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Mutations in signal transduction proteins increase stress resistance and longevity in yeast, nematodes, fruit flies, and mammalian neuronal cells

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Abstract

Mutations in Ras and other signal transduction proteins increase survival and resistance to oxidative stress and starvation in stationary phase yeast, nematodes, fruit flies, and in neuronal PC12 cells. The chronological life span of yeast, based on the survival of nondividing cells in stationary phase, has allowed the identification and characterization of long-lived strains with mutations in the G-protein Ras2. This paradigm was also used to identify the in vivo sources and targets of reactive oxygen species and to examine the role of antioxidant enzymes in the longevity of yeast. I will review this model system and discuss the striking phenotypic similarities between long-lived mutants ranging from yeast to mammalian neuronal cells. Taken together, the published studies suggest that survival may be regulated by similar fundamental mechanisms in many eukaryotes and that simple model systems will contribute to our understanding of the aging process in mammals.

Keywords: Aging; Yeast; Neuron; Ras; SOD; Oxidative damage; Stationary phase; G-protein

1. Introduction

Oxidative damage to macromolecules has been implicated in aging and certain aging-related diseases [1,40,56] and is believed to result from stochastic microenvironmental fluctuations in the balance between oxidants, such as O_2^-, H_2O_2, and ·OH, and antioxidants, including superoxide dismutases, peroxidases, and glutathione. However, the demonstrated ability of a single protein, such as Ras, to regulate the generation of reactive oxygen species, antioxidant defenses, and cell death in mammalian cells [11,44,61] raises the possibility that oxidative damage and aging may be regulated by a limited number of genes.

Caenorhabditis elegans (nematode), *Drosophila* (fruit fly), and mice are the three main model systems that are being genetically manipulated to experimentally address this topic [7]. *Saccharomyces cerevisiae* (yeast), thanks to straightforward genetic techniques and to the wealth of information available at the biochemical, molecular, and cellular level, is emerging as a novel and powerful model system to study the genetics of aging [14,22,35].

2. Budding life span and stationary phase

Yeast is a simple, unicellular, eukaryote for which extensive genetic and molecular biology are known. The entire genome has been completely sequenced and contains 5885 potential genes [9,71]. The similarities of a large number of signal transduction and other housekeeping proteins between yeast and humans have enhanced our understanding of human systems, thanks in part to the ability of mammalian proteins to functionally substitute for their yeast analogs. Examples include the antioxidant superoxide dismutase [35], Ras [16], and heat shock proteins [45]. In contrast to mammalian systems, the simplicity of genetic manipulations in yeast allows the removal or over expression of one or multiple genes to study the function of a particular protein [15]. In addition, this small eukaryote can be grown to large, stationary-phase populations of billions of organisms that can be used to screen for longevity mutations or to identify novel genes involved in the long-term resistance to insults, such as oxidative and thermal stress.

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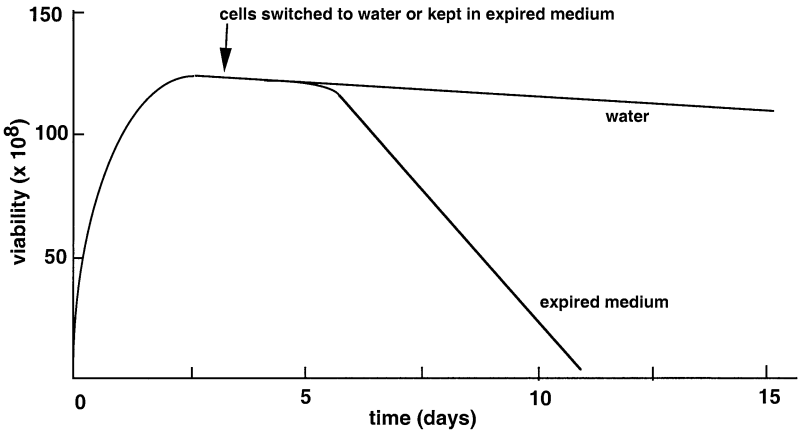

Fig. 1. The chronological life span of yeast. Typical survival curves for yeast stationary phase populations maintained in water or in expired medium. Cells are normally inoculated at a density of 1×10^6 cells/mL and grown in complete minimal medium at 30°C in a shaking incubator. After reaching a density of $2–4 \times 10^7$ cells/mL, external nutrients become scarce, growth slows down, and the population begins to store glycogen and other nutrients. By Day 4 (in strain EG103) cells enter the nondividing stationary phase characterized by decreased metabolic rates and increased protection against heat and oxidative stress. Mean survival, depending on the strain and the incubation medium (water vs. expired medium), ranges from 8 to 50 days. Viability in the culture is measured every 24 or 48 h by serially diluting aliquots of the population and plating onto rich medium plates. The ability of each live cell to form a colony is used to estimate viability in the culture.

