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T. Nyström
H. D. Osiewacz
(Eds.)

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Model Systems in Aging

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Model Systems in Aging

With 38 Figures, 2 in Color, and 9 Tables

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The cover illustration depicts pseudohyphal filaments of the ascomycete Saccharomyces cerevisiae that enable this organism to forage for nutrients. Pseudohyphal filaments were induced here in a wild-type haploid MATa S1278b strain by an unknown readily diffusible factor provided by growth in confrontation with an isogenic petite yeast strain in a sealed petri dish for two weeks and photographed at 100X magnification (provided by Xuewen Pan and Joseph Heitman).

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1 Conditional senescence in prokaryotes

Thomas Nyström

Abstract

Bacteria are immortal in the sense that their capacity for reproduction appears limitless as long as the environment supports growth. However, this reproductive ability is gradually lost upon famine conditions. The loss of reproductive ability is one of the first signs of physiological deterioration and is followed by a collapse of the membrane integrity. The underlying molecular mechanism behind this degenerative change and decline is something of an enigma in bacteriology. Still, recent analysis of the conditional deterioration of growth arrested *Escherichia coli* cells has revealed interesting similarities with the aging process of higher organisms. The similarities include oxidation of proteins and its target specificity, the role of antioxidants and oxygen tension, and an apparent antagonism between reproduction and survival activities. The analysis of the *E. coli* model system has also revealed a novel culprit in the oxidation of proteins, i.e. an intimate link between the fidelity of the translational apparatus and oxidative modification of proteins. This review summarizes and evaluates the models of bacterial conditional deterioration and relates them to contemporary theories of aging in higher organisms.

1.1 Introduction

The raison d'être for prokaryotes being mentioned in books on aging is that they serve as a prime example of organisms that do not age (e.g. Rose 1991; Holliday 1995). The reason for this is that their reproduction is accomplished by binary fission. Cytokinesis proceeds in a symmetrical fashion with a non-conservative dispersion of cytoplasmic material and damaged constituents are distributed equally, more or less, to both cells produced. As stated by Rose (1991), there is no adult form of bacterial cells and the population is not age-structured. However, it should be noted that these arguments are only applicable when considering an exponentially growing population of bacterial cells. Therefore, the statement that prokaryotes are examples of organisms that have evolved indefinitely long life spans (Rose 1991) is obscure. In fact, it can be argued that the bacterium as an individual ceases to exist at the time when cytokinesis is completed. Thus, the concept of a life span for dividing individual bacterial cells is meaningless and when instead applied to a population of growing cells in a clonal lineage becomes even less comprehensible.

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However, the terms life span, longevity, and bacterial age, can be useful when considering cells entering a non-proliferating state. Upon depletion of an essential nutrient already initiated cell division processes go to completion (reductive division). Thereafter, no further growth or proliferation processes will be initiated. At the time when reductive division is completed, the cells are defined as newborn. Progressive physiological alterations can subsequently be followed as a function of the chronological age of these individual growth arrested cells. The life span of such cells is far from indefinitely long; typically the mean life span of growth arrested *Escherichia coli* cells is around 3 to 5 days when starved for exogenous carbon (Ericsson et al. 2000; Ballesteros et al. 2001). Life span is most often analyzed with respect to the ability of individual cells to reproduce and form colonies on standard nutrient plates. The loss in plating efficiency has been described in microbial textbooks as the death phase of the bacterial growth cycle and such death has been argued to be the nearest bacteria come to a 'natural' death of the kind familiar among higher organisms (Postgate 1976). However, it should be stressed that it is a form of accidental death and is conceptually distinct from mandatory aging in higher organisms. Therefore, the term conditional senescence has been used to make such a distinction (Nyström 1995; 1999; 2002a; 2003) and one of the first signs of senescence is the failure of growth arrested cells to recover and reproduce once nutrients become available. In some cases, other markers of senescence have been analyzed, including membrane leakiness (Ericsson et al. 2000). In addition, it turns out that many molecular alterations occurring during conditional senescence of bacteria share features of the aging process of higher organisms (Nyström 2002b). These alterations and ideas concerning the underlying mechanisms will be reviewed here. In addition, special areas in which prokaryotic model systems may be instrumental for obtaining a detailed understanding of the nature and causation of degenerative processes at the cellular/molecular level will be highlighted.

1.2 The noble art of self-defense

Bacteria, like *E. coli*, display a remarkable capacity to defend themselves against the vicissitudes of their environment. This self-defense encompasses paradigm global regulatory networks, such as the heat shock and cold shock regulons, oxidative stress defenses, and the SOS response to DNA damaging agents. These regulatory networks allow the bacterial cell to survive, and sometimes even to reproduce, under a large variety of conditions that are lethal for higher organisms. The regulatory networks involved in defending the cells against a specific stress are usually induced by that specific stress condition (VanBogelen et al. 1990). Yet, global analysis of gene expression has shown that many stress networks, or part of networks, are induced during starvation for different essential nutrients when no other stress is imposed on the cells (Groat et al. 1986; Jenkins et al. 1988; VanBogelen et al. 1990; Matin 1991). This increased production of stress proteins render the starved and growth-arrested cell highly resistant to a variety of external

stresses (including heat, oxidative agents, and osmotic challenge); a phenomenon labeled starvation-induced cross protection (Jenkins et al. 1988; Matin 1991).

One model suggests that this induction of stress defense proteins prepares the starved cell for stress conditions that it may encounter in the future. It has been argued that such a response is sensible at the onset of starvation because energy and building blocks will become increasingly limited as cells progress into stationary phase making inducible responses less immediate and less effective (Kolter et al. 1993). Another model suggests that the induction of stress genes has a role in minimizing damage to target molecules caused by stasis *per se* (Matin 1991; Eisenstark et al. 1996; Dukan and Nyström 1998). It is suggested that stasis causes an increased damage of cellular components and that stress resistance develops because the cells are already exposed to the normal stress response signals. In support of this model, with respect to induction of heat shock genes during stasis, it was demonstrated that aberrant proteins are the likely candidates triggering induction of the heat shock regulon during both heat stress and stasis (Dukan and Nyström 1998). However, the signal (aberrant proteins) is in the latter case generated by a different pathway involving protein oxidation and translational errors (Dukan and Nyström 1998; Ballesteros et al. 2001). In addition, the role for many stress proteins in promoting stasis survival (Dukan and Nyström 1999; Benov and Fridovich 1995; Eisenstark et al. 1996) suggests that the starvation induced induction of stress regulons is not just for future provision but is actively participating in stasis physiology.

1.3 The nature of the bacterial defense against conditional senescence

Several of the genes required in the alleged defense against starvation-induced deterioration have now been identified and many of these encode proteins with functions related to oxidative management (Jenkins et al. 1986; Matin 1991; Kolter et al. 1993; Hengge-Aronis 1993; 2000; Nyström 2002a; 2003). The response of *E. coli* to starvation also includes an increased synthesis of specific glycolysis enzymes, concomitantly with a reduced production of TCA cycle enzymes (Nyström et al. 1996). In other words, the modulation of the synthesis of catabolic enzymes during aerobic carbon/energy starvation is remarkably similar to the response of cells shifted to anaerobiosis. *E. coli* can grow under aerobic and anaerobic conditions, deriving energy from a number of different respiratory pathways or from fermentation. Different genetic regulatory programs coordinately direct the cells' selection of the most efficient metabolic system in a particular environment. This selection ensures that electrons are channeled from donors to a terminal acceptor such that the drop in Gibbs energy is the maximal allowed under the particular growth condition (Iuchi and Lin 1991). This regulation, presumably, optimizes the metabolic systems used to maximize growth rate in a given environment. The ArcA/ArcB regulon is one of the global regulatory systems involved in this metabolic regulation (Iuchi and Lin 1991). The ArcA/ArcB pair makes up a two-

component regulatory system, which is activated when the environment contains no electron acceptors or only poor ones; ArcA being the regulator and ArcB the sensor component (Iuchi and Lin 1991).

The ArcA regulator is also a key player in starvation-induced modulations of gene expression and a Δ*arcA* mutant is impaired in several activities associated with the *E. coli* starvation response (Nyström et al. 1996). Specifically, the ArcA regulon appears to be involved in checking the rate of catabolic degradation of endogenous biomolecules. The respiratory activity is significantly higher during stasis in the Δ*arcA* mutant than the wild type parent as is the total metabolic activity and the fraction of total activity derived from aerobic respiration (Nyström et al. 1996). This ArcA-dependent response may be a defense mechanism mustered by the cell to protect itself against self-inflicted oxidative damage. In support of this notion, the reduction in the life span of growth-arrested cells caused by the *arcA* mutation can be suppressed by overproducing the superoxide dimutase SodA (Nyström et al. 1996). Thus, the ArcA dependent down regulation of reducing equivalent production and respiratory activity is suggested to reduce generation of reactive oxygen species (ROS) during starvation.

Primary oxidative defense proteins, including the superoxide dismutases (SodA and SodB), and catalases (KatE and KatG) are also induced in growth arrested cells, and denote the second line of defense, which further reduces ROS levels catalytically (Dukan and Nyström 1999). The elevated production of proteins involved in the reduction, repair, and proteolysis of oxidized proteins is a third mode of action in the bacterial defense against starvation-induced oxidation damage. The proteins of this defense system include peptide methionine sulfoxide reductase, glutathione reductase in concert with glutathione, thioredoxin, glutaredoxin, and heat shock chaperones (e.g. Eisenstark et al. 1996; Potamitou et al. 2002a; 2002b; Dukan and Nyström 1998; Nyström 2002a).

1.4 The failure of the bacterial self-defenses

As outlined in section 3, the data suggest that there is an increased demand for oxidation management in cells subjected to nutrient starvation and a significant number of the genes and regulons induced by stasis is part of such an induced defense machinery. However, like in aging organisms of eukaryotic origin, this system fails to fully combat stasis-induced oxidation of proteins. Oxidative modifications of proteins, such as carbonylation and illegitimate disulfide bond formation, increase during stasis in wild type *E. coli* cells (Dukan and Nyström 1998) and this oxidation affects specific proteins; e.g. the Hsp-70 chaperone, DnaK, the histone-like protein, H-NS, the universal protein, UspA, elongation factors, EF-Tu and EF-G, glutamine synthase, glutamate synthase, aconitase, malate dehydrogenase, and pyruvate kinase (Tamarit et al. 1998; Dukan and Nyström 1998; 1999). Some of these proteins have been demonstrated to be specifically carbonylated also in oxidation stressed yeast cells (Cabiscol et al. 2000), aging flies (Yan et al. 1997; Sohal 2002), and in Alzheimer's disease brain (Castegna et al. 2002).

In some cases, the levels of oxidatively damaged proteins have been shown to be associated with the physiological age or life expectancy of an organism rather than with its chronological age. For example, carbonyl levels are higher in crawlers (low life expectancy) than fliers in a cohort of houseflies of the same chronological age (Sohal et al. 1993). Similarly, using in situ detection of protein oxidation in single *E. coli* cells and a density gradient centrifugation technique to separate culturable and sterile cells of the same chronological age it was demonstrated that the proteins of sterile cells exhibit increased and irreversible oxidative damage (Desnues et al. 2003). In *E. coli*, starvation-induced sterility is rapidly followed by membrane malfunctioning and an irreversible loss of life supporting activities (Ericsson et al. 2000).

A question of interest is how the asymmetry in population damage is generated. Recent publications demonstrate that the expression of more genes than previously anticipated is affected during progression through the bacterial division cycle (Weitao et al. 2000; Laub et al. 2000). Possibly, a sudden arrest of growth at a time in the cycle when specific gene products, e.g. superoxide dismutases A and B, are present at low levels could generate a sub-population of cells experiencing increased damage during prolonged stasis. There is no direct evidence for this notion but it is interesting to note that the abundance of Sod is much lower in sterile *E. coli* cells and that the pattern of protein carbonylation is similar in sterile cells and cells lacking cytoplasmic Sod activity. For example, self-inflicted oxidation of proteins is enhanced in both *sod* mutants (Dukan and Nyström 1999) and nonculturable cells (Desnues et al. 2003). Moreover, the oxidation of specific proteins, such as H-NS, GltD and FabB, is similar in *sod* deficient cells in sterile wild type cells. In addition, the elevated expression of specific stress regulons in sterile cells can be mimicked by a diminished Sod activity (Desnues et al. 2003). Finally, *sodA sodB* mutants have been demonstrated to lose culturability at an elevated rate during stasis (Benov and Fridovich 1995; Dukan and Nyström 1999). Thus, it is possible that the loss of reproductive ability of some cells entering stationary phase is linked to the abundance of Sod in these individual cells. If so, the longevity of stationary phase *E. coli* cells might, like aging fruit flies (Sun and Tower 1999), be associated to the cellular availability of Sod.

A possible explanation for the failure of cells to combat stasis-induced oxidation comes from recent results demonstrating that this oxidation might occur by a route that eludes the classical oxidative defense pathways. The level of oxidized proteins increase upon treatment of cells with antibiotics and mutations causing increased mistranslation (Dukan et al. 2000). Interestingly, during these treatments, the rate of superoxide production and the activity of the superoxide dismutases and catalases are unchanged and the expression of oxidative stress defense genes do not increase (Dukan et al. 2000). In addition, it was demonstrated that the increased oxidation during these treatments was primarily the result of aberrant protein isoforms being oxidized (Dukan et al. 2000). In other words, increased protein oxidation can be the result of increased production of aberrant proteins and this does not appear to be sensed by the oxidative defense regulons and does not require increased generation of reactive oxygen species (Dukan et al. 2000). Moreover, two-dimensional gel electrophoresis of proteins demonstrated

that the sudden increase in protein oxidation during the early stages of stasis in *E. coli* is strongly associated with the production of aberrant protein isoforms that appear to be specific targets for oxidative modifications (Ballesteros et al. 2001). In addition, results showing that frameshifting (Barak et al. 1996), missense errors (O'Farrell 1978) and stop codon read-trough (Wenthzel et al. 1998; Ballesteros et al. 2001) increase in response to stasis in *E. coli* cells, suggest that protein oxidation in non-proliferating cells might be caused by an increased mistranslation. Indeed, protein oxidation is drastically attenuated in the early stages of stasis in *E. coli* cells harboring intrinsically hyper-accurate ribosomes (Ballesteros et al. 2001). Thus, the elevated oxidation of proteins in non-proliferating cells might be due to the abundance of substrates (aberrant proteins) available for oxidative attack rather than an increased production of reactive oxygen species. The classical oxidative stress defense proteins might be ineffective in counteracting such mechanisms of oxidation.

An alternative idea holds that a continued respiration in somatic G_0 cells or growth arrested bacteria will inevitably increase the levels of oxidized macromolecules because such cells have little ability to dilute any damage with *de novo* macromolecular synthesis. This proposal is in line with the rate of living hypothesis. In its simplest form, this model predicts that the higher the metabolic activity (i.e. respiration) in a non-growing system, the higher the protein oxidation and the shorter the life span. However, data concerning non-proliferating *E. coli* and yeast G_0 cells does not support this notion since the correlation between respiratory activity and protein oxidation in growth-arrested cells is poor or non-existent in the set of starvation experiments performed (Aguilaniu et al. 2001; Ballesteros et al. 2001). For example, phosphate starved cells exhibited the highest rates of respiration during growth arrest, yet protein oxidation is only marginally increased. In addition, the culture half-life is longer in the phosphate-starved cultures despite the continued high metabolic activity in these non-proliferating cells (Ballesteros et al. 2001). Again, this result is at odds with the rate of living hypothesis but not the free radical hypothesis of aging since phosphate starved cells exhibited very low levels of oxidized proteins. Thus, the rate of respiration in a non-growing microbial system does not, *per se,* determine the degree of oxidative damage to the proteins of the system.

1.5 A trade–off between maintenance and proliferation

Some evolutionary models of senescence propose that there is a trade-off between the resources an organism devotes to reproduction and growth and those devoted to cellular maintenance and repair (Kirkwood 1977). As an inevitable consequence, an optimal life history entails an imperfect ability to resist stress. In *C. elegans,* this trade-off can be altered by mutations in *DAF-16* such that transgenic animals carrying *DAF-16* alleles that slow down growth and reproduction live longer and are more resistant to extrinsic stresses (Hendersson and Johnson 2001). There are examples of such a trade-off also in *E. coli.* For example, Kurland and

Mikkola (1993) found that, in general, natural and laboratory *E. coli* isolates exhibiting fast growth and efficient ribosomes died more rapidly during starvation-induced stasis. Continuous cultivation in chemostats effectively selected for cells with faster growth rates with a concomitant increased efficiency of translation. However, the trade-off for this increased rate of reproduction was a reduced ability to withstand starvation-induced stasis (Kurland and Mikkola 1993).

The *E. coli* trade-off phenomenon has more recently been linked to the status of the *rpoS* gene, encoding σ^S. The σ^S transcription factor accumulates during stasis and directs the RNA polymerase to a large number of genes (Kolter et al. 1993; Hengge-Aronis 1993; 2000). There is a significant bias towards stress defense functions of these genes and these functions overlap with those of the *daf-16* regulated genes of *Caenorhabditis elegans* and the *RAS*/cAMP/PKA regulated genes in yeast (Larsen 1993; Johnson et al. 2000; Marchler et al. 1993). It is known that mutations in *rpoS* are common in many natural and laboratory *E. coli* strains and it was demonstrated that there is a selective advantage of losing σ^S function during growth (Notely-MacRobb et al. 2002). The loss of σ^S in populations growing under glucose-limited conditions is accompanied by an elevated expression of genes contributing to fitness; e.g. genes encoding glucose uptake systems that require the housekeeping sigma transcription factor σ^{70} (Notely-MacRobb et al. 2002). However, increased fitness is traded for a reduced stasis survival and stress resistance since σ^S is a master regulator required for these functions. This is a bacterial example of antagonistic pleiotropy, in which mutations that are beneficial for reproduction may be harmful during old age or stasis.

A molecular model for this antagonism, or trade-off, in *E. coli* has recently been presented that includes sigma factor competition for RNA polymerase binding and explains how the quality of the environment can be sensed and translated to intracellular signals that controls the allocation of resources between reproductive and maintenance activities. The model argues that the conflict between proliferation activities and maintenance could stem from the fact that RNA polymerase may be limiting for transcription and that sigma transcription factors compete for polymerase binding (Fig. 1). It has been shown that even a subtle overproduction of the housekeeping σ^{70} effectively shuts down maintenance genes requiring σ^S and the cells become sensitive to stresses (Farewell et al. 1998). In addition, overproduction of σ^S attenuates the expression of genes requiring σ^{70} (Farewell et al. 1998). This antagonism between sigma factors is highly regulated and is dictated by the nutritional quality of the environment and the alarmone ppGpp (Jishage et al. 2002). Mutants lacking ppGpp fail to induce σ^S–dependent genes upon the imposition of stress and starvation, a phenomenon that is explained by the fact that σ^S itself requires ppGpp for both its production (Gentry et al. 1993; Lange et al. 1995) and activity (Kvint et al. 2000). This activity appears to be linked to ppGpp facilitating the ability of σ^S to compete with σ^{70} for RNA polymerase binding (Jishage et al. 2002). Thus, ppGpp is priming the RNA polymerase (by direct binding to the β and β' subunits of the polymerase) in accordance with environmental signals such that the transcriptional apparatus will be primarily occupied

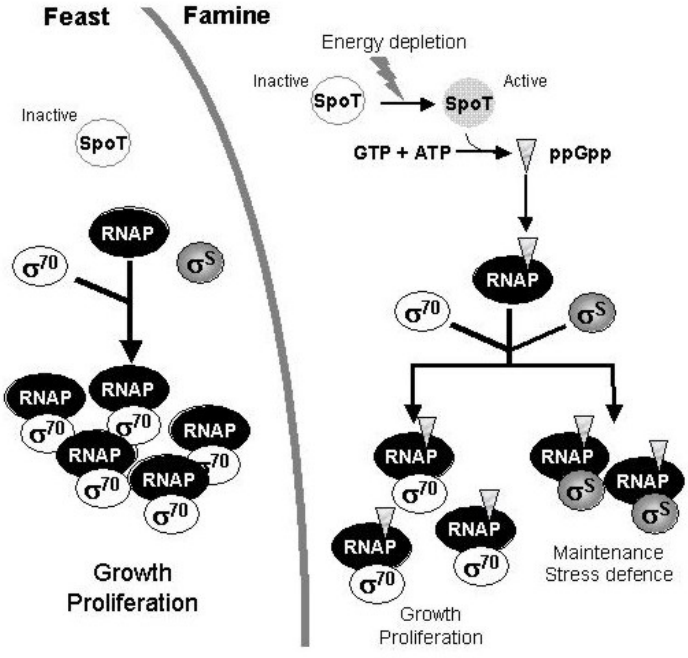

Fig. 1. Transcriptional trade-off between reproduction and maintenance. The model is based on data suggesting that RNA polymerase (RNAP) is limiting for transcription and that sigma transcription factors, e.g. σ^{70} (housekeeping sigma factor) and alternative sigma factors, such as σ^{S} compete for RNAP binding. This competition is regulated by the alarmone nucleotide ppGpp, which accumulates during starvation and stress (e.g. energy depletion). Two proteins, RelA and SpoT are responsible for the production of ppGpp and these proteins are activated by different conditions; SpoT being the one activated by energy/carbon starvation. The nucleotide ppGpp binds the core RNAP and is priming the RNAP in accordance with environmental signals such that the transcriptional apparatus will be primarily occupied with transcription of σ^{70}-dependent housekeeping genes (proliferation) as long as the ppGpp levels are low (Left panel: nutritional status of the environment favorable for growth). During energy limitation (Right panel), elevated ppGpp levels allow the alternative sigma factors, such as σ^{S}, required for expression of many maintenance genes, including those encoding oxidative stress defense proteins, to work in concert with σ^{70} by shifting the relative competitiveness of the sigma factors. In addition, ppGpp is required for the productions of σ^{S}. Nevertheless, even during growth arrest, a certain fraction of the RNAP is allocated to σ^{70}–dependent, housekeeping, gene expression, and genes requiring alternative sigma factors are not saturated.

with transcription of σ^{70}-dependent housekeeping genes as long as the ppGpp levels are low, which signals that the nutritional status of the environment is favorable for reproduction (Fig. 1). When conditions are less favorable for proliferation, elevated ppGpp levels allow the alternative sigma factors to work in concert with σ^{70} by shifting their relative competitiveness. Thus, the antagonistic pleiotropy

observed by Notley-McRobb et al. (2002) could be explained by the fact that more housekeeping σ^{70} proteins are allowed to bind RNA polymerase core in the total absence of any competing σ^S and more resources are, thereby, directed towards growth and reproduction-related activities.

One efficient way to increase the life span of rodents, worms, fruit flies, and yeast cells is to subject them to caloric restriction; i.e. a diet in which calories, often glucose, are limited by 30 to 40% compared with animals fed *ad libitum*. However, the mechanism by which caloric restriction retards aging is yet unclear (but see Lin et al. 2002). In *E. coli*, energy restriction is, in some yet unknown way, triggering the SpoT protein to produce ppGpp. As elaborated above, this allows an elevated expression of stress defense genes requiring alternative sigma factors, such as σ^S. In other words, the ppGpp/sigma factor competition model links the trade-off between reproduction and maintenance with nutrient availability and caloric restriction (Jishage et al. 2002). Thus, caloric (energy) restriction appears to cause a reallocation of resources, via signal transduction systems (e.g. Ras, Daf-16, σ^S) acting through hormone (insulin) or alarmone (ppGpp) control in organisms from bacteria to mammals.

The trade-off model could also explain why σ^S and its regulon genes are not able to fully combat stasis-induced deterioration, e.g., oxidative damage to proteins and other macromolecules. The model argues that sigma factors work in concert in a ppGpp-regulated fashion and that the housekeeping sigma factor competes with alternative sigma factors even during severe stress and growth arrest. Consequently, a certain fraction of the growth-arrested cell's resources is therefore allocated to activities related to proliferation rather than survival and oxidative stress defense. The benefit of such a regulatory system might be that the growth-arrested cell maintains the potential to respond rapidly, grow, and initiate proliferation should nutrients become available.

1.6 Protein oxidation and feed back catastrophe

Orgel (1963) has presented a conceptual and mathematical model explaining how an error feedback loop in macromolecular synthesis may cause an irreversible and exponential increase in error levels leading to an "error catastrophe". The feedback loop in Orgel's original model concerned ribosomes and translational accuracy such that errors in the sequences of proteins, which themselves functioned in protein synthesis (e.g. ribosomal proteins, elongation factors) might lead to additional errors. Such a positive feedback loop was argued to lead towards an inexorable decay of translational accuracy and, as a result, aging. The hypothesis is thus based on the assumption that mistranslated proteins can escape degradation and be incorporated into functional (but less accurate) ribosomes. However, several experimental and theoretical approaches, primarily using *E. coli* as a model system, has indicated that increased mistranslation does not cause a progressive decay in the proof-reading capacity of the ribosomes (see Gallant et al. 1997). The susceptibility of mistranslated proteins to oxidation may provide a molecular explanation

for this. It has been shown that oxidized proteins are more susceptible to proteolytic degradation than their non-oxidized counterparts (Bota and Davies 2002; Dukan et al. 2000; Starke et al. 1987). Thus, the rapid oxidation of an erroneous protein may ensure that such a polypeptide is directed to the proteolysis apparatus. This will effectively reduce incorporation of mistranslated proteins into mature machines (e.g. ribosomes and RNA and DNA polymerases) involved in information transfer. In this context, it should be pointed out that the reduced translation fidelity of growth-arrested cells is most likely the result of ribosomes being increasingly starved for charged tRNAs (empty A-sites are known to be slippery) rather than being intrinsically error-prone.

1.7 The evolution of aging; a prokaryotic perspective

The ultimate cause of aging is by evolutionary theorists accredited to the declining force of natural selection with respect to age after the onset of reproduction (e.g. Rose 1991). The rate at which the force of natural selection fades is directly related to the enmity of the environment. In addition, aging is argued to be ultimately associated to the separation of a soma and germ line and microorganisms, therefore, do not belong to the exclusive club of creatures that age. In fact, evolutionary theory regards aging as an evolved characteristic of the soma (Rose 1991) and the failure of the soma to reach immortality has been attributed to three possible mechanisms. One is mutation accumulation; in which potentially deleterious alleles may play havoc later in life provided they have little or no effect when natural selection is sufficiently powerful to overcome mutation pressure (Medawar 1952). The second theory, called antagonistic pleiotropy, highlights that alleles that are beneficial for fitness may be harmful at later ages. The greater power of selection at earlier ages gives a selective advantage of such alleles (Williams 1957). The third theory, the disposable soma theory, is in many respects similar to the antagonistic pleiotropy theory but is more specific in that it puts the spotlight on limited energy resources as the cause of antagonism, or trade-off, between reproduction and survival (Kirkwood 1977; Kirkwood and Holliday, 1979; Partridge and Barton, 1998). The theory argues that resources are limited and an increased investment in activities relating to reproduction will necessarily reduce allocation of resources to the maintenance of the soma. Conversely, investments required for an immortal soma will reduce Darwinian fitness.

The evolutionary theory of aging implies that aging evolved with the transition from vegetative to sexually reproducing organisms. It may, however, be counterproductive to think that the physiological/molecular mechanisms underlying the degenerative processes of aging evolved with the separation of a soma and germline. In fact, aging in higher organisms may well be unavoidably connected to their inheritance of the prokaryotic mode of metabolism and energy transfer. This mode is designed for efficient growth and vegetative reproduction but is incompatible with longevity during prolonged periods of growth arrest. Notably, the prokaryotic species capable of surviving such extended periods of growth arrest

do so by shutting down metabolism and forming dormant spores via a differentiation program. In contrast, the non-differentiating bacteria, like *E. coli*, are doomed to die during stasis and, as described in this review, oxidative damage appears to be one important factor in such stasis deterioration (referred to as conditional senescence). It may be argued that conditional senescence evolved with the acquisition of electron transport chains using, or working in the presence of, oxygen. The life span of growth arrested *E. coli* cells is greatly extended by omitting oxygen (100% viable anaerobically compared to 5% aerobically after 10 days of growth arrest; Dukan and Nyström 1999). In addition, disruption of *acnA* encoding aconitase, was recently shown to enhance the survival of stationary phase *Staphylococcus aureus* cells about 100-fold; a phenomenon that was speculated to result from a reduced oxidative load (Somerville et al. 2002). *E. coli* cells that lack all cytoplasmic superoxide dismutase activity grow remarkably well in rich media with a generation time only slightly reduced compared to wild type cells (Carlioz and Touati, 1986). However, in line with the argument that growth arrest imparts a special problem with respect to oxidation management, such *sod* mutants survive growth arrest extremely poorly (Dukan and Nyström 1999; Benov and Fridovich 1995).

The early eukaryotic cell acquired not only an efficient means of energy transfer, as a continuous history of aerobic respiration seems to have been the fate of most mitochondrial lineages (Kurland and Andersson 2000), but also a machinery that can cause serious cellular deterioration. Like *E. coli,* exponentially growing yeast cells can cope surprisingly well without their superoxide dismutases but are seriously defective in surviving proliferation arrest (Longo et al. 1996). It should be stressed that the rate of respiration in the yeast G_0 cells does not, *per se,* determine the life span or degree of oxidative damage to the proteins of the system (Aguilaniu et al. 2001). Instead, increased oxidation of target proteins is intimately associated to a transition to a state 4-type respiration in the growth arrested G_0 cells (Aguilaniu et al. 2001). However, as long as the eukaryote and its component organelles remain faithful to the simple prokaryotic way of life and self-renewal, deterioration and senescence will still be conditional.

In contrast, the eukaryotic organisms that sacrificed fissile reproduction and whose life history came to encompass a genetically programmed, developmental arrest of growth may have irreversibly entered the path of mandatory aging. Unless new defense systems evolved in such a multi-cellular organism to cope with the intrinsic problem of cellular growth arrest, the individual, or its soma, is destined to deteriorate and ultimately die. As suggested, the rate of deterioration of the soma may be dictated by resource allocation between reproduction activities and maintenance, which in turn is determined by the pressures of the environment (Kirkwood and Rose 1991; Partridge and Barton 1993). However, this trade-off between reproductive activities and somatic maintenance discusses mechanisms for how the rate of aging can be modulated in already aging organisms (see Holliday, 1998). The theory described here complements the evolutionary theory by arguing that the physiological/molecular mechanisms underlying mandatory aging in higher organisms may be associated to a prokaryotic mode of energy transfer trapped in a system with a finite growth phenotype. The question of why

evolution has failed to provide the soma with more efficient defense systems that can fully combat stasis-induced deterioration is adequately explained by the evolutionary theory of aging, which states that there is no need for such a defense in organisms with a soma distinct from the germ-line. It may be relevant to ask whether, in fact, it is energetically and thermodynamically possible for a growth arrested somatic cell, with little or no ability to dilute cellular damage, to maintain steady state levels of damage while simultaneously generating chemical fuel and maintaining a membrane potential via the electron transport chain. A mathematical/thermodynamic approach to this problem may be warranted.

1.8 Conclusion

Proponents of the stochastic deterioration theory argue that aerobic metabolism might be the Achilles heel of starving *E. coli* cells and that the loss of culturability is intimately linked to oxidative damage. This death, which seems to be the nearest they come to a 'natural' death of the kind familiar among higher organisms, is a consequence of growth arrest. Similar to aging in higher organisms (e.g. houseflies), life expectancy in a cohort of non-proliferating *E. coli* cells of the same chronological age is intimately correlated to the levels of oxidative damage. In addition, recent analysis of the nature of conditional senescence and the targets for oxidative damage suggests that the pathways of bacterial senescence and mandatory aging in higher organisms may have even more in common than first anticipated. For example, activities related to reproduction and maintenance appear to compete also in *E. coli* cells and trade-offs between these activities have been mechanistically linked to transcription factors competing for RNA polymerase. The outcome of this competition is dictated by the quality of the environment (e.g. caloric restriction) acting through the alarmone ppGpp. In addition, the *E. coli* model system has been instrumental in identifying a new culprit in stasis-induced oxidation of proteins; the specific oxidation of aberrant, malformed proteins produced under conditions of reduced translational fidelity.

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2 Aging and mitochondrial dysfunction in the filamentous fungus *Podospora anserina*

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Abstract

In the filamentous fungus *Podospora anserina*, mitochondria play a paramount role in life span control and aging. During aging, these organelles become damaged as the result of an age-dependent increase in the generation of mitochondrial reactive oxygen species (ROS). Remodelling of damaged proteins of the respiratory chain is limited to early phases in the life span. Only in these stages, the mitochondrial DNA (mtDNA) is intact. During aging, this DNA becomes efficiently rearranged by two kinds of reorganization processes. One process is specific for *P. anserina* and depends on the ability of a mobile mitochondrial intron to generate large sequence duplications. The other one, which occurs less frequently but seems to be conserved over a wide range of different systems, relies on naturally occurring short direct repeats in the mtDNA. Homologous recombination between these two types of repeated sequences leads to an almost quantitative reorganization of the mtDNA. Consequently, genes encoding proteins of the respiratory chain are deleted and can be recruited neither for remodelling of damaged organelles nor for the essential biogenesis of mitochondria during growth of the vegetation body. Data from various mutants provide clear clues that interventions leading to a reduced generation of mitochondrial ROS have an important impact on longevity. As one significant example, the switch from a cytochrome oxidase dependent respiratory chain to an alternative respiration was repeatedly demonstrated in different longevity mutants. In these cases, the induction of the alternative pathway is part of a mechanism that can compensate for mitochondrial dysfunction, which occur as the result of a defective cytochrome oxidase.

2.1 Introduction

The filamentous or mycelial fungi are a very diverse assembly of heterotrophic eukaryotes. In general, the individuum is represented by a mycelium, a vegetation body consisting of branched filaments, the so-called hyphae. In some fungal taxa, hyphae are subdivided by septae, incomplete cross-walls with a central porus allowing the movement of organelles and cytoplasm from one compartment to the other. Thus, although on first glance mycelia of these fungi seem to have a true cellular organization, they in fact represent single multinuclear (polyenergetic) compartments termed coenocytes or syncytia. Mycelia grow at the periphery by

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hyphal tip elongation (Bartnicki-Garcia 2002). In principle, growth can proceed indefinitely and may result in the formation of huge vegetation bodies. This has been impressively demonstrated for a mycelium of *Armillaria bulbosa* which was found to be ½ km in diamer (Smith et al. 1992). Fungi may thus form the biggest and oldest organisms on earth.

However, in sharp contrast to fungi capable to indefinite growth, there are a few species and strains reported which do not propagate indefinitely but become senescent and stop growing after a rather short period of vegetative growth. This holds true for strains of mycelial fungi like: *Ascobolus stercorarius*, *Aspergillus amstelodami*, *Aspergillus glaucus*, *Helminthelsporium victoria*, *Pestalozzia annulata*, *Podospora anserina*, *Podospora curvicolla*, *Neurospora crassa*, *Neurospora intermedia,* and *Sordaria macrospora* (Akins et al. 1986, Bertrand et al. 1980, Bertrand et al. 1985, Bertrand et al. 1986, Bertrand et al. 1993, Böckelmann and Esser 1986, Caten and Handley 1978, Chevaugeon and Digbeu 1960, de Vries et al. 1981, de Vries et al. 1986, Gagny et al. 1997, Griffiths and Yang 1995, Griffiths and Bertrand 1984, Handley and Caten 1973, Jinks 1956, Lazarus et al. 1980, Lazarus and Küntzel 1981, Lindberg 1959, Marcou 1961, Rieck et al. 1982, Rizet 1953a, Rizet 1953b). A few of them have been investigated in detail. In fact, they were the first systems in which clear mechanisms of aging emerged at the molecular level already more than twenty years ago (for review see: Esser and Tudzynski 1980, Griffiths 1992, Osiewacz 1990). Interestingly, these studies unravelled a paramount role of mitochondria in aging as they later became obvious also in other systems. Moreover, it subsequently turned out that different nuclear genes which are part of pathways involved in the biogenesis and function of mitochondria have an important impact on aging processes demonstrating that aging and longevity intimately depend on mitochondrial-nuclear interactions.

In this chapter, I shall concentrate on the role of mitochondria in aging of *Podospora anserina*. After a brief introduction into the biology of this filamentous ascomycete, I will summarize essential data and ideas from earlier investigations and will thereafter focus on more recent findings. Finally, I shall relate the conclusions derived from this eukaryotic aging model of low complexity to systems of higher organization. For more details concerning fungal senescence in general, the reader is referred to previous publications (Bertrand 2000, Esser and Tudzynski 1980, Esser et al. 1986, Griffiths 1992, Osiewacz 1990, Osiewacz 1997, Osiewacz 2002, Osiewacz and Borghouts 2000, Osiewacz and Kimpel 1999, Osiewacz and Scheckhuber 2002).

2.2 Life cycle and aging of *Podospora anserina*

Podospora anserina is a sessile ascomycete belonging to the family of Sordariaceae. The natural habitat is the dung of herbivores (Esser 1974, Rizet and Engelman 1949). Since this habitat dries out rather fast, conditions for life of cultures are restricted to a short period of time. Certainly, these conditions led to the evolution of a special strategy that guarantees *P. anserina* to survive even under these

unfavourable conditions. A short life cycle allowing an efficient reproduction is the answer to the problem. At least under laboratory conditions numerous ascospores are produced in about 10-12 days. They become actively discharged from the fruiting body. In nature, after release, the ascospores stick to the herbage in the neighbourhood of the culture from which they originate. Subsequent digestion by a herbivore and passage through its intestine provide a new substrate for progression through the next life cycle. Since the natural conditions are time limited, it makes no sense to invest much of the available energy into running efficient cellular maintenance systems that allow surviving longer periods of time. In fact, in *P. anserina* wild type strains, remodelling of damaged macromolecules appears to be actively prevented by a very efficient mechanism destructing the genetic information required for this process (see 6.3.2). Instead of investing into maintenance functions, the limited energy appears to be used to ensure a better efficient formation of reproduction units. According to the "disposable soma theory of aging" (Kirkwood 2002, Kirkwood and Holliday 1979), this strategy of energy investment is exactly expected for a sessile organism living on a habitat that is useful for life only for a short time.

In contrast to the closely related ascomycete *Neurospora crassa*, propagation of *Podospora anserina* strictly depends on sexual reproduction. This process is controlled by the two mating-types *mat*- and *mat*+. Usually, a mycelium that develops from a single ascospore is self-fertile. It contains both mating-type loci because in most cases, the ascospores produced in a perithecium are binucleate and each nucleus contains a different mating-type locus. Such a mycelium produces male gametes, termed microconidia or spermatia, which are *mat*+ and *mat*-, respectively. Spermatia of one mating-type fertilize the female gametangia containing the opposite mating-type. It thus appears as if *P. anserina* is a homothallic fungus allowing sexual reproduction to occur at a mycelium originating from just a single ascospore. However, a closer look makes clear that this is not the case and *P. anserina* in fact is pseudohomothallic. This becomes obvious when rarely occurring mononucleate ascospores, which are smaller than binucleate ascospores, are germinated and analyzed. These spores are produced in irregular asci when a spore wall surrounds only one nucleus during sporogenesis. In these cases, instead of four binucleate spores five (see Fig. 1) to eight-spored asci are generated. The small spores of irregular asci are homokaryotic and lead to mycelia containing only one mating-type. Consequently, they are not self-fertile demonstrating that *P. anserina* is in fact not homothallic. Only if such mycelia derived from one mononucleate ascospore come into contact with a culture of the opposite mating-type fertilization takes place. This breeding system has been termed secondary homothallism (Whitehouse 1949a, Whitehouse 1949b).

Although in nature, self-fertile mycelia are the vegetation bodies, which are normally generated, in the laboratory, frequently cultures are used which are generated from isolated mononucleate spores. The advantage of these cultures is that all perithecia formed after a cross of two strains of opposite mating-type are the result of the combination of the genetic material of the two parents and a subsequent meiosis. This situation allows a very efficient genetic analysis via classical

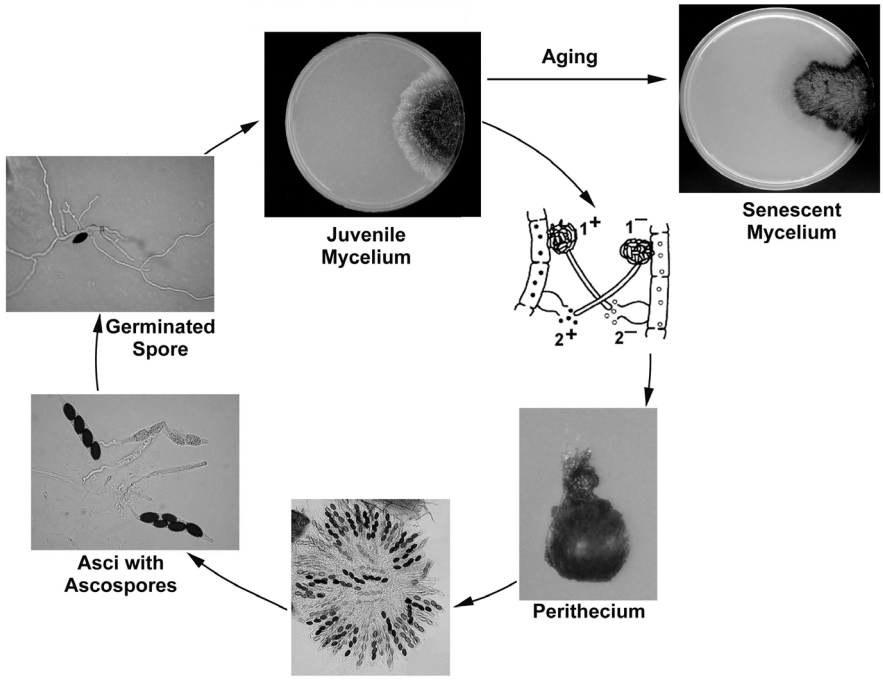

Fig. 1. Life cycle of *Podospora anserina*. Starting from a single ascospore a juvenile mycelium develops. If the ascospore is binucleate, as are formed in most cases (all spores in a regular four-spored ascus, the bigger spores in irregular asci), the mycelium is normally self-fertile. At such a mycelium, two types of female gametangia (protoperithecia) are formed, which only differ by the mating-type + or – (1+, 1-). In addition, two types of spermatia, which are either of mating-type + or – (2+, 2-) are produced. Protoperithecia of one mating-type fuse with spermatia of the opposite mating-type. After well-defined developmental processes including a dikaryotic stage, crozier formation, karyogamy, meioisis, postmeiotic mitosis, and sporogenesis a perithecium is formed. In this fruiting-body, numerous asci are located which are actively discharged. In nature, the ascospores are digested by an herbivore. After passage of the intestine, they germinate on dung. In the laboratory, single ascospores can easily be isolated and germinated on a specific germination medium. In the lower part of the figure, the content of an experimentally opened perithecium with numerous asci is shown. On the left, two mature asci were isolated. One ascus contains four binucleate ascospores; the other, irregular ascus contains five ascospores. Two of these spores are smaller than the other three and are mononucleate. Germination of these spores leads to mycelia, which do not reproduce sexually on their own. Only if two homokaryotic mycelia of the opposite mating-type come into contact, the sexual cycle proceeds in the explained way. Vegetative growth of a single culture takes place by mitotic nuclear divisions and elongation a branching of the hyphal tips. After a strain specific type, the morphology of the mycelium changes (e.g., darker pigmentation, reduced formation of aerial hyphae) and growth of the hyphae ceases.

tetrad analysis or single spore analysis. At this point one advantage of the *P. anserina* system for a formal genetic analysis should be emphasized. This is that the female gametangia, the protoperithecia, contain by far much more cytoplasm than their fusion partners, the spermatia. Consequently, most cytoplasm going into the ascospore is derived from the female partner of a cross. This situation allows the formation of reciprocal crosses and the discrimination of whether or not a phenotype is inherited by nuclear factors (mendelian inheritance) or whether it is encoded by extranuclear traits and "maternally" inherited. For more details concerning this aspect of the biology of *Podospora anserina* the reader is referred to (Esser 1974, Esser and Tudzynski 1980, Osiewacz 1996).

As mentioned above, *P. anserina* is one of the examples in which wild type strains are not able to indefinite vegetative propagation (Rizet 1953a, Rizet 1953b). After germination of an ascospore, a juvenile culture develops which growth by elongation of the peripheral hyphae (Fig.1). The resulting linear growth rate is strain-specific and depends on environmental factors (e.g., medium composition, temperature). Under defined growth conditions, the phenotype of the mycelium changes after a specific period of time. This time depends on the investigated strain and lies in the range of a few weeks to a few months. As one example, the wild type strain s, which has been most extensively analyzed, is characterized by a mean life span of 25 days when grown on rich-medium at 27°C. During this period of time, starting from a newly isolated and germinated ascospore, the culture initially growth with a linear growth rate of 7 mm/day until it reaches the senescent stage. At this stage, the growth rate slows progressively and the phenotype of the culture changes: the pigmentation of the mycelium increases, the formation of hyphae growing into the air, so-called aerial hyphae, decreases, and finally growth of a senescent culture stops completely and the hyphae die at the periphery. The hyphal tips of senescent cultures are more undulate and slender than those of younger cultures. Moreover, tips from senescent cultures were found to burst frequently (Delay 1963, Esser and Tudzynski 1980).

Importantly, the 'biological clock' counting the age of a culture can be reset during sexual reproduction. Crosses of two strains generated from two mononucleate ascospores of the opposite mating type lead to the generation of ascospores from which juvenile mycelia can be regenerated. This holds true even if none of the two parental strains is juvenile itself indicating some kind of 'filter' mechanism operating during sexual reproduction. Such a mechanism was experimentally demonstrated. It was shown to lead to the restoration of the wild type mtDNA via the eliminating of specific mtDNA mutations (Silliker et al. 1996, Silliker et al. 1997). The underlying details of that mechanism bear important clues. Unfortunately, they have yet to be unravelled.

2.3 The genetic basis of aging and longevity in *Podospora anserina*

2.3.1 Early evidence from genetic and physiologic investigations

Early genetic analyses led to the suggestion that during vegetative growth of *P. anserina* mycelia a 'determinant of senescence' accumulates. Under conditions which do not allow the migration of nuclei, this factor can be transferred to young cultures which immediately become senescent and die (Marcou 1961, Marcou and Schecroun 1959) The 'determinant of senescence' thus seems to be infectious and cytoplasmic. Reciprocal crosses further supported this conclusion. When spermatia from a juvenile culture of one mating-type were used to fertilize protoperithecia of a senescent culture of the opposite mating-type up to 90% of the progeny were senescent. In contrast, none of the progeny of a cross in which spermatia of a 'senescent' strain were used to fertilize protoperithecia of a 'juvenile' culture gave rise to 'senescent' cultures (Rizet 1957). This type of reciprocal differences is not consistent with the inheritance of nuclear factors and points to extranuclear traits controlling the onset of senescence. However, in addition, crosses of certain mutants with wild type strains also indicated that nuclear factors are involved in life span control as well. In reciprocal crosses of such strains, no reciprocal differences are observed and life span is inherited according to the Mendelian rules. Genetics thus suggests that life span and aging of *P. anserina* is controlled by both extranuclear and nuclear genetic factors (Esser and Keller 1976, Marcou 1961, Smith and Rubenstein 1973a, Smith and Rubenstein 1973b, Tudzynski and Esser 1979).

One line of subsequent investigations aimed to identify the nature of the extranuclear factor involved in the control of the onset of senescence concentrated on experiments using metabolic inhibitors. These experiments indeed gave important clues about the cellular compartment in which it is located, although the molecular mechanisms leading to the effect on life span are still not elucidated in detail. Inhibitors of mitochondrial ribosomes like kanamycin, neomycin, streptomycin, puromycin, and tiamulin, which were added to the growth medium resulted in a clear life span increase (Esser and Tudzynski 1977, Tudzynski and Esser 1977). The same was observed when compounds (e.g., ethidium bromide), which preferentially intercalate into mtDNA were used. Furthermore, growth of senescent *P. anserina* cultures on solid medium containing ethidium bromide could be rejuvenated (Koll et al. 1984). Finally, the use of inhibitors of the mitochondrial respiratory chain (e.g., mucidin, potassium cyanide) also resulted in an life span increase of cultures (Tudzynski and Esser 1977). Taken together, the early genetic and physiologic investigations strongly suggested that mitochondria play an important role in the control of life span in *P. anserina* but also demonstrated an impact and/or interactions of these organelles with the nucleus and with environmental conditions.

2.3.2. Rearrangements of the mtDNA during aging

In search of the extrachromosomal trait involved in life span control a covalently closed circular DNA species, termed plDNA, was found to accumulate in mitochondria of senescent cultures of wild type strains (Cummings et al. 1979, Stahl et al. 1978). In juvenile cultures, this element represents the first intron (the pl-intron) of the gene (*CoxI*) coding for the first subunit of the cytochrome oxidase (COX). During aging, the pl-intron becomes systematically liberated and amplified and, at the same time, large parts of the mtDNA become deleted (Belcour et al. 1981, Kück et al. 1981, Osiewacz and Esser 1984). After the initial identification of these events, a large body of evidence was generated suggesting that senescence in *P. anserina* is strictly dependent on the accumulation of plDNA. It consequently became consensus in the different laboratories working in this field that the liberation and amplification of plDNA is the cause of aging in *P. anserina*. More recent independent data demonstrated that *P. anserina* mutants may senesce independent of the amplification of plDNA. However, here, one has to take into account that the conditions differ significantly from those in nature. Mutant cultures may only be able to survive under those protected conditions (e.g. on agar plates providing sufficient nutrients for an unlimited time) found in the laboratory. In nature, these mutants, who often are characterized by a reduced growth rate, may not survive. In fact, it may well be that under natural conditions, the mechanism depending on the pl-intron mediated frequent mtDNA reorganization and amplification of the plDNA is the main mechanism of aging in this species. Still, the analysis of laboratory mutants is extremely useful since they identify other contributing mechanisms and allow dissection of the complete molecular network of pathways involved in aging and life span control.

The mechanism leading to the generation of plDNA and of the gross mtDNA reorganizations during aging of *P. anserina* cultures has been unravelled in some detail at the molecular level. It was demonstrated that the pl-intron is able to transpose to different positions in the intact mtDNA giving rise to the formation of repeated sequences of 2,5 kbp in size. This process depends on the activity of a reverse transcriptase which is encoded by an open reading frame on the pl-intron (Fassbender et al. 1994, Kück et al. 1985, Michel and Lang 1985, Osiewacz and Esser 1984). One site of integration of the pl-intron is the position directly downstream of the first *CoxI* exon (‘homing-like’ transposition), the site where the intron naturally is located. Following this route, tandem repeats of two or more intron copies – depending on the number of integration events – occur at this position in one mtDNA molecule. Subsequent intramolecular homologous recombination accounts for the generation of the plDNA accumulates as an oligomeric series of covalently closed circular molecules. Alternatively, transposition to an ‘ectopic’ site of the mtDNA containing a short acceptor sequence also leads to repeated pl-intron sequence in individual mtDNA molecules. In such molecules however, the repeated pl-intron sequences are separated by unique sequences. Homologous recombination between the pl-repeats now lead to the generation of DNA subcircles which do contain the unique mtDNA sequence located between the two pl-repeats (Borghouts et al. 2000, Sellem et al. 1993). If the corresponding

circles are not replicated, they become lost during subsequent mitochondrial divisions and thus deficient mtDNA's accumulate during aging of *P. anserina* cultures.

Apart from the very efficient pl-intron dependent recombination pathway, which seems to account for the majority of mtDNA reorganizations in wild type cultures, there are also pl-intron independent processes occurring. These processes were found to take place with a much lower frequency than the former processes and depend on short direct repeats as the are found dispersed in the mtDNA (Belcour et al. 1981, Jamet-Vierny et al. 1997a, Jamet-Vierny et al. 1997b, Kück et al. 1981, Kück et al. 1985).

Because of the efficient recombination activities, the mtDNA becomes almost quantitatively rearranged during aging of *P. anserina* wild type cultures. It consequently is not available for the expression of the different mtDNA encoded genes. In growing hyphal tips of older cultures thus the remodelling of impaired mitochondria and the biogenesis of new organelles is impaired leading to the senescence syndrome in these parts of a mycelium.

As mentioned above, the occurrence of the amplified plDNA appears to be a good marker of transposition processes and of subsequent homologous recombination which greatly contribute to the characteristic age-related mtDNA reorganisations observed during senescence of *P. anserina*. Various data demonstrate the role of mtDNA reorganizations and senescence in *P. anserina*, in particular in rapidly aging cultures of wild type strains. One example has been introduced above. Growth of senescent cultures on ethidium bromide containing substrate was found to lead to a rejuvenation of senescent cultures. At the molecular level, rejuvenation was found to be correlated with the recovery of intact wild type mtDNA (Koll et al. 1984). Additional data are derived from the analysis of different long-lived mutants or of transgenic strains. In such strains, a stabilization of the mtDNA was found due to different reasons. Examples are the exact or the partial deletion of pl-intron from the mtDNA (Begel et al. 1999, Schulte et al. 1988, Vierny-Jamet et al. 1982). In contrast to mutants in which parts of the *Cox1* coding region are deleted and which for this reason show a switch from a cytochrome c dependent respiration to an alternative respiration, the mutant with the exact pl-intron deletion respires via the standard respiratory chain utilizing cytochrome c oxidase. Interestingly, although the pl-intron is absent, the corresponding mutant is not immortal but characterized by an increased life span of about 50% demonstrating that it is not the pl-intron *per se*, which is responsible for the aging of *P. anserina* cultures (Begel et al. 1999). However, in all strains in which the pl-intron is deleted, no free plDNA is generated and the pl-intron dependent wild type specific mtDNA rearrangement pathway is impaired. Since the reorganization frequency following this molecular pathway is comparably low, the mtDNA appears to be stable in comparison to wild type mtDNA. In another extrachromosomal long-lived mutant, AL2, the processes leading to the amplification of the plDNA is delayed (Osiewacz et al. 1989). In this mutant, a linear plasmid (pAL2-1) encoding an RNA and a DNA polymerase interferes with the underlying processes (Hermanns et al. 1994, Hermanns et al. 1995, Hermanns and Osiewacz 1992, Hermanns and Osiewacz 1994, Hermanns and Osiewacz 1996, Osiewacz et al. 1989).

2.3.3 Nuclear mutations affecting mitochondrial functions

In addition to extrachromosomal mutants affecting the stability of the mtDNA, different nuclear long-lived mutants were demonstrated to modify the stability of the mtDNA. One example is the grisea mutant (Esser and Keller 1976, Prillinger and Esser 1977). In this mutant, the pathway leading to the duplication of pl-intron sequences is not affected. However, the last step, homologous recombination between repeated sequences, is impaired and no plDNA becomes amplified when strains are grown on standard growth medium (Borghouts et al. 2000). Interestingly, growing the grisea mutant in media containing increased copper concentrations reverts this molecular phenotype indicating that homologous recombination (Fig.2) between the duplicated sequences directly or indirectly depends on the availability of copper (Borghouts et al. 2000).

The demonstration that *P. anserina* strains exist which become senescent although they do not produce plDNA molecules clearly demonstrated that the amplification of the plDNA is not a prerequisite for aging (Borghouts et al. 1997, Silar et al. 1997). Instead it appears that the activity of this element leading to the generation of gross mtDNA reorganizations and to its own amplification is a process to guarantee efficient aging of cultures in a rather short time (see: 6.3.2). If the activity of the pl-intron for any reason is impaired, strains do not age rapidly and the culture becomes long-lived. The pl-intron thus appears to operate as some kind of genetic device acting as an accelerator of aging via the time-dependent generation of optimal conditions for efficient mtDNA reorganization. Consequently, at least one other basic mechanism is responsible for the aging of *P. anserina* cultures. The characterization of independent long-lived mutants provided important clues towards the identification of the relevant factors.

In different long-lived mutants and in one transgenic strain, the cyanide sensitive respiration chain utilizing complex IV with cytochrome oxidase (COX) as the terminal electron transferase is affected. In the above mentioned grisea mutant (Esser and Keller 1976, Prillinger and Esser 1977), the mutant phenotype is the result of a loss-of-function mutation in the *Grisea* gene (Borghouts et al. 1997, Osiewacz and Nuber 1996). This is a nuclear gene encoding the copper-modulated transcription factor GRISEA. The transcription factor is involved in a tight control of cellular copper levels. At low copper levels, GRISEA activates the transcription of different target genes including *PaSod2* and *PaCtr3* (Borghouts et al. 2002a, Borghouts et al. 2002b, Borghouts and Osiewacz 1998, Kimpel and Osiewacz 1999). The latter codes for a high affinity copper transporter able to transport copper efficiently across the plasma membrane even if its concentration in the environment is very low. In the grisea mutant, due to the loss-of-function mutation in the *Grisea* gene, high affinity copper-uptake is impaired leading to cellular copper-deficiency. Since copper is a cofactor of tyrosinase, an enzyme involved in melanin biosynthesis, the mutant displays a hypopigmentation phenotype: both, mycelia as well as the ascospores are grey instead of dark green to black. In addition, the delivery of copper to mitochondria and the assembly of complex IV requiring copper, are affected. Like in yeast and other systems, this process depends on copper chaperones binding copper immediately after it enters the cell. In yeast,

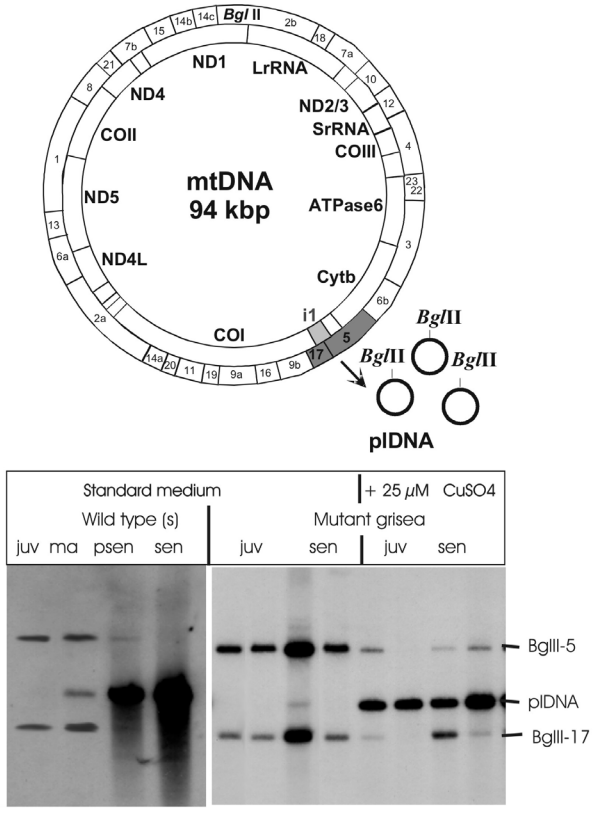

Fig. 2. Age-related mtDNA reorganizations in *Podospora anserina*. In juvenile strains, the mtDNA represents a circular molecule of 94 kb containing different genes (indicated by abbreviations) encoding parts of the respiratory complexes, two mitochondrial rRNAs and a set of tRNAs. In wild type strain s, the mtDNA becomes severely rearranged. One hallmark of this process is the liberation and amplification of the first intron in the cytochrome c oxidase subunit I gene. In juvenile cultures, the integrated intron can easily be detected by Southern Blot analysis using a pl-intron specific probe (e.g., *Bgl*II-5 and *Bgl*II-17 fragment). During aging of wild type strains, an additional fragment of 2.5 kbp shows up that corresponds to the lineraized circularized plDNA, which exactly is derived from the pl-intron sequence. At the same time, the two *Bgl*II fragments containing the integrated pl-intron and adjacent sequences disappear. This prominent wild type specific type of mtDNA reorganization event does not occur on standard growth medium of long-lived mutant grisea. DNA from two individual juvenile and senescent *grisea* cultures was analyzed. It can be clearly seen that an amplification of the plDNA does not take place during senescence of grisea cultures. On medium containing access copper, the wild type phenotype including the morphology, life span, and the ability to specifically amplify the pl-intron is restored in the mutant.

yCOX17, ySCOI, and most likely yCOX11 are involved in this process (Glerum et al. 1996, Hiser et al. 2000, Lode et al. 2000). Until now, in *P. anserina* only two genes of this pathway coding for a putative yCOX17 and an ySCOI homologue have been identified. PaCOX17 delivers copper to the mitochondrial intermembrane space (in preparation). Subsequently, PaSCOI may pass copper from PaCOX17 to COXI in complex IV.

Interestingly, the analysis of the molecular machinery involved in the control of copper homeostasis revealed an age-related alteration in cytoplasmic copper content. In the wild type levels of the transcript encoding the metallothionein of *P. anserina* rise during aging whereas those of PaCTR3 and PaSOD2 the expression of which is repressed by copper decrease. At the protein level, an age-dependent increase of cytoplasmic copper is indicated by the increased activity of the copper-dependent SOD1 of *P. anserina*. We surmise that the increase in cytoplasmic copper is due to its release from mitochondria during aging of cultures (Averbeck et al. 2001, Borghouts et al. 2002a, Borghouts et al. 2002b).

In principle, since *P. anserina* is an obligate aerobe and thus depending on mitochondrial ATP generation, a defect in the delivery of copper to mitochondria should be lethal. However, due to the existence of a specific backup system that is not dependent on copper this critical situation can be overcome. The corresponding response resembles the 'retrograde response' first described in *S. cerevisiae* (Liao and Butow 1993, Liao et al. 1987, Sekito et al. 2000), a mechanism able to compensate impairments of the respiratory chain. In the case of the grisea mutant, compensation results from the assembly of a cyanide resistant alternative oxidase (AOX) into the inner mitochondrial membrane. In contrast to COX, this enzyme requires iron instead of copper. The AOX pathway branches at the ubiquinone pool. The final step is the transfer of electrons from AOX to oxygen giving rise to the formation of water. Since the AOX is located upstream of complex III, the formation of the electron motive force is completely restricted to complex I. Consequently, two proton pumping sites are lost resulting in a lower production of ATP. However, since in the grisea mutant copper-deficiency is not complete and low amounts of copper seem to enter the cell via a low affinity uptake system, the mitochondrial respiratory chain of the grisea mutant seems to respire via both a copper-dependent COX (complex IV) and the iron-dependent AOX respiration. The life span of this mutant is increased by about 60%. In contrast, mutant ex1 respires exclusively via the AOX alternative pathway. Interestingly, this mutant appears to be immortal.

The induction of a retrograde response in *P. anserina* in different long-lived mutants is intriguing. However, what are the specific reasons for the observed differences in life span in different mutants? Answers to this question need a more detailed consideration of the molecular changes in the different mutants, which are found in addition to the switch from a COX-depending to an alternative respiration. In one study, in which the assembly of COX is impaired due to the disruption of the nuclear genes coding for subunit five (*Cox5*) of COX, the generation of ROS was found to be significantly lower than in the wild type strain respiring via the standard COX-dependent oxidase (Dufour et al. 2000). These data are in agreement with data from higher plants demonstrating a reduced generation of

ROS via an AOX-dependent respiratory chain (Wagner and Moore 1997) and link aging of *P. anserina* to the free radical theory of aging (Beckman and Ames 1998, Harman 1956, Harman 1988, Harman 1992, Harman 1998, Harman 2001, Miquel et al. 1992). According to the current view, in heterotrophic systems ROS are generated mainly at complex I and III of the respiratory chain. They lead to damage and malfunction of all types of biomolecules including nucleic acids, lipids, and proteins. In mitochondria of young organisms, only very low amounts of ROS are produced but these are enough to damage proteins of the respiratory chain. Therefore, the transfer of electrons increasingly becomes impaired and more and more ROS are generated. Finally, this kind of a 'vicious cycle' leads to severely dysfunctional mitochondria. Lowering mitochondrial ROS generation, as demonstrated in the long-lived *Cox5* inactivation strain mentioned above, appears to be also the reason for life span extension in the two mutants grisea and ex. The differences in the life span of these mutants, 39 days vs. greater than 10 years, seem to result from some basic differences. Both the exclusive respiration via the alternative oxidase and differences in the ROS scavenging system may be responsible. In the immortal ex mutant, the *Grisea* gene is functional and thus cellular copper levels are regulated utilizing transcription factor GRISEA. The same holds true for target genes of GRISEA not directly related to copper-uptake, transport, or storing. One example is *PaSod2* encoding the mitochondrial MnSOD. Moreover, copper is available at normal levels for the activation of the apoprotein of the cytoplasmic Cu/ZnSOD. Significantly, this enzyme appears to have also a scavenging function in mitochondria and not exclusively in the cytoplasm (Sturtz et al. 2001). Thus, in the ex mutant, the system directed against oxidative stress is operating while in mutant grisea it is impaired explaining the mortal long-lived phenotype of the grisea mutant (Borghouts et al. 2002a, Borghouts et al. 2002b).

2.3.4. A mitochondrial- nuclear network of interactions control life span in *P.anserina*

Until now two different general molecular aspects have been introduced to play a major role in life span control of *Podospora anserina*: the accumulation of mtDNA reorganizations and of defective mitochondria as a result of the age-related generation of increasing amounts of ROS. Are these processes operating independently or are they linked and part of a greater molecular network? Taking the different available data into account it indeed appears to be clear that a strong link exists. At this point, it needs to be recalled that senescence of *P. anserina* cultures occurs at the periphery of the mycelium at the hyphal tips. These are the areas in which most energy is required during hyphal tip elongating and branching. Consequently, there is a strong demand for functional mitochondria. Since the mycelium becomes progressively greater, the number of functional mitochondria needs to be increased via fission of existing mitochondria. This process depends on the co-ordinated expression of mitochondrial and nuclear encoded genes. However, since the mtDNA of *P. anserina* cultures is efficiently rearranged during aging no mtDNA encoded components can be provided for the biogenesis of mito-

chondria. Moreover, the remodelling apparatus of existing mitochondria, which during aging became impaired via the activity of ROS, is also affected due to the reorganization of the mtDNA. Specifically, damaged components of the respiratory chain cannot be degraded and replaced by newly proteins synthesized in the mitochondrion.

The mutant grisea is only one example in which the mutation of a nuclear gene affects mitochondrial functions having an impact on longevity. Since the vast majority of genes coding for different components of functional mitochondria are encoded by the nucleus, many other genes are controlling the biogenesis and function of mitochondria, which includes components of the respiratory chain, the whole set of enzymes of the citric acid cycle, all components of the protein import machinery of specific transporters, those of the biogenesis of Fe/S clusters, the enzymes involved in mtDNA replication and the expression of mitochondrial genes and of other essential mitochondrial functions. The transport of the various gene products and of cofactors like copper and iron into the different compartments of the organelle and the correct assembly of supra-molecular complexes appears to be of prime significance for the remodelling of existing mitochondria and for division of mitochondria in actively growing parts of a mycelium. At this time, only very limited data are available concerning age-related aspects of these basic processes. As mentioned above, the delivery of copper to the respiratory chain is one specific example but in addition to the few components (e.g., PaCTR3, PaCOX17, PaSCO1) identified in *P. anserina* others remain to be demonstrated and the role of the involved molecular pathways remains to be elucidated in more detail. The mitochondrial protein import machinery plays another important role. This is suggested by the analysis of a long-lived mutant in which the mutated gene was demonstrated to code for PaTOM70, a component of this machinery (Jamet-Vierny et al. 1997c).

2.4. Mitochondria: key players involved in mechanisms of aging conserved in organisms from simple unicellular up to highly complex organization

The initial demonstration of age-dependent mtDNA reorganization processes reproducibly occurring during aging of *P.anserina* cultures opened new avenues in experimental aging research. Related specific questions were subsequently addressed and investigated in other systems. In addition, in other filamentous fungi, rearrangements were demonstrated to correlate with senescence processes. However, since variable pairs of repetition sequences can be involved in such recombination processes, the resulting molecules are different in size. This holds true for senDNA's identified in senescent *Podospora curvicolla* strains (Böckelmann and Esser 1986) and in the 'ragged' mutants of *Aspergillus amstelodami*. Also in different laboratory mutants of *N. crassa* displaying a senescence-like degenerative phenotype intramolecular homologous recombination between pairs of short direct repeats were reported to lead to gross mtDNA reorganisations (Almasan and Mishra 1988, Bertrand et al. 1993, Gross et al. 1984). In addition to this type of

common mechanism, mtDNA rearrangements were found to occur utilizing different GC-rich palindromes able to form stem-loop secondary structures. These mtDNA rearrangements appear to be the result of single- or double-strand breaks and subsequent ligation processes (de Vries et al. 1981).

Apart from such processes in which sequences of the standard mtDNA are involved, another type of senescent-related mtDNA rearrangement was found in a number of natural isolates in the genus *Neurospora*. Here mtDNA reorganizations are generated by autonomous linear mitochondrial plasmids. In several *Neurospora* isolates from Hawaii, India, or China such elements have been isolated and characterized. One example is the Kalilo DNA, pKAL, a plasmid of 8.3 kbp containing long terminal inverted repeats and coding for a DNA and an RNA polymerase. Senescence of strains carrying this plasmid was reported to correlate with the integration of the plasmid into the mtDNA and the generation of giant repeats at the integration sites. Several integration sites were demonstrated in essential mtDNA sequences. However, integration of the element into the mtDNA occurs infrequently. Therefore, it is surprising that mtDNA molecules containing the integrated plasmid sequence that interrupts essential functions accumulates during senescence. The reason is unsolved.

Finally, in specific *Neurospora* strains, also circular mitochondrial plasmids were found to integrate into the standard mtDNA and subsequently lead to deletions and insertions. Interestingly, integration of the plasmids most likely proceeds via an RNA intermediate and via reverse transcription. Also in this system, dysfunctional mtDNA's accumulate and consequently lead to senescence (Akins et al. 1986).

It is striking that in different filamentous fungi different types of genetic elements affect the stability of the mtDNA leading to the development of degenerative phenotypes. However, the activity of comparable genetic traits may have different outcomes in different systems. Examples are the senescent inducing linear plasmids in *Neurospora* and the linear plasmid pAL2-1 of *P. anserina*. Whereas, pAL2-1 appears to stabilized the mtDNA leading to longevity of the corresponding *Podospora* strain, the kalilo plasmid, pKAL, from *N. intermedia* and other linear plasmids lead to increased mtDNA reorganizations and senescence. Moreover, there are a number of species known in which linear plasmids do not give rise to a particular phenotype (Kempken et al. 1992, Meinhardt et al. 1990).

The search for age-related DNA rearrangements in mammalian systems was first rather disappointing. However, initial data were obtained from a heteroduplex analysis using mtDNA from mice of different age. In these experiments, the number of single stranded loops in denaturated and reannealed mtDNA preparations increased when mtDNA of older mice was used. These data suggested an age-related increase in deletions/additions of short mtDNA sequences during aging (Bulpitt and Piko 1984). Utilizing PCR approaches this became demonstrated repeatedly both in tissues of healthy subjects and of those suffering from a range of different types of diseases (Kadenbach and Müller-Höcker 1990, Linnane et al. 1989, Linnane et al. 1990, Osiewacz and Hermanns 1992, Wallace 1989, Wallace 1993, Wallace 1999, Wallace 2001).

Rather recently, a crucial role of mitochondria in life span control emerged in aging models in which mitochondria were not thought to play a major role. It is one important finding that the retrograde response which is able to compensate impaired mitochondrial functions was first reported in this organism (Liao and Butow 1993, Liao et al. 1991, Sekito et al. 2000) and subsequently demonstrated to be effective in life span control (Kirchman et al. 1999). Moreover, very recent investigations suggested that also in yeast the accumulation of dysfunctional mitochondria appears to be causatively involved in life span control and aging (Lai et al. 2002). Furthermore, in the nematode *Caenorhabditis elegans*, mtDNA reorganizations were found to occur between short direct repeats by using long-extenuation PCR strategy. The rate by which the deletions occur were found to be significantly slower in the *age-1* long-lived strain as compared to wild type animals (Melov et al. 1995). More recently, the relevance of mitochondria in the aging of *C. elegans* was further underscored by the data obtained from an iRNA analysis of 5690 genes of the nematode. In this study, about 15% of the analyzed genes leading to an extended life span were found to code for mitochondrial functions (Lee et al. 2002). It thus appears that wherever an appropriate selection procedure or methodology is applied (e.g., heteroduplex analysis and PCR strategies to demonstrate age-related mtDNA deletions and point mutations *C. elegans* and in mammals, systematic iRNA analysis of large sets of genes in *C. elegans*, a screen to identify asymmetric segregation of mitochondria in yeast) an important if not causal role of mitochondria in aging processes is unravelled.

