 | <i>Ginkgo biloba</i> | Leaf | Idunn Technologies |
| PE9 | <i>Zingiber officinale Roscoe</i> | Rhizome | Idunn Technologies |
| PE10 | <i>Theobroma cacao L.</i> | Cacao nibs | Idunn Technologies |
| PE11 | <i>Camellia sinensis L. Kuntze</i> | Leaf | Idunn Technologies |
| PE12 | <i>Apium graveolens L.</i> | Seed | Idunn Technologies |
| PE13 | <i>Scutellaria baicalensis</i> | Root | Idunn Technologies |

| | | | |
|------|-----------------------------------|---------------|--------------------|
| PE14 | <i>Euterpe oleracea</i> | Fruit | Idunn Technologies |
| PE15 | <i>Withania somnifera</i> | Root and leaf | Idunn Technologies |
| PE16 | <i>Phyllanthus emblica</i> | Fruit | Idunn Technologies |
| PE17 | <i>Camellia sinensis</i> | Leaf | Idunn Technologies |
| PE18 | <i>Pueraria lobata</i> | Root | Idunn Technologies |
| PE19 | <i>Silybum marianum</i> | Seed | Idunn Technologies |
| PE20 | <i>Eleutherococcus senticosus</i> | Root and stem | Idunn Technologies |
| PE21 | <i>Salix alba</i> | Bark | Idunn Technologies |
| PE22 | <i>Glycine max L.</i> | Bean | Idunn Technologies |
| PE24 | <i>Calendula officinalis</i> | Flower | Idunn Technologies |
| PE25 | <i>Salvia miltiorrhiza</i> | Root | Idunn Technologies |
| PE27 | <i>Panax quinquefolium</i> | Root | Idunn Technologies |
| PE28 | <i>Harpagophytum procumbens</i> | Root | Idunn Technologies |
| PE29 | <i>Olea europaea L.</i> | Leaf | Idunn Technologies |
| PE30 | <i>Gentiana lutea</i> | Root | Idunn Technologies |
| PE31 | <i>Piper nigrum</i> | Fruit | Idunn Technologies |
| PE32 | <i>Aesculus hippocastanum</i> | Seed | Idunn Technologies |
| PE33 | <i>Mallus pumila Mill.</i> | Fruit | Idunn Technologies |
| PE34 | <i>Fragaria spp.</i> | Fruit | Idunn Technologies |
| PE35 | <i>Ribes nigrum</i> | Leaf | Idunn Technologies |
| PE36 | <i>Dioscorea opposita</i> | Root | Idunn Technologies |
| PE37 | <i>Cinnamomum verum</i> | Bark | Idunn Technologies |

Table 2.2. Properties of plant extracts that have been used in this study.

| Abbreviated name | Properties |
|-------------------------|---|
| PE1 | Extraction solvent: ethanol (75%)/water (25%). Extract ratio: 4/1. Composition: natural extract, maltodextrin. |
| PE2 | Extraction solvent: denatured ethanol (70%)/water (30%). Extract ratio: 10/1. Composition: natural extract (40-50%), gum arabic (50-60%). |
| PE3 | Extraction solvent: ethanol (60-80%)/water (40-20%). Extract ratio: 15-20/1. Composition: natural extract (80-100%), maltodextrin (0-20%). |
| PE4 | Extract ratio: 6-8/1. Composition: natural extract (28-38%), maltodextrin (60-70%), tricalcium phosphate (0-5%). |
| PE5 | Extraction solvent: denatured ethanol/water. Extract ratio: ~ 6/1. Composition: natural extract, maltodextrin, silica (0-1%). |
| PE6 | Extraction solvent: water (100%). Extract ratio: 4/1. Composition: natural extract, maltodextrin. |
| PE7 | Extraction solvent: ethanol (80%)/water (20%). Extract ratio: 40/1. Composition: natural extract (90-100%), maltodextrin (0-10%). |
| PE8 | Extraction solvent: ethanol/water. Extract ratio: 50/1. Composition: natural extract. |
| PE9 | Extraction solvent: ethanol/water. Composition: natural extract (96%), gingerols (4%). |
| PE10 | Natural powder/final product ratio: 2-3/1. Composition: natural powder. |
| PE11 | Extraction solvent: ethyl acetate (90%)/water (10%). Extract ratio: 6/1. Composition: natural extract (100%). |
| PE12 | Extraction solvent: ethanol (90%)/water (10%). Extract ratio: 8/1. Composition: natural extract, maltodextrin, modified starch, silica. |
| PE13 | Extraction solvent: ethanol/water. Extract ratio: 4/1. Composition: natural extract. |
| PE14 | Extraction solvent: ethanol/water. Extract ratio: 4/1. Composition: natural extract. |
| PE15 | Extraction solvent: water. Extract ratio: 9/1. Composition: withanolide glycoside conjugates (10%), oligosaccharides (32%), free withanolides (0.5%). |
| PE16 | Extraction solvent: water. Composition: hydrolysable tannins (>60%), including Emblicanin-A, Emblicanin-B, Punigluconin, Pedunculagin. |
| PE17 | Composition: tea polyphenols (>90%), including epigallocatechin gallate (>40%). |
| PE18 | Composition: flavonoids (>40%), including puerarin. |
| PE19 | Extraction solvent: ethanol/water. Composition: silymarin (>80%). |
| PE20 | Extraction solvent: water. Composition: eleutheroside B+E (>0.8%). |

| | |
|------|--|
| PE21 | Extraction solvent: ethanol/water. Composition: salicin (>25%). |
| PE22 | Composition: isoflavones (40%). |
| PE24 | Composition: lutein (>5%). |
| PE25 | Composition: tanshinones, isotanshinones, cryptotanshinone, isocryptotanshinone, dihydrotanshinone, hydroxytanshinones. |
| PE27 | Composition: ginsenosides (10%, by HPLC-UV), quintozone-free. |
| PE28 | Extraction solvent: ethanol/water. Extract ratio: 40/1. Composition: harpagosides (20%, by HPLC-UV). |
| PE29 | Extraction solvent: ethanol (70%)/water (30%). Extract ratio: 5-10/1. Composition: natural extract, maltodextrin, silica (0.2%). |
| PE30 | Composition: isogentisin (0.04%). |
| PE31 | Extraction solvent: ethanol. Extract ratio: 10/1. Composition: piperine (>90%). |
| PE32 | Composition: aescin (20%). |
| PE33 | Extraction solvent: ethanol (70%)/water (30%). Extract ratio: 120-130/1. Composition: natural extract (60-70%), maltodextrin (30-40%). |
| PE34 | Extract ratio: 5/1. Composition: natural extract, including polyphenols (>2%). |
| PE35 | Extraction solvent: water. Composition: polyphenols (15%, by HPLC-UV). |
| PE36 | Composition: diosgenine (>16%, by HPLC-UV). |
| PE37 | Extraction solvent: water. Composition: polyphenols (25%, by HPLC-UV). |

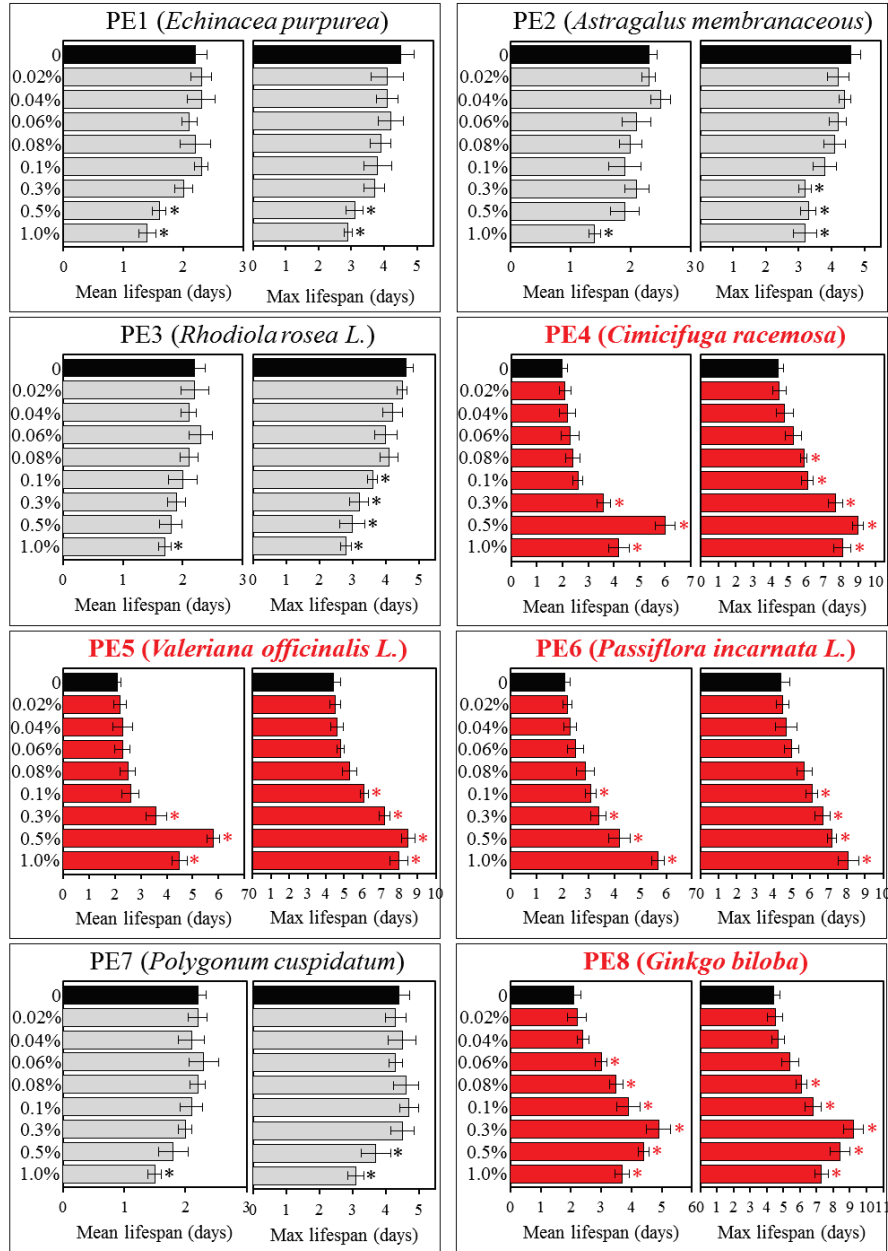


Figure 2.1. PE4, PE5, PE6 and PE8, but not PE1, PE2, PE3 or PE7, extend the CLS of WT yeast grown under non-CR conditions. WT cells were grown in the synthetic minimal YNB medium initially containing 2% glucose (non-CR conditions), in the presence of a PE or in its absence. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means \pm SEM (n = 6-21; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test). Note that PE1, PE2, PE3 and PE7 can shorten the CLS of WT yeast under non-CR conditions if added at high concentrations (n = 6; * $p < 0.05$; ; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test).

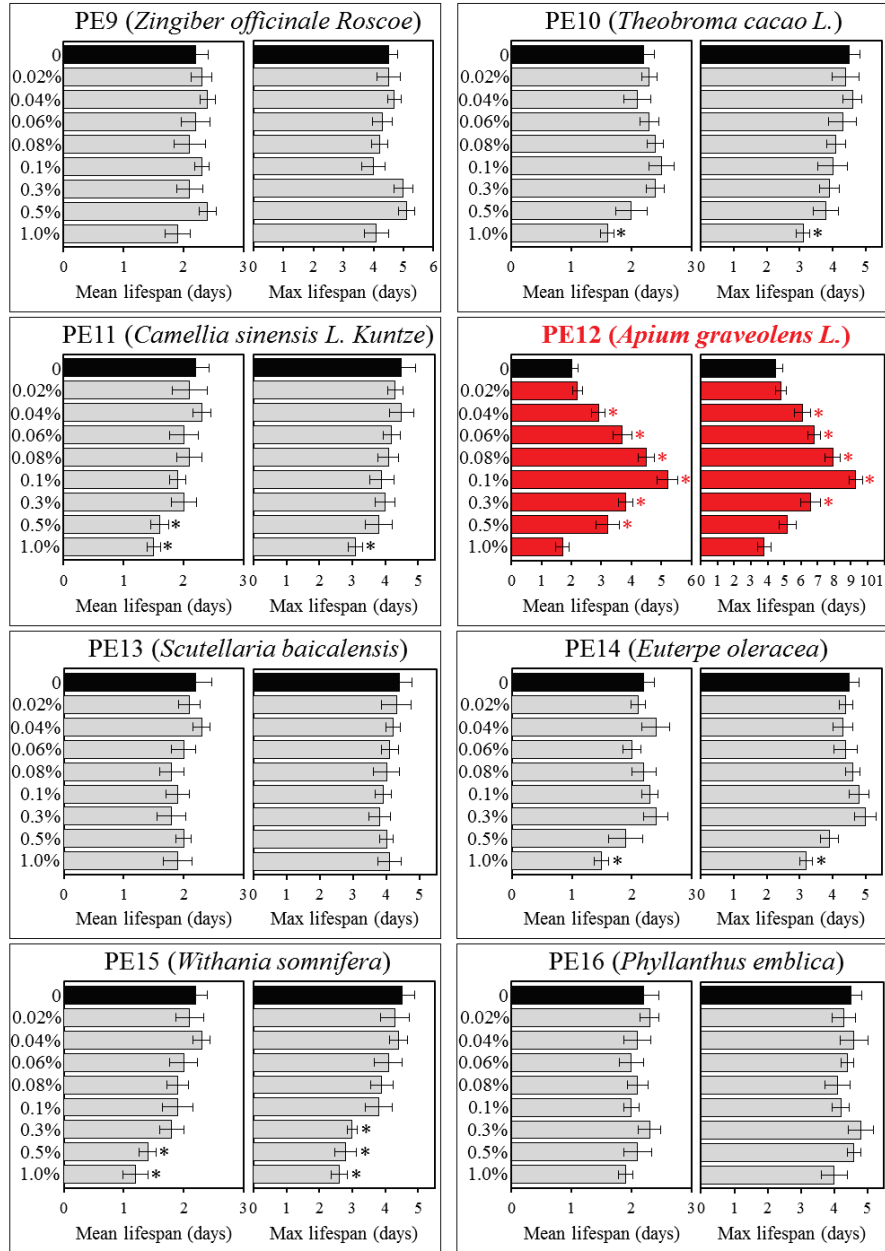


Figure 2.2. PE12, but not PE9, PE10, PE11, PE13, PE14, PE15 or PE16, extends the CLS of WT yeast grown under non-CR conditions. WT cells were grown in the synthetic minimal YNB medium initially containing 2% glucose (non-CR conditions), in the presence of a PE or in its absence. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means \pm SEM (n = 6-29; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test). Note that PE10, PE11, PE14 and PE15 can shorten the CLS of WT yeast under non-CR conditions if added at high concentrations (n = 6; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test).

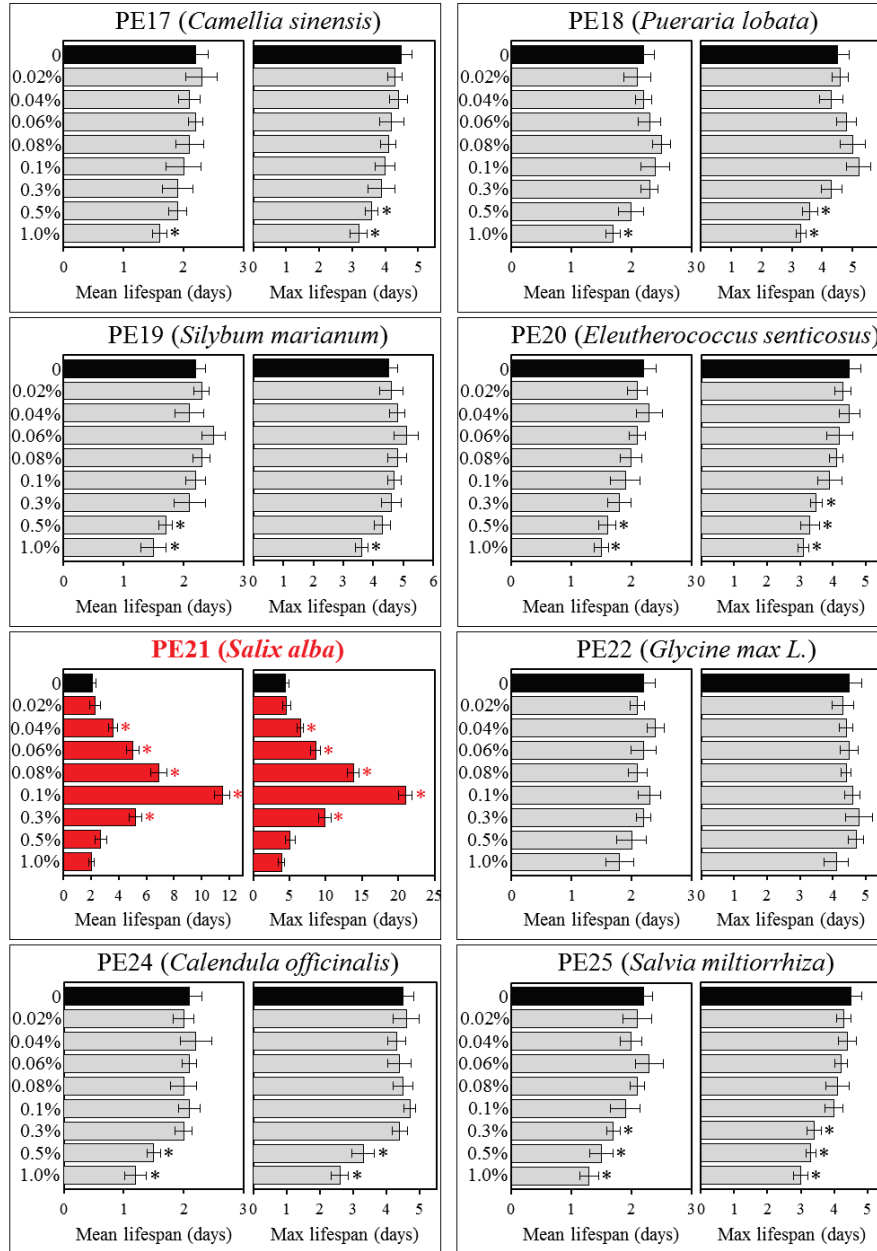


Figure 2.3. PE21, but not PE17, PE18, PE19, PE20, PE22, PE24 or PE25, extends the CLS of WT yeast grown under non-CR conditions. WT cells were grown in the synthetic minimal YNB medium initially containing 2% glucose (non-CR conditions), in the presence of a PE or in its absence. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means \pm SEM (n = 6-35; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test). Note that PE17, PE18, PE19, PE20, PE24 and PE25 can shorten the CLS of WT yeast under non-CR conditions if added at high concentrations (n = 6; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test).

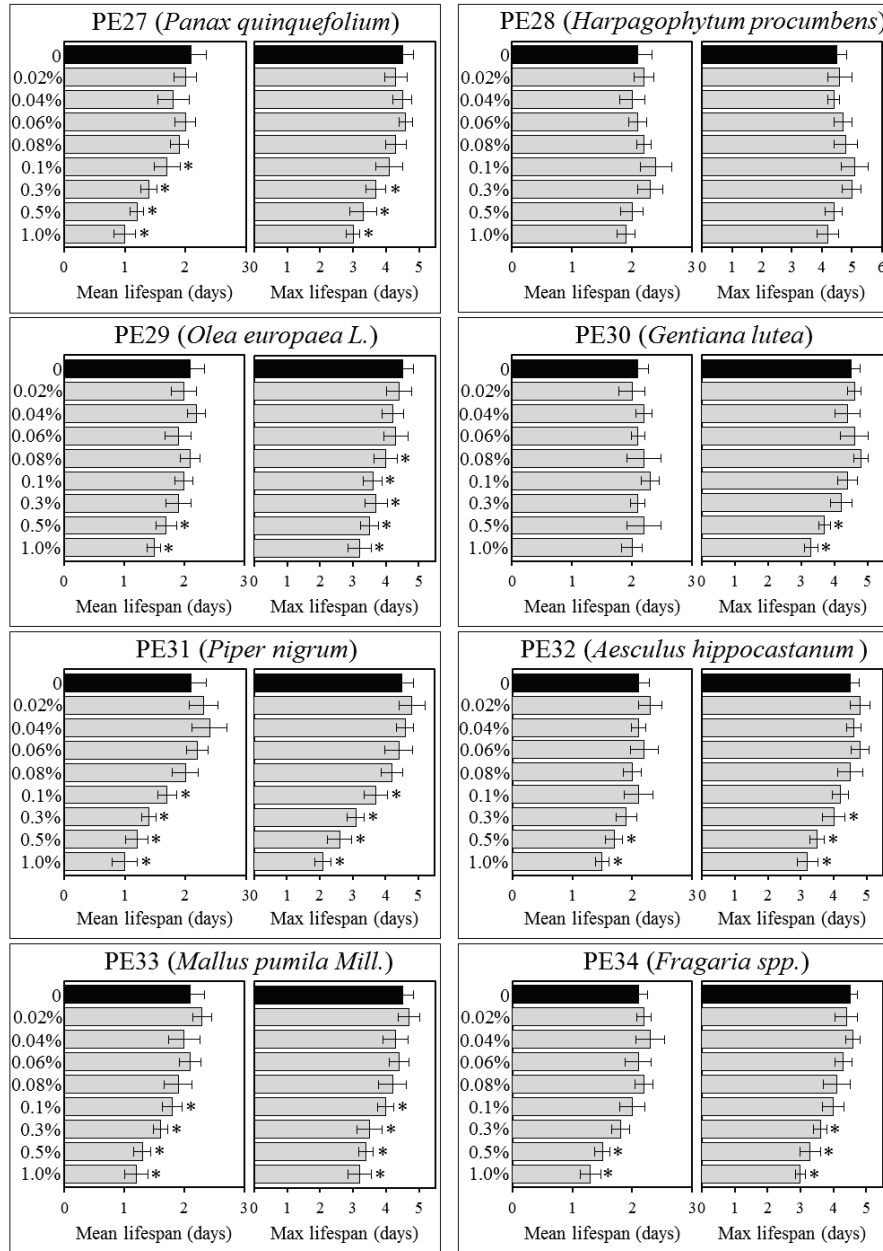


Figure 2.4. PE27, PE28, PE29, PE30, PE31, PE32, PE33 and PE34 do not extend the CLS of WT yeast grown under non-CR conditions. WT cells were grown in the synthetic minimal YNB medium initially containing 2% glucose (non-CR conditions), in the presence of a PE or in its absence. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means \pm SEM (n = 5-6; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test). Note that PE 27, PE29, PE30, PE31, PE32, PE33 and PE34 can shorten the CLS of WT yeast under non-CR conditions if added at high concentrations (n = 6; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test).