Aging in yeast is often measured by counting the finite number of buds that can be generated by a single mother cell maintained in the growth phase (budding life span) [48]. Published studies that used this paradigm suggest that replicative aging is caused by the accumulation of rDNA circles resulting in nucleolar fragmentation [59]. During the budding life span, each daughter cell must be separated from the mother cell by micromanipulation to prevent overcrowding and entry of the population in stationary phase [69]. In this nondividing phase, the cells can survive for long periods without dividing. Although the relationship between the budding life span and stationary phase survival is not clear [58], a recent study demonstrated that yeast cells maintained in stationary phase show a decrease in replicative life span after reentering the cell cycle [2]. The mean replicative life span was 27 after 1 day, 20 after 13 days, and 16 after 33 days in stationary phase, suggesting that nondividing yeast undergo senescence. Furthermore, one of the mutants with increased budding life span, *sir4–42*, was isolated by screening for cells able to survive longer than the wild-type in stationary phase [26]. However, other studies demonstrate that the over expression of the G-protein Ras2, which causes rapid death in stationary phase, increases the budding life span [62], whereas the deletion of Ras2, which doubles stationary phase survival (Longo et al., unpublished results), decreases budding life span [62]. The effect of Ras2 on budding life span was proposed to involve retrograde regulation; a regulation of signaling from the mitochondrion to the nucleus [29]. In summary, although some of the genes and mechanisms that regulate budding potential can also affect survival in stationary phase, further studies are necessary to clarify the relationship between these two paradigms.

Stationary phase is relatively well-understood from decades of study [70]. As the level of external nutrients decreases, yeast cells store glycogen and other nutrients intracellularly, decrease metabolic rates and protein synthesis, and develop increased thermotolerance and antioxidant defenses [70]. Cells can survive in stationary phase for days to months depending on the strain and on the incubation medium (Fig. 1) and can, under very low nutrient conditions, undergo meiosis and form spores able to survive for months to years, with metabolic rates lower than those of stationary phase cells [4].

Although the majority of multicellular eukaryotes, including the ones that can enter a hypometabolic state, such as nematodes, will spend most of their adult life with normal metabolic rates, yeast, however, either sporulate or unavoidably survive in the low metabolism stationary phase. In fact, “much of the microorganismal mass in the world is estimated to exist under nutrient-depleted conditions” [69]. Thus, it may be more correct to view the short growth phase of yeast as a hypermetabolic state aimed at quickly generating a large population and the long stationary phase as the normal metabolic state for long-term survival during which internal nutrient reserves are used slowly. The very low metabolism spore state, entered only by a minority of cells under extreme nutrient conditions, may be viewed as a true hypometabolic state, analogous to the nematode dauer larva (as recently pointed out by Kenyon and colleagues [5]).

3. Chronological life span of yeast

Most studies of yeast are performed by using logarithmically growing cells. However, the growth phase is not suited to study the accumulation of oxidative and other forms of macromolecular damage because individual cells

can only be exposed to a short period of stress, and damage is rapidly diluted by the synthesis of new macromolecules required for rapid growth. In fact, yeast cells grow well even in the presence of elevated concentrations of O_2^- and H_2O_2 [35,36]. By contrast, the long-term survival of cells maintained in stationary phase can serve as a valuable system to monitor long-term macromolecular damage and mortality [34–36,68]. This paradigm was termed the chronological life span to distinguish it from the budding life span. In most of the aging studies performed that use the chronological life span, cells are either grown and maintained throughout the study in minimal glucose medium, or they are transferred to water on Day 3 and washed every 48 h (Fig. 1) [13,35]. The cultures, grown in shaking flasks maintained at 30°C, normally reach a density of ~100 million cells/mL and, depending on the strain and the incubation medium, have a mean survival of 8–50 days (Fig. 1) [34,35].