2.5 Conclusions

The data and conclusions derived from intensive investigations of the fungal aging model *P. anserina* are an excellent example demonstrating that models of low complexity can indeed provide valuable clues to unravel complex mechanisms as those governing biological aging. Of coarse, one cannot expect that every detailed aspect identified in one system is exactly conserved in another organism. It is clear today that every organism and every individual ages differently. However, there are also conserved mechanisms of aging, which in principle – not in all details – are operating in a wide range of organisms (Martin et al. 1996). Clearly, efficient mtDNA reorganization processes as they reproducibly occur in wild type cultures of *P. anserina* are characteristic in this system but do not play a role in others. However, although these high frequency reorganization processes, which depend on the activity of a mobile intron, are not conserved among species, mtDNA rearrangements occurring between dispersed short direct repeats seem to be conserved. Moreover, from the early investigations with *P. anserina* one other more general aspect of aging became apparent: that is the accumulation of dysfunctional mitochondria. This aspect is still valid and is discovered even today in systems were – until now – it was not thought to play any role. The other way around, also the concepts developed for other systems (e.g., flies, rodents) have strongly influenced the unravelling of the mechanisms controlling aging in *P. anserina*. Specifi-

cally, the incorporation of the “free radical theory” of aging has modified and extended the view of mechanisms of aging operating in this aging model. Also in the future, it can be expected that travelling this route of research will be beneficial to unravel the complex network of molecular pathways involved in the control of longevity and aging in the different biological systems.

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3 Mitochondria, metabolism, and aging in yeast

S. Michal Jazwinski

Abstract

Quantitative and qualitative changes in metabolism take place when the lifespan is extended in yeast either by genetic or nutritional manipulation. In particular, remodeling of mitochondrial function occurs, and the relationship between this organelle and other cellular compartments moves to the fore. Two separate pathways, the retrograde response and calorie restriction, operate as metabolic mechanisms for life extension in yeast. Though distinct, they share common longevity effectors. The retrograde response is a compensatory measure for mitochondrial dysfunction, while calorie restriction is proposed to be a preventive response. The critical common feature in both appears to be the enhancement of the production of biosynthetic precursors for growth by the mitochondrial Krebs cycle and by the alternate glyoxylate cycle. The communication between the mitochondrion and other cellular compartments that is essential for delivery of these precursors is dependent on the maintenance of a sufficient mitochondrial membrane potential, which declines with age. In actively respiring cells, the transport of biosynthetic precursors is likely to fulfill an additional role. It can uncouple the mitochondria and thus lower the production of reactive oxygen species, which can cause deleterious age changes. When metabolic rates are low, this uncoupling mechanism would control the rate of respiration, while at higher metabolic rates ATP demand becomes dominant. It is likely that the essence of the role of mitochondrial function in aging summarized here is preserved across phyla.

3.1 Introduction

Metabolism would seem to be an important element contributing to yeast longevity and aging, at least in the case of the replicative lifespan of *Saccharomyces cerevisiae*, because the measure of this lifespan are the number of daughters produced by a mother cell before it dies (Mortimer and Johnston, 1959; Müller et al. 1980). All other things being equal, the greater that number is the more substantial the energy expenditure and biosynthetic activity that is required; that is, the total metabolic capacity is a measure of the lifespan as well. Hence, the fact that different yeast strains have different lifespans immediately discounts ‘rate of living’ (Pearl 1928) as a viable theory of aging in this organism, because there is no fixed total metabolic capacity available to all yeasts. There is no necessary connection between ‘living fast’ and yeast lifespan either, as longevity is separable from both

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cell division rate (Kim et al. 1999) and from the rate of cell size increase that attends the aging of the mother cell (Chen et al. 1990). This, however, does not mean that metabolic efficiency may not have a part in yeast longevity. There is also the variability in replicative lifespan between individual cells of a given yeast strain, with which it is necessary to contend. However, this facet of biological aging is universally encountered, and it enters into the common denominator regardless of the mechanisms being considered. This is where the element of chance enters, which has been modeled to show how a population of genetically identical individuals that are maintained under the same conditions becomes stratified (Jazwinski et al. 1998). This epigenetic stratification explains why these individuals differ in lifespan and why there are multiple and independent limiting factors for longevity in such populations (Jazwinski 2001).

Mitochondria play a prominent role in cellular metabolism, and in particular in energy metabolism. This would suggest that mitochondrial function has a crucial influence on yeast aging. However, *S. cerevisiae* is a facultative anaerobe, denying the mitochondrion its obligate central role in energy production. Not surprisingly then, it was shown that the lifespans of spontaneous and induced petites, yeasts missing part or all of their mitochondrial genome and thus lacking fully functional mitochondria, are not significantly different from that of the parent strain (Müller and Wolf 1978). So persuasive was this finding that a stark contrast was drawn between aging of *Saccharomyces* and aging of *Podospora* in which a mitochondrial etiology was apparent (Jazwinski 1996). A nucleocentric view of yeast aging has thus been dominant, creating a quandary regarding the metabolic determinants of aging. Fortunately, a resolution of this dilemma has recently emerged.

3.2 The retrograde response determines yeast lifespan

The first clear, experimental indication that metabolism is an important factor in yeast aging came from the implication of the retrograde response in lifespan determination (Kirchman et al. 1999). The retrograde response is an intracellular signaling pathway triggered by the lack of fully functional mitochondria, notably occurring in petites (Parikh et al. 1987). It involves interorganelle communication, because the mitochondrial dysfunction causes changes in nuclear gene expression. These nuclear genes encode a variety of mitochondrial, cytoplasmic, and peroxisomal proteins (Liao et al. 1991; Small et al. 1995; Chelstowska and Butow, 1995; Vélot et al. 1996). The net effect of these changes in gene expression is a profound remodeling of cell metabolism. There is a shift from the utilization of the full Krebs cycle to the use of the glyoxylate cycle, which allows the cell to synthesize biosynthetic intermediates from acetate. The two carbons of acetate are conserved in the glyoxylate cycle, while they are lost as carbon dioxide when the full Krebs cycle is operative. This is an example of metabolic efficiency. The maintenance of anaplerotic reactions (conversion of pyruvate to Krebs cycle intermediates) is also apparent in the retrograde response. There are effects indicating the mobilization of lipids as fuels. Cellular stress responses are also enhanced. Genes

encoding carrier proteins in the mitochondrial inner membrane and the ADP/ATP translocator are also induced. The global nature of the changes in gene expression has been demonstrated in gene microarray studies (Traven et al. 2000; Epstein et al. 2001). Interestingly, the induction of *ADH1* and/or *ADH2* as well as *GPD2* in the retrograde response (Epstein et al. 2001), which would suggest enhanced regeneration of NAD from NADH, could provide for an increase in Sir2p activity and thus increased longevity (Kaeberlein and Guarente 1999). This effect may be accentuated by the induction of *TNA1*, the nicotinic acid transporter, because nicotinic acid is a precursor of NAD. However, a significant change in *SIR2* mRNA levels is not among the many changes in gene expression that constitute the retrograde response (Epstein et al. 2001).

The changes in gene expression that constitute the retrograde response are caused by the activation of the Rtg1p-Rtg3p transcription factor, which binds to the retrograde response element in the promoters of responsive genes (Liao and Butow 1993; Jia et al. 1997; Rothermel et al. 1997). This is a heterodimeric transcription factor, which belongs to the basic, helix-loop-helix-leucine zipper family. Activation of Rtg1p-Rtg3p accompanies translocation from the cytoplasm to the nucleus, which requires the function of Rtg2p (Sekito et al. 2000). The Rtg1p-Rtg3p transcription complex is used by the cell under stressful conditions, particularly under metabolic duress due to reduction or complete loss of respiratory function. It replaces the Hap (heme-activated protein) transcription complex in controlling expression of Krebs cycle genes under these conditions (Liu and Butow, 1999). Rtg2p, on the other hand, enters the nucleus in separate events to become a part of the SLIK transcriptional co-activator complex (Pray-Grant et al. 2002).

The signal proximal to the mitochondrion in the retrograde response pathway is not known at present. However, the rest of this signaling pathway is rapidly being fleshed out (Fig. 1). The Lst8p negatively regulates the Rtg proteins, by stimulating the expression of the Ssy1p-amino acid sensor at the plasma membrane. Lst8p also acts downstream as an inhibitor between Rtg2p and Rtg1p-Rtg3p (Liu et al. 2001). The role of the Ssy1p is likely related to the repressive effect of glutamate, a rich nitrogen source, on the retrograde response (Liu and Butow, 1999; Komeili et al. 2000; Liu et al. 2001). Rtg2p also acts upstream of the nitrogen catabolism regulation pathway (Pierce et al. 2001), playing a special role in the cellular response to the quality of the nitrogen source. The retrograde response appears to be modulated, at least in part, by the target of rapamycin (TOR) kinase pathway (Komeili et al. 2000), which plays a crucial role in the response to nutrient quality. Mks1p, a negative regulator of the Ras-cAMP pathway, exists as a complex with Rtg2p, and it negatively regulates the retrograde response, acting between Rtg2p and Rtg1p-Rtg3p (Sekito et al. 2002). The activity of Mks1p is antagonized by Rtg2p, and both of these proteins lie downstream of TOR kinase (Dilova et al. 2002). Ras2p potentiates the retrograde response (Kirchman et al. 1999), and this action is likely mediated through the negative effect of the Ras-cAMP pathway on Mks1p. The retrograde response plays an important role in the metabolism of both nitrogen and carbon, judging by the signaling pathways that impinge upon it.

The retrograde response is not only induced in petites with defective mitochondrial DNA but also in nuclear petites that possess mutations in nuclear genes en-

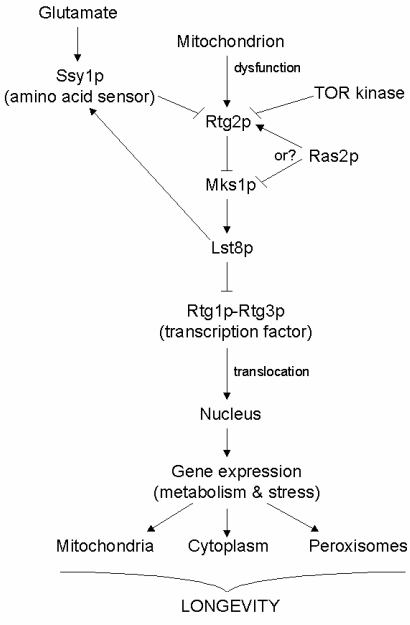

Fig. 1. The retrograde response signals mitochondrial dysfunction and extends yeast longevity. The retrograde response is elicited by mitochondrial dysfunction, which develops during normal aging in yeast. This signaling pathway from the mitochondrion to the nucleus results in changes in the expression of metabolic and stress response genes, whose products are destined for the cytoplasm, the mitochondria, and peroxisomes. The details of this signaling pathway are shown. The precise relationships between Mks1p and Lst8p in the respective portion of the pathway are not clear. It is also not known at present which one of the indicated interactions of Ras2p pertains, although this point may be mute because Rtg2p and Mks1p form a complex. ↓, stimulation/activation; ⊥, repression/inhibition.

coding components of the mitochondrial electron transport chain (Kirchman et al. 1999). Not surprisingly then, it is also induced when electron transport is blocked by antimycin A (Epstein et al. 2001). The participation of *MKS1* in retrograde signaling is based, among others, on the induction of the response in *mks1Δ* strains (Sekito et al. 2002; Dilova et al. 2002). The involvement of this gene in retrograde signaling explains the inhibitory effect of deletion of *RAS2* on the retrograde response (Kirchman et al. 1999) and the activating effect of the overexpression of this gene (P.A. Kirchman and S.M. Jazwinski, unpublished).

The lifespan-extending effect of the retrograde response was discovered as a consequence of analysis of the mechanism underlying the increased longevity of certain petite mutants (Kirchman et al. 1999). This increased longevity was not limited to mitochondrial petite mutants, but it was also found in mutants in nuclear genes that encode mitochondrial proteins. The life extension observed is not in-

consistent with the previously cited lack of effect of petite mutations on yeast longevity (Müller and Wolf 1978), because the life extension depends on the genetic background (Kirchman et al. 1999). This is sometimes cited as an argument against the generality of the effect of petite mutants on longevity. However, the issue is whether or not the retrograde response is induced in a petite in a particular genetic background (Kirchman et al. 1999). In fact, there is an extension of lifespan in petites in each of the four genetic backgrounds tested, under conditions in which the retrograde response is actived. It is these conditions that are background dependent and differ in these four strains. The key evidence for the causal role of the retrograde response in promoting yeast longevity is the dependence of the life extension observed in the petite on *RTG2* (Kirchman et al. 1999) and *RTG3* (J.C. Jiang and S.M. Jazwinski, unpublished). Furthermore, this lifespan extension in the petite is completely suppressed by deletion of *RAS2* (Kirchman et al. 1999). Overexpression of *RAS2*, on the other hand, extends yeast lifespan (Sun et al. 1994), and it also induces the retrograde response (P.A. Kirchman and S.M. Jazwinski, unpublished). However, the extent to which *RAS2* is expressed, as well as whether or not the cell is in a state of stress, affects the elicited response (Chen et al. 1990; Shama et al. 1998; Jazwinski 1999). Although much is known of the signaling events that constitute the retrograde response, it is not yet known which of the many induced changes in gene expression are necessary or sufficient for life extension.

The retrograde response is not a biphasic on-off switch for extended longevity. Instead, it acts in a continuous fashion, like a rheostat, responding to the severity of mitochondrial dysfunction by proportionately increasing the expression of retrograde responding genes and longevity in a commensurate fashion (Jazwinski 2000). Mitochondrial dysfunction accumulates with age in mammals (Shigenaga et al. 1997), and it has been recently shown to do so in yeast as well (Lai et al. 2002). This loss of mitochondrial function may be due to an increase in oxidative stress with age (Laun et al. 2001). Importantly, the activity of the retrograde response increases with age concomitantly with the increase in mitochondrial dysfunction (C. Borghouts, A. Benguria, J. Wawryn, and S.M. Jazwinski, submitted). Thus, we conclude that the retrograde response has a function during normal aging in yeast. This function appears to be to compensate for the accumulating mitochondrial dysfunction during aging. Perhaps, this is why yeasts live as long as they do.

The enhancement of longevity by the retrograde response (Kirchman et al. 1999) goes hand in hand with a pronounced increase in extrachromosomal ribosomal DNA circles (Conrad-Webb and Butow 1995), which are known to kill yeast cells (Sinclair et al. 1998). This conundrum has been resolved by the finding that the retrograde response sensor, Rtg2p, prevents the formation of these circles when it is not engaged in transmission of the retrograde signal (C. Borghouts, A. Benguria, J. Wawryn, and S.M. Jazwinski, submitted). When induced, the retrograde response not only compensates for the deleterious effects of the circles, but it activates life extension processes.

The retrograde response appears to possess broad significance as a metabolic mechanism determining longevity. The mitochondrial dysfunction displayed by a

mouse model of cardiomyopathy (Li et al. 2000) may be compensated by a mechanism akin to the retrograde response in yeast, allowing the animal to survive and function almost normally for a time. Some of the features of a retrograde response may operate in human longevity as well (De Benedictis et al. 2000; Bonafè et al. 2002). The loss of mitochondrial DNA that occurs in mammalian tissues with age may be compensated by the amplification of mitochondrial DNA itself, associated with mutations at the replication origin, which is governed by the nuclear genome (Zhang et al. 2003). This is yet another form of intergenome signaling that compensates for age changes (Poyton and McEwen 1996).

In *Caenorhabditis elegans*, mutations in genes encoding components of the respiratory chain, *clk-1* and *isp-1*, can extend longevity (Feng et al. 2001; Larsen and Clarke 2002). One possible mechanism underlying this effect is a reduction in oxidative stress. However, another plausible mechanism is the induction of a pathway of mitochondria-to-nucleus signaling like the retrograde response in yeast. In fact, the results of recent RNA interference-based schemes for the partial reduction of gene activity and the analysis of its effects on lifespan, one targeted (Dillin et al. 2002) and the other systematic (Lee et al. 2003), clearly demonstrate that mitochondrial dysfunction, even partial, can extend longevity. In fact, this life extension has been interpreted in terms of the retrograde response (Lee et al. 2003) or as a persistent regulatory adjustment that monitors mitochondrial activity (Dillin et al. 2002), which is largely an equivalent. The downregulation of any one of several genes encoding mitochondrial proteins causes the life extension observed in these studies. However, the common theme appears to be the disruption of the normal flow of protons and electrons in the mitochondrial inner membrane, as found in the yeast retrograde response. In other studies, the extension of lifespan by the *daf-2*/*daf-16* pathway is associated with a shift of metabolism from the Krebs to the glyoxylate cycle (Vanfleteren and DeVreese 1995), in similarity to the retrograde response in yeast. Thus, the life extension observed in the nematode appears to involve metabolic changes similar to those that occur during the extension of lifespan by the retrograde response in yeast. Although it is not a facultative anaerobe as yeasts are, adult *C. elegans* can survive and metabolize during extended periods of anaerobiosis (Föll et al. 1999). Thus, a partial reduction in respiratory activity should not pose a problem for this nematode. Compensation for aging deficits by a retrograde response mechanism is thus emerging as an important mechanism for life maintenance and extension in other organisms, in addition to yeast.

3.3 Calorie restriction extends longevity in yeast

The restriction of nutrient availability has been shown to affect the yeast replicative lifespan (Jiang et al. 2000). This effect possesses many of the features of calorie restriction in mammals, in which it has been known for many years to extend lifespan and to postpone the manifestations of aging (Masoro 1995). It has been proposed that calorie restriction in mammals involves changes in the way fuel is

metabolized (Masoro 1995). The demonstration that manipulation of the nutrient content of the growth medium affects yeast longevity provides further evidence for the essential role of metabolism in determining lifespan. In addition, it prompts the notion that the calorie restriction phenomenon has a cellular basis, which does not preclude an important role for systemic factors in multicellular organisms.

Extension of yeast longevity can be engineered by the reduction of either glucose or amino acids concentrations in the growth medium (Jiang et al. 2000). This indicates that changes in either carbon or nitrogen metabolism may elicit the response. Indeed, the possibility exists that two distinct mechanisms may be involved. On the other hand, there is also the alternative that one mechanism is operative, and the retrograde response is certainly a candidate. However, it has been clearly shown that calorie restriction and the retrograde response involve separate and distinct pathways, although some of the downstream longevity effectors of the two pathways are likely to be shared (Jiang et al. 2000). Among these shared downstream effectors are some of the metabolic and stress genes that are induced by the retrograde response and calorie restriction. Nevertheless, the concurrent operation of the retrograde response and calorie restriction pathways does not appear to be favored. Interestingly, calorie restriction can prevail over defects in retrograde signaling.

The identity of the signaling events in calorie restriction is becoming known (Fig. 2). Three pathways have been implicated. These are the Ras-cAMP, Gpr1p/Gpa2p (Lin et al. 2000), and Snf1p (Ashrafi et al. 2000) pathways. Unfortunately, it has not been demonstrated directly for any of these three pathways that they signal the glucose or amino acid limitation that enhances longevity. The proposed involvement of the Ras-cAMP pathway was based on a questionable role for Ras2p in glucose signaling (Thevelein and Winde 1999). The lifespan-curtailing effect of this pathway had been known already for some time (Sun et al. 1994). In contrast, the Gpr1p/Gpa2p pathway, which converges on adenylate cyclase as well, remains a plausible candidate for the glucose signaling pathway involved in life extension. The glucose sensor, Gpr1p, through the GTP-binding (G)-protein Gpa2p and adenylate cyclase, signals the activation of protein kinase A (Thevelein and Winde 1999). The Snf1 protein kinase has been implicated in yeast longevity (Ashrafi et al. 2000). The Snf1p pathway is required for the derepression of glucose-repressed genes, which allows yeasts to utilize alternative carbon sources (Johnston 1999). Thus, this pathway is activated when glucose concentration in the growth medium is reduced. It is surprising that the same lowering of glucose levels that extends lifespan (Lin et al. 2000) is known to activate Snf1 protein kinase, whose activity is known to curtail yeast longevity (Ashrafi et al. 2000). This dilemma requires resolution. As a start, it is necessary to consider the phenomenon of glucose repression in yeast.

High concentrations of glucose in the growth medium repress a wide array of genes, notably those involved in the utilization of alternate carbon sources (Johnston 1999). As glucose levels are lowered, these genes are de-repressed. The bulk of the cellular energy production is still by fermentation (glycolysis) rather than by respiration (Gancedo and Serrano 1989). Further reductions in glucose levels result in a larger respiratory component, until the cells fully rely on oxidative phos-

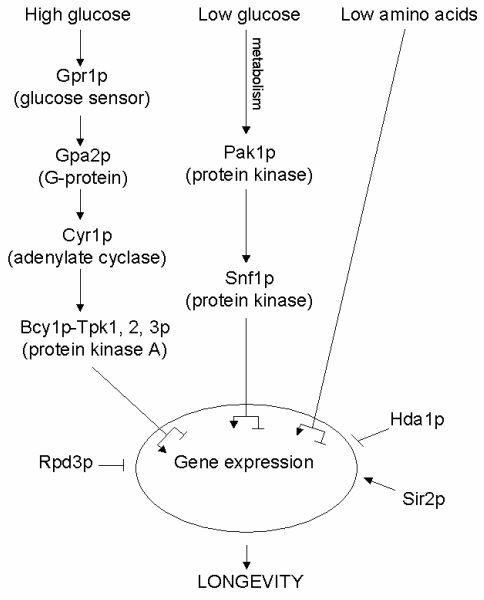

Fig. 2. Calorie restriction elicits changes in gene expression that prolong yeast life span. Limitation of nutrients, glucose or amino acids, activates signaling pathways that alter patterns of gene expression in the cell. These patterns of gene expression that influence life span are also modulated by histone deacetylase genes, which affect chromatin-dependent transcriptional silencing. G-protein, GTP-binding protein; Tpk1p, Tpk2p, Tpk3p, alternative catalytic subunits; ↓, stimulation/activation; ⊥, repression/inhibition.

phorylation. Any additional reduction in glucose concentration has no further effect. The life extension observed by Lin et al. (2000) was obtained at glucose levels at which the cells are released from glucose repression. These investigators also showed that deletion of *HXK2* caused an increase in lifespan. *HXK2* is required for glucose repression (Johnston 1999). Jiang et al. (2000) found that the reduction of the glucose concentration beyond the levels needed to de-repress the cells resulted in additional increases in lifespan. Indeed, the increases obtained were continuous up to the lowest glucose concentrations tested, which were 500-fold lower than those used in other studies (Lin et al. 2000; Lin et al. 2001, Lin et al. 2002). It therefore appears that additional gains in longevity occur on lowering glucose, even past the point when the cells are fully respiring. The relevance of the shift from fermentation to respiration that affects yeast longevity to the aging of higher organisms, which are aerobic, is not certain. More fundamentally, it is not yet clear whether the extended lifespan afforded by calorie restriction results from nutrient signaling events or from the changes in metabolism that are caused by the

lower substrate levels. Neither is it known whether calorie restriction is a positive signaling process or whether its effects are caused by the removal of the negative influences of high nutrient concentrations.

The major question concerning calorie restriction is the nature of the metabolic changes caused by reduced nutrient availability. This has been addressed in three studies. The global profiles of gene expression on moderate reduction of glucose levels (4-fold) that relieve glucose repression were compared with those that obtain on deletion of *HXK2* or overexpression of *HAP4* (Lin et al. 2002). Each of these treatments enhanced longevity in this study. The conclusion was that the major effects involved a shift away from glycolysis to respiration. This is not a surprising effect based on the known functions of the two genes. However, it is not clear whether enhanced respiration is causal in life extension or simply permissive of it. Interestingly, glucose reduction or *HXK2* deletion did not induce *HAP4* as might be expected, indicating the lack of congruence of these manipulations. Clearly, enhanced respiration does not extend lifespan. It was shown some time ago that yeast cells grown on a non-fermentable carbon source on which metabolism is respiratory do not have a longer lifespan than the same yeasts grown on a fermentable carbon source on which glycolysis is the major source of energy (Egilmez et al. 1990).

The results of Lin et al. (2002) summarized above are not consistent with those of Lin et al. (2001). The latter found that enhancement of glycolysis occurred on life extension following depression of Snf1p activity or moderate (4-fold) reduction of glucose levels, in stark contrast to the shift away from glycolysis noted by Lin et al. (2002). Under conditions of shortened lifespan, the gluconeogenic activator *HAP4* was induced, and this was associated with gluconeogenesis and glucose storage (Lin et al. 2001), which is inconsistent with the life extension found by Lin et al. (2002) through overexpression of *HAP4*. The difficulties with the interpretation of these studies and with the analysis of the signaling pathways in calorie restriction, as well as with other facets of the calorie restriction phenomenon in yeast are more fully discussed elsewhere (Jazwinski 2003).

In another approach to home in on the longevity effectors of calorie restriction in yeast, the manipulation of nutrient levels, both glucose and amino acids, was combined with genetic manipulations also known to affect lifespan (Jiang et al. 2002). These genetic manipulations involved three histone deacetylase genes, which are known, just like calorie restriction, to affect the global patterns of gene expression. These histone deacetylases exert these global effects by modifying the transcriptional silencing status of chromatin. The rationale was that there would be partial overlaps among the gene expression changes induced by manipulation of the histone deacetylase genes. From the effect on longevity of combining individual genetic manipulations with reduction of glucose or amino acids levels, it would be possible to decipher which of the overlapping patterns of gene expression might result in the life extension effect of calorie restriction. *RPD3*, *HDA1*, and *SIR2* were the three histone deacetylase genes studied, all three of which affect the yeast replicative lifespan (Kim et al. 1999; Kaeberlein et al. 1999). Deletion of *RPD3* extends lifespan, and there is no additional effect of calorie restriction (Jiang et al. 2002). Deletion of *HDA1*, on its own, has no effect but acts

synergistically with glucose limitation to enhance longevity. *SIR2* deletion shortens lifespan but does not prevent life extension by the reduction of glucose levels. However, *SIR2* deletion partially suppresses the extension of lifespan caused by reduction of amino acids levels. In other studies, deletion of *SIR2* was shown to suppress the life extension achieved by reduction of glucose levels (Lin et al. 2002). However, *SIR2* deletion was combined with the deletion of *FOB1* in those studies, adding an additional complication.

There are several conclusions from the analysis of calorie restriction with the use of histone deacetylase genes. Rpd3p affects both processes that are obligate effectors of calorie restriction and those processes that are synergistic with those effectors. Hda1p does not affect the obligate effectors, but it impinges on those processes that are synergistic with them. Sir2p plays a role similar to Hda1p; however, whereas the latter blocks processes synergistic with glucose restriction, Sir2p stimulates those processes that are enhanced uniquely by amino acid deprivation, albeit only partially in contrast to the effects mediated by Hda1p. The lifespan shortening effect of elimination of Sir2p appears to be caused by the loss of silencing at the ribosomal DNA locus (Smith and Boeke 1997; Kim et al. 1999). The processes affected by Hda1p and Sir2p are not likely identical, though there could be some overlap. From the known patterns of gene expression elicited by the deletions of *RPD3*, *HDA1*, and *SIR2* (Wyrick et al. 1999; Hughes et al. 2000; Bernstein et al. 2000), it was proposed that the major effectors of calorie restriction are involved in carbohydrate/energy metabolism, and in particular in mitochondrial function (Jiang et al. 2002). The expression of these effectors is modulated by the transcriptional state of chromatin, which is affected by the histone deacetylases. These histone deacetylases need not be part of the signaling pathways of calorie restriction. The available evidence argues against such a role for Sir2p. On the other hand, Rpd3p could conceivably be part of such a pathway, because it may interact with the nutrient-responsive TOR pathway through the rapamycin-binding proteins (Arévalo-Rodríguez et al. 2000).

SIR2 encodes an NAD-dependent histone deacetylase (Smith et al. 2000; Imai et al. 2000; Landry et al. 2000). The Sir2p has been purported to be part of the calorie restriction signaling pathway (Lin et al. 2000). It has been proposed that calorie restriction operates in yeast through a slowing of metabolism caused by limiting glucose, which thus results in the maintenance of NAD in the oxidized state, which is the form that is required for Sir2p deacetylase activity and transcriptional silencing (Lin et al. 2000). However, the enhanced respiration that occurs on limiting glucose can hardly be considered a slowing of metabolism as such. The NAD hypothesis has been modified to suggest that increased respiration is responsible for a higher NAD/NADH ratio in calorie-restricted cells (Lin et al. 2002). However, yeasts go to great lengths to maintain this ratio under a broad variety of conditions, using several different NAD regeneration systems for this purpose (Bakker et al. 2000). Thus, it is not likely that respiration creates a unique situation with respect to the redox state of this molecule. NAD is important in determining the lifespan of yeast, as manipulation of some of the genes that encode enzymes involved in the salvage pathway of NAD biosynthesis suggest (Lin et al. 2000; Anderson et al. 2002). However, NAD levels increase with age (Ashrafi et

al. 2000), and the increased activity of the salvage pathway alluded to above does not raise cellular NAD levels or the NAD/NADH ratio (Anderson et al. 2002). This may be due to the fact that this pathway resides in the cell nucleus. It is not clear how metabolism in other cellular compartments would affect the nuclear NAD pool, complicating the role of Sir2p as a sensor of cellular energy metabolism.

It is not clear at present whether calorie restriction extends lifespan by counteracting normal age changes in yeast or instead induces a qualitatively novel situation. On the other hand, the retrograde response represents a mechanism that extends longevity by compensating for deleterious age changes, as discussed earlier. In contrast, it has been proposed that calorie restriction is a preventive mechanism, which delays the onset of deleterious age changes (Jiang et al. 2000a). Although the possibility of the calorie restriction effect being mediated by NAD and the involvement of NAD in normal aging seem remote, Sir2p still could be involved in normal aging. If the level of the protein dropped with age, there would be a decline in its activity quite apart from any effect that changes in NAD levels could have. No evidence for changes in *SIR2* mRNA levels with age has been reported (Lin et al. 2001). Deletion of *RPD3* does not increase *SIR2* transcription (Bernstein et al. 2000; J.C. Jiang and S.M. Jazwinski, unpublished). Thus, the effects of any changes in *RPD3* expression are not mediated through changes in *SIR2* mRNA level, as has been implied (Guarente and Kenyon, 2000). Calorie restriction does not increase *SIR2* transcript levels either (Lin et al. 2002; J.C. Jiang and S.M. Jazwinski, unpublished).

In contrast to *SIR2* expression, the levels of *RPD3* and *HDA1* mRNAs fall with yeast age (Kim et al. 1999). This raises the possibility that yeasts live as long as they do in part because of gene expression changes caused by the loss of Rpd3 and Hda1 histone deacetylase activity. This is not, however, likely because simultaneous deletion of both *RPD3* and *HDA1* shortens mean lifespan by increasing the initial mortality of the cell population, while during the bulk of the lifespan mortality is identical to wild type (Kim et al. 1999). This increased initial mortality mirrors the normal pattern of decline in the expression of these two histone deacetylase genes during the lifespan, suggesting a requirement for the products of these genes early in life. Thus, deletion of both genes creates a pathological condition. The conclusion was reached that Rpd3p and Hda1p impact some of the same or similar life extension functions, but they also both impinge upon a set of processes essential for lifespan maintenance and viability (Kim et al. 1999; Jiang et al. 2002). Similar interpretations of cell pathology have subsequently been advanced for curtailment of lifespan in other yeast mutants (McVey et al. 2001). On the other hand, the marked extension of lifespan resulting from the deletion of *RPD3* alone is entirely consistent with the notion that this gene determines the characteristic lifespan of a yeast strain (Kim et al. 1999). Thus, *RPD3* may constitute a pacesetter for longevity. It has recently been shown that this gene plays such a role in *Drosophila* as well (Rogina et al. 2002), where *RPD3* and calorie restriction exhibit the same relationship as they do in yeast.

3.4 Mitochondria and aging

Most of the research into yeast aging has dealt with the question of why mother cells have a limited lifespan (reviewed in Jazwinski 2002). Little attention has been devoted to the mechanisms underlying the age asymmetry between the mother and its daughters, which results in these daughters having, in principle, the potential for a full replicative lifespan. The genetically-induced shortening of telomeres results in clonal senescence; however, there is no evidence that this is due to a loss of age asymmetry as such (Lundblad and Szostak 1989). Also, the induced amplification of extrachromosomal ribosomal DNA circles kills yeast cells, thus decreasing replicative lifespan, but this is not associated with either clonal senescence or with loss of age asymmetry (Sinclair et al. 1998). The loss of age asymmetry by definition would result in daughters being born having the same replicative age as their mothers have at the time of birth or cell separation. This, in turn, would result in clonal senescence, meaning the gradual demise of the entire cell lineage or pedigree. Recently, mutants in which age asymmetry is disrupted have been isolated (Lai et al. 2002). These mutants were obviously conditional, because the clonal senescence associated with the loss of age asymmetry would otherwise preclude their isolation.

The loss of growth potential caused by the clonal senescence is reversible when the mutant cells are shifted from the restrictive to the permissive temperature (Lai et al. 2002; C. Borghouts and S.M. Jazwinski, unpublished). The clonal senescence phenotype elicited in the mutant is subtle, because it takes many generations to develop and recovery is gradual. The phenotype is not simply due to the lack of synthesis of an essential molecule or cellular component at restrictive temperature, but rather to the loss of age asymmetry such that daughters are no longer born young and instead possess the age of their mothers. The mutation causing loss of age asymmetry was a single-base change in the *ATP2* gene (Lai et al. 2002). This gene encodes the β-subunit of mitochondrial F_1-ATPase. The mutation did not cause telomere shortening or the accumulation of extrachromosomal ribosomal DNA circles.

The clonal senescence phenotype was manifested only during growth on fermentable carbon sources, which suggests that the critical function of F_1-ATPase that is diminished in the mutant is not the synthesis of ATP driven by the $\Delta\Psi_m$, but rather the establishment of $\Delta\Psi_m$ powered by ATP hydrolysis. This is because on fermentable carbon sources yeasts generate the vast majority of their energy through glycolysis/fermentation (Gancedo and Serrano 1989). Still, mitochondrial function is essential, especially for the biosynthetic intermediates provided by the Krebs cycle. The metabolic communication between the mitochondrion and the cytoplasm is mediated by transporters in the inner mitochondrial membrane, whose activity is driven largely by the $\Delta\Psi_m$, which is generated during fermentation primarily by the reversal of the ATP biosynthetic reaction rather than by the greatly reduced activity of the mitochondrial electron transport chain.

But, how does this molecular change result in loss of age asymmetry and clonal senescence? Flow cytometry has demonstrated that there is a progressive decline

in $\Delta\Psi_m$ with the onset and progression of clonal senescence, followed by a reduction in mitochondrial mass (Lai et al. 2002). Young cells in the population begin to display a dearth of mitochondria. Fluorescence microscopic analysis has shown that the expression of the mutant phenotype is associated with changes in mitochondrial morphology and distribution in the cell, resulting in deficient segregation of mitochondria to daughter cells (Lai et al. 2002). The net result is the generation of cells totally lacking mitochondria, which become the dominant cell type as yeast clones become extinct. The results show that there is first a loss of mitochondrial activity followed by the physical disappearance of these organelles.

The studies with the *atp2* mutant indicate that the normal function of this gene is essential for the maintenance of age asymmetry. However, the deficits associated with this mutant are also encountered during the course of normal aging in wild-type yeast. There is a five-fold decline in $\Delta\Psi_m$ during the yeast lifespan with a fermentable carbon source available (Lai et al. 2002), which may be due to oxidative damage (Laun et al. 2001). Also, old yeast cells are prone to segregation of defective mitochondria to their daughters (Lai et al. 2002). These properties of mitochondria recapitulate the operation of the cytoplasmic senescence factor, whose manifestations intensify during yeast aging (Egilmez and Jazwinski 1989; Lai et al. 2002; Jazwinski 2003). Thus, mitochondrial dysfunction is very likely to be a cause of aging in yeast. This dysfunction expresses itself as a reduction in $\Delta\Psi_m$. It does not appear that defects in the electron transport chain, which generates $\Delta\Psi_m$, are a primary cause of aging when glucose is plentiful, because of the potent compensation provided by the retrograde response, as described earlier. Instead, it would seem to be the activity of F_1-ATPase that is the culprit. In conjunction with F_0, the F_1-ATPase can generate an electrochemical gradient across the mitochondrial inner membrane driven by ATP hydrolysis. In the absence of F_0, F_1-ATPase can hydrolyze ATP and thus cooperate with the ADP/ATP translocator to achieve the same effect (Dupont et al. 1985). In any case, the essential function is proposed to be the maintenance of a sufficient $\Delta\Psi_m$ to operate the transporters in the inner mitochondrial membrane that move biosynthetic intermediates between this organelle and the cytoplasm.

In general, the partition of active and undamaged cellular components to daughter cells is essential for the maintenance of age asymmetry. We have proposed (Lai et al. 2002; Jazwinski 2003) that there are 'filters' that support the accuracy of this process and that these 'filters' deteriorate with age. Their operation requires interorganelle communication and appropriate checkpoint controls. In higher eukaryotes, it is likely that stem cells are particularly dependent on these mechanisms. Recent studies showing preferential segregation of oxidatively-damaged proteins with mother cells (Aguilaniu et al. 2003) support our filter hypothesis.

The studies of age asymmetry have brought mitochondria and $\Delta\Psi_m$ to a central position in yeast aging (Fig. 3). The significance of mitochondrial function in yeast aging is also highlighted by the retrograde response, which compensates for the dysfunction of this organelle that develops during aging. In the retrograde response, this compensation is achieved by the activation of alternate pathways for

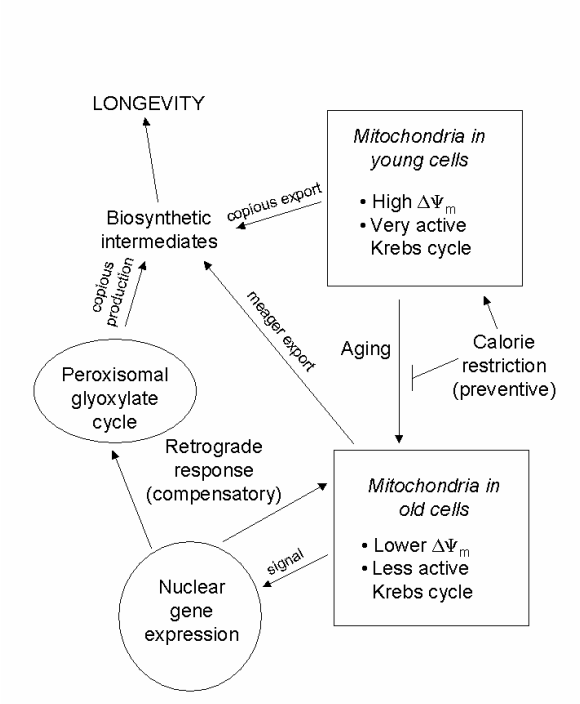

Fig. 3. Mitochondria play a role in both compensatory and preventive mechanisms of aging. This model emphasizes the role in longevity of biosynthetic intermediates provided by the Krebs and glyoxylate cycles and of mitochondrial membrane potential ($\Delta\Psi_m$), which is necessary for their export from mitochondria. The retrograde response induces expression of activities targeted to the cytoplasm, in addition to those imported into mitochondria and peroxisomes.

the provision of biosynthetic intermediates and cooperation with portions of the Krebs cycle. This cooperation requires communication between subcellular compartments and the activity of mitochondrial membrane transporters that are dependent on $\Delta\Psi_m$. The above considerations provide insights into the relative importance for longevity of the metabolic changes that occur on calorie restriction, which involve the remodeling of mitochondrial function. The critical metabolic events are proposed to be the enhancement of Krebs cycle activity for the provision of biosynthetic intermediates and the maintenance of a suitable $\Delta\Psi_m$ to allow their transport out of the organelle, rather than the intensification of respiratory activity as such. This enhanced respiratory activity represents an increase in the efficiency of energy production by the cell, and it serves the generation of the essential $\Delta\Psi_m$ exceptionally well. However, this function of the electron transport chain is not irreplaceable, because the $\Delta\Psi_m$ is readily maintained by the reversal of the

ATP-biosynthetic reaction catalyzed by F_1-ATPase, as noted above. Similarly, the activity of the electron transport chain is not obligatory for the maintenance of NAD in its oxidized form, because the cell possesses several alternative NAD regeneration systems, as mentioned earlier. Clearly, the electron transport chain is not the only available driving force for the synthesis of ATP, especially in yeasts growing on a fermentable carbon source such petites in which the retrograde response is induced. Thus, the differences between the retrograde response and calorie restriction are superficial. The net effect in each case is the provision of metabolites for the production of daughter cells, whose quantity is the measure of the lifespan. It is likely that the essence of this role of mitochondrial function in aging is preserved across phyla.

There is an additional aspect to the role of mitochondria and $\Delta\Psi_m$ in aging. To fulfill their role of providing biosynthetic precursors, mitochondria export a variety of compounds, notably Krebs cycle intermediates, across their inner membranes and into the cytoplasm. As discussed above, this function involves the activity of mitochondrial membrane carriers and is powered by $\Delta\Psi_m$. The activity of these carriers dissipates the $\Delta\Psi_m$, through the leak of protons across the mitochondrial membrane resulting in uncoupling of the mitochondria (Fig. 4). This uncoupling would reduce production of reactive oxygen species by the mitochondria (Bouillaud et al. 2001). Thus, the supply of biosynthetic precursors by mitochondria for the production of daughter cells, and hence extension of lifespan, brings with it a means for reduction of oxidative damage that can cause aging. This is all the more important as the yeasts become more and more dependent on respiration for energy production, as they are calorie-restricted to a greater and greater extent. Therefore, the provision of Krebs cycle intermediates, which is enhanced by calorie restriction, plays the primary role in extending lifespan, while the increased respiration, as such, is secondary. Furthermore, these considerations lead to the conclusion that any reduction in oxidative stress resulting from calorie restriction is a derivative of the metabolic changes precipitated by this mechanism of life extension.

I propose that the mechanism of limiting the production of reactive oxygen species by uncoupling of mitochondria is of increasing significance as the nutrient limitation becomes more profound during calorie restriction and goes beyond the point at which the metabolism of the yeast cells is fully respiratory. This is because the metabolic rate of the cells decreases as nutrients become more and more limited, and conditions of low cellular ATP demand predominate. At low rates of metabolism, most of the respiratory control is exerted by proton leak. In contrast, the high ATP demand (and ultimately the respiratory chain) dominates the control of respiration at rapid metabolic rates, resulting in a small depression of $\Delta\Psi_m$ and reduced production of reactive oxygen species. However, there are limits to which this latter protective effect can operate, based on the limits of inner mitochondrial membrane architecture. In sum, the limitation of the production of reactive oxygen species would play at least as important a role in aging as the antioxidant defenses and repair mechanisms. The production of reactive oxygen species is governed by the metabolic requirements of the cell, in both positive and negative ways.

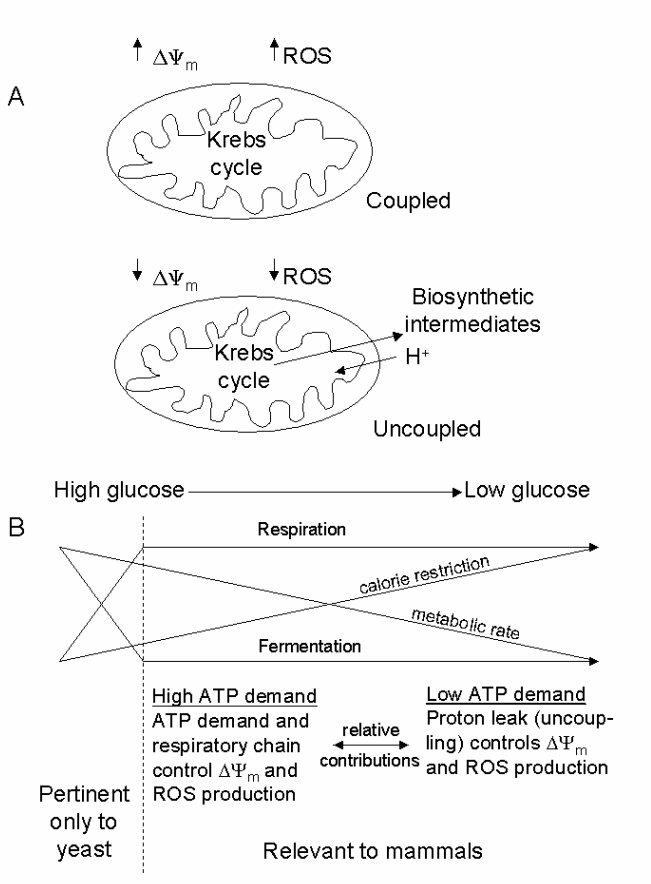

Fig. 4. Central role of Krebs cycle intermediates and $\Delta\Psi_m$ in mitochondrial function during aging. (A) All other things being equal, high $\Delta\Psi_m$ leads to elevated production of reactive oxygen species (ROS). The export of Krebs cycle intermediates as biosynthetic precursors for growth, which is a determinant of yeast life span, results in the concurrent leak of protons into mitochondria, also called uncoupling. This lowers $\Delta\Psi_m$ and reduces the production of ROS. (B) Lowering of glucose concentrations in the growth medium relieves glucose repression. As glucose is lowered further, the participation in energy production of respiration relative to fermentation increases. As calorie restriction is increased, metabolism becomes largely respiratory and metabolic rate declines. At high metabolic rates, ATP demand is high, and it predominates in control of $\Delta\Psi_m$ and ROS production. At low metabolic rates, ATP demand is low, and proton leak (uncoupling) controls $\Delta\Psi_m$ and ROS production. The relative contributions of these two mechanisms constitute a continuum. Only respiratory metabolism (to the right of the vertical, broken line) is relevant to mammals, while glucose-repressed and fermentative conditions (to the left) pertain to yeast only.

The low metabolic rates of yeast cells may be likened to the basal metabolic rate in higher eukaryotes. Upregulation of the basal metabolic rate, with attendant increased oxygen consumption, could occur to generate ATP in mitochondria that produce diminished amounts of reactive oxygen species, by virtue of increased uncoupling. Thus, higher basal metabolic rate may be protective and could be associated with longer life. Some evidence that indeed this may be the case in mice has been presented (Speakman et al. 2001). From the discourse above, it would follow that active metabolic rate could also be protective, but only up to a point.

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4 Yeast as a model for ageing and apoptosis research

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Abstract

Apoptosis is a form of programmed cell death with a crucial role in health and disease in metazoans. Recent findings demonstrate the existence of an apoptotic program also in unicellular eukaryotes. Oxygen stress as well as the expression of several conserved proapoptotic genes induce the apoptotic pathway in mammalian cells and yeast cells. The dying yeast cells show diagnostic markers of apoptosis.

Yeast provides a simple and powerful model for cellular ageing. Oxygen radicals are produced during mother cell-specific ageing of yeast cells. Postmitotic ageing during survival of stationary phase also is accompanied by oxidative stress. In both forms of ageing, the terminal yeast cells execute apoptosis. This may elucidate some questions about the oxygen theory of ageing in mammalian cells. The involvement of oxygen toxicity in the ageing processes of cells as widely divergent as human and yeast leads us to the proposal to use the powerful system of yeast molecular genetics to study the role of oxygen toxicity as a "public" mechanism of ageing.

It has became clear through recent studies of yeast and human cells, that apoptosis is not only a mechanism to prevent ageing of a tissue by removing irreversibly damaged cells which can then be replaced by regeneration. Apoptosis is also a mechanism of death for senescent cells.

Yeast cells are also an excellent system to test other current theories of ageing. Prominent among them are the theory according to which an ageing signal is produced by critically short telomeres and the theory according to which unrepaired DNA damage leads to premature ageing and apoptosis.

4.1 Introduction

In this review, we will summarize recent progress in two fast growing fields: mother cell-specific ageing and apoptosis in yeast. Chronological ageing (which occurs during survival of stationary phase) is only treated cursorily. Apoptosis (programmed cell death) was only recently established as an accepted fact in yeast and other monocellular eukaryotes (Madeo et al. 2002). Senescent yeast mother cells (Laun et al. 2001) as well as dying yeast cells in stationary phase (our unpublished data) display a number of apoptotic phenotypes. Likewise, it was recently

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shown that HUVEC (human umbilical vein endothelial cells) in culture die via apoptosis (Wagner et al. 2001). This was hard to accept to the ageing research community, since apoptosis was traditionally viewed as an anti-ageing mechanism (Warner 1999). While it is an obvious function of apoptosis to remove irreversibly damaged cells thus ensuring the well being of a tissue or organ, it is now also clear that apoptosis is a way to die when the limit of replicative ageing is reached. However, it is not completely clear whether apoptosis is the obligatory mechanism of death in replicative (and postmitotic) ageing, or whether apoptosis is just an epiphenomenon of ageing. Further experimentation is needed to clarify this question. The build-up of intracellular oxidative stress is a phenotype that is common to ageing and apoptosis. Oxygen toxicity seems to play a major role in both processes. Therefore, in a large part of this review, the role of oxygen toxicity and the cell's defence against oxidative stress in ageing and apoptosis is discussed.

Presently, we are facing a worldwide renaissance of ageing research. This has to do with the fact that human life expectancy at birth has increased considerably over the last 50 years in the industrialized countries resulting in an increasing amount of the lifetime of people spent during retirement. This has not only produced considerable socio-economic problems but has also resulted in growing awareness how little we know about the basic biology of the ageing process and has intensified clinical, biomedical, and biological ageing research. The questions that are the most interesting for us are: Which of the phenotypic traits that we observe in yeast ageing are common to all eukaryotic model systems for ageing, including humans? Which ones are accidental? Is apoptosis an essential component or is it a mere epiphenomenon of the ageing process? What is common and what is different in the apoptotic process of yeast and of higher cells? Why does a simple unicellular eukaryotic organism need the apoptotic program? What is the relation between replicative mother cell-specific ageing on the one hand and postmitotic ageing on the other hand in yeast? Several of these questions are under active investigation in yeast laboratories, but there are no definitive answers at the present. We must stress the fact that our treatment of yeast ageing is not comprehensive. Please consult the reviews written by Jazwinski et al. and by Osiewacz et al. (this volume).

Apoptosis is a form of programmed cell death with a central role in tissue homeostasis and maintenance in metazoans and is enormously important for human health. Constant turnover of cells driven by apoptosis is important for homeostasis in tissues and organs. During the last decade, apoptosis has become one of the most intensely researched topics in medical science. Some of the major roles of this cellular suicide program manifest itself during the ageing process.

Although the importance of apoptosis in stroke, neurodegenerative disorders, and cancer is increasingly evident, many details of its regulation and of the apoptotic phenotypes are poorly understood (for review see Uren and Vaux 1996). The complex regulatory network and the sometimes contradictory results obtained with human cell lines made application of an easier model system desirable. Apoptosis in yeast could provide a better understanding of certain aspects of the regulation of apoptosis. Through the last years, scientists were successful in iden-

tifying or characterizing new cell death regulators of humans using the yeast system. Moreover, many connections between yeast apoptosis and ageing emerged.

4.2 Model systems for ageing research

Why do we need simple model systems for the ageing process? This is because human genetics has not resulted in sufficient evidence to pinpoint a generally recognized mechanism for the ageing process of the organism and because, as we will discuss, the *in vitro* ageing process of human cells also is not telling us the most important and interesting facts about ageing of the organism. Family studies of longevity tell us that the heritability of lifespan is less than 50 per cent (Finch and Ruvkun 2001). The so-called premature ageing syndromes (progerias) only illuminate "segmental" aspects of ageing (Martin and Oshima 2000). Certain tissues or organs of those patients give the impression of an old age phenotype (skin, connective tissue) but others do not. The most prominent of the premature ageing syndromes (Werner's disease) is caused by a recessive mutation in a gene coding for a DNA helicase, which is probably involved in DNA repair. However, as there are a relatively large number of helicases on the genome and helicases are needed also for transcription, recombination, ribosome biogenesis, and many other processes, we presently cannot be absolutely sure about the importance of DNA repair (which aspects of the repair pathways?) for the ageing process.

50 years ago Hayflick discovered (Hayflick 1965) that human cells (fibroblasts) have a finite lifespan and age clonally *in vitro*. In this context, clonally means that all cells derived from a single cell stop dividing and reach the so-called Hayflick limit after a certain number of cell divisions. What was really intriguing about this finding was that fibroblasts derived from older individuals had a progressively shorter lifespan (Hayflick 1998). Cells (fibroblasts) derived from short-lived species likewise displayed a shorter lifespan *in vitro* (Hayflick 1998). However, it is by no means clear whether the Hayflick limit observed in cell culture is functionally relevant for *in vivo* ageing of the organism. Cells can escape the Hayflick limit by oncogenic point mutations. They will then grow indefinitely, but when transferred back into animals (mice) they give rise to tumours and display markers of tumour cells, like accumulation of chromosome mutations (inversions, translocations, aneuploidy).

One of the more prominent ageing theories states that the Hayflick limit is determined or signalled by progressive shortening of telomeres in cultured fibroblasts. Indeed overexpressing telomerase in those cells extends the Hayflick limit (Bodnar et al. 1998; for review see Hayflick 1998). Still, shortened telomeres were not observed in fibroblasts obtained through biopsies of centenarians (Mondello et al. 1999) and in the mouse, a homozygous telomerase deletion mutant did not show premature ageing but, quite unexpectedly, infertility and a number of other defects (Rudolph et al. 1999). This example shows that there exist serious difficulties to relate findings in cell culture with the organismic ageing process. However,

it was found in muscle satellite cells of patients suffering from muscle dystrophy, that the telomeres in those cells were shortened (to be discussed below).

4.3 Determination of replicative (mother cell-specific) lifespan in yeast

In yeast, two different physiological processes are called ageing. For both processes, parallels with the organismic ageing processes of higher organisms have been pointed out. First, there is postmitotic ageing, senescence, and ultimately death, which is equivalent to the changes occurring in stationary cells over relatively long periods of time. Stationary cells, depending on the exact conditions under which they become stationary, lose viability over a time period of weeks to months. Postmitotic ageing is also observed, for instance, in the brain, where neurons cannot be replaced (or hardly so) and therefore ageing of the brain depends on the survival and function of postmitotic neurons of the brain.

The second ageing process of yeast is the mother cell-specific ageing of growing cells (Mortimer and Johnston 1959). It occurs in the presence of nutrients and does not depend on starvation. Parallels in the ageing process of higher organisms are less obvious but will be discussed below (muscle ageing). Mother cell specificity means that only the mother cell ages in the asymmetric cell division process of yeast. Eventually the mother cell reaches a state of senescence, defined here as the terminal stage at which no further cell divisions are performed. On the contrary, the daughter cell resets the clock to zero and her lifespan is the same regardless of whether she is the first or the 10th daughter of an individual mother cell. Only the very last daughters of old mothers have a somewhat shortened lifespan (Kennedy et al. 1994). The lifespan distribution characteristic for a strain is determined by micromanipulating and counting all daughters of a set ("cohort") of virgin cells that were themselves isolated by micromanipulation. It takes about a week for a skilled worker to determine the lifespan distribution of a strain (Fig. 1b). This curve follows the Gompertz law, the same law that very well describes the lifespan distribution of humans! Lifespan depends only on the number of generations, not on calendar time (Jazwinski 1993). The median (the age, in generations, at which 50 per cent of the cells survive) is used to best describe the lifespan of the strain. The standard deviation of the median is given as an error bar in the graphs. The number of cells generally used in our laboratory for lifespan determinations is 60. In practice, it is important to determine whether two lifespan curves are significantly different. For instance, applying the non-parametric Wilcoxon test (Wilcoxon 1945), two of the lifespans shown in Fig.5, are not significantly different.

The median lifespan is well reproducible. On the other hand, the maximum lifespan of the strain is less well defined because of necessity the statistics are poor at the end of the lifespan curve when only a few cells survive.

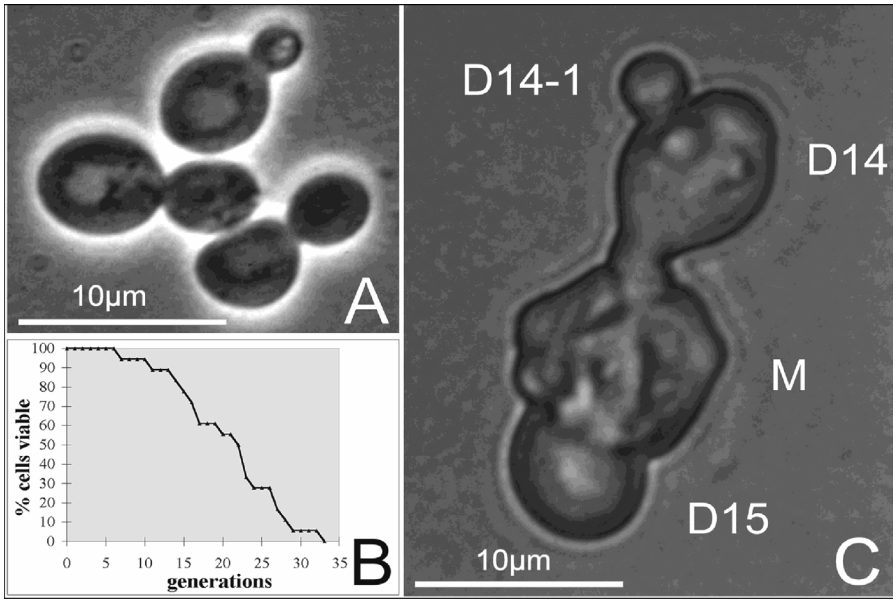


Fig. 1. A terminal stage of an old yeast mother cell isolated by micromanipulation and photographed in phase contrast (C). For comparison, a young cell is shown at the same magnification (A). M: mother cell, D15: the fifteenth (last) daughter; note that this cell cycle was not completed. D14: the fourteenth daughter; D14-1: the first granddaughter of D14; also this cell cycle was not completed. Note the surface changes of M. (B) A typical lifespan distribution obtained with 60 cells of the haploid strain W303 MATa. x-axis: number of cell generations, y-axis percent surviving cells. Source: Nestelbacher et al., Exp Gerontol 34:895-896 (1999), with permission of the publisher.

4.4 Mother cell specificity – a general eukaryotic phenomenon?

It is important to explain that the mother cell-specific ageing process of yeast cells, which was some time ago viewed as an exotic biological phenomenon, might be very common indeed. Even fission yeast which produces two daughters of equal size, in reality displays an asymmetric division and undergoes mother cell-specific ageing (Barker and Walmsley 1999). More importantly, the various stem cell populations of the human body do undergo asymmetric divisions resulting in one new stem cell and one cell that has made the first step on the way to differentiation. The two daughters resulting from such stem cell divisions may look morphologically similar, but they certainly differ in gene expression. In striated muscle but not in heart muscle a limited supply of satellite cells, which are equivalent to muscle stem cells are used for muscle growth and repair during our lifetime. This phenomenon is dramatically visible in patients suffering from muscular

dystrophies where the muscles undergo continuous cycles of degeneration and regeneration. It can also be observed in high-level sportspersons suffering from FAMS (Fatigued Athletes Muscle Syndrome) who have used up their supply through excessive training that damages many muscle fibres and consequently the continual growth and repair uses up the pool of stem cells. The lesson it teaches us is that even in the human body mother cell-specific ageing might occur. Otherwise, the supply of stem cells would not be exhaustible. Shortening of telomeres was observed in the satellite cells of patients suffering from muscle dystrophies, but not in muscle biopsies derived from old patients (Decary et al. 2000; Renault et al. 2002; Butler-Browne, pers. comm.).

4.4.1 Preparation of old cells

Biochemical investigation of the senescent phenotype requires an enrichment procedure that leads to sufficiently pure terminally senescent yeast mother cells. Purification of these cells, which occur in a batch grown yeast culture as a very minute fraction of all cells, has proven to be a major difficulty in yeast ageing research. All old cell preparations are contaminated with young cells, because in the cell cycle of budding yeast, the daughter adheres to the mother and is not separated immediately. Such mother/daughter aggregates behave like a large single cell during physical separation of large and small cells. The separation of daughters from their mothers at least in some favourable strains is more nearly complete in stationary phase. However, reaching the stationary phase should be avoided in order not to mix up the effects of mother cell-specific ageing, which is independent of nutrient availability, with the physiological effects of starvation. Egilmez et al. (Egilmez et al. 1990) separated young and old yeast cells in sucrose density gradients. Their fractions appear to be very pure and have led to the first phenotypic characterization of old cells based on a bulk cell preparation. Next, the magnetic bead technology was employed to immobilize yeast cells. The daughters are budded off and washed away, fresh growth medium is supplied either continuously or after certain times and in the end a population of yeast cells, still immobilized on the beads is obtained, that have a rather equal age structure (Laun 1999; Smeal et al. 1996). As we have shown (Laun 1999), the method is limited by the fact that the extensive cell surface alterations of very old (senescent) cells lead to the loss of these cells from the beads. Therefore, the method is excellent for enriching small amounts of cells of the 10^{th} or even 20^{th} generation in a strain with a median lifespan of 25 generations. It was however, not possible, to isolate significant numbers of senescent (terminally aged) cells on magnetic beads. Finally, we want to discuss the enrichment of senescent cells by elutriation centrifugation. This method was described in detail earlier (Laun et al. 2001) and is based in part on previous work by Woldringh et al. (1995). The strain to be investigated is batch grown for 20 hours (about 10 generations) without reaching stationary phase and the cells are separated into 6 fractions according to size by elutriation centrifugation. Fractions III and IV (large cells that are not damaged or broken) are re-inoculated into fresh medium and again grown for 20 hours. The elutriation pro cedure is repeated and

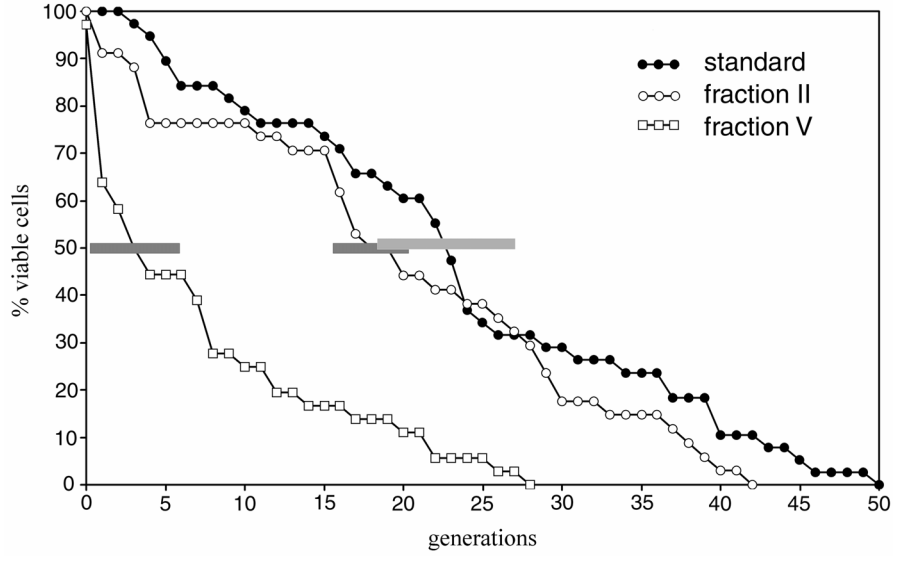


Fig. 2. Lifespans of elutriated wild type yeast fractions of strain JC482. Error bars: standard deviations of the median. Sample size: 40 cells in each experiment. x-axis: number of cell generations, y-axis percent surviving cells. Source: Laun et al., Mol Microbiol 39:1166-1173 (2001), with permission of the publisher.

fraction V (largest undamaged cells) and fraction II (virgin daughter cells) are washed and used for cytological and biochemical analysis. Fraction II cells are a convenient control because they were shown to be mostly virgin cells and first generation cells and have undergone the same purification procedure as the old cells, thus excluding artefacts of the purification procedure. Fraction V cells are to about 30 per cent final stage senescent cells. About one third of the cells stop dividing within the first cell cycle when tested in a standard lifespan test. As the lifespan curves of elutriated cell fractions show, fraction V still contains a considerable amount of young cells (represented by the tail of the curve), which originated from daughters adherent to their mothers (Fig. 2). A detailed description of these cells, which show all markers of yeast apoptosis, will be given later in this review.

4.5 The markers of yeast apoptosis

Apoptosis in eukaryotes is coordinated by a complex network of regulators and effectors, which can be triggered exogenously (e.g. by ethanol, oxidants, receptor ligands) and endogenously (e.g. by replication failure, inhibited DNA-repair or developmentally programmed cell death). Independently of the regulatory

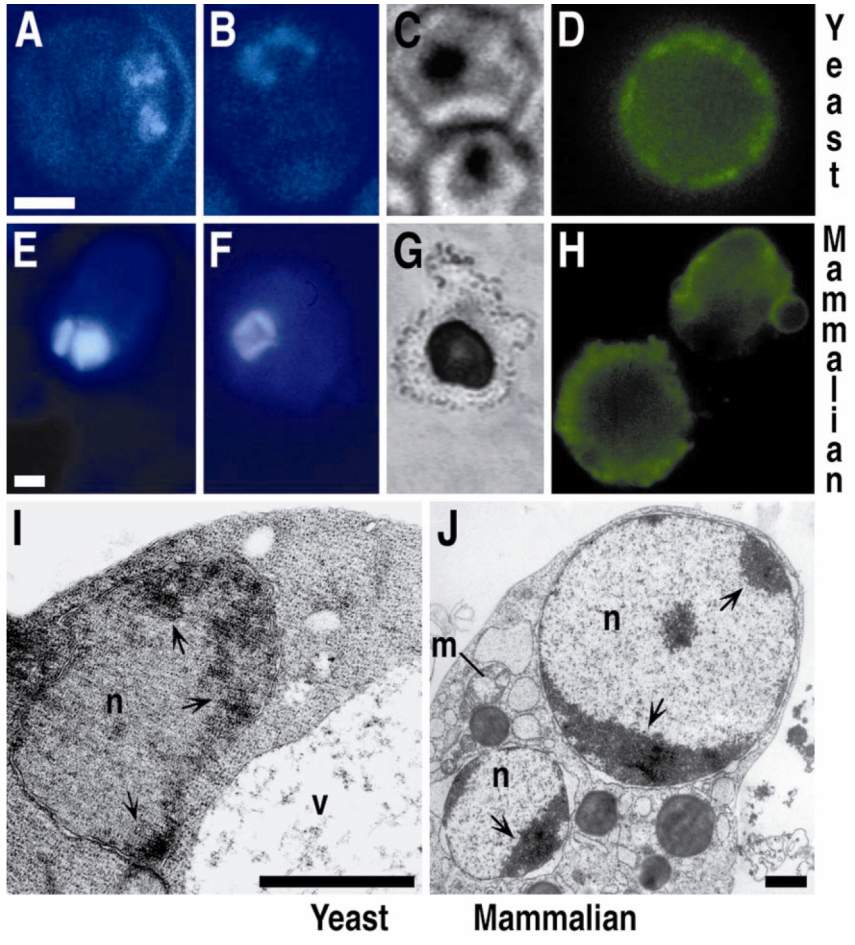


Fig. 3. Cytological markers of apoptosis in yeast and mammalian cells. *S. cerevisiae* treated with 1 mM H_2O_2 (A, B, C, I) or mutated in CDC48 ($cdc48^{S565G}$, D), porcine thyrocytes, treated with 30μM retinol (E, F, H, J), and human WISH cells treated 1 mM H_2O_2 (G) stained for chromatin with DAPI (A, B) or bisbenzimide (E, F), for DNA fragmentation with TUNEL assay (C, G), for the exposition of phosphatidylserine with FITC-labeled annexinV (D, H), or investigated by electron microscopy for chromatin condensation (I, J). Bars 10 μm (A-H), 1 μm (I, J). Source: Frohlich and Madeo, FEBS Lett 473:6-9 (2000), with permission of the publisher.

pathway, a characteristic phenotype occurs at the end of almost all apoptotic scenarios: Phosphatidylserine is externalized to the outer leaflet of the plasma membrane, chromatin condenses in a typical manner, DNA is cleaved into small fragments and cells break up into membrane-enclosed vesicles, the so called apoptotic bodies. Apoptosis had been assumed to be restricted to multicellular animals. For a unicellular organism like yeast, a suicide mechanism seemed useless, resulting

in the death of the whole organism, after all. When the complete genome sequence of *S. cerevisiae* became available in 1997, no homologues of the most central players in apoptosis, the caspases, members of the Bcl-2/Bax family or Apaf-1 were found, emphasizing the idea that apoptosis might be restricted to metazoan organisms.

Despite the seeming absence of conserved genes, we found a yeast mutant dying with a typical apoptotic phenotype: exposition of phosphatidylserine, margination of chromatin and formation of cell fragments (Madeo et al. 1997). The TUNEL test indicated massive DNA fragmentation (Fig. 3).

The apoptotic phenotype was caused by a point-mutation of *CDC48* (*cdc48*^{S565G}), coding for a *S. cerevisiae* protein belonging to the AAA family of membrane ATPases and involved in vesicle fusion. How can we explain a connection between vesicle fusion and apoptosis? This is not well known, however, Granot and colleagues (Levine et al. 2001) showed that Bax-triggered apoptosis in yeast can be blocked by enhancing vesicle trafficking. Moreover, a down-regulation of vesicular transport enhances the susceptibility of yeast cells to apoptosis providing an explanation for the *cdc48*^{S565G}-mediated apoptosis.

In case of *CDC48*, the yeast model system has already demonstrated its potential to identify new mammalian apoptotic regulators. In 1999, an anti-apoptotic role of the human *CDC48* ortholog VCP/p97 and of a related protein in *C. elegans* was described (Shirogane et al. 1999; Wu et al. 1999). A mutated form of VCP, in a manner similar to the *cdc48*^{S565G} mutation in yeast, dominantly induced apoptosis in B-cells. This makes CDC48/VCP the first apoptotic regulator originally discovered by its function in yeast apoptosis. Recently CDC48/VCP has been shown to act as a cell death effector molecule in brain, suggesting that its concentration is critical for neurodegeneration (Higashiyama et al. 2002). A comparison of apoptotic phenotypes in yeast and mammals is shown in Fig. 3. We will describe below, how the study of yeast apoptosis might also provide insight for yeast and mammalian ageing research.

4.6 The senescent phenotype in yeast includes diagnostic markers of apoptosis

We are using the term “ageing” for a gradual process over many cell generations, while the term “senescence” is used for the terminal state of aged cells in their last one or two cell cycles when a sharp decline in vitality is observed. Terminally senescent mother cells of yeast (Nestelbacher et al. 1999; Fig. 1c) can display dramatic changes in appearance and physiology, compared to young cells (Fig. 1a). Some of these changes are strikingly reminiscent of the changes seen in senescent endothelial cells (or the more commonly used fibroblasts) in culture.

Both the clonally aged HUVEC and the senescent yeast mother cells are much larger than young cells. Also, cytoskeletal abnormalities, especially in the actin cytoskeleton, are observed in both types of senescent cells (unpublished results, Fig. 4). Senescent yeast cells create internal oxidative stress (Laun et al. 2001, see

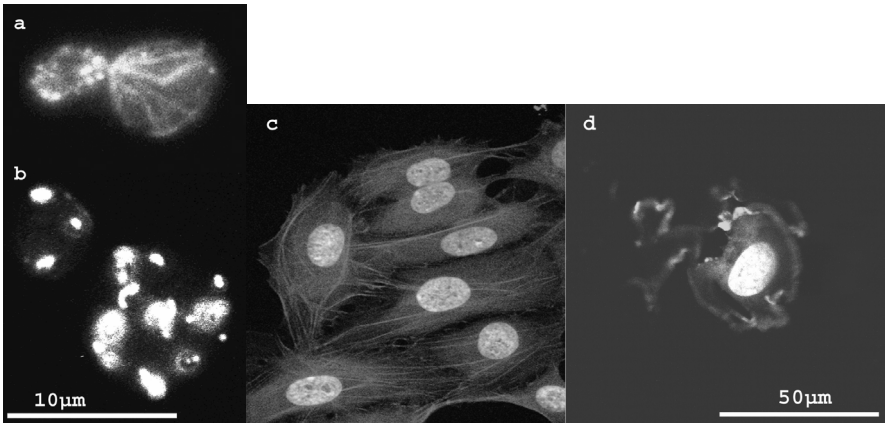


Fig. 4. a, b yeast cells, strain JC482 (unpublished data of A. Pichova); c, d HUVEC (unpublished, courtesy of G. Pfister and G. Wick). The fluorescent stain is specific for actin; in the HUVEC, nuclei were stained with DAPI. a: The typical dots and cables appearance of a young yeast cell with a small daughter cell. b: An old mother cell with her daughter in the upper left. The actin cytoskeleton is collapsed to a number of cytoplasmic patches. The two yeast cells are shown at the same magnification. c: Spindle shaped adherent young HUVEC. The actin cytoskeleton shows typical fibres. d: An old HUVEC in the process of detaching from the substrate. The larger part with the well-stained nucleus is rounded and detached and the smaller part of the cell (upper right) is still adherent. The cytoplasmic actin cytoskeleton looks patchy. c and d are shown at the same magnification.

also the next section) and consequently display a patchy actin cytoskeleton instead of the usual dots and cables picture (Fig. 4). Senescent HUVEC are not spindle-shaped but rather flat and spread out (G. Pfister, pers. comm.). A fraction of them display markers of apoptosis including markers of internal oxidative stress (Wagner et al. 2001). Those cells also display patchy actin cytoskeleton and start to detach from the glass surface (Fig.4). Nuclear chromatin stained with DAPI appears more diffuse than in young cells (Laun et al. 2001; Pichova et al. 1997). Frequently, we see cells without nucleus or with more than one nucleus due to nuclear fragmentation or, possibly, endomitosis. Perhaps as a consequence of the irregular cytoskeleton, the cell surface (plasma membrane) of HUVEC looks "folded" or shrunken and a similar picture is seen in yeast cells (transmission electron microscopy, our own unpublished data), and even the cell wall of senescent yeast cells looks folded (Fig.1c). When lifespans are determined by micromanipulation, it is consistently observed that cells that approach senescence need much longer to perform a cell division cycle. The same is true for cultured human cells.