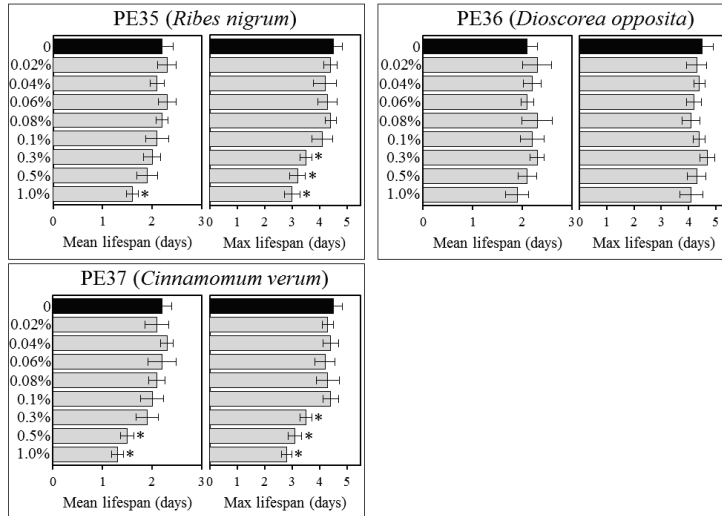


Figure 2.5. PE35, PE36 and PE37 do not extend the CLS of WT yeast grown under non-CR conditions. WT cells were grown in the synthetic minimal YNB medium initially containing 2% glucose (non-CR conditions), in the presence of a PE or in its absence. The mean and maximum lifespans of chronologically aging WT strain cultured under non-CR conditions without a PE or with a PE added at various concentrations are shown; data are presented as means \pm SEM ($n = 5-6$; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test). Note that PE 35 and PE37 can shorten the CLS of WT yeast under non-CR conditions if added at high concentrations ($n = 6$; * $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test).

PE6 from *Passiflora incarnata* L. (**Figure 2.1, Figure 2.6C, Figure 2.8A, Figure 2.8B**);
 4) 0.3% PE8 from *Ginkgo biloba* (**Figure 2.1, Figure 2.6D, Figure 2.8A, Figure 2.8B**);
 5) 0.1% PE12 from *Apium graveolens* L. (**Figure 2.2, Figure 2.6E, Figure 2.8A, Figure 2.8B**); and 6) 0.1% PE21 from *Salix alba* (**Figure 2.3, Figure 2.6F, Figure 2.8A, Figure 2.8B**). None of the six lifespan-prolonging PEs affected growth rates in logarithmic (L) and post-diauxic (PD) phases or impacted the maximum cell density in stationary (ST) phase of yeast cultures under non-CR conditions on 2% glucose (**Figure 2.9**). Thus, the observed lifespan extension by each of these PEs is unlikely to be caused by its ability to decrease growth rate or to make yeast more resistant to toxic substances accumulated during culturing in the synthetic minimal YNB medium.

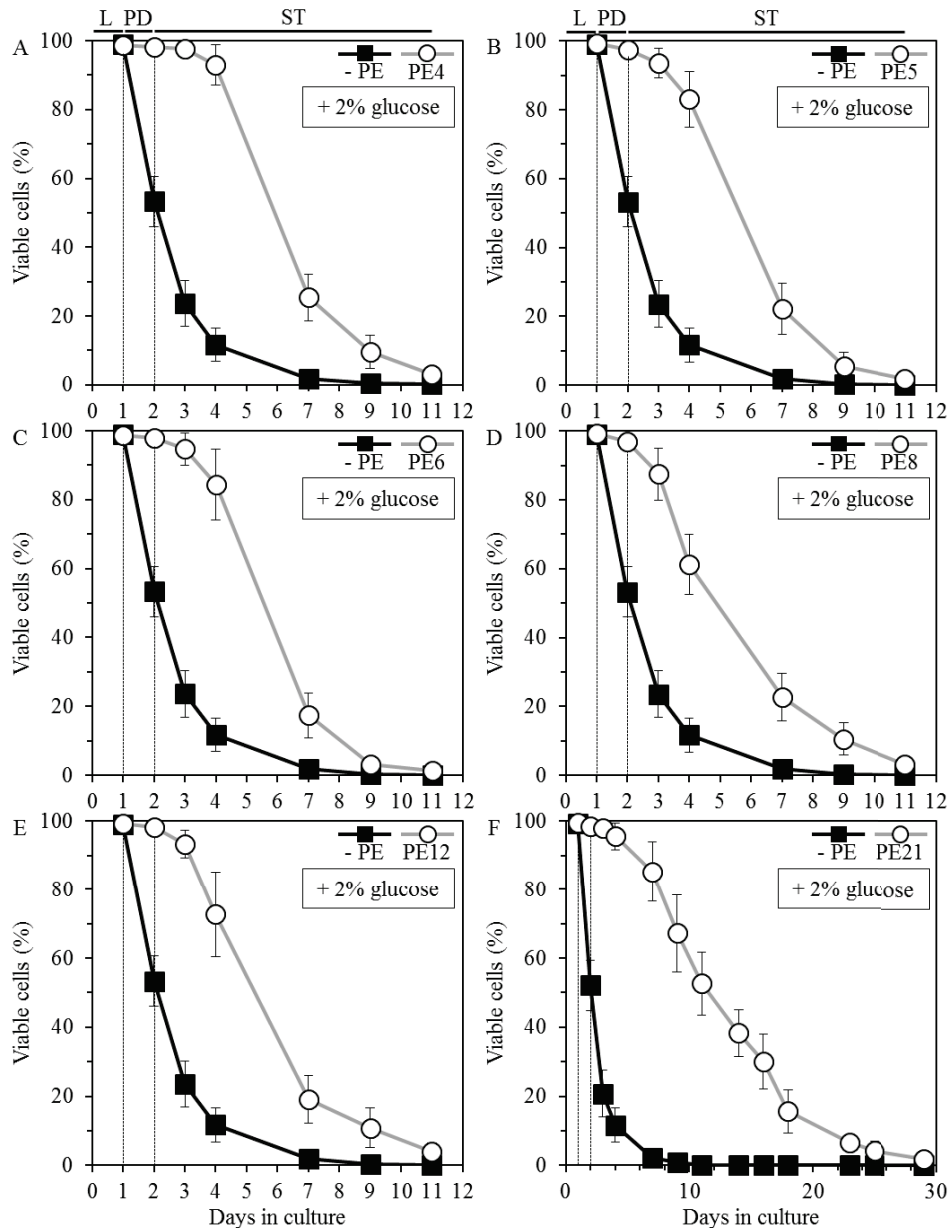


Figure 2.6. PE4, PE5, PE6, PE8, PE12 and PE21 extend the chronological lifespan (CLS) of yeast grown under non-caloric restriction (non-CR) conditions. Wild-type (WT) cells were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of a PE or in its absence. Survival curves of chronologically aging WT strain cultured with or without 0.5% PE4 (A), 0.5% PE5 (B), 1% PE6 (C), 0.3% PE8 (D), 0.1% PE12 (E) or 0.1% PE21 (F) are shown. Data are presented as means \pm SEM ($n = 21-35$). CLS extension was significant for each of the PEs tested ($p < 0.05$; the p values for comparing survival curves were calculated with the help of the GraphPad Prism statistics software). Abbreviations: Logarithmic (L), post-diauxic (PD) or stationary (ST) growth phase.

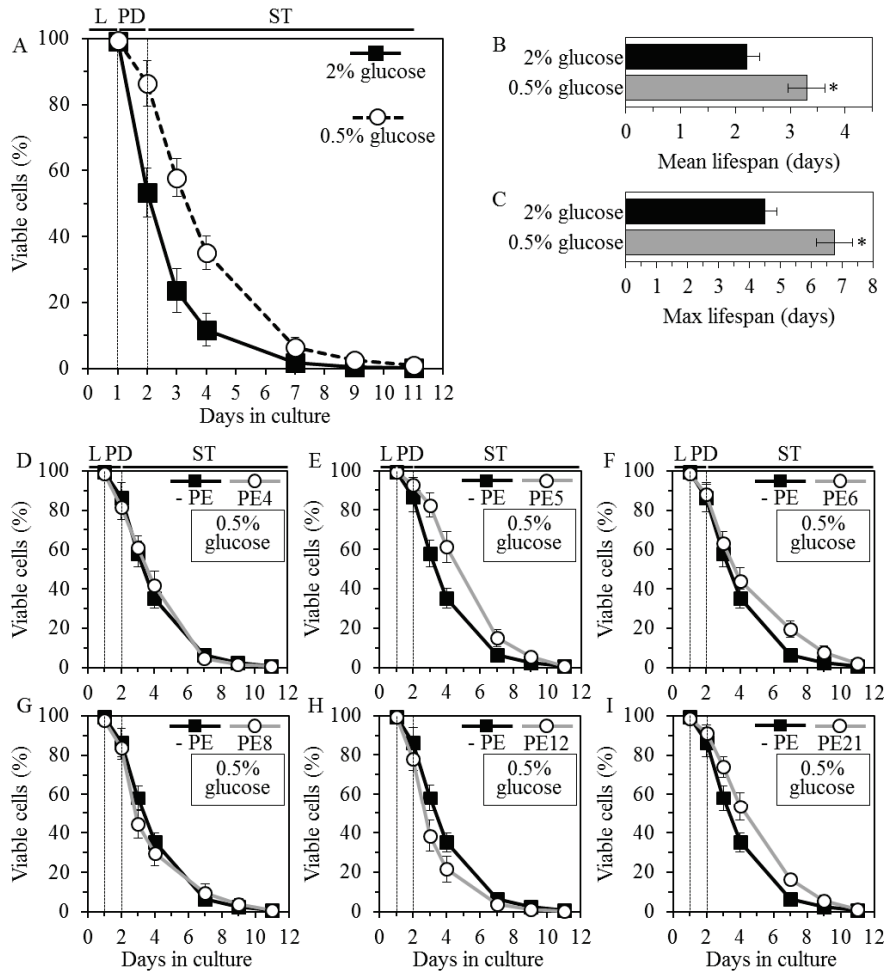


Figure 2.7. PE5 and PE21, but not PE4, PE6, PE8 or PE12, extend the CLS of yeast grown under CR conditions. WT cells were grown in the synthetic minimal YNB medium initially containing 0.5% glucose (CR conditions) or 2% glucose (non-CR conditions), in the presence of a PE or in its absence. Survival curves (A), the mean (B) and maximum (C) lifespans of chronologically aging WT strain cultured under CR or non-CR conditions in the absence of a PE are shown; data are presented as means \pm SEM ($n = 5-7$). CR caused significant extension of CLS (A) ($p < 0.05$; the p values for comparing survival curves were calculated with the help of the GraphPad Prism statistics software). CR extended both the mean (B) and maximum (C) lifespans ($* p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test). Survival curves of chronologically aging WT strain cultured under CR on 0.5% glucose with or without 0.5% PE4 (D), 0.5% PE5 (E), 1% PE6 (F), 0.3% PE8 (G), 0.1% PE12 (H) or 0.1% PE21 (I) are shown; data are presented as means \pm SEM ($n = 5-7$). CLS extension under CR on 0.5% glucose was significant for PE5 and PE21 ($p < 0.05$; the p values for comparing survival curves were calculated with the help of the GraphPad Prism statistics software). CLS extension under CR on 0.5% glucose was not significant for PE4, PE6, PE8 and PE12. Abbreviations: Logarithmic (L), post-diauxic (PD) or stationary (ST) growth phase.

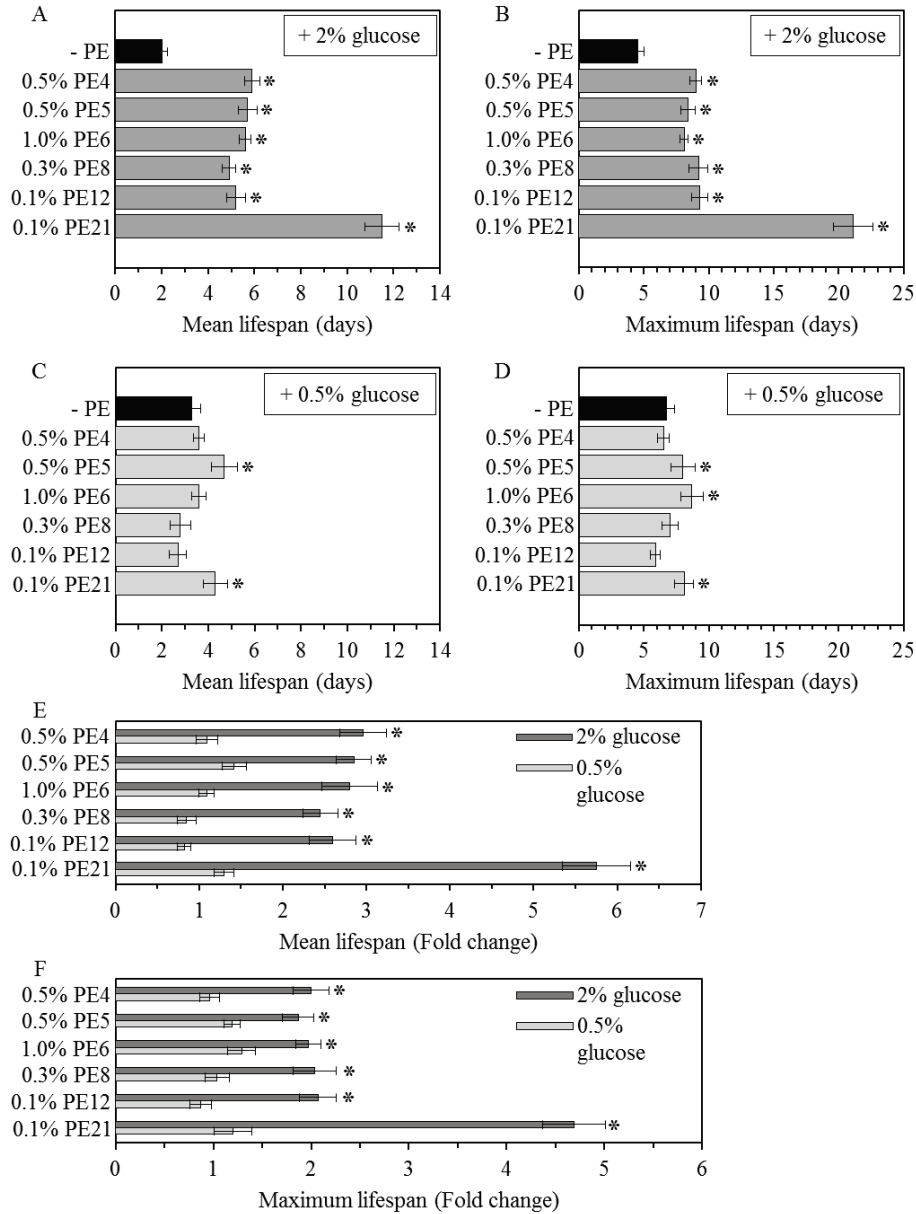


Figure 2.8. The longevity-extending efficacy under non-CR conditions significantly exceeds that under CR conditions for each of the six lifespan-prolonging PEs. WT cells were grown in the synthetic minimal YNB medium initially containing 0.5% glucose (CR conditions) or 2% glucose (non-CR conditions), in the presence of a PE or in its absence. The mean (A, C and E) and maximum (B, D and F) lifespans of chronologically aging WT strain cultured under CR (C, D, E and F) or non-CR (A, B, E and F) conditions in the absence of a PE or in the presence of 0.5% PE4, 0.5% PE5, 1% PE6, 0.3% PE8, 0.1% PE12 or 0.1% PE21 are shown; data are presented as means \pm SEM ($n = 5-7$; * $p < 0.05$). The extent to which each of the PE tested increases the mean and maximum lifespans under non-CR conditions exceeds that under CR conditions (* $p < 0.05$; the p values for comparing the means of two groups were calculated with the help of the GraphPad Prism statistics software using an unpaired two-tailed t test).

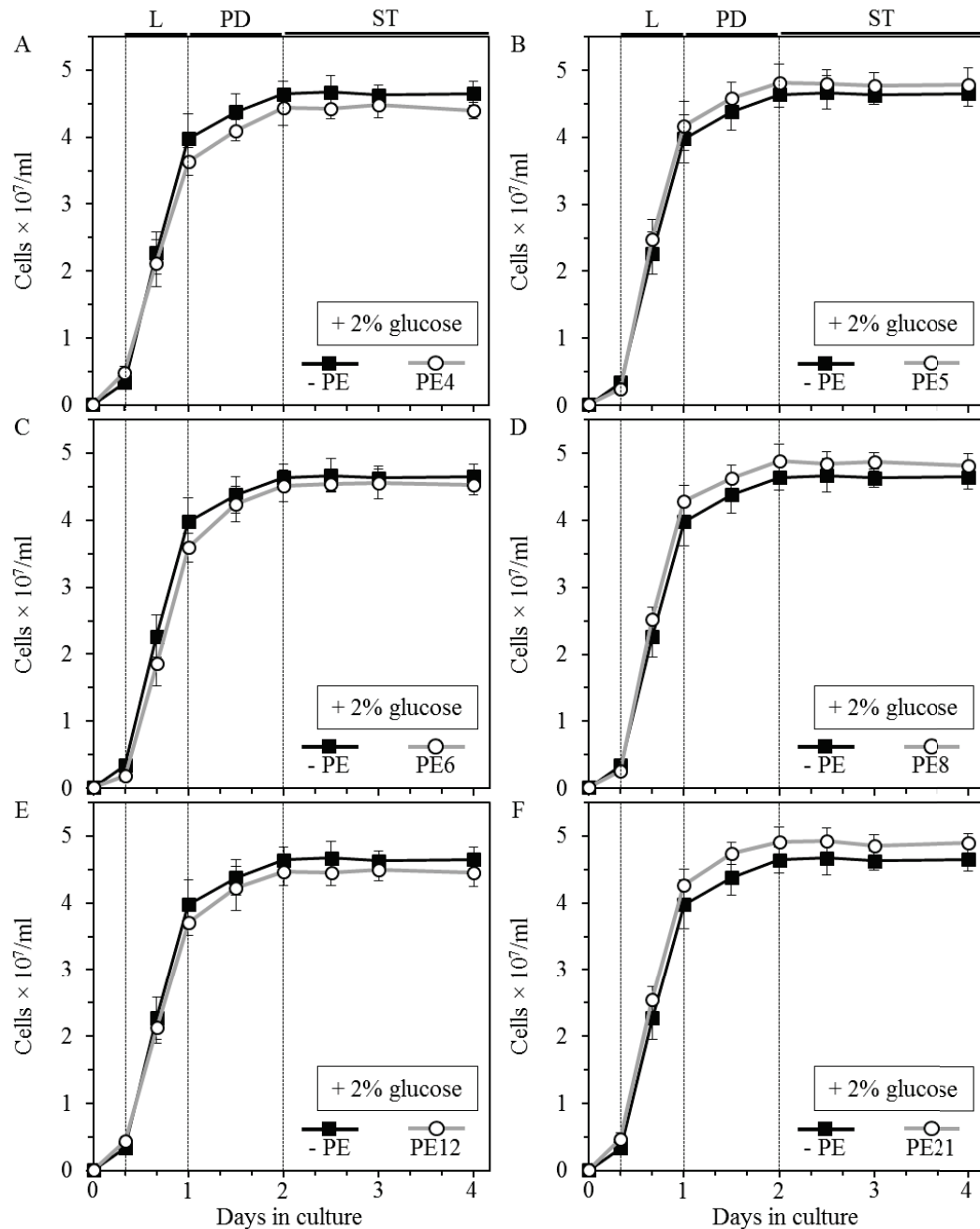


Figure 2.9. PE4, PE5, PE6, PE8, PE12 and PE21 do not cause significant effects on growth of WT yeast under non-CR conditions. WT cells were grown in the synthetic minimal YNB medium initially containing 2% glucose (non-CR conditions), in the absence of a PE or in the presence of 0.5% PE4 (A), 0.5% PE5 (B), 1% PE6 (C), 0.3% PE8 (D), 0.1% PE12 (E) or 0.1% PE21 (F). Kinetics of cell growth is shown (n = 8-14). Abbreviations: Logarithmic (L), post-diauxic (PD) or stationary (ST) growth phase.