These paradigms have allowed the simulation of two conditions commonly encountered by stationary phase yeast in the wild: 1) an environment with limited nutrient resources (expired medium) that causes a rapid increase in mortality after Day 5 (strain EG103) and that allows some growth to occur after the majority of cells are dead (usually >99.9%), and 2) an environment with low or no nutrients (water), that does not allow growth to occur, in which cells survive for extended time. Notably, the mechanisms that regulate survival under these environments appear to be similar because strains with increased survival in expired medium also survive longer in water.

4. Oxidative damage and longevity in yeast

Bacteria and yeast have been used for many years as simple model systems to study the function of antioxidant enzymes and to identify the sources of reactive oxygen species. Similarly to human cells, *S. cerevisiae* expresses a cytosolic CuZn superoxide dismutase (*SOD1*) and catalase (*CTT1*) as well as a mitochondrial Mn SOD (*SOD2*). The first report on the yeast chronological life span model system showed that cytoplasmic and mitochondrial superoxide dismutases, but not catalase or metallothionein, are required for the long-term survival of yeast. Sod2 was found to be required under both low and normal oxygen conditions, whereas cytoplasmic Sod1 was mainly required under normal aeration [35]. The expression of human *SOD1* in yeast *sod1* null mutants completely reversed the survival defects. These results are consistent with studies performed with the use of mice lacking *sod1*. Although *sod1* knockout mice showed few abnormalities, cultured fibroblast obtained from these mice were 80 times more sensitive to the superoxide generator paraquat than were wild-type cells. Furthermore these fibroblasts grew poorly in air [19], indicating that, as reported for yeast, Sod1 is only required under high concentration of oxygen or superoxide. The exposure of cultured cells to air, and therefore to a concentration of oxygen at least fourfold higher than that in the brain of live animals, may explain why *sod1* animals show few abnormalities in vivo, whereas cultured fibroblasts obtained from these animals have severe defects.

Table 1
Activity of mitochondrial enzymes in yeast and mice lacking Sod2

	Yeast[a] (Days 1–3)	Mice[b] (Days 4–6)
Survival	5–10 days	<10 days
Aconitase	↓ 67–96%	↓ 67–89%
Succinate dehydrogenase	↓ 52–84%	↓ 65–76%
Cyt c ox.	↓ 22–40%[c]	No change
ATPase	↓ 13–26%[c]	ND

[a] See reference 36.
[b] See reference 43.
[c] No change when adjusted for viability in addition to protein concentration.

Unlike most experimental organisms, yeast have the ability to grow either by respiration by using nonfermentable carbon sources or exclusively by fermentation by using glucose (low respiration). This simple feature, termed index of respiratory competence (IRC), is very useful when designing experiments to separate mitochondrial from extramitochondrial damage. Using the IRC, Longo et al. [36] were able to define the sequence of events that lead to the death of *sod2* mutants and were able to explore the role of mitochondrial damage in the mortality of wild-type yeast in stationary phase. The characterization of yeast *sod2* null mutants resulted in the identification of mitochondrial aconitase and succinate dehydrogenase, both 4Fe-4S cluster binding proteins, as the primary targets of mitochondrial superoxide [36]. These results are consistent with studies in *sod2* knockout mice in which the activity of mitochondrial aconitase was reduced by >67% in the heart and brain, and succinate dehydrogenase was reduced by >65% in the heart and skeletal muscle (Table 1**)**. As expected, in *sod2* mice the cells most severely affected by the increased concentration of mitochondrial superoxide were postmitotic. The similarities between stationary phase yeast and mice lacking either *SOD1* or *SOD2* suggest that the chronological life span of yeast is a valuable model system for the study of mechanisms of oxidative damage in mammalian cells, particularly postmitotic cells.

The over expression of CuZn SOD has been convincingly demonstrated to increase the life span of fruit flies. Orr et al. [51] showed that flies with extra copies of both CuZn SOD and cytosolic catalase, but neither gene alone, survived 30% longer than controls. However, more recent studies showed that the over expression of CuZn SOD alone is sufficient to extend the life span of fruit flies. The over expression of CuZn SOD in only the motor neurons extends life span by up to 40% [52]. A similar extension in longevity was obtained when the over expression of CuZn SOD alone was induced in adult flies by using the yeast recombinase system [63]. To test whether the over expression of antioxidant enzymes could also extend the life span

Table 2
Signal transduction mutations, stress resistance and longevity in eukaryotes