In order to observe biochemical and physiological changes in senescent cells, a method for enriching these old cells is also needed. This is no problem with cultured human cells (all of them senesce when the Hayflick limit is reached), but it is a major problem with yeast cells (see the preceding section of this review).

Using gradient purified old yeast mother cells, a slowing down of protein synthesis was observed in these cells (Egilmez et al. 1990).

The biochemical markers of oxidative stress-induced damage and of apoptosis will be discussed now.

Through recent research, a number of environmental situations that trigger yeast apoptosis and new genes that are involved in the process were elucidated. Most of them share human orthologues, so that the AAA protein Cdc48p did not remain the only apoptotic gene conserved between yeast and mammals.

Another AAA protein with a probable role in yeast apoptosis is the mitochondrial protease Yme1p. Expression of Bax leads to an apoptotic phenotype in yeast (Ligr et al. 1998) and activates Yme1p, resulting in a degradation of cytochrome c oxidase subunit 2 (Cox2p). The absence of Yme1p significantly delays Bax-induced cell death (Manon et al. 2001). In another publication, Manon and colleagues could show that Bax expression induces release of cytochrome c from mitochondria, a hallmark of mammalian apoptosis (Manon et al. 1997). Also UTH1, a gene possibly involved in autophagic death, is needed for Bax mediated killing in yeast (Camougrand et al. 2003). UTH1 is a good example for the close functional interrelationship between ageing (Austriaco 1996), apoptosis (Camougrand et al. 2003), oxidative stress defence (Bandara et al. 1998) and regulation of mitochondrial biogenesis (Camougrand and Rigoulet 2001; Camougrand et al. 2000) in yeast, because the gene was identified independently as a player in all of these processes. UTH1 seems to be a fungal-specific gene, as close sequence homologs are known in *S. pombe* and other fungi, but not in mammals.

Recently, Pavlov et al. (2001) found a novel high conductance channel of mitochondria. The activity of this channel is Bax dependent and is absent in mitochondria from yeast cells overexpressing antiapoptotic Bcl-2. The pore diameter inferred from the largest conductance state of this channel is approximately 4 nm, sufficient to allow diffusion of cytochrome c and even larger proteins. This channel was named mitochondrial apoptosis-induced channel and is a candidate for the outer membrane pore through which cytochrome c and possibly other factors exit mitochondria during apoptosis. The new conductivity channel was characterized only with biophysical methods. It is probably not structurally related to the previously described permeability transition pore, one component of which is the adenine nucleotide translocator of the inner mitochondrial membrane, which is believed to span both the outer and inner mitochondrial membrane and to be involved in cytochrome c release in apoptosis (Halestrap et al. 2002).

However, mitochondrial function is probably not needed for Bax mediated cell death in yeast as Kissova et al. (2000) found that Bax killed respiring wild type cells as well as a respiratory-deficient mutant lacking mitochondrial DNA (mtDNA) and anaerobically-grown yeast cells. Possibly, Bax kills yeast cells through mitochondria-dependent and independent pathways. Poliakova et al. (2002) demonstrated that the antiapoptotic protein $Bclx_L$ prevents the cytotoxic effect of Bax, but not Bax-induced formation of reactive oxygen species, in Kluyveromyces lactis, a yeast which, although not congeneric, is closely related to *S. cerevisiae*. The authors suggest that in *K. lactis* cells expressing Bax, ROS (reactive oxygen species) are not the *sine qua non* of cell death and that the antiapoptotic function of $Bclx_L$ is not limited to its antioxidant property.

Deletion of ASF1/CIA1, a gene coding for a histone chaperone, also results in yeast apoptotic cell death, following an arrest at the G2/M transition. Moreover reduction of the mitochondrial membrane-potential, dysfunction of the mitochondrial proton pump, and release of cytochrome c to the cytoplasm was observed (Yamaki et al. 2001). The human homologue of Asf1p/Cia1p, CIA, interacts with the largest subunit of TFIID, CCG1, which is involved in the regulation of apoptosis (Sekiguchi et al. 1995).

To identify genes whose function can be substituted by $Bclx_L$,Vander Heiden and colleagues (2002) performed a genetic screen in yeast. *S. cerevisiae* grows primarily by glycolysis when glucose is available, switching to oxidative phosphorylation when carbohydrate in the media becomes limiting during diauxic shift. Given that $Bclx_L$ appears to facilitate the switch from glycolytic to oxidative metabolism in mammalian cells, Vander Heiden et al. tested a library of yeast mutants for the ability to efficiently undergo diauxic shift in the presence and absence of $Bclx_L$. They identified several mutants that have a defect in growth when switched from a fermentable to a nonfermentable carbon source that is rescued by the expression of $Bclx_L$. These genes include the mitochondrial chaperonin TCM62, as well as uncharacterised genes. One of these uncharacterised genes, SVF1, promotes cell survival in mammalian cells in response to multiple apoptotic stimuli. The finding that TCM62 and the analogous human prohibitin gene also inhibit mammalian cell death following growth factor withdrawal implicates mitochondrial chaperones as regulators of apoptosis in yeast and mammals.

But what about the major mammalian killers in the apoptotic scenarios, the caspases?

We found that a caspase-related protease (Yca1p) mediates apoptosis in yeast (Madeo et al. 2002). Yca1p is processed in a caspase-typical manner and has proteolytic activity for caspase substrates. Overexpression of *YCA1* in synergy with oxidative stress efficiently triggers yeast cell death accompanied with markers of apoptosis. Conversely, *YCA1* disruption increases resistance against hydrogen peroxide-induced apoptosis.

Both, mother cell-specific ageing (see above) and chronological (post-mitotic) ageing in yeast cells lead to apoptosis, including DNA-fragmentation, externalisation of phosphatidylserine and generation of oxygen radicals (our unpublished data). Disruption of *YCA1* protects part of the culture from this ageing-induced cell death. *YCA1* belongs to the family of metacaspases described by Uren et al. (2000), which encompass members in fungi, plants, and protists. Indeed, observations of caspase activities have also been made in plants and protists. Woltering and colleagues (2002) could induce apoptosis accompanied with a burst of oxygen radicals in tomato cells by camptothecin, a classical apoptosis-inducing drug used in cancer therapy. Both camptothecin-induced cell death and the release of oxygen radicals were effectively blocked by application of caspase inhibitors (de Jong et al. 2002; Woltering et al. 2002). Hydrogen peroxide induces apoptosis-like death and caspase activity also in the protist pathogen *Leishmania donovani*. Again, these effects can be efficiently blocked by caspase inhibitors (Das et al. 2001) indicating that these effects may be due to *Leishmania metacaspases*.

Observations pointing to an apoptotic phenotype have also been made in *S. pombe*: The SpRad9 protein contains a stretch of amino acids with similarity to the Bcl-2 homology 3 death domain, which is required for SpRad9 interaction with human Bcl-2. Overexpression of Bcl-2 in *S. pombe* inhibits cell growth independently of *rad9*, but enhances resistance of *rad9*-null cells to methyl methanesulfonate, UV light, and ionizing radiation. The authors suggest that SpRad9 may represent the first member of the Bcl-2 protein family identified in yeast (Komatsu et al. 2000).

Ceramides and sphingosines are lipids which regulate apoptosis in mammals and possibly also in yeast. Some mammalian growth modulators including tumour necrosis factor alpha induce apoptosis or cell cycle arrest via ceramide which activates a specific protein phosphatase (Kishikawa et al. 1999). Ceramide induced G1 arrest of *S. cerevisiae* is also mediated via activation of a protein phosphatase (Nickels and Broach 1996). Sphingosines have recently been shown to induce apoptosis in another fungus, *Aspergillus nidulans*. The apoptotic phenotypes were dependent on mitochondrial function and protein synthesis, but independent of caspase activity and reactive oxygen species formation (Cheng et al. 2003).

4.7 Oxidative stress links ageing to apoptosis in yeast

ROS are well established as inducers of mammalian apoptosis, particularly during neurodegeneration. Treatment with low doses of H_2O_2 induces the apoptotic cascade in mammalian cell cultures. In addition, in neural cells deprived of nerve growth factor or potassium, ROS produced by the cell act as late signals of the apoptotic pathway, downstream of the action of Bax and caspases (Schulz et al. 1996).

In *S. cerevisiae*, exposure to low doses of H_2O_2 or oxidative stress by glutathione depletion induce apoptosis (Madeo et al. 1999). Inhibition of translation by cycloheximide prevents development of apoptotic markers in response to H_2O_2, indicating an active role of the cell in the death process.

Generation of intracellular ROS occurs during yeast apoptosis even in the absence of external oxidative stress. Yeast expressing Bax or $cdc48^{S565G}$ mutant cells are strongly stained after treatment with dihydrorhodamine 123, indicating positive intracellular redox potential and, possibly, an accumulation of ROS. The change in redox potential and/or the radicals appear to be necessary to induce the apoptotic phenotype in most of the scenarios, putting ROS at a central position in yeast apoptosis (Madeo et al. 1999). The toxic effect of Bax in yeast cells might be due to mitochondrial lipid peroxidation (Priault et al. 2002).

In mammalian cells, inhibition of proteasome-dependent proteolysis leads to either induction or repression of apoptosis, depending on the proliferative status of the cells. It has been suggested that in exponentially growing cells proteasomes continuously degrade an activator of apoptosis (Drexler 1997). To characterize which role the proteasome plays in yeast apoptosis, Ligr et al. (2001) screened for proteasomal substrates and found six genes which by overexpression in a

proteasome-deficient background trigger apoptosis. One of the genes, *SAR1*, is required for vesicular transport from ER to Golgi, hence is involved in a similar process as *CDC48*. Another one is *STM1*, a DNA-binding protein involved in DNA repair. *STM1* knock-out strains show enhanced resistance to oxygen stress. Ligr et al. suggest that Stm1 protein is an activator of apoptosis triggered by exposure of cells to low concentrations of H_2O_2.

The phosphoinositide 3-OH kinase (PI3K)/protein kinase B (PKB) signalling pathway has been shown to prevent apoptosis in a variety of mammalian cells. Jeon et al. (2002), could demonstrate clearly that phosphoinositide 3-OH kinase/protein kinase B inhibits apoptotic cell death induced by reactive oxygen species in yeast.

Finally, disruption of yeast caspase *YCA1* causes enhanced resistance to H_2O_2 (Madeo et al. 2002), linking the scenarios of oxygen stress-induced apoptosis to a "classical" apoptotic gene.

Another form of external stress resulting in apoptosis has been described by Ludovico et al. (2001). Yeast dies after treatment with low doses of acetic acid, showing markers of apoptosis. Induction of apoptosis by acetic acid probably functions via generation of oxygen radicals (Ludovico et al. 2002). Moreover, Ludovico et al. (2002) could demonstrate that cytochrome c is released in wild type cells after stimulation with acetic acid. Consistently, acetic acid-induced apoptosis is prevented in cells unable to synthesize cytochrome c and in respiratory-deficient cells.

Severin and Hyman (2002) demonstrated that mating type pheromone at a ten times higher concentration than needed to induce shmooing, induces apoptosis in yeast thus connecting the mating process of yeast to cell death processes. Pheromones induce apoptosis as documented by DNA-fragmentation and generation of oxygen radicals. Disruption of both genes coding for isoforms of cytochrome c prevents the cells from alpha factor induced apoptosis. Likewise, rho-zero cells do not undergo alpha-factor induced apoptosis. These results suggest that alpha-factor activates the mitochondrial apoptotic pathway (Severin and Hyman 2002). Recently, Mazzoni et al. (2003) demonstrated for the first time a connection between mRNA metabolism, oxygen stress and apoptosis in yeast. They demonstrated that the absence of factors involved in mRNA decapping such as LSM4, trigger apoptosis in yeast.

Cabiscol and colleagues (2002) could demonstrate that resistance to oxidative stress, and the labile iron pool are closely connected in yeast. Their results indicate that the role of Hsp60 in oxidative-stress defence is explained by protection of several Fe-S protein thus preventing the release of iron ions from these proteins and thereby averting further damage (Cabiscol et al. 2002). Free iron ions released from Fe-S protein might induce apoptosis in yeast by producing highly reactive hydroxyl radicals through the Fenton reaction. This might be a crossover between apoptosis and ageing, as it is known that, for instance, in aged rat brain the concentration of free iron ions increases leading to oxidative damage of lipids and, ultimately to cell death. These effects are effectively prevented by dietary caloric restriction (Choi et al. 1998).

Table 1. Endogenously and exogenously induced scenarios of yeast apoptosis

Scenario	Explanation	Reference
Endogenously induced yeast apoptosis		
point-mutation of CDC48 ($CDC48^{S565G}$)	AAA-ATPase essential for protein degradation vesicular fusion	Madeo et al. (1997)
deletion of ASF1/CIA1	Asf1p in a histone chaperone apoptosis induced following G2/M arrest	Yamaki et al. (2001)
overexpression of Yca1p	Yca1p is a metacaspase in *S. cerevisiae*	Madeo et al. (2002)
mother cell-specific ageing	ageing of single cells	Laun et al. (2001)
chronological (post-mitotic) ageing	ageing of the whole culture	Madeo, unpublished results
ceramide	induced G1 arrest of *S.cerevisae*	Nickels and Broach (1996)
overexpression of Sar1p in a proteasome-deficient background	Sar1p functions in vesicular fusion	Ligr et al. (2001)
overexpression of Stm1 in a proteasome-deficient background	Stm1p, a DNA-binding protein involved in DNA repair	Ligr et al. (2001)
phosphoinositide 3-OH kinase/protein kinase B	inhibits apoptosis induced by reactive oxygen species	Jeon et al. (2002)
mating pheromone in high concentration	prevented in cells unable to synthesize cytochrome c and in rho-zero cells	Severin and Hyman (2002)
deletion of LSM4	mRNA decapping factor	Mazzoni et al. (2003)
free iron ions released from Fe-S proteins	e.g. caused by deletion of Hsp60	Cabiscol et al. (2002)
degradation of Cdc6p	induced by the DNA damaging drug adozelesin may cause apoptosis through a uncoupling of DNA replication and mitosis	Blanchard et al. (2002)
inactivation of Cdc13p	results in abnormal telomeres depends on MEC1, a DNA-double strand break repair protein	Qi et al. (2003)
Exogenously induced yeast apoptosis		
Bax overexpression	apoptosis is prevented in absence of Yme1p (mitochondrial AAA-ATPase), Uth1p or enhanced vesicle trafficking	Ligr et al. (1998) Manon et al. (2001) Camougrand et al. (2003) Levine et al. (2001)
Bcl-2 overexpression	prevents cell death induced by Bax overexpression or by chronological ageing	Longo et al. (1997)
low doses of H_2O_2	oxygen stress	Madeo et al. (1999)
glutathione depletion	oxygen stress	Madeo et al. (1999)

Table 1. Continued

Scenario	Explanation	Reference
low doses of acetic acid	cell death is prevented in cells unable to synthesize cytochrome c and in respiratory-deficient cells	Ludovico et al. (2002) Ludovico et al. (2001)
osmotin	protein involved in plant defense depends on the RAS2/cAMP pathway	Narasimhan et al. (2001)
UV-irradiation	UV-dose dependent increase of the sub G1 population	Del Carratore et al. (2002)

In Table 1 we are summarizing the presently known conditions under which apoptosis is induced in yeast cells.

The involvement of the toxic side products of respiratory oxygen metabolism in ageing and in a number of human diseases was considered by Harman (Harman, 1956; for review see Harman 1998) when he formulated the "free radical theory of ageing" that also became known as the "oxygen theory of ageing" (Ames et al. 1993; Halliwell and Gutteridge 1999). Essentially, the theory states that one electron reduction of oxygen occurs relatively frequently in the respiratory chain (at the bc1 segment of electron transport) as a side product. This process yields superoxide radical, which is not in itself extremely reactive, but gives rise to ROS as follow-up products, which are toxic and mutagenic. One of the most important ROS is the OH radical that avidly reacts with DNA (producing 8-oxo-guanine, which is mutagenic), proteins (producing protein carbonyls), lipids, and other biomolecules. All these reactions have been implicated in ageing (Halliwell and Gutteridge 1999). Another important follow-up product of ROS, 4-hydroxy-2-nonenal, a product of peroxidation of multiply unsaturated fatty acids, was studied intensively in higher organisms, but apparently does not occur naturally in yeast cells which do not synthesize multiply unsaturated fatty acids. However, 4-hydroxy-2-nonenal is toxic for yeast cells. The eukaryotic cell has evolved a number of parallel and partly overlapping detoxification pathways for ROS. We are mentioning here the superoxide dismutase/catalase system that removes superoxide and hydrogen peroxide and the glutathione cycle (Grant et al. 1998) using reducing equivalents originating from NADPH to detoxify hydrogen peroxide and organic peroxides.

The oxygen theory of ageing is the only one out of a large number of ageing theories (not all of them mentioned in this review) for which experimental evidence has been supplied in all major model systems of ageing (Halliwell and Gutteridge 1999). The major facts are mentioned here briefly without going into detail, followed by a detailed discussion of yeast. ROS production in mitochondria increases with age and so do biochemical markers of oxidative damage in rodents, in flies, and in human cells. This is true at the organismic level and also at the cellular level in cultured cells reaching the Hayflick limit (Halliwell and Gutteridge 1999; Sitte et al. 2000). Especially in rodents, antioxidants in the diet improve survival and cognitive performance (Floyd 1991; Harman 1998). Overexpression of catalase and superoxide dismutase increases lifespan, and mutants selected for

longevity without selecting for oxidative stress resistance, nevertheless did show oxidative stress resistance in *Drosophila* (Arking et al. 2000; Orr and Sohal 1994; Sohal and Weindruch 1996). Perhaps the most striking fact in this connection is that it was found relatively recently that the most important long-lived mutants in *C. elegans* (those in the insulin-like growth factor signalling pathway) completely depend on functional defence against oxidative stress (Gems 1999; Taub et al. 1999). Restoring the insulin like growth factor signalling pathway in neurons was sufficient to restore the wild type lifespan, pointing to the fact that in higher organisms the ageing zeitgeber might be located in certain differentiated tissues, most likely in neurons, dictating ageing of the organism (Wolkow et al. 2000). However, the latter result has been challenged by subsequent work (C. Kenyon, pers. comm.) and was retracted by the authors (Taub et al. 2003).

In yeast, conditions that increase the load of the cells with ROS invariably decrease the replicative lifespan. This was shown by disrupting the two yeast catalase genes (Nestelbacher et al. 2000; Fig.5), by disrupting Cu/Zn-SOD (Barker et al. 1999), and by disrupting both yeast *SOD* genes, the mitochondrial Mn-SOD and the cytoplasmic Cu/Zn-SOD (Wawryn et al. 1999). Both *SOD*-disruption mutations reduced the lifespan and the effect was additive (Wawryn et al. 1999). Increasing the partial pressure of oxygen decreased the lifespan (Nestelbacher et al. 2000). Conversely, the biological antioxidant, reduced glutathione (1mM) in the growth medium used for lifespan determination restored the lifespan of catalase mutant strains to the normal one observed under standard conditions (Nestelbacher et al. 2000; Fig.5). Glutathione is the major "redox buffer" of the cell (Schafer and Buettner 2001) and is known to be taken up efficiently by yeast cells.

Using a method developed in our laboratory for the enrichment of senescent (terminal) yeast mother cells, we investigated cytological markers of oxidative stress and of apoptosis in senescent cells and, for comparison, in virgin daughter cells that had undergone the same elutriation procedure (Laun et al. 2001). Care was taken not to apply any external oxidative or starvation stress to these cells. Of course, atmospheric oxygen was present during workup. The remaining median lifespan of fraction V was only 3 generations as compared to the normal median lifespan of 22 generations in the virgin cells (Fig.2). About one third of the cells in the large cell fraction (fraction V) did enter a new cell cycle in the lifespan experiment, but did not complete it. Also one third of these cells showed inversion of the plasma membrane (annexin V test), diffuse nuclear chromatin, accumulation of DNA strand breaks (TUNEL test; Fig.6), and intense staining with dihydrorhodamine 123 (DHR) indicating a sufficiently positive redox potential in the cell to oxidize DHR (Fig.7). We conclude that these old wild type cells developed internal oxidative stress in the absence of any external stressors, just as the consequence of ageing. The morphology of the subcellular structures staining positively with DHR was clearly mitochondrial and no such staining was seen in young cells. As a control, the same cells were also stained with DASPMI, a mitochondrial specific fluorescent stain that does not depend on the redox potential (Fig. 7). Although these experiments are still not conclusive to prove a causal role for mitochondrial ROS in ageing, we hypothesize that the build-up of the markers of oxidative stress and apoptosis occurs only in senescence and during the last

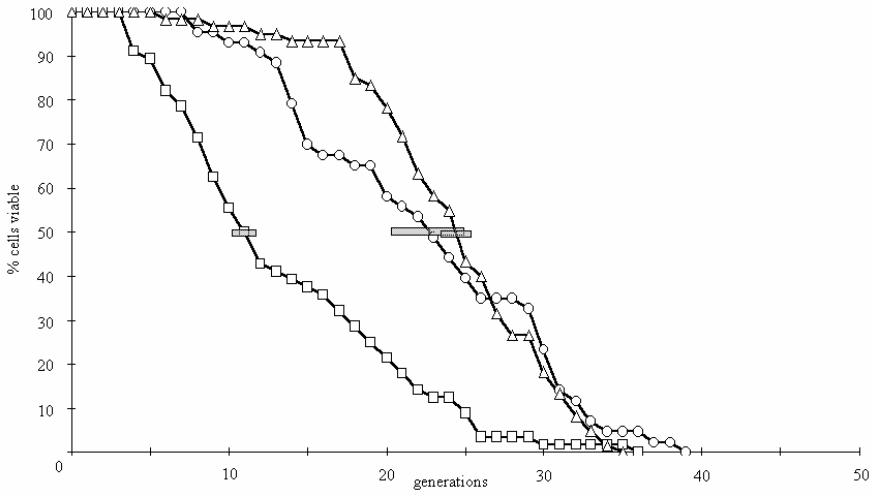
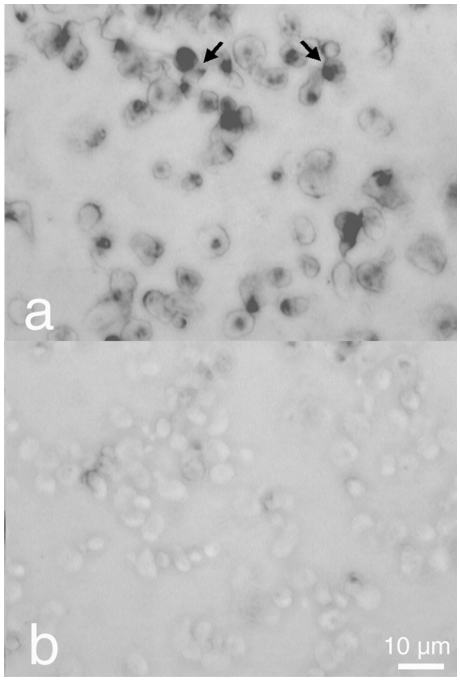


Fig. 5. Yeast strain W303 MATa, *ctt1::URA3*, *cta1::URA3*. Circles: ambient air, squares: 55% oxygen, triangles: 55% oxygen, 1mM GSH. The complete lack of catalase causes a large decrease in lifespan (in 55% oxygen), which is reversed to a normal lifespan by adding 1mM in the medium. The two curves for ambient air and for 55% oxygen plus 1mM GSH are not significantly different by applying the Wilcoxon non-parametric test. Source: Nestelbacher et al., Exp Gerontol 35:63-70 (2000), with permission of the publisher.

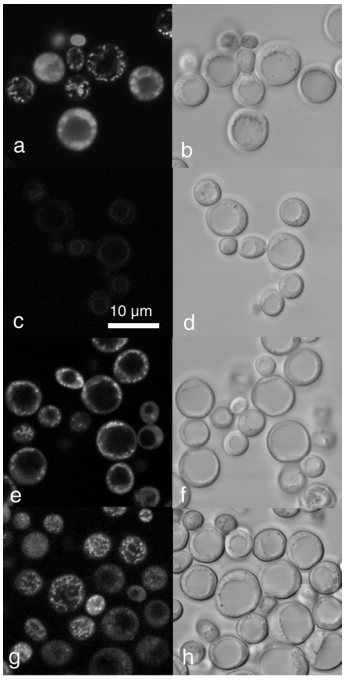
uncompleted cell cycle and is mediated through an active genetically controlled mechanism in response to still unknown signals. These signals could be triggered by the accumulation of a "death factor" in the mother cell, but not in the daughter cell. As to the nature of the death factor, only speculation is presently possible. It could be one or more radical species (superoxide) or simply the damaged cellular material. Whatever it is, it must have the capacity to create a signal in the cell and to be inherited very asymmetrically between mother and daughter. One example was given recently, showing that such asymmetric inheritance of damaged material is indeed possible. Immune fluorescence staining of mother/daughter cell pairs for protein carbonyls showed that the oxidized (carbonylated) proteins stay in the mother and are not transmitted to the daughter cell. The majority of the protein carbonyls are located to mitochondria (Aguilaniu et al. 2003).

A cautionary note is in order here: It is possible that DHR in our experiments is oxidized in the cytoplasm or at the plasma membrane of the cells and then moves to the mitochondria. Even staining with rhodamine, which does not need oxidation in the cell, stains mitochondria. Experiments addressing this important question are under way. Some recent experiments with yeast *RAS2* mutants would point to the fact that a non-mitochondrial mechanism for ROS production is feasible.

Experiments performed with respiratory-deficient rho-zero mutants and with anaerobiosis also can clarify the role of respiration in ageing. Naively, one would assume that by eliminating the respiratory chain, as is the case in rho-zero mutants, which depend on ATP produced in the glycolytic metabolism, would also



Left panel: Fig. 6. Yeast strain: JC482. TUNEL staining for DNA strand breaks. a: Fraction V old mother cells that were to about 30% in their final cell cycle and had entered apoptosis. Arrows mark mother daughter pairs that were both TUNEL-positive. b: Fraction II virgin cells of the same strain. TUNEL-staining is practically absent. Source: P. Laun et al., Mol Microbiol 39:1166-1173 (2001), with permission of the publisher.



Right panel: Fig. 7. Yeast strain: JC482. a, c fluorescent stain with DHR, e, g fluorescent stain with DASPMI, b, d, f, h phase contrast. a, b fraction V old yeast mother cells. Positive DHR stain indicates a positive internal redox potential. The stain is mitochondrial as shown by comparison with e and g. c, d fraction II virgin cells. DHR-positive cells are absent. e, f fraction V old mother cells. e: stained with DASPMI to show mitochondria. g, h young virgin cells. g: stained with DASPMI to show mitochondria. Source: P. Laun et al., Mol Microbiol 39:1166-1173 (2001), with permission of the publisher.

eliminate oxygen toxicity. However, this is not true. There are also cytoplasmic oxygen-dependent reactions that could give rise to oxygen toxicity (Rosenfeld et al. 2002). Rho-zero cells are DHR-positive under conditions where rho-plus cells are not, and the GSH/GSSG equilibrium of rho-zero cells is shifted towards a positive intracellular redox potential. Rho-zero strains are hypersensitive to chemicals inducing oxidative stress (our own unpublished observations, collaboration with the group of Ian Dawes). A likely explanation of these results is that oxygen toxicity arising from non-mitochondrial reactions plays a role in determining oxidative stress defence and lifespan in these strains.

The lifespan of rho-zero cells has been measured and compared to isogenic wild type cells by a number of laboratories (Berger and Yaffe 1998; Kirchman et al. 1999; Powell et al. 2000; and our own unpublished observations). The lifespan of rho-zero cells was found to be shorter than that of the corresponding wild type cells. In a widely used strain, W303, the median lifespan is 22 generations for wild type and 17 generations for the corresponding rho-zero strain (our own unpublished observations). Results with other strains are similar. However, in one case (Kirchman et al. 1999) a strain was described in which the lifespan is longer for the isogenic rho-zero derivative. This result is discussed in terms of the retrograde response (Jazwinski 2002; Small et al. 1995). The retrograde response in non-respiring yeast cells leads to a global metabolic shift, characterized, for instance, by a shift from the mitochondrial Krebs cycle to the peroxisomal glyoxylate cycle, which is conveniently monitored by measuring expression of *CIT2*, the citrate synthase isoenzyme characteristic for the peroxisome. The authors hold the view that in those strains where the retrograde response is sufficiently strong, the rho-zero strain has a longer lifespan compared to its rho-plus counterpart (see also the review written by Jazwinski, this volume).

4.8 Ageing and the *RAS* genes

Please consult Jazwinski (this volume) for a detailed treatment of this topic.

In yeast, the two homologs of the ras proto-oncogenes of higher organisms are *RAS1* and *RAS2*. Deletion of both genes is lethal, while deletion of *RAS1* has little effect and deletion of *RAS2* leads to inability to grow efficiently on non-fermentable carbon sources (Tatchell 1986; Thevelein and de Winde 1999). The two RAS genes were found to be differentially expressed during replicative ageing of yeast cells. Deletion and overexpression experiments showed antagonistic effects of the two genes on the replicative lifespan (Sun et al. 1994). Overexpressing the *RAS2* gene increased the lifespan, while a deletion of RAS2 slightly decreased the lifespan. The major and well-documented signal transduction pathway involving the yeast RAS genes is the well known RAS/cAMP/PKA pathway which controls entry into a new cell division cycle in response to nutrient availability and also the cell's response to various stress conditions (Thevelein and de Winde 1999). For instance, expression of the yeast catalase T gene, *CTT1*, is under strict control by this pathway, in response to heat stress or osmotic stress (Bissinger et al. 1989). Stress response is probably the link between the *RAS2* gene and ageing (Jazwinski 2002). Further experiments (using a version of *RAS2* defective in its effector domain) led Jazwinski to postulate that the effect of *RAS2* to increase lifespan and to protect yeast cells from the effect of chronic heat shock on lifespan were independent of cAMP (Sun et al. 1994). Thereby, possibly defining a new regulatory pathway in which the Ras proteins might be involved.

In yeast, the dominant "oncogenic" point mutation $RAS2^{gly18,val19}$ was extensively investigated which is homologous to the human Ha-ras-val12 mutation. Despite the fact that in tumours this mutation is frequently found, introducing this

mutation in isolation into human cultured cells leads to premature ageing and apoptosis (Serrano et al. 1997). This effect depends on oxygen; lowering the atmospheric oxygen completely prevents apoptosis and ageing under these conditions (Lee et al. 1999). This points to the fact that the effect of the dominant ras mutation might be through cell-internal creation of oxidative stress. We found (Pichova et al. 1997) that yeast *RAS2*^{gly18,val19} cells have a dramatically shortened lifespan which is partly restored by adding 1mM GSH to the growth medium (G. Heeren, unpublished). The elutriated fraction V cells of this strain display the same markers of apoptosis and oxidative stress as wild type cells, however, senescence is reached much earlier than in wild type (Pichova, unpublished). Exponentially growing cells of *RAS2*^{gly18,val19}, of wild type, of the *ras2::LEU2* deletion and of the corresponding rho-zero strains were investigated (Heeren, unpublished). Rho plus cells of the *RAS2*^{gly18,val19} strain showed a clear signal for superoxide radical anion in EPR (electron paramagnetic resonance) experiments. The corresponding rho-zero cells showed the same intensity of the superoxide signal, had the same extremely short lifespan and both strains showed strong staining with DHR in their mitochondria indicating a positive redox potential. As both, the superoxide-specific EPR signal and the positive intracellular redox potential are present even in rho-zero cells that have no functional respiratory chain, we hypothesize that extramitochondrial sources of oxidants are probably responsible for shifting the redox potential in *RAS2*^{gly18,val19} cells (unpublished data from our laboratory, collaboration with the group of Hans Nohl).

Recently, additional evidence for the involvement of RAS signalling in yeast apoptosis has been demonstrated. Bressan and colleagues showed that osmotin, a protein involved in the plant defence response induces apoptosis in yeast. Induction of apoptosis is correlated with intracellular accumulation of reactive oxygen species and depends on the RAS2/cAMP pathway (Narasimhan et al. 2001).

4.9 Caloric restriction and nutritional control of ageing

The elongation of lifespan by eating less (without serious malnourishment which of course shortens lifespan) was intensively studied in rodents (Finch and Ruvkun, 2001). It is debatable whether the manipulation of yeast growth media is comparable with caloric restriction in mammals. It should be noted that yeast lifespans should be determined on synthetic media, because complex media contain known and unknown substances that influence lifespan (for instance, GSH) in varying concentrations. The effects of low glucose, of different carbon sources, and of the reduction of amino acids in synthetic media was found to increase the lifespan. Furthermore, in this case, the phenomenon is strain-dependent.

Glucose repression of respiration and other metabolic pathways is a well-studied phenomenon in yeast that is highly strain-dependent. Several laboratories have found moderate effects of reducing the glucose concentration from the usual 2 per cent (these are repressing conditions in many strains) to the non-repressing 0.1 per cent, which resulted in an increase of lifespan (Jiang et al. 2000; Lin et al.

2000; our own unpublished results). The role which glucose repression might play in limiting lifespan was not studied in detail. In the same vein, the lifespan determined for a particular strain on 2 per cent glucose was compared to the lifespan determined on non-fermentable carbon sources. These carbon sources (ethanol, glycerol) lead to an increase in respiration. Their influence on lifespan is controversial and strongly depends on the strain used (Barker and Walmsley 1999; Muller et al. 1980; Van Zandycke et al. 2002; our own unpublished results).

Finally, a very strong influence on lifespan was found (Jiang et al. 2000) when in a strain prototrophic for amino acids, these amino acids which are normally contained in synthetic complete media, were left out. The elongation of lifespan measured under these conditions is big (2-fold). Here it could be argued that relieving the cells from nitrogen repression could be the mechanism, but further experiments addressed to this question have not been performed.

4.10 Ageing and accumulation of mutations, ERCs, and silencing

The accumulation of chromosomal mutations was discussed as a cause of postmitotic ageing in higher organisms (Finch and Ruvkun 2001; Halliwell and Gutteridge 1999). These mutations could be caused by the slow accumulation of ROS-induced damage. This kind of process is logically excluded for mother cell-specific ageing, since DNA replication is semi-conservative and the mother and daughter cell inherit the same chromosomal DNA sequence, and therefore, mutations. However, this kind of process is feasible for mtDNA. Of course, also mtDNA is replicated semi-conservatively. However, multiple copies of circular mtDNA exist in the cell, and only part of the mtDNA complement could be subject to accumulation of mutations. Moreover, it has been hypothesized that the process of mitochondrial mutation accumulation would lead to a vicious circle, in which mtDNA would be particularly prone to mutation because the ROS are believed to originate in mitochondria, and mutations in the mtDNA would lead to mutations in mitochondrially encoded components of the respiratory chain, which in turn would lead to further increase in mitochondrial mutations through increased ROS production. Such a picture, of necessity, would include the selective asymmetric distribution of damaged and undamaged mitochondria between mother and daughter cell. Preliminary evidence exists for such an asymmetric distribution of oxidatively damaged material between mother and daughter cell (Aguilaniu et al. 2003).

Loss of mitochondrial function in ageing cells of mammals (Ames et al. 1993) and yeast cells (Lai et al. 2002) has been demonstrated. However, overall, the hypothesis has never been rigorously tested.

A large number of papers deal with the role of extrachromosomal ribosomal DNA circles (ERCs) in yeast mother cell-specific ageing (Defossez et al. 1998; Guarente 2000; Sinclair and Guarente 1997). These authors originally described the ERCs as the cause of yeast ageing. ERCs arise as a product of recombination

between the rDNA repeats on chromosome XII of yeast. The resulting circular DNAs range from 1 repeat length of about 8.8 kb to several. They contain a functional origin of replication and in the cell division cycle behave like autonomously replicating plasmids without a centromere sequence. They therefore accumulate in the mother cell (as was shown previously for non-centromeric plasmids) and lead to enlarged nucleoli. Eventually, according to the theory put forward by the authors, the numerous ERCs titrate out the Sir2p (which binds to rDNA), leading to a deprivation of the sub-telomeric SIR complex, which is shown by the fact that sub-telomeric genes in these cells are not transcriptionally repressed like in young cells, but rather derepressed. This results in expression of the *HML* and *HMR* loci and in sterility of haploid aged cells. However, the titrating out of Sir2p also causes other more severe metabolic changes, like defects in chromosomal DNA replication, and leads to senescence. Several experimental results support the role of ERCs in ageing. For instance, the deletion of *FOB1* is a mutation which strongly suppresses recombination of rDNA (Defossez et al. 1999) and increases the median replicative lifespan of the strain by about 6 generations. Likewise, overexpressing *SIR2* increases the median lifespan (Kaeberlein et al. 1999). However, there are also serious difficulties with the ERC theory. The cells used for measuring the amount of ERCs were isolated by the magnetic bead method and were presumably still far from senescence. No adequate statistics was presented concerning the correlation of enlarged nucleoli with an increase of ERC DNA in young and old cells (Sinclair et al. 1997). Subsequent work showed (Kirchman et al. 1999) that under certain conditions (activation of the retrograde response) ERCs are massively accumulated although the lifespan is increased. It is important to note that in spite of intensive search ERCs were never found to be involved in ageing of higher cells or organisms (Guarente 2000).

The silencing protein, Sir2p, enhances silencing through its NAD-dependent histone deacetylase activity, by the same mechanism prevents ERC formation by repressing recombination of rDNA, and promotes longevity. *SIR2* is the founding member of a gene family now called the "sirtuin" family. It is highly conserved in eukaryotic cells and its involvement in ageing (and cancer) is being actively investigated presently (Bitterman et al. 2002; Rogina et al. 2002). It was hypothesized that *SIR2* might be the link between the nutritional status of the cell (represented by the available NAD) and chromatin silencing, not only in yeast (Guarente 2000). However, more recent research showed that the deacetylase activity of Sir2p is strongly inhibited by nicotinamide which might be the key metabolite regulating its activity (Anderson et al. 2003). In mammalian cells, seven members of the SIR2 family were identified, a function in ageing (but not in ERC formation) was found (Langley et al. 2002) and one member, SIRT3, was found to be located in mitochondria (Onyango et al. 2002) which could be significant given the involvement of mitochondria in ROS production and in programmed cell death.

4.11 Yeast ageing and telomeres

From about 1996 and onwards, the protein-coding genes coding for the catalytic (*EST2*) and accessory (other EST genes, and additional genes) subunits and the gene (*TLC1*) coding for the template RNA of yeast telomerase were identified. This in turn enabled the identification of the corresponding genes in higher organisms, using the genetic system of yeast (Shore 2001). These findings also enabled the workers in the field to rigorously test the telomere hypothesis of ageing, both in yeast and in higher organisms. In higher organisms (not to be discussed in detail here), the Hayflick limit of fibroblasts in the admittedly artificial situation of cell culture can indeed be increased by ectopically expressing telomerase and maintaining telomere length. These cells normally display progressively smaller telomeres when they clonally reach the Hayflick limit. The situation is, however, complicated by the fact that there is also at least one additional mechanism for maintaining telomere length by a process of recombination, and many immortalized cell lines, having escaped the crisis at the Hayflick limit, do not show telomerase activity. The mouse knock-out mutation for telomerase leads to the progressive shortening of telomeres but does not lead to organismic premature ageing. It leads, quite unexpectedly, after 6 generations with normal lifespan, to sterility and increased malignancies (Rudolph et al. 1999). In yeast, the loss of telomerase by deletion of *EST2* (or other essential subunits) leads to delayed mortality after about 70 cell generations (Lendvay et al. 1996). This kind of "senescence" is clonal (like the senescence of fibroblasts in culture) and clearly shows the essential function of telomeres for the yeast cell. However, this process of clonal ageing is not observed in wild type yeast and is, very probably, unrelated to the mother cell-specific ageing process of yeast cells. Old yeast mother cells isolated by the gradient method displayed a normal length of the telomeres (D'Mello and Jazwinski 1991).

SGS1 (slow growth suppressor 1) is a yeast gene interesting in several respects for the topic to be discussed here. It was discovered by Rothstein and co-workers (Gangloff et al. 1994) as a suppressor of a slowly growing topoisomerase mutant and subsequently shown to be needed for maintaining recombinational repair and the integrity of the rDNA repeats on chromosome XII of yeast. It also has a function in telomere maintenance in the absence of telomerase, i.e. it is involved in telomerase-independent telomere maintenance in certain yeast mutants that escape the "slow death" of the est2 deletion mutant. The gene sequence shows that *SGS1* is the only clear member of the RecQ family of ATP-dependent DNA helicases on the yeast genome. Its closest homologs on the human genome are the WRN and BLM DNA helicases. WRN is involved in a hereditary progeria (Werner's syndrome) while Bloom's syndrome presents with neurological symptoms and cancer predisposition, but not with progeria. Both genes were shown to be DNA repair helicases. The loss of sgs1 function leads to a drastically reduced mother cell-specific lifespan in yeast cells, which can be complemented by expressing the human BLM (but not WRN) cDNA in yeast cells deleted for sgs1 (Heo et al. 1999). For all of the above reasons, in particular because *SGS1* has a proven role in sup-

pressing excessive recombination of rDNA, the sgs1 mutant is an ideal test system for ERC hypothesis of ageing. However, the authors did not observe an increase of ERCs in the very short-lived sgs1 mutant or a decrease of ERCs after restoring the normal lifespan by expressing BLM. This shows that at least in the background and in the system tested by Heo et al. (1999), ERCs are not causative for mother cell-specific ageing. In another study, an increase in ERCs was found in an sgs1 deletion strain in a different genetic background (Sinclair et al. 1997). All this underlines the notion that yeast mother cell-specific ageing may be caused by a number of different and independent biochemical defects, depending on the strain background and the experimental parameters.

4.12 Yeast apoptosis, DNA-damage, and telomere structure

As we have shown above, the accumulation of chromosomal mutations cannot be causative for mother cell-specific ageing. Likewise, telomere shortening is not a cause of yeast mother cell-specific ageing (at least not in wild type cells). However, apoptosis in yeast, like in mammalian cells, is under control by the DNA-damage checkpoints. The intracellular signals, which are created by different types of DNA damage, lead to cell cycle arrest and enhance various repair processes, or alternatively, if the integrity of the genome is not restored within a certain time window, switch on the genetic program of apoptotic death. The biochemical nature of the relevant signals has only very recently started to be unravelled (Bakkenist and Kastan 2003). These topics are now briefly considered.

An apoptotic phenotype in yeast can be induced by DNA damage as indicated by a positive TUNEL test after UV irradiation. Interestingly, a UV dose-dependent increase in the sub-G1 population was found by flow cytometry. Sub-G1 cells were isolated by flow sorting and analysed by electron microscopy. This population showed condensed chromatin in the nucleus and cell shrinking (Del Carratore et al. 2002).

A p53-independent road to cell death which is conserved from yeast to mammals is the degradation of Cdc6p by a proteasome- and ubiquitin-dependent pathway induced by the DNA damaging drug adozelesin (Blanchard et al. 2002). Cdc6p is crucial for initiation of DNA-replication and for the coupling of DNA replication to mitosis. Apoptosis in proliferating mammalian cells is frequently associated with an uncoupling of DNA replication from mitosis due to the premature activation of mitotic cyclin-dependent kinases (for review see Guo and Hay, 1999). Destruction of Cdc6p by adozelesin may contribute to this uncoupling by abrogating checkpoints which require Cdc6p and other origin licensing proteins in yeast and mammals (Blanchard et al. 2002).

It is supposed today that the major part, if not all, human cancers cells display a defect in DNA-double strand break repair but do not undergo apoptosis as a consequence to unrepaired double strand breaks (for review see Rouse and Jackson 2002). Unrepaired double strand breaks are, therefore, potentially dangerous and

eukaryotic cells have developed efficient mechanisms to arrest the cell cycle and ultimately to trigger apoptosis, when double strand breaks occur. The potential induction of apoptosis after DNA-double strand breaks in yeast has not been investigated until recently. Qui et al. (2003), showed a connection between telomere length, DNA-double strand break repair and yeast apoptosis. Inactivation of the yeast telomere binding protein Cdc13p results in abnormal telomeres (exposed long G-strands) and activation of the DNA damage checkpoint. Inactivation of Cdc13p also induces apoptotic signals in yeast, as evidenced by caspase (Yca1p) activation, oxygen stress, and flipping of phosphatidylserine in the cytoplasmic membrane. These telomere-initiated apoptotic signals were shown to depend on *MEC1*, linking yeast apoptosis to one major signalling molecule involved in DNA-double strand break repair.

4.13 Why should a unicellular organism commit suicide? Physiological scenarios of yeast apoptosis are associated with the generation of oxygen radicals

The important role of ROS in the regulation of apoptosis may indicate origin and primary purpose of the suicide process. ROS occur during respiration in every aerobic organism. Because ROS are highly reactive and modify proteins, lipids and nucleic acids, ROS-induced cell damage is a frequent event. Cells have developed mechanisms to detoxify ROS and to repair oxidative damage, but this can only reduce, not completely prevent fatal cell damage. Most damaged cells will continue to metabolize for some time, even when they have lost the ability to proliferate. Or even worse: cells that suffer DNA-damage or have obtained mutations would proliferate, even if they endanger the genetic stability of the population. A rapid, active suicide of these cells would save metabolic energy for neighbouring cells, which, even in case of unicellular organisms, are mostly clonal relatives, genetically identical to the damaged cell. That way, suicide of a unicellular organism could provide an evolutionary advantage for its genome.

Higher eukaryotes evaluate cell damage (e.g., via the p53 system) to decide whether suicide is advisable. Before development of such a complex system, chemical reactivity of ROS themselves may have been used to trigger cellular suicide. To commit suicide in situations without external oxygen stress, cells developed mechanisms to produce these signals autonomously.

Chronological and replicative ageing might be physiological scenarios of yeast apoptosis as both are associated with the generation of oxygen radicals.

S. cerevisiae quickly lose viability in nutrient-depleted synthetic media (chronological ageing). Heterologous expression of Bcl-2, a human antiapoptotic protein delays the loss of viability (Longo et al. 1997). The cells on limiting resources indeed die with an apoptotic phenotype and an accumulation of ROS (Madeo, unpublished results). The source of the ROS is yet unidentified. As oxygen radicals are normal by-products of respiration, a specific modulation of the respiratory chain, potentially the release of cytochrome c, may have been devel-

oped to increase the output of ROS as needed. During a further refinement in the regulation of apoptosis, released cytochrome c itself became an apoptotic signal, perhaps in order to make the regulatory cascade less dependent on the redox state of the cell. With the development of multicellular organisms, a more flexible regulation of apoptosis became necessary, including responses to various external signals, resulting in additional regulatory steps upstream, downstream, or instead of ROS.

4.14 Genetic and genomic screens for yeast ageing mutants

In recent reviews (Jazwinski 2001; 2002), genes are listed that were published to be specifically involved in the ageing process. Many different biochemical functions are implicated showing that genetic studies in yeast have up to now not led to a simple picture for the causes or mechanisms of ageing. Similarly, genome-wide transcription studies have led to a very heterogeneous set of genes that are over- or underexpressed in old cells. This situation is somewhat different from the situation in *C. elegans*, where the genes constituting a main signalling pathway (the IGF1 pathway) give rise to the majority of long-lived mutants.

It makes sense to screen primarily for long-lived mutants, because as long as the field of yeast ageing is wide open (and it is!) we have to discover new and unknown cellular functions that will clarify ageing physiology. A long-lived mutant is a priori much more likely to shed light on the true ageing physiology, while short-lived mutants could reduce fitness in many different ways, which as a secondary effect could also shorten the lifespan of the cell.

It proved to be extremely difficult to set up a direct genetic selection for lifespan mutants. For such a system to work, one would have to purify very old mother cells to homogeneity. These cells after many generations could be plated out and only long-lived mutants would survive. However, because of the technical difficulties with these purifications (see section on preparation of old cells) there is presently no way for such a procedure.

Therefore, indirect methods were used. Jazwinski and co-workers (D`Mello et al. 1994; Egilmez et al. 1989) investigated genes corresponding to mRNA overexpressed in old cells and analysed by differential hybridisation. The *LAG1* (longevity assurance gene 1) was isolated using this method.

Another method that was widely used is pre-selection of mutants under stress conditions that kill the wild type cells and screening the stress-resistant mutants one by one for their lifespan. The stresses employed were starvation, heat, a combination of starvation and heat stress, and also oxidative stress using either one of a number of oxidants (menadione, hydrogen peroxide, tert-butyl hydroperoxide, diamide, etc.). A prominent example is the isolation of a mutant in the gene *SIR4* (Kennedy et al. 1995), coding for a member of the SIR complex of yeast. The mutant was described as semi-dominant, while the deletion of the gene caused a short lifespan. The phenotype of the mutant was pleiotropic, giving rise also to a co-

segregating mating defect that could be used to clone the gene. The implication of *SIR4* in ageing is intriguing, because also another member of the SIR complex (*SIR2*) plays a prominent role in ageing.

Finally, we want to present a method (S. Jarolim, work in progress), which could in principle improve previous methods because a large number of pre-selected mutants can be screened without doing individual lifespan determinations by micromanipulation. The method is based on a strain (Bobola et al. 1996) from the laboratory of K. Nasmyth. The strain is engineered to express a conditional mutant phenotype only on glucose. An essential gene (*CDC6*) is disrupted and another copy of *CDC6* is placed under the control of the mother cell-specific *HO*-promoter. Under these conditions, the essential *CDC6* gene is expressed only in the mother, not in the daughter cell. In addition, *CDC6* is present under control of the *GAL1/10* promoter. On glucose, the strain grows linearly (not exponentially) as long as the mother-cell-specific lifespan allows. All daughters produced under these conditions stop growing at the G2 phase of the cell cycle (as a mother cell with a large daughter). The final OD of the culture is therefore directly proportional to the number of cell division cycles that the strain has undergone. Placing 96 pre-selected mutants in a microtiter plate and monitoring the OD with an ELISA reader resulted in a simple lifespan measurement. The mutants were pre-selected on the oxidant molecules mentioned above and several hundred were tested for their lifespan on glucose, resulting in the isolation of some promising mutants which, at least on glucose, display about two-fold increase in lifespan.

Direct selection without pre-selecting resistance mutations proved to be not practicable, because the frequency of generation of mutants, many of them in the *ASH1* gene, influencing the regulation of the *HO*-promoter was too high. These mutants grew exponentially on glucose and were therefore easily recognized.

The only paper published at the time of writing covering genome-wide transcriptional analysis of ageing yeast cells is Lin et al. (2001). These authors compared transcript levels of wild type virgin cells and 7^{th} generation mother cells sorted on magnetic beads and find a metabolic shift away from glycolysis and towards gluconeogenesis in cells of the 7^{th} generation. This is further corroborated by investigating two deletion mutant strains, *sip2* and *snf4* under similar conditions. The mutation, which increases lifespan, *snf4*, shows a less pronounced shift towards gluconeogenesis, while *sip2* shows increased expression of gluconeogenetic genes and a shorter lifespan.

Work in progress in our laboratory aims at the genome-wide transcriptional analysis of senescent yeast mother cells. The large cell fraction V from our elutriation experiment that had been characterized in detail previously (Laun et al. 2001) was used for mRNA preparation. For comparison, the small, virgin, fraction II cells were used. Single stranded cDNA was prepared from these cells using established methods and the transcript levels corresponding to each one of about 6000 yeast genes were determined and compared between old and young cells by two different methods. In the first method, genomic filters supplied by J. Hoheisel (Hauser et al. 1998) and ^{33}P-labeling were used. In the second approach, using a different strain, the DNA-microarrays of Ontario Cancer Research corporation were used and equimolar mixtures of young and old cDNA labelled with Cy5-

CTP and Cy3-CTP, respectively, were applied. In each case, we obtained about 200 genes that were differentially expressed with a high quality measure (sum of those over- and underexpressed). More than 50 per cent of the genes found with the filter method were again found with the microarray method. The fact that the two sets of genes are not identical is for the most part explained by genes that could not be analysed on the filters because of lower data quality and also by strain differences. The gene set contains several genes with a known function in oxidative stress response, some known housekeeping genes and a large fraction of "orphan" (functionally unknown) genes. Testing oxidative stress resistance/hypersensitivity in deletion mutants corresponding to all of these genes resulted in a number of new genes involved in oxidative stress response. Determining lifespans in some promising cases is underway.

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Abbreviations

Cy5: FluoroLinkTMCy5 of Amersham Biochemicals
Cy3: FluoroLinkTMCy3 of Amersham Biochemicals
DAPI: Diamidinophenyl indole
DASPMI: 2-(4-(dimethylamino)styryl)-1-methylpyridinium iodide
DHR: Dihydrorhodamine 1,2,3
ELISA: enzyme-linked immuno-sorbent assay
EPR: electron paramagnetic resonance
ERC: extrachromosomal ribosomal DNA circle
FAMS: Fatigued Atheletes Muscle Syndrome
GSH: reduced glutathione
HUVEC: Human Umbilical Vein Endothelial Cell
IGF1: insulin-like growth factor 1
mtDNA: mitochondrial DNA

OD: optical density
ROS: reactive oxygen species
SOD: superoxide dismutase
TUNEL: TdT-mediated dUTP nick end labeling

5 Energy metabolism, anti-oxidant defense and aging in *Caenorhabditis elegans*

Bart P. Braeckman, Koen Houthoofd, Jacques R. Vanfleteren

Abstract

Food restriction and impaired gene function by mutation or RNAi treatment can extend the lifespan of *Caenorhabditis elegans* considerably. In contrast to the widespread belief, the antiaging action of these interventions is not due to a reduction of the rate of metabolism. Calorie restriction causes several alterations that are similar to those observed for the Ins/IGF mutants, but acts independently of this pathway. Life extension is associated with coordinated increases in superoxide dismutase and catalase activities in calorie-restricted worms and in mutants in the Ins/IGF transduction pathway. Mutation in any one of the *clk* genes does not result in a clear metabolic downregulation or upregulation of antioxidant enzymes. Lifespan extension in these mutants might be linked to their slow developmental rate during juvenile life, analogous to the effects caused by silencing of several genes with a mitochondrial function by RNAi treatment, and suggesting a regulatory system that makes various rates of juvenile life to persist during adulthood. The outcome would be slowing of a number of processes during adulthood, including aging.

5.1 Introduction

5.1.1 The biology of *Caenorhabditis elegans*

Caenorhabditis elegans (Rhabditida) is a terrestrial free-living nematode feeding primarily on bacteria. Natural populations consist predominantly of hermaphrodites. They produce both male and female gametes and can reproduce by self-fertilization. Low numbers of males also occur; they produce only spermatozoa and can fertilize hermaphrodites. The adult hermaphrodite is transparent, approximately 1.2 mm long and 50 μm wide. Like every nematode, the body plan of *C. elegans* is very simple and basically consists of two concentric tubes. The inner one is the alimentary canal (pharynx, intestine, and rectum) and the outer one has a protective (cuticle, hypodermis) and locomotory function (muscles, nerves). The space in between these two tubes, called pseudocoelom, is largely occupied by the gonads. In adult hermaphrodites, the bilobed gonad consists of two U-shaped arms of which one is extended anteriorly while the other is oriented posteriorly. The distal end of each arm contains the germ line nuclei. Along the tract of the ovi-

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duct, these cells undergo meiosis to form oocytes. When the oocytes pass along the spermatheca, they can be fertilized by amoeboid sperm cells. Finally, the zygote is protected by a vitelline membrane and a very tough chitinous eggshell. Usually the eggs reside in the oviduct for the next few hours and already start embryogenesis. Within 12 hours after the egg has been laid, a first stage larva hatches and starts to feed immediately when food is available. *C. elegans* is a filter-feeder, selectively eating bacteria by pharyngeal pumping. The larva develops rapidly over a total of four larval stages (called L1 to L4) that are separated by molts (Fig. 1). Under the old cuticle, a new one is synthesized and during a short period of lethargus (ceased pharyngeal pumping), the larva molts to the next developmental stage. During the L4 stage, gonadogenesis is completed and approximately 150 sperm cells are formed and stored in each spermatheca. After the final molt to adulthood, spermatogenesis is arrested and the gonads start producing oocytes. The haploid *C. elegans* gamete consists of 6 holocentric chromosomes (5 autosomes and one sex chromosome) each of similar size. In normal populations, almost all individuals are hermaphrodites and males occur at a frequency of only ~0.2%. Males arise spontaneously by X-chromosome nondisjunction during meiosis and therefore only contain one X chromosome. Males can be easily distinguished from hermaphrodites by their behavior and the shape of their tail, which is specialized for copulation and consists of spicules and a fan-like structure with sensory rays.

The life cycle of *C. elegans* is very short, taking approximately 2.5 to 3 days under optimal environmental conditions. The number of sperm cells produced during the L4 stage is a limiting factor on the progeny of an unmated hermaphrodite. Therefore, each hermaphrodite can lay about 300 self-fertilized eggs during the first 4 - 5 days of its adult life after which only oocytes can be produced. When mated by males, hermaphrodites can produce up to 1000 fertilized eggs. Under adverse conditions the L1 larva can molt to a predauer L2 larva (L2d) that, if harsh conditions persist, molts to a dauer larva. Dauer pheromone concentration (a measure of population density), food availability, and temperature are major factors playing a role in this developmental decision. The dauer larval stage is a special facultative diapause stage. Dauer larvae have a special morphology and an altered metabolism and they are resistant to a variety of stresses. Due to ceased pharyngeal pumping and a cuticular block of the buccal cavity, dauers are nonfeeding and rely on their internal fat stores for survival. Dauer larvae are very long-lived, often living four to eight times longer than adults. Dauer exit occurs when environmental conditions are favorable and a few hours after the commitment step, the animals molt to the L4 stage.

Caenorhabditis elegans can be maintained in a laboratory environment in several ways. The most common method is culture on agar plates seeded with *Escherichia coli*. The worms can also be cultured in monoxenic or axenic liquid media but under these conditions, they produce less offspring.

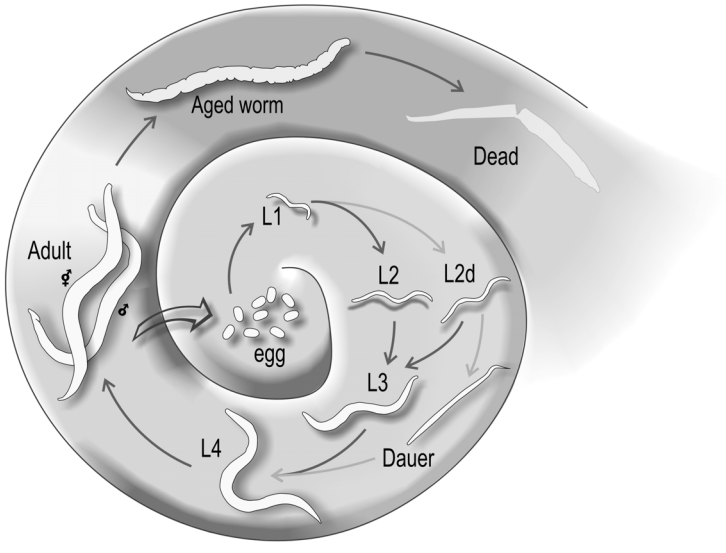

Fig. 1. Diagrammatic representation of the life cycle of *C. elegans*.

5.1.2 A massive amount of information

Dougherty, Nigon, and their colleagues provided the early information on the biology of the nematode model in the period between 1945 and 1965 (Dougherty 1960, Nigon 1965). In 1963, Sydney Brenner introduced *Caenorhabditis elegans* as a promising eukaryotic model in which to study fundamental problems of development and neurobiology. This species is a simple, yet multicellular, organism with a short life cycle. Because of its small size, *C. elegans* is very easy to culture in large numbers in a laboratory environment. This eutelic organism consists of a mere 1000 somatic nuclei but shows obvious differentiation such as epidermis, intestine, excretory system, nerve, and muscle cells. Brenners early work culminated in the milestone paper "The Genetics of *Caenorhabditis elegans*" (Brenner 1974). His early collaborators performed detailed analysis of the neuroanatomy (White 1986) and cell lineage (Sulston 1976, Sulston and Horvitz 1977, Sulston 1983) of the worm. This pioneering work attracted more scientists to the *C. elegans* research field. In 1988 and 1997, a large part of *C. elegans* research was summarized in the books "The nematode *Caenorhabditis elegans*" (Wood, 1988) and "*C. elegans* II" (Riddle et al. 1997), respectively. An important milestone was reached by the end of 1998; approximately eight years after the project was initiated, virtually the whole 97-megabase *C. elegans* genome was sequenced (The *C. elegans*

sequencing consortium 1998). Together with the initiation of this project, a *C. elegans* database (ACeDB) was set up as a data-managing tool. In 2000, a web-based version of this database, called Wormbase (http://www.wormbase.org), became available and is currently a popular resource providing information on many aspects of the worm. It now includes the completed genome sequence, a physical map, the complete cell lineage and parts list, a virtual anatomical worm atlas, an RNAi knockout database, a microarray and expression database, an extensive list of publications and author information, and many more data. Recently, Brenner, Horvitz, and Sulston, three pioneers of *C. elegans* research were awarded the 2002 Nobel Prize in Physiology and Medicine.

5.1.3 *C. elegans* as a model in aging research

The first use of nematodes in aging research was reported in 1970 when Gershon published a paper on age-dependent alteration of enzyme activity in *Turbatrix aceti*, a nematode that, like *C. elegans*, belongs to the family of Rhabditidae. This animal was used essentially during the 70's as a model for the study of age-dependent changes in enzyme activity and conformation (Gershon and Gershon 1970; Zeelon et al. 1973; Rothstein 1977; 1980; 1982). Apart from the early descriptive data on *C. elegans* lifespan (Maupas 1900), the first study on *C. elegans* aging was published by Beguet and Brun in 1972 (i.e. before the classical Brenner paper of 1974), and reported on the influence of parental age on F1 fecundity (Beguet and Brun 1972). Zuckerman and coworkers also conducted biogerontological studies on the close relative *Caenorhabditis briggsae* during the 70's (Zuckerman and Himmelhoch 1980). The first classical paper on *C. elegans* aging, however, was published in 1976 and described the non-aging characteristics of the dauer stage (Klass and Hirsh 1976). Although Gershon (1970) first proposed nematodes in general as being excellent models to study aging, Klass (1977) emphasized the potential of *C. elegans* in particular by stating: "The free-living nematode *Caenorhabditis elegans* is an excellent experimental system for the study of aging. The present study identifies some of the major biological and environmental factors influencing lifespan as a prelude to more detailed genetic and biochemical analyses." In the early eighties, Johnson and Wood (1982) found that polymorphic genes can influence lifespan in natural populations of *C. elegans*. This work was a prelude to extensive research on the genetics of aging that would be conducted during the next two decades (discussed in detail below).

Currently, the most prominent models in biogerontology are (from simple to complex systems): yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and rodents (mouse/rat). In this series, *C. elegans* is ranked as the simplest multicellular organism but in spite of its simple structure, it consists of highly differentiated organs such as muscle, intestine, gonads, and a neuronal system. Apart from the popular model systems, important aging studies were also performed on *Escherichia coli* (Dukan and Nyström 1998), *Podospora anserina* (Osiewacz and Borghouts 2000; Osiewacz 2002), primates (Lane et al; 1996; Kayo et al. 2001; Mattison et al. 2003), and some other species.

Working with *Caenorhabditis elegans* as a model in aging studies offers several advantages over other systems. Aging experiments often require large synchronized populations that do not mix with next generations. Such populations can be easily obtained for nematodes by isolating eggs with a bleaching method (Sulston and Hodgkin 1988), consequently growing the synchronized population to adulthood and adding the DNA-synthesis inhibitor 5'-fluorodeoxyuridine to prevent progeny (Hosono 1978; Houthoofd et al. 2002a). Using worms harboring a temperature-sensitive mutation that renders sterility can also prevent F1 generation. Both generation time and lifespan of *C. elegans* are extremely short, allowing the researcher to manage a considerable number of experiments in a short time. The possibility of selfing in hermaphrodites as well as cross-fertilization by males makes this organism ideal for genetic analysis. The strains of interest can be stored in liquid nitrogen indefinitely, reducing the risk of unwanted genetic changes over time as well as reducing the work of maintaining stock cultures. The major advantage, however, is the availability of an impressive number of long-lived mutants that were generated over the last 15 years (for extensive lists, see http://sageke.sciencemag.org/cgi/genesdb and http://ibgwww.colorado.edu/tj-lab/frame_worm1.html). Many of these relay external (environmental) or internal signals (from the reproductive system) and function in, or are linked to, an insulin/IGF-like signal transduction pathway; other genes have a mitochondrial function and/or regulate temporal functions (the clock genes). These groups of long-lived mutants will be discussed in detail in section 5.3.

5.1.4 General concepts and theories that link metabolism to aging

It has been long known that among homeothermic animals there is an inverse correlation between lifespan and specific metabolic rate (Rubner 1908), and that poikilothermic species such as flies also show tight inverse correlations between metabolic rate and lifespan at different temperatures (Loeb and Northrop 1917). Pearl (1928) combined both observations in his Rate-of-Living hypothesis, which assumes that lifespan is inversely proportional to metabolic rate. However, in later studies, many exceptions to this rule were found to accept this idea as a universal phenomenon (for an extensive monograph, see Finch 1990).

Since the publication of Harman's paper in 1956 on the role of free radicals in aging, this hypothesis has gained more attention every year (Harman 1956). The central idea is that very reactive free radicals damage macromolecules, thereby impairing or abolishing their function. The gradual accumulation of such damaged molecules and concurrent decrease in cellular and systemic function would underlie the molecular mechanism of aging. The major cellular sources of free radicals are the reactive oxygen species (ROS) generated in the mitochondria as inevitable by-products of oxidative metabolism. The primary type of ROS in the mitochondria is the superoxide radical/anion, which is mainly produced at the mitochondrial complexes I and III (Herrero and Barja 1997; Turrens 1997). Free radical production rises with the degree of reduction of the respiratory chain carriers. Thus in state 4 respiration (or when complex IV is blocked), electron flow is im-

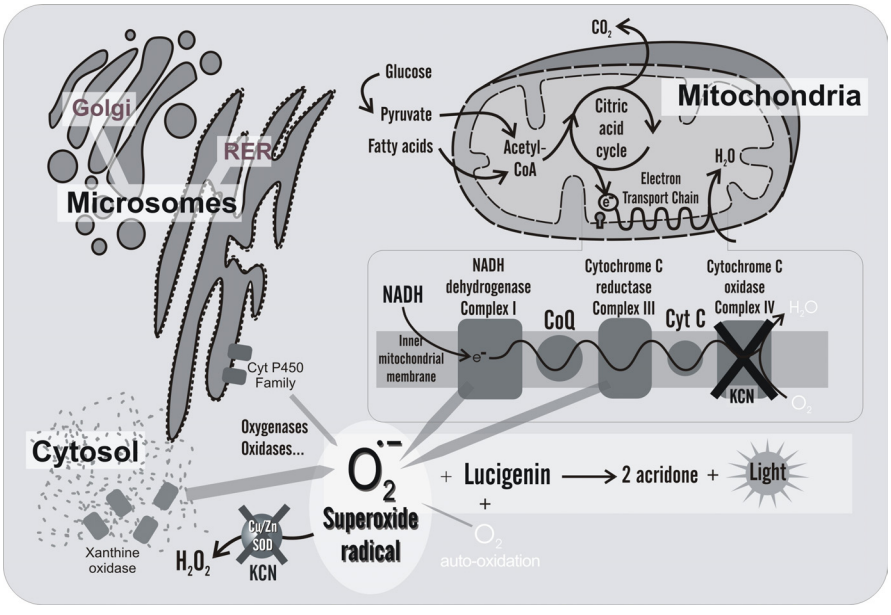

Fig. 2. Major sites of superoxide production in the cell and rationale of the lucigenin assay.

peded and electrons leak to oxygen, producing superoxide. During state 4 to state 3 transition electron flow resumes, the degree of reduction of the respiratory chain decreases, and complex III stops producing free radicals. Complex I continues to generate free radicals likely because its degree of reduction remains sufficiently high, due to its closer proximity to the substrate (Herrero and Barja 1997). There is much consent that it is the lipid–soluble molecule ubiquinone (UQ) that leaks electrons to oxygen. Ubiquinone is a two-electron carrier and during the brief period between the first and second electron transfer from the FeS cluster in complex I to UQ, the carrier exists as a semiquinone radical ($UQH^{\bullet}$). The fully reduced ubiquinol (UQH_2) transfers its first electron to the FeS cluster in complex III, while the second electron is transferred to cytochrome b-566 of the same complex. The latter will transfer its electron to the cytochrome b-562 subunit, which, in turn, uses this electron to reduce another semiquinone to UQH_2 (Voet and Voet, 1995). During this UQ-cycle, the semiquinone radical intermediate can again react with molecular oxygen to form the superoxide anion (Fig. 2). The superoxide anion can be converted to hydrogen peroxide in a dismutation reaction, catalyzed by the enzyme superoxide dismutase (SOD). In eukaryotes, two types of this SOD concur: manganese containing enzymes (MnSOD) reside in the mitochondrial matrix, and copper and zinc containing isoforms (Cu/ZnSOD) are present in the cytoplasm, the mitochondrial intermembrane space, lysosomes, peroxisomes, the nucleus and the extracellular space (Halliwell and Gutteridge, 1999). Hydrogen peroxide, in turn, can be converted into water and molecular oxygen by catalase. In the presence of metal cations like Fe(II) and Cu(I), hydrogen peroxide can, instead of being detoxified by catalase, react with superoxide in a Fenton-like reac-

tion yielding the extremely reactive hydroxyl radical ($OH^{\bullet}$). Reactive oxygen species are known to react with a variety of cellular components and are considered a threat to cellular homeostasis. Free radicals can also function in signaling cascades and defensive mechanisms, however.

A combination of Harman's (1956) Oxidative Damage theory and Pearl's (1928) Rate-of-Living hypothesis was readily made. By directly linking free-radical production to metabolic rate, it was obvious that organisms with high metabolic rates should accumulate oxidative damage faster, resulting in accelerated aging and early death (see also section 5.5.2). Currently, this idea remains very popular among many gerontologists.

The discovery of long-lived mutants in *C. elegans* allowed researchers to test this 'live fast, die young' concept straightforwardly by measuring metabolic rates in these mutants using wild type as a control. These tests have been performed and will be discussed in detail in section 5.3.1.3 and 3.2.3.

5.2 Measuring energy metabolism in *C. elegans*

5.2.1 Setting up age-synchronous cohorts

A small non-synchronous culture can be synchronized by dissolving the gravid worms in a mixture of sodium hydroxide and bleach, while retaining the viable eggs (Sulston and Hodgkin 1988; Braeckman et al. 2002a). The eggs are allowed to hatch overnight in S buffer and the first-stage larvae are then transferred to a lawn of *E. coli* cells on nutrient agar. The worms are allowed to develop and shortly after the molt to the fourth larval stage, they are rinsed off the plates and suspended in S buffer containing cholesterol, at densities not exceeding 2,000 worms/mL. The cultures are shaken in Fernbach flasks and fed frozen *E. coli* cells. FUdR (5-fluorodeoxyuridine) is added to suppress reproduction. *E. coli* is added daily as needed to maintain the initial bacterial concentration. Alternatively, age-synchronous mass cultures can be kept on plates during their whole lifespan, but it is more difficult to keep food supply constant in this situation. To avoid variation in food uptake, worms can be cultured in axenic medium, but this culture method likely imposes dietary restriction (Houthoofd et al. 2002c; see also section 5.4.5).