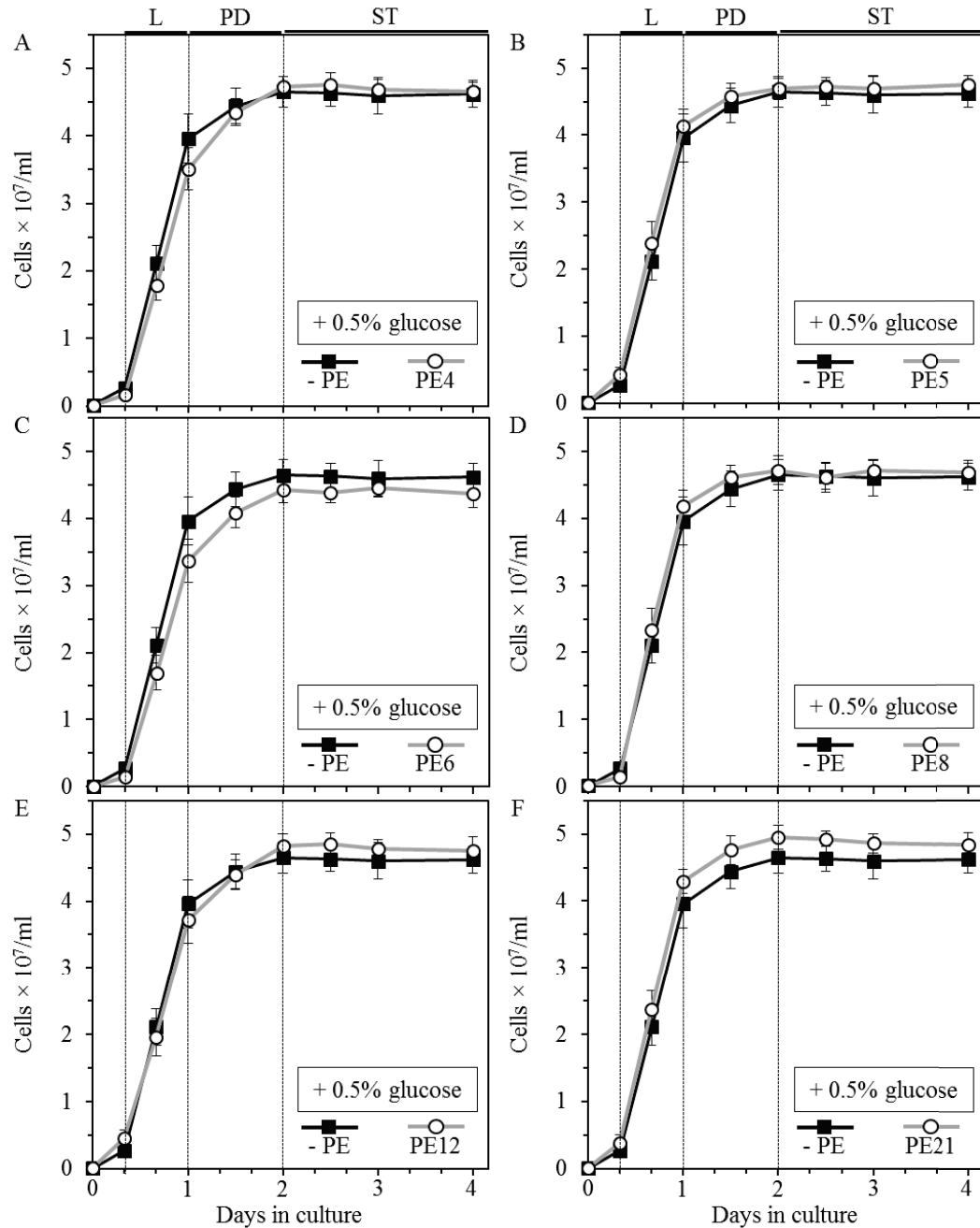


Figure 2.10. PE4, PE5, PE6, PE8, PE12 and PE21 do not cause significant effects on growth of WT yeast under CR conditions. WT cells were grown in the synthetic minimal YNB medium initially containing 0.5% glucose (CR conditions), in the absence of a PE or in the presence of 0.5% PE4 (A), 0.5% PE5 (B), 1% PE6 (C), 0.3% PE8 (D), 0.1% PE12 (E) or 0.1% PE21 (F). Kinetics of cell growth is shown (n = 6-9). Abbreviations: Logarithmic (L), post-diauxic (PD) or stationary (ST) growth phase.

2.3.2 For each of the six lifespan-prolonging PEs, the longevity-extending efficacy under CR conditions is significantly lower than that under non-CR conditions

Chronologically aging yeast grown under CR conditions on 0.5% glucose are known to live longer than yeast cultured under non-CR conditions on 2% glucose. Such ability of the CR diet to extend CLS has been reported for yeast cultured in media of various nutrient compositions [2, 4, 5]. I found that, if the CR diet is administered by culturing yeast in the YNB medium initially containing 0.5% glucose, it significantly increases both the mean and maximum CLS of *S. cerevisiae* (**Figure 2.7A – Figure 2.7C**). I discovered that 0.5% PE5 and 0.1% PE21 (but not 0.5% PE4, 1.0% PE6, 0.3% PE8 or 0.1% PE12) extend the mean CLS of yeast grown under CR conditions (**Figure 2.7D – Figure 2.7I, Figure 2.8C**). I also revealed that 0.5% PE5, 1.0% PE6 and 0.1% PE21 (but not 0.5% PE4, 0.3% PE8 or 0.1% PE12) extend the maximum CLS of yeast grown under CR conditions (**Figure 2.7D – Figure 2.7I, Figure 2.8D**). Akin to their effects under non-CR conditions, none of the six lifespan-prolonging PEs influenced growth rates in L and PD phases or altered the maximum cell density in ST phase of yeast cultures under CR conditions on 0.5% glucose (**Figure 2.10**). Importantly, each of the six lifespan-prolonging PEs extended both the mean and maximum CLS of yeast cultures under non-CR conditions on 2% glucose to a significantly higher extent than those of yeast under CR at 0.5% glucose (**Figure 2.8A – Figure 2.8F**). This observation indicates that each of these PEs could mimic the longevity-extending effect of CR.

2.3.3 Each of the six longevity-extending PEs is a geroprotector which delays the onset and slows the progression of yeast chronological aging by eliciting a hormetic stress response

As I found, PE4, PE5, PE6, PE8, PE12 and PE21 greatly extend the mean CLS of yeast cultured under non-CR conditions (**Figure 2.6A, Figure 2.8A, Figure 2.8B, Figure 2.1 – Figure 2.3**). Mean lifespan is believed to be directly proportional to the survival rates of organisms in the population during development and maturity stages of organismal aging; mean lifespan is likely to be under control of certain extrinsic (environmental) factors [22, 28 - 30]. Thus, it is conceivable that PE4, PE5, PE6, PE8, PE12 and PE21 decrease the extrinsic rate of yeast chronological aging prior to cell entry into quiescence or senescence.

Furthermore, I revealed that PE4, PE5, PE6, PE8, PE12 and PE21 also substantially increase the maximum CLS of yeast grown under non-CR conditions (**Figure 2.1 – Figure 2.3, Figure 2.6A, Figure 2.8A**). Maximum lifespan is believed to reflect the duration of “healthy” life period (i.e. healthspan) during quiescence/senescence stage of organismal aging; maximum lifespan is likely to be controlled by certain intrinsic (cellular and organismal) longevity modifiers [1, 22, 28, 29, 31, 32]. One could therefore conclude that PE4, PE5, PE6, PE8, PE12 and PE21 also decrease the intrinsic rate of yeast chronological aging after cell entry into quiescence or senescence.

My analysis of the Gompertz mortality function further validated the above conclusion that PE4, PE5, PE6, PE8, PE12 and PE21 significantly reduce the rate of yeast chronological aging. Indeed, I found that each of these longevity-extending PEs

causes a substantial decrease in slope of the Gompertz mortality rate (also known as mortality rate coefficient α) and a considerable increase in the mortality rate doubling time (MRDT) (**Figure 2.11A – Figure 2.11G**). Such changes in the values of α and MRDT are characteristic of interventions that decrease the rate of progression through the process of biological aging [22, 23 - 26].

Noteworthy, my analyses of how different concentrations of PE4, PE5, PE6, PE8, PE12 and PE21 impact yeast longevity under non-CR conditions revealed that each of them causes a so-called “hormetic” stress response in chronologically aging yeast with respect to longevity. Indeed, the dose-response curves (i.e. the curves that reflect relationships between PE concentrations and mean or maximum CLS) for PE4, PE5, PE8, PE12 and PE21 were inverted U-shaped, whereas the dose-response curve for PE6 was J-shaped (**Figure 2.1 – Figure 2.3**). Such nonlinear and biphasic dose-response curves denote a hormetic kind of stress response, in which 1) lower (hormetic) concentrations of a chemical compound increase the survival of a cell or an organism by stimulating biological processes that allow to maintain cellular or organismal stress at a level which is below a threshold of toxicity; while 2) higher concentrations of this chemical compound decrease the survival of a cell or an organism by creating stress which exceeds such threshold [7, 45 - 48].

2.4 Discussion

In studies described in this chapter of my Thesis, I performed a screen for PEs capable of extending longevity of the chronologically aging yeast *S. cerevisiae*. My screen revealed six PEs (which I call PE4, PE5, PE6, PE8, PE12 and PE21) that can significantly

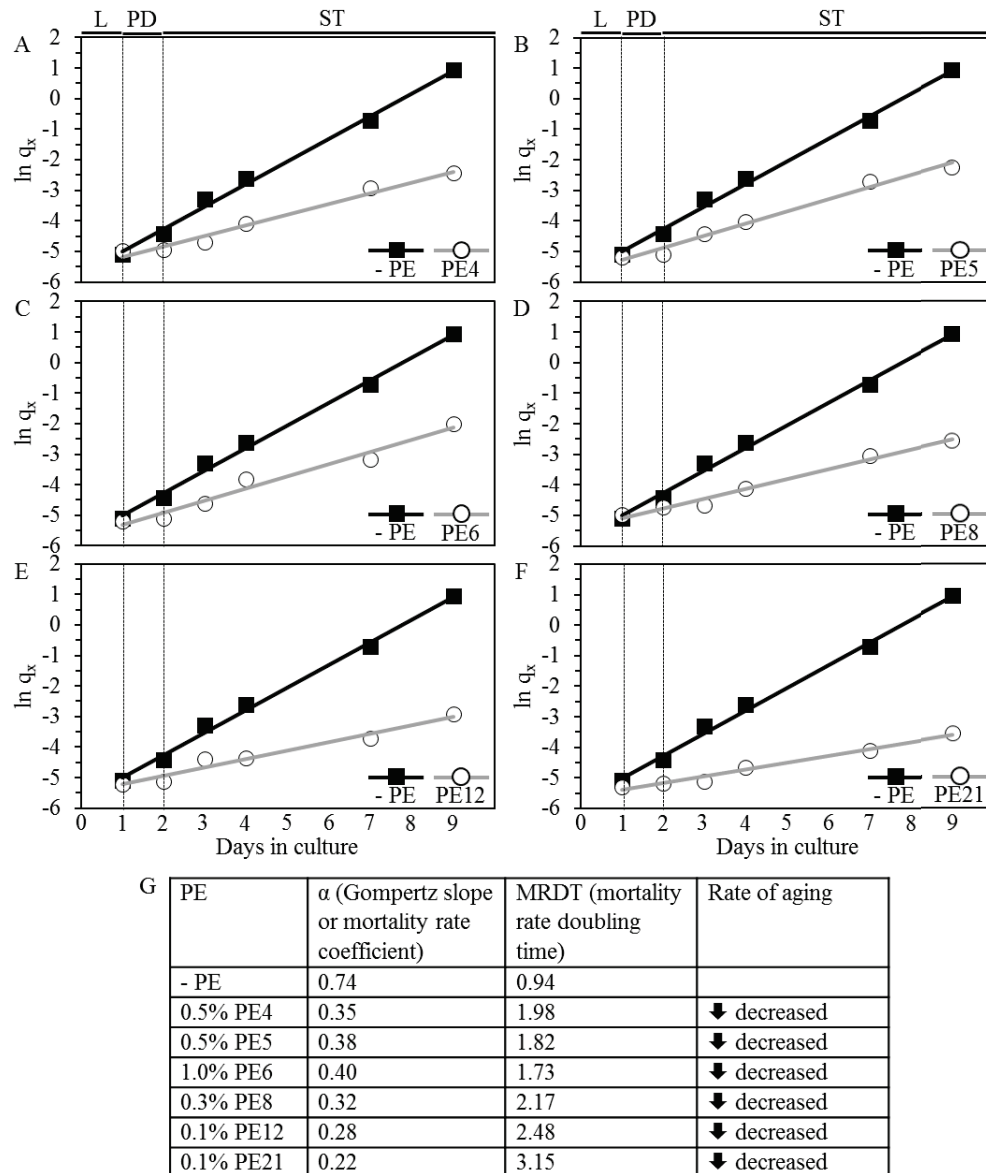


Figure 2.11. Analysis of the Gompertz mortality function indicates that PE4, PE5, PE6, PE8, PE12 and PE21 significantly decrease the rate of chronological aging in yeast. WT cells were grown in the synthetic minimal YNB medium initially containing 2% glucose, in the presence of a PE or in its absence. Survival curves shown in Figs. 1A - 1F were used to calculate the age-specific mortality rates (q_x) of chronologically aging WT yeast populations cultured with or without 0.5% PE4 (A), 0.5% PE5 (B), 1% PE6 (C), 0.3% PE8 (D), 0.1% PE12 (E) or 0.1% PE21 (F). Each of these longevity-extending PEs caused a substantial decrease in slope of the Gompertz mortality rate (also known as mortality rate coefficient α) and a considerable increase in the mortality rate doubling time (MRDT) (G). The values of q_x , α and MRDT were calculated as described in Materials and methods. Abbreviations: Logarithmic (L), post-diauxic (PD) or stationary (ST) growth phase.

increase yeast CLS. I demonstrated that each of these PEs is a geroprotector which delays the onset and slows the progression of yeast chronological aging by eliciting a hormetic stress response. My findings imply that the efficiency of longevity extension by PE4, PE5, PE6, PE8, PE12 or PE21 greatly exceeds that for any of the 42 chemical compounds known to increase lifespan in yeast, filamentous fungi, nematodes, fruit flies, daphnias, mosquitoes, honey bees, fish, mammals and cultured human cells (**Table 2.3**). Indeed, under non-CR conditions these longevity-extending PEs increase the mean and maximum CLS of yeast by 145% - 475% and 80% - 369%, respectively (**Figure 2.8A, Figure 2.8B, Table 2.3**); the corresponding rows in **Table 2.3** are highlighted in yellow. In contrast, any of the 42 currently known lifespan-extending chemical compounds has been shown to extend cellular and/or organismal lifespan in evolutionarily distant eukaryotes much less efficiently, within the 5% to 75% range (**Table 2.3**) [45 – 105]. Only two chemical compounds, spermidine under non-CR conditions and lithocholic acid under CR-conditions, have been reported to exhibit the lifespan-extending efficiencies that are comparable to those for PE4, PE5, PE6, PE8 and PE12 (**Table 2.3**) [72, 73, 102]. Specifically, both these pharmacological interventions were demonstrated to increase the RLS and/or CLS of yeast and human peripheral blood mononuclear cells by 83% - 200%; the corresponding rows in **Table 2.3** are highlighted in green. Of note, PE21 appears to be the most potent longevity-extending pharmacological intervention presently known. It increases the mean and maximum CLS of yeast by 475% and 369%, respectively (**Figure 2.8A, Figure 2.8B, Table 2.3**).

In the future, it would be important to further explore the following key aspects of the mechanisms through which each of the six longevity-extending PEs slows biological aging.

First, it is intriguing to identify the individual chemical compounds responsible for the ability of each of these PEs to delay the onset and decrease the rate of yeast chronological aging. Such identification is already underway in the Titorenko laboratory; of note, it is conceivable that only some combinations of certain chemicals composing these PEs (but not individual chemical compounds *per se*) can be responsible for their extremely high efficiencies as aging-delaying interventions.

Second, it is important to investigate how various combinations of the six longevity-extending PEs with each other and with presently known aging-delaying chemical compounds alter the extent of CLS extension in yeast. These studies may identify such combinations of various pharmacological interventions that impose substantial additive or synergistic effects on the efficiencies with which organismal lifespan and healthspan can be prolonged.

Third, the ongoing studies in the Titorenko laboratory and in the laboratories of his collaborators indicate that the six longevity-extending PEs also extend longevity of other eukaryotic model organisms, delay the onset of age-related diseases and/or exhibit anti-tumor effects. In this regard, it needs to be mentioned that genetic, dietary and pharmacological interventions known to delay aging in yeast and other eukaryotes have been shown to selectively kill cultured human cancer cells and/or decrease the incidence of cancer [106 - 119]. The challenge for the future is to define mechanisms through which the six geroprotective PEs prolong healthy lifespan and decelerate tumorigenesis.

Table 2.3. Percent increase of lifespan by geroprotective PEs discovered in this study and by longevity-extending chemical compounds that have been previously identified.

| PE or chemical compound | % increase of lifespan* [reference] | Organism exhibiting lifespan increase |
|--------------------------------|---|--|
| 0.5% PE4 | 195% (mean CLS) [this study] 100% (max** CLS) [this study] | <i>S. cerevisiae</i> |
| 0.5% PE5 | 185% (mean CLS) [this study] 87% (max CLS) [this study] | <i>S. cerevisiae</i> |
| 1.0 % PE6 | 180% (mean CLS) [this study] 80% (max CLS) [this study] | <i>S. cerevisiae</i> |
| 0.3% PE8 | 145% (mean CLS) [this study] 104% (max) [this study] | <i>S. cerevisiae</i> |
| 0.1% PE12 | 160% (mean CLS) [this study] 107% (max CLS) [this study] | <i>S. cerevisiae</i> |
| 0.1% PE21 | 475% (mean CLS) [this study] 369% (max CLS) [this study] | <i>S. cerevisiae</i> |
| Acteoside | 24 - 25% (female); 16 - 18% (male) (mean OLS) [45] 9 - 13% (female); 9 - 15% (male) (max OLS) [45] | The fruit fly <i>Drosophila melanogaster</i> |
| Butein | 55% (mean RLS) [46] | <i>S. cerevisiae</i> |
| Caffeic acid | 11% (mean OLS) [47] | The nematode <i>Caenorhabditis elegans</i> |
| Caffeine | 8% (median CLS) [48] | <i>S. cerevisiae</i> |
| | 46% (mean CLS) [49] 17% (max CLS) [49] | The yeast <i>Schizosaccharomyces pombe</i> |
| | 52% (mean OLS) [50]; 37% (mean OLS) [51] 29% (median OLS) [50] | <i>C. elegans</i> |
| Catechin | 15% (mean OLS) [52] 14% (median OLS) [52] | <i>C. elegans</i> |
| Celastrol | 13% (mean OLS) [53] | Mouse model of amyotrophic lateral sclerosis |
| Curcumin, tetrahydrocurcumin | 13% - 39% (mean OLS) [54] | <i>C. elegans</i> |
| | 26% (females; mean OLS); 16% (males; mean OLS) [55, 56] 75% (median OLS) [57] | <i>D. melanogaster</i> , including 5 different models of the Alzheimer's disease |
| | | |
| Crocin | 37% - 58% (max OLS) [58] | Dalton's lymphoma ascites bearing mice |
| Cryptotanshinone | 34% (mean CLS) [59] | <i>S. cerevisiae</i> |
| Cyanidin | 2% - 6% (max RLS; untreated cells); 14 - 21% (max RLS; | WI-38 human diploid |