Gene(s)	Protein function	Life span (% increase)	Increased resistance to
S. cerevisiae (chronological life span)			
*ras2*Δ[a]	G-protein	100%	Oxidants, heat, starvation
↑ *SOD1-SOD2*[a]	Antioxidants	10–33%	Oxidants
C. elegans			
age-1[a]	PI3K	65%	Oxidants, heat, starvation
daf-2[a]	Insulin receptor-like	100%	Oxidants, heat, starvation
Drosophila			
Mth[a]	G-protein coupled rec.	35%	Oxidants, heat, starvation
↑ *SOD1*[a]	Antioxidant	30–40%	Oxidants
Neuronal cells (PC12)			
p21RAS[a]	G-protein	>100%	Oxidants, serum withdrawal

[a] See text.

of yeast, Longo et al. constructed yeast strains expressing several-fold higher concentrations of these antioxidant enzymes. In yeast, the over expression of *SOD1* and *SOD2* together increased viability in stationary phase (Longo et al., unpublished results). The over expression of each Sod alone had a significant, but modest, effect on survival.

Yeast has been used for many years as a model system to understand the function and mechanism of action of human proteins. The anti-apoptotic human Bcl-2 protein was over expressed in yeast *sod* mutants and in wild-type cells to investigate its mechanism of action and to understand whether yeast may have components of a programmed cell-death pathway [34]. The results suggested that human Bcl-2 partially reversed several defects of yeast that lacked *sod1* or both *sod1* and *sod2*, which is consistent with the demonstrated antioxidant function of Bcl-2 in mammalian cells [24]. Bcl-2 over expression increased the long-term viability and growth in 100% oxygen of *sod1* and *sod1sod2* mutants [34]. Bcl-2 also delayed the death of wild-type cells by ~2 days in stationary phase, raising the possibility that portions of an apoptotic pathway are present in yeast. This hypothesis was recently supported by studies that show that the expression of the human pro-apoptotic protein Bax causes cell death in yeast, which can be reversed by co-expression of Bcl-2 [42,57]. Furthermore, the depletion of glutathione or the treatment of yeast cells with hydrogen peroxide was shown to induce a form of apoptosis that was blocked by inhibiting protein synthesis and by incubating the cells under low oxygen [37].

5. Control of survival by signal transduction proteins in nondividing cells

Central signal transduction proteins, by virtue of controlling a wide range of cellular functions, are ideal candidates as regulators of survival in simple eukaryotes. Mutations in signal transduction proteins were found to increase survival in *C. elegans* [23,27,28,47] and in *Drosophila* [32]. A mutation that increases the chronological life span, as well as the thermotolerance and antioxidant defenses, was recently identified in yeast (Longo et al., unpublished results). This work suggests that the G-protein Ras2, which is highly conserved in many organisms and functionally interchangeable between yeast and human cells, decreases stress protection and survival in yeast (Table 2). In two strains lacking the *ras2* gene, mean survival increased by >100% compared to wild-type. In both strains, *ras2* null mutations caused increased resistance to superoxide toxicity. Although over expressing various combinations of antioxidant enzymes increased survival, the effect was much smaller compared to that observed in *ras2*, indicating that changes in multiple systems are necessary to achieve a major extension in longevity.

The Ras2/protein kinase A (PKA) pathway (Fig. 2) negatively regulates a number of genes that contain the *st*ress *r*esponse *e*lement (STRE) in their promoters [38,55] through its action on transcription factors Msn2 and Msn4 [12,60]. Among the genes reported to be regulated in this manner are those for several heat shock proteins, catalase (*CTT1*), polyubiquitin (*UBI4*) and the DNA damage-inducible gene *DDR2* [41]. The CuZn SOD promoter also contains a potential STRE sequence. In the yeast strain SP1, the deletion of *ras2* caused a twofold increase in SOD activity and increased resistance to the superoxide-generating agent paraquat (Longo et al., unpublished results). *ras2* mutations were also shown to double the expression of *SOD2* in the yeast strain JC482 [8]. Over expression of Msn2 and Msn4 was shown to increase survival, in stationary phase, and thermotolerance [41]. These results are consistent with the data obtained by using *ras2* mutants (Longo et al., unpublished results), and indicate roles for Msn2, Msn4, and STRE response elements in life span regulation in the pathway downstream of Ras2. Taken together, these studies suggest that, in the presence of all essential nutrients, yeast Ras2 promotes growth, while downregulating the expres-