Samples are usually harvested at daily intervals and washed with Percoll and sucrose to remove dead worms and bacteria, respectively (Fabian and Johnson 1994; Braeckman et al. 1999; Sulston and Hodgkin 1988). As the proportion of dead worms in the aging cultures increases, the Percoll treatment may fail to yield at least 95% of live worms, and sampling should be discontinued. The cleaned worms are concentrated by mild centrifugation and can be used for the metabolic assays.

5.2.2 Oxygen consumption

Oxygen is the ultimate acceptor of the electrons flowing through the electron transport chain in the inner mitochondrial membrane. In this reaction, molecular oxygen is reduced to water. There are also other cellular processes that consume molecular oxygen (e.g. reactions involving oxygenases and oxidases), but they are quantitatively far less important in actively respiring cells. Accurate measurement of minute changes of oxygen concentration in air is technically difficult, if not impossible, because of the high concentration of atmospheric oxygen. For that reason direct measurements of oxygen consumption in *C. elegans* are usually performed with Clark type electrodes using worms that are suspended in liquid.

5.2.3 Carbon dioxide production

In the TCA (tricarboxylic acid) cycle, carbons are oxidized, NAD and FAD cofactors are reduced and CO_2 is released. The reduced cofactors can deliver their electrons to the electron transport chain, where they are finally accepted by molecular oxygen. Apart from the TCA cycle, there are other processes that release CO_2 to some extent. Estimates of metabolic rates based on CO_2 measurements may deviate from those based on oxygen consumption, depending on the nature of the metabolized substrate (fat, carbohydrate, protein) and the proportion of the energy fluxes through alternative biochemical pathways (pentose phosphate pathway, glyoxylate pathway etc.). However, an advantage of this approach is that it allows monitoring of worms that are exposed to an atmospheric environment, which is more consistent with their normal environmental conditions (Van Voorhies and Ward 1999). Simultaneous measurement of O_2 or CO_2 during the worm's life has the potential to uncover subtle metabolic shifts.

5.2.4 Microcalorimetry

Metabolic heat produced by living tissue can be measured most accurately by isothermal microcalorimetry. In this method, samples are maintained in an essentially isothermal state and the release of heat is directly quantified. Aerobic metabolism can be divided into two interdependent processes: catabolism (in which substrate carbon is oxidized to CO_2, yielding ATP and reducing equivalents) and anabolism (in which substrates are converted into new biomass or used for maintenance or work). Heat production rate determinations measure the sum of catabolic and anabolic rates in terms of energy.

5.2.5 ATP measurements

The ATP content is a measure of the instantly available energy. ATP can be measured most accurately by monitoring the amount of light emitted when luci-

ferin reacts with oxygen in the presence of luciferase. This reaction is driven by ATP present in the sample. The ratio [ATP]/[ADP][P_i] is an indicator of the energy status of the cell. Energy charge is an alternative indicator defined by the following ratio: [ATP]+0.5[ADP] / [ATP]+[ADP]+[AMP]. This entity controls the rate of many metabolic reactions.

5.2.6 The lucigenin assay

A lucigenin assay was developed to measure the maximal scope for metabolic activity (Braeckman et al. 2002a). In this assay, a mixture of NADH, NADPH, KCN and lucigenin is added to freeze-thawed worms (Fig. 2). The reducing equivalents will feed the electron transport chain at a maximal rate making it independent of the TCA cycle. At the other end of the chain, complex IV is blocked by KCN, forcing the other complexes towards a reduced state. In this state, ubiquinone will easily leak electrons to molecular oxygen to form superoxide anions (Nicholls 2002) as described in section 5.1.4. These are not scavenged by Cu/ZnSOD because the activity of this enzyme is completely suppressed by KCN as well. MnSOD, a cyanide-insensitive SOD isoform, is present in *C. elegans*, but its activity levels are very low (Vanfleteren 1993). Lucigenin easily penetrates the freeze-thawed worms and is first monovalently reduced by endogenous reductase(s) after which it can react with superoxide. This reaction generates two acridone molecules and a photon, which can be detected. The amount of lucigenin-mediated luminescence provides a reliable estimate of the potential of nematode tissue for metabolic activity, immediately prior to freeze fixation. Any change in the scope for metabolic activity in live nematode tissue (e.g. an age-dependent decrease) will be highlighted by a corresponding change of luminescence output in the light production assay. Currently, this method shows the most impressive age-related decline (20x or more) of any one biochemical or physiological characteristic tested in *C. elegans*.

5.2.7 Data normalization

A major methodological question is how to normalize the experimental data. It has been argued that metabolic data of aging studies need to be normalized to worm number (expressed as per individual), given that aging is a single organismal trait (Van Voorhies and Ward 1999; Van Voorhies 2003). We do not concur with this reasoning, which has, in addition, the trivial outcome that the largest individuals will yield the largest scores if everything else but size is kept constant. Age-dependent changes of metabolic rate occur at the (sub)cellular level, and size differences (if not excessive) can be alleviated by normalizing to biomass. Scaling of rates of metabolism in terms of unit metabolically active mass (often called specific metabolic rate) provides a theoretical base for comparing metabolic rates. As the amount of metabolically active mass is usually unknown, various proxies, including volume, wet weight, dry weight, and protein content have been widely

used, and they are all acceptable as long as they have a fixed relation to the metabolically active biomass and to each other, independent of age. In practice, correlation is usually fair. However, non-congruencies may occur and confound correct evaluation of the data. Since biomass varies over age and genotype, scaling to number or biomass readily results in widely different metabolic profiles and interpretation of the data.

When the body sizes compared differ substantially, simple scaling to body mass may no longer be appropriate, because the rate of energy expenditure shows a negative allometric relationship with body mass, and the data may need to be corrected for size differences using the Brody-Kleiber equation (Braeckman et al. 2002b). The issue of normalization was recently discussed at length in Braeckman et al. (2002b) and Van Voorhies (2002a).

5.3 Genes that influence lifespan in C. elegans

5.3.1 Insulin/IGF-like signaling mutants

5.3.1.1 Elucidation of the pathway

The first screen for lifespan mutants was carried out on 8000 F2 clones descending from an original set of 200 mutagenised L3 worms that carried the temperature-sensitive sperm-defective mutation *fer-15* (Klass 1983). Eight clones were found showing extended lifespan, but all had an unhealthy appearance. Klass concluded that the lifespan extension was caused by a caloric restriction, rather than an explicit genetic effect. Few years later, Friedman and Johnson (1988) found that one of the mutant lines of the Klass study lost its sick appearance, but remained long-lived after it was backcrossed with the wild type. The mutant strain was designated *age-1* and was the first longevity mutant described for *C. elegans*. Initially, the closely linked *fer-15* mutation was still present in this mutant, and it was suggested that the reduced hermaphrodite self-fertility was a trade-off for the long-lived phenotype. In a later study, the *fer-15* mutation was crossed away and the resulting *age-1* mutant was still long-lived and had only marginally reduced fertility (Lithgow et al. 1994; Lithgow et al. 1995). The discovery that single mutations could profoundly extend longevity disproved the earlier discouraging predictions raised by some evolutionary biologists (reviewed in Gavrilov and Gavrilova 2002) and opened the hunt for other 'gerontogenes' hidden within in the *C. elegans* genome. It was soon reported that mutations in the gene *daf-2* can give rise to active and fertile adults that live twice as long as wild type, and that another gene of the dauer formation pathway, *daf-16*, is required for this effect to occur (Kenyon et al. 1993). Genetic analysis of Daf mutations allowed Gottlieb and Ruvkun (1994) and Dorman et al. (1995) to conclude that *daf-2*, *daf-23, daf-18*, and *daf-16* operate in a common pathway to determine lifespan. Experiments conducted by Larsen et al. (1995) resulted in a similar pathway including the genes: *daf-2*, *daf-12*, *daf-16*, *daf-18*, and *daf-23*. In this study, the double mutant *daf-2;daf-12* showed an impressive fourfold lifespan extension compared to wild type.

Later studies showed that *daf-23* and *age-1* are allelic and the gene name *age-1* was retained (Malone et al. 1996). Although dauers are characterized by their extended lifespan, not all *daf* genes that are involved in dauer formation influence adult lifespan. Mutations in genes of a separate TGF-β signaling pathway have no effect on lifespan.

After lining up several genes that influence lifespan in *C. elegans*, the next step was to define their identity and biological function. In 1996, the first of these genes was cloned and characterized: *age-1* encodes a homologue of the p110 catalytic subunit of a mammalian phosphatidylinositol-3-OH kinase family member (Morris et al. 1996). This indicated the possible involvement of neuroendocrine signaling in lifespan determination. Only one year later, it was revealed that *daf-2* was an insulin receptor-like gene (Kimura et al. 1997) and this receptor protein was placed upstream of AGE-1 in a signaling cascade. Obviously, the next target for identification was the downstream gene *daf-16*. Only two months later, Ogg et al. (1997) and Lin et al. (1997) reported almost simultaneously that this gene encoded a Forkhead transcription factor. Since *daf-16* activity was necessary for the lifespan extension conferred by mutation in *age-1* or *daf-2*, it was expected that this transcription factor controlled several genes that were involved in an enhanced life maintenance program. During the next years, other elements were added to the signaling cascade. AKT-1 and AKT-2 were shown to act between AGE-1 and DAF-16 and are redundant Akt-PKB serine/threonine homologues that are predominantly expressed in neurons (Paradis and Ruvkun 1998). In the same year, Ogg and Ruvkun (1998) reported that DAF-18 is a homologue of the human tumor suppressor PTEN and shows 3-phosphatase activity toward phosphatidylinositol-3,4,5-phosphate. Therefore, DAF-18 can be considered as a functional antagonist of AGE-1. The enzyme that transduces signals from AGE-1 to AKT-1/AKT-2, a 3-phosphoinositide-dependent kinase, was identified by Paradis et al. (1999). It was named PDK-1 after the mammalian homologue Akt/PKB kinase PDK1 and exhibited an expression pattern similar to that of AKT-1/AKT-2. At that moment the signaling pathway from the DAF-2 receptor to the DAF-16 transcription factor was known in quite some detail (Fig. 3), but the major effectors up- and downstream remained undiscovered. A first clue on lifespan regulating genes that act upstream of DAF-2 came from Ailion et al. (1999), who found that mutation in the genes *unc-31* and *unc-64* extends lifespan in a *daf-16*-dependent fashion. These genes both encode homologues of the mammalian neuronal proteins, CAPS (Ca^{2+}-dependent activator protein for secretion) and syntaxin (involved in synaptic transmission), respectively. The simplest model to explain lifespan extension in both *unc* mutants is that these genes are directly or indirectly involved in Ca^{2+}-dependent secretion of an insulin-like ligand for the DAF-2 receptor.

The search for the insulin-like signal eventually led to the identification of 37 predicted "insulin" encoding genes (Duret et al. 1998, Gregoire et al. 1998, Kawano et al. 2000, Pierce et al. 2001). GFP-reporter constructs for 14 of the *in*s genes showed that these genes are expressed in the larval and adult (except *ins-2*) stages. *ins-1, ins-18, ins-9* and *ins-22* are expressed primarily in subsets of neurons throughout most of the life cycle. However, it is not clear yet which peptide

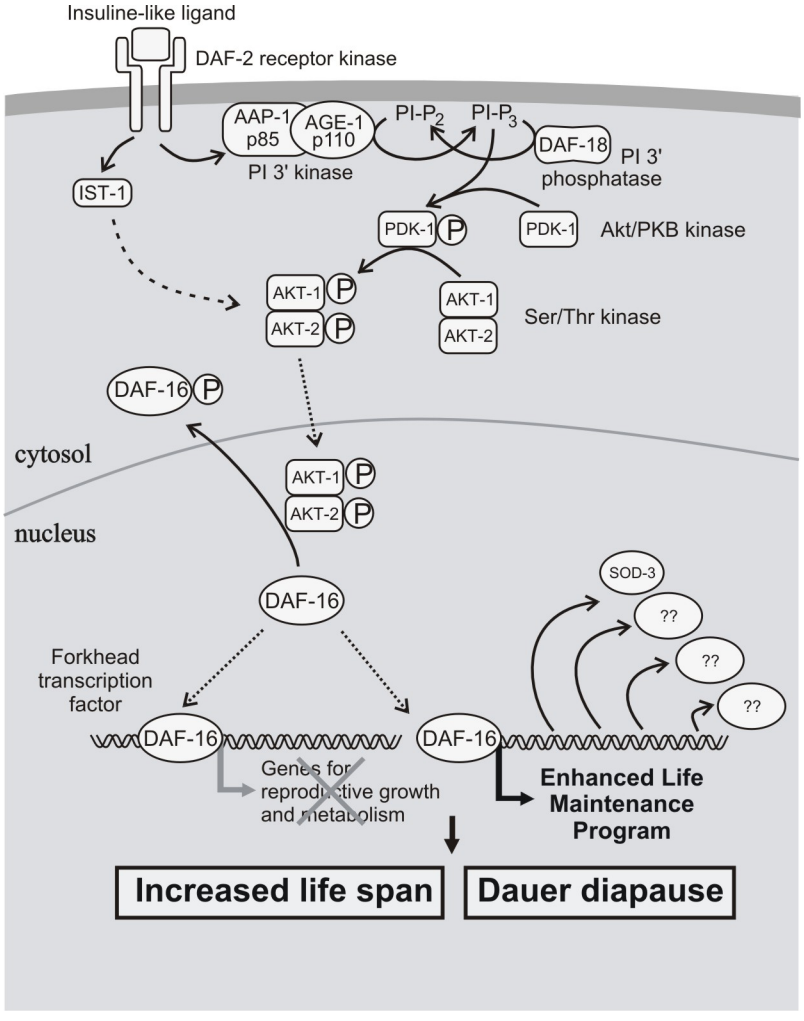

Fig. 3. Schematic diagram of the Ins/IGF pathway in *C. elegans*. Activation of the insulin-like receptor DAF-2 results in the activation of the PIP-3-kinase, which produces PIP_3. PIP_3 activates the PDK-1 and AKT-1/AKT-2 kinases, which in turn phosphorylate the Forkhead transcription factor DAF-16. Phosphorylated DAF-16 resides in the cytoplasm, where it is inactive.

functions in dauer formation and life extension upon binding onto DAF-2. It is likely that there is much redundancy among the 37 INS peptides. Some of these, including INS-1 seem to antagonize, rather than activate, DAF-2 signaling (Pierce et al. 2001). INS-18 has been reported to act as an agonist (Kawano et al. 2000), as well as an antagonist (Pierce et al. 2001).

At the other end of the signal transduction pathway, the search for genes that act downstream of *daf-16* yielded the first results as well. *daf-2* mutants express

the dauer-specific superoxide dismutase-3 (SOD-3), a mitochondrial MnSOD isoform, constitutively during their whole lifespan. The *sod-3* expression is also dependent on the activity of the *daf-16* forkhead transcription factor (Honda and Honda 1999). It is generally believed that aging is, at least in part, caused by the non-specific deleterious actions of reactive oxygen species (ROS), obligatory by-products of oxidative metabolism. One type of these harmful ROS molecules is the superoxide anion, which is converted into hydrogen peroxide by SOD. It is tempting to hypothesize that lifespan of *C. elegans* is regulated, or at least influenced, by an insulin-like signal in the neurons that results in the expression of a ROS-scavenging enzyme. A second gene under regulation of *daf-16* is *old-1* (formerly *tkr-1*), a transmembrane tyrosine kinase, which is upregulated in the long-lived *daf-2* and *age-1* mutants. It is stress inducible, expressed in a variety of tissues, and, most importantly, *old-1* activity is required for the lifespan extension conferred by mutation in *age-1* and *daf-2* (Murakami and Johnson 2001). Currently it is not clear whether *old-1* activity is necessary for the *daf-16*-dependent expression of *sod-3*. It is obvious that *daf-16* controls many more than these two genes. Recently it was found that HSP16A increases lifespan in *C. elegans* in a *daf-16* dependent way (Walker and Lithgow 2003). In another study, at least 8 genes with very diverse biological functions were found to be under *daf-16* control (Yu and Larsen 2001). Whole genome expression approaches including SAGE (Jones et al. 2001) and microarraying (Lund et al. 2002) are being increasingly used to track changes in gene expression during dauer diapause and aging. Using the microarray technique, McElwee et al. (2003) found a large set of genes that were differentially expressed in *daf-16(+)* and *daf-16(-)*. The function of these genes ranged from metabolism and energy generation to stress response. Expectedly, *sod-3* and several heat shock proteins were identified. Also, a protease with unknown function was found to be necessary for the *daf-2* Age phenotype. Seventeen orthologous genes from *C. elegans* and *Drosophila* bearing a DAF-16 binding site in the promotor region were found using in silico comparative genomics (Lee et al 2003a). Six of these genes were differentially expressed in *daf-2* and *daf-2;daf-16* worms, suggesting that they were regulated by the Insulin/IGF-like signaling pathway. Three genes conferred lifespan extension when knocked down with RNAi and were homologous to an ABC transporter, hydroxyphenylpyruvate dioxygenase and retinoblastoma binding protein 2.

5.3.1.2 Localization and timing of insulin/IGF-like signals

Using genetic mosaic analysis, Apfeld and Kenyon (1998) found that the presence of only a fraction of *daf-2(-)* cells in a worm was sufficient to cause lifespan extension. This experiment showed that *daf-2* influences longevity systemically and that some *daf-2(-)* cells in the worm can override *daf-2(+)* cells to become long-lived suggesting that *daf-2* signaling resides in specific cells or tissues. This idea was confirmed by Wolkow et al. (2000) by using cell or tissue-specific expression of *daf-2(+)* and *age-1(+)* in animals that were otherwise *daf-2(-)* or *age-1(-)* mutants. By limiting expression of the wild type allele to neurons the Age phenotype was rescued. However, tissue specific expression in muscle or intestine did not

rescue wild type lifespan. These experiments confirmed the assumption that *daf-2* signaling occurs through the neurons. The involvement of neuronal signaling in *daf-2*-dependent longevity increase was also confirmed by another set of experiments. Several mutants suffering impaired sensory perception showed a *daf-16*-dependent extension of lifespan. This important observation showed that lifespan might be regulated by environmental cues. These sensory neurons are probably not directly involved in the secretion of the *daf-2* ligand. It was found that an acetylcholine pathway is involved suggesting the presence of a synaptic signal (Tissenbaum et al. 2000).

Apart from the environmental cues, gonadal signals also regulate lifespan in a *daf-16*-dependent fashion in *C. elegans*. Hsin en Kenyon (1999) ablated the Z2 and Z3 cells, (the germline precursor cells) of young worms and found that lifespan was extended by an impressive 60%. This longevity increase was *daf-16* dependent seeing that the effect did not occur in *daf-16* null mutants. Thus, a signal from the germ cells inhibits *daf-16* activity and is life-shortening in wild type. A few years earlier, however, it was found that, when somatic as well as germline precursor cells (Z1 through Z4) were ablated, no lifespan extension could be observed (Kenyon et al. 1993). This suggests that the somatic gonad is able to provide a life-extending signal that counterbalances the life-shortening signal from the germ cells. Germline ablated *daf-2* mutants outlived *daf-2* mutants indicating that the *daf-2* signaling pathway and the germline signals act independently on *daf-16* to influence longevity. By using DAF-16::GFP fusion proteins it was shown that *daf-2* signaling keeps DAF-16 out of the nucleus; when the signal is halted (e.g. by mutation in *daf-2*), the transcription factor DAF-16 migrates to the nucleus where it may activate an enhanced life maintenance (ELM) program (Henderson and Johnson 2001). However, nuclear localization of DAF-16 by itself is not sufficient for activation of the program. It was shown that, by changing the putative AKT target phosphorylation sites to alanine, DAF-16^{A} migrated to the nucleus but this modification did not result in a longevity phenotype (Lin et al. 2001).

The nuclear hormone receptor DAF-12 is also required for the life extension in germ-line ablated animals to occur (Hsin and Kenyon 1999). DAF-12 interacts with the Ins/IGF transduction pathway to regulate dauer formation and longevity. Loss-of-function mutations in *daf-12* and constitutive mutations in *daf-2* have antagonistic activities in dauer formation, but act synergistically to regulate lifespan: some *daf-2;daf-12* doubles live much longer than the *daf-2* single mutants, although the *daf-12* single mutants are slightly short-lived (Larsen et al. 1995; Gems et al. 1998; Antebi et al. 2000, Gerish et al. 2001).

In summary, the lifespan determination by *daf-16* is regulated by environmental as well as gonadal signals. Apart from lifespan regulation, *daf-2* signaling is also important in the dauer decision and, therefore, it should act before the L3 stage. Is early *daf-2* signaling capable of influencing lifespan at adulthood? Or is *daf-2* signaling separated in time; an early dauer decision signal and a later lifespan signal? This question was addressed by switching *daf-2* off and on during the life cycle of *C. elegans*. This was achieved by using RNAi to knock down *daf-2* activity, followed by knocking down the dicer gene (*dcr*, involved in RNAi silencing) to re-

store *daf-2* activity at the desired time point (Dillin et al. 2002a). The results of this experiment showed that early *daf-2* signals are required for the dauer decision only, whereas *daf-2* activity during adulthood determines lifespan. Moreover, the *daf-2* signaling pathway also controls reproduction, although independently of lifespan.

5.3.1.3 Insulin/IGF-like signaling and energy metabolism

Predictions. One of the possible predictions of the oxidative damage theory of aging is that metabolic rate should be lowered in long-lived Ins/IGF pathway mutants (see also section 5.1.4). An alternative hypothesis is that oxidative damage is restricted via upregulation of the anti-oxidant system, which, in turn, would slow down the aging process. Metabolic activity as well as anti-oxidant activity of SOD and catalase was tested in dauers and Ins/IGF mutants.

Energy metabolism in dauers. Ins/IGF-like signaling plays an important role in dauer diapause, fertility and lifespan determination. Can the increased longevity of *daf-2* mutants be ascribed to the anachronistic expression of a dauer-specific program during adulthood? Dauer larvae show several metabolic shifts compared to normal L3 larvae. The activity of several TCA cycle enzymes is substantially repressed resulting in a relative increase of the contribution of glyoxylate cycle activity to energy metabolism (O'Riordan and Burnell 1989; O'Riordan and Burnell 1990; Wadsworth and Riddle 1989). In the glyoxylate cycle, isocitrate is converted directly into succinate and glyoxylate (catalyzed by isocitrate lyase) instead of α-ketoglutarate (catalyzed by isocitrate dehydrogenase). As a result, both decarboxylation steps of the TCA cycle are bypassed. Malate synthase then condenses glyoxylate and a second acetyl-CoA molecule to malate, which is further processed in the TCA-cycle. The glyoxylate cycle utilizes fat as fuel and produces small amounts of energy, but most importantly, it is a source of biosynthetic intermediates. Consistent with this data, several studies point out that dauers have reduced metabolic rate and ATP levels (Anderson 1978, Houthoofd et al. 2002a, Vanfleteren and De Vreese 1996, Figure 4A-C). Wadsworth and Riddle (1989) measured exceptionally low ATP concentrations in dauers using (^{31}P) NMR. Possibly much ATP was lost during the perchloric acid treatment in these experiments.

Do Ins/IGF signaling mutants show dauer-like metabolic profiles? Since Ins/IGF mutants harbor a mutation in a gene involved in dauer formation, one might expect them to show a low dauer-like metabolism during adulthood as well. Vanfleteren and De Vreese (1996) measured slightly higher oxygen consumption rates, normalized to protein, over the entire adult life trajectory in *age-1* mutants, relative to wild type worms. Similar results were obtained in a later study, using *daf-2* mutants, but after these were adjusted for size differences wild type and mutant worms had essentially identical rates of metabolism (Braeckman et al. 2002b Figure 5A).

These results were challenged by Van Voorhies and Ward (1999), who reported that CO_2 production was drastically reduced in *daf-2* adults. However, these authors normalized their data to worm number, although the strains examined had

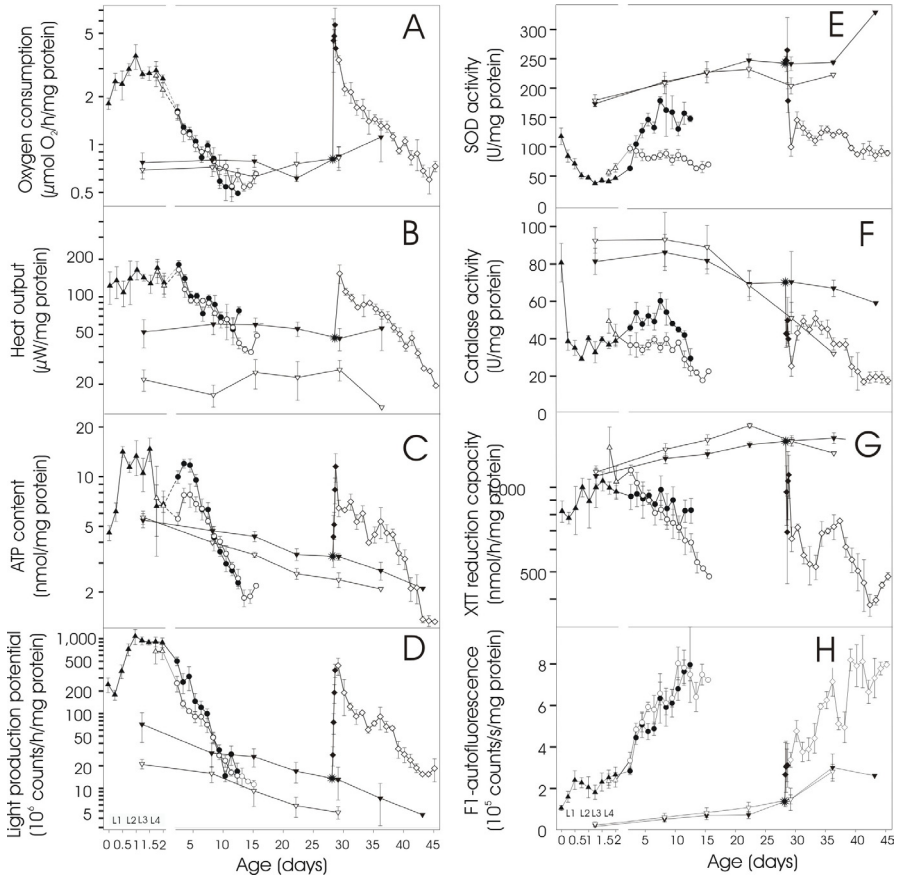

Fig. 4. Metabolism of wild type dauers and adults. A: Respiration rate, B: Heat production, C: ATP content, D: Light production potential, E: SOD activity, F: Catalase activity, G: XTT reduction capacity, H: Oxidized flavins (F1 autofluorescence). Triangles: juvenile stages, inverted triangles: dauers, circles: adults, diamonds: post dauer worms, asterisk: food addition to elicit dauer exit. Open symbols represent liquid cultures, closed symbols represent cultures grown on agar plates. For experimental details, see Braeckman et al. 2002a and Houthoofd et al. 2002a.

widely differing body masses. Metabolic aspects may contribute to the discrepancy as well. For example, less CO_2 is produced when fat is combusted relative to carbohydrate, because of the more reduced state of fatty acids. This is important because *daf-2* and *age-1* mutants have a metabolic shift to fat production and utilization, as previously mentioned. Furthermore, it can reasonably be assumed that these mutants have enhanced phosphoenolpyruvate carboxykinase activities, and re-use CO_2 in an anaplerotic reaction, as dauers do (O'Riordian and Burnell, 1989). The presumed CO_2/O_2 ratio would also be strongly biased, if the activity of the pentose phosphate shunt (which produces CO_2 without consuming O_2) differed

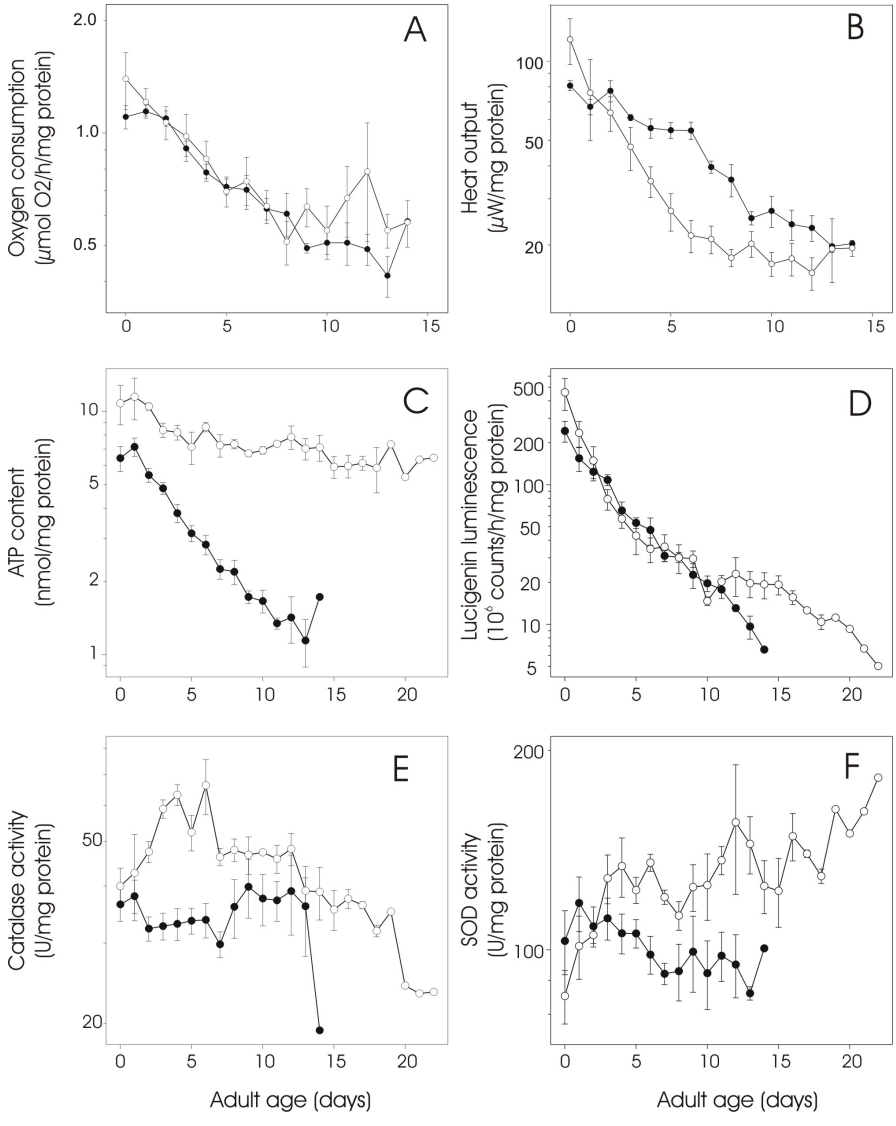

Fig. 5. Metabolism of wild type (closed circles) and *daf-2 (e1370)* (open circles) adults. A: Respiration rate, B: Heat production, C: ATP content, D: Light production potential, E: Catalase activity, F: SOD activity.

among the strains compared. The reader is referred to Braeckman et al. (2002b) and Van Voorhies (2002a, b) for a comprehensive discussion.

An extensive study by Houthoofd et al. (in preparation) suggests a shift in the *daf-2* metabolism, rather than a mere increase or decrease. The oxygen consumption of *daf-2* mutants is similar to wild type over a large part of the adult life while

heat production is definitely decreased, especially around mid-age (Figure 5A-B). The low calorimetric/respirometric ratio (C/R-ratio) suggests an increase of catabolic efficiency. This phenomenon is expected to enhance ATP synthesis in the *daf-2* mutants. Consistent with this expectation, very high ATP levels were detected in these mutants (Dillin et al. 2002b; Houthoofd et al. in preparation, Figure 5C). The relationship involving mitochondrial efficiency and standing ATP concentrations is likely to be much more complex, however. Firstly, since ATP is usually made as needed, this would implicate that ATP production and consumption are uncoupled in these mutants. Higher ATP levels might then as well derive from reduced ATP consumption rates for anabolic reactions. Secondly, a causal connection between the low C/R ratio and high ATP levels has not been established. Thirdly, the fact that the C/R ratio deviates from wild type only around mid-age, while ATP is high over the entire adult lifespan, indicates that other factors may be involved.

Antioxidant protection in dauers and in Ins/IGF-signaling mutants. Based on the comparable respiration rates of *daf-2* grossly similar superoxide fluxes are likely to be expected (disregarding the difference in C/R ratio). Why then are these mutants long lived? One possible explanation is that these animals are more resistant to oxidative stress. Increased SOD activity in dauers was already described two decades ago (Anderson 1982). The comprehensive study by Houthoofd et al. (2002a) pointed in the same direction: catalase as well as SOD activities are conspicuously upregulated during dauer diapause and fall sharply as dauers exit from diapause (Figure 4E-F). This study also suggests that the redox state of dauer tissue is very negative as assessed from the high capacity of reducing XTT and the low levels of oxidized flavins recorded (Figure 4G-H). A more negative redox potential may help dauers to protect themselves against oxidative damage.

Like dauers, adult *daf-2* and *age-1* mutants exhibit enhanced SOD and catalase activities (Figure 5E-F cfr 3.1.3.2.) that likely underlie the increased resistance of these animals to H_2O_2 and paraquat (generates superoxide) (Larsen 1993; Vanfleteren 1993; Vanfleteren and De Vreese 1995). *daf-2* mutants, but not wild type animals, express the dauer-specific mitochondrial MnSOD also in the adult stage (Honda en Honda 1999). *daf-16* largely suppresses the upregulation of SOD and catalase in *daf-2* mutants (Houthoofd et al. in preparation), which is consistent with other evidence that *daf-16* controls expression of SOD (Murakami and Johnson 2001). It was also reported that *ctl-1*, a gene encoding a putative cytoplasmic catalase, is necessary for the lifespan of *daf-2* mutants to be extended (Taub et al. 1999), but this publication has been withdrawn (Nature 421, retractions, p764, 2003).

In summary, metabolic rate shows little alteration, but anti-oxidant defense is upregulated in long-lived Ins/IGF pathway mutants. It has been suggested that mitochondrial uncoupling might be an important mechanism to reduce superoxide generation (Brand 2000), but this is energetically unfavorable. *daf-2* mutants apparently solve this problem by rising their levels of SOD and catalase. This would allow high coupling efficiency and perhaps cause the high ATP levels measured in *daf-2* mutants.

5.3.2 Slowing down physiological rates

5.3.2.1 The Clock genes

The *clk* mutants were isolated in a genetic screen for maternal-effect mutations affecting development and behavior. The *clk* genes control the timing of a wide range of physiological processes in *C. elegans*. Mutations in any one of these genes cause, besides individual deregulation, mean increases in adult lifespan and cell cycle length, and a slowing down of development and behavioral activity (pharyngeal pumping, defecating, egg laying, and moving). Although mutations in any of these genes cause similar phenotypes, the proteins encoded by the *clk* genes seem unrelated.

CLK-1, a mitochondrial protein that is conserved among eukaryotes (Ewbank et al. 1997) is a putative hydroxylase involved in the biosynthesis of nonaprenylated ubiquinone (UQ_9) in *C. elegans* (Branicky et al. 2001; Hekimi et al. 2001; Jonassen et al. 2001; Miyadera et al. 2001). Although UQ_9 has a function as an electron carrier in the mitochondrial electron transport chain, respiration in *clk-1* mutants is not dramatically lowered (Braeckman et al. 1999; Felkai et al. 1999). Instead, the biosynthetic intermediate DMQ_9 seems to be capable of functioning as an efficient electron carrier in this mutant (Miyadera et al. 2001). Because *clk-1* mutants are not able to grow on a UQ-less diet, it was concluded that DMQ_9 could not replace UQ_9 for essential non-mitochondrial functions required for development (Hihi et al. 2002; Jonassen et al. 2001). *Clk-1* mutants grown on a UQ-replete diet, however, do survive and live longer when fed on a UQ-less diet during adulthood only (Larsen and Clarke 2002). Apart from its involvement in UQ_9 synthesis, CLK-1 also shows binding activity specific to the O_L region of mitochondrial DNA in *C. elegans*. Since ADP inhibits binding activity, it is speculated that CLK-1 is involved in an ADP-dependent regulation of mtDNA replication (Gorbunova and Seluanov 2002).

clk-2 encodes a homologue of the yeast Tel2p protein (Ahmed et al. 2001; Bénard et al. 2001; Lim et al. 2001) that binds to telomeric repeats *in vitro* (Kota and Runge 1999). In yeast, this gene regulates telomere length and is involved in gene silencing in subtelomeric regions *in vivo* (Runge and Zakian 1996). Recently, it was found that *clk-2* is allelic with *rad-5,* a DNA damage checkpoint gene (Ahmed et al. 2001). Against any expectation, RAD-5/CLK-2::GFP fusion proteins were observed in the cytoplasm and seemed to be excluded form the nuclei in *C. elegans* (Bénard et al. 2001). Given this localization, RAD-5/CLK-2 must act either indirectly or at very low concentrations in the nucleus, well below the GFP detection threshold. Even more controversial is the role of this gene in telomere length regulation in *C. elegans*; some reports claim that telomere length is elongated in *clk-2* mutants (Bénard et al. 2001), while others contradict these findings (Lim et al. 2001). A third study, however, puts these results in perspective by stating that telomere length varies considerably among wild type and that in *clk-2* it is not altered altogether (Ahmed et al. 2001).

The mutant *gro-1* was originally isolated as a slow-growing strain and only later it was grouped with the other *clk* mutants. *gro-1* encodes isopentenylpyro-

phosphate:tRNA transferase, an enzyme that transfers an isopentenyl moiety to the adenosine on the 3' side of a tRNA anticodon terminating in U (Lemieux et al. 2001). This modification enhances the efficiency and fidelity of the mitochondrial translation process (Björk et al. 1999). *gro-1*, the fifth member in a five-gene operon, can be translated into two different proteins by alternative translation initiation. The longer form, containing a mitochondrial targeting sequence, is mainly directed to the mitochondria while the short form is partitioned between the cytoplasm and the nucleus. The *gro-1* mutant phenotype results from failure of the mitochondrial protein since GRO-1::GFP that was localized to the mitochondria, was sufficient to rescue the mutant phenotype (Lemieux et al. 2001).

The less intensively studied *clk-3* gene still awaits molecular characterization. *clk-3* behaves like a typical *clk* mutant but its body size is unique. While *clk* mutants usually are slightly smaller than wild type, *clk-3* has a giant phenotype showing a 30% volume increase over wild type (Braeckman et al. 2002c).

5.3.2.2 Other genes related to the Clk phenotype

Recently, other genes have been reported to show a Clk-like phenotype when hypo- or nullomorphous. Mutation in the gene *isp-1*, which encodes an iron-sulfur protein of complex III in the mitochondrial electron transport chain, is reported to result in a slow and long-lived (Age) phenotype (Feng et al. 2001). In a systematic RNAi screen of chromosome I genes, several genes involved in the respiratory chain were found to yield a Clk-like phenotype (Dillin et al. 2002b). When their expression was inhibited using RNAi, several physiological processes slowed down and lifespan of the treated worms was extended.

5.3.2.3 Energy metabolism of clk mutants

Predictions. Most of the genetic alterations that result in a Clk phenotype are linked to some kind of mitochondrial modification. *clk-1* is a gene involved in the synthesis of ubiquinone, a lipid soluble molecule with one of its functions being an electron carrier in the respiratory chain. *gro-1* is involved in the fidelity of mitochondrial translation. *isp-1* is directly involved in electron transport over the mitochondrial membrane. Other genes that function in the electron transport chain (*nuo-2*, *cyc-1*, *cco-1*) as well as the ATP-synthase complex (*atp-3*) yielded a similar phenotype (Dillin et al. 2002b). Currently, only *clk-2* cannot be linked directly to mitochondrial performance. Considering this data, it is very tempting to hypothesize that metabolic rate in all Clk's is compromised. The low mitochondrial activity would generate less free radicals and thus cause less molecular damage, which in turn slows down the aging process. The decreased mitochondrial function would generate less ATP necessarily causing a slow phenotype. This model is sensible and appealing, but it needs experimental verification.

Energy metabolism in *clk-1*, *clk-2*, *clk-3*, and *gro-1*. The first data addressing this metabolic issue proved that succinate cytochrome *c* reductase activity was not lowered dramatically in *clk-1* mutants (Felkai et al. 1999). Also, respiration rates in *clk-1* and *gro-1*, measured by oxygen consumption, were not widely different

from wild type. Moreover, ATP-levels were obviously not lower in the *clk-1* mutant; it appeared that these levels were even higher at advanced age (Braeckman et al. 1999). In a recent extensive study covering several aspects of energy metabolism of the four 'classical' Clk mutants, *clk-1*, *clk-2*, *clk-3*, and *gro-1*, it was confirmed that for all these mutants energy metabolism was not significantly different from wild type (Braeckman et al. 2002c). It was then expected that like in the Ins/IGF mutants, the Age phenotype in Clks could correlate to an increased oxidative stress resistance. Quantification of SOD and catalase activity did not confirm this speculation, however. While catalase levels were basically unchanged, SOD activity was lowered in *clk-1*, *clk-2*, and *clk-3* (Braeckman et al. 2002c). Ablation experiments revealed that the life-extending effect of mutation in *clk-1* only occurs when the somatic gonad is present (Dillin et al. 2002a). On the other hand, gonad-ablated *clk-1* mutants still showed the slow phenotype. This important finding shows that the Age and Slow phenotypes can be uncoupled. Moreover, the fact that only the somatic gonad is necessary to evoke lifespan extension in *clk-1* puts the role of mitochondrial performance in *clk-1* lifespan extension in doubt. The wild type-like patterns of energy metabolism in *clk-1* mutants are consistent with this data.

Energy metabolism in other Clk-like mutants. Mutation in the iron-sulfur protein *isp-1* probably causes a slow down of the electron transport chain because cyanide-sensitive oxygen consumption was decreased (Feng et al. 2001). Like in *daf-2*, the dauer-specific *sod-3* gene was highly upregulated during adulthood in the *isp-1* mutant, which might result in lower oxidative damage underlying its slow aging phenotype. Although the behavioral phenotype of *isp-1* is similar to that of the classical Clk's, its biochemical and physiological characteristics are widely different.

The RNAi knock-downs of *nuo-2*, *cyc-1*, *cco-1* and *atp-3* all showed decreased ATP levels consistent with their compromised respiratory chain elements (Dillin et al. 2002b). These authors provided evidence, however, that the lifespan extension was independent of this hypometabolic state. This interesting study will be discussed in more detail in section 5.5.

5.3.2.4 Interaction between Clk and the Ins/IGF pathway

The family of Clk genes as well as the genes involved in Ins/IGF signaling influence aging. Whether or not they define completely separated pathways is still controversial. By analyzing survival data of *clk-1*, *daf-16* and the double mutant *clk-1;daf-16* it was concluded that mutation in *daf-16* could not suppress lifespan extension in *clk-1* and that therefore *clk* and *daf* pathways influenced lifespan in an independent fashion (Lakowski and Hekimi 1996). In the same year, Murakami and Johnson (1996) reported that the lifespan extension conferred by mutation in *clk-1* was abolished in the *clk-1;daf-16* double mutant. Lifespan experiments in axenic culture, supplied with heat-killed bacteria as a dietary UQ source, pointed in the same direction suggesting that the longevity increase in *clk-1* was *daf-16* dependent (Braeckman et al. 1999). This controversy was discussed in more detail in a recent paper on genetic interaction studies (Gems et al. 2002). Other than life-

span studies, shed some light on the interaction of both genes, however, *clk-1* does not reach adulthood in a UQ-less standard axenic medium, but an additional *daf-16* mutation fails to restore this capability (Vanfleteren and Braeckman 1999). Another interaction between these genes was found at the level of oxidative stress resistance (Honda and Honda 1999). Adults of the single mutant *clk-1* do not show any *sod-3* expression at all while in the long-lived *daf-2* the expression of this ROS scavenger is upregulated during the entire adult lifespan. In the *clk-1 daf-2* double mutant, however, *sod-3* expression is potentiated far beyond the *daf-2* level. This extreme *sod-3* expression correlated with an impressive extension of longevity and resistance to oxidative stress. *sod-3* expression in *daf-2* can be suppressed completely by mutation in *daf-16*. It would be interesting to find out whether the triple mutant *clk-1 daf-2; daf-16* would be able to express *sod-3* to some extent. RNAi directed against *clk-1* did not result in nuclear localization of DAF-16::GFP, suggesting that both genes act in different pathways (Henderson and Johnson 2001).

5.3.3 Short-lived mutants

Studying long-lived mutants enables us to focus on genes that are life shortening in wild type. By clarifying the gene's biological function, localizing its activity in the worm and studying its effect over time, important processes that underlie the mechanisms of aging may be revealed. This approach is generally unbiased by pre-existing hypotheses since the genetic longevity screen (whether by mutation or RNAi) is blind and the identity of the genes showing an Age phenotype is the second step in the process. Data on short-lived mutants should be interpreted much more carefully because early death does not necessarily imply premature aging. Almost certainly, the majority of mutations that shorten lifespan cause pathologies reflecting the absence or inadequacy of molecules required for vital biological functions. Moreover, distinguishing premature aging from pathology in *C. elegans* is not a simple task when the phenotypes are very subtle.

Despite these reservations, one short-lived *C. elegans* mutant gained quite some attention in biogerontology. *mev-1* (methylviologen-sensitive) was found to be short-lived (Ishii et al. 1990) and hypersensitive to oxygen (Hosokawa et al. 1994). SOD activity in this mutant was about half of wild type activity (Ishii et al. 1990;1994; Adachi et al. 1998; Yanase et al. 2002). This mutant also tended to accumulate more lipofuscin (fluorescent aggregates of oxidized lipids and lipoproteins that gradually accumulate with age and are considered being a good biomarker for aging) and at a greater rate than wild type (Hosokawa et al. 1994). Therefore, it was concluded that *mev-1* suffered accelerated aging. As a result of its oxygen-sensitivity, *mev-1* accumulates high amounts of carbonylated proteins (Adachi et al. 1998; Ishii et al. 2002; Yasuda et al. 1999), the concentration of which is frequently used as a biomarker for aging. The gene *mev-1* encodes the cytochrome b large subunit of succinate dehydrogenase (complex II), which catalyses electron transport from succinate to ubiquinone (Ishii et al. 1998). Isolated mitochondria, as well as submitochondrial particles of *mev-1* animals, yield

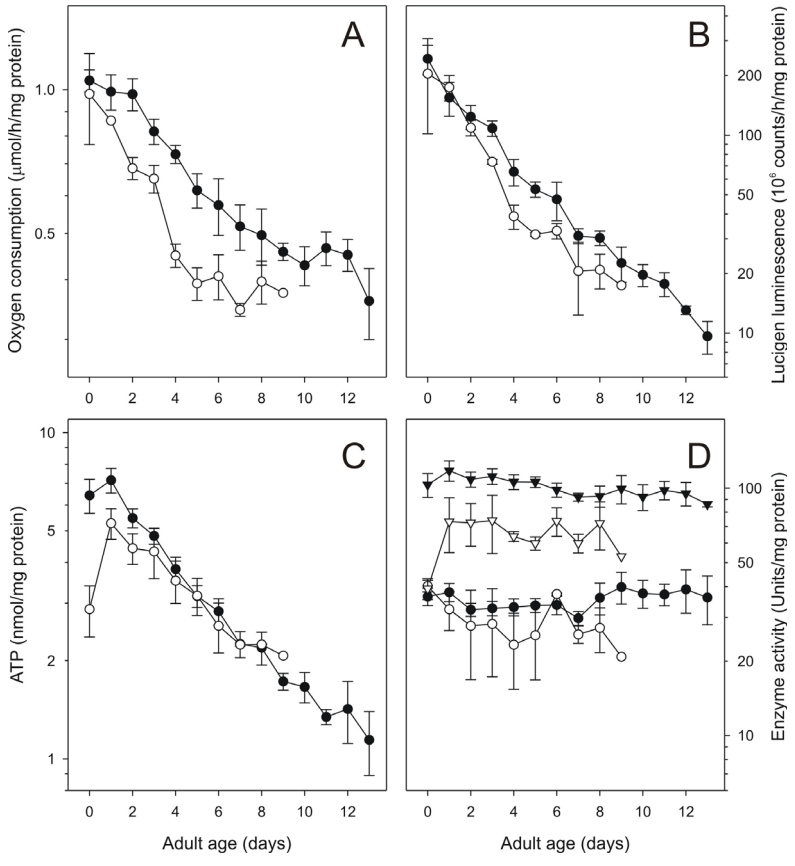

Fig. 6. Measures of metabolism and antioxidant enzyme activities in *mev-1* mutants. A: oxygen consumption, B: heat production, C: ATP content, D: catalase activity (circles) and SOD activity (triangles). Closed symbols: wild type, open symbols: *mev-1 (kn1)*.

significantly more superoxide anions than wild type. Together with the fact that these mutants also show drastically lowered concentrations of reduced glutathione, it can be suggested that *mev-1* worms are oxidatively challenged (Senoo-Matsuda et al. 2001).

Respiration as well as lucigenin luminescence (maximal electron transport capacity) in this *mev-1(kn1)* is, as expected from its complex II insufficiency, lower than the control over a large part of its lifespan (Figure 6A-B). However, ATP levels are wild type-like (Figure 6C and Senoo-Matsuda et al. 2001), possibly because the mitochondrial defect is counterbalanced by increased lactic fermentation (Senoo-Matsuda et al. 2001). The lowered SOD activity reported earlier (Ishii et al. 1990;1994; Adachi et al. 1998; Yanase et al. 2002) was also found in a detailed longitudinal study (Figure 6D) suggesting that the *mev-1* mutant is vulnerable to the excess of superoxide that it produces. Finally, catalase expression (measured as mRNA of both *ctl-1* and *ctl-2*) was reported to be surprisingly high in *mev-1*

(Yanase et al. 2002). In our longitudinal study, no upregulation of catalase activity could be measured (Figure 6D) indicating that either catalase is under strict translational control or that *mev-1* mutants show considerable variation of catalase levels, depending on the experimental conditions.

Another short-lived nematode *gas-1* (general anesthetic-sensitive) also shows hypersensitivity to the deleterious effects of free radicals and hyperoxia (Kayser et al. 2001). *gas-1* encodes a homologue of the 49-kDa iron protein subunit of complex I in the mitochondria. As expected, *gas-1* animals have a reduced complex I activity, but they exhibit increased complex II-dependent metabolism. Although *gas-1* and *mev-1* seem to be functionally related and they show common phenotypes, they clearly differ in other features. Unlike *gas-1*, *mev-1* is insensitive to volatile anesthetics but it is hypermutable (Hartman et al. 2001). The exact role of *gas-1* in aging is still unknown.

5.3.4 Males, not mutants but genetically different

C. elegans males have a normal set of autosomes but they have only one sex chromosome as a result of meiotic non-disjunction. This two-fold difference in X-chromosome number is able to trigger a developmental process that differs completely from the usual hermaphrodite development. The molecular mechanisms underlying many aspects of sex determination and male development and mating behavior have been revealed and are reviewed in Meyer (1997) and Emmons and Sternberg (1997).

An early study on male lifespan noted that mated *C. elegans* males live shorter than hermaphrodites because of the energy cost of producing additional sperm (Van Voorhies 1992). Frischknecht and Wedekind (1993) challenged this conclusion given that no lifespan extension was found in unmated *spe-26* (defective spermatogenesis) relative to unmated wild type males. Later, it was shown that mating reduces the lifespan of the hermaphrodites, but that male lifespan is unaffected by mating with hermaphrodites, and no sperm cost is involved (Gems and Riddle 1996). Surprisingly, solitary males lived longer than male groups showing male-male interaction, indicating that mating behavior between males reduces lifespan (Gems and Riddle 2000). The same study reported that the long lifespan of unmated males was independent of *daf-2* but dependent on *daf-16* activity. This might point to some male specific signal upregulating *daf-16* independently of *daf-2*, resulting in an extended constitutional male lifespan. An alternative explanation is that hermaphrodites possess a life-shortening signal that antagonizes *daf-16*, which is consistent with the results of Hsin and Kenyon discussed in section 5.3.1.2. Currently no metabolic data is available on male *C. elegans* populations. In addition, the importance of *daf-16* controlled ROS-scavenging enzymes on male longevity remains unexplored.

5.4 Environmental factors that influence aging

5.4.1 Stress resistance and longevity correlate.

During their entire lives, organisms have to cope with continuously fluctuating environmental features such as temperature, food availability, light, xenobiotics, pH and salinity (aquatic organisms) or humidity (terrestrial organisms). In order to survive the biggest oscillations, organisms are equipped with a physiological defense system. This stress response is used to prevent or repair damage to vital biomolecules. It has long been hypothesized that this system plays a key role in longevity (Kirkwood 1977, Kirkwood and Rose1991). This prediction seemed to be confirmed by the observation that the long-lived mutant *age-1* is resistant to hydrogen peroxide (Larsen 1993) and the free radical generator paraquat (Vanfleteren 1993) and that *age-1* and *daf-2* are both resistant to heat shock (Lithgow et al. 1994; Lithgow et al. 1995), UV (Murakami and Johnson 1996) and heavy metals (Barsyte et al. 2001). Exposing wild type animals to sublethal heat shocks induced thermotolerance and a small but significant lifespan increase (Lithgow et al. 1995). Recent work showed that over-expression of both HSP70F (Yokoyama et al. 2002) and HSP16A (Walker and Lithgow 2003) causes lifespan increase in *C. elegans*. Thus, an increase in thermotolerance (by genetic or environmental manipulation) appears to be causally connected to lifespan extension (reviewed by Muñoz 2003). As anticipated, stress resistance in Ins/IGF signaling mutants is dependent on *daf-16* activity (Murakami and Johnson 1996). Later on, the animals overexpressing *old-1* were added to the list of long-lived worms that have an increased thermotolerance (Murakami and Johnson 1998). However, thermotolerance by itself is not enough to extend lifespan, since the heat resistant *daf-4* and *daf-7* mutants are not long-lived (Lithgow et al. 1995). In *clk* mutants, the correlation between resistance to multiple stress factors and longevity is also fair (Johnson et al. 2001). An early study showed that the *e2519* allele of *clk-1* is resistant to UV stress in a *daf-16*-dependent fashion (Murakami and Johnson 1996), corroborating the interaction between the *clk-1* and the Ins/IGF pathway discussed in section 5.3.2.4.

How are longevity and stress resistance mechanistically connected? The Oxr (oxidative stress resistance) phenotype of Ins/IGF signaling mutants can be readily associated with their high SOD and catalase levels (Honda and Honda 1999; Houthoofd et al. in preparation; Larsen 1993; Vanfleteren 1993). The *clk-2* mutant shows a surprising pattern of stress resistance; despite its Age phenotype, it is sensitive to UV (*clk-2* is allelic to *rad-5* and encodes a DNA-damage checkpoint protein) but highly resistant to the free-radical generator juglone (Johnson et al. 2001). The juglone resistance might underlie the Age phenotype of *clk-2*, but cannot be attributed to increased SOD activity (Braeckman et al. 2002c). The Itt (intrinsic thermotolerance) phenotype of *age-1* and *daf-2* mutants can be linked to their enhanced expression of heat shock proteins (Lithgow and Walker 2002; Walker et al. 2001). *daf-2* and *age-1* resistance to heavy metals is correlated with enhanced expression of small cysteine-rich heavy-metal detoxifying proteins

called metallothioneins (Barsyte et al. 2001). Similar data on *clk* mutants is still lacking.

5.4.2 Hormesis

Hormesis is a very widespread biological phenomenon in which low doses of substances or treatments are known to have beneficial or stimulatory effects while they can be harmful at higher doses. Experiments on worms treated with sublethal heat pulses showed that mild and brief hyperthermia was able to extend longevity (Butov et al. 2001; Michalski et al. 2001; Lithgow et al. 1995; Yashin et al. 2001). Other treatments, such as hyperoxia and exposure to juglone resulted in the same hormetic effect. However, UV and ionizing radiation did not yield a hormetic effect (Cypser and Johnson 2002). The hormetic effects are believed to be a consequence of the successful activation of a stress response such as the upregulation of heat shock proteins and antioxidants (Verbeke et al. 2001). The almost universal beneficial effect of caloric restriction may be a special case of hormesis and will be discussed separately in section 5.4.5.

5.4.3 SOD and catalase mimetics

The oxidative damage theory of aging predicts that a dietary supply of antioxidants should postpone the aging process. Experiments in which *C. elegans* cultures were treated with vitamin E did not increase lifespan to a considerable extent (Adachi and Ishii 2000; Harrington and Harley 1988). Metalloporphyrin antioxidants exhibit catalytic SOD activity *in vitro*, making them much more powerful than the non-recyclable Vit E (Batinic-Haberle et al. 1997). Another class of synthetic catalytic compounds was designed to ameliorate oxidative stress in several disease models. These salen manganese compounds, designated EUK-8 and EUK-134, have high SOD activity and some catalase activity *in vitro* as well as *in vivo*, (Baudry et al. 1993). These compounds were administered to *C. elegans* cultures and were found to extend their lifespan by a mean of 44% (Melov et al 2000). These results suggested that oxidative stress is indeed an important lifespan determinant and that aging can be attenuated by pharmacological intervention. Oddly, no concentration effect of the mimetics was observed in this study. In a recent study, Keany and Gems (2003) replicated these experiments using EUK-8 from two independent sources and did not find any lifespan extension in *C. elegans*. On the contrary, higher doses of EUK-8 seemed to be toxic and to shorten worm lifespan. These results are consistent with similar experiments on houseflies (Bayne and Sohal 2002) and our own data on *C. elegans* (unpublished observations).

5.4.4 Temperature

C. elegans is an exothermic organism and its lifespan is negatively correlated with temperature in a physiologically acceptable range. Early work on temperature dependence and lifespan in nematodes was done using the close *C. elegans* relative *Turbatrix aceti* (Vogel 1974). A few years later, similar experiments were carried out for *C. elegans* and yielded the same correlation (Klass 1977). The temperature-dependency of development and lifespan may be interpreted as a mere thermodynamic feature since one would expect that, within certain limits, the rate of biochemical reactions should increase with temperature. Consequently, metabolic rate would also be expected to be positively correlated with temperature. By measuring CO_2 production, this correlation was experimentally verified (Van Voorhies and Ward 1999). In this study, however, metabolic rate was expressed per worm and possible size variation was not accounted for (see also section 5.2.7). Most intriguingly, *clk* mutants are unable to adjust their growth rate to temperature changes (Wong et al. 1995) suggesting that rate of development is not a mere thermodynamic, but a genetically controlled effect. Later studies revealed that apart from development, also behavioral rates, like defecation, were not adapted to temperature shifts in *clk* mutants (Branicky et al. 2001). It would be interesting to test whether the rate of aging (and thus lifespan) does not respond to temperature shifts in these *clk* mutants as well.

5.4.5 Caloric restriction

5.4.5.1 Introduction

Reducing caloric uptake (caloric restriction, CR) to approximately 60% of an ad libitum diet extends lifespan significantly in a wide variety of animals (Masoro 2000). From the moment of its discovery (McCay et al. 1935), CR has been extensively tested in several species ranging from yeast (Lin et al. 2000) to primates (Weindruch 1996, Mattison et al. 2003). Using a dilution series of bacteria in liquid culture medium, Klass (1977) was the first to illustrate the CR effect on *C. elegans*. The mechanism by which CR extends lifespan is still unknown but considering the universality of this phenomenon, it most likely involves an ancient molecular pathway. Several hypotheses have been forwarded to explain the beneficial effects of CR (reviewed in Masoro 2000), one of the most popular being the reduction of oxidative damage. It has been shown that CR decreases the rate of oxidative damage to DNA (Sohal et al. 1994), lipids (Matsuo et al. 1993), and proteins (Dubey et al. 1996). This made researchers to conclude that CR acts through a reduction of oxidative damage via a decrease of metabolic rate (Sohal and Weindruch 1996). An alternative hypothesis is that caloric restriction induces a life maintenance program that evolved to permit survival and to secure reproductive capacity under conditions of short-term food shortage, until conditions would improve again (Harrison and Archer 1989; Holliday 1989; Masoro and Austad 1996; Shanley and Kirkwood 2000).

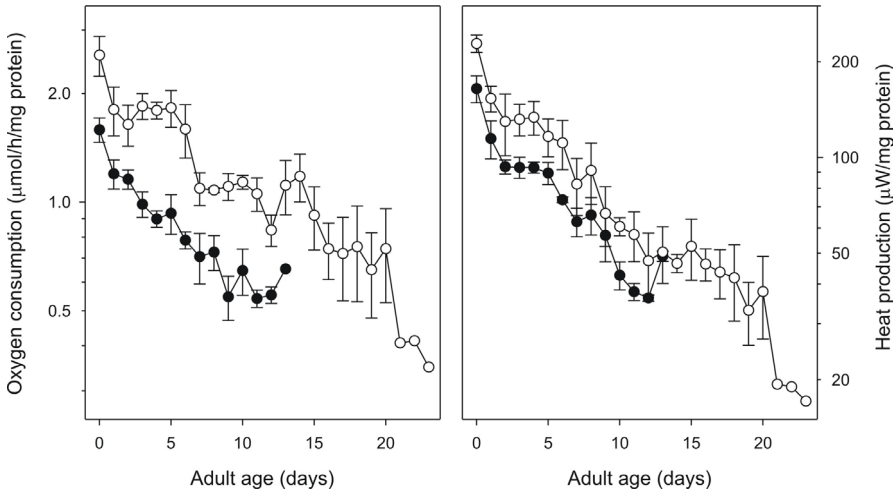

Fig. 7. Effect of caloric restriction caused by *eat* mutation on the rate of metabolism in *C. elegans*. Left: Oxygen consumption, right: heat production. Closed circles: Wild type (N2), open circles: *eat-2(ad1113)*.

5.4.5.2 Calorie restriction, metabolism, and stress resistance in C. elegans

A recent detailed study reports that, like in rats, the calorie-restricted *C. elegans* is not confined to a hypometabolic state (Houthoofd et al. 2002b). In this study, a set of worms were subjected to a bacterial gradient in liquid culture while another set harbored the *eat-2* mutation which reduces food intake by decreased pharyngeal pumping. The Eat mutants exhibited signs of a significant hypermetabolic state, measured as increased oxygen uptake and heat dissipation rates (Fig. 7). ATP-levels of Eat mutants did not differ significantly from the control strain. Heat fluxes, but not oxygen consumption rates, tended to decrease with increasing food supply in wild type and germline defective *glp-4* worms (Houthoofd et al. 2002b). ATP levels showed a clear positive correlation with bacterial concentration in the culture medium. The discrepancy between oxygen consumption, heat output, and ATP levels can be caused by several phenomena. For example, it cannot be ruled out that the difference between the oxygen consumption and heat dissipation profiles is due to a methodological artifact generated by the different conditions under which heat and oxygen measurements took place. The reduction of ATP with shrinking food supply might be due to increased ATP consumption for de novo synthesis of biomolecules that are otherwise supplied by the food. Since it was clear that metabolic rates were not reduced in calorie-restricted worms, Houthoofd et al. (2002b) measured the activity of the anti-oxidant enzymes catalase and SOD in the same experimental batch. The activity of both enzymes was upregulated in the Eat mutants that were kept in liquid culture but there was only a small (though significant) negative correlation between anti-oxidant enzyme activity and food

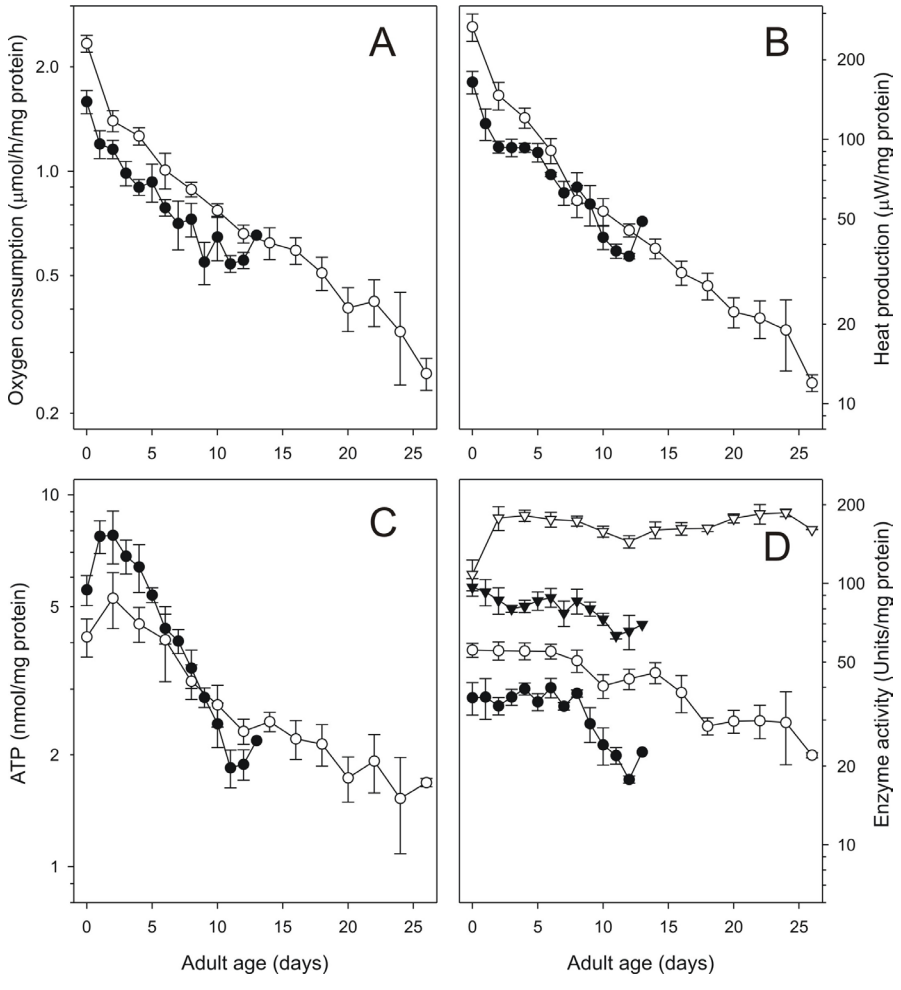

Fig. 8. Comparison of metabolic parameters of worms grown under monoxenic and axenic culture conditions. A : Oxygen consumption, B: heat production, C: ATP content, D: catalase activity (circles) and SOD activity (triangles). Closed symbols: wild type worms grown monoxenically, open symbols: wild type worms grown in axenic medium.

supply for wild type. For *glp-4*, this negative correlation was found only for SOD activity.

Worms cultured in axenic medium (a sterile mixture of 3% yeast extract, 3% soy peptone and 0.5 mg/ml hemoglobin) resemble calorie-restricted worms in several ways; they develop more slowly, are slender, have decreased and postponed fecundity, and most importantly, they live about twice as long. Clearly, the animals suffer a nutrition deficit in axenic culture, although they can be cultured indefinitely in this medium if serially transferred to fresh medium. Thus, no essential nutrients are lacking. Therefore, this culture medium has been used as an

alternative tool to study the effects of food restriction (axenic dietary restriction, ADR) on longevity in *C. elegans* (Houthoofd et al. 2002c). The results of this report revealed similar but not identical biochemical profiles to the previous study (Houthoofd et al. 2002b), suggesting that BDR (restriction by lowering bacterial food supply) and ADR are related and trigger similar survival responses. Both oxygen consumption rates and heat production were upregulated in worms that were cultured axenically (Figure 8A-B). ATP content was initially (i.e. in very young adults) low but the age-dependent decrease was less sharp as compared to ATP profiles from worms cultured on bacteria, resulting in higher ATP levels at an advanced age (Figure 8C). These low initial ATP levels were explained by the assumption that de novo synthesis of essential biomolecules, lacking in axenic medium, consumes considerable amounts of ATP. Catalase as well as SOD activities were upregulated over the entire lifespan of axenically cultured worms (Figure 8D).

The above-mentioned studies suggest that metabolism is not downregulated in food-restricted animals, but a stress response is likely to be activated instead, although to different extents depending on the 'type' of food restriction (Eat mutants, bacterial dilution series, axenic culture medium). Are worms submitted to dietary restriction more stress resistant? A series of worms cultured on a wide range of food supply were examined for H_2O_2 and paraquat resistance (Houthoofd et al. 2002b). Sensitivity to H_2O_2 remained unaltered over the entire food dilution range, which might reflect the minor effect on catalase upregulation that was observed in a similar experiment. Surprisingly, paraquat resistance was much higher at an abundant food concentration, not reflecting the SOD activity pattern that was found over the bacterial dilution series. A likely explanation is that paraquat-mediated superoxide anion formation, which is dependent on endogenous reductase and NADH/NADPH levels, was much higher in restricted cultures. Worms cultured in axenic medium were found to be much more resistant to heat stress than worms that were grown on a normal bacterial diet (Houthoofd et al. 2002c). It is very likely that, in axenic medium, a heat shock response is triggered, similar to what is found for longevity mutants such as *age-1* (see also section 5.4.1). It would be interesting to find out whether or not ADR also triggers oxidative stress resistance as suggested by the induction of high catalase and SOD activity.

5.4.5.3 Does caloric restriction act through the Ins/IGF signaling pathway?

In CR, the intake of calories (mainly carbohydrate and fat) is reduced. The Ins/IGF signaling pathway responds to insulin-like molecules and insulin is a regulator of glucose metabolism. Therefore, it is tempting to speculate that calorie restriction acts through an Ins/IGF signaling pathway. Apfeld and Kenyon (1999) showed that *C. elegans* mutants that are unable to sense food live longer and this lifespan extension was found to be *daf-16* dependent. The gene Sir-2 mediates lifespan extension in yeast in response to CR (Lin et al. 2000). Overexpressing the *C. elegans* homologue *sir-2.1* also extends worm lifespan, and Tissenbaum and Guarente (2001) suggest that *sir-2.1* silences genes upstream of *daf-16* in response

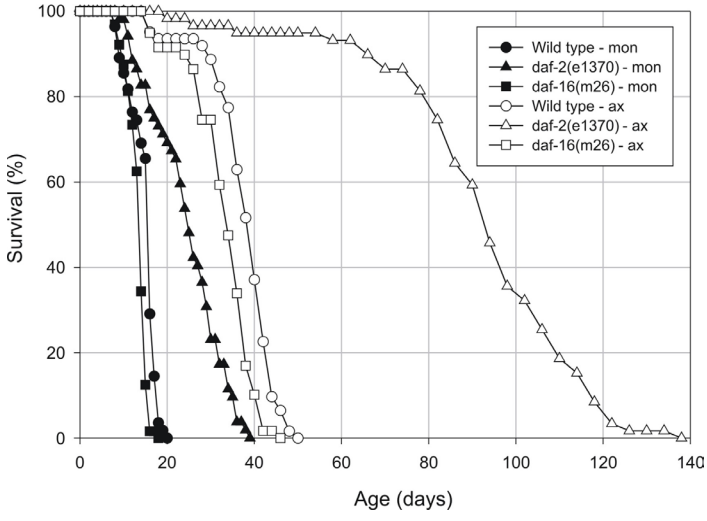

Fig. 9. Growth in axenic medium extends the lifespan of Ins/IGF mutants.

to CR. These experiments suggest that CR acts via Ins/IGF signaling. However, a group of other studies support the opposite hypothesis. Lifespan extension in worms bearing the *eat-2* mutation was shown to be independent of *daf-16* activity (Lakowski and Hekimi 1998). Moreover, the lifespan extension in *daf-2* and *eat-2* was additive, suggesting that they act through independent pathways. In another study, no nuclear localization of the DAF-16::GFP fusion protein could be observed in *eat-2* (Henderson and Johnson 2001). A recent *C. elegans* study showed that both ADR and BDR act synergistically with the Ins/IGF signaling pathway to extend lifespan, stress resistance and antioxidant activities (Houthoofd et al. 2003). The results of the survival experiments in this study confirmed the independency of longevity increase due to Ins/IGF-signaling and ADR (Fig. 9). In monoxenic culture, mutation in *daf-2* resulted in a life expectancy increase of about 69% over wild type. Axenic culture, however, increased *daf-2* longevity with an extra 274%. The combination of such genetic and environmental factors resulted in the longest lifespan of *C. elegans* ever reported (90.9 days, which is 6- to7-fold wild type lifespan in monoxenic culture at 24°C). In the same experiment, it was shown that *daf-16* activity was not required for longevity increase under ADR. Although less pronounced, similar results were obtained using bacterial dietary restriction. ADR is known to induce enhanced stress resistance (Houthoofd et al. 2002c) and experiments were run to assess if this is an Ins/IGF-dependent effect. Wild type animals and Ins/IGF-signaling mutants were grown monoxenically and axenically and subsequently subjected to heat stress (35°C). Thermotolerance of all tested strains was upregulated in axenic medium revealing that Ins/IGF signaling and axenic culture confers heat resistance via independent pathways (Fig. 10). In a similar experiment in which paraquat resistance was tested, ADR resulted in a modest increase of stress resistance, most likely due to sequestration of paraquat, and perhaps superoxide, by the bacteria in the monoxenic control experi-

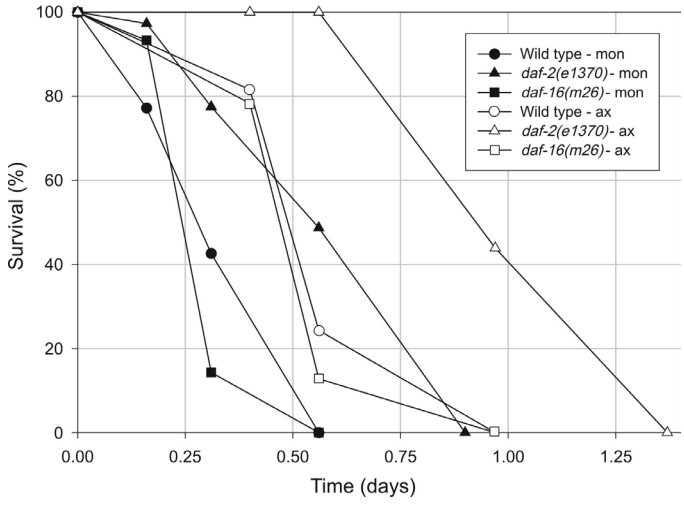

Fig. 10. Growth in axenic medium increases the heat resistance (35°C) of Ins/IGF mutants.