| | | |
|---------------------------|---|--|
| | prematurely aging cells pre-treated with H ₂ O ₂) [60] | fibroblasts |
| Diallyl trisulfide | 13% (mean OLS) [61] | <i>C. elegans</i> |
| Ellagic acid | 9% (mean OLS); 11% - 13% (median OLS) [62] | <i>C. elegans</i> |
| Epigallocatechin gallate | 10% - 14% (mean OLS) [63] | <i>C. elegans</i> |
| Epicatechin | 42% (mean OLS) [64] 3% (max OLS) [64] | <i>D. melanogaster</i> |
| | 8% (mean OLS) [64] | Obese diabetic mice |
| Ferulsinaic acid | 18% (mean CLS) [65] 42% (max OLS) [65] | <i>C. elegans</i> |
| | | |
| Fisetin | 31% (mean RLS) [46] | <i>S. cerevisiae</i> |
| | 6% (mean OLS) [66] | <i>C. elegans</i> |
| | 6% (median OLS) [66] | |
| Fucoxanthin | 14% (mean OLS) [67] 24% (max OLS) [67] | <i>C. elegans</i> |
| | 33% - 49% (females; median OLS); 33% (males; median OLS) [67] | <i>D. melanogaster</i> |
| | 22% - 27% (females; max OLS); 12% - 17% (males; max OLS) [67] | |
| Gallic acid | 12% (mean OLS) [62] 14% (median OLS) [62] | <i>C. elegans</i> |
| | | |
| HDTIC-1, HDTIC-2 | 14% - 38% (max RLS) [68] | Human fetal lung diploid fibroblasts |
| Icariin, icaraside II | 31% (mean OLS) [69] | <i>C. elegans</i> |
| Kaempferol | 10% (mean OLS) [66] 6% (median OLS) [66] | <i>C. elegans</i> |
| | | |
| Lipoic acid | 21% (median OLS) [70] | <i>C. elegans</i> |
| | 12% (females; average OLS); 15% (females; median OLS) [71] | <i>D. melanogaster</i> |
| | 4% (males; average OLS); 4% (males; median OLS) [71] | |
| Lithocholic acid | 146% (mean CLS) [72, 73] 100% (max CLS) [72, 73] | <i>S. cerevisiae</i> |
| | | |
| Metformin | 40% (median OLS) [74] | <i>C. elegans</i> |
| | 38% (mean OLS) [75] | Mice |
| | 10% (max OLS) [75] | |
| Methionine sulfoximine | 78% (mean CLS) [76] 63% (max CLS) [76] | <i>S. cerevisiae</i> |
| | | |
| Mianserin | 25% (mean OLS) [77] | <i>C. elegans</i> |
| Myricetin | 15% (mean OLS) [78] 17% (median OLS) [78] 24% (max OLS) [78] | <i>C. elegans</i> |
| | | |
| | | |
| Nordihydroguaiaretic acid | 10% (median OLS) [79] 32% (max OLS) [79] | Mouse model of the Alzheimer's disease |
| | 12% (median OLS) [80] | Male mice |
| | 12% (mean OLS) [81] | <i>D. melanogaster</i> |

| | | |
|----------------------|--|--|
| | 42% - 64% (mean OLS) [82] | Mosquitoes |
| Oleuropein | 15% (max RLS) [83] | Human embryonic fibroblasts |
| Phloridzin | 35% (mean RLS) [84] 41% (max RLS) [84] | <i>S. cerevisiae</i> |
| Propyl gallate | 41% (median OLS) [70] | <i>C. elegans</i> |
| Quercetin | 60% (mean CLS) [85] | <i>S. cerevisiae</i> |
| | 15% (mean OLS) [86] | <i>C. elegans</i> |
| | 18% (mean OLS) [87] | |
| | 14% (median OLS) [88] | |
| | 5% (max RLS) [89] | Human embryonic fibroblasts |
| Rapamycin | 16% (mean RLS) [90] | <i>S. cerevisiae</i> |
| | 36% (mean CLS) [91] | |
| | 17% (mean OLS) [92] | <i>D. melanogaster</i> |
| | 23% (max OLS) [92] | |
| | 13% (females; mean OLS); 9% (males; mean OLS) [93] 14% (females; max OLS); 9% (males; max OLS) [93] | Mice |
| Reserpine | 64% (mean OLS); 50% (max OLS) [94] | <i>C. elegans</i> |
| | 52% (mean OLS) [95] | <i>C. elegans</i> model of the Alzheimer's disease |
| Resveratrol | 61% (mean RLS) [46] | <i>S. cerevisiae</i> |
| | 10% (mean OLS) [96] | <i>C. elegans</i> |
| | 20% (females; mean OLS); 16% (males; mean OLS) [96] | <i>D. melanogaster</i> |
| | 56% (median OLS) [97] | The short-lived fish <i>Nothobranchius furzeri</i> |
| | 59% (max OLS) [97] | |
| | 38% (mean OLS) [98] | The honey bee <i>Apis mellifera</i> |
| | 4% (max OLS) [99] | Mice on a high-calorie diet |
| Rosmarinic acid | 10% (mean OLS) [47] | <i>C. elegans</i> |
| SkQ1 | 38% (mean CLS) [100] | The fungus <i>Podospora anserina</i> |
| | 16% (max CLS) [101] | |
| | 69% (mean CLS) [101] | The crustacean <i>Ceriodaphnia affinis</i> |
| | 64% (max CLS) [100] | |
| | 13% (females; mean OLS) [100] | <i>D. melanogaster</i> |
| | 7% (females; max OLS) [100] | |
| | 43% (mean OLS) [100] 58% (max OLS) [100] | p53-Deficient mice |
| | 52% (mean OLS) [100] 34% (max OLS) [100] | Tumor-bearing immunodeficient mice |
| Sodium nitroprusside | 60% (max RLS) [101] | Human peripheral blood mononuclear cells |
| Spermidine | 200% (mean CLS) [102] | <i>S. cerevisiae</i> |

| | | |
|---------------|--|--|
| | 183% (mean RLS) [102] 17% (max RLS) [102] | |
| | 15% (mean OLS) [102] 14% (max OLS) [102] | <i>C. elegans</i> |
| | 30% (mean OLS) [102] 8% (max OLS) [102] | <i>D. melanogaster</i> |
| | 178% (max RLS) [102] | Human peripheral blood mononuclear cells |
| Tannic acid | 18% - 25% (mean OLS) [50, 62, 103]; 18% (median OLS) [62, 103] 59% (max OLS) [50] | <i>C. elegans</i> |
| Taxifolin | 26% (median OLS) [70] | <i>C. elegans</i> |
| Trolox | 15% (median OLS) [70] | <i>C. elegans</i> |
| Tyrosol | 21% (mean OLS); 21% (median OLS) [104] 11% (maximum OLS) [104] | <i>C. elegans</i> |
| Valproic acid | 35% (mean OLS) [105] 42% (max OLS) [105] | <i>C. elegans</i> |

* Chronological or replicative lifespan (CLS or RLS, respectively) of cell cultures or organismal lifespan (OLS).

** Abbreviation: max, maximum.

3 Six plant extracts delay yeast chronological aging through different signaling pathways

3.1 Abstract

Studies described in chapter 2 of my Thesis have revealed six plant extracts that slow yeast chronological aging more efficiently than any chemical compound yet described. The rate of aging in yeast is controlled by an evolutionarily conserved network of integrated signaling pathways and protein kinases. In studies described in this chapter of my Thesis, I assessed how single-gene-deletion mutations eliminating each of these pathways and kinases affect the aging-delaying efficiencies of the six plant extracts. My findings imply that these extracts slow aging in the following ways: 1) plant extract 4 (PE4) decreases the efficiency with which the pro-aging TORC1 pathway inhibits the anti-aging SNF1 pathway; 2) plant extract 5 (PE5) mitigates two different branches of the pro-aging PKA pathway; 3) plant extract 6 (PE6) coordinates processes that are not assimilated into the network of presently known signaling pathways/protein kinases; 4) plant extract 8 (PE8) diminishes the inhibitory action of PKA on SNF1; 5) plant extract 12 (PE12) intensifies the anti-aging protein kinase Rim15; and 6) plant extract 21 (PE21) inhibits a form of the pro-aging protein kinase Sch9 that is activated by the pro-aging PKH1/2 pathway.

3.2 Materials and Methods

Yeast strains, media and growth conditions

The wild-type strain *Saccharomyces cerevisiae* BY4742 (*MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0*) and single-gene-deletion mutant strains in the BY4742 genetic background (all from Thermo Scientific/Open Biosystems) were grown in a synthetic minimal YNB medium (0.67% (w/v) Yeast Nitrogen Base without amino acids) initially containing 2% (w/v) glucose and supplemented with 20 mg/l histidine, 30 mg/l leucine, 30 mg/l lysine and 20 mg/l uracil. Cells were cultured at 30°C with rotational shaking at 200 rpm in Erlenmeyer flasks at a “flask volume/medium volume” ratio of 5:1.

Aging-delaying PEs

0.5% (w/v) PE4 from *Cimicifuga racemosa*, 0.5% (w/v) PE5 from *Valeriana officinalis* L., 1.0% (w/v) PE6 from *Passiflora incarnata* L., 0.3% (w/v) PE8 from *Ginkgo biloba*, 0.1% (w/v) PE12 from *Apium graveolens* L. and 0.1% (w/v) PE21 from *Salix alba* were used. A 20% (w/v) stock solution of each PE in ethanol was made on the day of adding this PE to cell cultures. For each PE, the stock solution was added to growth medium with 2% (w/v) glucose immediately following cell inoculation into the medium.

CLS assay

A sample of cells was taken from a culture at a certain day following cell inoculation and PE addition into the medium. A fraction of the sample was diluted in order to determine the total number of cells using a hemacytometer. Another fraction of the cell sample was

diluted and serial dilutions of cells were plated in duplicate onto YEP (1% yeast extract, 2% peptone) plates containing 2% glucose as carbon source. After 2 d of incubation at 30°C, the number of colony forming units (CFU) per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each culture, the percentage of viable cells was calculated as follows: (number of viable cells per ml/total number of cells per ml) \times 100. The percentage of viable cells in mid-logarithmic growth phase was set at 100%.

Gompertz mortality function analysis

The age-specific mortality rate (q_x) [22, 24, 25], Gompertz slope or mortality rate coefficient (α) [23, 24, 25], and mortality rate doubling time (MRDT) [24, 26] were calculated as previously described. The value of q_x was calculated as the number of cells that lost viability (i.e. are unable to form a colony on the surface of a solid nutrient-rich medium) during each time interval divided by the number of viable (i.e. clonogenic) cells at the end of the interval. The natural logarithms of the values of q_x for each time interval were plotted against time. The value of α was calculated as the slope of the Gompertz mortality line, whereas the value of MRDT was calculated as $\ln 2/\alpha$.

Statistical analysis

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean \pm SEM. The p values for comparing the means of two groups (using an unpaired two-tailed t test) and survival curves (using a two-tailed t test) were calculated with the help of the GraphPad Prism statistics software.

3.3 Results

3.3.1 The rationale of my experimental approach

PE4, PE5, PE6, PE8, PE12 and PE21 may have different effects on signaling pathways and/or protein kinases integrated into the longevity-defining network. To identify the pathways and kinases through which each of these PEs slows yeast chronological aging, I assessed such effects. Specifically, I elucidated how mutations eliminating these signaling pathways and protein kinases affect the efficiency with which each of the six PEs extends yeast CLS. **Table 3.1** shows the single-gene-deletion mutations used in this study. This table also demonstrates how each of the mutations impacts different longevity-defining signaling pathways and protein kinases, and how it alters yeast CLS. I investigated the effects of the following single-gene-deletion mutations shown in **Table 3.1**: 1) *tor1Δ*, which impairs the pro-aging TORC1 pathway and increases CLS [120, 121]; 2) *ras2Δ*, which weakens the pro-aging PKA pathway and extends CLS [123]; 3) *rim15Δ*, which eliminates the anti-aging protein kinase Rim15 and shortens CLS [122]; 4) *sch9Δ*, which removes the pro-aging protein kinase Sch9 and increases CLS [122]; 5) *pkh2Δ*, which weakens the pro-aging PKH1/2 pathway and extends CLS [124, 125]; 6) *snf1Δ*, which impairs the anti-aging SNF1 pathway and decreases CLS [126, 129]; and 7) *atg1Δ*, which deteriorates the anti-aging ATG pathway and shortens CLS [127, 128].

A logical framework for identifying signaling pathways and/or protein kinases controlled by each of the six longevity-extending PEs is schematically depicted in **Figure**

3.1. Pro-aging signaling pathways or protein kinases A and B in this figure are displayed in black color, whereas their anti-aging counterparts C and D are shown in grey color. One could envision that if PE(x) extends yeast CLS by inhibiting a pro-aging pathway/protein kinase A, this PE: 1) is unable to extend longevity of the ΔA mutant strain lacking this signaling pathway/protein kinase (**Figure 3.1B**); 2) exhibits an additive or synergistic longevity-extending effect with the ΔB mutation, which eliminates the pro-aging signaling pathway/protein kinase B (**Figure 3.1B**); and 3) is able to prolong longevity of the ΔC or ΔD mutant strain, which lacks the anti-aging signaling pathway/protein kinase C or D (respectively), but to a lesser extent than that of wild-type (WT) strain (**Figure 3.1B**). It is also plausible that that if PE(y) extends yeast CLS by activating an anti-aging pathway/protein kinase C, this PE: 1) displays an additive or synergistic longevity-extending effect with the ΔA or ΔB mutation, which eliminates the pro-aging signaling pathway/protein kinase A or B (respectively) (**Figure 3.1C**); 2) is incapable of increasing longevity of the ΔC mutant strain deficient in the anti-aging signaling pathway/protein kinase C (**Figure 3.1C**); and 3) can extend longevity of the ΔD mutant strain, which lacks the anti-aging signaling pathway/protein kinase D, although not as considerably as that of WT strain (**Figure 3.1C**).

Table 3.1. Single-gene-deletion mutations used in this study and their known effects on longevity-defining signaling pathways and longevity of chronologically aging yeast.

| Single-gene-deletion mutation | Protein eliminated | Longevity-defining signaling pathway(s) affected | Effect on longevity |
|--------------------------------------|---------------------------|---|----------------------------|
| <i>tor1Δ</i> | Tor1 | TORC1 | ↑ Extended [120, 121] |
| <i>ras2Δ</i> | Ras2 | PKA | ↑ Extended [123] |
| <i>rim15Δ</i> | Rim15 | TORC1, PKA, PKH1/2 | ↓ Shortened [122] |
| <i>sch9Δ</i> | Sch9 | TORC1, PKA | ↑ Extended [122] |
| <i>pkh2Δ</i> | Pkh2 | PKH1/2 | ↑ Extended [124, 125] |
| <i>snf1Δ</i> | Snf1 | SNF1 | ↓ Shortened [126, 129] |
| <i>atg1Δ</i> | Atg1 | ATG | ↓ Shortened [127, 128] |

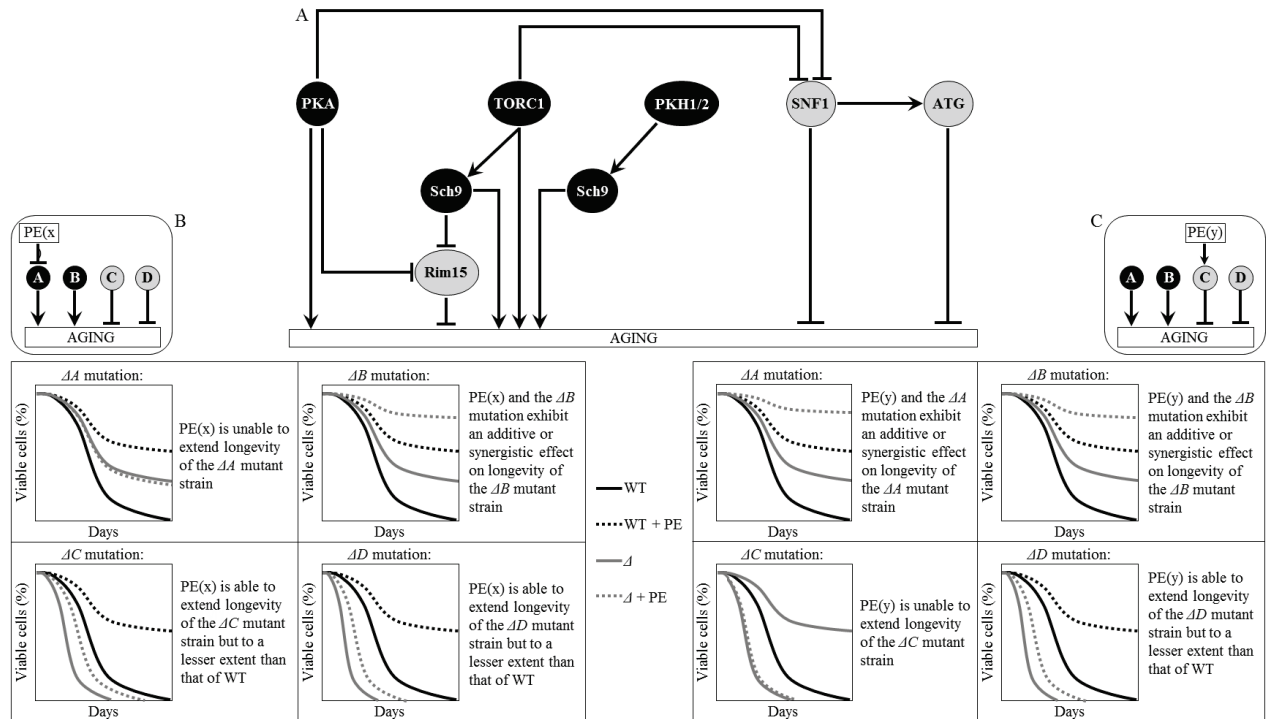


Figure 3.1. A logical framework for identifying signaling pathways and/or protein kinases controlled by the longevity-extending PE(x) and PE(y). (A) A schematic depiction of a network which integrates several signaling pathways and protein kinases to define the rate of yeast chronological aging. Pro-aging signaling pathways or protein kinases A and B are shown in black color. Anti-aging signaling pathways or protein kinases C and D are displayed in grey color. Abbreviations are as in Fig. 1. (B) Predicted effect of PE(x), which extends yeast CLS by inhibiting a pro-aging pathway/protein kinase A, on longevity of the ΔA , ΔB , ΔC or ΔD mutant strain lacking a signaling pathway/protein kinase A, B, C or D. (C) Predicted effect of PE(y), which extends yeast CLS by activating an anti-aging pathway/protein kinase C, on longevity of the ΔA , ΔB , ΔC or ΔD mutant strain lacking the corresponding signaling pathway/protein kinase. Abbreviation: WT, wild-type strain.

3.3.2 PE4 delays chronological aging by attenuating the inhibitory effect of

TORC1 on SNF1

PE4 exhibited additive longevity-extending effects with the *ras2Δ*, *sch9Δ* and *pkh2Δ* mutations, which eliminate different signaling pathways/protein kinases comprising the longevity-defining network (Figure 3.2A, Table 3.2, Table 3.3, Figure 3.3; note that data for the mock-treated WT strain and for the WT strain cultured with

PE4 are replicated in all graphs of **Figure 3.2A** and **Figure 3.3**). PE4 caused a decrease in slope of the Gompertz mortality rate (also known as mortality rate coefficient α) and an increase in the mortality rate doubling time (MRDT) for *ras2Δ*, *sch9Δ* and *pkh2Δ* (**Figure 3.4**; note that data for the mock-treated WT strain and for the WT strain cultured with PE4 are replicated in all graphs of this figure). Such changes in the values of α and MRDT are characteristic of interventions that decrease the rate of biological aging [23-26, 28, 29]. Thus, PE4 delays yeast chronological aging independently of the pro-aging PKA pathway, the pro-aging PKH1/2 pathway or the pro-aging protein kinase Sch9.

PE4 extended longevities of the *rim15Δ* and *atg1Δ* mutant strains, although to a lesser extent than that of WT strain (**Figure 3.2A**, **Table 3.2**, **Table 3.3**, **Figure 3.3**). PE4 reduced the value of α and elevated the value of MRDT for *rim15Δ* and *atg1Δ*, though not as significantly as for WT (**Figure 3.4**). Hence, PE4 slows yeast chronological aging not through the anti-aging protein kinase Rim15 or the anti-aging ATG pathway.

PE4 was unable to extend the CLS of *tor1Δ* and *snf1Δ* (**Figure 3.2A**, **Table 3.2**, **Table 3.3**, **Figure 3.3**) and did not alter the values of α or MRDT for these mutant strains (**Figure 3.4**). I concluded that PE4 delays yeast chronological aging via the pro-aging TORC1 pathway and the anti-aging SNF1 pathway, by weakening the known [130, 131] inhibitory effect of TORC1 on SNF1 (**Figure 3.2B**).