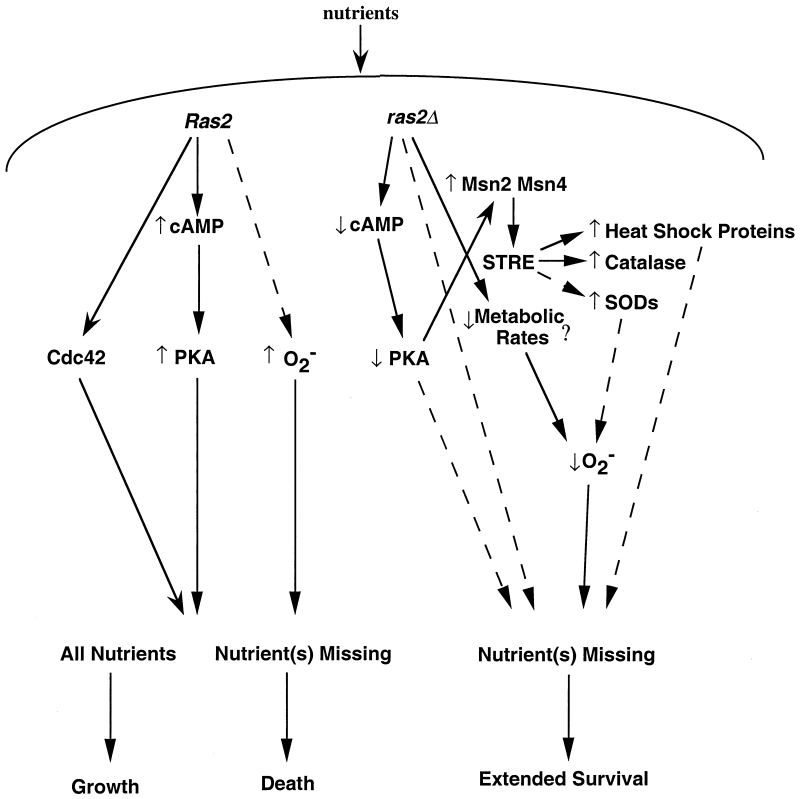

Fig. 2. The Ras pathway, protection, survival, and death in *S. cerevisiae*. In the presence of the nutrients necessary for growth, in wild-type yeast (*RAS2*) the activation of both Ras1 and Ras2 in turn increases cyclic AMP generation [66] and activates protein kinase A [65] as well as a cyclic AMP-independent pathway [49,53]. Activation of Ras promotes growth when all the required nutrients are available, but causes loss of viability when essential nutrients are absent [66]. *ras2* null mutations (*ras2*Δ) increase glycogen accumulation [64], SOD activity (Longo et al., unpublished results; [8]), catalase activity [55], and thermotolerance [38], and increase chronological survival by twofold (see text). Metabolic rates are similar to that of wild-type before and after the entry into the hypometabolic state. However, in *ras2*Δ mutants the entry into the hypometabolic state occurs 1–2 days earlier than in wild-type (Longo et al., unpublished results). In the presence of all essential nutrients, the activity of Ras1 is sufficient for growth in *ras2*Δ mutants [25]. The Ras2/PKA-dependent regulation of stress response and glycogen accumulation is mediated by transcription factors Msn2 and Msn4 [12,60] through the induction of genes that contain a response element (STRE) in their promoter, such as SOD, catalase, and several heat shock genes (see text). The life-extension effect of the double over expression of Sod1 and Sod2 suggest that physiological concentrations of cytoplasmic and mitochondrial O_2^- act synergistically to decrease survival in wild-type strains (Longo et al., unpublished results). The increased resistance to superoxide toxicity and the induction of multiple protection systems through STREs are likely to mediate the extension of survival in *ras2* mutants (see text). It is not known whether in addition to downregulating antioxidant enzymes, Ras2 activity causes an increased generation of superoxide, as shown in mammalian neuronal PC12 and other cells (see text). Dashed lines indicate possible functions; solid lines indicate known functions (see text for details and further references).

sion of antioxidants and heat shock proteins (Fig. 2). When essential nutrient are missing, Ras2 promotes the loss of viability that appears to be mediated in part by superoxide (Longo et al., unpublished results; [66]). It is not clear whether superoxide causes death in yeast only by damaging macromolecules directly, and through the generation of the highly reactive hydroxyl radical, or by acting as a signal transduction messenger that promotes death. Recent results suggest that activation of Ras increases superoxide generation, which in turn functions as a mitogenic signaling molecule in fibroblasts and PC12 cells [20,21,44]. Although a signaling role for superoxide has not been demonstrated in yeast, we cannot exclude that superoxide may act both as a toxic oxygen species that causes direct macromolecular damage and as a signal transduction messenger that promotes either apoptosis or necrosis.