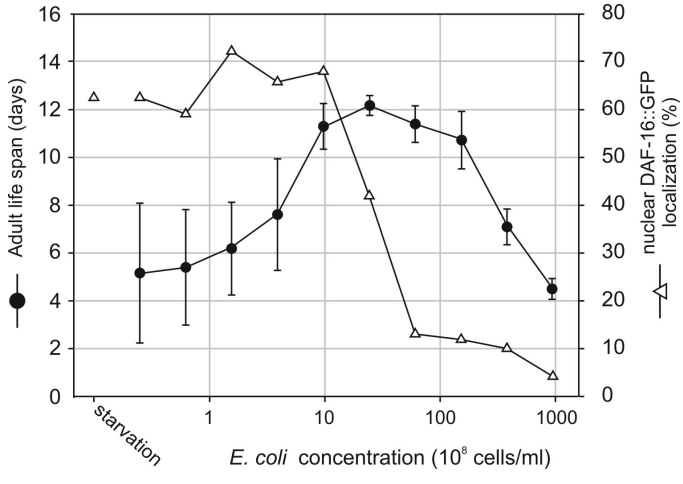

Fig. 11. Localization of DAF-16::GFP and lifespan of worms grown in different concentrations of *E. coli* in S buffer.

ment. On the other hand, it was clear that expression of the antioxidant enzyme activities of catalase and SOD was upregulated independently by Ins/IGF signaling and ADR treatment (Houthoofd et al. 2003). If CR acts through the Ins/IGF signaling pathway, DAF-16::GFP should relocate to the nucleus. Worms grown in axenic medium did not show obvious nuclear localization of DAF-16::GFP, however. A more complex picture arose in experiments on bacterial dietary restriction (Fig. 11); at bacterial concentrations higher than 6 x 10^9 cells/ml, DAF-16::GFP was not localized in the nucleus. When bacterial concentration was lowered to 2.4 x 10^9 cells/ml, about 42% of the worms showed nuclear DAF-16::GFP; this diet

yielded the greatest longevity increase. At lower bacterial concentrations, worms were starved, lifespan was decreased dramatically, and most worms showed nuclear localization of DAF-16::GFP. It was hypothesized that mild food reduction (CR) causes the activation of a longevity assurance program, independent of Ins/IGF signaling. In contrast, severe reduction of food (near starvation) likely elicits a *daf-16* dependent stress response.

5.5 Aging and mitochondria

5.5.1 Mitochondria and lifespan determination

The free radical theory of aging is still one of the leading aging theories and much effort has been spent to find a causal link between the rate of aging and free radical damage (reviewed in Golden et al. 2002). In aerobic organisms, the major sources of free radicals are the mitochondria and therefore these important organelles are assumed to have the largest impact on the aging process. A recent extensive study reported that RNAi inactivation of about 1.8% of the genes of chromosome I caused a reproducible longevity increase (Lee et al. 2003b). Interestingly, about 15% of this subgroup was specific for mitochondrial function, which is a tenfold over-representation, compared to the random distribution of biological functions over the six chromosomes. These results stress the importance of mitochondrial energetics in lifespan determination. Nevertheless, apart from the large proportion of genes involved in mitochondrial function, the remaining 85% of lifespan affecting genes have other cellular functions.

5.5.2 Metabolic rate and free radical generation

It is often assumed that mitochondrial superoxide production increases with increasing electron flow rates over the electron transport chain. In this view, high aerobic respiration should coalesce with increasing free radical generation. This, however, seems not to be the case (Korshunov et al. 1997; Brand 2000; Nicholls 2002). Metabolic control is complex and each step exerts some control over the total flux through the pathway (such as the electron transport chain, proton leak, and ATP synthesis). In fact, an inverse relationship exists between cellular energy demand and ROS generation; at low rates of respiration, membrane potential is very high which, in turn, results in high ROS generation. When respiration rates are high, the membrane potential drops slightly, but enough to dramatically decrease ROS generation. With this interesting fact in mind, the simple direct correlation between metabolic rate, free radical generation and lifespan is not tenable.

5.5.3 Mitochondrial impairment results in complex phenotypes

During a systematic RNAi screen of chromosome I genes, several genes involved in the respiratory chain were found to cause Clk and Age phenotypes when RNAi inactivated (Dillin et al. 2002b). RNAi knockouts for components of complex I, III and IV were found to be long-lived (see also Lee et al. 2003b) and showed a slow Clk phenotype, independent of *daf-16*. In these animals, respiration and ATP levels were lowered, consistent with a decreased function of a compromised electron transport chain (Dillin et al. 2002b; Lee et al. 2003b). Dillin et al. tested whether the hypometabolic state by itself is responsible for the extended longevity. They came to the surprising conclusion that it is not. By restricting the RNAi treatment to adulthood only, they found that animals were hypometabolic but lifespan did not differ from the control. Thus, hypometabolism per se, is not a determinant of lifespan (which is consistent with the results on *mev-1* metabolism discussed in section 5.3.3). Even more striking was the fact that, when RNAi was imposed only during development, worms kept their hypometabolic state during adulthood and still lived longer. Apparently, it is the metabolic status during development that influences lifespan, yet through an unknown mechanism.

Manipulating mitochondrial function does not result in a single well-defined phenotype. Depending on where and how severe the activity is changed; metabolic rate and lifespan can be altered to different extents and seemingly independently (Table 1).

5.6 Conclusions

Studying the genome of *C. elegans* revealed much of its potential function in aging. Altering the genome by mutation taught us that several genes are able to influence lifespan dramatically. Subsequently, several reports were published on the functional response of the genome during the aging process; microarrays were used to scan the transcriptome in order to detect differential expression of genes over age. Unfortunately, no clear picture could be detected yet. However, it is the metabolome that represents the base reality of cellular function (Fell, 2001). Studying metabolism in age cohorts of *C. elegans* reveals what is happening at the ultimate bottom end of cell biological hierarchy (i.e. the genome generals may have authority, nevertheless the metabolite soldiers are the ones that fight the war of aging). What did the metabolic studies teach us about the aging process in *C. elegans*? Against the anticipation of many researchers, long-lived *C. elegans* mutants do not show a lowered metabolic rate. Notwithstanding the low metabolic rate of 'enduring' dauer larvae, long-lived Daf mutants have metabolic rates that are similar to wild type although a shift towards higher catabolic efficiency seems to occur. This fact falsifies the rate-of-living hypothesis but not the free radical theory of aging. The free radical production might not be lower in long-lived Ins/IGF mutants, but their scavenging system is certainly more active and this prevents excessive oxidative damage. Dauers also have a powerful active anti-

Table 1. Phenotypes of Clk mutants and worms with compromised mitochondrial function.

Gene	Identity / Function	Phenotype				Reference
		Clk	**Age**	**energy metabolism**	**SOD**	
clk-1	ubiquinone synthesis - mtDNA replication?	+	+	0	-	a,b,c
clk-2	DNA-damage checkpoint protein	+	+	0	-	b,d
clk-3	?	+	+	0	-	b,e
gro-1	isopentenylpyrophosphate:tRNA transferase	+	+	0	0	b,f
isp-1	Iron-sulfur protein of complex III (electron transport)	+	+	-	?	g
mev-1	cytochrome b560 unit of complex II	?	-	-	-	h, i
gas-1	subunit of complex I	?	-	?	?	j
nuo-2	subunit of complex I	+	+	-	?	j
atp-3	subunit of complex V (ATP synthase)	+	+	-	?	j
cyc-1	subunit of complex III	+	+	-	?	j
cco-1	subunit of complex IV	+	+	-	?	j
D2030.4	B18 subunit complex I	?	+	-	?	k
T02H6.11	subunit of complex III	?	+	-	?	k
F26E4.6	VIIc subunit of complex IV	?	+	-	?	k
F26E4.9	Vb subunit of complex IV	?	+	-	?	k
W09C5.8	IV subunit of complex IV	?	+	-	?	k
B0261.4	mitochondrial ribosomal subunit	?	+	-	?	k
T06D8.6	cytochrome c heme lyase	?	+	-	?	k
F13.G3.7	mitochondrial carrier	?	+	-	?	k
K01C8.7	mitochondrial carrier	?	+	-	?	k
F28B3.5	1-acyl-glycerol-3-phosphate acyltransferase	?	+	-	?	k
F57B10.3	Phosphoglycerate mutase	?	+	-	?	k

[a]Ewbank et al. (1997), [b]Braeckman et al.(2002), [c]Gorbunova and Seluanov (2002), [d]Ahmed et al. (2001), [e]Wong et al. (1995), [f]Lemieux et al. (2001), [g]Feng et al. (2001), [h]Ishii et al. (1998), [i]Braeckman et al., this work, [j]Dillin et al.(2002b), [k]Lee et al.(2003b)

oxidant system and their low metabolism may be more related to the economic use of their finite fat stores rather than to extend longevity per se. Caloric restriction yields biochemical patterns similar to those observed for the Ins/IGF mutants, but it acts independently of this pathway because both life-extending interventions act synergistically. For Clk mutants, the picture is less clear. Mutation in any of the

Clk genes does not result in a clear metabolic downregulation or an upregulation of free radical scavengers. Lifespan extension in these mutants might be linked to their slow developmental rate analogous to the effects observed by a recent RNAi study (Dillin et al. 2002b). However, in this study, the RNAi treated animals were hypometabolic.

Will the study of a simple multicellular organism such as *C. elegans* provide any clues on how humans age? Considering the universal occurrence of aging and senescence throughout the animal kingdom, aging may have, like other complex processes such as development, a similar molecular basis in every animal. Recently, components of the Ins/IGF signaling pathway have been found to influence lifespan in yeast (Lin et al. 2000), *Drosophila* (Clancy et al. 2001), and mice (Holzenberger et al. 2003) as well (reviewed in Kenyon, 2001). These promising reports validate the presence of *C. elegans* at the biogerontological frontier. Not all aging-related phenomena found in *C. elegans* can be readily transferred to other models or humans, however. Some important findings such as the gonadal lifespan signal could not be reproduced in some other nematode species (Patel et al. 2002). Nevertheless, the immense possibilities that *C. elegans* offers over other, more complex models, should be used to explore the basic molecular mechanisms of aging. This will certainly lead to several hypotheses that can be tested more specifically in more complex systems.

Acknowledgements

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6 Do green plants age, and if so, how?

Howard Thomas

Abstract

Time-dependent ageing-like processes in green plants are discussed and compared to gerontological changes in animals and other organisms. The question of plant ageing is inseparable from the issue of the developmental architecture of plants. Modular structure and fractal, recursive patterns of plant development mean that the concept of an individual, and hence of whole-organism ageing, is ambiguous. Selective disposal of cells, tissues and organs, a major determinant of perenniality and hence lifespan, is a morphogenetic and adaptive tool that superficially resembles, but is fundamentally different from, ageing. The contrast between autotrophs and heterotrophs in the relation between resource acquisition and allocation is discussed, particularly partitioning between reproductive and vegetative development. Genetic, environmental, and epigenetic factors influencing ageing-like behaviour, including senescence, stress responses, somatic mutation and phase change, are considered. Finally, mechanisms are proposed for the origin of ageing as an intrinsic property of living cells.

6.1 Introduction

6.1.1 Why are plants of interest to gerontologists?

Green plants occupy a special niche in the field of gerontology. The yellowing, withering, and falling of leaves and other plant parts have been tropes for human ageing since poets and artists first indulged in what Ruskin (1856) called the 'Pathetic Fallacy'. Kerr, Wyllie, and Currie (1972) maintained this tradition when they introduced the term apoptosis, Greek for leaf drop, to describe programmed cell death in humans and animals. A second reason for gerontological interest in plants concerns the vast range of lifespan in the botanical world, from ephemerals that survive for a few weeks to the oldest living individuals on the planet (considered to be bristlecone pines of southeast California - Schulman 1958, Johnson and Johnson 1978). Comparing such extremes of longevity may be expected to provide insights into genetic and physiological factors underlying biological ageing. The relationship between reproduction and senescence is another feature of plant life cycles that seems to connect with patterns of ageing in many animal species. On the face of it, the death of the whole plant following flowering and fruiting in annuals and monocarpic perennials is similar in principle to suicidal reproduction

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in semelparous animals such as Pacific salmon, mayflies and many cephalopods (Patnaik et al. 2004, Carey 2002, Rocha et al. 2001).

6.1.2 The semantics of senescence and death

There is much confusion about the precise meanings of the various terms associated with terminal events in the lives of plants and their parts. The most striking symptom of plant senescence is the yellowing of green tissues, which in turn signifies radical alterations in the plastids of green cells. Thomas et al. (2003) pointed out that the conversion of chloroplasts to gerontoplasts is reversible, a property of senescing cells which, with other characteristic features, identifies this phase of plant development as a process of transdifferentiation or metaplasia and not one of deterioration. Both in its reversibility and in its absolute dependence on the maintenance of viability for initiation and progression, senescence is fundamentally different from programmed cell death. The timing and location of senescence is determined not only by transcription of senescence-related genes (Yoshida 2003), but also by regulatory events at the post-transcriptional and post-translational levels (Thomas and Donnison 2000, Dangl et al. 2000). Senescence reversibility means these processes must be under play-stop-rewind control, in contrast to the propagating one-way destructiveness of cell death mechanisms. Senescence and ageing are terms often used interchangeably in gerontology. In plants, senescence has a specialised meaning that relates to ageing in the sense that it is a time-based process of physiological change, but this change is not intrinsically, inevitably, or irreversibly deteriorative. This is in marked contrast to plant cell death, to which plant senescence is at best only distantly related (Thomas et al. 2003).

6.1.3 Criteria of viability and ageing

Related to the issue of the meanings of senescence, ageing, and death in the specific context of the plant life cycle is the question of how to tell if a plant, or one of its parts, is in an ageing condition. What criteria can be applied? What biomarkers are there that can be screened to provide an index of ageing? Genomics technologies may be expected to be informative and it is certainly true that characteristic differences in transcription pattern can be identified in pre-senescent and ageing human fibroblasts (Linskens et al. 1995). But what would such a readout of cells, tissues, or organs say about ageing in plants? For example, wood formation is a tissue death process and has a distinctive transcriptional profile (Hertzberg et al. 2001); but it is doubtful whether anything useful about ageing for a whole tree could be inferred from the molecular events associated with building the major part of its body, even if most of that body is itself dead. Interestingly, lengths of tracheids in the wood of individual bristlecone pines have been shown to have carried on increasing for more than 2000 years (Baas et al. 1986).

6.1.4 Distinguishing symptoms from causes of ageing

It would be useful to have a term, the equivalent of "gerontology", to cover the study of the causes and symptoms of dying as distinct from ageing. Classical Greek provides a suitable etymology. From Acheron, the river that runs through the chasm of the underworld, comes "acherontic", tottering on the brink of death ("...an old acherontic dizzard, that hath one foot in the grave..." - Burton 1624) - hence "acherontology". Examples of acherontological processes in plants include many kinds of post-harvest deterioration, malting, retting, and ensiling, as well as pre-digestive autolysis following herbivory as described by Beha et al. (2002).

Acherontology and gerontology are confronted with the same dilemma. Of the phenomena they define, which are symptoms and which are causes? For example, is vacuolar lysis in post-senescent leaf cells the agent of cell death or the consequence of lost viability? Is there such a condition as "slightly dead"? Is ageing the slow accumulation of acherontological events? How much of the active research area defined as Programmed Cell Death is really concerned with acherontology, and how many of the processes and mechanisms described are really post-mortem necrochemical changes (Thomas et al. 2003)?

6.1.5 Issues in plant ageing

In spite of the visibility and extreme expression of senescence and longevity in the plant kingdom, insights into ageing processes in plants do not seem to have had a particularly productive influence on understanding of human or animal ageing. Partly this is an inevitable consequence of the relatively tiny research effort on plants compared with that taking place in the biomedically-driven field of gerontology. But perhaps more significantly, it is questionable whether the mechanisms of ageing in plants are related other than very distantly to those of animals, or even whether plants undergo ageing in any gerontologically-recognisable sense (Thomas 2002). This conclusion arises from consideration of unique structural, functional, and genetic characteristics that equip plants to avoid, resist or exploit the inevitability of ageing.

6.2 Individual or population?

6.2.1 Body plan

Plants and animals differ in some fundamentals of organisation and development - for example, there is no differentiation into germline and soma in plants (Walbot 1985). Also of particular significance for ageing is the body plan, which in plants, is continuously expanding by the repetitive proliferation of structural units. Variation in the spatial arrangement of modules, or in the timing of initiation and development of these units, accounts for the vast range of plant form and life cycle

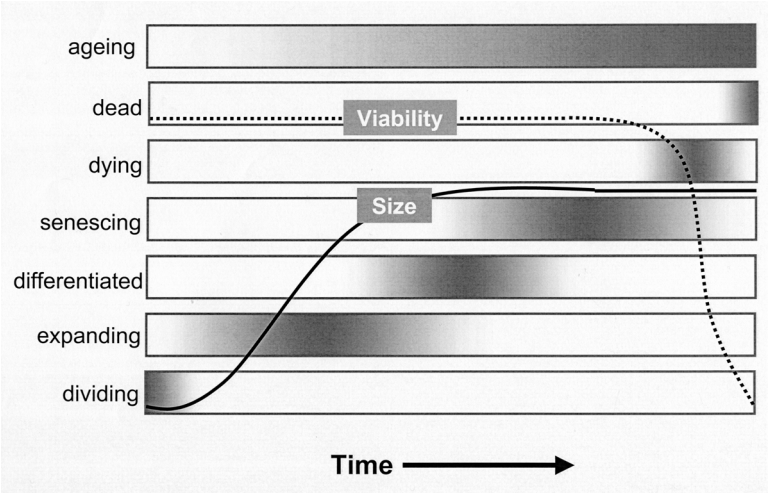

Fig. 1. Stages in the life-history of a plant and its parts. The sequence of events is reiterated at all levels in the hierarchy of plant organisation, from cells, tissues, and organs through to individual plants, communities, and even whole floras (Leopold 1975). Note that ageing includes, but is not limited or defined by, terminal senescence and death phases

(White 1979, Room et al. 1994). Individual plants behave as competing populations of genetically equivalent organs, interacting through hormonally-controlled vascular connections and internal competition for resources, a mode of organisation that ensures adaptation of the plant to heterogeneous environments (Sachs et al. 1993).

6.2.2 Fractal development

Plants are constructed from metamers, repeated units that are morphological homologues. In general, an organ or structural module follows a typical growth-curve reaching an asymptotic maximum and at some later stage, there is a period of senescence followed by death (Fig. 1). Just as organs pass through this sequence, so too do individual cells that make up its tissues. Organs, in turn, combine to impose the initiation-growth-senescence-death progression on the entire plant. As each metamer of an intact plant moves through the ageing sequence, its chemical composition, physiology, and complement of transcribed genes will change in a characteristic way and will provide measures of progress from birth to death. The interaction between different modules is the key to whole-plant longevity and the expression of mono- and poly-carpy (Thomas et al. 2000).

In this sense, plant development is fractal, a mode of organisation that has allowed the morphogenesis of real and imaginary plants to be dynamically modelled according to relatively simple heuristic principles (van Groenendael 1985, Prusinkiewicz and Lindenmayer 1990). Lindenmayer (L-) and similar systems share with fractals the property of emergence, in which complex structures are created

from simple starting data (Krieger 1996), and may therefore have some mechanistic basis as well as empirical value. For the purposes of the present discussion, it is enough to note that an important feature of the developmental hierarchy is the inclusion of a defined period of senescence at each level of organisation. The question of the relationship of this phase of cell, organ, and organism development to ageing is not straightforward and will be addressed in more detail later. For now, we propose that ageing comprises the time-based changes that occur throughout the life of the structural unit, which means that it includes, but is not identical with or bound by, the period of senescence (Fig. 1).

6.2.3 Plants as populations of parts

An individual plant at any given time will generally comprise a number of structural units each of which will be at a different point in its developmental or, we might say, ageing sequence. This has led some plant scientists, notably J L Harper and his colleagues, to argue that a plant is not an individual but rather a population of parts (Harper and White 1974, White 1979). Treating leaves, for example, as an age-structured population allows the application of analytical methods developed for demographic studies of individual organisms (Harper 1989a). Implicit in this approach is the idea that modules compete with each other (for resources, space, light and so on). Moreover, survival of individual structures will be determined by the intra-organismal equivalent of ecological fitness, and the higher-order organisation of the plant body (including, by extension, its ageing pattern) can be approached as a population phenomenon (Harper 1989b, Eissenstat and Yanai 1997).

6.2.4 What is an individual?

If a single plant is structured demographically as a population of metamers, can it be regarded as an individual for the purposes of understanding its ageing behaviour? This is a key question if we are to discover the mechanistic basis for the extremes of longevity observed in the plant kingdom. A human community that has survived in a particular place for 4000 years would be remarkable for anthropological reasons, but a 4000-year-old person would be a true gerontological wonder. The individual-as-population concept of plant organisation would argue that bristlecone pine is more like the former than the latter.

In many ways, a plant resembles a colonial animal, and not only in form. Martinez (1998) measured extremely low mortality rates in three hydra cohorts over four years and failed to find evidence for decline in reproductive rates over this period. It was concluded that, by constantly renewing the tissues of its body, this colonial metazoan probably escapes the deteriorative processes that increase the probability of death with increasing chronological age, and may therefore be potentially immortal. Interestingly, colonial cnidarians such as corals have been shown to contain lytic bodies that seem to have a role in digestion and cell senescence and these are located in the endosymbiotic algae that occupy the host cells

(Hawkridge et al. 2000). As described subsequently, expression of normally latent autolytic potential is a feature of senescence and cell death processes in terrestrial plants, so this may be another aspect of development and body organisation that plants and colonial animals share.

6.2.5 Scaling up and scaling down

Observation of ageing behaviour at a high level of organisation in order to identify causative time-dependent physiological processes in sub-components is a "scaling down" issue. There is an equivalent "scaling up" question concerned with the degree to which ageing is a holistic or gestalt phenomenon or else the sum of autonomous ageing events undergone by constituent structural units.

In some circumstances, events contributing to overall mortality are clearly cell- or organ-autonomous. For example if pathological cellular incidents such as hypersensitive reaction (Dangl et al. 2000) propagate extensively enough, death of the whole plant may occur. However, it is a much more subtle matter to determine whether whole-plant (or in deference to the metapopulation model, whole-module) ageing is an expression of the concerted ageing of individual cells (Kaplan and Hagemann 1991). The extreme totipotency of cultured plant cells is difficult to reconcile with the Swim-Hayflick concept that the limited lifespan of isolated animal cells in vitro is the cellular expression of the ageing process (Swim 1959, Hayflick and Moorhead 1961). Telomere attrition and apoptosis may offer plausible mechanistic explanations for cellular longevity (Bree et al. 2002); but the status and significance of both phenomena in plants are highly uncertain (see later).

There is strong evidence that individual organs of animals age at a rate determined by intrinsic factors rather than by whole-organism properties. For example, heterochronic transplantation in mammals shows that the transferred organ retains the age characteristics of the donor rather than the recipient (Krohn 1966, Hollander 1970). A contemporary perspective on this issue is given by the debate following the discovery (Shiels et al. 1999) that Dolly, the cloned sheep, showed signs of premature ageing. There are countless examples of the cloning of whole plants from single cells, excised tissues, or entire organs; but clear evidence is absent that reduction in vigour or lifespan in regenerants can be related directly to the age of the plant from which the original cell or tissue was taken. Although, there are plenty of observations of somaclonal variation contributing to ageing-like changes in vitro (Kaeppler et al. 2000). Organ transplantation studies have been carried out on tobacco plants. Leaves of flue-cured tobacco varieties senesce much slower than those of burley varieties, heritable behaviour which is related to differences in the efficiency with which nitrogen fertilizer is utilized by the two types (Crafts-Brandner et al. 1987). Nitrogen use efficiency is under simple two-locus genetic control (Henika 1932, Stines and Mann 1960). Crafts-Brandner et al. (1988) carried out reciprocal leaf grafting experiments with flue-cured and burley cultivars. They found that the grafted leaf retained the compositional characteristics of the donor variety but senescence rate was determined by the genotype of

the stock. These experiments indicate that intrinsic genetic control of organ senescence exerted indirectly through resource accumulation and use can be overridden by factors that coordinate senescence at the integrated, whole-plant level. The contrast in behaviour of transplanted organs exemplifies fundamental differences between animals and plants in their relationships to resources.

6.3 Ageing and plant life-form

6.3.1 Meristems

Plant anatomy and morphology are generated by differential cell division and expansion initiated in meristems (Meyerowitz 1997). Longevity depends on the activities of terminal shoot and root meristems and the lateral meristems at each node on the plant axis. A meristem may be indeterminate (capable of continued initiation of organs while retaining a population of uncommitted proliferative cells) or determinate (differentiating a terminal set of organs and simultaneously losing generative capacity). Death of a determinate meristem is frequently preceded by a (sometimes reversible) period of proliferative arrest (Bleecker and Patterson 1997). The apical meristems of perennials remain indeterminate for more than one growth season. With notable exceptions (Battey and Lyndon 1990, Thomas et al. 2000) the apical meristem of a vegetative shoot is commonly indeterminate; when it becomes reproductive and switches from generating vegetative structures such as leaves to reproductive structures (flower parts), it becomes determinate. The interaction between meristem determinacy and the sequential or progressive programmed senescence of lateral organs determines the longevity of the axis.

6.3.2 Annuality and perenniality

Raunkiaer (1934) introduced a classification of plant life-forms based on the survival of apical meristems in their active or dormant forms (Table 1). The various life-forms are characterised by the extent to which shoot axes persist (phanerophytes, chamaephytes), retrench (hemicryptophytes, cryptophytes), or die outright (therophytes) at the end of the growing season. Annuals and many biennials are therophytes. Meristem determinacy is an important, but not the only, factor in determining a plant's position in the Raunkiaer classification. The formation of resting structures and the progressive programmed senescence and death of organs are critical also.

Active chamaephytes and certain hemicryptophytes, for example creeping species like clover, are horizontal perennials (Thomas 1994). They forage for resources in their environment (Stephens and Krebs 1987, Grime and Hodgson 1987, Van Kleunen and Fischer 2001) by apical proliferation, elongation growth and subsequent tissue senescence, death and decay (Gallagher et al. 1997, Turner

Table 1. The classification of plant life-forms (based on Raunkiaer 1934)

Life-form	Definition	Types included
Phanerophytes	Generally tall plants visible throughout the year, carrying surviving buds or apices at least 25 cm up from the ground. Examples are trees, large shrubs and lianas	a) Evergreens without bud covering b) Evergreens with bud covering c) Deciduous with bud covering d) Less than 2 m high
Chamaephytes	Low growing plants visible all year round, bearing perennial buds between ground-level and 25 cm up. Examples include shrubby tundra species.	a) Suffruticose (woody at the base, herbaceous above) chamaephytes that bear erect shoots which die back to the portion that bears the surviving buds b) Passive chamaephytes with persistent weak shoots that trail on or near the ground c) Active chamaephytes that trail on or near the ground because they are persistent and have horizontally directly growth d) Cushion plants
Hemicryptophytes	The surviving buds or shoot apices are situated at or just below the soil surface. Includes perennial grasses, many forbs, and ferns.	a) Protohemicryptophytes with aerial shoots that bear normal foliage leaves, but of which the lower ones are less perfectly developed b) Partial rosette plants bearing most of their leaves (and the largest) on short internodes near ground level c) Rosette plants bearing all their foliage leaves in a basal rosette
Cryptophytes	At the end of the growing season, die back to bulbs, corms, rhizomes, or similar underground (in some species, underwater) structures. For example lilies, onions, garlic, potatoes, and similar forbs.	a) Geocryptophytes or geophytes which include forms with: (i) rhizomes; (ii) bulbs; (iii) stem tubers; and (iv) root tubers b) Marsh plants (helophytes) c) Aquatic plants (hydrophytes)
Therophytes	Plants that complete their life cycle from seed to seed and die within a season, or that germinate in fall, and reproduce and die in the spring of the following year.	

and Pollock 1998). The plant survives for as long as young proliferating tissues can keep ahead of the wave of senescence and tissue death behind them. The shoots of phanerophytes (shrubs and trees) also move out into the environment but do so in the vertical plane. Older tissues become senescent and die, but do not undergo post-mortem decay, persisting instead in the form of wood. Root systems forage through the soil and pass through the apical proliferation-growth-senescence-death sequence rather like inverted shoots of vertical perennials (Spaeth and Cortes 1995; Eissenstat and Yanai 1997). We may conclude that Raunkiaer's life-forms, as they relate to degree of annuality or perenniality, are a direct expression of the extent to which proliferation at apical meristems outpaces a pursuing wave of (programmed) tissue senescence and death.

Annual plants, which grow, reproduce, and die in a single season, seem to obey the "live fast, die young" rule (Kaufmann 1996). Biennials, which generally devote the first year to vegetative growth and the second year to reproduction and death, have life cycles that are qualitatively no different from those of annuals. There are also species such as *Agave*, which may survive for many years in the vegetative condition but then produce flowers and fruits and die. In all these cases of monocarpy (semelparity), there is clearly a relationship between reproduction and whole-organism death.

Amongst polycarpic (iteroparous) perennial species, where flowering and whole-plant senescence are not obligately linked, the range of lifespans is striking, ranging from less than 10 years in some herbaceous species to more than 2000 years in woody conifers (Table 2). Asexual reproduction propagates clones, which often remain attached to the parent plant and can proliferate to establish community-sized "individuals" of extraordinary longevity, maybe in excess of 10000 years (Table 2). In this respect, clonal plants resemble the huge underground hyphal networks of certain fungi, some of which may be even older (Smith et al. 1992). Clonal behaviour like this really does stretch the concept of organismal individuality beyond breaking-point and may not be especially illuminating when it comes to fathoming the functional basis of ageing.

6.3.3 Body piercing and body sculpture

The structural complexity of plants arises from repetition and variation in time and space between metamers (White 1979, Room et al. 1994). Structural modules turn over, that is, there is a flux of metamers through the plant body. In the case of shoots, this usually takes the form of recruitment by propagation of new metamers at terminal meristems, progression partly or entirely through the age-structured strata of the modular plant body and ultimate loss through programmed senescence and death (Thomas 1992). Turnover is, of course, a central factor in biology at all levels of organisation, from the subcellular (metabolism determines pool sizes and fluxes of intermediates through synthesis, interconversion, and breakdown) to the demographic (population structures defined by births, deaths and migrations). Indeed, Leopold (1975) visualised a continuum of biological turnover running from molecules to entire floras, with turnover at each level in this

Table 2. Maximal lifespans of individual and clonal plants (Nooden 1988)

Species	Age (years)
Single plants	
Bristlecone pine (*Pinus longaeva*)	4,600
Giant sequoia (*Sequoia gigantea*)	3,200
Huon pine (*Dacrydium franklinii*)	2,200+
Common juniper (*Juniperus communis*)	2,000
Stone pine (*Pinus cembra*)	1,200
Queensland kauri (*Agathis microstachya*)	1,060
European beech (*Fagus sylvatica*)	600-930
Olive (*Olea europaea*)	700
Scots pine (*Pinus silvestris*)	500
Pear (*Pyrus communis*)	300
Black walnut (*Juglans nigra*)	250
European ash (*Fraxinus excelsior*)	250
Apple (*Pyrus malus*)	200
English ivy (*Hedera helix*)	200
Arctic willow (*Salix arctica*)	130
Flowering dogwood (*Cornus florida*)	125
European white birch (*Betula verrucosa*)	120
Quaking aspen (*Populus tremuloides*) (ramet)	100
European grape (*Vitis vinifera*)	80-100
European cyclamen (*Cyclamen europaeum*) (tuber)	60
Scots heather (*Calluna vulgaris*)	42
Myrtle whortleberry (*Vaccinium myrtillus*)	28
Spring heath (*Erica carnea*)	21
European elder (*Sambucus racemosus*)	20
Eurasian solomon seal (*Polygonatum multiflorum*) (root stock)	16-17
Scandinavian thyme (*Thymus chamaedrys*)	14
Crossleaf heather (*Erica tetralix*)	10
Broadleaf solomon seal (*Polygonatum latifolium*) (root stock)	8
Yellow wood anemone (*Anemone ranunculoides*) (root stock)	7
Clonal plants	
Huckleberry (*Gaylussacia brachycerium*)	13,000+
Creosote (*Larrea tridentata*)	11,000+
Quaking aspen (*Populus tremuloides*)	10,000+
Bracken (*Pteridium aquilinum*)	1,400
Velvet grass (*Holcus mollis*)	1,000+
Sheep fescue (*Festuca ovina*)	1,000+
Red fescue (*Festuca rubra*)	1,000+
Ground pine (*Lycopodium complanatum*)	850
Lily of the valley (*Convallaria majalis*)	670+
Reed grass (*Calamagrostis epigeios*)	400+
Black spruce (*Picea mariana*)	330+

hierarchy conceived as being the integral of the turnover processes occurring below it, with an overlay of emergent properties. This view is essentially fractal and is of recurring value in attempting to define the relationship between plants, their parts, and the ageing process.

A unique feature of the modular developmental architecture of plants is the ultimate disposability of each and every structural unit. Disposability is programmed into plant development, which in turn implies the timely operation of programmes for the senescence and death of component cells, tissues and organs. Selective senescence and death is a creative force throughout the life of the plant and its parts (Bleecker and Patterson 1997). For example, pervasion by holes and tubes ensures the surface area:volume ratio of a tissue or organ is sufficient to sustain vital transport and exchange processes. The rigid wall immobilizes plant cells, which means that hole and tube formation by cell migration, such as occurs during gastrulation in animals, is not possible. Instead, tissue perforation in plants, like disposal of individual metamers, comes about through controlled, localised cell death. Thus, selective cell and tissue death are critical for plant architecture, adaptation, and life cycle. For example, hypersensitive response cell death is fundamental for plant reactions to biotic and other stressful challenges (Heath 2000). Localised cell death in apices and primordia is also decisive for the generation of organ form (e.g .Calderon-Urrea and Dellaporta 1999). Plant morphogenesis is not just origami - it employs scissors too.

6.3.4 Origins of lysigeny and schizogeny

The capacity for controlled autolysis is present even in single-celled and filamentous plants (Park et al. 1999, Moriyasu 1995) so it is reasonable to conclude that it probably arose very early in plant evolution. Certainly, the first terrestrial plants that colonised the land were already actively exploiting lysigeny (intracellular dissolution of protoplasm) and schizogeny (cell separation) to differentiate conducting tissues and shed reproductive structures and other parts (Raven 1986, Edwards 1993). The secretory pathway within plant cells (Hadlington and Denecke 2000) is critical for lysogeny and schizogeny. The vacuole represents "inner space", into which lytic enzymes and other components are secreted. The vacuole's role in terminal processes of cell development is more than simply to act as a leaky bag of aggressive catabolic enzymes (Thomas et al. 2003). Vacuolar accumulation of phenylpropanoid pigments accounts for the colour changes that occur in ripening fruit such as strawberry and in highly pigmented senescent leaves such as those of maples. The final products of chlorophyll catabolism are directed to the vacuole (Matile et al. 1999; Thomas et al. 2001). Sequestration of pigments and catabolites provides direct or indirect protection from photodamage (Matile et al. 1999, Feild et al. 2001). The vacuole also defends against pathogens and pests and is the destination for some of the pathogenesis-related proteins encoded by certain senescence up-regulated genes (Hanfrey et al. 1996; Thomas and Donnison 2000). At one time, it was thought that senescence in green plant cells resulted from vacuoles flooding the cytosol with hydrolases or even engulfing whole organelles such as chloroplasts. More plausibly, vacuoles perform a non-lytic protective role during senescence and only at the extreme stage of cell death (see Fig. 1) does release of vacuolar hydrolases occur. Autolysis of cell contents in the death phase of *Zinnia* tracheid transdifferentiation exemplifies the sequential control of vacuolar

function (Fukuda 1996). In schizogeny, the secretory system directs lytic enzymes to the "outer space" of the apoplast, where cleavage of bonds in the extracellular matrix weakens cell-cell adhesion, leading to cell separation and ultimate shedding of the tissue or organ (Roberts et al. 2002)

6.4 Ageing in relation to resource allocation

6.4.1 Ageing in autotrophs

Plant body-plan and life-form follow radically distinct design specifications from those of animals. Fundamentally different expressions of ageing in plants and animals are a direct consequence of contrasting structural and developmental principles. Another definitive difference between plants and animals concerns the acquisition and internal allocation of energy and raw materials. Heterotrophs have constantly to trade off investment in repair and maintenance against growth and reproduction, and the major theories of ageing have their mechanistic basis in this relationship (Kirkwood 2002). Clearly, no plant can survive if denied light, water, or nutrients for long enough (though tolerance of such deprivation can be astonishingly high in some cases - see Thomas and Sadras 2001). Furthermore, the productivity of many natural plant communities is commonly limited by one or more of these environmental inputs. Nevertheless, the appropriation and utilisation of resources by green plants follow sufficiently different rules from those of heterotrophs that they call into question the generality of "trade-off" theories of biological ageing. Obeso (2002) reported a number of case studies where the cost-of-reproduction model did not fit observations. For example, dioecious woody perennials seemed to fit the hypothesis, but dioecious herbs did not. Obeso concluded that plants were able to compensate for reproductive costs through the plasticity of assimilation and growth responses, somatic architecture and physiological integration. Thomas and Sadras (2001) described examples of apparently deliberate "inefficiency" in some characteristic developmental and metabolic processes in plants and argued that the evolutionary legacy of promiscuous resource capture has driven the adoption of apparently wasteful developmental and physiological adaptations.

6.4.2 Ageing as a starvation or neglect process

Individuals in a plant community interact with each other in a number of ways. They compete for nutrients, water, light, and space (Grime 2001). They sometimes conduct chemical warfare by exuding allelopathic compounds (Harborne 1993). There is evidence that they can communicate pathological danger to each other by emitting volatile signals (Tscharntke et al. 2001). There are parallels in the behaviour of metamers within an individual plant. For example, leaves are both assimilatory and storage organs. During senescence, the photosynthetic function declines

and redistribution of reserves becomes the dominant activity. Nutrient status is amongst the most important internal factors triggering the transition from assimilatory to mobilisation function. Young developing plant parts have a high requirement for N. When the appetites of growing tissues cannot be satisfied by import from the rhizosphere alone, N will be sourced from older tissues and distinctive senescence patterns will arise (Thomas et al. 2002). Transfer of nutrients from leaves to seeds and fruits is a feature of reproductive senescence. During seasonal senescence of deciduous trees, N is relocated to storage structures such as bark. N export to the growing apex is related to progressive or sequential senescence in the vegetative phase of development.

Although there is a clear relationship between senescence pattern and internal distribution of nutrients in the whole plant, it is doubtful that the latter is the cause of the former. This is discussed further in the context of reproduction-triggered senescence. On the other hand, there are instances when physical factors might influence viability and ageing by limiting supply of raw materials. For example, as a tree grows, increasing distances between the roots and the extremities of the crown impose increasing stress on the hydraulic functions of the vascular system. Ryan and Yoder (1997) considered this to be more likely than nutrient allocaton, respiratory patterns or increasing mutational load as a determinant of tree growth and form (and, by implication, ageing). Hubbard et al. (1999) showed that declining photosynthesis in older ponderosa pine trees is associated with decreases in hydraulic conductance and whole-tree sap flow. Significant as this mechanism might be for certain phanerophytes (Table 1), it clearly cannot be a general cause of plant ageing. Lanner and Connor (2001) could find no evidence for age-related deterioration in the function of xylem and phloem in bristlecone pines over the age range 23 to 4713 years.

6.4.3 Reproductive development and ageing

Senescence, as a consequence of exhaustion or starvation, was one of the original hypotheses proposed for the mechanism of monocarpy (Molisch 1938). It is certainly true that seed development depends on a supply of current fixed carbon, which in turn requires maintenance of the photosynthetic apparatus in source leaves. But for the growth of the same seeds, reduced nitrogen compounds must be provided to support synthesis of enzymes and storage proteins. The leaves that supply photosynthate are also the major potential sources of mobilised reduced N. In moncarpic species like soybean or sunflower (which have high-protein seeds), there is a clear functional conflict between the photosynthetic and protein storage functions of the foliage (Sadras et al. 1993).

Nevertheless, the evidence against nutrient diversion as a cause of whole-plant death in monocarpic species is strong and has been often reviewed (see, for example Thomas 1992, 2002, Nooden et al. 1997). An alternative hypothesis states that young sinks seek to satisfy their requirement for recycled nutrients by exporting a "death hormone" (Wilson 1997), which promotes senescence and remobilisation in source tissues. A related hypothesis proposes that older leaves are out-competed

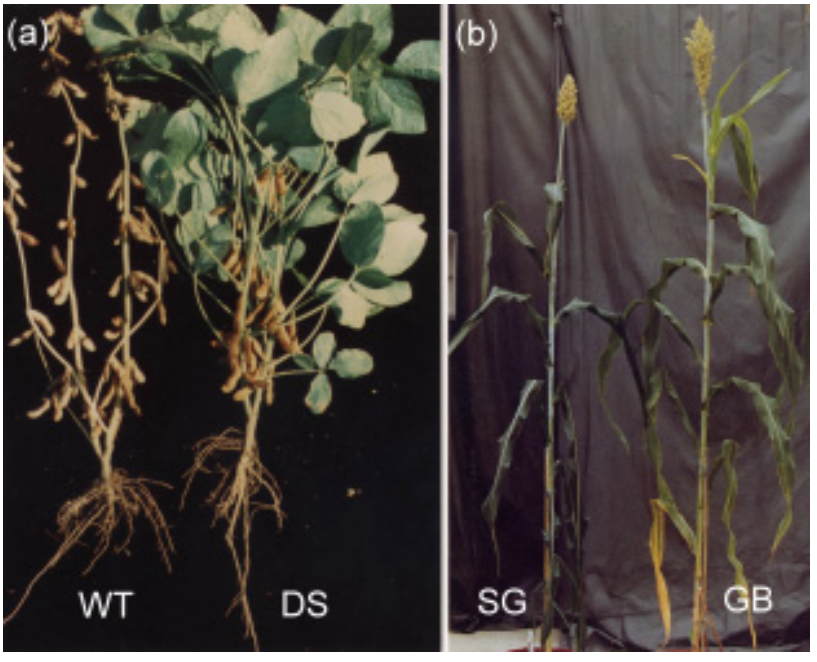

Fig. 2. Genetic disturbance of the relationship between reproduction and total senescence of vegetative parts. (a) Wild type (WT) soybean and an individual (DS) with alleles for early flowering and determinate growth habit, in which fruit set occurs without leaf senescence and abscission (Abu-Shakra et al. 1978, Thomas and Smart 1993). (b) Go-brown (GB) and stay-green (SG) lines of sorghum, a species in which retention of green leaf area during grain fill has been achieved by introgression of genes from polycarpic land race germplasm (Thomas and Howarth 2000).

by young tissues for "anti-death" hormones in the transport system (Nooden and Letham 1993). In many species, ethylene has some of the characteristics of the senescence-promoting hormone (Jing et al. 2002), while cytokinins exhibit many of the senescence-inhibiting properties of an anti-death hormone (Hwang and Sheen 2001). Surgical, physiological, and transgenic experiments have provided reasonable evidence in support of roles in senescence for either or both regulators (Dangl et al. 2000).

The nutrient-diversion and (anti-) death hormone hypotheses for the integrative control of senescence in the whole plant may not be fundamentally different, because many translocated raw materials (sucrose, nitrate for example) have both a nutritional and a morphogenetic role in plants. It is significant in this connection that a number of senescence-enhanced genes have been shown to be hormone and carbohydrate-regulated (Ono et al. 2001, Yoshida 2003). Thus, internal competition for resources is a factor in plant ageing by virtue of its direct influence on interacting developmental programmes, one of which specifies a senescence syndrome that is intrinsic, with variations, to every element in the modular plant body.

6.4.4 Evolution of reproductive habit in relation to ageing

Even in a species like soybean, which physiological models suggest may be obligately self-destructive at seed-set (Sinclair and deWit 1975), reproduction and overall senescence are readily unlinked by genetic means (Guiamét et al.1991, Thomas and Smart 1993, Nooden et al. 1997, Fig. 2). It is clear that the link has been repeatedly forged and broken during evolution, as annuality/moncarpy/semelparity has given way to perenniality/polycarpy/iteroparity within taxonomic groupings. In Charnov and Schaffer's (1973) view, the annual or ephemeral habit is favoured in hostile environments. Perennials invest in long-lived vegetative biomass rather than big-bang monocarpism and so tend to outcompete annuals in more benign habitats by closing the canopy and shading them out. The development of molecular systematics has allowed evolutionary trends in life-history within taxa to be reconstructed. Bena et al. (1998) have developed such a scheme for the genus Medicago and presented evidence that the ancestral form was a selfing annual, from which there has been recurrent evolution towards perenniality and outcrossing. Self-fertilization, which is much more common in annual than in perennial plants, may itself be an important factor in evolution of the annual habit (Zhang 2000). Silvertown et al. (2001) mined data on lifespan and fecundity for 65 species of polycarpic perennials to test hypotheses about the evolution of senescence and life-history traits. A conclusion from this work is that plant senescence rate is independent of initial mortality rate (in contrast to the relationship in animals) but positively related to reproductive lifespan (though fecundity generally did not decline with age). The implication that the risk of death increases with each additional cycle of reproduction is consistent with the quantitative genetic regulation of monocarpy/polycarpy as described by Thomas et al. (2000). Interestingly, these analyses suggested strongly that the clonal habit, particularly where clones fragment rather than remain physiologically integrated, is an effective means by which plants have escaped the evolution of senescence.

6.5 Genetics and epigenetics of plant ageing

6.5.1 Time and entropy

In engineering, the term stress describes an environmental factor which, when applied to an object or system, invokes a corresponding strain. Biologists have requisitioned the concept of stress and used it, not always very fastidiously, to describe the experience of non-optimal environments by living organisms. In a sense, time is a stress, though it differs from all other stresses in that, except at absolute zero, it is always present. Even when all controllable environmental influences are fixed or excluded, time accumulates as thermal time. Ageing is the biological response to time-stress. The physiology of individuals and their substructures is envisaged as reacting to non-optimal environments by invoking specific stress genes, stress

proteins, and stress metabolites. By the same token, it could be argued that ageing expresses the activities of time-stress genes and their products (Thomas 1994).

Viable organisms and their components can adopt any of three different strategies to deal with environmental stress: avoidance, resistance, or exploitation. Therophytes (Table 1) are stress-avoiders, whereas phanerophytes survive by resisting stress. All plants exploit stress to some degree or other - for example low seasonal temperature is used for time-measurement by winter-dormant structures such as seeds and buds. Indeed, many or even most plants are absolutely dependent on environmental deviations from optimality to cue normal progress through their developmental cycles. Moreover, as Thomas (1992) has pointed out, a common plant adaptation to stress is to mimic the state that lack of adaptation would have imposed. Thus, a winter dormant deciduous tree looks like a dead tree.

By analogy, for an organism not to succumb to time-stress (and hence ageing), it has the choice of avoidance, resistance, or exploitation. Time-stress can be avoided by outrunning it: that is, by growing, developing, and differentiating (avoid growing old by staying young). It can be resisted, by building-in structural and functional durability and by repairing wear and tear. Or time-stress can be pre-empted, through the adoption of programmed senescence as a developmental and adaptive resource so that ageing and death take place on the organism's own terms. Accordingly, genes with functions in ageing are of three kinds. Ageing avoidance genes include all the programmes for embryological development, structural and functional specialisation, and maturation. Ageing resistance genes regulate metabolic homeostasis, balanced turnover, macromolecular repair and maintenance, and resilience towards pathological influences such as diseases or free radicals. Pre-emptive or suicide genes function in the purposeful destruction of cells, tissues, and organs in defiance of entropy (Thomas 1994).

6.5.2 Programmes for cell death and senescence

We have seen, there are many ways by which plant cells and tissues can die. They may be programmed to die during normal development as part of processes that create complex organ shapes and specialized cell types. Alternatively, there may be necrotic death resulting from exposure to environmental deviations beyond the tissue's adaptive limits (Pennell and Lamb 1997). Another route to inviability is pathological programmed hypersensitivity (Heath 2000). A common fate for plant biomass is to be ingested by vertebrates or invertebrates, during which a distinctive type cell death process is triggered with far-reaching ecological implications (Kingston-Smith and Theodorou 2000). Then there is senescence in its special plant sense, a component of normal development intimately associated with, but significantly different from, autolytic, and/or pathological cell death. Figure 3 summarises the interrelationships and semi-independence of the physiological (senescence, cell specialisation) and acherontological (biotic and abiotic stress) routes to plant cell death (Thomas and Donnison 2000).

The genetic programs underlying these modes of impending mortality are becoming better understood. Some recent reviews covering regulation of the

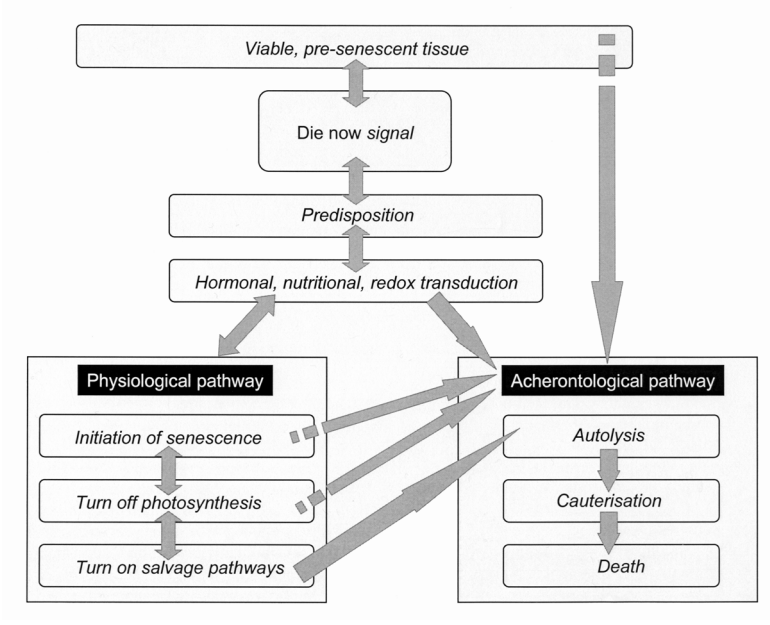

Fig. 3. Terminal processes in the life of plant tissues. The physiological (senescence) route and the acherontological (pathological) course can work in series or in parallel, making it possible for death to occur without senescence, or (through reversal of transdifferentiation) for senescence to happen without death. It is not known how much, if any, of the regulatory and metabolic machinery of senescence and cell death are common to the two pathways (modified from Thomas and Donnison 2000).

initiation and progress of programmed death in plants are: Jones and Dangl (1996), Pennell and Lamb (1997), Dangl et al. (2000), Ono et al. (2001), Yoshida (2003). Programs for cell death and senescence will not be discussed in detail here, but the relationship of such mechanisms to the wider issue of ageing may be noted. As discussed previously, in some cases, components of the syndrome of declining viability are likely to be at least partially acherontological. Others contribute directly or indirectly to the generation of form and complex function. The senescence of leaves and other green tissues is a (trans)differentiation rather than a deteriorative process. Take away these elements of programmed cell senescence and death in plants and very little remains that could be identified with a gerontological role.

6.5.3 Non-optimal environments and ageing

Seasonal plant senescence symbolises ageing, even though it is not itself a true gerontological process. Senescence happens to viable cells, tissues, and organs and, as a physiological activity, is responsive to non-optimal environmental condi-

tions. Seasonal senescence is strategic and anticipates future stress, exemplified by deciduous trees that sense declining day lengths after midsummer and initiate mass leaf senescence in preparation for winter. Senescence may also be tactical, deployed when an unpredictable environmental challenge is experienced, such as pathogen attack. A given stress may have one effect on the initiation of tactical or strategic senescence and a completely different influence on execution of the syndrome. For example, drought often invokes premature foliar senescence but reduces the rate of yellowing. If the stress develops quickly or severely enough it can overwhelm the tactical or strategic deployment of programmed senescence and divert the tissue directly into the acherontological pathway leading to death (Fig. 3).

Plants and their parts employ this kind of pre-emptive proxy ageing response to deal with many of the environmental challenges that are thought to contribute to gerontological changes in animals. For example, ultraviolet radiation induces deteriorative changes in plant tissues, including enhanced expression of genes associated with senescence (John et al. 2001). Reactive oxygen has moved centre stage in the drama of ageing mechanisms. On the animal side, there is a substantial body of evidence for pathological oxidation as a major player at the cellular level (Martin et al. 1996, Lithgow 2000, Finkel and Holbrook 2000). There is also a rapidly expanding literature on a range of effects of reactive oxygen in plant physiological processes, including claims that oxidative damage has a leading role in senescence and ageing. It is necessary for the present author to declare a prejudice: he is deeply sceptical about most of these claims and sees fundamental difficulties in most models of plant ageing based on reactive oxygen. Within plant cells, there are many sources of reactive oxygen species and in some cases; these become more active with age (e.g. Munné-Bosch and Alegre 2002). For example, superoxide anion or its disproportionation product are normal products of metabolism in peroxisomes, chloroplasts, and other cell compartments. Oxidative metabolism is active in and necessary for plant senescence to proceed normally. Senescence, which is an energy-demanding process, can be immediately suspended by treatment with respiratory poisons. In a more subtle way, senescence is also sensitive to cellular redox conditions (Chen et al. 1998). During senescence, peroxisomes redifferentiate into glyoxysomes (Nishimura et al. 1993) a transition that changes the profile of superoxide-producing and antioxidant enzymes (del Rio et al.1998). In these examples, reactive oxygen species act as components of regulated, integrated signalling and metabolic mechanisms in coherent, viable cells. When they break free of control and promote rapid destruction of cells through the propagation of free radical cascades, they become agents of acherontological change, which, as argued repeatedly, is distinct from ageing. Finally, it is an ironic fact that plants are generally so replete with defences against harmful build-up of reactive oxygen species that curative or anti-ageing claims are often made for cosmetics, herbal remedies, and functional foods containing plant products. It might therefore be argued (perhaps not wholly seriously) that it is difficult to see how reactive oxygen species could be effective in promoting the ageing of such well-defended organisms.

6.5.4 Chimeras and somatic mutations

The indeterminate meristems of a long-lived perennial plant would be expected to have accumulated somatic mutations and it is reasonable to conclude that these will contribute to the ageing and ultimate death of the whole individual. The age-related increase in the frequency with which chimeras and sports arise is evidence that such mutational events do occur; but the case for somatic mutation as a mechanism of whole-plant ageing is weak. There is good evidence that mutations of this sort can be important sources of adaptive fitness (Gill et al. 1995; Salomonson 1996; Pineda-Krch and Fagerstrom 1999). New genotypes better adapted to variable environments can arise, for example by a surge of transposon activation in meristems (Chaparro et al 1995; Walbot et al 1998). Klekowski (1988) modelled the genetic load characteristics of two fern species differing in the longevity of clonal genotypes based on the propagation of mutations in a microbial cell culture. Such models of genetic mosaicism in plants and other organisms with a modular, clonal architecture (Fischer and Van Kleunen 2001) show that intraorganismal selection is effective in purging most deleterious somatic mutations (eg Pineda-Krch and Fagerstrom 1999, Byers and Waller 1999, Orive 2001). The mutational load hypothesis of plant ageing has been put to the severest test by Lanner and Connor (2001) who assessed the frequency of mutations in the pollen, seed, and seedlings of bristlecone pines up to more than 4700 years old. They found no statistically significant relationship between mutation frequency and age of individual.

6.5.5 Telomeres and telomerases

Shortening of telomeres occurs during human differentiation and ageing (Harley et al.1990). The reverse transcriptase-type enzyme telomerase is responsible for maintenance of telomeres (Lundblad 1998). Loss of telomerase activity results in progressive reduction in telomere length until, at a critical point; chromosomal fusions and rearrangements become frequent enough to cause replicative senescence (Counter et al. 1992). All the elements of the telomere-telomerase system are present in plants, and there have been a few observations of apparent age-related shortening of telomeres in some species (Kilian et al. 1995). Nevertheless, mutagenic manipulation of plant telomerase activity has failed to show any consistent consequence for ageing or related processes. Riha et al. (2001) generated *Arabidopsis* mutants lacking telomerase that were able to survive for up to 10 generations. Beyond the 5th generation, there was progressive accumulation of severe cytogenetic defects, including end-to-end chromosome fusions and anaphase bridges. These late-generation plants exhibited malformations of organs and meristems and ultimately arrested in a vegetative and partially de-differentiated condition. Interestingly, the lifespans of mutants at this terminal stage were longer than comparable wild type individuals. The authors conclude that fundamental differences between animals and plants in their response to telomere disruption reflect differences in developmental and genomic architecture.

6.5.6 Phase-change

By all observable criteria, bristlecone pine shows no sign of undergoing an intrinsic ageing process (Lanner and Connor 2001). The present discussion concludes that this is true of green plants in general. Nevertheless, there is one aspect of plant development that arguably has some kind of relationship to ageing, namely phase-change, also referred to as maturation or heteroblasty (Grenwood 1995). Poethig (1990) described four phases or maturation stages in the life cycle: embryonic; post-embryonic juvenile; adult vegetative; adult reproductive. Each phase is associated with a characteristic package of morphological and physiological traits. If juvenile tissue of, for example, ivy (Bauer and Bauer 1980) is cultured and plants are regenerated from it, these plants have a stable juvenile phenotype; similarly, mature tissue yields regenerants with mature characteristics. Although juvenile and mature types are genetically identical, it is often so difficult to bring about reversion by external treatment that the phenotype appears to be fixed by some kind of epigenetic mechanism resembling genomic imprinting (Martienssen 1998, Kierszenbaum 2002). Studies of gene expression have identified a few phase-specific transcripts, a number of phase-change mutants have been described, and a vegetative-phase regulator has recently been described (Berardini et al. 2001). The latter was identified as the product of the *Arabidopsis* gene *SQUINT*. It is a homologue of cyclophilin 40, a component of the Hsp90 chaperone complex found in animals and yeast as well as plants. There is a long way to go before it will be possible even to frame a hypothesis about the mechanisms underlying phase change; but this area seems in some respects closer to the field of ageing research than many of the superficially related terminal developmental and acherontological plant processes that have been covered in this discussion.

6.6 Valediction

6.6.1 Poise

Within cells, aggressive lytic enzymes and metabolites are often physically separated from cytosol, for example by sequestration in lysosomes, vacuoles, zymogen bodies or the apoplast (Bursch 2001, Matile 1997, Donepudi and Grutter 2002; Lazure 2002, Hoson 2002). Of course, this does not mean that cytosol is therefore a benign environment in which macromolecules and their ligands enjoy a life free from the threat of destruction. On the contrary, the fidelity and fitness of molecules, complexes, and cell structures are continuously being tested by cytosolic systems that prowl the cell and pick off damaged, badly folded, mis-assembled, idle, inappropriate, or superfluous components (Thomas 1997). This means that cytosol is in reality a severe and stringent milieu and its survival critically depends on metabolic poising, which we recognise as cell viability. This poising is like that of a tightrope walker, inching along a potentially endless high-wire. For cells, the wire is time, and in the end, poise will not be enough, and the ropewalker will fall

one way or the other. Is this, writ large, what we call ageing? If so, and the author considers it could well be, then ageing is a non-negotiable property of protoplasm and inseparable from viability. Life proceeds and proliferates because living organisms have the means to re-establish poise. Sex is one way. For example, reinstatement of cellular poise can be clearly seen in pollen development, where cells undergo what Dickinson and Heslop-Harrison (1977) strikingly referred to as "cytoplasmic restandardization". Through a different route, involving intraorganismal cell selection at the somatic level, cytoplasmic stability is sustained in terminal meristems.

6.6.2 It's not what you do...

One of the great biological principles is that the development, adaptation, and survival of living organisms are the results of closing down options. The genome represents the impractical unedited totality of what the organism is capable of. Successful organisms do more than possess, express, and pass on the right genes - they refrain from expressing inappropriate potential. Music provides an analogy. Western music uses the 12 notes of the chromatic scale. Imagine sitting at the keyboard of an organ. Simultaneously hold down the 12 keys corresponding to the chromatic scale. Cease playing 3 minutes later. Within that cacophonous 3 minute block of sound are all possible 3-minute musical works. But "Tea for two" (Tatum 1933) is 3 minutes of musical genius, and why? Overwhelmingly, because of the notes that were not played. So it is with living organisms - the genome is the chromatic scale, the surviving organism is the harmonised musical line. The selectivity that orchestrates expression of genomic potential comprises cellular processes that repress and destroy. Might it not be that ageing is the long-term revelation of these negative, but nonetheless essential, forces that animate the machinery of living matter?

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7 Mammalian and bird aging, oxygen radicals, and restricted feeding

Gustavo Barja

Abstract

In this chapter, the relationship of aging with oxidative stress is reviewed. Endogenous tissue antioxidants do not determine aging because they correlate inversely with the maximum longevity of animals and the experimental modification of their levels can change survival and mean but not maximum lifespan. On the other hand, long-lived mammals or birds consistently show low rates of mitochondrial oxygen radical generation and a low degree of membrane fatty acid unsaturation, which can be responsible for their slower rates of aging. Oxidative damage to mitochondrial DNA is also lower in long-lived than in short-lived species. Caloric restriction, the only known manipulation, which decreases the rate of aging, also lowers mitochondrial ROS generation and oxidative damage to mitochondrial DNA in rodents. Such decrease in mitochondrial ROS production has been localized in complex I in rat heart and liver.

7.1 Introduction. Characteristics of the aging process

Aging is a degenerative process which progressively deteriorates biological systems, increasing their susceptibility to disease and finally to death. Aging causes a multitude of changes which affect a large number of aspects including morphology, physiology and behaviour at all levels of organization, molecular, cellular, tissular, organic and that of the whole individual. In humans, they include the easy to recognize external features such as greying of hair, wrinkling of skin, and changes in body shape and posture, as well as disorganization of tissular elements, decreases in maximum functional capacities, and higher susceptibility to stress, pathogenicity, and death. Although it is not a disease in itself, aging strongly increases the susceptibility to suffer many degenerative diseases. All those changes are thought to originate by a much smaller number of causes operating throughout the life of the individual. The early proposal by Denham Harman (1956) that free radicals are among those main causes is increasingly receiving support from published scientific studies. In this chapter, present information concerning the mitochondrial free radical theory of aging will be summarized and the relevance for aging of the rate of mitochondrial free radical attack to main targets like mitochondrial DNA (mtDNA) will be emphasized, focusing on mammals and birds.

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Table 1. Six traits of the aging process

Number	Trait	Reference
1	Aging is progressive	(1)
2	Aging is endogenous	(1)
3	Aging is universal	(1)
4	Aging is deleterious	(1)
5	Aging is irreversible	
6	Aging is best observed in post-mitotic cells	(2)

(1) Strehler 1962; (2) Miquel and Economos 1980

Other chapters of this book deal with invertebrate models like *C. elegans*, *Podospora anserina,* and yeast.

Gerontological knowledge is based on many kinds of studies including aging experiments, comparisons between species with different maximum longevities, comparisons between young and old animals, and theoretical approaches. Understanding the significance of those studies has been greatly facilitated by the enunciation by B.L. Strehler (1962) of the so-called "four rules of aging". They state that aging is progressive, universal, endogenous, and deleterious. The rules of aging are described below in a slightly modified form including some examples to illustrate them. These traits (Table 1) can be very useful to better design future research about aging and to help to interpret the already available data.

Aging is a progressive process. The rate of aging is approximately similar at different ages and thus aging changes occur throughout life in an approximately progressive way. Examples of this are the declines (Dice 1993; Masoro 1995) in many maximum physiological capacities with age (e.g. cardiac output, lung vital capacity, force and elasticity of the muscle-skeletal system, nervous coordination, etc.), with the accompanying progressive decrease in reserve capacities and homeostasis against may causes of stress. A consequence of this trait is that true causes of aging must be present throughout life in order to lead to those progressive changes. Thus, although the majority of aging studies look for changes in discrete parameters in old individuals, if they are causes of aging they must be present already in young adult individuals. Moreover, they should be present throughout the life of the mature adult (either young or old) at roughly the same levels. Thus, a decline in the levels of a putative antiaging substance occurring only at old age cannot obviously be a cause of aging. Also, an increase in a putative cause of aging with age would be incompatible with a progressive aging since it will lead to accelerated aging in old individuals. What should increase with age are the final consequences of the aging process, not the causes themselves.

Aging is an endogenous process. Even if animals are exposed to optimum conditions throughout life, they still experience the aging process at the rate typical of their species, because the causes of aging are endogenous. This basic concept is very useful to eliminate external factors when looking for causes of aging during scientific research. Thus, already on theoretical grounds, dietary antioxidants as vitamin E (or vitamin C in humans) cannot be a cause of the extraordinarily high longevity of human beings, whereas endogenously generated factors like reactive

oxygen species (ROS) can, in principle, be causes of aging. Similarly, external radiation (e.g. UV) cannot be considered a cause of aging even if it damages the skin of exposed individuals, because it is not endogenous and responsible for degenerative changes of the skin during aging of all the individuals of the species, exposed or not to UV radiation. The endogenous character of aging also means that, ultimately, the rate of aging and the maximum longevity of a species is primarily determined by its genes, not by the environment. This should be kept in mind when classifying theories of aging e.g. as stochastic versus genetically determined. Free radical theories are usually classified as stochastic due to the random nature of damage inflicted by endogenously generated ROS, but mitochondrial ROS are produced in the electron transport system of the respiratory chain, composed of proteins and lipids whose structures are genetically determined. Thus, the mitochondrial free radical theory of aging could also be classified as a genetic theory if the rate of ROS production of each animal species is, as it seems, genetically programmed.

Aging is a universal process. The concept of universality of aging originally referred to the occurrence of the aging process in all the individuals of a given species. This concept can also be extended to many animal species including at least the sexually reproducing metazoa (Bell 1984). Since aging probably has multiple causes, it is possible that different species exhibit different aging mechanisms quantitatively or even qualitatively. It is however logical to think that the closer the phylogenetic distance between species, the more similar their mechanisms of aging would be. This is generally accepted otherwise the vast majority of aging research would not be done in animals with the objective of extrapolating its deductions to humans. Thus, it is progressively more probable that aging mechanisms can be extrapolated to humans by studying poikilothermic vertebrates, homeothermic vertebrates, mammals, or primates than studying invertebrates like nematodes or insects, even though these last animals undoubtedly have other advantages for aging studies. The universal character of aging, thus, broadly conceived, is also the justification of the use of the comparative approach between animals of different species showing different maximum longevities. They help to search for causes of aging looking for them by comparing young adult individuals, in which they must be already present (see above). They also allow to eliminate traits suspected as causes of aging if they do not correlate with the maximum longevity of the different species included in the comparison or if they do not correlate with it in the appropriate sense (e.g. the inverse correlation observed between endogenous antioxidants and maximum longevity; see section 2). Thus, comparative studies constitute strong Popperian "falsity" tests after which the tested aging theory can survive and be strengthened or be rejected. Comparative aging studies help to select traits (and thus their determining genes) with possibilities of being causes of aging and longevity. They can be very useful before performing long-term expensive aging experiments in large numbers of control and experimental mammals to ascertain if the experimental manipulation of the selected trait can decrease the rate of aging and increase the maximum longevity. They can thus save a huge amount of resources and avoid losing a long time performing experimental aging studies about traits without real possibilities of being causes of aging. Various par-

ticular examples of the value of the comparative approach when testing the free radical theory of aging are given in the next sections. Comparative aging studies are based on the fact that differences in maximum longevity between species are primarily genetic, whereas differences in "longevity" between individuals of a given species is to a great extent environmental (heritability of human "longevity" is generally agreed to be only around 30%). In this second case, the term "longevity" of individuals undoubtedly has a very different meaning, simply the mean time from birth to death of the individuals, which is under the influence of a multitude of environmental factors like infections, accidents, exposure to toxic chemicals in air or food, or predation pressure.

Aging is obviously detrimental to the individual. As age advances, molecular and cellular deterioration leads to declines in maximum tissue functions thus decreasing homeostasis and capacity to adaptively react to stress. At the level of the species, the detrimental character of aging is not so obvious, in a sense, since it contributes to a continuous replacement of individuals in the population. This maintains a constant flux of new variability, which is the fundamental substrate of natural selection allowing adaptation to changing environments. Another trait of aging is its irreversible character. Aging changes individuals from young to old, but never on the reverse direction, at least by natural means. Theories of aging should also be able to explain this irreversibility.

Aging is best observed in post-mitotic cells. Among the somatic cells of the adult, the ones in which age changes manifest more clearly are the post-mitotic ones, like neurons, cardiac myocytes, or skeletal muscle fibres, whereas cells which divide continuously, like those of skin germinal epithelium, bone marrow or intestinal enterocytes are not altered during aging of the organism. In other organs, like liver, kidney, and many glands there are cellular divisions only when necessary for regeneration or repair and they do not change extensively during aging. The kinds of cells can profoundly affect the final outcome of e.g. their DNA mutations: while in post-mitotic cells they can lead to aging, in mitotic cells they can generate cancer, and in the germ line they can promote embryonic abnormalities or even evolutionary change. Thus, studies centred on aging should be ideally performed on post-mitotic cells in order to be fruitful, whereas studies performed in mitotic tissues would be of less relevance for aging. The important concept that aging is mainly patent in post-mitotic cells has been timely emphasized (Miquel and Economos 1980) in relation to the mitochondrial theory of aging. The reasons why cells with mitotic activity do not show aging-related changes are probably multiple. One is that in actively dividing tissues there is cell growth previously to cell division, the whole process leading to dilution of cell contents including their degenerative changes. At the same time, mitotic tissues maintain constant size by growth of new cells and death of heavily damaged cells (e.g. skin, enterocytes, or blood cells), thus leading to constant tissue renewal. This cellular selective process is not possible in post-mitotic tissues of old animals which are known to be mosaics of healthy and damaged cells. A similar phenomenon probably occurs in many other continuously dividing rodent "immortal" cell lines, free-living unicellular animals, or bacteria. They are commonly said to be "immortal", but what is immortal is the continuity of the group of cells, not the individual cells themselves

which are being constantly replaced, damaged cells being substituted by undamaged ones through selective cell death at least under conditions of constant total cell mass.

7.2 Antioxidants and longevity

Since free radicals are damaging substances that were proposed to be involved in aging (Harman 1956), it was originally thought that antioxidants, by eliminating free radicals, could also control the rate of aging. Antioxidant rather than endogenous prooxidant factors have been intensively studied in the past in relation to aging. The idea that aging could be a result of a decrease in antioxidants in old age was soon discarded when it was observed that the changes in endogenous antioxidants as a function of age did not follow a consistent pattern, showing decreases, increases, or lack of changes in old animals depending on the particular antioxidant, tissue or animal strain (Barja de Quiroga et al. 1992; Benzi and Moretti 1995). Early comparative studies, however, led some authors to suggest that antioxidants were longevity determinants (Tolmasoff et al. 1980). This was based on comparative studies of mammals with widely different maximum lifespan potentials (MLSPs), which showed that some tissue antioxidants like CuZnSOD (superoxide dismutase) were positively correlated with MLSP although only after dividing their value by the metabolic rate of the whole animal (Tolmasoff et al. 1980). Since the animals that were compared greatly differed in body size, and larger mammals have lower metabolic rates, the positive correlations observed with MLSP were mainly due to the lower oxygen consumption per Kg of the long-lived animals (which is well known since the early work of Max Rubner almost one century ago), not to higher levels of antioxidants. When tissue antioxidants were directly studied as a function of MLSP without that mathematical transformation, 10 out of the 12 independent investigations performed in seven different laboratories showed that endogenous antioxidant enzymes and low molecular weight antioxidants are negatively correlated with maximum longevity (see Pérez-Campo et al. 1998 for review), while in two studies no correlation was evident (Tolmasoff et al. 1980; Sohal et al. 1990a).