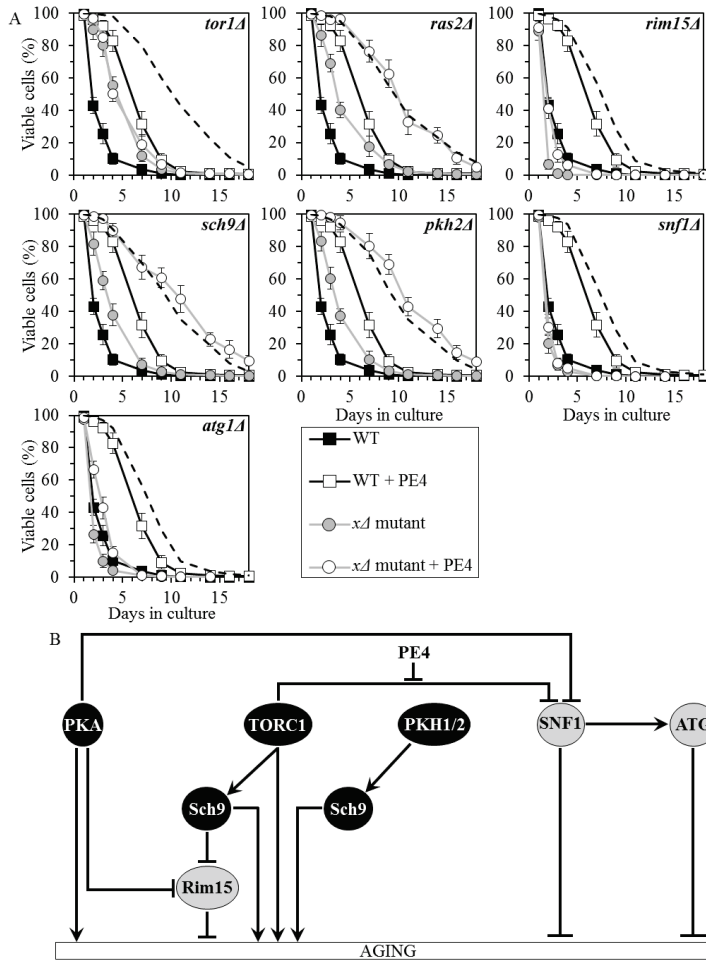


Figure 3.2. PE4 extends yeast CLS by weakening the restraining action of TORC1 on SNF1. (A) Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.5% PE4 (ethanol was used as a vehicle at the final concentration of 2.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves of chronologically aging WT and mutant strains cultured with or without 0.5% PE4 are shown. Data are presented as means \pm SEM ($n = 7$). The dotted line indicates the predicted survival curve of a particular mutant strain cultured with PE4 if this PE exhibits an additive longevity-extending effect with the mutation. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.5**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.5**. Data for the WT strain cultured with PE4 are replicated in all graphs of this Figure. (B) The effect of PE4 on the signaling pathways and protein kinases comprising the longevity-defining network. This effect is inferred from the data presented in (A), **Table 3.2**, **Table 3.3**, **Figure 3.3** and **Figure 3.4**. Abbreviations: as in the legend to **Figure 1.7**.

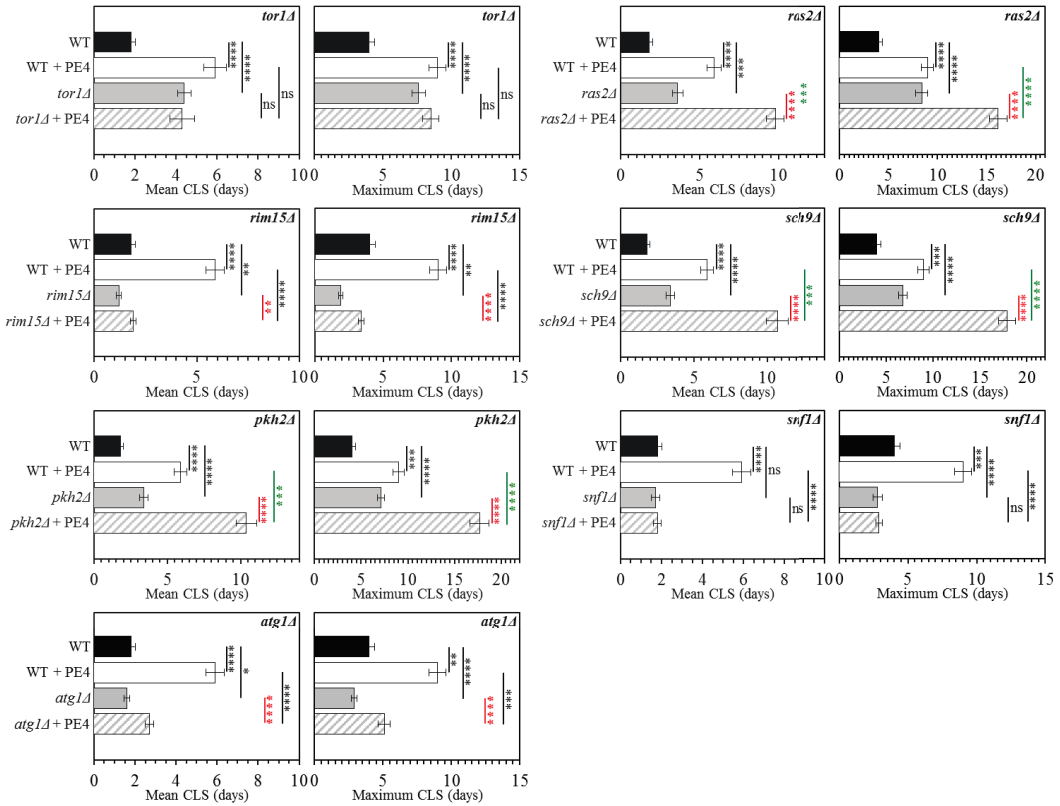


Figure 3.3. PE4 is unable to extend the chronological lifespans (CLS) of the *tor1Δ* and *snf1Δ* mutant strains, and exhibits additive CLS-extending effects with the *ras2Δ*, *sch9Δ* and *pkh2Δ* mutations. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.5% PE4 (ethanol was used as a vehicle at the final concentration of 2.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.2A** were used to calculate the mean and maximum CLS for WT and mutant strains cultured with or without 0.5% PE4. Data are presented as means \pm SEM ($n = 7$; ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). The ability of PE4 to cause a significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$) increase in the CLS of a particular mutant strain is displayed in red color. The ability of a combination between PE4 and a particular mutation to cause a significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$) increase in CLS-extending efficiencies of each other (i.e. the ability of such combination to exhibit an additive extending effect on yeast CLS) is displayed in green color. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.6**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.6**. Data for the WT strain cultured with PE4 are replicated in all graphs of this Figure.

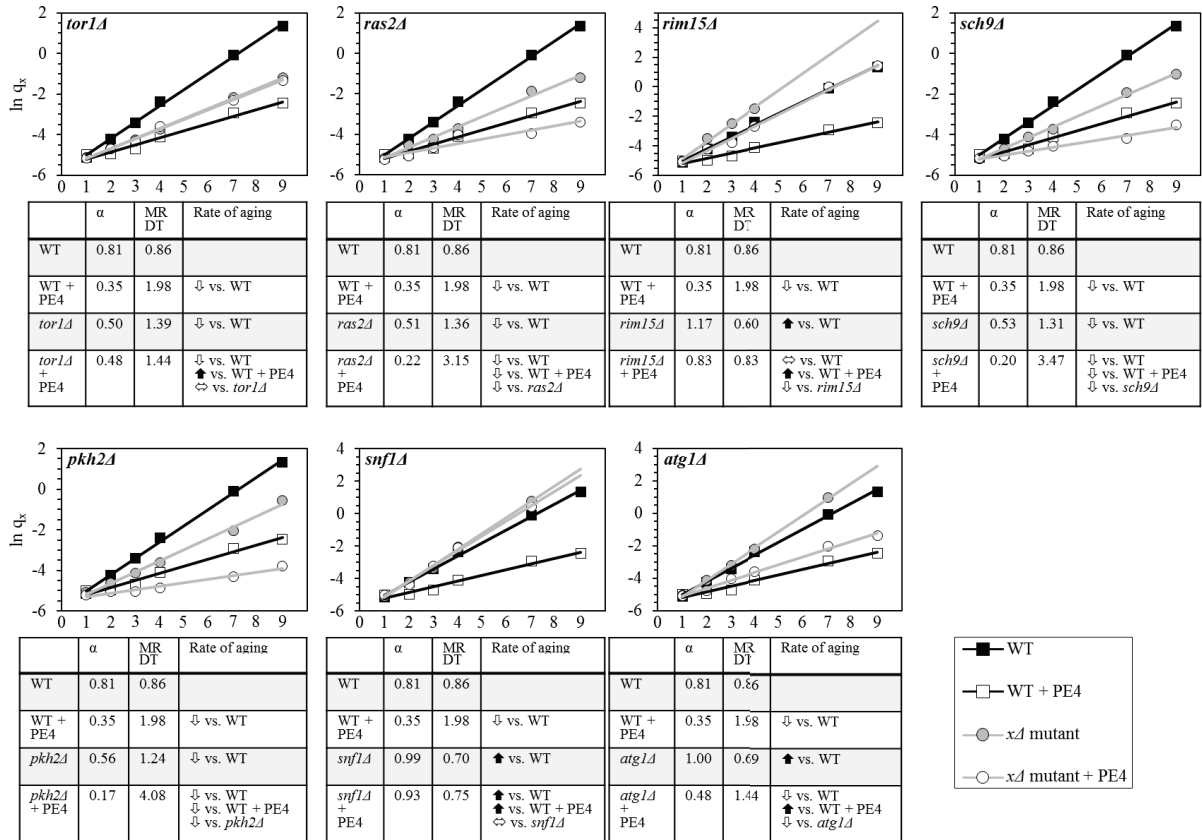


Figure 3.4. Analysis of the Gompertz mortality function indicates that PE4 delays yeast chronological aging by attenuating the inhibitory effect of TORC1 on SNF1. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.5% PE4 (ethanol was used as a vehicle at the final concentration of 2.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.2A** were used to calculate the age-specific mortality rates (q_x), the Gompertz mortality rates (also known as mortality rate coefficient α) and the mortality rate doubling times (MRDT) for WT and mutant yeast populations cultured with or without 0.5% PE4. The values of q_x , α and MRDT were calculated as described in Materials and methods. Data for the mock-treated WT strain are replicated in all graphs of this Supplementary Figure and in all graphs of **Figure 3.7**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.7**. Data for the WT strain cultured with PE4 are replicated in all graphs of this Figure.

Table 3.2. *p* Values for pairs of survival curves of a yeast strain cultured with or without the indicated plant extract (PE). Survival curves shown in **Figure 3.2A – Figure 3.17A** were compared. The survival curve of a strain cultured with the indicated PE was considered statistically different from the survival curve of the same strain cultured without it if the *p* value was less than 0.05; such *p* values are displayed in red color. For each pair of survival curves with the *p* value less than 0.05, the survival rate of the strain cultured with the indicated PE was higher than the survival rate of the same strain cultured without it. The *p* values for comparing pairs of survival curves were calculated as described in Materials and methods.

| Strain without PE | Same strain with the indicated PE | | | | | |
|-------------------|-----------------------------------|----------|----------|----------|----------|----------|
| | PE4 | PE5 | PE6 | PE8 | PE12 | PE21 |
| WT | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| <i>tor1Δ</i> | 0.8899 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| <i>ras2Δ</i> | < 0.0001 | 0.3664 | < 0.0001 | 0.41888 | < 0.0001 | < 0.0001 |
| <i>rim15Δ</i> | 0.0042 | 0.0168 | < 0.0001 | 0.0184 | 0.6453 | < 0.0001 |
| <i>sch9Δ</i> | < 0.0001 | < 0.0001 | < 0.0001 | 0.0006 | < 0.0001 | 0.0306 |
| <i>pkh2Δ</i> | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| <i>snf1Δ</i> | 0.5873 | 0.0075 | < 0.0001 | 0.7124 | 0.0132 | < 0.0001 |
| <i>atg1Δ</i> | 0.0027 | 0.0061 | < 0.0001 | 0.0086 | 0.0208 | < 0.0001 |

Table 3.3. *p* Values for pairs of survival curves of the wild-type (WT) and mutant strain, both cultured in the presence of the indicated PE. Survival curves shown in **Figure 3.2A – Figure 3.17A** were compared. The survival curve for the WT strain cultured with the indicated PE was considered statistically different from the survival curve for the mutant strain cultured with this PE if the *p* value was less than 0.05. The *p* values less than 0.05 are displayed in red color if the survival rate of the mutant strain cultured with the indicated PE was higher than the survival rate of the WT strain cultured with this PE. The *p* values less than 0.05 are displayed in blue color if the survival rate of the mutant strain cultured with the indicated PE was lower than the survival rate of the WT strain cultured with this PE. The *p* values for comparing pairs of survival curves were calculated as described in Materials and methods.

| PE4 | <i>tor1Δ</i> + PE4 | <i>ras2Δ</i> + PE4 | <i>rim15Δ</i> + PE4 | <i>sch9Δ</i> + PE4 | <i>pkh2Δ</i> + PE4 | <i>snf1Δ</i> + PE4 | <i>atg1Δ</i> + PE4 |
|----------|--------------------|--------------------|---------------------|--------------------|--------------------|--------------------|--------------------|
| WT + PE4 | 0.0827 | 0.0002 | < 0.0001 | 0.0004 | 0.0007 | < 0.0001 | < 0.0001 |

| PE5 | <i>tor1Δ</i> + PE5 | <i>ras2Δ</i> + PE5 | <i>rim15Δ</i> + PE5 | <i>sch9Δ</i> + PE5 | <i>pkh2Δ</i> + PE5 | <i>snf1Δ</i> + PE5 | <i>atg1Δ</i> + PE5 |
|----------|--------------------|--------------------|---------------------|--------------------|--------------------|--------------------|--------------------|
| WT + PE5 | < 0.0001 | 0.0724 | < 0.0001 | 0.0008 | < 0.0001 | < 0.0001 | 0.0004 |

| PE6 | <i>tor1Δ</i> + | <i>ras2Δ</i> + | <i>rim15Δ</i> | <i>sch9Δ</i> + | <i>pkh2Δ</i> + | <i>snf1Δ</i> + | <i>atg1Δ</i> + |
|-----|----------------|----------------|---------------|----------------|----------------|----------------|----------------|
|-----|----------------|----------------|---------------|----------------|----------------|----------------|----------------|

| | | | | | | | |
|-------------|---------------------|---------------------|----------------------|---------------------|---------------------|---------------------|---------------------|
| | PE6 | PE6 | + PE6 | PE6 | PE6 | PE6 | PE6 |
| WT + PE6 | < 0.0001 | < 0.0001 | 0.0364 | 0.0008 | < 0.0001 | < 0.0001 | < 0.0001 |
| PE8 | <i>tor1Δ</i> + PE8 | <i>ras2Δ</i> + PE8 | <i>rim15Δ</i> + PE8 | <i>sch9Δ</i> + PE8 | <i>pkh2Δ</i> + PE8 | <i>snf1Δ</i> + PE8 | <i>atg1Δ</i> + PE8 |
| WT + PE8 | < 0.0001 | 0.0124 | < 0.0001 | 0.0032 | < 0.0001 | < 0.0001 | 0.0008 |
| PE12 | <i>tor1Δ</i> + PE12 | <i>ras2Δ</i> + PE12 | <i>rim15Δ</i> + PE12 | <i>sch9Δ</i> + PE12 | <i>pkh2Δ</i> + PE12 | <i>snf1Δ</i> + PE12 | <i>atg1Δ</i> + PE12 |
| WT + PE12 | 0.0003 | 0.0011 | < 0.0001 | 0.0018 | < 0.0001 | 0.0004 | 0.0012 |
| PE21 | <i>tor1Δ</i> + PE21 | <i>ras2Δ</i> + PE21 | <i>rim15Δ</i> + PE21 | <i>sch9Δ</i> + PE21 | <i>pkh2Δ</i> + PE21 | <i>snf1Δ</i> + PE21 | <i>atg1Δ</i> + PE21 |
| WT + PE21 | 0.0036 | 0.0062 | 0.0109 | < 0.0001 | 0.0044 | 0.0009 | 0.0086 |

3.3.3 PE5 slows chronological aging by impeding two branches of the PKA pathway

PE5 displayed an additive longevity-extending effect with the *sch9Δ* mutation, and increased yeast CLS in synergy with the *tor1Δ* and *pkh2Δ* mutations (**Figure 3.5A**, **Table 3.2**, **Table 3.3**, **Figure 3.6**; note that data for the mock-treated WT strain and for the WT strain cultured with PE5 are replicated in all graphs of **Figure 3.5A** and **Figure 3.6**). PE5 decreased the values of α and increased the values of MRDT for chronologically aging cultures of strains carrying each of these three mutations (**Figure 3.7**; note that data for the mock-treated WT strain and for the WT strain cultured with PE5 are replicated in all graphs of this figure). Therefore, PE5 slows aging not through TORC1, PKH1/2 or Sch9.

PE5 increased CLS of the *rim15Δ*, *snf1Δ* and *atg1Δ* mutant strains, however to a lesser extent than that of WT strain (**Figure 3.5A**, **Table 3.2**, **Table 3.3**, **Figure 3.6**). PE5

decreased the value of α and increased the value of MRDT strains carrying each of these mutations, although not as considerably as for WT (**Figure 3.7**). Thus, PE5 slows aging independently of Rim15, SNF1 and ATG.

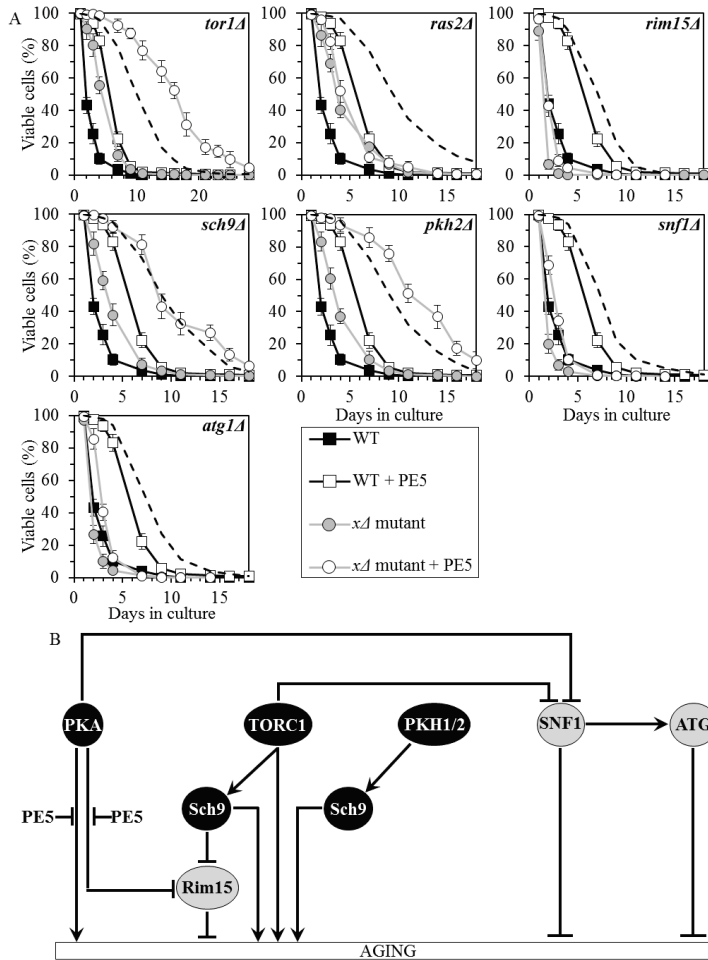


Figure 3.5. PE5 extends yeast CLS by weakening two branches of the PKA signaling pathway. (A) Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.5% PE5 (ethanol was used as a vehicle at the final concentration of 2.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves of chronologically aging WT and mutant strains cultured with or without 0.5% PE5 are shown. Data are presented as means \pm SEM ($n = 7$). The dotted line indicates the predicted survival curve of a particular mutant strain cultured with PE5 if this PE exhibits an additive longevity-extending effect with the mutation. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.2**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.2**. Data for the WT strain cultured with PE5 are replicated in all graphs of this Figure. (B) The effect of PE5 on the signaling pathways and protein kinases comprising the longevity-defining network. This effect is inferred from the data presented in (A), **Table 3.2**, **Table 3.3**, **Figure 3.6** and **Figure 3.7**. Abbreviations: as in the legend to **Figure 1.7**.

PE5 was unable to extend the CLS of *ras2Δ* (**Figure 3.5A**, **Table 3.2**, **Table 3.3**, **Figure 3.6**) and did not alter the values of α or MRDT for this mutant strains (**Figure 3.7**). Hence, PE5 delays aging by weakening two branches of the PKA signaling pathway (**Figure 3.5B**). One of these branches involves the Rim15-independent processes of autophagy inhibition and protein translation activation in the cytosol, whereas the other branch attenuates the Rim15-driven establishment of an anti-aging transcriptional program of numerous nuclear genes [129 - 141].