There are many phenotypic similarities between long-lived yeast *ras2* mutants and long-lived mutants of certain higher eukaryotes (Table 2). *C. elegans age-1* and *daf-2* mutations, which have been shown to increase the life span in adult organisms by 65–100%, occur in signal transduction genes that are involved in regulating the formation of the hypometabolic, long-lived dauer larva [23,27,28]. Analogously to *ras2* mutations in yeast, these longevity mutations in nematodes also cause increased accumulation of nutrients, thermotolerance, and antioxidant defense [28,30, 33,67]. Recently, the over expression of *SOD1* was shown to increase survival in *Drosophila* by up to 40% [51,52,63], and a *Drosophila* line with a mutation in the G-protein coupled receptor homolog *MTH* gene displayed a 35% increase in life span [32]. *Drosophila mth* mutants are resistant to starvation and superoxide toxicity. The striking similarities between long-lived mutants of organisms as phylogenetically distant as yeast, nematodes, and fruit flies

suggest that longevity may be regulated by similar mechanisms in many eukaryotes (Table 2).

6. Do signal transduction proteins regulate survival in mammals?

The phenotypic similarities between long-lived eukaryotic mutants (Table 2) raises the possibility that analogous mutations may also affect the survival of mammals. Interestingly, two of the yeast genes found to have the most profound effect on survival, *SOD1* and *RAS2*, have DNA sequences that are >60% identical to their mammalian homologs and can be functionally substituted by them [35, 46]. Although, the possibility that mutations in G-proteins affect mammalian longevity has not been addressed at the organismal level, several studies performed with the use of mammalian cells suggest that Ras activity increases the generation of superoxide and decreases survival in neurons. Neuronal apoptosis increases in mice that lack a negative regulator of Ras [18], whereas inhibition of p21Ras rescues naive and neuronally differentiated PC12 cells from apoptotic death (Table 2) [6]. Analogously to yeast lacking Ras2, the inhibition of p21Ras in PC12 cells increases resistance to oxidative stress [61] and survival after serum withdrawal [6]. Inhibition of p21Ras in PC12 cells also prevents superoxide generation induced by treatment with epidermal growth factor (EGF) [44]. Ras also mediates apoptosis in T cells [10] and in human epithelial cells [17] and induces replicative senescence in human diploid fibroblasts by increasing intracellular levels of reactive oxygen species [31], suggesting that its ability to cause cell death is not limited to neuronal cells.

However, Ras activation was also shown to prevent cell death in rat sympathetic neurons [50] and in endothelial cells [54]. These apparently contradictory roles of Ras in the induction and prevention of cell death are consistent with multiple functions of mammalian Ras. Activation of Ras may cause or prevent cell death depending on the activity of other signal transduction pathways and on the relative activity of proteins acting downstream of Ras, such as MAP kinase (MAPK), Rac1, and protein kinase B, which have been implicated in both pro-apoptotic and anti-apoptotic signaling [3,11,39,72].

7. Conclusion

The involvement of signal transduction proteins that affect longevity in the regulation of thermotolerance, resistance to oxidative stress, and accumulation of reserve nutrients in yeast, nematodes, and flies suggest that longevity is regulated, or at least strongly influenced, by similar mechanism in many eukaryotes. It would be very surprising if the increased resistance to starvation heat and oxidative stress shared by all the long-lived mutants (Table 2) was just a coincidence. In yeast, the Ras pathway plays a central role in cell growth, but decreases survival in nondividing organisms. In neurons, Ras functions in a pathway that mediates cell growth and differentiation, but can induce cell death. Although Ras function and apoptosis have not been demonstrated to decrease longevity in mammals, the disruption of the delicate balance between multiple-signal transduction pathways may be responsible for the age-dependent increase in oxidative damage, loss of certain cellular functions, and death of postmitotic cells. Thus the central role of Ras and other signal transduction proteins in cellular functions ranging from growth, to differentiation, to death makes these signaling proteins ideal candidates as longevity assurance genes. Further biochemical and genetic studies in simple and rodent model systems should soon reveal the extent of the conservation of the role of signal transduction-dependent regulation of multiple protection systems in the longevity of eukaryotes.

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