Experimental studies of antioxidant supplementation generally agree with the results of those comparative investigations. Out of 16 available studies on lifelong experimental modification of antioxidant levels, performed in large samples of animals using dietary supplementation, pharmacological induction, or transgenic techniques to increase the tissue antioxidants, 4 found some increase in MLSP (Epstein and Gershon 1972; Miquel 1975; Heidrick et al. 1984; Orr and Sohal 1994). Whereas in the 12 remaining studies, MLSP did not change (Kohn 1971; Clapp et al. 1979; Enesco and Verdone-Smith 1980; Ledvina and Hodanova 1980; Porta et al. 1980; Bozavic and Enesco 1986; Harris et al. 1990; López-Torres et al. 1993a,b; Orr and Sohal 1992; Seto et al. 1990; Staveley et al. 1990). The general trend for a lack of effect is even more evident in vertebrate animals, where out of eight investigations, only one described a small increase (12%) in

MLSP in mice (Heidrick et al. 1984). The seven remaining studies found no effect in frogs, mice, or rats (Kohn 1971; Clapp et al. 1979; Ledvina and Hodvanova 1980; Porta et al. 1980; Harris et al. 1990; López-Torres et al. 1993a,b; Orr and Sohal 1992; Seto et al. 1990; Staveley et al. 1990). In addition, 1.5- to 4-fold overexpression of CuZnSOD in different tissues of transgenic mice produces a strongly pathological phenotype affecting many vital organs and leads to similar or shorter MLSP and higher resistance to stress than in the control mice (Huang et al. 2000). In another investigation, 4- to 13-fold CuZnSOD overexpression in mice generated an array of neurodegenerative changes including swelling and vacuolization of mitochondria in neurons, axonal degeneration and loss of spinal motoneurons during aging (Jaarsma et al. 2000). A lack of positive effect of GSH-reductase overexpression on MLSP together with increased resistance to stress has been recently described in *Drosophila melanogaster* (Mockett et al. 1999), whereas overexpression of MnSOD in mice increased (Wispe et al. 1992) or did not change (Ho 1994) resistance to hyperoxia. In the case of mean lifespan, an increase in its value in antioxidant-treated or antioxidant-induced animals was a much more frequent finding in the studies described above. Those increases in mean lifespan suggest that antioxidants can non-specifically protect against many causes of early death - they can increase survival - especially when the experiments are performed under suboptimum conditions. Part of this effect can be due to the capacity of antioxidants to inductively react and then protect against increases in oxidative stress of exogenous origin. These protective effects will be important in avoiding early death in human populations that live in suboptimum environmental conditions and have less than rectangular survival curves. However, the general lack of effect of antioxidants on MLSP indicates that they do not slow the intrinsic aging process. Many experiments recently performed in SOD knockout mice also support this concept. Thus, aging rate does not seem to change in homozygous CuZnSOD (Reaume et al. 1996; Shefner et al. 1999), extracellular SOD (Carlsson et al. 1995), GSH-peroxidase (Ho et al. 1997a), or heterozygous MnSOD (Tsan et al. 1998) knockout mutant mice; the effects are limited to lack of modification (Tsan et al. 1998; Ho et al. 1997b) or increased susceptibility to higher than normal stress (Reaume et al. 1996; Carlsson et al. 1995; Ohlemiller et al. 1999; de Haan et al. 1998). In the case of homozygous MnSOD knockout mice, the lack of this enzyme produces a strongly pathological phenotype, very different from normal aging, and leads to death with dilated cardiomyopathy a few days after birth (Li et al. 1995; Melov et al. 1999a). Thus, although possibly affecting survival and resistance to stress, neither increasing nor decreasing tissue antioxidants changes the fundamental rate of aging or maximum longevity.

7.3 Mitochondrial oxygen radical generation, maximum longevity, and caloric restriction

If antioxidants do not control aging, what is the oxidative stress-related parameter involved in such control? The negative correlation of endogenous antioxidant lev-

els with MLSP led to hypothesize that the rate of free radical production should also be smaller in long-lived than in short-lived animals (López-Torres et al. 1993c; Barja et al. 1994a). A general compensation between H_2O_2 generation and scavenging would occur both in short- and long-lived animals, allowing tissue homeostasis and thus short-term survival in both cases, the cellular ROS turnover being high in the first and low in the second kind of animals. But the local concentration of ROS near targets relevant for aging (like mtDNA) situated close to the places of ROS generation (the inner mitochondrial membrane) would be lower in long-lived animals due to their lower rates of ROS production; this would result in a lower rate of oxidative damage to mtDNA and a slow rate of aging (Barja et al. 1994a). The rates of mitochondrial ROS generation and the level of oxidative DNA damage were studied in mammals and birds with different longevities in order to test that hypothesis.

Initial studies on the possible relationship between mitochondrial free radical production and longevity compared mammals with different MLSPs strongly differing in body size: mouse, rat, guinea pig, rabbit, pig, and cow (Sohal et al. 1990b). These are species, which "follow" the rate of living theory, the inverse relationship between basal specific metabolic rate (flux of calories per unit time per unit body weight) and MLSP (Rubner 1908; Pearl 1928). The results showed a negative exponential correlation between liver submitochondrial particle $O_2^{\cdot-}$ production (Sohal et al. 1989) or mitochondrial H_2O_2 production (Sohal et al. 1990b) and MLSP in those mammals. Similar negative relationships in kidney and heart mitochondria of the same species plus hamsters were found afterwards (Ku et al. 1993). However, since the included species followed the rate of living theory, the results obtained could also be interpreted, in principle, as a correlate of that phenomenon: the species with short MLSP could show high mitochondrial H_2O_2 production simply because their rates of mitochondrial oxygen consumption were also higher. Positive correlations between mitochondrial oxygen consumption and oxygen radical production and between mitochondrial oxygen radical production and metabolic rate were indeed found by the authors in those species (Ku et al. 1993). Therefore, those studies cannot discard the possibility that the correlations observed between mitochondrial oxygen radical production and MLSP can be due to the correlation observed between mitochondrial oxygen radical production and metabolic rate. This last rate could be also correlated, in turn, with many other unknown factors, some of which possibly causing aging, since hundreds of different reactions occur at an accelerated rate when the metabolic rate is high. This is why the mitochondrial H_2O_2 production of birds, animals with both a high rate of oxygen consumption and a high MLSP was studied. The possession of an extraordinarily high longevity in relation to body size or metabolic rate, compared to the majority of mammals following the rate of living phenomenon, is shown only by three groups of homeothermic vertebrates (Prinzinger 1993): birds, bats, and primates. It was therefore hypothesized that, if a low rate of oxygen radical production contributes to a slow aging rate in birds, their mitochondria should show a low rate of H_2O_2 production in spite of the high rate oxygen consumption of these animals (López-Torres et al. 1993c; Barja et al. 1994a). It was indeed found, that the succinate-supported rate of H_2O_2 production of crude brain, lung, and liver mi-

tochondria was lower in pigeons (MLSP = 35 years) than in rats (MLSP = 4 years), even though metabolic rate and body size are of a similar order of magnitude in these two vertebrate species (Barja et al. 1994b). A lower succinate-supported H_2O_2 production was also independently described in the pigeon than in the rat in heart, crude brain and kidney mitochondria (Ku and Sohal 1993). Posterior studies found also lower H_2O_2 production in pigeon than in rat heart mitochondria with pyruvate/malate (complex I-linked substrate) and succinate (Herrero and Barja 1997), and in pigeon than in rat nonsynaptic brain mitochondria with pyruvate/malate (Barja and Herrero 1998). The lower rate of oxygen radical generation of pigeon *versus* rat mitochondria, observed in all the tissues studied, agrees with the mitochondrial free radical theory of aging, taking into account the MLSPs of the two species selected.

Nevertheless, the lower rate of mitochondrial ROS of pigeons could be related, in principle, to other characteristics of this particular animal species distinct from their high longevity. Trying to test if birds in general have a low rate of oxygen radical production, similar studies were performed in other birds belonging to different orders than that of pigeons (Columbiformes). With this purpose, heart mitochondria were isolated from mice (MLSP = 3.5 years), canaries (MLSP = 24 years; Order Passeriformes), and parakeets (MLSP = 21 years; Order Psittaciformes). These three vertebrate homeothermic species also show, like in the rat-pigeon comparison, similar values of metabolic rate and body size but a very different MLSP. The results showed that the rate of H_2O_2 production of pyruvate/malate-supplemented heart mitochondria is also significantly lower in canaries and parakeets than in mice (Herrero and Barja 1998). This suggests that a low rate of oxygen radical production is a general characteristic of the mitochondria of birds, exceptionally long-lived animals. A closer interspecies comparison has shown that mitochondrial H_2O_2 production is also lower in the white-footed mouse *Peromyscus leucopus* (MLSP=8 years) than in the house mouse *Mus musculus* (MLSP=3.5 years; Sohal et al. 1993), whereas the metabolic rate is similar again in these two species. Noticeably, the different investigations performed in mammals and birds show that mitochondrial ROS generation is always lower in long-lived than in short-lived animals, which agrees with the mitochondrial free radical theory of aging. A negative correlation between mitochondrial ROS production and longevity has been also described in five insect species (Sohal et al. 1995). On the other hand, in the majority of the bird-mammal comparisons, the difference in mitochondrial H_2O_2 production between species was generally smaller than their difference in MLSP. This would be consistent with the idea, prevalent in gerontology, that aging is due to more than one single major cause. The opposite kind of comparison can also be of value: mammals with similar MLSPs but different metabolic rates and body sizes, like the rat and the mouse, should show similar rates of mitochondrial oxygen radical generation if this is a main determinant of aging rate. Similar rates of H_2O_2 production have been found indeed in heart mitochondria of rats and mice (Herrero and Barja 1997, 1998). This is consistent with their very similar MLSPs (3.5 and 4 years), whereas the metabolic rate is 3-fold higher and the body size is around 15-fold lower in the mouse than in the rat. Apart from the rate of mitochondrial ROS production, only

another known characteristic connects aging and oxidative stress: long-lived mammals and birds constitutively have low degrees of fatty acid unsaturation in their cellular membranes in most tissues. Unsaturated fatty acids are the cellular macromolecules most sensitive to free radical damage, and this sensitivity increases strongly as a function of the number of double bonds per fatty acid. Thus, the low degree of unsaturation of the cellular membranes of long-lived animals strongly decreases their susceptibility to lipid peroxidation and lipid peroxidation-derived damage to other macromolecules (see Pamplona et al. 2002 for review).

What are the causes of the low H_2O_2 production of bird mitochondria? Various studies have shown that, in many cases, bird mitochondria generate less ROS per unit oxygen consumption (they have a lower free radical leak) than those of rodents of similar size and metabolic rate (Barja et al. 1994b; Herrero and Barja 1997; 1998; Barja and Herrero 1998). In the case of nonsynaptic brain mitochondria, the mechanism responsible for this was the lower degree of electronic reduction of the complex I oxygen radical generator observed in the bird (Barja and Herrero 1998). In heart mitochondria, a second additional mechanism to decrease ROS production operates or not depending on the bird species: a low rate of mitochondrial oxygen consumption per mg of mitochondrial protein, evolutionarily compensated with a larger heart size to allow the maintenance of a cardiac output at rest similar to that of mammals (Herrero and Barja 1997, 1998). Canary heart emphasized the first mechanism (low free radical leak), parakeet heart emphasized the second one (low mitochondrial oxygen consumption and a large heart size), and pigeon heart mitochondria exhibited both mechanisms. Decreasing the free radical leak is a very interesting way of slowing the rate of mitochondrial oxygen radical production for future hypothetical manipulations, because the ultimate purpose of basic gerontological studies is to decrease the rate of human aging without lowering the metabolic rate, and thus the general level of activity. Besides, the presence of different values of free radical leak in the mitochondrial respiratory chain of different animal species shows that the amount of electrons diverted to ROS generation is not a fixed percentage of total electron flow, as it is frequently assumed. The species-specific character of the free radical leak indicates that mitochondrial oxygen radical production is not a simple "by-product" of mitochondrial respiration. Instead, the rate of oxygen radical generation seems to be a parameter regulated at a different level in each animal species in relation to its maximum longevity.

Another relevant issue is the place in the respiratory chain where such species-specific regulation of free radical generation takes place. The site in the respiratory chain where the longevity-related difference in mitochondrial ROS production occurs has been localized in two species with very different MLSPs. Although complex III semiquinones of heart mitochondria can produce ROS (Boveris and Cadenas 2000), in agreement with data from submitochondrial particles (Takeshige and Minakami 1979; Turrens and Boveris 1980), it was observed that complex I also contains an important ROS generator in intact functional heart and non-synaptic brain mitochondria (Herrero and Barja 1997; 1998; Barja and Herrero 1998). This was recently confirmed by another laboratory (Genova et al. 2001). But although both complexes I and III can produce ROS, it was found that

the respiratory complex responsible for the lower ROS generation of pigeon in relation to rat heart mitochondria is complex I, not complex III, because the difference in ROS production between both species with succinate as substrate disappears after addition of rotenone (Herrero and Barja 1997). Further studies localized the ROS generator in the electron pathway inside complex I in heart and brain mitochondria between the ferricyanide reduction site and the rotenone binding site, which discards the flavin and suggests that the source of ROS can be the complex I FeS clusters situated in that area (Herrero and Barja 2000). Recent studies, studies using different methodologic approaches, have reached a similar conclusion (Genova et al. 2001; Kushnareva et al. 2002).

Restriction in the intake of food calories is the only known experimental manipulation that can increase MLSP in mammals. It was recently investigated if changes in mitochondrial ROS production are also implicated on the life extension effect of caloric restriction. This was studied both on the short-term (6 weeks) and the long-term (1 year) in the heart of young and old rats and new information concerning whether, where, and how mitochondrial ROS generation is modified in caloric restriction emerged (Gredilla et al. 2001a). Short-term caloric restriction did not change any of the parameters measured. However, long-term caloric restriction significantly decreased the rate of mitochondrial ROS generation with pyruvate/malate as substrate (Gredilla et al. 2001a). The decrease in mitochondrial ROS generation in caloric restricted rats generally agrees with a previous report in mice (Sohal et al. 1994). In contrast, neither the expression (Weindruch et al. 2001) nor the activity (Sohal et al. 1994) of the antioxidant enzymes SOD, catalase, or GSH-Px are modified in a consistent way by caloric restriction. On the other hand, in contrast with a previous report in mice (Sohal et al. 1994), the effect of caloric restriction in rats was not to avoid increases in ROS production with age. Instead, mitochondrial ROS production was strongly decreased by caloric restriction below the basal levels of young animals fed ad libitum (Gredilla et al. 2001a). This is consistent with the possibility that such decrease can be a basic mechanism responsible for the slow aging rate of caloric restricted animals. On the other hand, although heart mitochondria produce ROS at complexes I and III, the site where caloric restriction decreases oxygen radical generation was located exclusively at complex I, because the decrease in H_2O_2 production occurred with pyruvate/malate but not with succinate plus rotenone as substrate (Gredilla et al. 2001a). This finding is reminiscent of what was found when comparing pigeon and rat heart mitochondria. The mechanism allowing the decrease in ROS production in caloric restriction was not a simple decline in mitochondrial O_2 consumption since it did not change. Instead, the percentage free radical leak was lowered by caloric restriction due to a decrease in the degree of electronic reduction of the complex I generator in the steady state. This is again similar to one of the mechanisms responsible for interspecific differences in ROS production between species with different MLSPs (see above). Essentially similar results were subsequently observed in rat liver mitochondria (López-Torres et al. 2002) with the main difference that the decreases in ROS production occurred already after six weeks of caloric restriction (Gredilla et al. 2001b). A decrease in electronic reduction of the complex I generator can be due to an increase in its concentration since the same

electron flow would be distributed among more complex I copies. Recent microarray data show that gene transcripts of various complex I and IV subunits are increased by 2- to 4-fold after caloric restriction in rat skeletal muscle (Sreekumar et al. 2002).

In summary, since there are many animal species with longevities much higher or lower than predicted by the rate of living theory of aging inversely relating metabolic rate and maximum longevity, that theory lacks universal application. On the contrary, all the comparative studies performed to date between different mammals or between mammals and birds show that long-lived species have low rates of mitochondrial oxygen radical production. Therefore, mitochondrial oxygen radical generation seems to be a better correlate of longevity and aging rate than metabolic rate. In species following the rate of living phenomenon, the inverse correlation between metabolic rate and MLSP seems to be a simple consequence of the similar free radical leak of these animals. When comparing longevities of different species, which cannot be explained based on the rate of living phenomenon, the absence of correlation between metabolic rate and MLSP can be due to the presence of different free radical leaks in their respiratory chains. But in all species, a low rate of free radical production correlates with a slow aging rate. That decrease in ROS production occurs at complex I of the respiratory chain, at least in some long-lived species and can be due to a low degree of electronic reduction of the complex I generator. Mitochondria from caloric restricted rats also show decreases of ROS generation specifically at complex I.

7.4 Oxidative damage to mitochondrial DNA, longevity and caloric restriction

Strongly reactive free radicals oxidize molecules present at or very close to their places of generation, and mtDNA is situated very close to the site of ROS production, the inner mitochondrial membrane. Since long-lived vertebrates have low rates of mitochondrial free radical production, this should be reflected on their levels of oxidative damage to mtDNA. Therefore, 8-oxodG was measured in the heart and brain mtDNA and nuclear DNA (nDNA) of eight mammals differing 13-fold in MLSP. The results showed that the steady-state level of 8-oxodG in the mtDNA is negatively correlated with MLSP in both organs (Barja and Herrero 2000). Furthermore, the 8-oxodG level of nDNA did not correlate with MLSP in any organ. Thus, the correlation of oxidative damage to DNA with maximum longevity is specific for mtDNA. 8-oxodG was also generally lower in the heart and brain mtDNA of long-lived birds when compared to short-lived mammals, whereas again this was not the case in nDNA (Herrero and Barja 1999). The results of those investigations also showed that 8-oxodG is various fold higher in mtDNA than in nDNA in the heart and brain of all the 11 species of mammals and birds studied. This agrees with studies performed in the liver or rodents (Richter et al. 1988), and human brain samples obtained postmortem (Mecocci et al. 1993).

Table 2. Summary of oxidative stress-related changes generally observed in long-lived and in caloric restricted animals

	Long-lived animals	Calorie restriction
Endogenous antioxidants	↓	↓↔↑
ROS production	↓	↓
mtDNA oxidative damage	↓	↓
mtDNA mutations	↓	ND
nDNA oxidative damage	↔	↓↔
Free radical leak	↓↔	↓
Fatty acid unsaturation	↓	↓↑

ND - not determined. ROS production can determine mtDNA oxidative damage and then the rate of accumulation of mtDNA mutations. The free radical leak is the percentage of total electron flow directed to free radical generation at respiratory chain. The low ROS production of long-lived animals can be due to a low free radical leak or to a low rate of oxygen consumption depending on the animal species considered.

Caloric restriction decreases mitochondrial ROS generation. Then, if mitochondrial ROS production is a strong determinant of oxidative damage to mtDNA, 8-oxodG should also be lower in the mtDNA of the restricted animals. In agreement with this, oxidative damage to mtDNA was significantly lower in the heart of the long-term restricted old rats in which mitochondrial ROS production was diminished (Gredilla et al. 2001a). This decrease in 8-oxodG occurred also only in mtDNA, not in nDNA. Conversely, when the rats were restricted only for 6 weeks, neither mitochondrial ROS generation nor 8-oxodG in mtDNA changed. These results further support the idea that a causal relationship exists between these two parameters. Additional investigations showed that caloric restriction also decreases both mitochondrial ROS production and 8-oxodG in mtDNA in rat liver (Gredilla et al. 2001b; López-Torres et al. 2002). The decrease in ROS production and 8-oxodG steady-state levels in mtDNA suggests that the flux of oxidative damage (attack and repair) through the mtDNA of caloric restricted animals must be lower than in the ad libitum-fed controls, like in the case of long-lived versus short-lived animals. In agreement with this possibility, it has been observed that the expression of several DNA repair genes (Fornace et al. 1989; Payne and Chu 1994; Petrini 1999) is decreased in caloric restriction.

In summary, the studies performed to date show that oxygen radicals damage more intensely mtDNA than nDNA, and that the steady-state level of oxidative damage to mtDNA increases as a function of the rate of mitochondrial ROS generation. This conclusion strengthens the mitochondrial free radical theory of aging. It agrees with the concept that mitochondrial ROS production near or even in contact with mtDNA attacks this informational molecule in a way, which determines aging rate (Barja et al. 1994b; Barja 1999). This can occur through the accumulation of mtDNA mutations in postmitotic tissues during aging (Muscari et al. 1996; Nagley and Zhang 1998; Ozawa 1999; Napiwotzki et al. 1999; Pesce et al. 2001). The rate of accumulation of somatic age-related mutations in mtDNA is indeed much faster in short- than in long-lived animals (Wang et al. 1997; Melov et al.

1999b). Thus, the rate of mitochondrial ROS production of each animal seems to determine the rate of ROS attack to and flux of oxidative damage through mtDNA (Barja 1999), and then, its rate of accumulation of mtDNA mutations and aging rate (Table 2). Such a model agrees with the results obtained in comparisons between animals with different maximum longevities as well as with the investigations performed in caloric restriction models.

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8 Aging and the programmed death phenomena

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Abstract

Biochemical mechanisms of the programmed death phenomena are considered at levels of unicellular organisms, mitochondria, cells, groups of cells, and organs. Some examples of programmed death of multicellular organisms are also discussed. A concept is developed that aging, being inherent in the great majority of eukaryotic organisms, is programmed, operating as a mechanism accelerating evolution. It is assumed that genetic changes which are too small to be a subject of natural selection in strong young individuals, become essential with age, and, hence, can be selected at older (but still reproductive) ages when the organism is weakened by aging. This weakening is suggested to be due to a decrease in the antioxidant defence so that aging facilitates evolutionary improvement of such a defence. A tentative scheme of the programmed aging is described. It postulates involvement of a kind of biological clock, aging or juvenile hormones, reactive oxygen species, telomeres, some specific intracellular aging-mediating proteins (p53, p66Shc), oxidation of mitochondrial DNA and proteins, nuclear DNA demethylation, etc. It is concluded that the programmed aging concept should be regarded as an alternative to the traditional point of view considering aging as being due to inevitable damage to long-term operating complex living systems.

8.1 Introduction: The "Samurai" law of biology

Living creatures hate spontaneous events. They try to control all the processes occurring in their bodies. A question arises whether death, the most dramatic event of the life story, can also be, to some degree, under the control of the organism. If it were the case, we might speak about programmed death at the organismal level. Let us to define such a hypothetic mechanism as *phenoptosis* by analogy with apoptosis, the programmed death of the cell (Skulachev 1999a; b). In this review, I shall summarize some pieces of evidence in favour of the existence of phenoptosis within the framework of a more general principle called "The Samurai Law of Biology": "*It is better to die than to be wrong*"; or in more detail:

"Complex biological systems are equipped with programs of self-elimination. These suicide mechanisms are actuated when the system in question appears to be dangerous for the system of higher position in the biological hierarchy" (Skulachev 2000a).

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Considering application of this principle to living systems of increasing complexity, from mitochondria to cells, tissues and organs, we shall come, after all, to the entire organism. Here an attempt will be made to explain aging as a result of operation of a death program functioning at the organismal level.

8.2 Phenoptosis as a tool to purify a population from genetically-damaged individuals

8.2.1 One more line of DNA defence?

Genomes of the modern organisms are the result of a billion years of biological evolution. Histories of animal and plant species can be millions of years long. How can cellular DNA be almost unchanged during such a long time, always being a target for, say, reactive oxygen species (ROS) produced in the same cell? It is quite obvious that any living organism must possess a very sophisticated system responsible for maintenance of intactness of its DNA.

It is generally assumed that the system in question includes measures (i) to prevent oxidative and any other injury to the genome, and (ii) to repair damage if it does appear. However, the above problem is so serious that it would be hardly surprising if biological evolution created special mechanisms that carry out self-elimination of individuals, which cannot guarantee effective defence of their genomes. Such cruel regulations seem to be necessary since individuals with changed genomes can dramatically affect the fate of a population even if they amount to only a very small part the population. Muir and Howard (1999) published an excellent example illustrating this statement. A fish with an inserted human growth hormone gene was studied. The transgenic animals were found to have increased growth rate. Just this effect was expected. Moreover, it was shown that in a mixed population of modified and unmodified animals, the larger modified males attracted four times as many mates as their smaller rivals. However, only two-thirds of the modified animals survived to reproductive age. Thus, the modification decreases the reproductive potential of the fish. Calculations showed that the whole population would become extinct within 40 generations if 60 transgenic fish joined a population of 60,000 fish. Even a single transgenic individual could have such an effect, although extinction would take a longer time. This study gave a quantitative description to practices applied for many years as a defence against some insects. To the insect population, some sterilized males were added, which resulted in extinction of the population since the balance between reproduction and death of insects proved to be shifted toward death.

Lewis wrote in his recent review: “It is quite possible that the main danger unicellular organisms face are not competitions, pathogens, or lack of nutrients, but their own kin turning into ‘unhopeful monsters’ causing death of the population” (2000). The above-mentioned work of Muir and Howard clearly shows that this statement should be extended to multicellular animals for which an “anti-monster” defence is perhaps even more important than for a bacterium because of the much

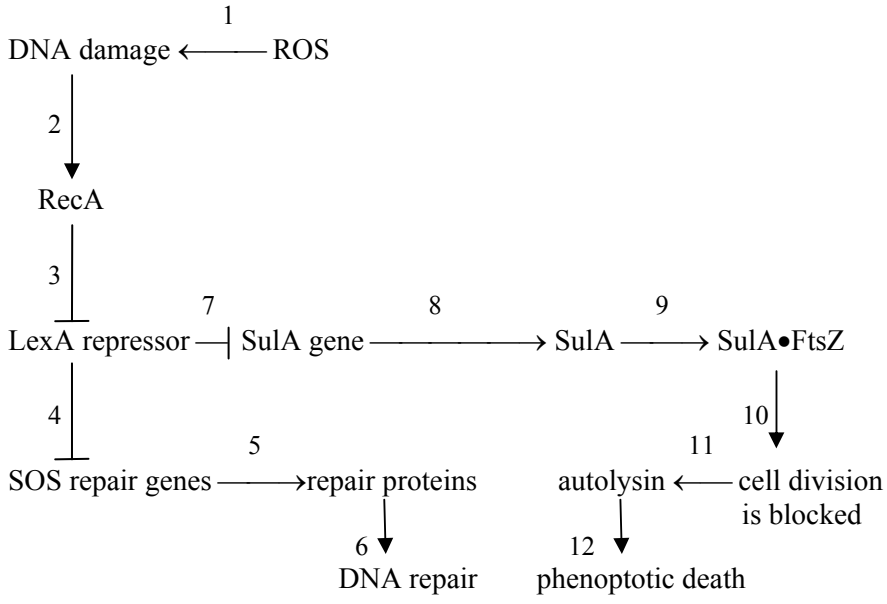

Fig. 1. Regulation of DNA repair, cell division, and phenoptosis in bacteria. ***1***. DNA damage caused by ROS (or by any other agents) activates RecA protein (***2***), which hydrolyses the LexA repressor (***3***), a protein that arrested the SOS repair genes (***4***), and the SulA gene (***7***); ***5***, formation of the DNA repair proteins; ***6***, DNA repair; ***8***, formation of SulA protein, which binds the FtsZ protein (***9***) required for cell division ***(10)***. Moreover, SulA (directly or due to arrest of division) is assumed to actuate the autolysin-mediated phenoptosis (***11, 12***) (Modified from Lewis, 2000).

higher complexity of their organization. The very fact that a simple change in dose of a single gene in a small minority of organisms composing a population can be sufficient to kill this population suggests that a mechanism should exist ridding populations of genetically damaged individuals. Phenoptosis might, in principle, serve as such a mechanism.

In bacteria, RecA is a protein that monitors damage to DNA in bacteria. DNA damage is shown to stimulate specific proteolytic activity of RecA, which is directed to LexA, a repressor protein (Walker 1996). Hydrolyses of LexA results in derepression of genes encoding (i) proteins of SOS DNA repair and (ii) the short-lived protein SulA. The latter binds FtsZ, a protein that forms a division ring, so cell division appears to be blocked. Surprisingly, the *sulA*$^-$ mutant is many-fold less sensitive to the same DNA damage (caused by quinolone antibiotics) than the wild-type bacterium (Piddock and Walters 1992). Lewis (2000) suggested that SulA, besides binding FtsZ, sends a signal for cell suicide. He hypothesized that such a phenoptotic signal is realized by means of activation of an autolysin, a peptidoglycan hydrolase causing decomposition of the cell wall and, in turn, phenop-

tosis of bacteria reaching the stationary growth phase or treated with some antibiotics. Another possibility is that a long-term arrest of the cell division *per se* is a suicide signal (Fig. 1). Lewis concluded that "*sulA*⁻ mutants have an enormous survival advantage over the wild type, yet the immediate benefit of greater survival of *sulA*⁻ cells is apparently outweighed by the longer-term disadvantage due to the loss of the ability to eliminate defective cells" (Lewis 2000).

The SulA story is instructive in that a unicellular organism commits suicide due to DNA damage long before this damage *per se* becomes incompatible with the life of the organism. This means that a cell usually dies when DNA is damaged because of a suicide signal rather than because of a dysfunction of DNA. Such a strategy is consistent with the "Samurai" principle formulated above. This insures the genetic program against dangerously modified DNA that can result from its accidental damage. Here Nature seems to follows a principle of one of Moliere's characters, who prefers to die according to all the rules than to recover against the rules.

8.2.2 Some other cases of phenoptosis in bacteria

Hochman (1997), Lewis (2000), Engelberg-Kulka et al. (2001) and Prozorov (2002) listed some other cases that can be referred to phenoptosis of unicellular procaryotes. Among these are (i) active lysis of the mother cell of *Bac. subtilis* and *Streptomyces* during sporulation, which is required to release spores; (ii) development of bacteroids in *Rhizobium*; (iii) lysis of some cells of *S. pneumoniae* to release DNA, which is picked up by other cells that did not lyse; (iv) lysis of the colicin-forming *E. coli* cells to release a colicin killing bacteria of other strains; (v) toxin/antitoxin systems; (vi) lysis of *S. pneumoniae* caused by penicillin. In the latter case, a mutant was selected which was resistant not only to lysis by penicillin, but also by several other antibiotics acting on quite different targets. In spite of the absence of the *lysis* response, all the antibiotics tested were shown to inhibit *growth* of the mutant bacteria just as that of the wild type, a fact showing that the antibiotics were able to act normally against their targets in the mutant cells.

In *E. coli*, three suicide mechanisms that are activated by the appearance of a phage in the cell interior have been described. One of them is the formation of ion-permeable channels in the bacterial membrane, whereas the two others are the activation of a protease or ribonuclease specifically cleaving a protein ($EF\text{-}T_u$) or an RNA ($tRNA^{Lys}$), respectively, i.e., components required for protein synthesis (Raff, 1998; Skulachev 1999a,b).

8.3 How evolution could occur in spite of operation of multiple DNA defence systems?

The error rates in the DNA, RNA, and protein syntheses were estimated to be 10^{-10}, 10^{-5}, and 10^{-4} (Ninio 1991). This was the reason for Radman et al. (1999) to

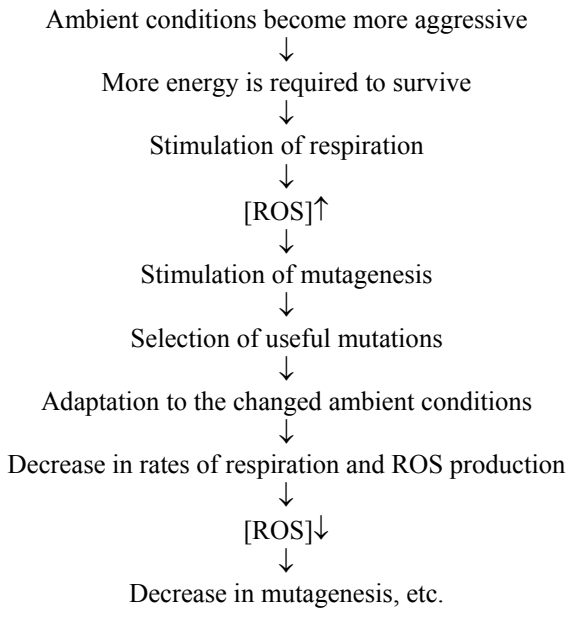

Fig. 2. ROS-mediated acceleration of evolution

state that "the DNA molecule is … the most stable molecular memory system known". Among mutations, only one per 10^4 has a chance to be adaptive, i.e., to give some selective advantage if we deal with bacteria (Taddei et al. 1997). Such relationships for sure make a great problem for adaptation of the living creatures to changing conditions, and hence, for their progressive evolution.

Most probably, the solution was found in that specialized mechanisms helping the living creature to evolve were invented in the course of evolution. One of them operates in such a way that *the rate of evolution increases when conditions worsen.* This can be done by means of at least four different mechanisms, namely (i) an increase in the mutation rate, (ii) a stimulation of DNA recombination processes, (iii) an increase in the reproduction rate, and (iv) a decrease in the lifespan entailing an increase in the rate of change of generations. There are some reasons to assume that in prokaryotes, mechanisms (i) and (ii) are operative, in unicellular eukaryotes mechanisms (ii) – (iv) seem to be dominating, whereas in animals and plant mechanisms (iii) and (iv) are mainly employed.

For aerobic organisms, an increase in mutagenesis under adverse conditions might be a direct consequence of the increased respiration rate when more energy is required to survive in a more hostile environment. A higher respiration requires a larger amount of respiratory chain enzymes to be involved. This increases the probability for these enzymes to be attacked by O_2, resulting in generation of $O_2^{\cdot-}$ and other ROS. The [ROS] increase, in turn, increases probability of DNA damage, i.e., a mutation. More mutations accelerate the appearance of new traits. If a useful trait appears, it can be conserved by means of natural selection. This facili-

tates survival and, hence, decreases respiration, [ROS] and mutagenesis as shown in Figure 2 (Skulachev 1999c; 2001a).

As a result of evolution, this simple scheme was modified such that the mutation rate proves to be under control. In bacteria, special mutator genes were invented. Being normally repressed, they are derepressed in an SOS response-mediated fashion (Radman et al. 1999).

It was found that deterioration of the ambient conditions results in accumulation of ppGpp (so-called stringent response), which mediates an increase of the mutation rate by some unknown mechanism (Rudner et al. 1999). The same effect may be a result of a signalling via cAMP, SOS system, and catabolite repression system as well as of mutations in genes encoding the mismatch repair enzymes, which edit both replication and recombination of DNA (Radman et al. 1999).

8.4 Phenoptosis in yeast

Interesting examples of phenoptosis have been documented in unicellular eukaryotes. Fröhlich and co-workers (1999) reported that in the yeast *Saccharomyces cerevisiae* small amounts of H_2O_2 caused cell shrinkage, appearance of phosphatidyl serine in the outer membrane leaflet, chromatin condensation and margination, and cleavage of DNA, all these events resembling the H_2O_2-induced apoptosis in multicellular organisms. A protein synthesis inhibitor suppressed the death program initiated by H_2O_2. Similar relationships were revealed by Corte-Real and co-workers who studied acetic acid instead of H_2O_2 as a toxic agent (Ludovico et al. 2001). Phenoptosis was accompanied by cytochrome *c* release from mitochondria (Ludovico et al. 2002).

A programmed death apparently took place in experiments of Longo et al. (1997), when the yeast *S. cerevisiae* was kept in expired minimal medium. Expression of the animal antiapoptotic protein Bcl-2 prolonged the life of the yeast cells under these conditions. Death did not occur within the measured period of time (12 days) if pure water was used in place of the expired minimal medium. Similar relationships (except for the Bcl-2 effect) were revealed with some bacteria. It was suggested that such strategy represents an altruistic suicide of the majority of cells to allow a small number of cells to survive in the minimal media. This cannot help when nutrition is completely absent (water instead of a minimal medium), so the cells use a strategy other than phenoptosis.

In 2002, the yeast programmed death concept received direct support. (1) Madeo et al. (2002) disclosed a caspase-like activity which is stimulated by H_2O_2 or aging and is required for the protein-synthesis-dependent death of yeast. Thus, a specific cell death-mediating protein was identified for the first time in *S. cerevisiae*. (2) Severin and Hyman (2002) discovered that death of yeast, induced by a high level of a yeast pheromone, is programmed. In particular, the death was prevented by cycloheximide and cyclosporin A. It required mitochondrial DNA, cytochrome *c* and the pheromone-initiated protein kinase cascade. When haploids of opposite mating types were mixed, some cells died, the inhibitory pattern being

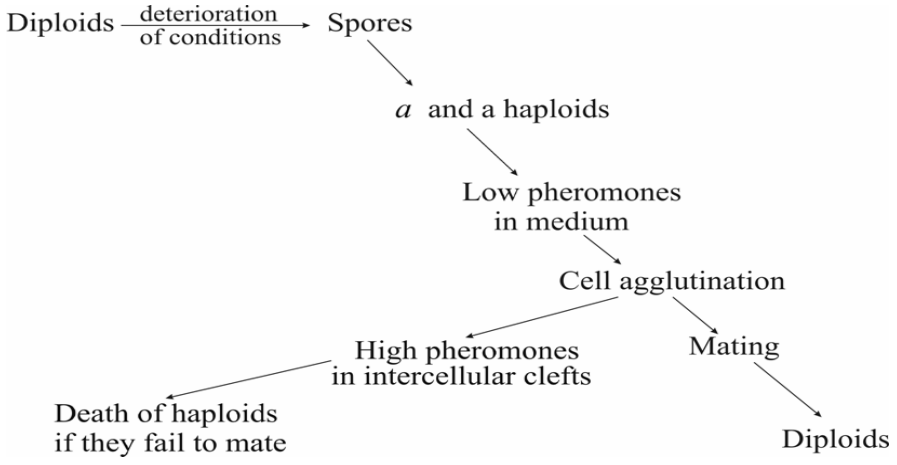

Fig. 3. The life cycle of yeast: oscillation between long-lived and short-lived modes. Deterioration of ambient conditions induces the vegetative-to-sexual reproduction switch mediated by excretion of pheromones to the medium. Low concentrations of pheromones cause cell agglutination and mating of α and *a* haploid cells. Those cells that were agglutinated but failed to mate are killed by high concentrations of pheromones appearing in narrow clefts between agglutinated cells. This mechanism purifies the yeast population from such cells and accelerates change of generations in the population. (From Skulachev [2002a]).

the same as in the case of the killing by pheromone. Inhibition of mating was favourable for death. Thus, pheromone not only activated mating but also eliminated yeast cells failing to mate. This programmed death (a) switched the yeast population from vegetative to sexual reproduction (cf. stimulation of recombination in bacteria under adverse condition, see above, Section 8.3) and (b) shortened the lifespan and, hence, accelerated changing of generations. As a result, the probability for appearance of new traits, when ambient conditions worsen, could be enhanced (Fig. 3).

In this context, observation by Reznik (1997) can be mentioned. Guppies that had not previously encountered predation were subjected to predation for five years; these animals were found to reach fertility at an earlier age and had a shortened lifespan.

8.5 The programmed death phenomena in higher organisms: mitoptosis, apoptosis, and organoptosis

8.5.1 Mitoptosis, programmed elimination of mitochondria

In 1992, my co-worker Dr. D.B. Zorov suggested that animal mitochondria possess a mechanism of self-elimination (Zorov et al. 1992). This function was ascribed to the so-called permeability transition pore (PTP). The PTP is a rather large non-specific channel located in the inner mitochondrial membrane. The PTP is permeable for compounds of molecular mass < 1.5 kDa. The PTP is usually closed. A current point of view is that PTP opening results from some modification and conformation change of the ATP/ADP antiporter, the protein normally responsible for exchange of extramitochondrial ADP for intramitochondrial ATP. Oxidation of a cysteine residue in the antiporter seems to convert it to the PTP in a way that is catalyzed by another mitochondrial protein, cyclophilin (Zoratti and Szabo 1995; Halestrap et al. 1997; Costantini et al. 2000). When opened, the PTP makes impossible the performance of the main mitochondrial function, i.e., coupling of respiration with ATP synthesis. This is due to the collapse of the membrane potential and pH gradient across the inner mitochondrial membrane, mediating respiratory phosphorylation. Membrane potential is also a driving force for import of cytoplasmic precursors of mitochondrial proteins. Moreover, it is strictly required for the proper arrangement of mitochondrially-synthesized proteins in the inner membrane of the mitochondrion. Thus, repair of the PTP-bearing mitochondrion ceases, and the organelle should perish (Skulachev 1994; 1996a; b; 1998b).
It is noteworthy that the above scheme of elimination of a mitochondrion does not require any extramitochondrial proteins. It can be initiated by a signal originating from a particular mitochondrion, such as reactive oxygen species (ROS) produced by the mitochondrial respiratory chain. ROS seem to oxidize the crucial SH-group in the ATP/ADP-antiporter, thereby actuating the elimination process. This is why one can consider this effect as the programmed death of the mitochondrion (mitochondrial suicide). For this event, I coined the word *mitoptosis* (Skulachev 1998a; see also Cohen et al. 2002). I also suggested that the biological function of mitoptosis is ridding the intracellular mitochondrial population of those that have become dangerous for the cell because their ROS production exceeded their ROS scavenging capacity. This situation may well be a particular case of the above-mentioned “Samurai” law.

As was shown by Lemasters and co-workers (Elmore et al. 2001), the action of glucagon on hepatocytes can be a good model to study mitoptosis. In this case, addition of glucagon to the cell culture resulted in the cyclosporine A-sensitive PTP opening (revealed by collapse of the membrane potential) followed by autophagia. It was found that only dead mitochondria having no membrane potential are consumed by autophagosomes, special intracellular vesicles surrounded by two membranes. Then autophagosomes are acidified and acquire acid hydrolases by fusion with lysosomes. During this process, the inner membrane of the autophagosome disappears (Dunn 1990).

In fact, Lemasters' experiments exemplify a case when elimination of mitochondria is initiated by an extramitochondrial stimulus (a hormone). However, the same phenomenon can be caused when a mitochondrion overproduces ROS that damage mtDNA. In this case, ROS-induced PTP (Bernardy 1999) might be a mechanism initiating mitochondrial suicide and eliminating ROS-damaged mtDNA from the mitochondrial gene pool (Elmore et al. 2001).

8.5.2 Massive mitoptosis results in apoptosis

Opening of the PTP leads to an osmotic imbalance between the mitochondrial matrix and cytosol, swelling of the matrix and, consequently, the loss of integrity of the outer mitochondrial membrane, thus releasing the intermembrane proteins into the cytosol. Among them, five proteins are of interest in this context: cytochrome *c*, **a**poptosis-**i**nducing **f**actor (AIF), the ***s***econd ***m***itochondrial apoptosis-***ac***tivating protein (Smac; also abbreviated DIABLO), procaspase 9 and endonuclease G. All these proteins are somehow involved in apoptosis (for review, see Skulachev 2002b).

AIF was the first mitochondrial component for which the ability to activate apoptosis was revealed (Susin et al. 1996). AIF is a flavoprotein of sequence resembling that of dehydroascorbate reductase, an enzyme found in the intermembrane space of plant mitochondria (Susin et al. 1999). The reductase regenerates ascorbate, the main water-soluble antioxidant, from dehydroascorbate, using NADH as the reductant. One might speculate that AIF was originally used by mitochondria as an antioxidant enzyme and later was employed by the cell as an apoptosis-activating protein (Skulachev 2000b). When released from mitochondria, AIF goes to the nucleus and activates a nuclease that decomposes nuclear DNA (Susin et al. 1999).

The route of cytochrome *c*-mediated cell death was shown to be more complicated (Liu et al. 1996; Yang et al. 1997; Kluck et al. 1997; Skulachev 1999a). In cytosol, cytochrome *c* combines with a cytosolic protein called **ap**optosis **p**rotease-**a**ctivating **f**actor 1 (Apaf-1) and dATP. The complex, in turn, combines with an inactive protease precursor, procaspase 9, to form the "apoptosome". In the apoptosome, procaspase 9 is converted to active caspases 9. When formed, caspase 9 attacks procaspase 3 and cleaves it to form active caspase 3, a protease that hydrolyzes certain enzymes occupying key positions on the metabolic map. This causes cell death. At least in some tissues, procaspase 3, like some other procaspases, is also localized mainly in the intermembrane space (Samali et al. 1999).

Smac, the third proapoptotic protein of the intermembrane space, was recently shown to bind cytoplasmic ***i***nhibitors of ***a***poptosis-activating ***p***roteins (IAPs), which suppress the activities of caspases 9, 3, and others. Smac•IAP complexes lack the antiapoptotic activity inherent in the free IAPs (Du et al. 2000; Verhagen et al. 2000). As to endonuclease G, also hidden in the intermembrane space, it also, when released from this space, goes to the nucleus and attacks DNA (Parrish et al 2001; Li et al 2001; Wildak et al 2001; Davies et al 2003).

Considering these data, the following scenario of the final steps of the defence of a tissue from mitochondrion-produced ROS seems to be the most likely. ROS induce PTP opening and, consequently, release of cytochrome *c* and other proapoptotic proteins from mitochondria to the cytosol. If this occurs in a small fraction of ROS-overproducing mitochondria, these mitochondria die. The cytosolic concentrations of proapoptotic proteins released from the dying mitochondria appear to be too low to induce apoptosis. [Apparently this was the case in the above-mentioned experiments of Lemasters when glucagon stimulated mitoptosis of a small number of mitochondria in hepatocytes but no apoptotic markers appeared (Elmore et al. 2001)]. If, however, more and more mitochondria become ROS-overproducers, the concentrations in question reach a level sufficient for the induction of apoptosis. This results in purification of the tissue from the cells whose mitochondrial population produces too many ROS (Skulachev 2001a).

It should be stressed that dysfunction of mitochondria *per se* can be a reason for cell death in tissues where phosphorylating respiration is the major source of ATP. However, apoptosis caused by cytochrome *c* and other intermembrane "death proteins" occurs much earlier than the mitochondrial dysfunction results in exhaustion of ATP. On the contrary, dATP and/or ATP are required for apoptosis (e.g., to actuate the apoptosome formation, see above). It looks as if cytochrome *c* and other mitochondrial "death proteins", when they appear in large amounts in the cytosol, represent a signal for the cell that something is dramatically wrong with its mitochondrial population. Such a cell commits suicide, following the "Samurai" law.

In 1994, I suggested that mitoptosis is an event preceding apoptosis (Skulachev 1994). In the same year, Newmeyer et al. (1994) published the first indication of a requirement of mitochondria for apoptosis. Quite recently, Tolkovsky and her coworkers presented direct proof of the mitoptosis concept (Fletcher et al. 2000; Xue et al. 2001). In the first set of experiments, axotomized sympathetic neurons deprived of neuron growth factor were studied. It was found that such neurons died within a few days, showing cytochrome *c* release and other typical features of apoptosis. However, the cells survive if a pan-caspase inhibitor Boc-Asp (O-methyl)-CH_2F (BAF) was added a day after the growth factor deprivation. The cell survival was due to the fact that the mitochondrion-linked apoptotic cascade was interrupted downstream of the mitochondria. Electron microscopy showed that in the majority of such cells *all the mitochondria disappear within 3 days* after the BAF addition (Fletcher et al. 2000). Later, the same group reported (Xue et al. 2001) that a similar effect could be shown using such classical experimental models of apoptosis as HeLa cells treated with staurosporine. Again, addition of BAF to the staurosporine-treated cells resulted in that (i) the cells lived longer and (ii) mitochondria disappeared on the time scale of days. This was shown to be accompanied by disappearance of mitochondrial DNA and as well as the cytochrome oxidase subunit IV encoded by nuclear DNA. On the other hand, nuclear DNA, Golgi apparatus, endoplasmic reticulum, centrioles, microtubules, and plasma membrane remained undamaged. Mitoptosis was prevented by overexpression of antiapoptotic protein Bcl-2, which is known to affect mitochondria upstream from the cytochrome *c* release.

Apparently, disappearance of mitochondria in the apoptotic cells without BAF could not be seen since the cells die too fast to reveal massive mitoptosis and subsequent autophagia of dead mitochondria. On the other hand, inhibition of apoptosis at a post-mitochondrial step prevented fast death of the cells so there was time for mitoptosis to be completed. It should be stressed that both initial and terminal steps of mitoptosis are required for mitochondria-dependent apoptosis to proceed. As was shown in our group (Shchepina et al. 2002), the TNF-induced apoptosis of HeLa cells is accompanied by cyclosporin A-sensitive mitochondrial swelling, decomposition of long mitochondria to small supercondensed bodies engulfed after all to autophagosomes. It was shown that apoptosis does not proceed when fission of the extended mitochondrial profiles (Frank et al. 2002) or autophagia (Jia et al. 1997) are inhibited.

It is noteworthy that, besides the above-described scenario, there is an alternative and more delicate mechanism of mitochondria-linked apoptosis, which does not abolish oxidative phosphorylation and does not kill mitochondria. It consists in some modification of porin localized in the outer mitochondrial membrane. Modified porin becomes permeable for proteins that are released from the intermembrane space to cytosol. The modification in question can be carried out by (i) proapoptotic protein Bax migrating from cytosol to mitochondria in response to some apoptogenic stimuli (Shimizu et al. 1999) or (ii) oxidation of porin by superoxide (Madesh and Hajnocky 2001). It seems probable that the mitoptotic (PTP-linked) and the mitoptosis-independent (porin-linked) mechanisms are actuated by the mitochondrion-produced ROS and extramitochondrial ROS, respectively.

Independently of the mechanism of release of mitochondrial intermembrane proteins, all the above-mentioned types of apoptosis employ mitochondria as amplifiers of a suicide signal. A precedent is described when such a function, rather than oxidative phosphorylation, appears to be the main function for these organelles. During differentiation of eosinophiles, mitochondria were shown to be required for apoptosis. As to the cellular respiration, it was cyanide-resistant and could not produce membrane potential. All the energy-linked functions, including formation of membrane potential on the inner mitochondrial membrane, were supported by hydrolysis of glycolytic ATP (Peachman et al. 2001).

On the other hand, sometimes apoptosis was found to be mitochondria-independent. This usually occurs when an apoptotic stimulus is very strong and can be realized without mitochondrion-mediated amplification (for reviews, see Skulachev 1998b; 1999a; Cohen et al. 2002).

8.5.3 Programmed death at supracellular level: bystander effect

Several cases were described when apoptotic cells formed clusters in tissues *in vivo* or in cell monolayers *in vitro* (so-called death of bystander cells) (Shao et al. 2000; Kagawa et al. 2001; Adachi et al. 2002; for refs., see also Reznikov et al. 2000). In 1998, I suggested that H_2O_2 produced by an apoptotic cell serves as an apoptogenic signal to the bystander cells surrounding the apoptotic cell (Skulachev 1998c). In this way, I explained a role of H_2O_2 in antiviral defence of the tis-

sue. At least in some cases, infected cells have been shown to produce H_2O_2 not only to commit suicide but also to induce apoptosis in nearly cells that are most probable targets for the virus during the expansion of the infection. As a result, a "dead area" is organized around the infected cell. In 2000, Bakalkin and co-workers (Reznikov et al. 2000) confirmed this suggestion, applying a combined experimental and mathematical analysis to investigate the bystander phenomenon. Monolayers of human osteosarcoma Saos-2 cells were studied. Apoptosis was induced by serum deprivation. The number of apoptoses around an apoptotic cell was found to be two to three times greater than around a viable cell. The bystander effect disappeared when catalase, the H_2O_2 scavenger, was added to the growth medium. In the same study, it was found that apoptosis of Saos-2 cells is accompanied by great increase in the H_2O_2 production. The H_2O_2 concentration proved to be strongly elevated in both the apoptotic cells and the serum-deprived medium where these cells were grown. When H_2O_2 was added to intact Saos-2 cells, they entered apoptosis.

Quite recently, we extended the Bakalkin et al. observation to human cervical carcinoma HeLa cells treated with other apoptogens, namely TNF or staurosporine. The protein synthesis inhibitor emetine was added to prevent the NF-κB-mediated antiapoptotic effect of TNF. Experiments performed by Drs. O. Ivanova and L. Domnina clearly showed clustering of apoptotic cells. Dr. A. Alexeevsky and Mr. D. Alexeevsky suggested a computer program Clud ("ClusterDetector") to (i) calculate number of apoptotic cells contacting with a given apoptotic cell in the HeLa cell monolayer (an experimental value) and (ii) compare this value with a theoretical one obtained assuming occasional distribution of apoptotic cells in the monolayer. The result confirmed development of the bystander effect during apoptosis (Alexeevsky et al. in preparation).

Another series of experiments with HeLa cells was performed in our group by Drs. O. Pletjushkina and E. Fetisova. The HeLa cells were grown on two glass coverslips. One of them was treated with staurosporine to induce apoptosis. Then the glass was removed from the staurosporine-containing medium and put to the fresh one side by side to the second glass, which was not staurosporine-treated. It was found that apoptotic cells appeared in the intact monolayer, the number of apoptotic cells decreasing with increase of the distance from the first glass. Such an effect was strongly suppressed by catalase (for review, see Skulachev 2003).

Thus, we can conclude that the hypothesis on H_2O_2-mediated bystander effect is experimentally proved. There are some indications that such a phenomenon is of great importance in humans at infarction and stroke (Skulachev 1999a) as well as in plants for the so-called hypersensitive response (Neill et al. 2002).

8.5.4 Organoptosis, programmed elimination of an organ

It is obvious that massive apoptosis of cells composing an organ should eliminate the organ. This process can be defined as "organoptosis" (Skulachev 1999a). For example, consider the disappearance of the tail of a tadpole when it converts to a frog. Addition of thyroxine (a hormone known to cause regression of the tail in

tadpole) to severed tails surviving in a special medium was shown to cause shortening of the tails that occurred on the time scale of hours. The following chain of events was elucidated (Kashiwagi et al. 1999):

thyroxine $\rightarrow$ $NO^{\cdot}$ synthase induction $\rightarrow$ $[NO^{\cdot}]\uparrow \rightarrow [H_2O_2]\uparrow \rightarrow$ apoptosis $\rightarrow$ organoptosis (1)

Mechanisms of the $[H_2O_2]$ increase caused by $NO^{\cdot}$ remain to be revealed. This may be inhibition by $NO^{\cdot}$ of the main H_2O_2 scavengers, catalase and glutathione peroxidase (for refs., see Kashiwagi et al. 1999). Another possibility consists in affecting the mitochondrial respiratory chain. As was shown by Borevis and his colleagues, $NO^{\cdot}$ inhibits the respiratory chain of mitochondria or submitochondrial particles *in vitro* at levels of cytochrome oxidase and cytochrome *b*. This was shown to be accompanied by H_2O_2 production, which was as fast as in the presence of antimycin A (Poderoso et al. 1996, 1999a). Later the groups of Boveris and Cadenas showed the same phenomenon in isolated beating rat heart (Poderoso et al. 1998). They also analyzed non-enzymatic mechanisms of $CoQH_2$ interaction with $NO^{\cdot}$ as well as with $NO^{\cdot}$ derivatives (Poderoso et al. 1999b). Among them, three reactions of semiquinone formation were identified. Semiquinone is in turn an excellent one-electron donor for O_2, converting O_2 to $O_2^{\cdot -}$. As to the mechanism of cytochrome *b* inhibition, it may be due to the effect of peroxynitrite ($ONOO^-$) formed from $NO^{\cdot}$ and $O_2^{\cdot -}$ (Guidarelli and Cantoni 2002).

8.6 Phenoptosis of multicellular organisms that reproduce only once

In some of such species, the organism is constructed in a way predetermining death shortly after reproduction. Remember mayflies. Their imagos die in few days since they cannot eat due to lack of a functional mouth, and their intestines are filled with air. In the mite *Adactylidium*, the young hatch inside the mother's body and eat their way out (Kirkwood and Cremer 1982), just as spores formed inside a *Bac. subtilis* cell are released to the outer medium by lysis of the cell (see above, Section 8.2.2). However, much more often a special phenoptotic program is switched on immediately after the act of reproduction. The male of some squids dies just after transferring his spermatophore to a female (Nesis 1997). The female of some spiders eats the male after copulation. Bamboo can live for 15-20 years reproducing vegetatively, but then, in the year of florescence, dies immediately after the ripening of the seeds (for discussion, see Kirkwood and Cremer 1982, Bowles 1998).

8.7 Phenoptosis and defence against pathogens

A higher organism seriously infected by a dangerous pathogen is an unwanted guest for the community, just as a phage-infected *E. coli* cell is for a population of these bacteria (see Section 8.2.2). From a community point of view, quick death of such an individual might be a cruel but radical solution to the problem, being the last line of the anti-epidemic defence of the population. This means that a population of organisms possessing a mechanism to purify the community from badly infected individuals will have reproductive advantage compared with that lacking such a mechanism (Skulachev 1999a; 2002b).

All the features of septic shock indicate that the death of an ill individual is well organized by the macroorganism itself, the role of the pathogen being rather passive. "Endotoxin", causing sepsis, is a lipopolysaccharide (LPS) forming the outer layer of the wall of Gram-negative bacteria. The toxicity of endotoxin is absolutely dependent on the presence of (i) an endotoxin-binding protein in blood and (ii) some receptors in the plasma membrane of the patient's cells. Sepsis is accompanied by massive formation of TNF and other cytokines that induce apoptosis of these cells. Knockout of genes coding for these proteins as well as inhibition of the receptors (for reviews, see Klosterhalfen and Bhardai 1998; Fenton and Gelonbock 1998) or of caspases (Hotchkiss et al. 1999) decrease the toxicity of LPS. In fact, LPS looks like a signal of the appearance (in blood and tissues) of Gram-negative bacteria, which may be especially dangerous because their LPS-containing wall protects them from attack by the antibacterial systems of the macroorganism. This effect apparently represents a generalized response of an organism to *any* Gram-negative bacteria, including non-infectious ones. This explains why sepsis can be non-contagious (Skulachev 1999b; 2002b).

Certainly, phenoptosis is the last line of defence for a community of organisms against infection. If the amount of pathogens in an individual is not too high, the same LPS signal is used by the organism to attract leucocytes to the infected region of a tissue. The formation of cytokines by leucocytes is LPS-dependent. Moreover, LPS induces synthesis of mitochondrial uncoupling proteins that are apparently responsible for hyperthermia, bringing the body temperature to a value that is non-permissive for the bacteria (Cortez-Pinto et al. 1998). If the extent of infection is not too high, these measures are useful for the organism. This is why the common opinion concerning sepsis is that it represents an over-use by the macroorganism of its defensive antimicrobial tools. However, such a point of view fails to explain why a control mechanism to prevent this potentially very dangerous system from killing the organisms has not been invented during the evolution of these organisms.

Within the framework of our concept, the patient–pathogen relationships can be described as the interaction of the three types of mechanisms: (i) attack of the patient's cells by pathogen toxins, (ii) activation of antibacterial defence of the macroorganism, and (iii) generation of a phenoptotic signal when the level of infection exceeds some critical value. It seems possible that effects of the diphtheria, *Pseudomonas aerogenose*, cholera, pertussis, murine plague, and some other tox-

ins can also be attributed to phenoptosis (Skulachev 2002b). (For mathematical description of situation when dramatic shortening of the lifespan of infected individuals appears to be favourable for survival of the population, see Kirchner and Roy 1999, 2002).

8.8 Aging as slow phenoptosis

8.8.1 Some history

In 1818, Arthur Schopenhauer wrote: "Nature is always ready to let the individual fall, and the individual is accordingly not only exposed to destruction in a thousand ways from the most insignificant accidents, but is even destined for this and is led towards it by nature herself, from the moment that individual has served the maintenance of the species" (Schopenhauer 1969). Later Charles Darwin put forward a concept assuming that biological evolution proceeds exclusively in the direction favourable for individuals. However, he clearly recognized serious problems created by such a concept when, e.g., sexual selection is considered (quite recently, this aspect was nicely analyzed by Goldsmith, 2003).

In 1881, August Weismann developed, in fact, the Schopenhauer principle, postulating the programmed death of organisms (published in English in 1889). He wrote: "Worn-out individuals are not only valueless to the species, but they are even harmful, for they take the place of those, which are sound... I consider that death is not a primary necessity, but that it has been secondarily acquired as an adaptation. I believe that life is endowed with a fixed duration, not because it is contrary to its nature to be unlimited, but because the unlimited existence of individuals would be a luxury without any corresponding advantage" (Weismann 1889).

Weismann's hypothesis on aging as an adaptive mechanism was later strongly attacked as an anti-Darwinism. Medawar (1952) assumed that aging could not have developed during the course of biological evolution. Medawar stressed that, under natural conditions, the majority of organisms die before they become old (see also Comfort 1979). This point of view was recently criticized by Bowles (1998, 2000) and Goldsmith (2003). It is hardly right since the *aging* starts long before it appears to be an immediate reason for the *death*. Indirectly, age-dependent weakening of an organism can well be the reason for, e.g, death due to an attack by predators, pathogens, etc, (see Skulachev 2001a and the next Section). Loison et al. (1999) and Bonduriansky and Brassil (2002) showed that both long-lived ungulates (roe deer, bighorn sheep, isard) and short-lived antler fly suffer senescence under natural conditions.

Weismann's concept of programmed death received support in 1972 when Kerr Wyllie and Currie published their famous paper "Apoptosis: a basic biological phenomena with wide-ranging implication in tissue kinetics". Later numerous observations clearly demonstrate that apoptosis is involved in ontogenic development, anticancer defence, immune response, etc. In 2002, a Nobel Prize in Physiology and Medicine was given to S. Brenner, H. R. Horvitz and J. Sulston for

studies on *C. elegans*, including identification of genes of an apoptotic program. Quite recently, some pieces of evidence were obtained that a suicide program operates at the subcellular level (mitoptosis) as well as at supracellular levels (bystander effect and organoptosis) (see above, Sections 5.1, 5.3 and 5.4). For unicellular organisms, programmed death was experimentally proved (Sections 2.1, 2.2 and 4).

All these observations stimulate us to revisit Weismann's concept of aging as a program, which now looks like the next logical step in the range of such events as mito-, apo-, and organoptosis.

8.8.2 Why aging is slow: the hares-vs.-fox case

Slow rate of aging allows this process to serve as a specialized mechanism accelerating evolution. Let me consider an example. Two young hares differing "intellectually" have equal chances to escape from a fox since both of them are running faster than a fox. This situation is nicely described by the Russian proverb: "Syla est' – uma ne nado". ("If you are strong, it is not necessary to be clever"). However, with age the clever hare acquires some advantage, which becomes of crucial importance when the rate of running of hares lowers to that of a fox. Now the clever hare has a better chance to escape and, hence, to produce leverets, than the stupid hare. This in turn will be favourable for selection of clever hares.

The above relationships presume that (i) aging of brain does not develop much faster than that of skeletal muscles and (ii) muscles become weaker with age when reproduction is still possible. This is certainly the case for humans since here "aging atrophy of muscles begins around 25 years" (Lexell et al. 1988). Goldsmith (2003) wrote: "Because even a relatively minute deterioration will cause a statistically significant increase in death rate, one suspects that the evolutionary effects of aging in wild mammals begin at relatively young ages." As Loison et al. (1999) mentioned, observed death rates in wild mammals increase beginning at puberty.

Thus, it appears that slow aging allows a small useful trait (which is not essential for a young organism) to be identified and selected. This must facilitate progressive evolution since it is obvious that, as a rule, great advantages appeared because of developing small positive changes (Skulachev 2001a; 2002b; 2003; Goldsmith 2003).

8.8.3 Why rate of aging of different species varies over very wide limits? Aging as a part of the *r*-strategy.

As an adaptive mechanism facilitating evolution, the aging program should be mobilized when conditions deteriorate (like pheromone-induced shift to sexual reproduction and shortening of the lifespan in yeast, see Section 8.4). This may explain why "the cellular tool kit that existed about 600 million years ago allowed the evolution of lifespans ranging up to one million-fold difference in length" (Finch and Austad 2001).

In evolution, two strategies were identified depending on degree of adaptation of the species to the ambient conditions, namely (i) for bad conditions, the ***r***-strategy (short lifespan and high rate of reproduction), and (ii) for good conditions, the ***K***-strategy (long lifespan and low rate of reproduction) (MacArtur and Wilson 1967). In higher animals, the ***r***- and ***K***-strategies can be exemplified by comparing mammals and birds. Austad and his colleagues wrote: "Many birds live up to three times longer than mammals of equivalent body mass. The slow aging rates typical of the class Aves are paradoxical given their high metabolic rates (2-2.5 times higher, with lifetime energy expenditures up to 15 times higher), body temperature (approximately 3°C higher), and blood glucose levels (two- to four-fold higher)" (Holmes et al. 2001). Bowles (1998) mentions a bird species, which suddenly dies at about 50 without any features of aging. Among birds, some species were found that show no age-specific increase in mortality, no endocrine senescence, and even an increase (!) in reproduction effort with age (for review, see Holmes et al. 2001). This means that the program of aging in these birds is practically not operating. The reason for this is that birds, who recently occupied such a new and extensive area as the air ocean, so strongly improved conditions of their life that can allow themselves to employ an extreme case of the ***K***-strategy. As to mammals, they are shifted, compared with birds, to the ***r***-strategy.

8.8.4 Mutations prolonging life

A prediction of the concept regarding aging as a program consists in that there should be mutations damaging this program and, hence, prolonging life. In literature, one can already find numerous cases of this kind. In *Caenorhabditis elegans*, mutations in over 50 genes have been identified, which result in longer lifespan (Johnson et al. 2002; Herndon et al. 2002). Some of these mutations arrest developments at the long-lived dauer diapause stage whereas others prolong the life with no dauer response induced. Among the latter, the most demonstrative example was reported by Hekimi and co-workers (Lakowski and Hekimi 1996; Ewbank et al. 1997; Hekimi and Guarente 2003) who succeeded in extending by a factor 5.5 the lifespan of *C. elegans* if two genes were knocked out (see below, Section 8.8.4.2).

8.8.4.1 p66Shc as an aging-mediating protein in mammals

In mice, Pelicci and his colleagues (Migliaccio et al. 1999; Trinei et al. 2002; Napoli et al. 2003) have reported that (i) animals lacking a particular 66-kDa protein (p66Shc) lived 30% longer and are less sensitive to paraquat-induced oxidative stress, and (ii) fibroblasts derived from these mice did not respond to an H_2O_2 ad-

dition by initiating apoptosis.[1] P66Shc proved to be a component of the following apoptotic pathway:

$$H_2O_2 \text{ or UV} \rightarrow \text{DNA damage} \rightarrow \text{p53} \rightarrow \text{p66S} \rightarrow \text{release of mitochondrial cytochrome } c \text{ to cytosol} \rightarrow \text{apoptosis} \quad (2)$$

In support of this scheme, Trinei et al. (2002) reported that treatment of fibroblasts with such DNA-damaging agents as H_2O_2 or the ultraviolet light (UV) resulted in (i) an increase in the p66Shc level due to its stabilization, (ii) a burst of intracellular ROS formation, (iii) cytochrome *c* release, and (iv) apoptotic cell death. In $p53^{-/-}$ cells, no p66Shc stabilization occurred. An overexpression of p53 was found to induce an increase in [p66Shc] and apoptosis but in $p66Shc^{-/-}$ cells it failed to initiate the programmed death. On the other hand, the cell cycle arrest, also mediated by p53, was still present in $p66Shc^{-/-}$ cells. Further studies revealed that the $p66Shc^{-/-}$ mice (a) show unchanged probability of cancer and (b) have decreased oxidative damage of both mitochondrial and nuclear DNA *in vivo* in lung, liver, spleen, skin, skeletal muscles, and kidney. No measurable effect was found in brain and heart (Trinei et al. 2000). Such a tissue specificity is in line with the level of p66Shc in various organs, which is the lowest in brain and heart (Cattaneo and Pelicci 1998; Trinei et al. 2002).

It is noteworthy that p66Shc is stabilized by ROS (H_2O_2) via p53 and, on the other hand, stimulates ROS generation during execution of the suicide program (see eq. 2). According to Nemoto and Finkel (2002) a fork head transcription factor is related to such regulation.

8.8.4.2 Deacetylase, insulin, and insulin-like growth factor-1

It was the Russian gerontologist V. M. Dilman who postulated that aging is under hormonal control in such a way that the hypothalamus-insulin interrelations play the central role (Dilman 1971; 1978; 1994; Dilman and Anisimov 1979; Dilman et al. 1979). Further studies on yeast, worms, flies, and mice confirmed the idea of controlled aging and revealed some details of this mechanism (for reviews see Anisimov 2003; Hekimi and Guarente 2003; Longo and Finch 2003; Tatar et al. 2003).

In yeast, it was found that lifespan is increased with higher doses of the gene called *sir2* (encodes protein SIR2, a NAD^+-dependent histone deacetylase). This enzyme suppresses activity of rDNA genes encoding ribosomal RNA. It lowers the amount of extrachromosomal rDNA cycles accumulating during aging of yeast that are presumably toxic. It is also possible that the observed longevity is a consequence of some other effect(s) of SIR2 (for references, see Hekimi and Guarente 2003).

[1] Compare with an observation of Kirkwood and co-workers (1982) who found a positive correlation of the lifespan of various mammals and the *in vitro* resistance of their fibroblasts to oxidative stress.

In *C. elegans*, the *sir2* ortholog, *sir-2.1*, proved to be involved in increasing the lifespan. This was accompanied by strong increase in resistance of the worm to ROS and the negative regulation of signal transduction pathway involving insulin-like hormone(s) (see Hekimi and Guarente 2003). In flies, some indications of involvement of an insulin-like hormone in shortening the lifespan were also reported (reviewed by Tatar et al. 2003).

As to mammals, it was shown that the Sir2p ortholog, SIRT1, binds to and deacetylates p53. This down-regulates the activity of p53 on damage-response target genes, including genes that induce apoptosis (Hekimi and Guarente 2003).

As already mentioned, the most dramatic (by factor 5.5) mutation-linked increase in lifespan was observed in 1996 by Lakowski and Hekimi on *C. elegans*. Genes *daf-2* and *clk-1* were mutated. A single *daf-2* mutation prolonged life by 2-3 times (Kenyon et al. 1993; Lakowski and Hekimi 1996). It could occur without arresting development at the dauer stage (Kenyon et al. 1993). The corresponding DAF-2 protein was found to share 35 and 34% of its amino acid sequence with a human insulin receptor and insulin growth factor-1 receptor (IGF-1R), respectively (for reviews, see Kimura et al. 1997; Roush 1997). The receptor in question is a transmembrane tyrosine kinase. As to *clk-1* gene, it encodes the enzyme catalyzing a final step of CoQ synthesis so that in the mutant a CoQ precursor, demethoxyubiquinone (DMQ), accumulates. DMQ seems to be a better antioxidant than CoQ and its redox cycle is less prone to ROS production (Miyadera et al. 2002).

Quite recently, Holzenberger et al. (2002) showed that $Igf1r^{+/-}$ mice lived 26% longer than the wild type. Independently, Blüher et al. (2003) reported that the tissue-specific knockout of the insulin receptor in adipocytes resulted in 18% increase in the mouse lifespan. According to data of Holzenberger et al. (2002), the $Igf1r^{+/-}$ mice showed higher resistance to paraquat and 50% lower level of p66Shc. The authors hypothesize that "p66Shc is, together with insulin receptor substrate (IRS), a major cytoplasmic signal transduction molecule for IGF-1R" (Holzenberger et al. 2002). If it is the case, this means that p66Shc receives signals not only from an intracellular regulator (p53) but also from an extracellular one (IGF-1).

It is noteworthy that both $Igf1r^{+/-}$ mice and adipose insulin receptor mice did not develop dwarfism or hypofertility. This is in contrast to the long-lived $Prop1^{df/df}$ and $Pit1^{dw/dw}$ mutants that are sterile dwarfs displaying impaired pituitary gland development and low level of growth hormone and other pituitary hormones (Flurkey et al. 2001; Brown-Borg et al. 1996).