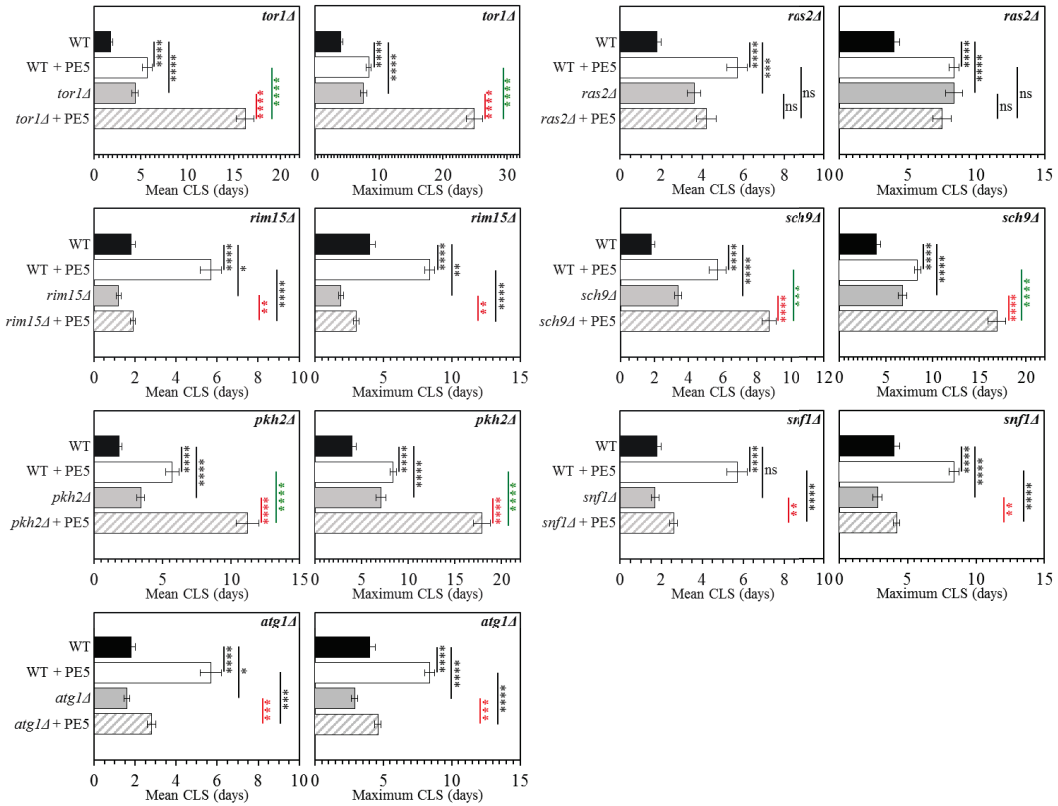


Figure 3.6. PE5 is unable to extend the chronological lifespan (CLS) of the *ras2Δ* mutant strain, exhibits an additive CLS-extending effect with the *sch9Δ* mutation, and increases yeast CLS in synergy with the *tor1Δ* and *pkh2Δ* mutations. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.5% PE5 (ethanol was used as a vehicle at the final concentration of 2.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.5A** were used to calculate the mean and maximum CLS for WT and mutant strains cultured with or without 0.5% PE5. Data are presented as means \pm SEM ($n = 7$; ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). The ability of PE5 to cause a significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) increase in the CLS of a particular mutant strain is displayed in red color. The ability of a combination between PE5 and a particular mutation to cause a significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) increase in CLS-extending efficiencies of each other (i.e. the ability of such combination to exhibit an additive or synergistic CLS-extending effect) is displayed in green color. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.3**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.3**. Data for the WT strain cultured with PE5 are replicated in all graphs of this Figure.

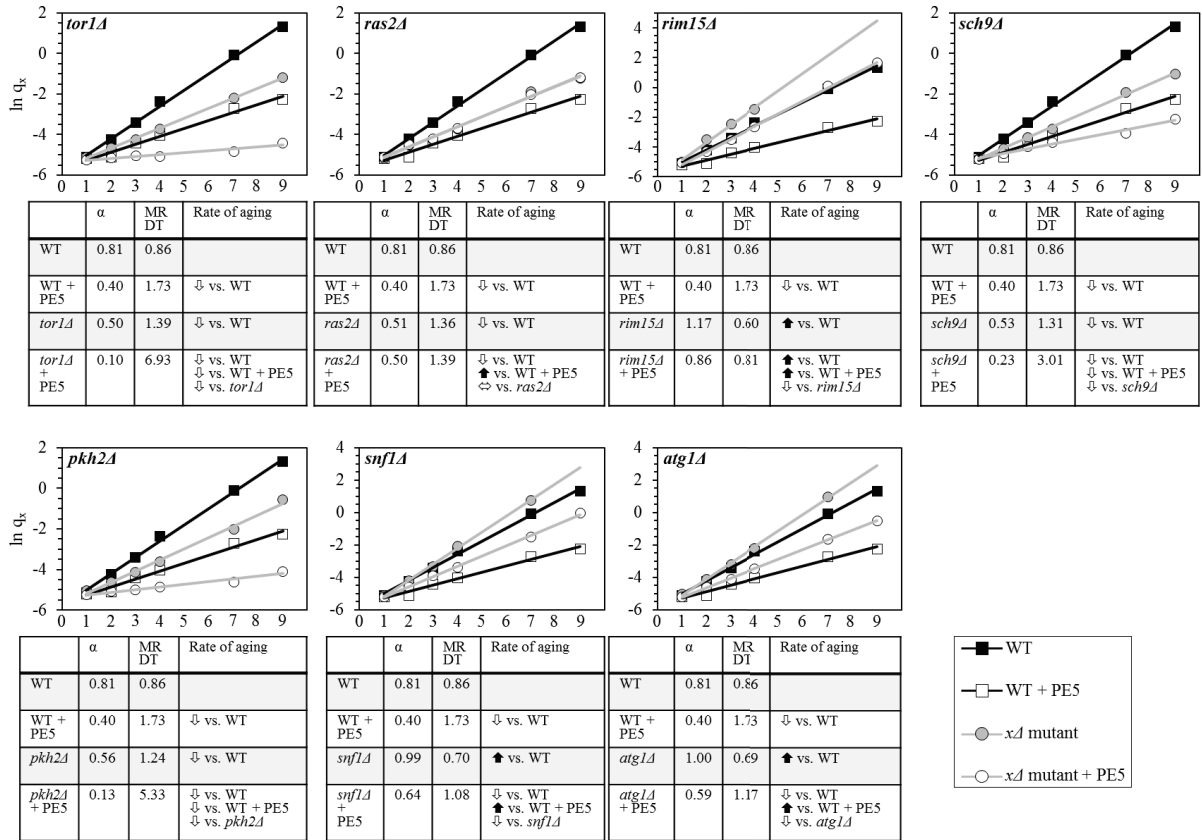


Figure 3.7. Analysis of the Gompertz mortality function indicates that PE5 delays yeast chronological aging by attenuating two branches of the PKA signaling pathway. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.5% PE5 (ethanol was used as a vehicle at the final concentration of 2.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.5A** were used to calculate the age-specific mortality rates (q_x), the Gompertz mortality rates (also known as mortality rate coefficient α) and the mortality rate doubling times (MRDT) for WT and mutant yeast populations cultured with or without 0.5% PE5. The values of q_x , α and MRDT were calculated as described in Materials and methods. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.4**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.4**. Data for the WT strain cultured with PE5 are replicated in all graphs of this Figure.

3.3.4 PE6 delays chronological aging by coordinating processes that are not integrated into the network of longevity-defining signaling pathways/protein kinases

PE6 exhibited additive longevity-extending effects with the *rim15Δ*, *sch9Δ* and *atg1Δ* mutations, and extended longevity synergistically with the *tor1Δ*, *ras2Δ*, *pkh2Δ* and *snf1Δ* mutations (**Figure 3.8A**, **Table 3.2**, **Table 3.3**, **Figure 3.9**; note that data for the mock-treated WT strain and for the WT strain cultured with PE6 are replicated in all graphs of **Figure 3.8A** and **Figure 3.9**). PE6 lowered the values of α and raised the values of MRDT for chronologically aging cultures of strains carrying each of these seven mutations (**Figure 3.10**; note that data for the mock-treated WT strain and for the WT strain cultured with PE6 are replicated in all graphs of this figure). Thus, PE6 delays aging by activating anti-aging processes and/or inhibiting pro-aging processes that are not assimilated into the network of presently known signaling pathways/protein kinases (**Figure 3.8B**).

Although *rim15Δ*, *snf1Δ* and *atg1Δ* exhibited decreased CLS in the absence of PE6, this PE extended the CLS of each of these mutant strains to a greater extent than that of WT strain (**Figure 3.8A**, **Table 3.2**, **Table 3.3**, **Figure 3.9**, **Figure 3.10**). It is possible that the efficiency with which PE6 activates anti-aging processes and/or inhibits pro-aging processes outside of the network in the absence of Rim15, Snf1 or Atg1 may exceed such efficiency in the presence of any of these anti-aging proteins.

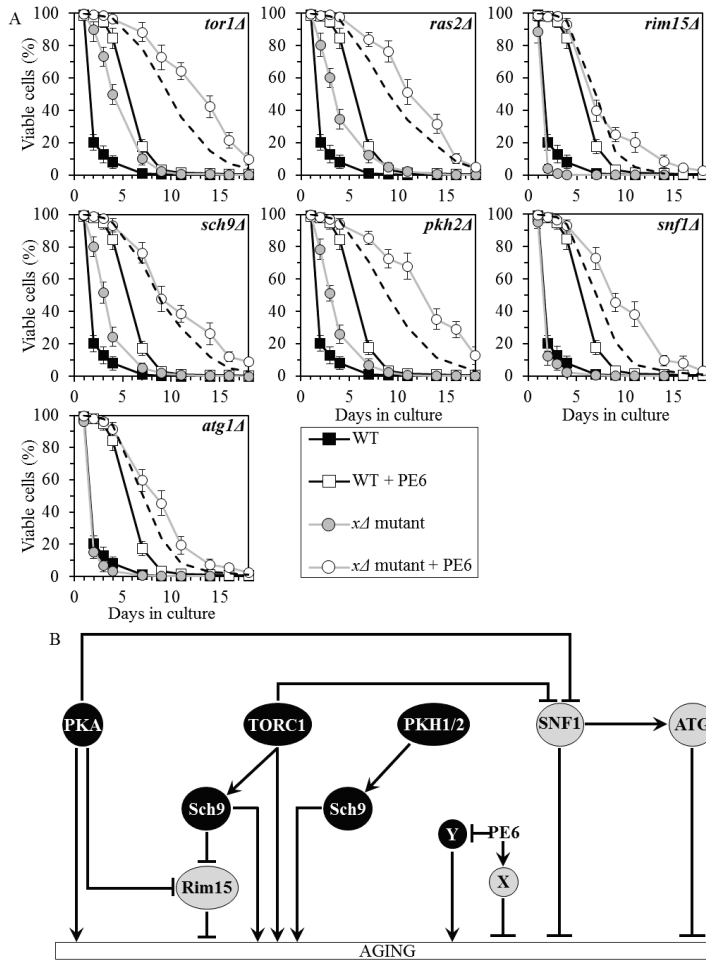


Figure 3.8. PE6 extends yeast CLS independently of presently known longevity-defining signaling pathways/protein kinases. (A) Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 1.0% PE6 (ethanol was used as a vehicle at the final concentration of 5.0%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves of chronologically aging WT and mutant strains cultured with or without 1.0% PE6 are shown. Data are presented as means \pm SEM ($n = 8$). The dotted line indicates the predicted survival curve of a particular mutant strain cultured with PE6 if this PE exhibits an additive longevity-extending effect with the mutation. Data for the mock-treated WT strain and for the WT strain cultured with PE6 are replicated in all graphs of this Figure. (B) The effects of PE6 on anti-aging and/or pro-aging processes that are not controlled by the network of presently known signaling pathways/protein kinases. These effects are inferred from the data presented in (A), Table 3.2, Table 3.3, Figure 3.9, Figure 3.10. Abbreviations: as in the legend to Figure 1.7.

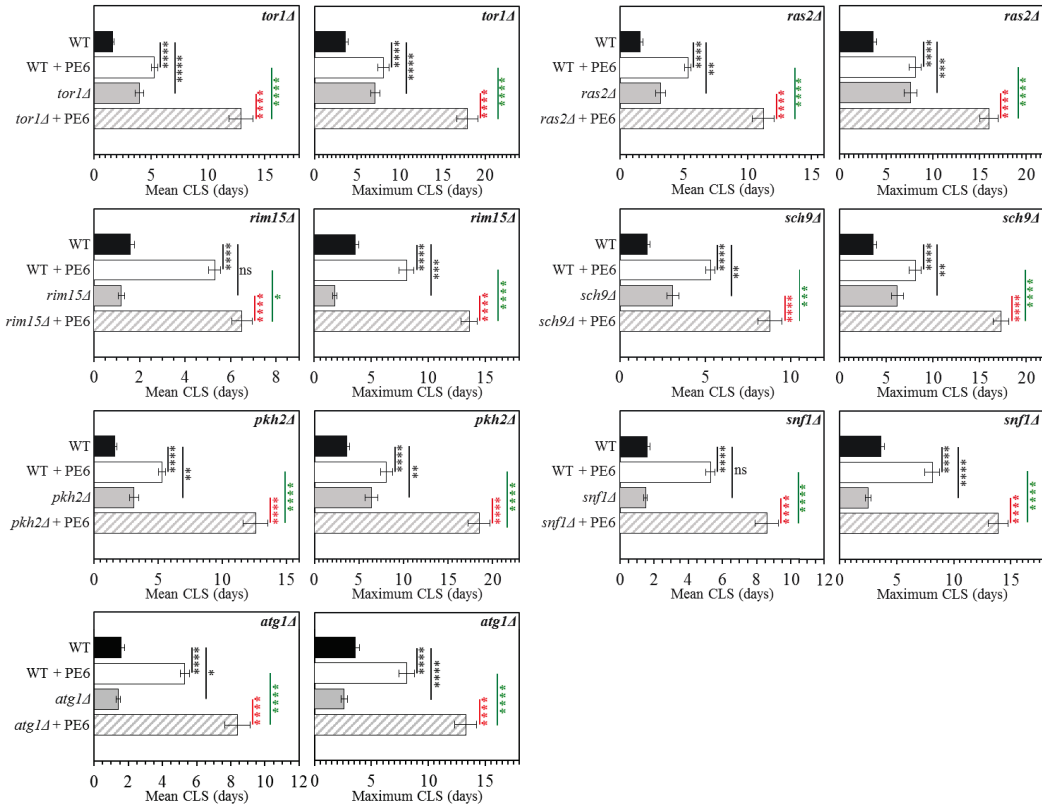


Figure 3.9. PE6 exhibits additive CLS-extending effects with the *rim15Δ*, *sch9Δ* and *atg1Δ* mutations; PE6 also increases yeast CLS in synergy with the *tor1Δ*, *ras2Δ*, *pkh2Δ* and *snf1Δ* mutations. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 1.0% PE6 (ethanol was used as a vehicle at the final concentration of 5.0%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.8A** were used to calculate the mean and maximum CLS for WT and mutant strains cultured with or without 1.0% PE6. Data are presented as means \pm SEM ($n = 8$; ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). The ability of PE6 to cause a significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$) increase in the CLS of a particular mutant strain is displayed in red color. The ability of a combination between PE6 and a particular mutation to cause a significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$) increase in CLS-extending efficiencies of each other (i.e. the ability of such combination to exhibit an additive or synergistic CLS-extending effect) is displayed in green color. Data for the mock-treated WT strain and for the WT strain cultured with PE6 are replicated in all graphs of this Figure.

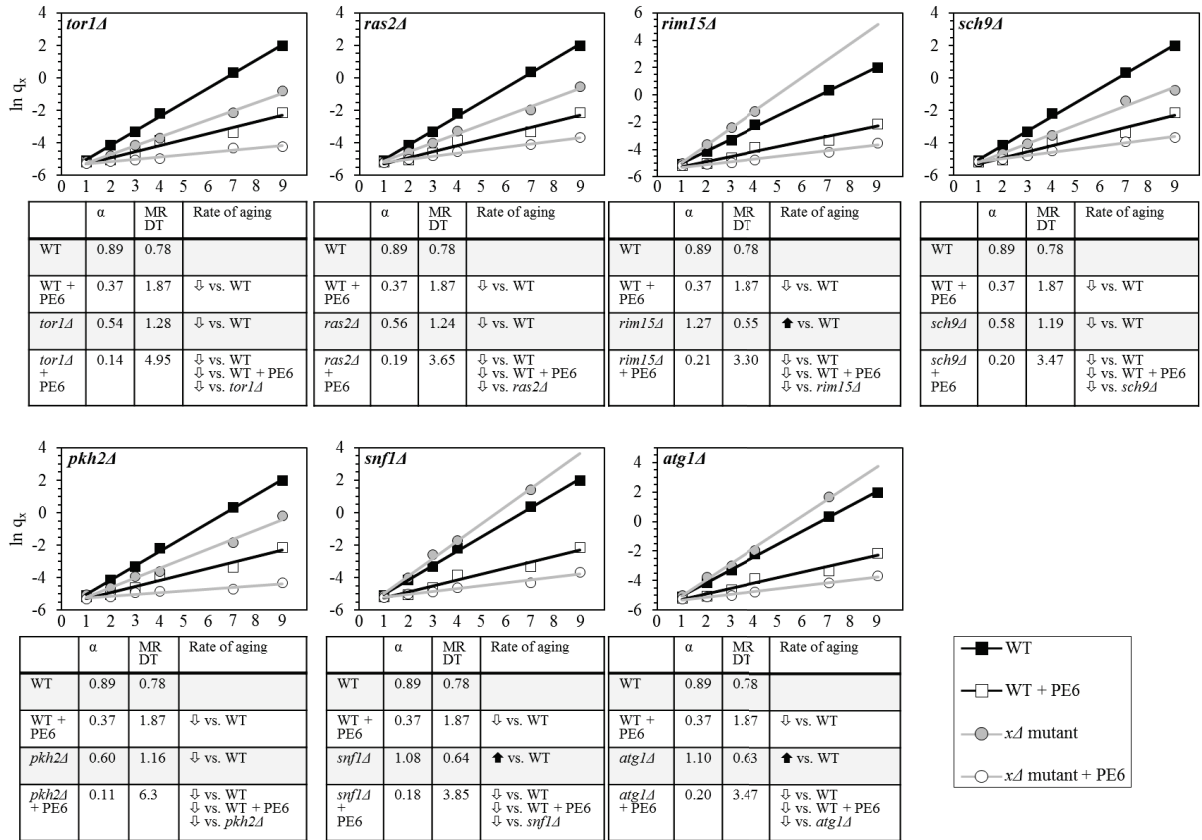


Figure 3.10. Analysis of the Gompertz mortality function indicates that PE6 delays yeast chronological aging independently of presently known longevity-defining signaling pathways/protein kinases. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 1.0% PE6 (ethanol was used as a vehicle at the final concentration of 5.0%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.8A** were used to calculate the age-specific mortality rates (q_x), the Gompertz mortality rates (also known as mortality rate coefficient α) and the mortality rate doubling times (MRDT) for WT and mutant yeast populations cultured with or without 1.0% PE6. The values of q_x , α and MRDT were calculated as described in Materials and methods. Data for the mock-treated WT strain and for the WT strain cultured with PE6 are replicated in all graphs of this Figure.

3.3.5 PE8 slows chronological aging by weakening the inhibitory effect of PKA on SNF1

PE8 displayed an additive longevity-extending effect with the *sch9Δ* mutation, and increased yeast CLS in synergy with the *tor1Δ* and *pkh2Δ* mutations (**Figure 3.11A**, **Table 3.2**, **Table 3.3**, **Figure 3.12**; note that data for the mock-treated WT strain and for the WT strain cultured with PE8 are replicated in all graphs of **Figure 3.11A** and **Figure 3.12**). PE8 decreased the values of α and increased the values of MRDT for chronologically aging cultures of strains carrying each of these three mutations (**Figure 3.13**; note that data for the mock-treated WT strain and for the WT strain cultured with PE8 are replicated in all graphs of this figure). Therefore, PE8 slows aging independently of Sch9, TORC1 and PKH1/2.

PE8 increased CLS of the *rim15Δ* and *atg1Δ* mutant strains, though not as considerably as for WT (**Figure 3.11A**, **Table 3.2**, **Table 3.3**, **Figure 3.12**). PE8 lowered the values of α and raised the values of MRDT for chronologically aging cultures of strains carrying each of these mutations (**Figure 3.13**). Hence, PE8 delays aging not through Rim15 or ATG.

PE8 was unable to extend the CLS of *ras2Δ* and *snf1Δ* (**Figure 3.11A**, **Table 3.2**, **Table 3.3**, **Figure 3.12**) and did not alter the values of α or MRDT for these mutant strains (**Figure 3.13**). Thus, PE8 slows aging via PKA and SNF1, by attenuating the known inhibitory action of PKA on SNF1 (**Figure 3.11B**).

3.3.6 PE12 delays chronological aging by stimulating Rim15

PE12 increased yeast CLS synergistically with the *pkh2Δ* mutation, and displayed additive longevity-extending effects with the *tor1Δ*, *ras2Δ* and *sch9Δ* mutations (**Figure 3.14A**, **Table 3.2**, **Table 3.3**, **Figure 3.15**; note that data for the mock-treated WT strain and for the WT strain cultured with PE12 are replicated in all graphs of **Figure 3.14A**

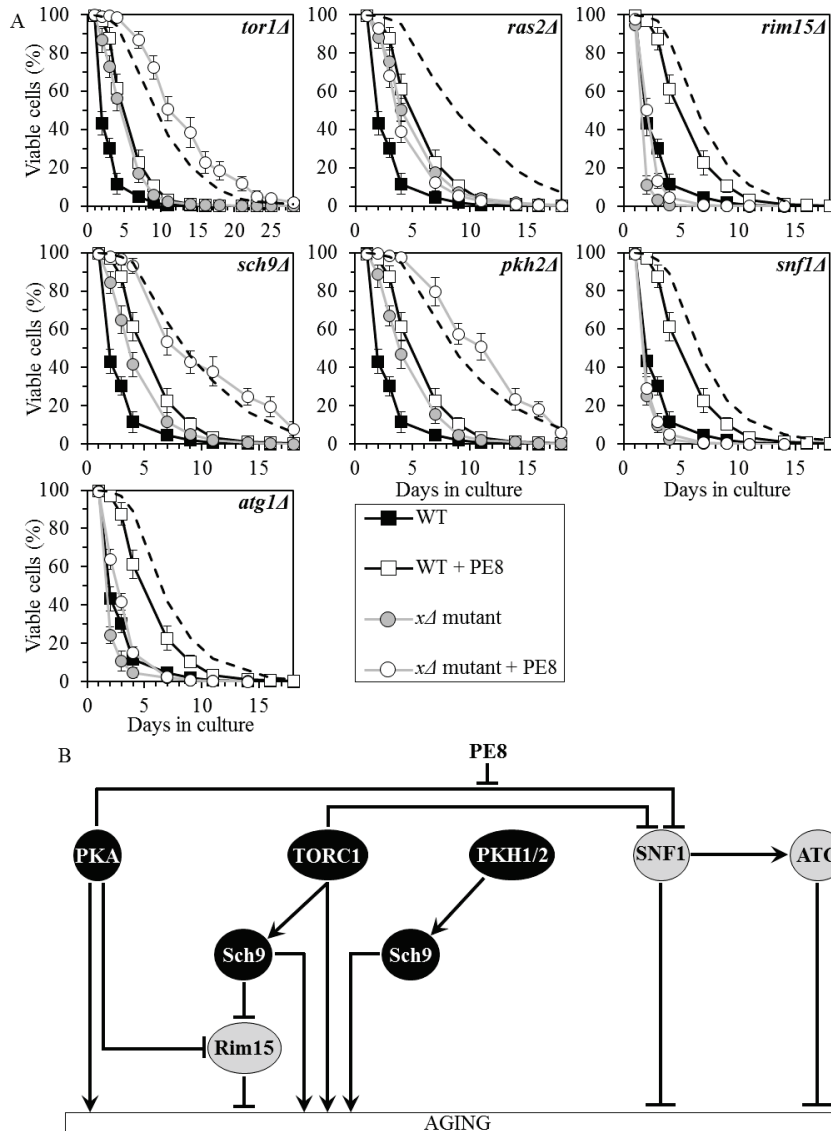


Figure 3.11. PE8 extends yeast CLS by attenuating the inhibitory effect of PKA on SNF1. (A) Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.3% PE8 (ethanol was used as a vehicle at the final concentration of 1.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves of chronologically aging WT and mutant strains cultured with or without 0.3% PE8 are shown. Data are presented as means \pm SEM ($n = 6$). The dotted line indicates the predicted survival curve of a particular mutant strain cultured with PE8 if this PE exhibits an additive longevity-extending effect with the mutation. Data for the mock-treated WT strain and for the WT strain cultured with PE8 are replicated in all graphs of this Figure. (B) The effect of PE8 on the signaling pathways and protein kinases integrated into the longevity-defining network. This effect is inferred from the data presented in (A), Table 3.2, Table 3.3, Figure 3.12, Figure 3.13. Abbreviations: as in the legend to Figure 1.7.

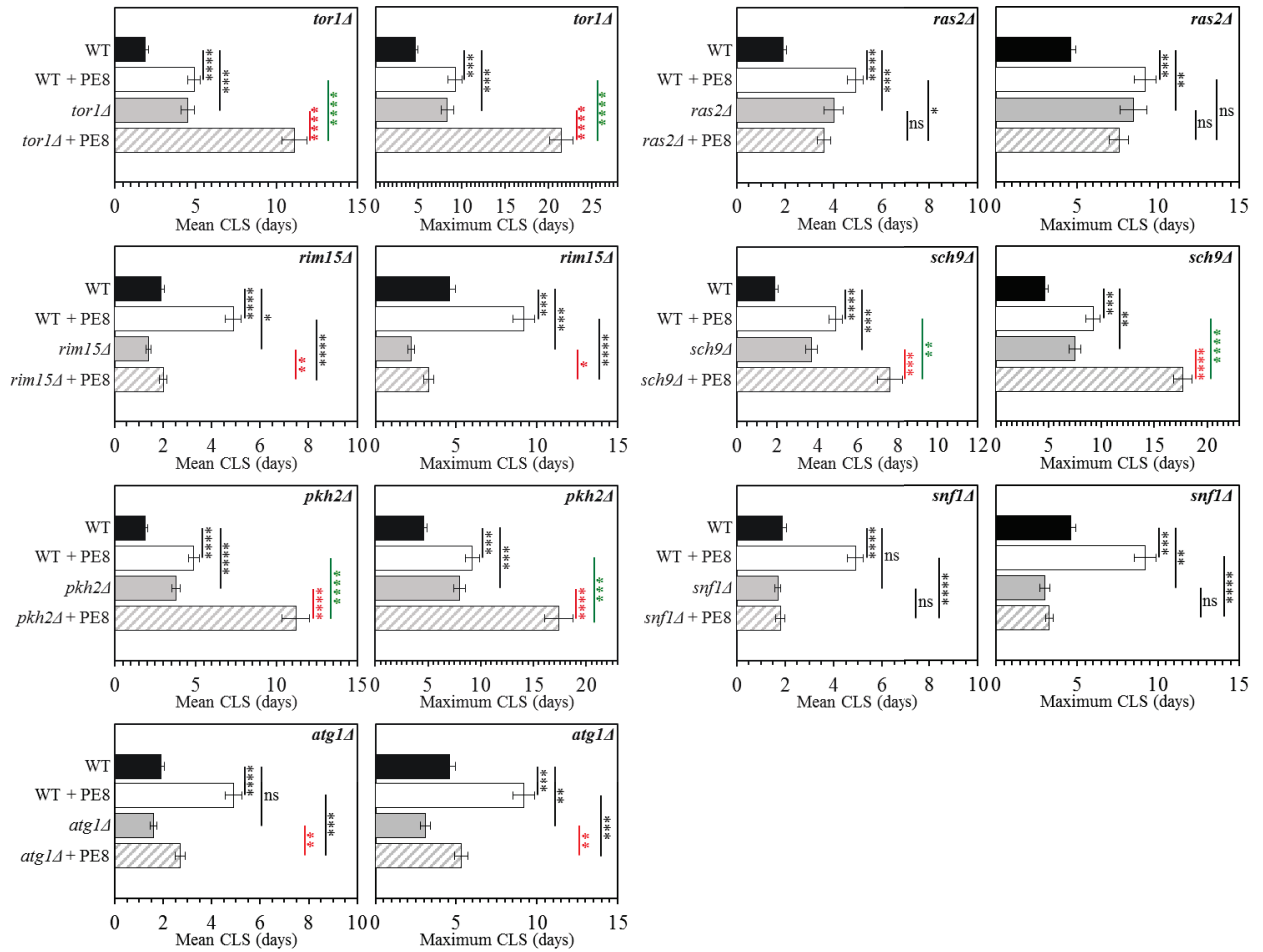


Figure 3.12. PE8 is unable to extend the chronological lifespans (CLS) of the *ras2Δ* and *snf1Δ* mutant strains, exhibits an additive CLS-extending effect with the *sch9Δ* mutation, and increases yeast CLS in synergy with the *tor1Δ* and *pkh2Δ* mutations. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.3% PE8 (ethanol was used as a vehicle at the final concentration of 1.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.11A** were used to calculate the mean and maximum CLS for WT and mutant strains cultured with or without 0.3% PE8. Data are presented as means \pm SEM ($n = 6$; ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). The ability of PE8 to cause a significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) increase in the CLS of a particular mutant strain is displayed in red color. The ability of a combination between PE8 and a particular mutation to cause a significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) increase in CLS-extending efficiencies of each other (i.e. the ability of such combination to exhibit an additive or synergistic CLS-extending effect) is displayed in green color. Data for the mock-treated WT strain and for the WT strain cultured with PE8 are replicated in all graphs of this Figure.

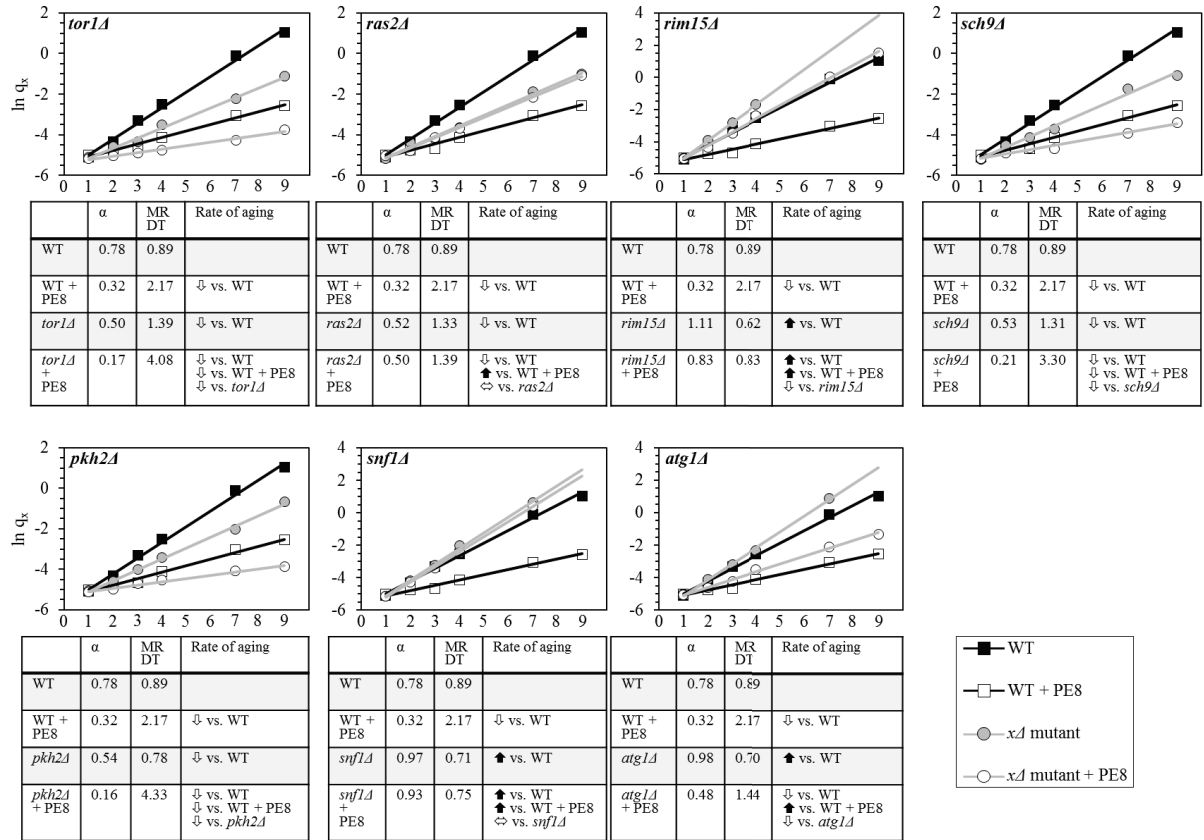


Figure 3.13. Analysis of the Gompertz mortality function indicates that PE8 delays yeast chronological aging by attenuating the inhibitory effect of PKA on SNF1. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.3% PE8 (ethanol was used as a vehicle at the final concentration of 1.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.11A** were used to calculate the age-specific mortality rates (q_x), the Gompertz mortality rates (also known as mortality rate coefficient α) and the mortality rate doubling times (MRDT) for WT and mutant yeast populations cultured with or without 0.3% PE8. The values of q_x , α and MRDT were calculated as described in Materials and methods. Data for the mock-treated WT strain and for the WT strain cultured with PE8 are replicated in all graphs of this Figure.

and **Figure 3.15**). PE12 reduced the values of α and augmented the values of MRDT for chronologically aging cultures of strains carrying each of these four mutations (**Figure 3.16**; note that data for the mock-treated WT strain and for the WT strain cultured with PE12 are replicated in all graphs of this figure). Hence, PE12 slows aging not through PKH1/2, TORC1, PKA or Sch9.

PE12 extended longevity of the *snf1 Δ* and *atg1 Δ* mutant strains, although to a lesser extent than that of WT strain (**Figure 3.14A**, **Table 3.2**, **Table 3.3**, **Figure 3.15**). PE12 decreased the value of α and increased the value of MRDT strains carrying each of these mutations, however not as considerably as for WT (**Figure 3.16**). Therefore, PE12 delays aging independently of SNF1 and ATG.

PE12 was unable to extend the CLS of *rim15 Δ* (**Figure 3.14A**, **Table 3.2**, **Table 3.3**, **Figure 3.15**) and did not alter the values of α or MRDT for this mutant strain (**Figure 3.16**). Hence, PE12 slows aging by activating Rim15 (**Figure 3.14B**).

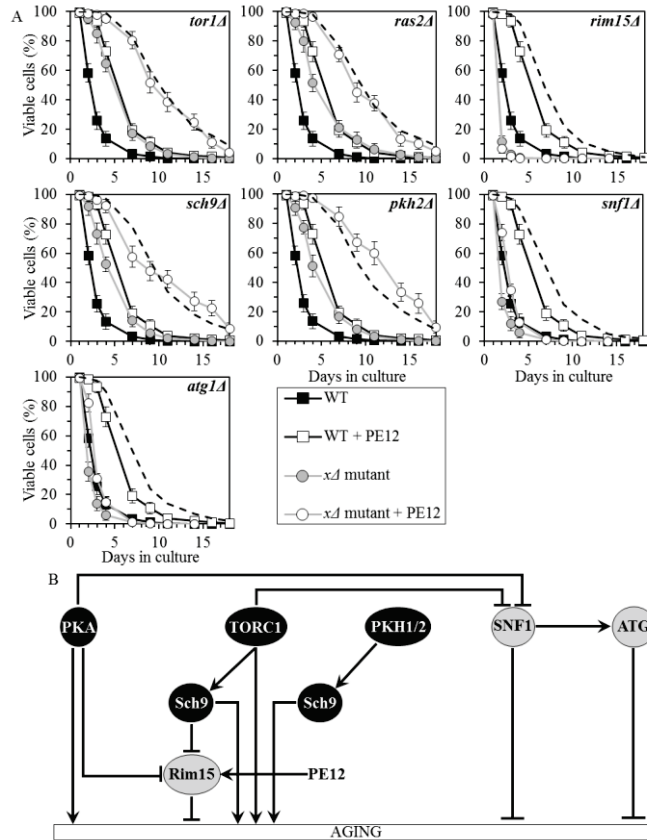


Figure 3.14. PE12 extends yeast CLS by stimulating Rim15. (A) Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.1% PE12 (ethanol was used as a vehicle at the final concentration of 0.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves of chronologically aging WT and mutant strains cultured with or without 0.1% PE12 are shown. Data are presented as means \pm SEM (n = 8). The dotted line indicates the predicted survival curve of a particular mutant strain cultured with PE12 if this PE exhibits an additive longevity-extending effect with the mutation. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.17**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.17**. Data for the WT strain cultured with PE12 are replicated in all graphs of this Figure. (B) The effect of PE12 on the signaling pathways and protein kinases integrated into the longevity-defining network. This effect is inferred from the data presented in (A), **Table 3.2**, **Table 3.3**, **Figure 3.15**, **Figure 3.16**. Abbreviations: as in the legend to **Figure 1.7**.

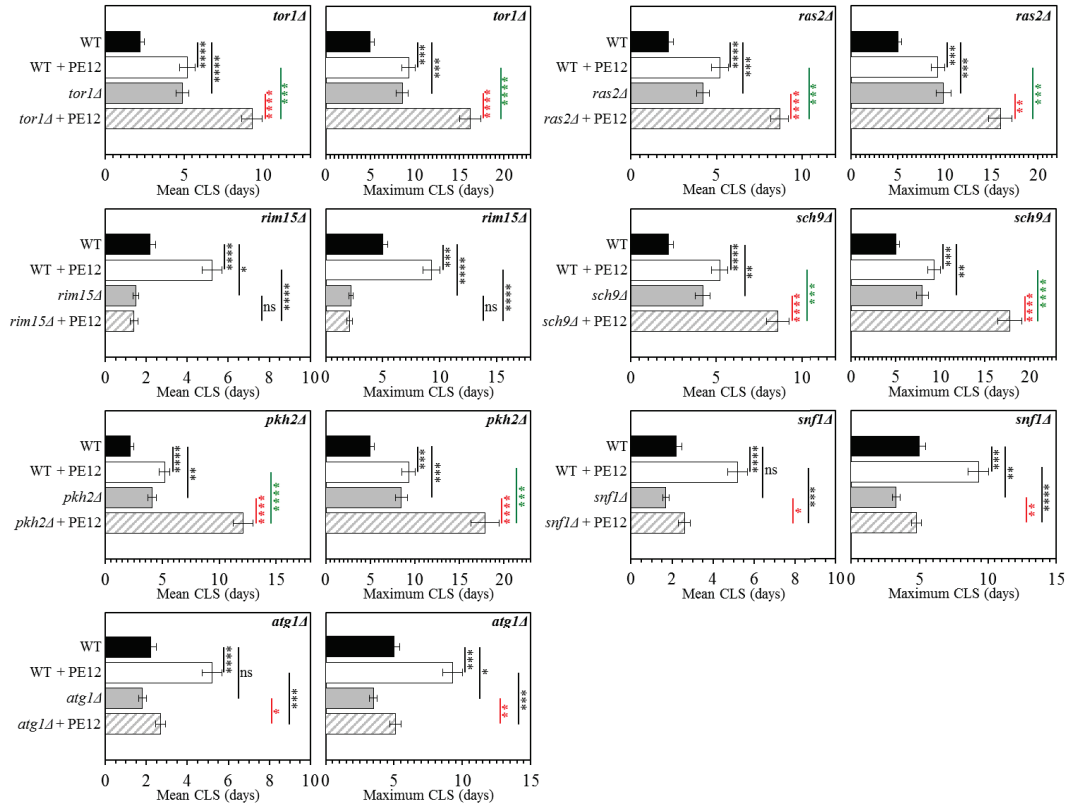


Figure 3.15. PE12 is unable to extend the chronological lifespan (CLS) of the *rim15Δ* mutant strain, exhibits additive CLS-extending effects with the *tor1Δ*, *ras2Δ* and *sch9Δ* mutations, and increases yeast CLS in synergy with the *pkh2Δ* mutation. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.1% PE12 (ethanol was used as a vehicle at the final concentration of 0.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.14A** were used to calculate the mean and maximum CLS for WT and mutant strains cultured with or without 0.1% PE12. Data are presented as means \pm SEM (n = 8; ns, not significant; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001). The ability of PE12 to cause a significant (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001) increase in the CLS of a particular mutant strain is displayed in red color. The ability of a combination between PE12 and a particular mutation to cause a significant (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001) increase in CLS-extending efficiencies of each other (i.e. the ability of such combination to exhibit an additive or synergistic CLS-extending effect) is displayed in green color. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.18**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.18**. Data for the WT strain cultured with PE12 are replicated in all graphs of this Figure.

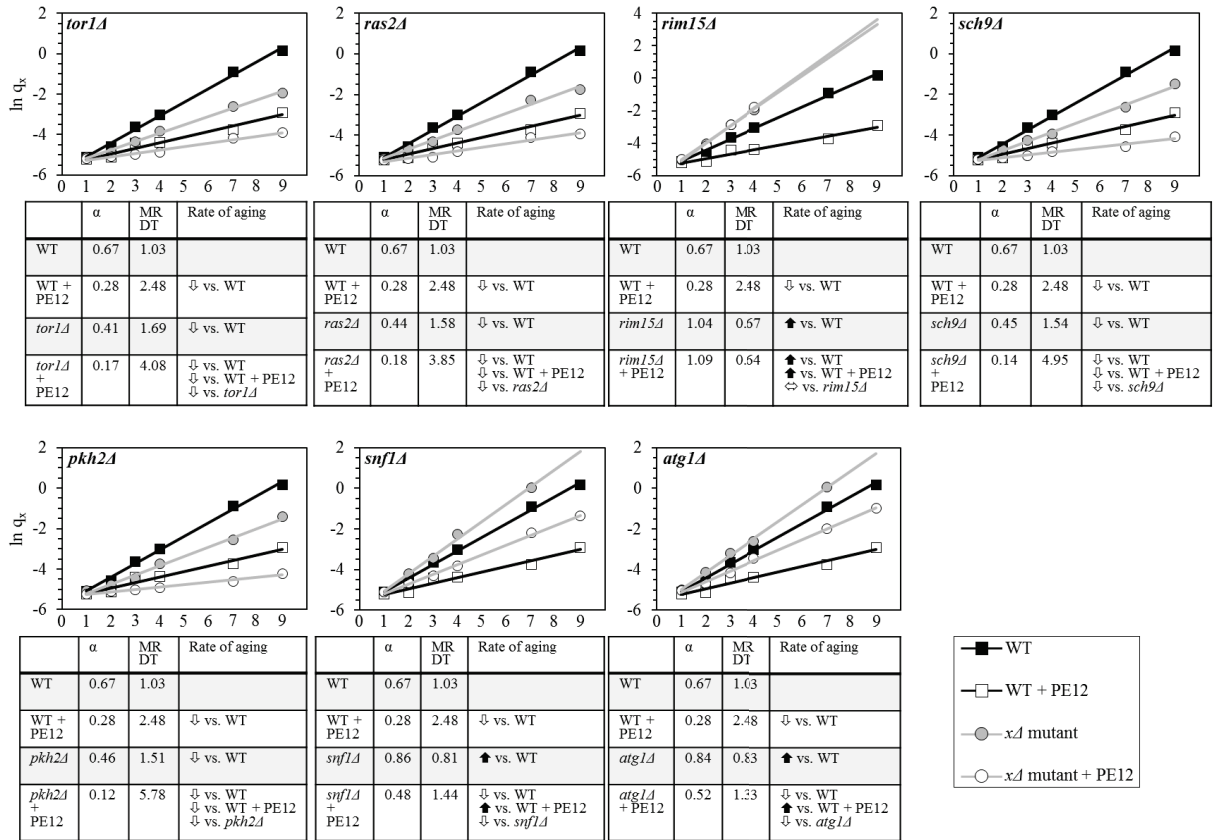


Figure 3.16. Analysis of the Gompertz mortality function indicates that PE12 delays yeast chronological aging by stimulating Rim15. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.1% PE12 (ethanol was used as a vehicle at the final concentration of 0.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.14A** were used to calculate the age-specific mortality rates (q_x), the Gompertz mortality rates (also known as mortality rate coefficient α) and the mortality rate doubling times (MRDT) for WT and mutant yeast populations cultured with or without 0.1% PE12. The values of q_x , α and MRDT were calculated as described in Materials and methods. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.19**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.19**. Data for the WT strain cultured with PE12 are replicated in all graphs of this Figure.