8.8.5 Paradox of Donehower's mice

In the first issue of *Nature* magazine in 2002, Donehower and co-workers (Tyner et al. 2002) described mice with a deletion in the *p53* gene that expresses a truncated mRNA encoding a carboxyl-terminal 24 kDa fragment of p53. Heterozygote mutant mice ($p53^{+/m}$) exhibited, for some unknown reason, enhanced p53 activity, and resistance to spontaneous tumours. In fact, none of the thirty-five $p53^{+/m}$ mice

developed overt, life-threatening tumours, whereas over 45% $p53^{+/+}$ and over 80% $p53^{-/-}$ mice, respectively, developed such tumours. Surprisingly to the authors, the $p53^{+/m}$ mice lived *shorter* by about 20%. Shortening of the lifespan proved to be a result of the fact that aging of the $p53^{+/m}$ mice started earlier than that of $p53^{+/+}$ mice. The aging in question included such traits as reduction in body weight; loss of mass of liver, kidney, and spleen; lymphoid and muscle atrophy; osteoporosis; hunchbacked spine, and grey hair.

Quite recently, Serrano and co-workers (Garcia-Cao et al. 2002) tried to reproduce the Donehower data using another approach. Instead of deleting a part of the p53 gene and heterozygote mice studies by the Donehower group, the authors obtained mice carrying supernumerary copies of the p53 gene. Formally speaking, such an approach is more accurate. However, the resulting increase in the p53 activity level proved, most probably, to be smaller than in the Donehower model. The anticancer effect was less demonstrative. Seventeen per cent of mutant mice still died with tumours. No early aging was revealed by Serrano and his colleagues.

It remains obscure why the Donehower procedure was more efficient in enhancing the p53 activity than an increase in number of copies of the p53 gene employed by Serrano. Apparently, this was a result of very complicated pattern of regulation of the p53 activity (for discussion, see Campisi 2003; Skulachev 2003). In any case, comparison of data in the two above groups may be explained assuming that the p53-mediated life shortening is seen at higher p53 activity than the anticancer effect of this protein. These relationships are hardly surprising if we take into account that any anticancer effect must *increase* the lifespan provided that other conditions are unchanged.

8.9 Possible mechanisms of slow age-dependent phenoptosis

If aging is a specialized mechanism to facilitate evolution, it seems probable that it was discovered at some stage of this evolution. There are some indications that this is the case. Senescence is not typical for prokaryotes. Usually, neither time nor the numbers of divisions are counted by bacteria or archaea. It does not mean that they have no mechanisms of programmed death. In Sections 8.2.1 and 8.2.2, we already considered such mechanisms. All of them are independent of age, which in fact is not known for these types of microorganisms. “Senescence” of bacterial cultures is as a rule *conditional:* it occurs only if the growth conditions worsen, as was indicated in the recent review by Nyström (2002). This review is an excellent summary of various pieces of evidence that bacterial suicides have something in common with the programmed death of the eukaryotic cell, which is hardly surprising since the eukaryotic aging program includes some components (e.g., ROS) of earlier evolutionarily systems already employed in the bacterial programmed death.

8.9.1 End-underreplication of linear DNA as an ancient molecular mechanism of aging

It was suggested that, historically, the living cell invented a specialized mechanism of aging when a linear DNA substituted for a circular one inherent in the majority of prokaryotes (Olovnikov 1971; 1973; Bowles 1998). This event immediately resulted in a specific kind of DNA aging, a process consisting of replication-linked shortening of DNA. Such shortening inevitably accompanies replication of linear DNA, since even now the replicative complex operates with linear DNA in the same way as it does with circular DNA. To produce an exact copy of a template, this complex should have some nucleotide residues to the left and to the right from the place where it combines with DNA. This is always the case if it deals with a circular DNA. However, with a linear DNA, the operation of this mechanism results in underreplication of the ends of the DNA molecule, as was first indicated by Olovnikov (1971; 1973). The question arises why eukaryotes, during many millions of years of evolution, failed to improve this most important enzyme to adapt it to linear DNA, while at the same time they solved many much more difficult problems. According to Olovnikov (1973) and Bowles (1998), it happened first of all because the DNA underreplication is a mechanism applied by primordial unicellular eukaryotes to accelerate the change of generations by shortening of the lifespan.

Apparently, this mechanism was eventually perfected such that special non-coding sequences (telomere repeats) were added to the ends of linear DNA. The shortening of the telomere could be used by the cell to count cell divisions without damaging those DNA sequences that encode RNA. Thus, the old (genetic) DNA function was separated from the new one, i.e., cell division counting (Skulachev 2002b).

8.9.2 Telomeres in yeast

On the face of it, a possible role of the telomere in aging should be first of all studied in unicellular eukaryotes like yeast where the limited lifespan was discovered as early as in 1959 by Mortimer and Johnson. Unfortunately, even in such simplest eukaryote the aging mechanism seems to be more complex than telomere shortening due to end-underreplication. It was found that the yeast mother cell forms limited (usually about 30) numbers of buds converting to daughter cells. After this, the mother cell dies by a phenoptotic mechanism apparently resembling apoptosis of multicellular eukaryotes (Laun et al. 2001). As D'Mello and Jazwinski (1991) reported, no change in the telomere length was observed in *Saccharomyces cerevisiae* cells that had completed up to 83% of the mean lifespan. It does not mean, however, that yeast telomeres are not involved in lifespan control. Guarente and co-workers (Kennedy et al. 1995) concluded that SIR4 protein shortens the yeast lifespan when sitting on the telomere and prolongs it when bound to some other chromosome region(s) (see also the next Section).

8.9.3 Telomeres in animals

The most convincing evidence that just telomere length is critical for the cell lifespan was obtained in studies on animals.

1. Telomerase, an enzyme increasing the telomere length, is active in the embryonic, stem, germ, intestine, epithelial and cancer cells but is inactive in other somatic cells undergoing a finite number of divisions (Collins et al. 1995; Lingner et al. 1996; Counter et al. 1997).
2. The above statement, being valid for the great majority of "aging-competent" animals, is invalid for rainbow trout and lobster (Klapper et al. 1998) that are always growing and showing no typical traits of the aging syndrome. In these animals, very active telomerase is permanently present in all tissues.
3. There is a good correlation between the length of telomeres and the replicative capacity of primary human fibroblasts taken from a range of different individuals (Allsopp et al. 1992; Harley et al. 1990). Cells from individuals with premature aging syndrome (progeria) display the shortest telomeres and weakest replication capacity. This was shown for Hudchinson-Gilford progeria (Allsopp et al. 1992) and Werner's syndrome (Wyllie et al. 2000).
4. Telomeres from lymphocytes or peripheral blood leukocytes have been shown to shorten with age of humans (Schwartz et al. 1993; Vaziri et al. 1993).
5. An increase in the telomere length by activating telomerase in somatic cells, which lack telomerase when grown in primary culture causes telomere lengthening and cell immortalization (Bodnar et al. 1998; Counter et al. 1998). Conversely, inhibition of telomerase entails telomere shortening and cell senescence (Yu et al. 1990).

All these observations have been, in fact, predicted by Olovnikov's original hypothesis of aging (1971, 1973; see also Allsopp et al. 1992) postulating that telomere end-underreplication is the molecular mechanism of aging.

However, during the last few years some data have been published showing that the situation is not so simple. Besides the above results on yeast, some indications were revealed that certain telomere-linked parameters other that its length can also contribute to development of aging (Blackburn 2000; Wright and Shay 2002; Karlseder et al. 2002). Blackburn (2000) mentioned the following observations made in different laboratories supporting the above statement:

1. Cultures of fibroblasts (which lack active telomerase) from older people reached senescence *in vitro* no faster than fibroblasts taken from younger people (Cristofalo et al. 1998).
2. In some cell types, telomeres can be longer in an old person than in another, younger person (Harley 1997; Allsopp et al. 1992).
3. In two budding yeasts, *S. cerevisiae* and *Kluyveromyces lactis*, cells lacking telomerase ceased dividing after some period of telomere shortening. Conversely, cells expressing certain hypomorphic but catalytically fully active telomerase continued to divide even when their telomeres became and remained much shorter than the shortest telomeres in the telomerase-lacking cells (Prescott et al. 1997; Roy et al. 1998). Similar results were obtained in human endothelial cells or fibroblasts lacking telomerase. When telomerase was ex-

pressed, cells continue to divide not only because divisions resulted in smaller telomere shortening, but also due to the fact that the very presence of telomerase allowed cells to divide although their telomeres were shorter in length than that critical for the telomerase-lacking cells (Zhu et al. 1999; Ducray et al. 1999). All these data can be accounted for assuming that telomere-bound telomerase protein *per se* somehow attenuates senescence-inducing signal generated by a short telomere.

Another indication pointing to the above conclusion was recently published by Smogorzewska and De Lange (2002) who showed that removal of another telomere-bound protein, TRF2, induced immediate premature senescence of cultured mammalian cells.

The above-considered facts suggest that telomere really controls the cellular replicative aging but such control includes several factors, and the telomere length is only one of them.

8.9.4 Mice without telomerase

The situation is even more complicated when one considers aging of the entire organism. An attempt to directly attack the problem, carried out by DePinho and coworkers (Rudolph et al. 1999), failed unequivocally to solve the problem but, nevertheless, resulted in very interesting findings. Knockout of a gene required for formation of active telomerase in mouse was shown to result in appearance of mutant animals surviving during five generations with no changes in the lifespan. However, mice of the six-generation proved to be infertile, short-lived (18 months instead of 24 months in control) and had 20-25% smaller body weight. Loss of telomere function did not elicit a full spectrum of aging symptoms but some of them were revealed. In particular, aged skin phenotype was observed (greying hair, alopecia, some typical changes in the skin histology). Certainly, one may argue that mice have very long telomeres (by the way, much longer than humans, a good illustration that there is no direct correlation between the telomere length and the lifespan of the organism).

On the face of it, the above data indicate that telomere shortening can, in principle, limit the mouse lifespan but it requires sixth generations even without any telomerase activity. If this is the case, the role for telomere shortening in the programmed aging of the organism should be reduced to defence of a population from appearance of very long-lived individuals that might arise due to mutation in an alternative, telomere-independent mechanism of aging.

On the other hand, it is possible that shortening of telomeres in the germ and stem cells, which seems inevitable for telomerase$^{-/-}$ mice, does not correlate with telomere shortening in the cells responsible for measurement of time by an organism. A precedent is already described that there is a sophisticated system, other than end-underreplication, which shortens telomeres in certain cells. At an early stage of development of barley germs, the telomere suddenly loses 50 kb. Then it loses an additional 20 kb during growth of the spike (McKnight et al. 1997). It is not excluded that in the hypothetical “chronometer” cells (see below, Section

8.9.6.1) the telomere length is initially decreased to a standard value which is smaller than telomere length in the germ cells, and only after six generations the germ cell telomere becomes below this value. It seems also possible that in different organs and tissues aging is initiated when different values of critical telomere shortening were achieved. Perhaps, in this manner one might explain why some aging features (e.g., hair colour) appeared in the third generation of the telomere$^{-/-}$ mice whereas infertility and decrease in the lifespan require six generations (Rudolph et al. 1999).

Another possibility is that in the mouse genome two or even several aging programs are encoded, each of them being responsible for aging of a group of organs. Indirect evidence in favour of the above speculations was quite recently obtained by Cawthon et al. (2003). The authors measured telomere length in blood leucocytes of 143 humans. Using this parameter, they formed two groups of people, namely individuals from the top and bottom halves of the telomere length distribution. It was found that the mortality rate in the short telomere group was almost two-fold higher than in the long one. Effects on the organ-specific mortality proved to be quite different. Mortality due to pneumonia and some other infection diseases was 8.5 times higher in the short telomere group, mortality due to heart-related diseases was 3.2 times higher, whereas that due to cerebrovascular diseases and cancer were only 35 and 43% higher, respectively. These data suggest that the rate of telomere-related aging is quite different in different organs and tissues.

8.9.5 Alternative functions of DNA end-underreplication

It seems quite possible that in modern multicellular organisms telomere shortening, besides aging, is employed to limit the number of divisions of somatic cells [the Hayflick limit (Hayflick 1965)]. Such a limit may be of importance for normal functioning of tissues, including anticancer defence. It is generally accepted that malignization of somatic cells is accompanied by reactivation of telomerase in these cells. Respectively, knocking out of a gene required for telomere formation inhibits in some cases cancerogenesis (Chin et al. 1999). On the other hand, there are some indications that the intact telomere is unfavourable for the development of other kinds of cancer (Rudolph et al. 1999; for discussion see de Lange and Jacks 1999). In any case, it is obvious that underreplication of the telomere DNA appeared already in unicellular eukaryotes, i.e., long before carcinogenesis, so their application in anticancer defence must represent the modification of another, much older function (Skulachev 2002b).

8.9.6 A tentative general scheme of organismal aging

Recently, Olovnikov (2003) suggested that, instead of the telomere, a special short double-stranded DNA molecule is employed by organisms to measure “biological time” and to make a decision when the aging program should be switched on. This

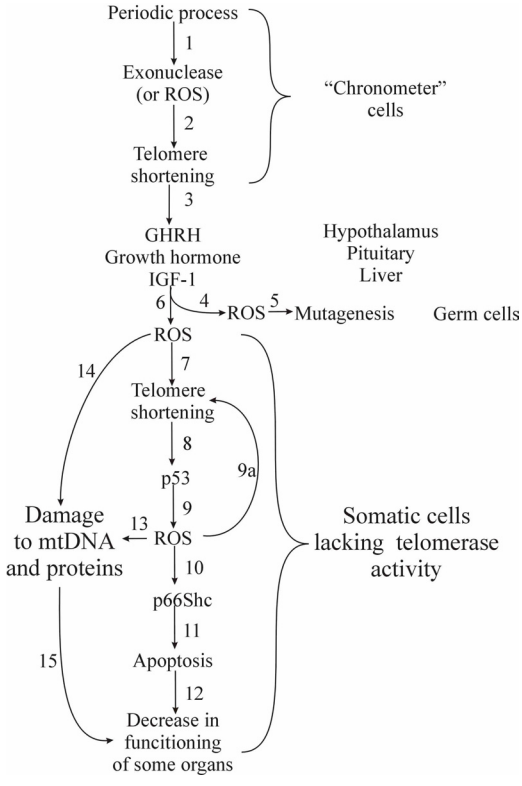

Fig. 4. A tentative scheme of programmed aging of organism. (For explanation, see the text).

hypothetical DNA called "chronomere" was postulated to be of identical sequence with a DNA region between the telomere and the major part of the chromosome. The next postulate of Olovnikov's new concept consists in that the "chronomere" DNA shortens in response to periodic release of some hormone by a gland involved in the mechanism of the biological clock.

In my opinion, however, possibilities of the original version of telomere concept of aging (Olovnikov 1971, 1973, 1997; see also Allsopp et al. 1992) are not yet exhausted, so today the chronomere hypothesis looks like an unnecessary complication. Below a tentative scheme will be presented trying to explain aging as a program. This scheme (Fig. 4) is composed of 15 discrete steps that will be considered in the next sections.

8.9.6.1 Chronometer (steps 1, 2)

Like any concept of programmed aging, our scheme needs a time-measuring mechanism. Such a biological clock, or chronometer, might be composed of (i) a periodical biochemical process and (ii) a system counting the number of periods.

For the process in question, a lunar cycle seems appropriated if the lifespan of the organism is as long as many years. A precedent of such a cycle is well known. This is the menstrual cycle. During the chronometer cycle (Fig. 4, step 1) changes should occur in concentration of a compound which activates special exonuclease (step 2) shortening telomere in the specialized chronometer cells (step 3)[2].

8.9.6.2 The chronometer cells produce a juvenile hormone (step 3)

It is assumed that an aging hormone is formed when the chronometer cell telomeres are short, or, alternatively, the cells procedure a juvenile hormone (e.g., dehydroepiandrosterone) when the telomere is long. The hormone in question operates in a tissue-specific fashion, initiating (or preventing) aging of cells in the target tissues. It seems probable that two or three hormones are included in series to amplify the primary aging signal.

In *C. elegans*, it was shown that "signals from both neurons and the gonad appear to negatively regulate longevity" (Murakami et al. 2000). Wolkow et al. (2000) analyzed what cells of *C. elegans* are responsible for shortening the life due to operation of genes *daf*-2 (insulin receptor-like gene) and *daf*-1 (the downstream phosphoinositol 3-kinase gene). The authors restored the *daf*-2 pathway signalling in three types of cells of worms lacking these genes. It was found that the lifespan shortens only if the restoration was done in neurons, not in muscle or intestine.

According to Akifiev and Potapenko (2001), it is DNA of neural ganglions that serves as the compound initiating aging in flies. As to higher animals, apparently one should search for the chronometer DNA in some specific brain cells. We already mentioned epiphysis when we discussed the possible role of melatonin (see the preceding Section). Hypothalamus (see Dilman 1971, 1978, 1994; Dilman and Anisimov 1979; Dilman et al 1979; Tatar et al 2003) and hypophysis (see Bowles

[2] Existence of an exonuclease specifically attacking telomere was discussed by Makarov et al. (1997). Another possibility consists in that the telomere shortening in the chronometer cells is caused by ROS (see below, Section 9.6.5). In this case, we should assume that the ROS level oscillates due to periodical change in rates of ROS production or ROS removal. An intriguing variation of this theme is that the chronometer cells are located in epiphysis and the ROS level oscillations occur due to changes in concentration of melatonin, a potent antioxidant. In this case, we deal with a daily, rather than with a monthly, rhythm. On the other hand, it seems also possible that the postulated periodic process results in division of the chronometer cell (one division per one cycle) and the DNA shortening in the chronometer cell is a simple consequence of end-underreplication. However, it seems essential that in any case it is DNA that performs the function of "the wild ass' skin " just as in the primordial eukaryotes who invented aging by opening the prokaryotic cyclic DNA. DNA is the only polymer molecule in the living cells, which functions for many years (e.g., in non-dividing brain cells of the long-lived mammals, one and the same DNA molecule is involved in the RNA formation during all the time of existence of the adult). This property seems to make DNA indispensable time-measuring compound in the chronometer cells.

1998; Tatar et al 2003) should be also mentioned in this context. In the former and the latter cases, role of the growth hormone releasing hormone (GHRH) and the growth hormone are of special interest.

It seems probable that the signal of the brain hormones is then multiplied in some way to be executed by cells of peripheral tissues. IGF-1 produced by liver and some other tissues looks like a good candidate to the list of mediators of the aging signal (see Section 8.8.4.2).

8.9.6.3 Hormone-stimulated ROS formation (steps 4 and 6)

An example of such a kind was indicated in Section 8.5.4 when the thyroxine-induced $NO^{\bullet}$- and H_2O_2-mediated organoptosis of the tadpole tail was described. As to the suggestion on an age-dependent ROS increase, there are very numerous reports that this is the case in eukaryotic cells from yeast to humans, as was originally suggested by Harman (1956) (for refs., see Skulachev 1997, 1999a; 2002b; Dillin et al 2002).

8.9.6.4 ROS-increased mutagenesis in germ cells (step 5)

In germ cells, the [ROS] increase (step 4) is assumed to be limited and its effect is confined to some increase in the nuclear DNA mutagenesis (step 5). Telomere length is not affected since telomerase is always active in germ cells. Mutagenesis in question might be the basis of genetic variations if the species is at such a stage of its evolution when the ***r***-strategy is employed. If the ***K***-strategy is employed, steps 4 and 5 can be absent.

In fact, the above-proposed role of ROS in mediating the aging program may be regarded as a modification of the primordial dependence of ROS formation upon the rate of aerobic metabolism, which proved more intensive when ambient conditions became more aggressive (see above, Section 8.3). As was mentioned in this section, such a relationship could be a mechanism to increase mutagenesis and, hence, genetic variability when conditions worsen.

8.9.6.5 ROS-induced telomere shortening in somatic cells (step 7)

In the telomerase-lacking somatic cells, the aging hormone-induced [ROS] increase (Fig. 4, step 6) is postulated to entail telomere shortening. It was shown by Von Zglinicki and co-workers (1995) that oxidative stress stimulates by a factor more than ten the telomere shortening rate in human fibroblasts in culture. It was also found that the GGG triplet in the human telomere-composing TTAGGG repeat is especially sensitive to cleavage by oxidative damage (for refs., see Von Zglinicki 2000). Moreover, oxidative stress increases the frequency of S1 nuclease-sensitive sites in telomeres (Von Zglinicki et al. 1995; Von Zglinicki 2000). In contrast to the rest of genome, these sites induced in fibroblasts by H_2O_2 are never completely repaired if the cells do not proliferate (Petersen et al. 1998). Direct experiments of Ren et al. (2001 a, b) showed that $OH^{\cdot}$ induces telomere shortening in

cell cultures. This initiated apoptosis in telomerase-lacking cells. (For reviews, see Von Zglinicki 2000, 2002; Serra et al 2000).

8.9.6.6 Telomere shortening is recognized by p53 (step 8)

There are many indications that this is the case. As was shown by Stansel et al. (2002), p53 specifically binds to some parts of telomeres, i.e., to the single strand overhangs and t-loop junctions. Milyavsky et al. (2001) reported that $(TTAGGG)_{40}$ oligonucleotide strongly stabilizes p53 while other sequences do not affect it. The authors suggested that 5'-**PuPuPuC(A/T)(T/A)G**PyPyPy-3' (the consensus p53 binding sequence), which is recognized by the protein "core" domain, resembles the **TTAGGGTTAG**GG telomere motive. According to Von Zglinicki (2000), in telomere, t-loop formation hinders access of the repair proteins to the telomere, leading to accumulation of single-strand breaks. Such single strands are strong inducers of the p53 pathways.

It is well established that p53, when it finds defects in DNA structure, induces operation of genes responsible for (i) synthesis of the DNA repair enzymes, (ii) cell cycle arrest and (iii) apoptosis (for reviews, see Oda et al. 2000; Lloyd 2002; Wahl and Carr 2001). Apparently, p53 recognizes breaks in a telomere as a DNA damage but repair enzymes failed to reach the damaged place. Thus, the first of the above p53 functions cannot be realized whereas two others can. In line with this statement, DePinho and his colleagues (Chin et al. 1999) found that survival of telomerase-deficient mice was increased by knocking out the p53 gene. The cells from the telomerase$^{-/-}$, p53$^{+/+}$ mice proved to be prone to growth arrest and apoptosis. Such effects were strongly attenuated in cells from the telomerase$^{-/-}$, p53$^{-/-}$ animals (see also Leri et al. 2003).

Another piece of evidence for involvement of p53 in aging was obtained when the Werner syndrome was studied. Within the framework of our concept, this disease, like other cases of progeria, represents a case of premature initiation of the aging program. As was recently found, such an effect can be due to a mutation in a gene called WRN. The WRN gene encodes a DNA helicase. Various types of the DNA damage were found to entail translocation of the Werner helicase from the nucleolus to nucleoplasmic foci (Blander et al. 2002). It was found that the Werner helicase directly interacts with p53 (Nehlin et al. 2000) (about other cases of progeria, see Saretzki and Von Zglinicki 2002; Hasty et al. 2003).

There are many observations that at least one of the p53-induced apoptoses is amplified by mitochondria. This effect is mediated by activation of the proapoptotic protein Bax and inactivation of antiapoptotic protein Bcl-2 (reviewed by Thomas et al. 2000), which in turn result in cytochrome *c* release to cytosol from the mitochondrial intermembrane space, activation of Apaf-1, and caspases 9 and 3(see above, Section 8.5.2).

8.9.6.7 ROS-induced ROS formation (step 9)

It is well known that some step(s) of the ROS-initiated mitochondria-mediated apoptosis are accompanied by a burst in formation of the secondary ROS. This

phenomenon was called by Zorov et al. (2000) "ROS-induced ROS release". The level of the secondary ROS is always much higher than that of the primary ROS. The mechanism of such an effect is not clear. Most probably it is caused by several reasons such as (i) an inhibition of the respiratory chain due to the cytochrome *c* release, resulting in lowering of O_2 consumption and, hence, and in an increase in the $O_2^{\cdot-}$ production by the initial and middle steps of the respiratory chain; (ii) exhaustion of the mitochondrial pool of antioxidants due to their release from mitochondria; (iii) de-energization of the mitochondrial membrane making impossible $NADP^+$ reduction by the energy-linked transhydrogenase and, hence, NADPH-supported regeneration of antioxidants, etc. Quite recently, Blasco and co-workers (Ramirez et al. 2003) showed that various DNA-damaging agents initiate massive telomere shortening, mitochondrial de-energization, ROS formation and apoptosis. An artificial uncoupler caused strong [ROS] increase and appearance of short telomeres.

Strong increase in [ROS] (Fig. 4, step 9) entails further telomere damage and shortening (step 9a). Thus, step 7 – 9a represent, in fact, an autocatalytic system with a positive feedback.

8.9.6.8 Role of p66Shc (steps 10 and 11)

Another consequence of the secondary ROS formation consists in activation of p66Shc. The structure of p66Shc strongly indicates that it belongs to a group of adaptor proteins responsible for transmission of a signal from the plasma membrane receptors to intracellular targets (Migliaccio et al. 1999). I suggested that a p66Shc-served receptor monitors the level of phosphatidyl serine (PS) in the outer leaflet of the plasma membrane (Skulachev 2000b, 2001a). Normally PS is absent from the outer leaflet being pumped to the inner leaflet by a PS-importing ATPase (for refs., see Skulachev 2000b). However, at an early stage of apoptosis, PS appears in the outer leaflet (most probably due to its oxidation by ROS, see Kagan et al. 2000). Appearance of phosphatidyl serine in the outer membrane leaflet and its recognition by p66Shc might be one more positive feedback mechanism facilitating apoptosis.

In any case, it is already clear that p66Shc is involved in apoptotic cascade downstream of the secondary ROS and p53. In the $p53^{-/-}$ cells, H_2O_2 failed to induce apoptosis (Trinei et al. 2002). H_2O_2 was also shown to be ineffective in the $p66Shc^{-/-}$ cells. On the other hand, in $p66Shc^{-/-}$ cells, p53 proved to be quite competent in the cell cycle arrest caused by H_2O_2. Apparently, proapoptotic p53 function is the only one that is absent from the $p66Shc^{-/-}$ cells whereas other functions of this protein are still present (Trinei et al. 2002). It is already revealed how p53 enhances p66Shc level. This occurs due to stabilization of p66Shc (Trinei et al. 2002). As to possible molecular mechanism of proapoptotic effect of p66Shc, this requires further investigation. It might include formation of tertiary ROS if a p66Shc signal reaches mitochondria (not shown in Fig. 4). Quite recently, the Pelicci's group reported about strong decrease in a high fat diet-induced systemic and tissue oxidative stress in $p66Shc^{-/-}$ mice. This was accompanied by lowering

of plasma density lipoprotein oxidizability and of altherogenesis caused by such a diet (Napoli et al. 2003).

8.9.6.9 Tissue-specific decline of physiological functions (step 12)

It is remarkable that the level of p66Shc is quite different in various tissues. As already mentioned, it is practically absent in the brain while its level is very low in heart, higher in skeletal muscles and kidney and much higher in skin, lung, spleen, and liver (Cattaneo and Pelicci 1998; Trinei et al. 2002). This predicts that the aging should be slowest in brain and fastest in skin, lung, etc., which is in line with an observation that it was first of all skin that showed aging features in telomerase$^{-/-}$ mice (see above, Section 8.9.4). In this way, I suggest, differential effect of aging on various organs is realized. This in turn leads to reproductive advantage of "good brain" animals according to the "hare-vs.-fox" rule (see above, Section 8.8.2).

It should be stressed that aging of brain should be slow not only because of absence of p66Shc but also due to the fact that telomeres are not shortened with age in this tissue. The same seems to be true for retina, heart, and skeletal muscle whereas in skin fibroplasts, liver, satellite cells, etc., age-dependent telomere shortening was revealed (Decary et al. 1997; Michelson 2001; Takubo et al. 2000).[3]

Another anti-aging effect in brain might be related to uncoupling proteins (UCPs). In fact, brain is the only organ where three antioxidant UCP species are present, i.e., UCP2, 4 and 5 (for refs., see Goglia and Skulachev 2003). Moreover, in the rat brain, aging was shown to be accompanied by an increase in the Bcl-2/Bax ratio whereas cytosolic cytochrome *c* is not enhanced; in heart, this ratio decreases and cytosolic cytochrome *c* rises. This means that on aging the brain and heart cells become less and more sensitive to apoptotic stimuli, respectively (Pollack et al. 2002). In *C. elegans*, Herndon et al. (2002) revealed "remarkable preservation of the nervous system, even in advanced old age, in contrast to a gradual, progressive deterioration of muscle, resembling human sarcopenia."

8.9.6.10 Role of damage to mtDNA and proteins, and telomere-independent aging mechanisms (steps 13-15)

As indicated by Melov (2000), "transgenic approaches in invertebrate models… have shown that the endogenous production of ROS due to normal physiological

[3] Paradoxically, telomere shortening occurs even in cells possessing active telomerase. This is the case for human intestine epithelial cells (esophageal and colonic mucosa) dividing once per 3-7 days. Apparently, in these cells the telomerase-mediated telomere elongation appears to be slower than the telomere shortening. As a result, the net telomere shortening rate in the intestine cells is 60-67 bp in 1 year. Values of the same order of magnitude were shown not only for human fibroblasts and lymphocytes (50-150 bp/year) but also for hepatocytes (55 bp/year) that divide once per 1.3-1.7 year (Takubo et al. 2000).

processes is a major limiter of life span". This ROS production can be localized both upstream (steps 4 and 5) and downstream (step 9) of telomere shortening (see Fig. 4). Above we already considered the telomere-linked organ weakness developing with age. However, it is already obvious that there are also other mechanisms producing the same effect with no telomere and age-linked telomere shortening involved. In tissues where such a shortening occurs, the primary ROS should play only a small role since the secondary ROS (step 9) must be produced at much higher rate. On the other hand, in tissues with no telomere shortening only the primary ROS seem to be formed. They may also be damaging if we deal with sufficiently long periods of time.

In any cell, two major targets for ROS should be mentioned, i.e., mitochondrial (mt)DNA and cellular proteins. Damage to any DNA is always especially dangerous for the cell because of the highest position occupied by this molecule in the biochemical hierarchy. As to mtDNA, it is much more vulnerable than nuclear DNA. It is localized in the mitochondrial matrix, i.e., near ROS-producing redox-proteins of the inner membrane; mitochondria lack histones covering nuclear DNA; the DNA reparation system in mitochondria is considerably weaker than that in the nucleus. Most probably, it is damage to mtDNA that is responsible for lethal effect of knocking out of the mitochondrial Mn-superoxide dismutase gene. Under the same conditions, knocking out of genes encoding the cytosolic and extracellular Cu,Zn-superoxide dismutases resulted in mild non-lethal phenotype (for reviews, see Melov 2000, 2002). Similar data were obtained on *C. elegans* where a mutation resulting in an enhancement of the mitochondrial Mn-superoxide dismutase was shown to prolong the lifespan (for refs., see Honda and Honda 2002).

Barja and co-workers (Pamplona et al. 1998) reported that the number of double bonds and the peroxidability index of fatty acid residues of the liver mitochondrial phospholipids are negatively correlated with the lifespan (mammals ranging in maximum lifespan from 3.5 to 46 years were studied). This is mainly due to a shift from the highly unsaturated docosahexaenoic acid to less unsaturated linolenic acid.

Lipid peroxidation may well be related to the potent antioxidant effect of minor uncoupling proteins, namely UCP 2, 3, 4, and 5. As we recently suggested (Goglia and Skulachev 2003), these UCPs can operate as pumps ridding the inner membrane leaflet of highly aggressive fatty acid peroxides. Such an effect should be membrane potential-driven. In line with this hypothesis, Santos et al. (2003) have reported that lowering of the mitochondrial membrane potential correlated with degree of oxidation of mtDNA in the H_2O_2-treated fibroblasts. As to nuclear DNA, it proved to be H_2O_2-resistant independently of membrane potential. Another membrane potential-dependent mitochondrial defence mechanism can be related to the energy-consuming transhydrogenase, which reduces $NADP^+$ to NADPH, the latter being a substrate of the SS-glutathione-reductase (for review, see Skulachev 1999a). In any case, the lifespan was shown to inversely correlate with rate of the ROS production by isolated mitochondria (reviewed by Honda and Honda 2002).

Besides mtDNA, ROS are known to attack proteins both inside and outside mitochondria. Quite recently, in the Nyström's laboratory it was described a striking phenomenon of segregation of oxidized proteins between the yeast mother cell and the bud (Aguilaniu et al. 2003; see also Hlavata et al. 2002). It was found that replicative aging of yeast is accompanied with massive oxidation of proteins in the mother cell whereas proteins in the daughter cell (the bud) appear to be intact. Previously in the same group, it has been shown that protein oxidation correlates with the levels of transcriptional or translational errors (Dukan et al. 2000).

Ryazanov (2001) assumed that it is the degree of protein oxidation that determines rate of aging of various organisms. He compared data for humans, rats, and flies and concluded that in all these cases a strong increase in the level of protein oxidation occurs at ages corresponding to 50% maximal lifespan.

It is not clear yet whether the age-dependent protein oxidation is related to mitochondria. As to telomere shortening, it may be involved in aging in tissues like liver but not in heart and brain when the length of telomeres remains constant during all the life. In heart, nevertheless, mtDNA seems to be strongly damaged with age. Ozawa (1997) reported that 89% of heart mtDNA contained some deletions in a woman of 97 whereas in a newborn child no deletions were found. Remarkably, the old woman died because of stomach cancer, not heart failure. Apparently, it was glycolysis that mainly compensated for the oxidative phosphorylation decrease under conditions of mitochondrial dysfunction. Certainly, maximal capacity of the heart as well as skeletal muscles strongly lower with age (see, e.g., Lexell et al. 1988; Reid and Durham 2002) and this occurs due to not only mitochondrial dysfunction in remaining cells but also decline in the cell number per organ. Weakness of an organ inevitably entails larger functional loading per cell in this organ, an event, stimulating further development of the stress usually accompanied by an elevated ROS production. Thus, one more step of the aging-linked ROS-induced ROS production seems to be organized. Such an effect will be the stronger in organs that are the primary targets of the programmed aging mechanism whereas such organ as brain should be protected.

8.10 Acute phenoptosis as the terminal step of the aging program

Aging as a specialized mechanism stimulating evolution is reasonable only during the reproductive period of life. When this period is over, further existence of the old individuals looks counterproductive. They die like bamboo, which already produced seeds, and this death is most probably programmed. However, in some species such an acute phenoptosis occurs before reproductive ability is exhausted. In Section 8.8.3, I already stated that the lifespan is optimizes depending on what the strategy, ***r*** or ***K***, and to what degree, is employed by the species at the given stage of its evolution. If the strategy in question is shifted to ***r***, acute phenoptosis may kill the organism even during the reproductive period. Such phenoptosis can be organized because of the aging process developing in an organ of vital impor-

tance, or, alternatively, as activation of a special suicide system. One more possibility is inactivation of a system defending the organism of, say, pathogens or other enemies. Fir dies at about 70 since it looses its resistance to fir crest. For higher animals, a similar mechanism consists in such lowering of activity of the immune system that the organism cannot survive pneumonia or some other infection diseases.

As to suicide mechanisms of acute phenoptosis, here infarction, stroke, and cancer should be mentioned. To some degree, infarction and stroke can be regarded as hyperapoptotic diseases whereas cancer as hypoapoptotic one. It was revealed that in infarction and stroke rather small initial necrotic region is surrounded by large region where cells die by means of apoptosis (for review, see Skulachev 1999a). The organism dies if the latter event makes impossible the functioning of heart or brain. In the case of cancer, malignization requires, as a rule, mutations resulting in decrease in level of the proapoptotic p53 or, less frequently, in increase in level of Bcl-2 or some other antiapoptotic factors (reviewed by Chumakov 2000 and Skulachev 2002b). Such events occur with age because of ROS-induced mutations in corresponding genes. This may explain age dependence of the great majority of types of cancer (Anisimov 1983, 1998, 2003). It is noteworthy that mutations in the p53 gene take place much more often than one could expect taking into account overall probability of mutations in nuclear DNA of mammals (Chumakov 2000).

It remains obscure what mechanisms are involved in initiation of acute phenoptosis at the age when the reproductive period of life is over. Most probably, a decrease in production of sex hormones plays here a major role (see, e.g., Bowles 1998).

Human aging represents a special case. In particular, women live for a long time after the reproductive period is over (menopause). Lewis (1999) proposed that "transmission of knowledge from grandparents to progeny serves as a driving force for extending human longevity… In early human societies, older individuals who are no longer useful could increase their reproductive success by activating a programmed aging mechanism, which would result in channelling of resources of progeny". It is not known yet how such an aging program, if it exists, is actuated. Apparently, this occurs when an old man or woman recognizes that he (she) is no more useful for the kin. Actually, there is a correlation between human mortality and psychological factors, such as a lack of emotional support and low mastery, i.e., belief that one is able to control his or her own life (Penninx et al. 1997).

8.11 Problems of genetic conservatism vs. variability and immortality

Bowles (1998, 2000) and Goldsmith (2003) discussed the possibility that females and males are mainly responsible for genetic conservatism and variability, respectively. One may suggest similar distribution of functions between young and old individuals. Within the framework of the above concept, it seems obvious that a

young organism with non-modified germ cell DNA and equally powerful organs corresponds much better to the conservative function, i.e., to reproduction of progeny typical for the present stage of evolution of the species. On the other hand, an older individual who is characterized by some changes in the germ cell DNA and by a weakness developing with different rate in different organs ("the hares-vs.-fox case", Section 8.8.2) is good to produce progeny slightly differing from the dominating phenotype. In this context, it should be emphasized that the old organisms (organs, cells) differ from each other much more strongly than the young ones. Such a difference is especially demonstrative if we consider a substance of aging, the telomere. Remember the well-known fact of great variations in the measured values of telomere length.

Existence of programmed aging presumes that there are mechanism(s) preventing immortality, otherwise immortal mutants who lost the aging program would crowd out the mortal wild type from a population. In this context, two aspects should be regarded.

1. There are parallel metabolic pathways leading to aging. In Fig. 4, this was exemplified by telomere dependent (steps 7-13) and independent (steps 14, 15) systems of programmed aging.[4] This should strongly decrease probability of appearance of immortal individuals since this requires not a single gene but several independent genes to be mutated.
2. Even more important, the aging program seems to be organized in such a way that some of its components are bifunctional, being simultaneously required for death (aging) and life (anticancer defence).

An excellent example of such a situation was given by the already discussed (see Section 8.8.5) experiments of Donehower and colleagues (Tyner et al. 2002) who have shown that an increased p53 activity appears to be simultaneously of anticancer and pro-aging effects. This means that dysfunction of the p53 gene, which potentially might cause immortality due to switching off an aging mechanism,

[4] Such a possibility was discussed in detail by Bowles (1998) who considered, besides the telomere- and ROS-linked programmed aging mechanisms, the DNA demethylation. Methylation of the DNA cytosine by DNA methylase results in formation of 5-methylcytosine residues. This inhibits transcription of the corresponding genes being a mechanism of suppression of activity of these genes (for review, see Cooney 1999). It was found that aging is accompanied by DNA demethylation (Vanyushin et al. 1973; Wilson et al 1987; Mazin 1993a,b; see also Cooney 1999). In fact, the animal genome loses practically all 5-methylcytosines during the life, the rate of the loss being inversely proportional to maximal lifespan of the species (Mazin 1993a). The same occurs in cell cultures, again the rate being inversely proportional to the cell lifespan (Hayflick limit) (Mazin 1993 a,b). It was also shown that oxidation by ROS of the guanine DNA residues to 8-hydroxyguanine strongly inhibits methylation of adjacent cytosines (Weitzman et al. 1994). Antioxidants, on the other hand, cause DNA hypermethylation (Romanenko et al. 1995). According to Panning and Jaenisch (1996), DNA hypomethylation activates Xist gene expression in X chromosome, which correlates with a dramatic stimulation of apoptosis. All these observations may be summarized by the following chain of age-related events:

ROS → DNA demethylation → apoptosis → aging (3)

does not prolong the lifespan since an abrogation of the anticancer defence takes place. In fact, in the same experiments, it was shown that $p53^{-/-}$ mice live very much shorter than the wild type because of great increase in probability of cancerogenesis (Tyner et al. 2002; for discussion, see Sharlees and DePinho 2002; Skulachev 2002b).

Thus, the above example may explain how aging mechanisms, when they appeared, could be retained during evolution: p53 is required for slow phenoptosis (age-dependent weakening of the organism) but it prevents one of the most widespread types of acute phenoptosis (cancer) (Skulachev 2002b). Perhaps, just in this way we should answer a question put by Weismann more than a century ago when he formulated his paradoxical concept of death as adaptation: "There cannot be the least doubt that the higher organisms, as they are now constructed, contain within themselves the germs of death… The question arises as to how this has come to pass" (Weismann 1889).

Special attention should be paid to the role of oxygen in aging as well as in biological evolution in general. It seems probable that great evolutionary success of aerobic organisms versus anaerobes is at least partially due to that ROS are major mutagens responsible for DNA changes. On the same reason, however, ROS are the major danger for the life based upon DNA-linked information storage.

In Figure 4, it is assumed that also today ROS are performing their dual role, being responsible for DNA changes causing further evolution of this molecule and for its irreversible decompositions. Destructive role of ROS is still the great problem for any aerobe. This is why such a specialized mechanism of evolution as aging is arranged in such a way that it always tries to better the multifaceted antioxidant system of organism. In other words, ROS operate like the fox in our hares-vs.-fox case (see Section 8.8.2), so the evolution is always directed toward more robust antioxidant defence. This appears to be a direct consequence of that execution of the aging signal results in lowering of the level of antioxidant defence in organelles, cells, tissues, and organs. Such a lowering could be a result of in an increase in ROS formation and/or a decrease in amount of ROS scavengers. If such an effect is organ-specific, it also stimulates evolution of this organ according to the hares-vs.-fox rule. The above relationships explain why ROS appear several times in the general scheme of aging shown by Figure 4.

8.12 Conclusion

In this paper, I tried to present a tentative general scheme of aging of various organisms because of operation of some genetically-determined programs. Doing this, I have based a recent finding of the self-elimination systems inherent in cells (including unicellular organisms) as well as some sub- and supracellular systems. It appeared that such a scheme could really be constructed without making any improbable assumptions. The concept is already supported by many pieces of indirect evidence. The main obstacle in accepting seems to be of psychological matter. We still believe (i) to some biologists-evolutionists that aging does not occur in

wild nature and (ii) to the common sense that the death, being an alternative to the life, cannot, in principle, be programmed by a living creature. However, it becomes clear that both these statements are hardly right. (1) In ontogenesis of modern eukaryotic organisms, aging starts so early that for sure it can affect their very fate. (2) Programmed death is already described among many unicellular organisms and some multicellular ones; moreover, it is clearly demonstrated at the suborganismal levels in the animal, plant, and fungal species studied.

Simple logic clearly shows that aging may be employed by living creatures as a special mechanism facilitating evolution. In fact, it allows small changes, which are inessential for a strong young organism to be recognized and selected at older ages when an organism is weakened by age. Such a weakening is due to controlled lowering of efficiency the organizmal antioxidant systems, an event directing evolution toward a high ROS resistance.

The negative opinion concerning possibility of existence of the programmed death of organism was questioned in some quite recent publications. In *Nature* magazine, Lithgow and Gill (2003) discussing extension of the lifespan of mice by knocking out of *ifg-1r* gene, entitled their paper "Cost-free longevity in mice?" They stressed that hormone-mediated shortening of the lifespan can hardly be accounted for without assuming that aging is somehow programmed. In the February 28 (2003) issue of *Science*, a series of articles appeared directly related to the programmed aging problem. In all of them, schemes of hormonal cascades resulting in aging are presented (Hekimi and Guarente 2003; Tatar et al. 2003; Longo and Finch 2003). Nevertheless, none of the authors directly claims that he accepts the programmed death paradigm. Hekimi and Guarente (2003), the World champions in prolonging the life of *C. elegans*, wrote: "Lifespan, therefore, appears to be regulated in these situations", but immediately added: "in spite of the fact that it is not the feature shaped adaptively by natural selection". Dear colleagues, you should not apologize for the former part of your conclusion.

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9 The human Werner Syndrome as a model system for aging

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Abstract

The aging process is one of the most challenging biological pathways to understand. Applications of model systems for aging have contributed significantly to the recent understanding of the aging process. Characteristics of the human Werner syndrome resemble those of normal aging in many ways. The premature aging disease Werner syndrome exhibits many symptoms of genomic instability, and the Werner syndrome protein (WRN) interacts with many proteins that participate in maintaining genomic integrity and repairing damaged DNA. This illustrates a strong correlation between aging and genomic instability. This review summarizes the biochemistry and the main DNA repair pathways of WRN and their impacts on genomic stability and aging.

9.1 Premature aging models: RecQ helicases stand out

The human Werner syndrome (WS) is a segmental progeroid syndrome, and is considered an excellent model for human aging. WS is an autosomal recessive disorder in which the *WRN* gene is mutated, and displays age-associated diseases such as atherosclerosis, non-insulin-dependent diabetes mellitus, ocular cataracts, osteoporosis, and increased incidence of certain forms of cancer, especially sarcomas (Martin et al. 1996). WRN belongs to the RecQ helicase family, which comprises a conserved group of proteins implicated in several aspects of DNA metabolism (Karow et al. 2000). Among the family members, three helicases, WRN, Bloom syndrome (BS) protein (BLM) and Rothmund-Thomson syndrome (RTS) protein (RTN) are associated with heritable human diseases.

One major theory of aging is based on the observation that DNA damage accumulates as individuals become older. WS, BS, and RTS display chromosomal aberrations (Table 1), and all three proteins responsible for these syndromes exhibit mutations including nonsense, frameshift, and splicing defects. Such mutations lead to truncated proteins, resulting in loss of their nuclear localization (see below). The truncated, mis-located BLM and WRN are targets of protein degradation, and the loss of these human RecQ proteins may result in chromosomal instability. Cellular localization of the mutant RTN is currently unclear. Translocation mosaicism is common in both WS and RTS, and hyper-recombination is one of

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Table 1. Human RecQ helicases and the genetic diseases

	WRN	**BLM**	**RTS**	**RecQ1**	**RecQ5**
Syndrome	**Werner**	**Bloom**	**Rothmund-Thomson**	**Unknown**	**Unknown**
Genomic instability	Translocation mosaicism, large deletions, hyper-recombination	Hyper-recombination, sister-chromatid exchange	Translocation mosaicism	-	-
Chromosomal location	8p12	15q26.1	8q24.3	12p12	17q25.2-25,3
Size (aa)	1432	1417	1208	649	991
Cases	~1100[1]	~200[2]	~300[1]	-	-
Types of mutation	Nonsense, frameshift, splicing defect	Nonsense, frameshift, splicing defect, missense	Nonsense, frameshift, splicing defect	-	-

[1]Worldwide cases, since discovery.
[2]Worldwide cases, since 1975.

the common characteristics between WS and BS. One unique cellular feature of BS is an elevated rate of sister chromatid exchange, which has been used for diagnosis. Large chromosomal deletions are commonly observed in WS.

WS was first reported in 1904 by a German ophthalmologist, Otto Werner. The average life expectancy of WS patients is 46 ± 11.6 years, and the major cause of death is malignant cancer and cardiovascular infarction. Based on the reported cases, the incidence rate for WS is about 1/1,000,000, and 75% of the patients are of Japanese descent (Goto 1997). Table 2 compares the clinical features of WS and normal aging. WS patients display a remarkable number of clinical common features with older adults. However, WS patients do not display severe neurodegeneration, and short stature and soft tissue calcification common in WS patients are not associated with normal aging. Our recent cDNA microarray analysis shows that the gene expression patterns between WS and normal aging are very similar (Kyng et al. submitted), supporting the general view that WS is one of the best models for segmental progeroid. Although BS and RTS also show some signs of segmental progeria, they are not as prominent as those of WS. BS patients are diagnosed with cancer at an average age of 24, which is usually the main cause of death. Such early death may prevent BS patients from developing symptoms of normal aging as those observed in patients with WS. Clinically, patients with RTS exhibit symptoms which partially overlap with normal aging including cataracts

Table 2. Comparisons of WS clinical features with normal aging

Major clinical features	WS	Normal aging
Graying and loss of hair	Yes	Yes
Wrinkling of the skin	Yes	Yes
Cataract	Yes	Yes
Malignancies, especially sarcomas	Yes	Yes
Atherosclerosis	Yes	Yes
Osteoporosis	Yes	Yes
Type II diabetes	Yes	Yes
Hypogonadism	Yes	Yes
Hyaluronic aciduria	Yes	Yes
Hypertension	Yes	Yes
Short stature	Yes	-
Soft tissue calcification	Yes	-
Laryngeal atrophy	Yes	-
Central nervous system degeneration	-	Yes
Inheritance	Autosomal recessive	Polygenic

and greying and loss of the hair (Lindor et al. 1996). However, RTS is extremely rare, and caution should be taken when one considers RTS a case of RecQ helicase gene mutation, as only a subset of those patients show mutations in the RTS gene. In light of these limitations, WS is the best model to study premature aging among those with RecQ helicase gene mutations.

9.2 Biochemical aspects of WRN: domains, protein-protein interactions, and post-transcriptional modifications

WRN is a 1432 amino acid nuclear protein that possesses four conserved regions, two targeting sequences, and three catalytic activities (Figure 1). This protein has 3'→ 5' helicase, 3'→ 5' exonuclease and ATPase activities (for review, see Brosh, Jr. and Bohr 2002). DNA helicases promote the separation of complementary strands of the DNA duplex, providing access for proteins to the template during replication, recombination, and repair (Lohman and Bjornson 1996). 3' → 5' exonucleases play a proofreading role for DNA replication, repair, and recombination (Shevelev and Hubscher 2002). ATPase activity provides the required energy for the WRN helicase activity. Because of the multifunctional property and the many protein-protein interactions of WRN, this protein may be a modulator of DNA metabolism.

The N-terminal part of WRN (first 500 amino acids) is composed of the DNA exonuclease domain (Huang et al. 1998; Kamath-Loeb et al. 1998) and an acidic region (Yu et al. 1996). WRN is unique among the human RecQ helicases in that it contains an exonuclease domain (for review, see Karow et al. 2000). This do-

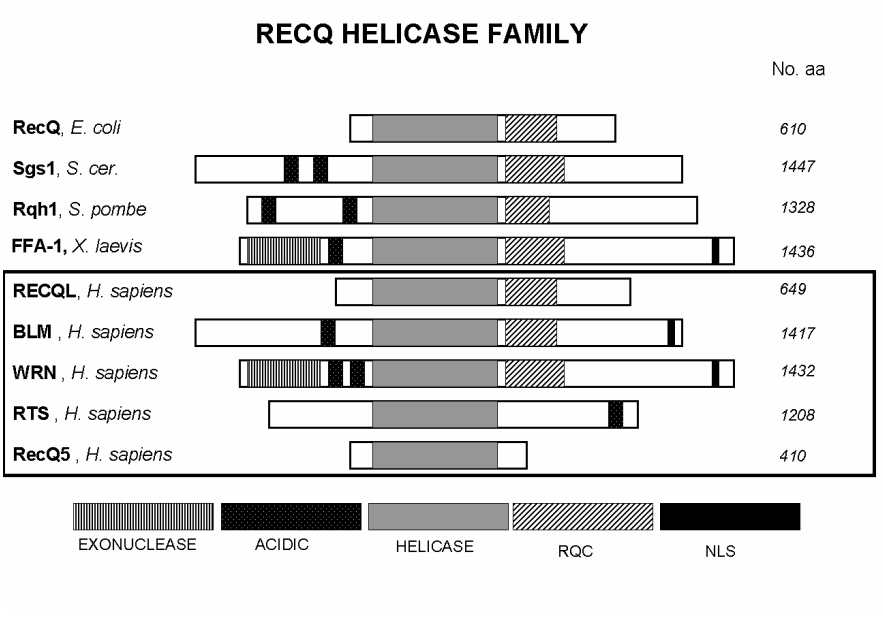

Fig. 1. Domains of the RecQ helicase family. The human family members are shown in the box.

main alone (first 370 amino acids), when expressed as a recombinant protein, is fully active, indicating that other WRN regions are not necessary for its exonuclease activity (Huang et al. 2000; Xue et al. 2002). The middle part of WRN contains an ATPase-dependent DNA helicase domain (Gray et al. 1997; Suzuki et al. 1997). This domain is conserved among other members of the RecQ helicase family (Fig. 1). Finally, the C-terminal part of WRN (last 500 amino acids) contains the RQC (RecQ conserved) and the HRDC (helicase, RNaseD conserved) domains (Yu et al. 1996) and two targeting sequences (nuclear localization signal, NLS; nucleolar targeting sequence, NTS) that direct the protein to the nucleus and, specifically, to the nucleolus (Matsumoto et al. 1997; von Kobbe and Bohr 2002). The NLS is located at the end of the C-terminal region of WRN (amino acids 1370-1375) (Matsumoto et al. 1997), whereas the NTS is in the RQC domain (amino acids 949-1092) as visualized in living cells (von Kobbe and Bohr 2002). Another recent report shows that the two amino acids, Arg1403 and Lys1404, in the C-terminal region are required for the nucleolar localization of WRN using fixed cells (Suzuki et al. 2001). However, both reports show that correct nucleolar localization of WRN requires both the NLS and NTS in the same polypeptide. The mouse WRN does not localize to the nucleolus, and the amino acids Arg1403 and Lys1404 in human WRN are not conserved in mouse WRN. When cells express a human WRN mutant that lacks the NTS, this mutant WRN is excluded from the nucleolus. These results may link WRN nuclear import and nucleolar localization to a convergent pathway (von Kobbe and Bohr 2002). Although a yeast hybrid-

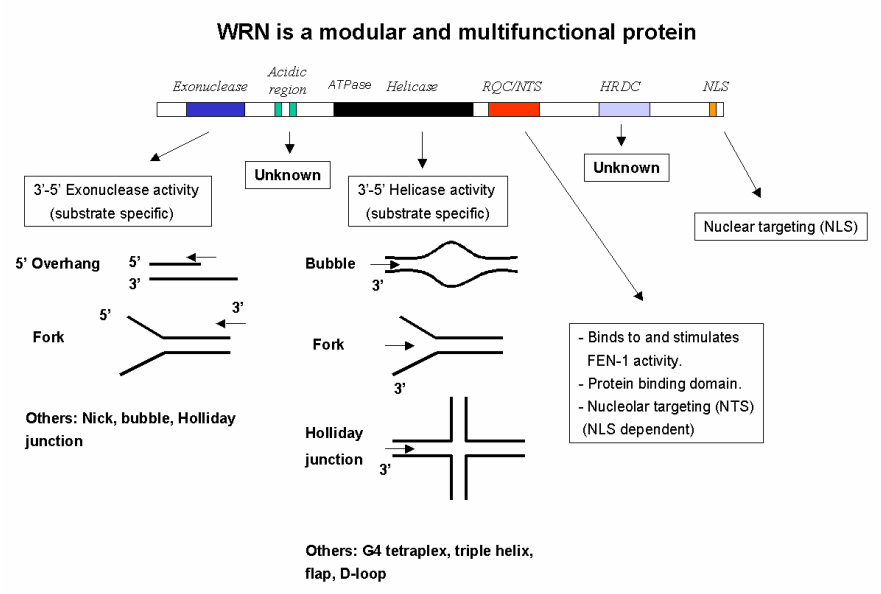

Fig. 2. Catalytic activities and identified functions of the domains and regions found in the WRN protein. The identified substrates for WRN exonuclease and helicase activities are shown. To date, there is no known function for the acidic region and the HRDC domain. The small RQC/NTS domain contains the nucleolar targeting sequence and may be the major region for WRN to bind to its protein partners.

protein reporter assay showed that a direct repeat of 27 amino acids in the acidic region may enhance the role of WRN in transcription (Balajee et al. 1999), the precise function(s) of the acidic region and the HRDC domains remain to be elucidated.

The exonuclease and helicase activities of WRN depend on the structure of the substrates (Shen et al. 1998; Shen and Loeb 2000) (see Figure 2) and, in some cases, can work in a coordinated manner on the same substrate (Opresko et al. 2001; Machwe et al. 2002). WRN catalytic activities have been tested using a spectrum of DNA structures *in vitro*, including the Holliday junction that resembles recombination intermediates (Mohaghegh et al. 2001; Constantinou et al. 2000), the forked duplex that resembles a replication fork (Opresko et al. 2001), the bubble-containing substrate that resembles DNA repair intermediates (Shen and Loeb 2000), and a D-loop that resembles telomere ends (Orren et al. 2002). The capability of WRN to digest or unwind these substrates supports a role for WRN in many facets of DNA metabolism such as recombination, replication, DNA repair, and telomere maintenance. The WRN exonuclease digests recessed 3'OH-bearing and forked substrates efficiently, but it does not digest blunt ended, recessed 5'OH-bearing, and ssDNA substrates. However, pre-incubation of WRN

with Ku makes it capable of digesting blunt ended and ssDNA substrates (Li and Comai 2001) and allows WRN to bypass oxidative DNA lesions (Orren et al. 2001). The WRN helicase can also unwind DNA bubbles, forks, Holliday junctions, G4 tetraplexes, triple helices, but does not unwind blunt ended or recessed 3'OH-bearing duplexes.

The catalytic activities of WRN are modulated by its binding to other proteins and/or by phosphorylation. The N-terminal region of the protein (first 120 amino acids) binds several proteins including the Ku heterodimer (Ku70, Ku80), BLM, and telomeric repeat binding factor 2 (TRF2) (Karmakar et al. 2002b; Li and Comai 2001; von Kobbe et al. 2002; Opresko et al. 2002). The DNA-PK complex consists of the large DNA-PKcs subunit and the Ku heterodimer bound to DNA. It is proposed that Ku is recruited to the broken DNA ends, which in turn recruit DNA-PK_{CS} and other factors to repair DNA by non-homologous end-joining (NHEJ) (see below). Binding of WRN to Ku dramatically stimulates the exonuclease activity of WRN (Cooper et al. 2000), and this stimulation is abrogated when WRN is Ser/Thr phosphorylated by the catalytic subunit of the DNA-PK complex (DNA-PKcs) (Karmakar et al. 2002a; Yannone et al. 2001). In contrast, binding of BLM or p53 to WRN inhibits its exonuclease activity (von Kobbe et al. 2002; Brosh et al. 2001), and there is no effect of TRF2 on WRN exonuclease activity (Opresko et al. 2002). The helicase activity of WRN is stimulated by replication protein A (RPA) (Brosh, Jr. et al. 1999) and, modestly, by the telomere binding protein TRF2 (Opresko et al. 2002). WRN helicase activity is also enhanced when incubation with the Nbs1 protein that is mutated in Nijmegen breakage syndrome (Cheng et al. submitted). Currently, the mechanism of the stimulation of WRN helicase due to protein-protein interactions is not fully understood. In addition to direct protein-protein interactions, phosphorylation of WRN by the DNA-PK complex or by the c-Abl tyrosine kinase inhibits the exonuclease and helicase activities of WRN (Karmakar et al. 2002a; Yannone et al. 2001; Cheng et al. 2003).

The RQC domain of WRN contains the NTS and binds to proteins including flap endonuclease-1 (FEN-1), BLM, poly(ADP)ribose polymerase 1 (PARP-1), and TRF2 (Brosh, Jr. et al. 2001b; von Kobbe et al. 2002; Opresko et al. 2002; von Kobbe et al. submitted). We have recently shown that the WRN RQC domain also binds to the c-Abl tyrosine kinase, and association of WRN with c-Abl is disrupted after bleomycin treatment, followed by a re-distribution of WRN to the nucleoplasm (Cheng et al. 2003). Similarly, if the NTS is missing, WRN localizes to the nucleoplasm instead of the nucleolus where it normally resides (von Kobbe and Bohr 2002). In addition, the FEN-1 endonuclease cleavage activity is dramatically stimulated when it binds to the RQC domain of WRN (Brosh Jr et al. 2001b). Thus, this highly conserved domain appears to play an important role in mediating WRN protein interactions and in regulating the nucleolar targeting of the protein. The detailed functional significance of protein-protein interactions in association with the RQC domain remains to be elucidated.

In light of the known functions of WRN and its protein partners, WRN appears to participate in many pathways of DNA metabolism, which may depend on the stage of cell cycle and on the cell type. Conformational changes, due to post-

translational modifications of WRN and the proteins WRN binds to, may alter the accessibility of WRN such that it can be transported to different nuclear regions. This context could explain why the catalytic activities and the nuclear trafficking of WRN are affected by physical interaction with other proteins. Thus far, WRN has been found to be modified by phosphorylation at Ser/Thr (Karmakar et al. 2002a; Yannone et al. 2001) and Tyr (Cheng et al. 2003) residues, sumoylation (Kawabe et al. 2000), and acetylation (Blander et al. 2002). These post-translational modifications are likely to modulate the localization and catalytic activities of WRN. Phosphorylation of WRN, either by the DNA-PK Ser/Thr kinase or by the c-Abl Tyr kinase, inhibits both the exonuclease and helicase activities. The nucleolar localization of WRN is modulated by acetylation and tyrosine phosphorylation (Blander et al. 2002; Gray et al. 1998). Although the biological significance of WRN modification by sumoylation is unknown, this event may occur in the promyeloytic leukemia (PML) bodies where they co-localize with WRN (Johnson et al. 2001). Sumoylation of many other components of the PML bodies is critical for their proper nuclear localization (Seeler and Dejean 2001). In addition, a recent report showed that DNA damage-induced WRN acetylation is associated with WRN re-localization (Blander et al. 2002). This acetylation event is likely to be linked to WRN tyrosine phosphorylation, as c-Abl is required for some DNA damage-induced acetylation events (Costanzo et al. 2002).

Post-translational modification may also affect the nature of the protein-protein interaction. We have recently found that treatment of cells with DNA strand breaks inducers disrupts the association between WRN and c-Abl, but increases the association of WRN with the Mre11 complex (Cheng et al. submitted). Another factor that affects protein-protein interactions with WRN is the stage of cell cycle. For example, BLM is predominantly expressed in the S phase, and, in yeast, the Mre11 complex contains two other proteins in meiotic cells (Usui et al. 1998). Another consideration is the strength of the interaction. We found that, using a RQC domain of WRN fused to GST to pull down proteins from nuclear extracts of unstressed cells, PARP-1 is the most prominent protein associated with this WRN domain (von Kobbe et al. submitted). A stronger interaction between two proteins may imply a more important functional significance. Taken together, it is not surprising that WRN interacts with many proteins, as the preference of WRN to interact with its protein partners changes in response to many factors. Much debate has surrounded the nature and the physiological importance of the many interfaces of WRN. However, based on the known functions of the several proteins with which WRN interacts, the consensus that WRN participates in many aspects of DNA metabolism is well justified. In particular, WRN appears to participate in a number of DNA repair pathways and clearly has an important function in this general process. Important issues to be addressed are whether these post-translational modifications are necessary for the biological functions of WRN, and where the sites of those modifications in the WRN amino acid sequence are located. In this scenario, it makes sense to speculate that WRN participation in different facets of DNA metabolism is dependent on WRN location and modification under specific cellular conditions. Answering these questions will lead to a better

understanding of the precise cellular roles for WRN, as well as provide possible insight into the mechanism of the observed aging phenotypes in WS.

9.3 Linkage between the sub-cellular distribution and function of WRN

In exponentially growing human cells, WRN localizes primarily in the nucleolus where it may participate in rRNA metabolism (Gray et al. 1998; Marciniak et al. 1998; von Kobbe and Bohr 2002; Machwe et al. 2000b). One recent study showed that WRN associates with a RNA polymerase I component, RPA40, and that inhibition of rRNA transcription by actinomycin D re-localizes WRN to the nucleoplasm (Shiratori et al. 2002). However, another study found no role for WRN in ribosomal transcription, while it clearly functioned in RNA polymerase II-mediated nuclear transcription (Balajee et al. 1999). WRN also re-localizes to the nucleoplasm as nuclear foci or nuclear diffuse in response to DNA damage. Inducers that result in WRN re-distribution include etoposide, 4-nitroquinoline 1-oxide (4-NQO), bleomycin, γ-irradiation, camptothecin, and mitomycin C (Gray et al. 1998; von Kobbe and Bohr 2002; Sakamoto et al. 2001). Nuclear localization of WRN changes at different stages of the cell cycle, as evidenced by the nuclear diffusion of WRN in the G_0 and S-phase cells (Shiratori et al. 2002; Brosh Jr. et al. 2001). The linkage between the nuclear localization and the functional role of WRN remains to be elucidated; however, it appears that WRN plays critical roles in different nuclear regions.

Nucleoplasmic WRN may be recruited to sites of DNA damage, and some of the DNA repair foci are likely to be coincident with damaged replication forks (McGlynn and Lloyd 2002). Sites of DNA damage are foci where local concentrations of DNA repair proteins are enriched. They include γH2AX, Rad51, the Mre11 complex (containing Mre11, Rad50 and Nbs1/Xrs1) and BRCA1 foci. However, those foci may not necessarily co-localize, as the Mre11 foci are distinct from the Rad51 foci at an early stage after the formation of DNA double strand breaks (Nelms et al. 1998). After hydroxyurea treatment which arrests cells in S-phase, WRN re-distributes and co-localizes with RPA and p53 (Constantinou et al. 2000; Brosh, Jr. et al. 2001). Moreover, it has been shown that a significant portion of WRN co-localizes either with Rad51 after exposure to camptothecin (Sakamoto et al. 2001) or with Nbs1 in response to γ-irradiation or mitomycin C (Cheng et al., submitted). Clearly, re-localization of WRN from the nucleolus to the sites of DNA damage in the nucleoplasm appears to be required for WRN to play a functional role in the DNA damage response.

What are the signals that trigger WRN to leave the nucleolus? We have recently found a novel physical and functional interaction between WRN and c-Abl that may contribute to DNA damage-induced WRN re-localization (Cheng et al. 2003). The ubiquitous c-Abl belongs to the non-receptor tyrosine kinase family, and the nuclear c-Abl is activated upon treatment with DNA-damaging agents including ionizing radiation, mitomycin C, cisplatin, etoposide, camptothecin, and ara-C

(Wang 2000; Kharbanda et al. 1995; Liu et al. 1996; Yoshida et al. 2002). After treatment of cells in culture with bleomycin, which induces DNA strand breaks, WRN dissociates from c-Abl, and this event precedes WRN re-localization. We also observed that the formation of γH2AX foci precedes WRN re-localization (Cheng et al. unpublished). These events imply that, in response to DNA damage, the foci formation and WRN re-localization are distinct events, and proteins in the foci may recruit WRN. Importantly, this bleomycin-induced WRN re-localization can be attenuated by an Abl kinase inhibitor. These lines of evidence strongly support the notion that WRN can be sequestered by c-Abl in the nucleolus. At early S phase, either during the normal cell cycle or after being halted by DNA damage, c-Abl is activated, which, in turn, releases WRN to the sites of DNA damage and/or replication forks. However, other c-Abl-independent pathways may retain the remaining WRN protein in the nucleolus for rRNA metabolism after DNA damage, as a portion of WRN is usually resistant to re-localization. This c-Abl-mediated WRN re-localization may explain why WRN is constitutively distributed throughout the nucleus in chronic myeloid leukemia where Abl kinase is highly active (Sakamoto et al. 2001). Future studies should be designed to delineate the detailed mechanism of WRN tyrosine phosphorylation, and how the status of this WRN modification on its physical and functional interaction with other proteins in the nucleoplasm.

9.4 DNA repair defects in WS

In Figure 3, we categorized selective agents that induce many types of DNA lesions, and the four major mammalian DNA repair pathways involved: base-excision repair (BER), nucleotide-excision repair (NER), recombinational repair, and mismatch repair. The sensitivity of WS cells to DNA-damaging agents further supports a role for WRN in DNA repair. WS cells show marked hypersensitivity to 4-NQO, topoisomerase inhibitors, and DNA interstrand cross-linking agents (reviewed in Brosh, Jr. and Bohr 2002). In addition, a mild sensitivity of WS cells to ionizing radiation has been demonstrated (Yannone et al. 2001; Saintigny et al. 2002). In contrast, WS cells display no or little sensitivity to ultraviolet light, bleomycin, hydroxyurea, or alkylating agents (reviewed in Shen and Loeb 2001). However, recent reports showed that $WRN^{-/-}$ chicken cells are hypersensitive to the alkylating agent methyl methanesulfonate (MMS) (Imamura et al. 2002), and, after treatment of mammalian cells with bleomycin, WRN forms distinct foci in the nucleoplasm (Sakamoto et al. 2001). Whether these responses of WRN to MMS or bleomycin are cell type-specific remains to be determined. These observations reinforce the notion that WRN participates in many aspects of DNA repair in response to different cellular conditions.

Various types of DNA repair defects have been found in WS cells. We have previously shown that transformed WS lymphoblasts exhibit a decreased rate of repairing actively transcribed genes following UV exposure. However, this defect is not observed in primary WS fibroblasts (Webb et al. 1996). Recent reports

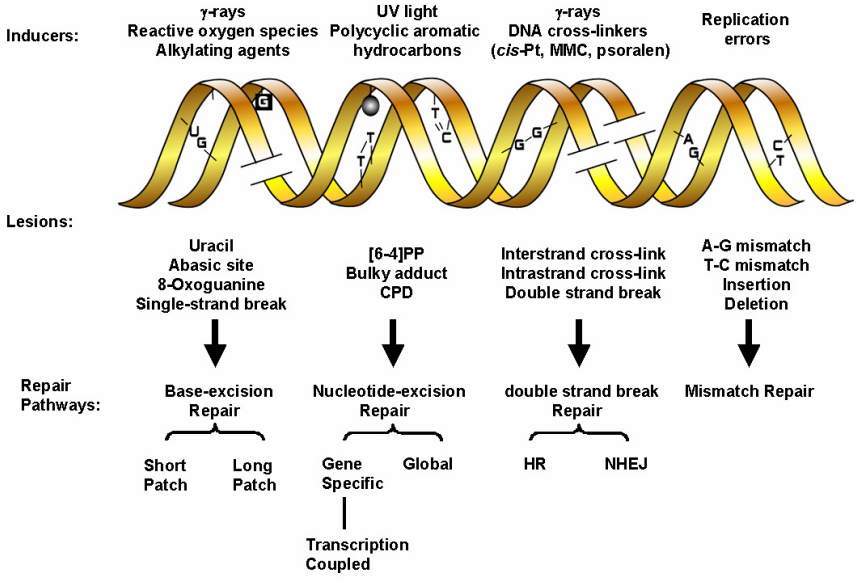

Fig. 3. The major types of DNA lesions, the genotoxic reagents that induce them, and the major pathways that repair them. Cis-Pt, *cis*-platinum; CPD, cyclobutane pyrimidine dimers; HR, homologous recombination, NHE, non-homologous end-joining; MMC, mitomycin C

showed that WS cells are particularly sensitive to compounds that induce DNA cross-links (Poot et al. 2001). A current focus in WRN research is to explore the mechanism by which WRN functions in DNA double strand repair and in the resolution of stalled replication forks. Homologous recombination (HR) and NHEJ may be involved in both of these events. By transfecting cells with linearized plasmids or shuttle vectors, it was demonstrated that WS cells are as efficient as control cells in DNA end-joining (Runger et al. 1994; Bohr et al. 2001). However, WS cells showed elevated rates of mutation by PCR sequencing after the cells were transfected with a plasmid carrying a mutagenesis marker near the ligation site (Runger et al. 1994). By sequencing the regions across the joined ends, Oshima et al. (2002) found that WS cells showed extensive degradation of DNA ends before ligation, particularly at the 5'-recessed ends. Although the precise mechanism is unknown, WRN appears to be of great importance in maintaining genomic stability via the NHEJ pathway. Recently, WS cells were found to exhibit hypersensitivity to *cis*-platinum following mitotic recombination, and WRN may function in resolving recombination intermediates in a Rad51-dependent manner (Prince et al. 2001; Saintigny et al. 2002). Understanding the mechanism by which WRN participates in the DNA repair pathways may provide insight into the genetic instability and the accelerated aging in WS. The three major DNA repair pathways that WRN participates in are discussed in more detail below. To date,

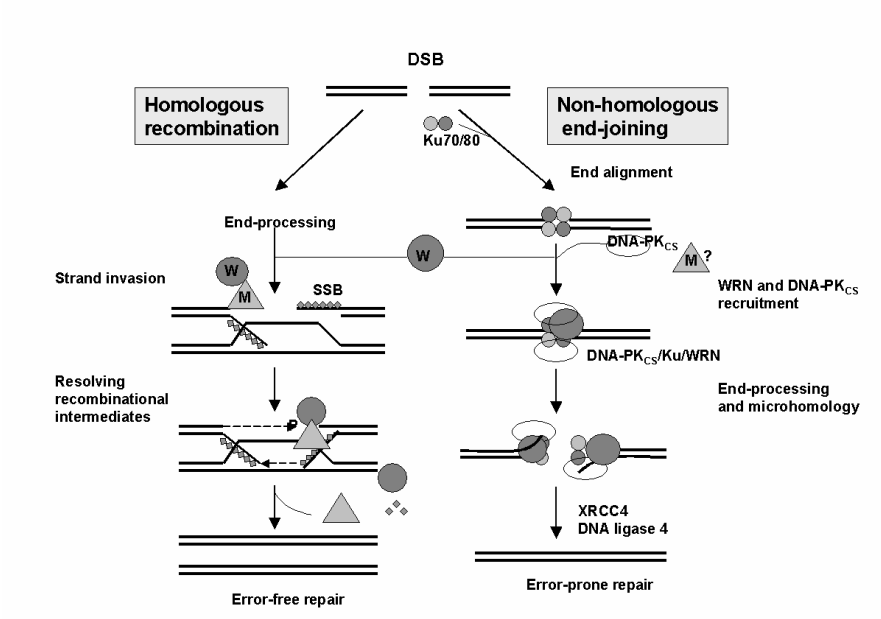

Fig. 4. Schemes of homologous recombination and non-homologous end-joining pathways for repairing DNA double strand breaks (DSB). M, the Mre11 complex; W, the Werner syndrome protein; SSB, single strand binding protein. The Mre11 complex may also participate in the non-homologous end-joining pathway.

there is no evidence of the involvement of WRN in the nucleotide excision repair pathway.