3.3.7 PE21 slows chronological aging by inhibiting a PKH1/2-sensitive form of Sch9

PE21 increased yeast CLS in synergy with the *pkh2Δ* mutation, and displayed additive longevity-extending effects with the *tor1Δ* and *ras2Δ* mutations (**Figure 3.17A**, **Table 3.2**, **Table 3.3**, **Figure 3.18**; note that data for the mock-treated WT strain and for the WT strain cultured with PE21 are replicated in all graphs of **Figure 3.17A** and **Figure 3.18**). PE21 decreased the values of α and increased the values of MRDT for chronologically aging cultures of strains carrying each of these three mutations (**Figure 3.19**; note that data for the mock-treated WT strain and for the WT strain cultured with PE21 are replicated in all graphs of this figure). Thus, PE21 slows aging independently of PKH1/2, TORC1 and PKA.

PE21 increased CLS of the *rim15Δ*, *snf1Δ* and *atg1Δ* mutant strains, although to a slightly lesser extent than that of WT (**Figure 3.17A**, **Table 3.2**, **Table 3.3**, **Figure 3.18**). PE12 lowered the values of α and raised the values of MRDT for chronologically aging cultures of strains carrying each of these mutations, however somewhat less considerably than those for WT (**Figure 3.19**). Hence, PE12 delays aging not through Rim15, SNF1 or ATG.

PE21 extended the CLS (**Figure 3.17A**, **Table 3.2**, **Table 3.3**, **Figure 3.18**), decreased the value of α (**Figure 3.19**) and increased the value of MRDT (**Figure 3.19**) significantly less efficiently for *sch9Δ* than it did for WT. I therefore concluded that PE21 slows aging by attenuating a PKH1/2-sensitive form of Sch9 (**Figure 3.17B**).

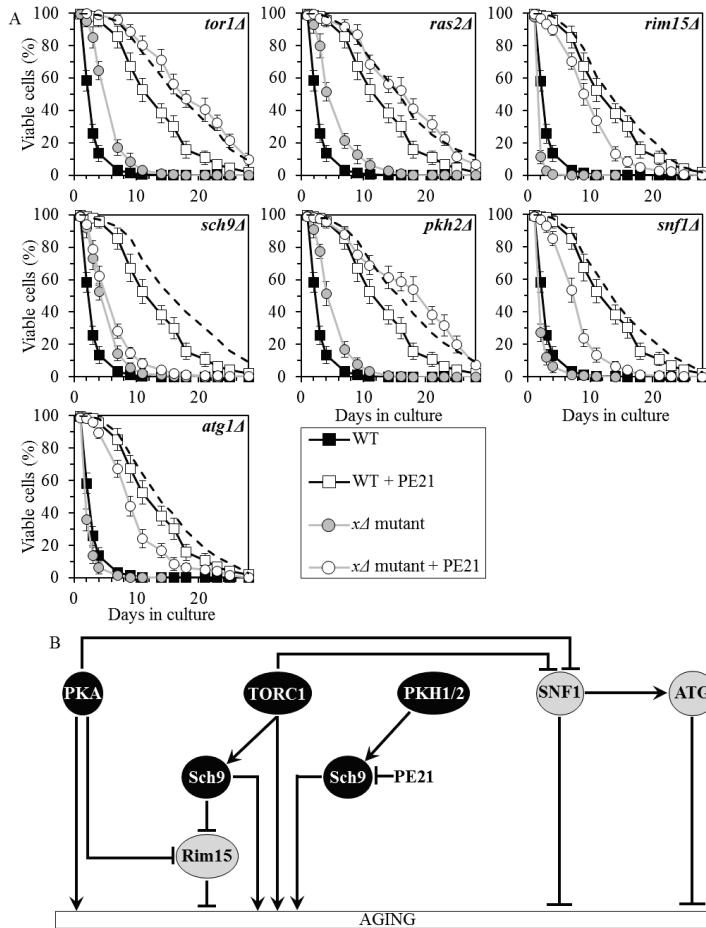


Figure 3.17. PE21 extends yeast CLS by attenuating a PKH1/2-sensitive form of Sch9. (A) Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.1% PE21 (ethanol was used as a vehicle at the final concentration of 0.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves of chronologically aging WT and mutant strains cultured with or without 0.1% PE21 are shown. Data are presented as means \pm SEM ($n = 8$). The dotted line indicates the predicted survival curve of a particular mutant strain cultured with PE21 if this PE exhibits an additive longevity-extending effect with the mutation. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.14**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.14**. Data for the WT strain cultured with PE21 are replicated in all graphs of this Figure. **(B)** The effect of PE21 on the signaling pathways and protein kinases integrated into the longevity-defining network. This effect is inferred from the data presented in **(A)**, **Table 3.2**, **Table 3.3**, **Figure 3.18**, **Figure 3.19**. Abbreviations: as in the legend to **Figure 1.7**.

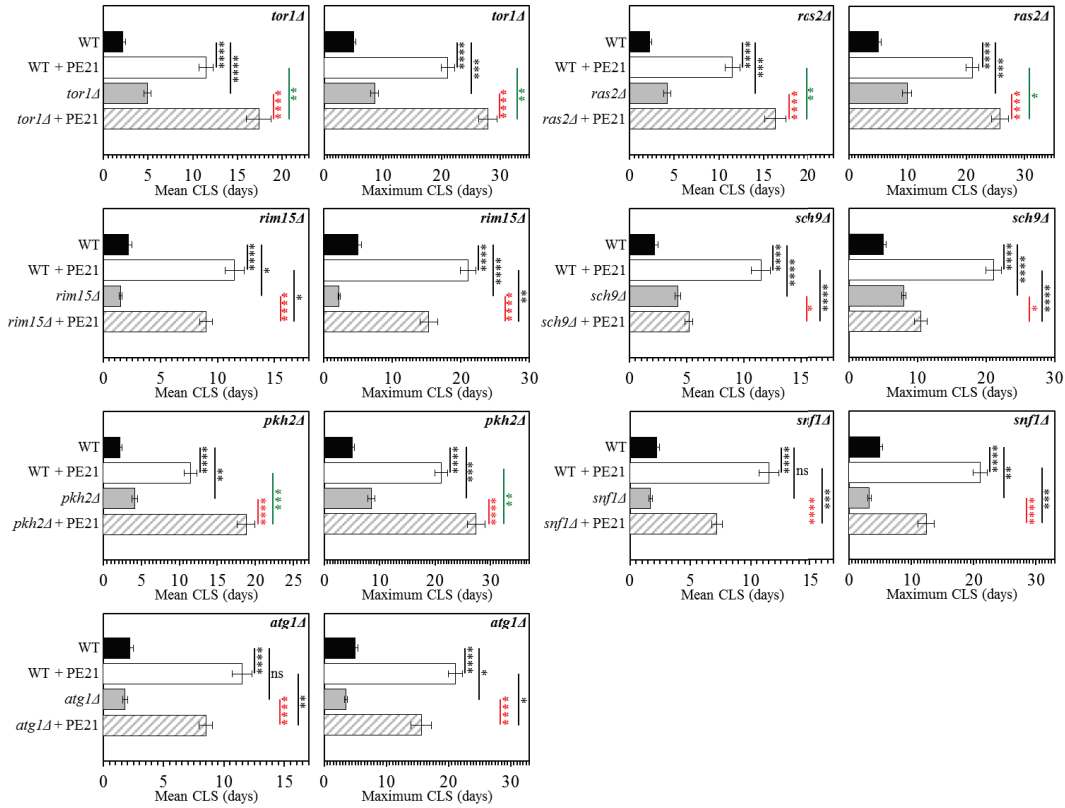


Figure 3.18. PE21 extends the chronological lifespan (CLS) of the *sch9Δ* mutant strain significantly less efficiently than that of the wild-type (WT) strain, exhibits additive CLS-extending effects with the *tor1Δ* and *ras2Δ* mutations, and increases yeast CLS in synergy with the *pkh2Δ* mutation. Cells of the WT and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.1% PE21 (ethanol was used as a vehicle at the final concentration of 0.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.17A** were used to calculate the mean and maximum CLS for WT and mutant strains cultured with or without 0.1% PE21. Data are presented as means \pm SEM ($n = 42$ for WT; $n = 5-7$ for WT with PE21 and mutants strains with or without PE; ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). The ability of PE21 to cause a significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$) increase in the CLS of a particular mutant strain is displayed in red color. The ability of a combination between PE21 and a particular mutation to cause a significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$) increase in CLS-extending efficiencies of each other (i.e. the ability of such combination to exhibit an additive or synergistic CLS-extending effect) is displayed in green color. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.15**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.15**. Data for the WT strain cultured with PE21 are replicated in all graphs of this Figure.

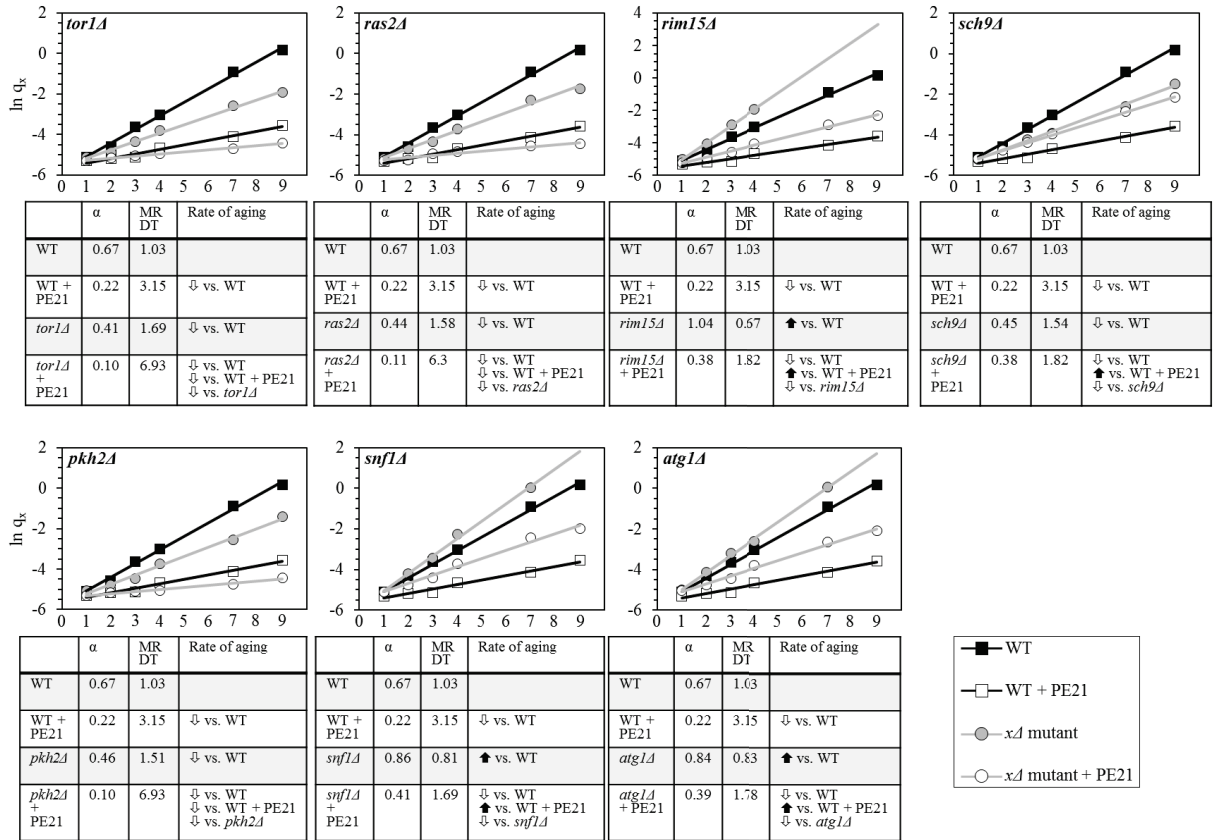


Figure 3.19. Analysis of the Gompertz mortality function indicates that PE21 delays yeast chronological aging by inhibiting a PKH1/2-sensitive form of Sch9. Cells of the wild-type (WT) and indicated mutant strains were grown in the synthetic minimal YNB medium (0.67% Yeast Nitrogen Base without amino acids) initially containing 2% glucose, in the presence of 0.1% PE21 (ethanol was used as a vehicle at the final concentration of 0.5%) or in its absence (cells were subjected to ethanol-mock treatment). Survival curves shown in **Figure 3.17A** were used to calculate the age-specific mortality rates (q_x), the Gompertz mortality rates (also known as mortality rate coefficient α) and the mortality rate doubling times (MRDT) for WT and mutant yeast populations cultured with or without 0.1% PE21. The values of q_x , α and MRDT were calculated as described in Materials and methods. Data for the mock-treated WT strain are replicated in all graphs of this Figure and in all graphs of **Figure 3.16**. Data for each of the mock-treated mutant strains presented in this Figure are replicated in the corresponding graphs of **Figure 3.16**. Data for the WT strain cultured with PE21 are replicated in all graphs of this Figure.

3.4 Discussion

A hypothetical model for how the six PEs delay yeast chronological aging via the longevity-defining network of signaling pathways/protein kinases emerges from my analysis. This model is depicted schematically in **Figure 3.20**. The model suggests that these PEs delay aging as follows: 1) PE4 attenuates the inhibitory effect of TORC1 on SNF1; 2) PE5 weakens both the Rim15-dependent and Rim15-independent branches of the PKA signaling pathway; 3) PE6 activates anti-aging processes and/or inhibits pro-aging processes that are not integrated into the network of signaling pathways/protein kinases; 4) PE8 attenuates the inhibitory effect of PKA on SNF1; 5) PE12 activates Rim15; and 6) PE21 inhibits a PKH1/2-sensitive form of Sch9. Thus, geroprotective chemical compounds from some plants can slow yeast chronological aging by targeting different hubs, nodes and/or links of the longevity-defining network that integrates certain evolutionarily conserved signaling pathways and protein kinases. In the future, it would be important to validate the above hypothesis by investigating how each of the six aging-delaying PEs impacts the physical links that connect individual hubs and nodes of the chronological aging network shown in **Figure 3.20**. These links are known to be mainly activating or inhibiting phosphorylations and dephosphorylations of certain target proteins that are transiently or permanently reside in various cellular locations, including the plasma membrane, vacuole, nucleus, mitochondria or cytosol [120 – 141].

I found that each of the six PEs delays aging through different signaling pathways and/or protein kinases (**Figure 3.20**). It is possible therefore that if these PEs are mixed in various combinations, some of the combinations may display additive or synergistic

effects on the aging-delaying efficiencies of each other. The ongoing studies in the Titorenko laboratory explore this possibility.

My study also revealed that certain combinations of PE4, PE5, PE8, PE12 or PE21 and the *tor1Δ*, *ras2Δ*, *pkh2Δ* or *sch9Δ* mutation (each of which impairs a pro-aging

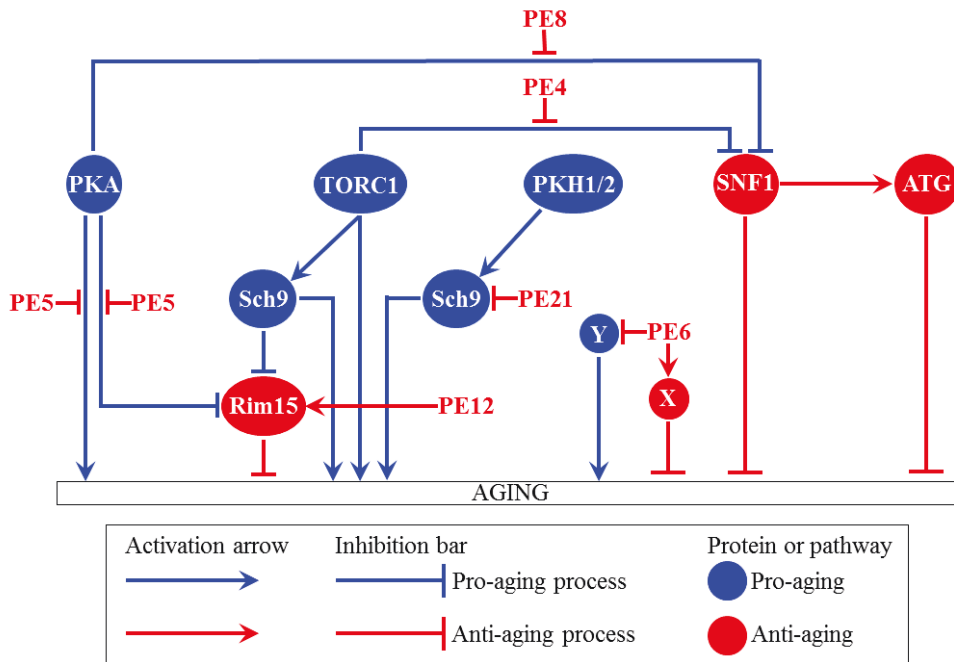


Figure 3.20. A model for how PE4, PE5, PE6, PE8, PE12 and PE21 delay yeast chronological aging via the longevity-defining network of signaling pathways/protein kinases. Activation arrows and inhibition bars denote pro-aging processes (displayed in blue color) or anti-aging processes (displayed in red color). Pro-aging or anti-aging signaling pathways/protein kinases are displayed in blue or red color, respectively. Please see text for additional details.

signaling pathway or protein kinase) markedly increase aging-delaying proficiencies of each other. Furthermore, all combinations of PE6 and mutations impairing either anti-aging or pro-aging signaling pathways/protein kinases display additive or synergistic effects on the extent of aging delay. It is known that the network of longevity-defining signaling pathways/protein kinases is controlled by such aging-delaying chemical compounds as resveratrol, rapamycin, caffeine, spermidine, myriocin, methionine

sulfoxide, lithocholic acid and cryptotanshinone [132, 141 – 154]. One could envision therefore that certain combinations of these chemical compounds and the six PEs may have additive or synergistic effects on the aging-delaying proficiencies of each other. The ongoing studies in the Titorenko laboratory address the validity of this assumption.

The evolutionarily conserved nutrient-sensing signaling pathways that accelerate chronological aging in yeast (**Figure 3.20**) are known to stimulate chronological senescence and geroconversion of post-mitotic human cells; these pathways are likely to expedite organismal aging and cancer development in humans [155 - 161]. Moreover, genetic and pharmacological manipulations that attenuate these signaling pathways and delay chronological aging in yeast are known to decelerate chronological senescence and geroconversion of post-mitotic human cells; it is believed that these manipulations may also delay organismal aging and tumorigenesis in humans [155 - 161]. Thus, some of the six geroprotective PEs that slow down yeast chronological aging through these signaling pathways (**Figure 3.20**) may prolong healthy lifespan and decelerate tumorigenesis.

The challenge for the future is to assess whether any of the six PEs can delay the onset and progression of chronic diseases associated with human aging. Among such diseases are arthritis, diabetes, heart disease, kidney disease, liver dysfunction, sarcopenia, stroke, neurodegenerative diseases (including Parkinson's, Alzheimer's and Huntington's diseases), and many forms of cancer [141, 143, 144, 146, 162 – 179]. Because the major aspects of aging and age-related pathology are conserved across phyla [142 - 146, 163 – 165], it is noteworthy that this study, recent findings [154] and some ongoing research in the Titorenko laboratory have revealed several features of the six PEs as potential interventions for decelerating chronic diseases of old age. These features are

the following: 1) the six PEs are caloric restriction (CR) mimetics that imitate the aging-delaying effects of the CR diet in yeast under non-CR conditions; 2) they are geroprotectors that slow yeast aging by eliciting a hormetic stress response; 3) they extend yeast longevity more efficiently than any lifespan-prolonging chemical compound yet described; 4) they delay aging through signaling pathways and protein kinases implicated in such age-related pathologies as type 2 diabetes, neurodegenerative diseases, cardiac hypertrophy, cardiovascular disease, sarcopenia and cancers; and 5) they extend longevity and delay the onset of age-related diseases in other eukaryotic model organisms. The potential of using the six aging-delaying PEs for delaying the onset of age-related diseases in humans is further underscored by the fact that the Health Canada government agency classifies these PEs as safe for human consumption and recommends to use five of them as health-improving supplements with clinically proven benefits to human health [180].

4 References

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