9.4.1 The homologous recombination pathway

DNA damage in the form of DNA double strand breaks (DSBs) compromises the integrity of cells. To prevent the broken ends from the subsequent adverse effects, eukaryotic cells have evolved two conserved repair pathways: HR and NHEJ. The term 'illegitimate recombination' found in the literature is a conceptual synonym of 'NHEJ'. Several lines of biochemical and genetic evidence strongly support the notion that the HR pathway is important in maintaining genomic stability. DNA recombination inevitably occurs in the physiological processes of meiotic DNA replication and V(D)J recombination, which can be resolved by the HR pathway. This repair pathway also protects cells from DSBs generated from exogenous agents including ionizing radiation and DNA cross-linkers. Genetic and biochemical studies have established a central role for Rad51 and its paralogs in HR. Rad51 paralogs include Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3. It is generally accepted that Rad51 plays a central role, and that the paralogs assist Rad51 in the

process of the recombinational repair (Baumann and West 1998). Figure 4 presents the current understanding of the key steps for the HR pathway, and how WRN may participate in this repair pathway. Generally, repair of DNA via HR starts with nuclease digestion to generate single-stranded DNA tails, which is coated and stabilized by single strand binding proteins, such as Rad51 and RPA. Then, the stabilized single strand tails search homologous sequence in the undamaged sister chromatid, and DNA synthesis enzymes complete the repair process. A recent report provided convincing evidence for an essential role of Nbs1 in HR, but not in NHEJ (Tauchi et al. 2002). Interestingly, HR is likely to be conducted separately by the Rad51- and Nbs1-depedent pathways because, after severe ionizing radiation treatment, sites of DSBs that contain either Rad51 or Nbs1 (together with Mre11 and Rad50) are not coincident (Nelms et al. 1998). It has been shown that WRN co-localizes with Rad51 after camptothecin treatment (Sakamoto et al. 2001) or with Nbs1 after ionizing radiation or mitomycin C treatment (Cheng et al. submitted). Salient questions to be addressed are whether pathways of WRN-mediated recombinational repair via Rad51 or Nbs1 are parallel and mutually exclusive, and whether Rad51 and Nbs1 interact with WRN at different stages of the HR event.

Cells from WS patients are hypersensitive to DNA cross-linkers and, to a lesser extent, to ionizing radiation (Yannone et al. 2001; Saintigny et al. 2002). The rate of HR in yeast is increased when the human RecQ orthologue Sgs1 is mutated, and this defect is suppressed by overexpressing human WRN (Yamagata et al. 1998). In addition, defects in Sgs1 have been shown to result in hyper-recombination in yeast (Myung et al. 2001). These lines of evidence from yeast studies suggest a role for human WRN in HR. Direct evidence for a role of WRN in HR is provided by two recent reports, which strongly support a function for WRN in resolving recombination intermediates in a Rad51-dependent manner (Saintigny et al. 2002; Prince et al. 2001). Viable mitotic recombinants progeny are reduced by more than 20-fold in WS cells transfected with a *neo* gene selection vector (Prince et al. 2001). Interestingly, the generation of viable recombinant progeny in WS cells was rescued by the expression of either WRN or the bacterial resolvase protein RusA. In addition, the suppression of Rad51-dependent recombination significantly reversed the DNA damage hypersensitivity in WS cells (Prince et al. 2001). Specifically, recombination initiation and rates appeared normal in WS cells in the absence of cell division (Prince et al. 2001). In addition, we have recently found an interaction between WRN and the Mre11 complex after mitomycin C and ionizing radiation treatments (Cheng et al. submitted). Elucidation of these two WRN interactions should advance our understanding underlying the mechanism of the hypersensitivity to DNA cross-linkers in WS cells.

One hallmark of BS cells is an increased rate of sister chromatid exchange (reviewed in German 1993). It is interesting that the two RecQ proteins, WRN and BLM, interact physically and functionally (von Kobbe et al. 2002). However, the biological significance of the inhibition of the WRN exonuclease activity by its binding to BLM is still unclear. As the Ku stimulation of WRN exonuclease activity may drive cells to choose the error-prone NHEJ pathway, inhibition of WRN exonuclease activity by BLM may insure that cells choose the error-free HR

pathway. In comparison with cells deficient in the individual genes, sensitivity to genotoxic agents including 4NQO, camptothecin, MMS and UV are increased synergistically in the *WRN*$^{-/-}$/*BLM*$^{-/-}$ chicken cells (Imamura et al. 2002). Along with their similarities in helicase activities and substrate specificities in vitro, WRN and BLM may function in the HR pathway in a complementary manner. In support of this notion, it has been suggested that these two RecQ helicases may act at different steps in the HR pathway (review see Franchitto and Pichierri 2002), and this pathway may be important to resolve the stalled replication forks (for reviews see Thompson and Schild 2002). This notion is further supported by recent observations that both WRN and BLM co-localize with Rad51 when cells are arrested in S phase (Sakamoto et al. 2001; Sengupta et al. 2003). The WRN and BLM helicases may partially overlap and be redundant, which may explain why mutations in either of their genes are not lethal. Future experiments are required to delineate the potential interaction between WRN and BLM in the HR pathway, and the significance of this interaction in human aging.

A recent report showed that WRN co-localizes with Rad51 and RPA in response to camptothecin treatment in the K562 cells where the Abl kinase activity is constitutively activated (Sakamoto et al. 2001). As stated above, Rad51 and RPA are required for the HR pathway. Recent publications provide evidence for a role of Abl and its ortholog Arg tyrosine kinase in the HR pathway. It has been shown that the Rad51-dependent HR can be stimulated by the Abl kinase, as evidenced by transfecting this kinase or kinase-inactive vectors into a recombination reporter cell line (Slupianek et al. 2001). A recent paper shows that Arg is required for ionizing radiation-induced Rad51 foci formation (Li et al. 2002). Our recent result showed that WRN physically interacts with and is functionally inhibited by c-Abl, providing possible insight into the mechanism of the WRN-Rad51 interaction in the HR pathway (Cheng et al. submitted). The strand exchange activity of Rad51 is inhibited by c-Abl tyrosine phosphorylation in vitro (Yuan et al. 1998). However, correct Rad52 foci formation after ionizing radiation treatment requires Abl kinase activity (Kitao and Yuan 2002), and tyrosine phosphorylation of c-Abl enhances the complex formation between Rad51 and Rad52 (Chen et al. 1999). Therefore, delineating the mechanism of WRN regulation by c-Abl in the HR pathway will advance our understanding of the genomic instability phenotypes observed in WS cells.

9.4.2 The non-homologous end-joining pathway

Recent publications showed that WRN might function in the NHEJ pathway of DSB repair. This repair pathway is operated mainly by the DNA-PK complex, which is composed of the Ku heterodimer, the DNA-PK$_{cs}$, and DNA. NHEJ joins broken ends directly without a DNA template, mainly due to the contribution of the Ku heterodimer that recognizes the ends in a sequence-independent manner. Recent reports showed that WRN interacts physically and functionally with the components of the DNA-PK complex. Deficiencies in either Ku or DNA-PKcs renders cells hypersensitive to ionizing radiation, due to defects in NHEJ-

mediated repair of DSBs (reviewed in Smith and Jackson 1999). WS fibroblasts display a mild, but significant, sensitivity to ionizing radiation, compared to control fibroblasts (Yannone et al. 2001; Saintigny et al. 2002). These findings suggest that WRN may interact with the DNA-PK complex to repair DSBs by the NHEJ pathway.

Several independent studies have found that the Ku heterodimer associates with WRN in the cell by co-immunoprecipiation experiments, and binds directly to WRN in vitro using purified proteins (Cooper et al. 2000; Li and Comai 2000; Karmakar et al. 2002b). Functionally, Ku strongly stimulates the 3' $\rightarrow$ 5' exonuclease activity of WRN, not only by increasing its processivity (Cooper et al. 2000; Karmakar et al. 2002b), but also by expanding the spectrum of substrates that are degraded by the WRN exonuclease (Li and Comai 2000). Ku also promotes WRN exonuclease digestion past DNA adducts that normally block progression of the enzyme on the substrates (Orren et al. 2001). Structurally, the Ku protein has a strong tendency to bind broken DNA ends (Walker et al. 2001). It is possible that Ku binds to the broken DNA ends and recruits WRN to process the damaged DNA.

WRN was found to associate with another component of the DNA-PK complex, DNA-PK_{CS}, by co-immunoprecipitation experiments in three laboratories. This kinase belongs to the phosphatidylinositol-3-kinase family that phosphorylates substrate proteins at Ser/Thr residues. After formation of the DNA-PK complex, the kinase activity of DNA-PKcs is activated. The WRN interaction with the DNA-PK complex is mediated by binding with Ku (Karmakar et al. 2002a; Li and Comai 2002), and/or potentially through interaction with DNA-PKcs according to one study (Yannone et al. 2001). WRN has been shown to be a substrate for the DNA-PK complex in vitro (Yannone et al. 2001; Karmakar et al. 2002a). Specifically, we found evidence for DNA-PK-dependent phosphorylation of WRN after treating cells with bleomycin or 4-NQO (Karmakar et al. 2002a), two cancer therapeutic drugs that cause DNA strand breaks. Therefore, WRN, along with the components of the DNA-PK complex, may function coordinatively at the broken DNA ends via a phosphorylation-dependent mechanism. Consistent with this, purified WRN, Ku and DNA-PKcs have been found to form a complex on DNA by gel shift analyses (Yannone et al. 2001; Karmakar et al. 2002a). WRN association with, and phosphorylation by, the DNA-PK complex inhibits the exonuclease and helicase activities of WRN; however, whether this inhibition requires Ku is still unclear. We observed that this inhibition requires the activated DNA-PKcs in association with Ku and DNA (Karmakar et al. 2002a), while Yannone et al. (2001) reported that physical interaction with DNA-PKcs alone is sufficient to inhibit the two catalytic activities of WRN. Clues for this discrepancy may be provided if one considers the status and forms of post-translational modification of the purified proteins.

Defects in DSB repair can lead to chromosomal translocation, a cellular defect associated with WS. The finding that WRN interacts with the DNA-PK complex may shed light on the mechanism of this defect in WS cells. In figure 4, we propose a model by which WRN may participate in joining two broken DNA ends. The NHEJ- mediated repair of DSBs is likely to be initiated by Ku binding to both

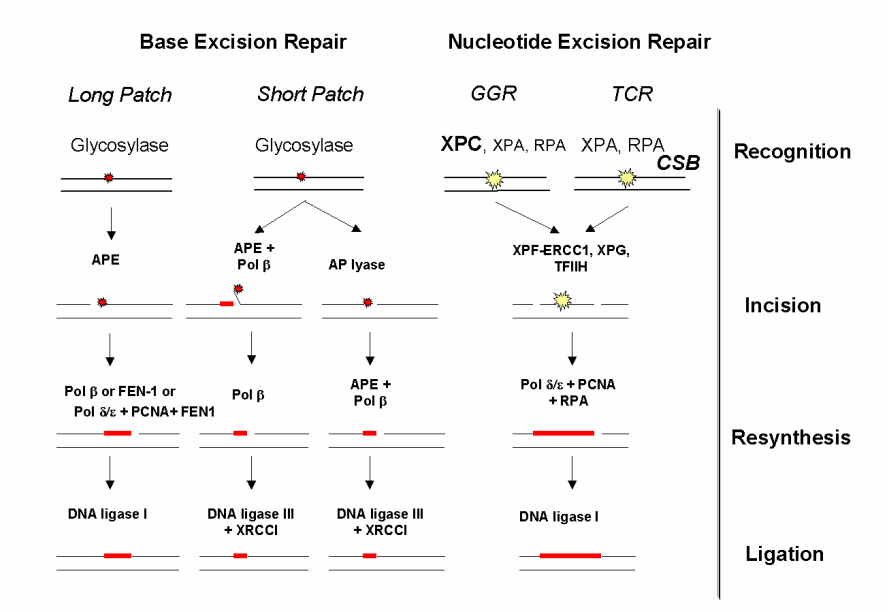

Fig. 5. Schemes of base excision repair and nucleotide excision repair pathways. Each of them has two sub-pathways. APE, apurinic/apyrimidinic endonuclease endomuclease; XRCC1, X-ray repair cross-complementing group 1; CSB, Cockayne syndrome protein.

of the broken DNA ends and bringing them into close proximity. It has been demonstrated that the DNA-bound Ku has an increased tendency to self-associate (Cary et al. 1997; Ramsden and Gellert 1998). Once bound to DNA, Ku can recruit DNA-PK_{CS} and WRN, and perhaps other helicases and nucleases to process the broken ends. This event reveals microhomology for the subsequent process of strand annealing. The WRN exonuclease, and WRN stimulation of FEN-1 flap cleavage (Brosh Jr. et al. 2001b) may act to remove the resulting flaps and to process the ends for proper ligation. The activated DNA-PK complex may limit the extent of end degradation by inhibiting the activity of WRN exonuclease, thereby preventing extensive deletions. However, more promiscuous nucleases may substitute in the absence of WRN. Consistent with this view, in a cellular assay for NHEJ, WS cells displayed extensive deletions at the non-homologous joined ends, compared to normal cells (Oshima et al. 2002). Future studies are required to determine whether WRN is a direct in vivo substrate for DNA-PK_{CS}, and whether this type of WRN phosphorylation is linked to the NHEJ pathway in cells undergoing DNA double strand break repair.

9.4.3 The base excision repair pathway

One major theory of aging is that its phenotypic changes are due to the accumulation of oxidative products during the aging process (Beckman and Ames 1998). 4-

NQO generates a wide spectrum of DNA adducts as well as oxidative damage (Gebhart et al. 1988; Ogburn et al. 1997), and ionizing radiation produces a diverse range of DNA damage including strand breaks and oxidative base modifications (Weinfeld et al. 2001; Wallace 1998). Hypersensitivity of WS cells to these damaging agents suggests that WRN may participate in some aspects of oxidative DNA damage repair.

BER is important for the repair of a wide variety of DNA damage including oxidized, alkylated, deaminated, and hydrolyzed bases (reviewed in Wilson, III and Thompson 1997; Lindahl 2000). The BER repair pathway (Figure 5) involves the sequential actions of several proteins and is coordinated by protein-protein and protein-DNA interactions (Wilson and Kunkel 2000). Generally, glycosylases recognize damaged DNA bases and incise them, followed by abasic site-specific endonuclease cleavage to generate nicks. Then, DNA synthesis takes place from the single strand break site. Extracts from WS cells have been shown to efficiently repair a plasmid containing an abasic (AP) site (Bohr et al. 2001) which is generally repaired by short-patch BER, indicating that WS cells are proficient in this process. However, the ability of WS cell extracts to repair lesions requiring long-patch BER in vitro remains to be investigated. In addition, it will be important to find DNA-damaging agents that enrich for lesions specifically repaired by the long-patch BER pathway. Due to the small percentage of lesions that are repaired by long-patch BER, it may be difficult to identify significant differences in sensitivities to oxidative DNA damaging agents in WS cells. In support of the notion that WRN may participate in BER at the stage of damage recognition, progression of the WRN exonuclease is blocked by some oxidative DNA modifications such as AP sites, 8-oxoguanine, and 8-oxoadenine (Machwe et al. 2000a). Pausing at sites of oxidative lesions may be an early step in the DNA damage recognition process, and WRN may serve to recruit DNA repair proteins to the site of the lesion via protein-protein interactions. After the damage has been sensed, WRN may serve additional roles through its helicase and exonuclease activities. Furthermore, based on the importance of maintaining genomic integrity following insult by reactive oxygen species, there likely exist many back-up processes in BER. However, based on the sensitivity of WRN-deficient cells to agents which produce oxidative DNA damage, it is likely that WRN participates in vivo to coordinate interactions between BER proteins, to unwind repair intermediates, and/or to stimulate flap cleavage.

WRN has been shown to interact physically and/or functionally with several proteins involved in the long-patch sub-pathway of BER including DNA polymerase δ (Pol δ), proliferating cell nuclear antigen (PCNA), RPA, and FEN-1. WRN interacts physically with Pol δ and recruits it to the nucleolus (Szekely et al. 2000). Furthermore, WRN has been shown to stimulate nucleotide incorporation by Pol δ (Kamath-Loeb et al. 2000). A physical interaction between WRN and PCNA has been identified; however, a functional interaction has yet to be determined (Lebel et al. 1999). RPA stimulates the WRN helicase to unwind long duplex DNA substrates that WRN alone is not able to unwind (Brosh, Jr. et al. 1999; Shen et al. 1998). WRN interacts physically with FEN-1 and stimulates its DNA flap cleavage activity (Brosh, Jr. et al. 2001b). However, because these proteins

participate in both replication and long-patch BER, an association with WRN does not reveal whether this interaction is important for one or both pathways. Distinguishing a role for WRN in DNA repair, we have recently identified a physical interaction between WRN and DNA polymerase beta (Pol β) (Harrigan et al. 2003), a central participant in BER. Furthermore, WRN stimulates Pol β strand displacement DNA synthesis (Harrigan et al. 2003), suggesting a novel mechanism by which WRN may function in DNA repair.

WRN has been shown to unwind alternate DNA structures including triplex DNA (Brosh, Jr. et al. 2001a), Holliday junctions (Constantinou et al. 2000), and DNA tetraplexes (Fry and Loeb 1999)(Figure 2). WRN also enables Pol δ to synthesize past hairpin and tetraplex structures of the $d(CGG)_n$ trinucleotide repeat sequence. Furthermore, WRN helicase activity is essential to alleviate pausing of Pol δ at these tetraplex regions (Kamath-Loeb et al. 2001). Triplets of bases repeated in tandem can form a variety of structures including single-strand hairpin loops, which may result in slippage and expansion by DNA polymerases. The weak strand displacement activity of Pol β results in the expansion of CAG/CTG triplet repeats (Hartenstine et al. 2002). Furthermore, weak strand displacement activity during DNA repair at strand breaks may enable short tracts of repeat sequences to be converted into longer, more mutable stretches associated with neurological diseases, including Huntington's disease, myotonic dystrophy, spinocerebellar ataxia and spinal and bulbar muscular atrophy. As polymerase-initiated DNA synthesis errors most likely play a central role in human aging and disease, it is interesting to speculate that WRN may increase the fidelity of polymerases (such as Pol β and Pol δ) via unwinding of alternate structures or through stimulation of strand displacement DNA synthesis (Pol β).

Pol β does not have an intrinsic editing function, and thus makes frequent errors, at the level of 1 per 4000 nucleotide incorporation events (Lindahl 2000). Unless special processes exist to increase the accuracy of BER, error-prone repair may result in mutations and cancer. One possible mechanism for increasing the fidelity of repair is a separate editing enzyme that would remove nucleotides misincorporated by Pol β. WRN may complement Pol β for its lack of proofreading activities. WRN is a 3'→5' exonuclease, and the sequence of the WRN exonuclease is homologous to the 3'→5' proofreading domain of E. coli DNA polymerase I (Mian 1997). Furthermore, WRN has been shown to remove 3' mismatches (Kamath-Loeb et al. 1998; Huang et al. 2000) and is one of several proteins which has been suggested to serve this proofreading function (Kamath-Loeb et al. 1998; Shevelev and Hubscher 2002). It remains to be determined whether WRN increases the fidelity of Pol β and/or serves as a proofreading enzyme for Pol β during repair events.

Although WRN may not be an essential component of BER, it may play an important role during the repair of certain lesions during long-patch BER. For example, oxidized abasic residues may function as suicide substrates in the formation of protein-DNA crosslinks, as has been demonstrated for 2-deoxyribonolactone residues and Pol β (DeMott et al. 2002). These protein-DNA crosslink intermediates may not be readily repaired by short-patch BER and may require alternative

modes of repair. WRN may also function to unwind such intermediates and/or stimulate FEN-1 cleavage of these protein-DNA substrates. The demonstrated sensitivities of cells lacking WRN to oxidizing and alkylating agents suggest WRN functions in the repair of oxidative DNA damage. The importance of the WRN helicase to unwind DNA repair intermediates, the exonuclease to remove incorrectly inserted bases, and physical interactions with protein participants of BER will serve to unravel the precise function of WRN in BER.

9.5 Transcriptional defects in WS

The established role of helicases in transcription suggests that the WRN helicase may play a role in transcription. As discussed in Section 9.2, G4 tetraplex structures are substrates for WRN. This type of DNA structure may exist in vivo in telomeres and rDNA, supporting a role for WRN in ribosomal DNA transcription. RNA polymerase I is responsible for the transcription of ribosomal RNA in the nucleolus, while the major function of RNA polymerase II is to transcribe messenger RNA in the nucleoplasm. In unstressed cells, WRN resides in the nucleolus, and its appearance is correlated with the transcriptional activity in the nucleolus (Gray et al. 1998). It has been shown that the SV-40 transformed WS lymphoblasts exhibit a reduction in the RNA polymerase II-dependent global or regional transcription, and WRN stimulates RNA polymerase II-dependent transcription (Balajee et al. 1999). A recent report showed that treatment of primary cells with an inhibitor of RNA polymerase I results in the re-localization of WRN from the nucleolus to the nucleoplasm (Shiratori et al. 2002). Further analysis by measuring [^{3}H]-uridine incorporation showed that levels of labelled rRNA are decreased in primary WS cells in comparison to the control cells. Clearly, the role of WRN in transcription requires further investigation.

9.6 Replicational defect in WS

Somatic WS cells exhibit impaired DNA replication that results in an extended S-phase (Poot et al. 1992). The WRN orthologue FFA-1 in Xenopus laevis is required for the formation of replication foci, supporting a role for the human RecQ helicases in replication (Yan et al. 1998). Indeed, co-immunoprecipitation experiments demonstrated that WRN is associated with PCNA and the p50 subunit of polymerase δ, two components of the multi-protein replication complex (Lebel et al. 1999; Szekely et al. 2000). In addition, it has been shown that WRN co-purifies with the replication complex and re-locates and migrates along Holliday junctions, a form of stalled replication sites (Constantinou et al. 2000; Lebel et al. 1999). Kamath-Loeb et al. (2001) reported that polymerase δ-mediated DNA synthesis is impeded by certain distorted structures, such as G4 tetraplexes, but this blockage is reversed by WRN, suggesting a role of WRN in resolving stalled replication.

The linkage between recombination repair and the restart of replication forks is increasingly evident (for review, see McGlynn and Lloyd 2002). However, recombination may not be necessary for DNA replication if the progression proceeds continuously (Courcelle and Hanawalt 2001). WRN may coordinate these two aspects of DNA metabolism. In eukaryotic cells, replication forks can be assembled at recombinational intermediates in the form of a displacement loop (D-loop) (Malkova et al. 2001; Holmes and Haber 1999), and this D-loop DNA structure has recently been identified as a substrate for WRN (Orren et al. 2002). One possible function of WRN at the stalled replication forks is to stimulate the FEN-1 endonuclease (Brosh, Jr. et al. 2001b), an nuclease necessary for processing of Okazaki fragments during strand displacement synthesis on the lagging strand. In addition, the WRN helicase is capable of unwinding an RNA-DNA heteroduplex (Suzuki et al. 1997), suggesting that WRN may displace the RNA primer of the Okazaki fragment.

The Nbs1 protein is the mammalian homolog of the yeast Xrs1. During S phase, Nbs1 forms foci with PCNA (Maser et al. 2001b; Maser et al. 2001a), a protein that plays a central role in DNA replication. PCNA has been shown to co-localize with many types of "DNA repair" foci, including Rad51, Nbs1, γH2AX, and BRCA1, after hydroxyurea or ultraviolet irradiation exposure (Ward and Chen 2001; Scully et al. 1997; Limoli et al. 2000). These results are consistent with the finding that Nbs1 foci appear when the progression of replication forks are inhibited in UV-irradiated XP cells (Limoli et al. 2002). The γ-H2AX foci co-localize with Nbs1 and stalled replication forks (Kobayashi et al. 2002), suggesting the formation of DSBs at the stalled replication forks, which must be resolved to re-initiate replication (for review see Michel 2000). We have recently shown that the co-localization and association between WRN and Nbs1 are increased after ionizing radiation and mitomycin C treatment (Cheng et al. submitted). In light of the essential roles for WRN and Nbs1 in the HR pathway (Tauchi et al. 2002; Saintigny et al. 2002), these two proteins likely work together to resolve stalled replication fork after DNA damage via the HR pathway.

9.7 Telomere defects in WS

Genetic instability and the reduced replicative lifespan observed in WS cells are consistent with defects in telomere metabolism. Telomeres protect chromosome ends from being recognized as DNA DSBs. A progressive decline in telomere length occurs with each cell division. Once the telomere length is shorter than certain tolerable levels, replicative senescence is triggered. Loss of telomere function can also lead to genomic instability. Telomere-associated senescence can be bypassed by the expression of telomerase, which extends telomeres (for review see Campisi et al. 2001). Since the expression of exogenous telomerase in WS fibroblasts extends the cellular lifespan (Wyllie et al. 2000; Ouellette et al. 2000), the premature senescence observed in WS cells may be related to the telomere-associated senescence. The expression of exogenous telomerase also partially re-

verses the hypersensitivity of WS cells to 4-NQO (Hisama et al. 2000). However, the reduced proliferative capacity of WS cells cannot be explained simply by the acceleration of telomeric loss. Although WS fibroblasts demonstrate accelerated rates of telomere shortening, the mean telomere lengths are longer in WS cells compared to those of the controls, when the cells are senescent (Kruk et al. 1995; Schulz et al. 1996). Increasing evidence indicates that changes in telomere structure, rather than telomere length, induce cellular senescence (Karlseder et al. 2002).

Consistent with a role for WRN in telomere maintenance, WS fibroblasts were observed to be defective in repair of UV lesions at telomeres (Kruk et al. 1995). Defects in other repair enzymes including the Ku heterodimer, DNA-PKcs, as well as the Mre11 complex also result in dysfunctional telomeres, as manifested by accelerated telomere shortening and/or telomere end fusions (Bailey et al. 1999; Samper et al. 2000; Gilley et al. 2001; Ranganathan et al. 2001). WRN also interacts with these enzymes in DSB repair (see sections 9.4.1 and 9.4.2). How these repair proteins function in telomere maintenance is not well understood; however, one of the common functions may be in a cellular response to dysfunctional and/or damaged telomeres. Furthermore, Ku interacts with the critical telomere binding proteins TRF1 and TRF2 (Song et al. 2000; Hsu et al. 2000), and Nbs1 interacts with TRF2 (Zhu et al. 2000). Both TRF1 and TRF2 regulate telomere length (Smogorzewska et al. 2000), and defects in TRF2 induce telomere end fusions and either growth arrest or apoptosis (Karlseder et al. 1999). We have found that WRN also interacts with TRF2 by co-immunoprecipitation, co-localization, and in vitro with purified proteins (Opresko et al. 2002). In addition, TRF2 interacts with another RecQ family member, BLM, and promotes DNA unwinding by the WRN and BLM helicases in vitro (Opresko et al. 2002). Collectively, these studies suggest that RecQ helicases may function in a protein complex at telomeres, in cellular pathways that impact telomere integrity.

Another potential biological significance of the WRN interaction at telomeric ends is to resolve DNA secondary structures. Electron microscopy studies have shown that telomeres in mammalian cells form t-loop structures in which the single-stranded 3' tail invades the homologous duplex telomere region, creating the D-loop (Griffith et al. 1999). Consequently, the telomeric end is protected and sequestered, which in turn, prevents its recognition as a DSB. The formation of telomeric t-loops in vitro requires TRF2 (Stansel et al. 2001). Presumably, structures at telomeric ends must be resolved for telomerase, the DNA replication forks, and DNA repair proteins to gain access to the terminal region of the telomere. WRN and BLM proteins are likely candidates to participate in this process, since they unwind various DNA secondary structures, recombination intermediates and G4 tetraplexes, which are found in the telomeric G-rich sequence (Mohaghegh et al. 2001).

Further support for a role of WRN in telomere metabolism is derived from studies in yeast. The RecQ homolog in S. cerevisiae, Sgs1p, participates in a telomerase-independent pathway for telomere lengthening (Johnson et al. 2001). This mechanism, termed alternative lengthening of telomeres (ALT), is predicted to involve recombination. Evidence for a similar pathway has been found in te-

lomerase-negative immortalized mammalian cells (Bryan et al. 1997). These cells are characterized by long heterogeneous telomeres and distinct nuclear foci referred to as ALT-associated PML bodies, which contain Rad52, Rad51, RPA, TRF1, TRF2, telomeric DNA (Yeager et al. 1999), Ku (d'Adda et al. 2001) and Nbs1 (Wu et al. 2000). Both WRN and BLM were also found to localize in AA-PML bodies (Yankiwski et al. 2000; Johnson et al. 2001; Opresko, et al. 2002). WRN and BLM interact physically and/or functionally with many of the proteins in these foci, suggesting that these RecQ helicases may act in a protein complex at telomeric ends in recombination pathways.

9.8 Conclusion and perspectives

As WRN has been shown to interact with a number of proteins and to participate in many DNA repair pathways, the in vivo function of this protein is likely to be a coordinator of the response to DNA damage. It is thought that the ATM kinase is an immediate sensor following DNA damage, especially DNA DSBs. A recent report showed that ATM may be activated by changes in the chromatin structure, and that the ATM activation is unlikely to occur by its direct binding to DNA strand breaks (Bakkenist and Kastan 2003). Oxidative stress has been implicated in the induction of chromatin remodelling (Rahman 2002) and it is possible that all the major types of DNA damage, including DNA DSBs, cross-links, and base oxidative modification, lead to chromatin remodelling. In this context, it is tempting to propose that WRN may be activated following chromatin remodelling. Subsequently, WRN is activated together with other DNA proteins specifically to repair damages via the NHEJ, HR, or BER pathways. The activation of ATM by chromatin remodelling may have other impacts on the phenotypes of WS, as a recent report showed that ATM deficiency accelerates aging and induces telomere dysfunction (Wong et al. 2003). In addition, our recent results directly link WS to the Nijmegen breakage syndrome and the AT-like disorder, two human recessive genetic disorders with mutations in Nbs1 and Mre11, respectively. These two proteins play pivotal roles in DNA DSBs, and thus connect WRN to the NHEJ and HR pathways. The cellular and clinical phenotypes of Nijmegen breakage syndrome are very similar to ataxia telangiectasia, which has been proposed to be another model system for premature aging. The DNA damage-induced post-translational modifications of WRN and other proteins that WRN interacts with, also play important roles in the regulation of WRN function. This notion is supported by the fact that WRN can be Tyr and Ser/Thr phosphorylated, sumoylated, and acetylated. Mutations of the *WRN* gene result in the human premature aging syndrome, and the WRN protein also interacts directly or indirectly with proteins (ATM, BLM, and Nbs1) potentially capable of delaying the aging process. Collectively, WRN may convergently coordinate pathways of DNA repair by protein-protein interactions and following post-translational modification, and plays a central role in slowing down the aging process.

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Abbreviations

ALT: alternative lengthening of telomeres
AP: abasic
BER: base-excision repair
BLM: Bloom syndrome protein
BS: Bloom syndrome
D-loop: displacement loop
DNA-PK: DNA-dependent protein kinase
DNA-PK_{cs}: the catalytic subunit of DNA-PK

DSB: double strand break
FEN-1: flap endonuclease-1
HR: homologous recombination
MMS: methyl methanesulfonate
NHEJ: non-homologous end-joining
NLS: nuclear localization sequence
4-NQO: 4-nitroquinoline 1-oxide
NTS: nucleolar targeting sequence
PARP-1: poly(ADP)ribose polymerase-1
PCNA: proliferating cell nuclear antigen
PML: promyolytic leukemia
Pol β: DNA polymerase beta
RPA: replication protein A
RTN: Rothmund-Thomson syndrome protein
RTS: Rothmund-Thomson syndrome
RQC: RecQ conserved
TRF2: telomeric repeat factor 2
WRN: Werner syndrome protein
WS: Werner syndrome

10 Role of subcytotoxic stress in tissue ageing

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Abstract

Most commonly stress-induced premature senescence (SIPS) is defined as the long-term effects of subcytotoxic stress on proliferative cell types, including appearance of features of replicative senescence. An integrative model of signal transduction can explain the occurrence of SIPS. Cells in SIPS display differences at the level of protein expression which are either common with replicative senescence, or are specific 'molecular scars' of the stress. These proteins with changes of expression level affect energy metabolism, defence systems, redox potential, cell morphology and transduction pathways. In vivo, cells capable of proliferation are often exposed to various stresses which nature depends on their location in the body, or on particular conditions such a local asymptomatic microinflammation. Several studies already showed the occurrence of prematurely senescent cells in vivo. Lastly, human ageing is characterised by an increase in proinflammatory cytokines, favouring the installation of a pro-inflammatory state and most likely SIPS.

10.1 Life, evolution, stress, and ageing

Cellular and molecular biologists, whether working on human, animal, or plant cells, agree that " any environmental factor potentially unfavourable to a living organism " is a stress. It is also generally acknowledged that " if the limits of tolerance are exceeded and the adaptive capacity is over-worked, the result may be permanent damage or even death " (Larcher 1987). The first phase of the stress response is characterised by a deviation of the functional norms, decline of vitality, and excess of catabolism over anabolism. The second phase is the restitution phase or resistance phase with launching of adaptation and repair processes. The third phase is either the end phase or the regeneration phase. The end phase is a stage of exhaustion or long-term response when stress intensity is too heavy, leading to overcharge of the adaptation capacity, damage, chronic diseases, senescence or even death. The regeneration phase allows full restoration of all physiological functions when the stressor is removed (Lichtenhaler 1998).

When seen from an evolutionary perspective, stress represents an evolutionary force. Indeed living systems have been exposed to stress since the dawn of bio-

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logical time. Stress participates in the evolution through natural selection of the fittest. The theory of disposable soma, analogically derived from the Darwinian theory of evolution, proposes that organisms with the longest lifespan secure their longevity through investment in a more durable soma, including enhanced stress response (Kirkwood and Austad 2000). In other words, longer lifespan means evolution-driven better adaptation to stress.

Most modern theories of ageing propose that damage accumulates with time due to increase in damaging events with age and decreased defence and repair capabilities, reaching eventually a " global failure of maintenance " (Holliday 1988) as prefigured in the generalised theory of catastrophic error proposed by Orgel (Orgel 1973). The so-called theory of free radicals proposed in the nineteen fifties is the most popular theory of ageing (Harman 1956). This theory is based on the discovery that oxygen free radicals, or reactive oxygen species, can alter most biomolecules. Indeed, oxidative stress is probably the most common stress affecting cells. The origin of reactive oxygen species can be exogenous (UV, ionisating irradiations, xenobiotics, etc.) and reactive oxygen species are also produced continuously by the cell in normal physiological conditions. The principal source of reactive oxygen species in the cell is the oxidative metabolism linked to respiration (Chance et al. 1979). Oxidative damage of all sorts accumulate with ageing like carbonylated proteins (for a review: Sohal and Weindruch 1996), lipofuscin (Sohal and Brunk 1989), 8-hydroxydeoxyguanosine (Chen et al. 1995, Homma et al. 1994), deletions in the mitochondrial genome (Yen et al. 1994) and accumulation of lipid peroxides (Yen et al. 1994). An extensive review of the accumulation of oxidative damage with ageing was published by Beckman and Ames (1998).

10.2 Testing the theories of ageing

Animal transgenesis opened the possibility to test the theories of ageing that consider damage and stress response as essential beacons in biogerontology. Most mutants of *Caenorhabditis elegans* with increased longevity are more resistant to various kinds of stress such as UV, oxidative, or heat shock (for a review: Murakami et al. 2000). Transgenic *Drosophila melanogaster* overexpressing Cu/Zn superoxide dismutase has a 34% longer lifespan when compared to control animals (Orr and Sohal 1994). Overexpression of human Cu/Zn superoxide dismutase in motor neurones of *Drosophila* prolongs its lifespan by 40% (Parkes et al. 1998). The knocking out of the murine $p66^{shc}$ gene prolongs the lifespan of mice and enables the resistance of mouse embryonic fibroblasts to apoptosis induced by H_2O_2 and UV. $p66^{shc}$ is namely involved in triggering mitochondrial bursts of reactive oxygen species leading to apoptosis, independently of the role of this protein as an adaptor protein involved in the signalling triggered by mitogenic factors (Migliaccio et al. 1999). Overexpression of the murine methionine sulfoxide reductase in the nervous system of *Drosophila* increases its general activity and prolongs its reproductive capabilities (Ruan et al. 2002). Extended longevity in mice lacking the insulin receptor in adipose tissue seems to protect these ani-

mals against the deleterious effects of fat accumulation (Blüher et al. 2003). This suggests that overfeeding due to ad libitum feeding of laboratory animals, pets, or humans with a functional receptor to insulin in adipose tissue might have noxious effects.

It was also tested in vitro whether the survival of cells from long-lived mammalian species to oxidative and non-oxidative stress is increased when compared to short-lived mammalian species. Stress resistance seen in terms of cell survival after stress at cytotoxic concentration was positively correlated with mammalian longevity (Kapahi et al. 1999). However, the link existing between resistance to cytotoxic stress and the way normal ageing proceeds is not obvious. The fundamental question is why and how do cells slowly accumulate irreversible modifications, either spontaneously due to molecular decay in normal physiological environment, or in response to abnormal stress, reaching eventually a critical threshold of error leading to irreversible degeneration, functional losses and death (Toussaint et al. 1991). In vitro models provided a way to study how irreversible modifications, particularly modifications due to reactive oxygen species, take place with ageing. Among these models, the most popular is indubitably the model of Hayflick, also called " ageing under glass " or replicative senescence (Shay and Wright 2000). Human diploid fibroblasts (HDFs) divide a finite number of times in culture, counted in terms of cumulative population doublings (CPDs) (Hayflick 1965, Hayflick and Moorhead 1961). The phrase 'replicative senescence' is now widely used. The occurrence of replicative senescence has been demonstrated for many other cell types such as keratinocytes, vascular smooth muscle cells, endothelial cells, adrenocortical cells, lymphocytes, chondrocytes, etc. Notable exceptions are embryonic germ cells and the large majority of tumour-derived cells (for a review: Campisi 1999). In vivo the proliferative capacity of HDFs is never completely exhausted. HDFs of centenarians are still able to divide in vitro, sometimes for a number of CPDs that renders them undistinguishable from explants of HDFs of young donors (Tesco et al. 1998). In other words, only a minority of cells is likely to be senescent in tissues of aged persons. This has been called the 'Hayflick mosaic' (Toussaint et al. 2002b). It was proposed that the presence of a minority of senescent cells in a tissue could interfere with the homeostasis of somatic tissues, thereby participating in tissular ageing (Shay and Wright 2000).

Many genes undergo expression changes at the mRNA (messenger RNA) level along in vitro ageing. These genes code for growth inhibitors, growth factors, receptors, cytokines, proteins involved in DNA synthesis, structure and repair, antioxidant enzymes, proteins from the respiratory chain, etc. (for lists see Cristofalo et al. 1998, Shelton et al. 1999). A few proteomic studies have been performed as concerns replicative senescence. Toda et al. established a database of protein expression changes along different stages of in vitro ageing of TIG-3 HDFs (http://www.tmig.or.jp/2D) (Toda et al. 1998). Another proteomic analysis allying two-dimension gel electrophoresis and mass spectrometry identified proteins undergoing changes in expression level in senescent rat embryo fibroblasts (Benvenuti et al. 2002). A similar study was performed with WI-38 fetal lung HDFs and showed many discrepancies between replicative senescence of human and rat diploid fibroblasts (Dierick et al. 2002b, Dierick et al. 2002c).

According to several authors the 'biomarkers' of in vitro replicative senescence also appear during in vivo ageing. These biomarkers are for instance an enlarged cell size (Bayreuther et al. 1988), senescence-associated ß-galactosidase activity (SA ß-gal) (Dimri et al. 1995), deletions in the mitochondrial DNA (Corral-Debrinski et al. 1992, Dumont et al. 2000b, Hayakawa et al. 1993, Liu et al. 1998, Zhang et al. 1999), lower induction of *c-fos* proto-oncogene (Seshadri and Campisi 1990), decreased induction of heat shock proteins (Blake et al. 1991, Bonelli et al. 1999, Choi et al. 1990, Deguchi et al. 1988, Fargnoli et al. 1990), and increased metalloproteinase activity degrading the extracellular matrix (for a review: Campisi 1999). However most of these 'biomarkers' also appear after proliferative cells are exposed to subcytotoxic stress.

10.3 Senescence of proliferative cell types and stress

The narrowest definition of senescence covers irreversible growth arrest triggered by telomere shortening which counts cell generations of cells lacking endogenous telomerase (Wright and Shay 2001) and which is due to the inborn mechanisms of DNA duplication. Other authors enlarge this definition to a functional definition encompassing any kind of irreversible arrest of proliferative cell types induced by damaging agents or cell cycle deregulations caused for instance by overexpression of proto-oncogenes (Serrano and Blasco 2001). Notably, most 'biomarkers' of replicative senescence also appear after exposure to subcytotoxic oxidative stress and DNA damaging agents. According to this enlarged definition, telomere-dependent replicative senescence due to the inborn mechanisms of DNA duplication is only one of the types of cellular senescence and represents the ultimate barrier against cell immortalisation. All other forms of senescence may also represent barriers against immortalisation as long as irreversible growth arrest is observed like oncogene-induced or stress-induced premature senescence linked with telomere shortening or not. The different forms of senescence could be seen as sharing both irreversible growth arrest and concomitant or subsequent phenotypic changes.

Single telomere length analysis is a polymerase chain reaction-based approach that accurately measures the full spectrum of telomere lengths from individual chromosomes. Using this technique, Baird et al. observed bimodal distributions of telomeres in normal fibroblasts. These distributions resulted from inter-allelic differences of up to 6.5 kb. Most telomeres shorten in a gradual fashion consistent with simple losses through end replication, and the rates of erosion are independent of allele size. Superimposed on this are occasional, more substantial changes in length, which may be the consequence of additional mutational mechanisms. Notably, some alleles show almost complete loss of TTAGGG repeats at senescence (Baird et al. 2003).

Telomere-dependent replicative senescence due to the inborn mechanisms of DNA duplication is already different from senescence due to oxidative stress-induced accelerated telomere shortening (for a review:von Zglinicki 2002). Most

studies on replicative senescence were performed with cells cultivated at 20% O_2. This represents an oxidative stress when compared to the much lower in vivo physiological pressure of O_2 (Wright and Shay 2001), with accelerated telomere shortening (von Zglinicki 2002). Each cell type or cell strain from the same or different species often have different sensitivities to chronic exposure to O_2 levels higher than those encountered in vivo (for a review: Wright and Shay 2001). Unless most experiments are carried out again at the respective O_2 tension characterising specific tissues of different species, interspecies comparisons of the maximum number of CPDs are on shaky grounds since O_2 tension influences telomere shortening. Even from an intraspecies point of view, a given strain of HDFs cultured under 3% O_2, i.e. close to physiological O_2 tension, may achieve up to 20 more CPDs than at 20% O_2 (Chen et al. 1995). Under 20% O_2, a significant proportion of dermal HDFs rapidly switch from a mitotic to a post-mitotic phenotype, which is prevented at 4% O_2 (Alaluf et al. 2000). Other interesting experiments show that the growth potential of rat mammary epithelial cells is enhanced when Cu/Zn superoxide dismutase, catalase, and vitamin E and/or low O_2 tension are used during the cell dissociation period. Antioxidants and low O_2 tension in culture after the cell dissociation period fail to improve the growth potential (Lin et al. 1991).

10.4 Stress-induced premature senescence

Stress-induced premature senescence (SIPS) was coined in 1999 at the EMBO Workshop of Molecular and Cellular Gerontology held in Switzerland (Brack et al. 2000). From the definitions given above, replicative senescence in cultures exposed to 20% O_2 is already a form of stress-induced premature senescence. More commonly, SIPS can be defined as the long-term effects of subcytotoxic stress on proliferative cell types, including appearance of many features of replicative senescence, like irreversible growth arrest of (a majority of) the cell population. Many proliferative cell types, immortalised or not, undergo SIPS after exposure to subcytotoxic stress (for a review: Dierick et al. in press-a). Let us review briefly the biomarkers of replicative senescence that were checked in SIPS.

10.4.1 Preliminaries: individual morphology of HDFs as a tool for SIPS

Observing the individual morphology of thousands of HDFs with in vitro and in vivo ageing, Bayreuther's group defined a classification of seven morphological types they called "morphotypes". The three first morphotypes are mitotic fibroblasts while the last 4 morphotypes are post-mitotic fibroblasts. HDFs at early CPDs are very rich in mitotic fibroblasts I and mitotic fibroblasts II. Then mitotic fibroblasts III accumulate along CPDs. After the last CPD, when the cells are kept as post-mitotic cultures, the proportion of post-mitotic fibroblasts increases until

occurrence of a short-lived process of cell degeneration (Bayreuther et al. 1988). This group exposed various strains of HDFs to repeated stress as different as UV, mitomycin C, or strong electromagnetic fields. They observed that, from two to three days after the last stress, the HDFs acquired senescence-like morphological features. They observed an acceleration of the transition through the sequence of the seven morphotypes. Similar morphological data were acquired after stress at subcytotoxic doses of *tert*-butylhydroperoxide (*t*-BHP) and ethanol (Toussaint et al. 1992; Toussaint et al. 1995). Two-dimension gels were run after isolation of relatively pure clones of HDFs at a given morphotype after repeated subcytotoxic stress. Interestingly, some proteins underwent similar expression changes in HDFs in the same morphotype either undergoing in vitro senescence or obtained after exposures to stress (Rodemann 1989; Rodemann et al. 1989a; Rodemann et al. 1989b). The technology available at the end of nineteen eighties was not sensitive enough to identify the proteins corresponding to these spots from a reasonable number of two-dimension gels. Bayreuther viewed these observations as evidence of a programmed process of differentiation. However did it prove that the process of evolution through the various morphotypes is not a continuous process ? Indeed isolating relatively pure populations of HDFs at successive moments of their in vitro ageing allows the appearance on two-dimension gels only of (a subpopulation of) the proteins representing the most homogeneous part of the populations of cells. In other words, the amount of the proteins specific to HDFs, which might represent intermediaries between two defined morphotypes, would be so tiny that these proteins would be undetectable on two-dimension gels (Dierick et al. 2002a).

10.4.2 SA ß-gal and stress

The proportion of HDFs positive for SA ß-gal activity in a culture increases with the number of CPDs. SA ß-gal activity is detected in several cell types when senescent in vitro: human keratinocytes (Dimri et al. 1995), endothelial cells (Kurz et al. 2000), hepatocytes (Sigal et al. 1999), melanocytes (Medrano et al. 1995), osteoblasts (Michishita et al. 1999), uroepithelial cells (Reznikoff et al. 1996), retinal pigment epithelial cells (Matsunaga et al. 1999), human and rabbit smooth muscle cells (van der Loo et al. 1998), etc. Cells positive for SA ß-gal are found in vivo in human skin biopsies (Dimri et al. 1995). Increased proportions of SA ß-gal positive cells are also observed in SIPS of different cell types induced by many different stressors like UVB (Chainiaux et al. 2002a), *t*-BHP, and H_2O_2. An excellent correlation exists between the increase of the proportion of HDFs positive for SA ß-gal activity and the proportion of the morphotypes as defined above, both with increasing number of CPDs and in SIPS of HDFs (Toussaint et al. 2000a). The ß-galactosidase activity detected at pH 6 in senescent cells is a manifestation of activity of the classic acid lysosomal ß-gal at suboptimal pH (Kurz et al. 2000). This residual activity becomes detectable at pH 6 in senescent cells due to rise in the amount of the lysosomal enzyme related to the previously reported increase in number and size of lysosomes in senescent cells (Brunk et al. 1973, Robbins et al.

1970). Lastly, the appearance of SA ß-gal activity in H_2O_2-induced SIPS depends on the activation of mitogen-activated protein kinase p38 ($p38^{MAPK}$) stress response protein and transcription factor ATF-2 (ATF-2) (Frippiat et al. 2002).

10.4.3 Mitochondrial DNA deletions

Mitochondria imprison several copies of a 16,569 bp-long circular genome coding for a majority of the proteins involved in ATP production. Over 150 deletions in the DNA of the mitochondrial genome are known. The most frequent of these deletions is 4,977-bp long. Its frequency increases with in vivo ageing of different organs of mice (Brossas et al. 1994), rat (Gadaleta et al. 1992), monkey (Lee et al. 1993) and human such as skin (Pang et al. 1994, Yang et al. 1994), skeletal muscle (Lee et al. 1994), heart (Liu et al. 1998), kidney (Liu et al. 1998), liver (Yen et al. 1991), and lung (Fahn et al. 1996). One of the reasons proposed for the age-related increase of the frequency of the 4,977 bp-long mitochondrial genome deletion is life-long exposure to reactive oxygen species. The frequency of this deletion in different rat tissues is positively correlated to their respective metabolic rate (Filser et al. 1997). This frequency also increases with the number of in vitro CPDs undergone by HDFs (Dumont et al. 2000b). This deletion is also detected in SIPS generated by subcytotoxic doses of *t*-BHP (Dumont et al. 2000b). In vivo there is a preferential increase in frequency in natural sunlight-exposed skin compared with non-exposed skin from given individuals (Pang et al. 1994).

10.4.5 Regulation of the cell cycle and telomere shortening

p53 triggers the overexpression of the cyclin-dependent kinase inhibitor $p21^{WAF-1}$ protein which expression level increases dramatically during the two to three last passages of HDFs before senescence. This level declines when senescence is reached. During this period, cyclin-dependent kinase inhibitor $p16^{INK-4a}$ protein level gradually rises. In senescent HDFs, $p16^{INK-4a}$ is a major inhibitor of cyclin-dependent kinase 4 and 6 kinase activities (Alcorta et al. 1996). The retinoblastoma protein becomes subsequently hypophosphorylated (Medema et al. 1995). Hypophosphorylated retinoblastoma protein inactivates the E2F transcription factor, which controls the expression of genes necessary for the cells to start the S phase (for a review: Campisi 1999). $p16^{INK-4a}$ and $p21^{WAF-1}$ ectopic expression in HDFs at early CPDs induce a dramatically reduced proliferative capacity, an altered cell size and cell shape, an underphosphorylation of the retinoblastoma protein, an increased expression of plasminogen activator inhibitor 1 and SA ß-gal activity (McConnell et al. 1998). Most human primary cells express low levels of telomerase and are therefore subjected to a progressive erosion of their telomeres with each cell generation. Age-related telomere shortening has been observed in vitro (Harley et al. 1990) and in vivo (Allsopp et al. 1992, Hastie et al. 1990). A dramatic consequence of telomere shortening is the appearance of chromosomal fusions (for a review: Serrano and Blasco 2001). The activation of p53 is seen as a

mediator of replicative senescence of human cells induced by short telomeres (Humphries et al. 1998, Vaziri and Benchimol 1996). In mice, the shortest telomere, not average telomere length, is critical for cell viability and chromosome stability (Hemann et al. 2001). In HDF clones, the onset of replicative senescence is significantly correlated with the mean telomere length but, strikingly, not with chromosomes with the shortest telomere length (Martens et al. 2000). Telomere loss due to the inborn mechanisms of DNA duplication is accelerated by mild oxidative conditions, leading to growth arrest at a number of CPDs inversely proportional to the level of mild oxidative conditions above physiological conditions (for a review: von Zglinicki 2002). Cultivation of WI-38 HDFs under 40% O_2 hyperoxia induces the appearance of a senescent morphology and a G1/S growth arrest after 1 to 3 population doublings. The telomere shortening rate of these HDFs under normoxia is 90 bp per population doubling and is 500 pb per population doubling in hyperoxia. In both cases, the HDFs irreversibly stop dividing when the telomere length reaches 4 kb (von Zglinicki et al. 1995). These results favour a role of telomere shortening in the appearance of SIPS.

In another model where SIPS is induced by acute exposure to H_2O_2 in IMR-90 HDFs, growth arrest, senescent morphology, and SA ß-gal activity appear without marked telomere erosion (Chen et al. 2001). In this case it seems that telomeres shortening is not necessary for subcytotoxic stress to induce SIPS. Of course, this does not rule out the role of DNA damage in SIPS, but simply other mechanisms of growth arrest than critical telomere loss intervene, which prevent cells from duplicating their DNA, resulting in no telomere loss.

Lastly, three laboratories analysed the induction of SIPS in telomerase-expressing HDFs compared to their parental cell strains. HDFs overexpressing the catalytic subunit of human telomerase do not undergo telomere shortening and seem to have an infinite proliferative lifespan in vitro, without presenting so far any (other) characteristic of transformed cells (Bodnar et al. 1998). BJ, WI-38, IMR-90, HCA2, and LF1 HDFs expressing or not the catalytic subunit of human telomerase were exposed to subcytotoxic doses of H_2O_2, UV, UVB or gamma-irradiation in conditions inducing SIPS. After stress, all these cell lines displayed biomarkers of replicative senescence like loss of replicative potential, increase in SA ß-gal activity, senescence-like morphology, overexpression of $p21^{WAF-1}$, hypophosphorylated retinoblastoma protein. A slight telomere shortening was measured after stress both in BJ HDFs expressing the catalytic subunit of human telomerase or not (de Magalhaes et al. 2002; Gorbunova et al. 2002; Matuoka and Chen 2002). These results suggest that SIPS can be induced by subcytotoxic doses of UVB or H_2O_2 independently of critical telomere shortening.

10.4.6 Signal transduction and gene expression in SIPS

An integrative model has been proposed to explain the occurrence of H_2O_2-induced SIPS in IMR-90 fetal lung HDFs. This model is based on a positive feedback loop engaged between the activation of the stress-activated protein kinase $p38^{MAPK}$ and the overexpression of Transforming Growth Factor-ß1 (TGF-ß1).

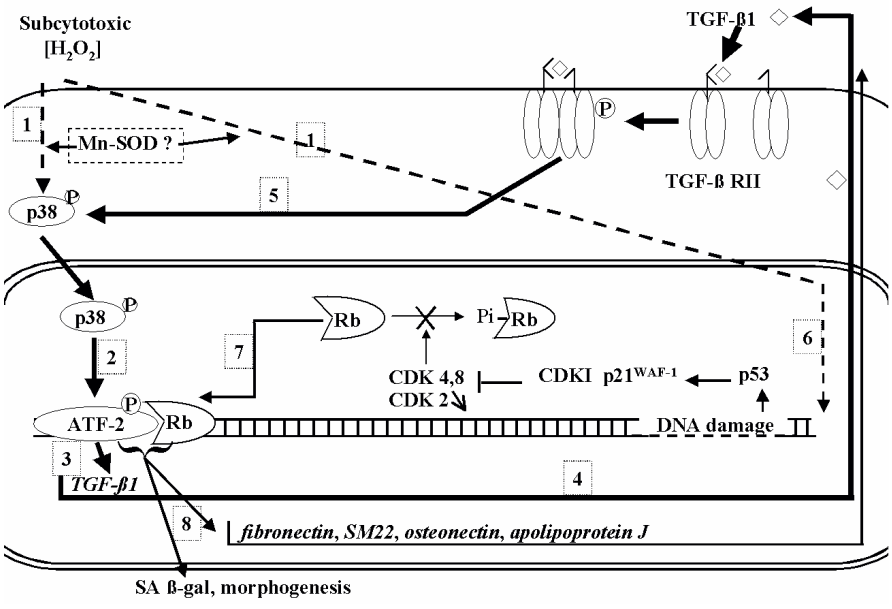

Fig. 1. Integrative simplified model for the establishment of hydrogen-peroxide induced premature senescence of human diploid fibroblasts. This model is based on a positive feedback loop engaged between the activation of the stress-activated protein kinase $p38^{MAPK}$ and the overexpression of Transforming Growth Factor-ß1 (TGF-ß1). H_2O_2 activates $p38^{MAPK}$ by phosphorylation immediately after the onset of exposure. Once activated, $p38^{MAPK}$ phosphorylates and activates the ATF-2 transcription factor, which then participates in the immediate growth arrest and triggers the overexpression of TGF-ß1, which reactivates $p38^{MAPK}$. Cyclin-dependent kinase inhibitor $p21^{WAF-1}$ is overexpressed due to DNA damage, inhibiting the cyclin D- cyclin-dependent kinase 4 and 6 complexes kinase activities, explaining in part why the retinoblastoma protein becomes hypophosphorylated. In addition, kinase activity of cyclin-dependent kinase 2 is decreased at 72 hours after stress, also explaining in part why the retinoblastoma protein becomes hypophosphorylated, this is responsible for long-term growth arrest. At 24 hours after stress, phosphorylated ATF-2 and hypophosphorylated retinoblastoma protein start to interact. This complex induces the appearance of biomarkers of senescence: senescence-like morphology, SA ß-gal activity and overexpression of the senescence-associated genes fibronectin, apolipoprotein J, osteonectin, and SM22. In addition, TGF-ß1 was shown to induce the overproduction of H_2O_2 in IMR-90 HDFs probably due to the activation of a NAD(P)H oxidase (Thannickal and Fanburg 1995), thereby, generating a constant oxidative stress which likely explains the sustained activation of the $p38^{MAPK}$ pathway as well as the overexpression of TGF-ß1. Unpublished data suggest that the mitochondrial Mn-superoxide dismutase, which produces hydrogen peroxide, is overexpressed at 72 hours after the initial stress, thereby, maintaining a production of hydrogen peroxide and subcytotoxic oxidative stress well after the initial stress.

Note that TGF-ß1 is also overexpressed by AG04431 skin HDFs after repeated subcytotoxic exposures to UVB (Chainiaux et al. 2002b). H_2O_2 activates $p38^{MAPK}$ by phosphorylation immediately after the onset of exposure. Once activated, $p38^{MAPK}$ phosphorylates and activates ATF-2 (Waas et al. 2001), which then participates in the immediate growth arrest and triggers the overexpression of TGF-ß1. At 24 hours after stress, phosphorylated ATF-2 and hypophosphorylated retinoblastoma protein start to interact. This complex induces the appearance of biomarkers of senescence (Frippiat et al. 2002). Incubation of IMR-90 HDFs with TGF-ß1 also induces SIPS with senescence-like morphology, SA ß-gal activity and overexpression of the senescence-associated genes fibronectin, apolipoprotein J, osteonectin, and SM22 (for Smooth Muscle 22) (Dumont et al. 2000b, Frippiat et al. 2001).

Antibodies against TGF-ß1 or TGF-ß1 receptor II incubated for three days after H_2O_2 subcytotoxic stress abolish the induction of these biomarkers (Frippiat et al. 2001). $p38^{MAPK}$ and ATF-2 remain phosphorylated for at least 72 hours after the initial stress. Neutralising antibodies against TGF-ß1 prevent the sustained phosphorylation of $p38^{MAPK}$ and ATF-2. This indicates that TGF-ß1 sustains the long-term activation of $p38^{MAPK}$ whereas it does not prevent the immediate $p38^{MAPK}$ phosphorylation, which takes place just after stress (Frippiat et al. 2002). It is known that TGF-ß1 activates the $p38^{MAPK}$ pathway (Hanafusa et al. 1999). Along the same line of evidence, inhibition of $p38^{MAPK}$ or ATF-2 by chemical inhibitor or by antisense oligonucleotides prevents the overexpression of TGF-ß1 and blocks the appearance of the H_2O_2-induced senescent-like phenotype (Frippiat et al. 2002) (Fig. 1). In addition, TGF-ß1 induces the overproduction of H_2O_2 in IMR-90 HDFs probably due to the activation of a NAD(P)H oxidase (Thannickal and Fanburg 1995), generating thereby a constant oxidative stress which likely explains the sustained activation of the $p38^{MAPK}$ pathway as well as the release of TGF-ß1. Unpublished data suggest that the mitochondrial Mn-superoxide dismutase, which produces hydrogen peroxide, is overexpressed at 72 hours after the initial stress, maintaining thereby a production of $H_2O_2$and subcytotoxic oxidative stress well after the initial stress. Cyclin-dependent kinase inhibitor $p21^{WAF-1}$ is overexpressed in H_2O_2- and *t*-BHP- induced SIPS, inhibiting the cyclin D- cyclin-dependent kinase 4 and 6 complexes kinase activities, explaining in part why the retinoblastoma protein becomes hypophosphorylated, and interacts with ATF-2 (for reviews : Toussaint et al. 2002a, Toussaint et al. 2000c). The mRNA level, protein level and kinase activity of cyclin-dependent kinase 2 are decreased at 72 hours after subcytotoxic exposure to H_2O_2, independently of $p21^{WAF-1}$ (Frippiat et al. 2003).

Recently, Volonte et al. showed upregulation of caveolin-1 in NIH-3T3 mouse embryonic fibroblasts after subcytotoxic H_2O_2 stress. Premature senescence induced by H_2O_2 is greatly reduced in mouse embryonic fibroblasts harbouring antisense caveolin-1 and cells overexpressing caveolin-1 show reduced proliferative lifespan, senescent-like morphotypes and an increase in SA β-gal activity. It has also been shown that caveolin-1 is tyrosine phosphorylated in mouse embryonic fibroblasts by $p38^{MAPK}$ after H_2O_2 or UV stress. Taken together, these results support the hypothesis that upregulation of caveolin-1 may represent a fundamental

step in mediating SIPS and may be part of a $p38^{MAPK}$-dependent signal transduction cascade activated by cellular stress (Volonte et al. 2001, Volonte et al. 2002).

Apolipoprotein J has been described as a survival gene against cytotoxic stress. In order to study whether apolipoprotein J would be protective against cytotoxicity SIPS and replicative senescence in human fibroblasts, a full-length complementary DNA (cDNA) of apolipoprotein J was transfected into WI-38 HDFs and SV40-transformed WI-38 HDFs. The overexpression of apolipoprotein J resulted in an increased cell survival after *t*-BHP and ethanol stress at cytotoxic concentrations. In addition, when WI-38 HDFs were exposed to 5 subcytotoxic stresses with ethanol or *t*-BHP, i.e. in conditions inducing SIPS, a lower induction of 2 biomarkers of SIPS was observed in HDFs overexpressing apolipoprotein J. Apolipoprotein J senescence–related overexpression is proposed to have antiapoptotic rather than antiproliferative effects. Indeed no effect of apolipoprotein J overexpression was observed on the proliferative lifespan of HDFs, even if apolipoprotein J overexpression triggered osteonectin and fibronectin overexpression. Osteonectin overexpression was shown to decrease the mitogenic potential of platelet-derived growth factor but not of other common growth-inducing conditions, in WI-38 HDFs. An increase in fibronectin level could protect the cells against apoptosis through the reinforcement of their anchorage to their substrate since fibronectin is a major component of the extracellular matrix that is responsible for anchorage of the cells (Dumont et al. 2002).

10.5 Proteomics and SIPS

The level of transcripts of many genes is altered in senescent cells (Doggett et al. 1992, Frippiat et al. 2000, Hara et al. 1993, Linskens et al. 1995, Shelton et al. 1999). Other studies restricted to a limited set of genes undergoing expression changes in replicative senescence showed similar changes in SIPS induced by hyperoxia, H_2O_2 or *t*-BHP and in replicative senescence (for a review Toussaint et al. 2002b). However, these studies do not consider the weakness of the correlation between a level of transcripts and the abundance of the corresponding proteins (Gygi et al. 1999).

A proteomic study based on two-dimension gel electrophoreses compared WI-38 fetal lung HDFs undergoing SIPS induced by *t*-BHP or ethanol, or replicative senescence (Dierick et al. 2002b). HDFs in SIPS display differences at the level of protein expression which are either common with replicative senescence, specific to the type of stress, or to SIPS regardless of the type of the stress. The stress-induced changes of the level of expression of specific proteins, which appear several days after stress whenever stress-specific or common to several kinds of stress were baptised ‘molecular scars’ (Brack et al. 2000).

Thirty proteins were identified which showed changes of expression level specific or common to replicative senescence and SIPS. These changes affect different cell functions including energy metabolism, defence systems, maintenance of the redox potential, cell morphology, and transduction pathways. Nine of the pro-

teins identified are involved in the energy metabolism, seven of these being overexpressed in replicative senescence and/or SIPS. Particularly pyruvate kinase is overexpressed in replicative senescence and also in SIPS induced by *t*-BHP or ethanol (Dierick et al. 2002c). An increased pyruvate kinase protein content in senescent HDFs could explain the increase of the glycolytic activity and the subsequent higher production of lactate observed in these cells more than twenty years ago (Goldstein et al. 1982). Pyruvate kinase is a key regulatory enzyme of glycolysis. Pyruvate is produced by pyruvate kinase and does not seem to be totally consumed by the cycle of tricarboxylic acids in senescent HDFs since lactate accumulates in these cells. An increased glycolytic activity could counteract at least partially the age-associated decrease in the mitochondrial ATP regeneration due to accumulation of damage in the mitochondrial membranes and DNA.

Peroxiredoxin VI was identified in several spots of two-dimension gel electrophoresis that had approximately the same molecular weight, different isoelectric points and displayed changed integrated intensity on two-dimension gels in replicative senescence and *t*-BHP induced premature senescence. Purified recombinant peroxiredoxin VI has both a peroxidase (Fisher et al. 1999) and a phospholipase A_2 activity. This bifunctionality allows peroxiredoxin VI to participate in the repair/elimination of peroxidised membrane phospholipids and represent a general antioxidant enzyme (Chen et al. 2000a). Peroxiredoxin VI is overexpressed at the protein and mRNA level in HDFs in replicative senescence and in *t*-BHP-induced SIPS (Dierick et al. 2002b, Dierick et al. 2002c). Ectopic overexpression of peroxiredoxin VI in WI-38 HDFs prior exposure to cytotoxic doses of *t*-BHP favours cell survival after stress (Dierick et al. 2003).

Two proteins with differences of expression level were identified in SIPS induced by *t*-BHP and by ethanol and not in replicative senescence. Firstly, 14-3-3 tau protein, which can block the cell cycle (Aitken 1995, Sladeczek et al. 1997) and could play a role in the cell cycle arrest was observed in SIPS (Dierick et al. 2002c). Secondly, a 30-35 kD isoform of actin was found (Dierick et al. 2002c). Fragments of actin are known to play a role in the reorganisation of the cytoskeletton in conditions generating stress fibres (Mashima et al. 1999). The isoform of actin identified in this work could participate in the reorganisation of actin into stress fibres observed in SIPS (Chen et al. 2000b).

Among the other proteins overexpressed in replicative senescence, creatine kinase BC and Heat Shock Protein 27 were identified. Glucose-6 phosphate dehydrogenase was identified as underexpressed in replicative senescence. A senescent-like phenotype and oxidative stress have been observed in HDFs deficient in glucose-6-phosphate dehydrogenase (Ho et al. 2000). A decrease in glucose-6-phosphate dehydrogenase content favours the consumption of glucose-6-phosphate by glycolysis since it becomes less consumed by the pentose phosphate pathway. Indeed glucose-6-phosphate dehydrogenase is the key regulator of the pentose phosphate pathway, which is the main producer of cellular NADPH and ribose-5-phosphate (Kletzien et al. 1994). Glutathione reductase consumes NADPH to reduce the thiol group of glutathione. A 40 % decrease of glucose-6-phosphate dehydrogenase content could explain the decrease of the concentration of reduced glutathione observed in senescent HDFs (Yuan et al. 1996). Selenium-

dependent glutathione peroxidase oxidises glutathione when detoxifying hydroperoxides (for a review: Michiels et al. 1994). A shortage of NADPH leads to a decrease of the activity of selenium-dependent glutathione peroxidase. This could participate in the increase of the oxidant status observed taking place during the in vitro ageing of HDFs (Houben et al. 1984). Ribose-5-phosphate is a precursor of the synthesis of the bases of DNA, which shortage could be linked to the absence of DNA duplication observed in replicative senescence**.** Table 1 shows the comparative changes in mRNA or protein level observed in SIPS and replicative senescence in the studies described in this paper.

A complementarity exists between transcriptomics and proteomics of SIPS. Based on the efficient technology of low-density cDNA arrays, our laboratory constructed SENECHIP, a low-density cDNA array platform for studying stress and ageing. This array represents genes involved in cell proliferation, apoptosis, stress response, inflammatory processes, protein turnover, and energy metabolism. The selection of these genes was based on scrupulous analysis of the literature and in-house investigations namely by proteomics, and reverse transcription-polymerase chain reaction differential display (Dierick et al. 2002a). As low-density cDNA arrays are much cheaper than high-density arrays, this platform allows kinetic and dose/response studies. The combination of toxicotranscriptomics of SIPS, and toxicoproteomics of SIPS represents a valuable toxicology tool for detecting the long-term effects of subcytotoxic concentrations of molecules in Research & Development departments. This kind of screening in vitro can be carried out before in vivo testing, indubitably decreasing the budgetary and ethical burden of in vivo toxicology (Toussaint et al. 2000b, Toussaint et al. 2002c).

10.7 Oxidative stress, DNA damage, pro-inflammatory cytokines, and SIPS

Many different oxidative stressors and DNA damaging agents can induce biomarkers of replicative senescence in many different cell types. Dierick *et al.* (Dierick et al. in press) presented an exhaustive list of the different models of stress, on normal non-transformed and transformed cell types, which can be considered as triggering SIPS. Induction of a senescence-like phenotype in transformed cells and particularly of irreversible cell cycle arrest is seen as an interesting concept in combating cancer.

Stimulation of cells with cytokines can also induce SIPS as shown by the effect of repeated stimulation of WI-38 HDFs with Interleukin-1α or Tumor Necrosis Factor-α (Dumont et al. 2000a, Toussaint et al. 1996). In this case transient overproduction of reactive oxygen species acting as secondary messengers, and/or activation of $p38^{MAPK}$ could be the real inducers of SIPS.

Table 1. List of the changes in gene expression identified in different models of SIPS (stress and cell type). The columns " SIPS " and " RS " indicate the sense of variation of the level of protein or mRNA expression in SIPS and replicative senescence respectively when compared to non-stressed cells at early CPDs. " up " stands for upregulation, " down " for downregulation, " no ch. " for no change, and " n.t. " indicates that this molecular marker has not been tested in this condition in the corresponding study.

Gene/protein	level	SIPS	RS	stress	cell type	reference(s)
p21$^{Waf-1/SDI-1/Cip1}$	prot.	up	up	UVB	melanocyte (human)	(Medrano et al. 1995)
				H_2O_2	IMR-90 lung HDFs	(Chen et al. 2000b)
		up	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2000)
				t-BHP	WI-38 lung HDFs	(Dumont et al. 2000b)
	mRNA	up	up	hyperoxia	BJ foreskin HDFs	(Saretzki et al. 1998)
					MRC-5 lung HDFs	(Saretzki et al. 1998)
		up	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2000)
c-fos	prot.	up	up	UVB	melanocyte (human)	(Medrano et al. 1995)
	mRNA	down	down	hyperoxia	BJ foreskin HDFs	(Saretzki et al. 1998)
hypophosphorylated Rb	prot.	up	up	UVB	melanocyte (human)	(Medrano et al. 1995)
		up	up	H_2O_2	IMR-90 lung HDFs	(Chen et al. 2000b)
		up	up	t-BHP	WI-38 lung HDFs	(Dumont et al. 2000b)
stanniocalcin / MMP-1	mRNA	up	up	hyperoxia	BJ foreskin HDFs	(Saretzki et al. 1998)
					MRC-5 lung HDFs	(Saretzki et al. 1998)
collagen-1α	mRNA	down	down	hyperoxia	BJ foreskin HDFs	(Saretzki et al. 1998)
					MRC-5 lung HDFs	(Saretzki et al. 1998)
IFN-γ / PAI-2 / Mn-SOD	mRNA	up	up	hyperoxia	BJ foreskin HDFs	(Saretzki et al. 1998)
elastin	mRNA	up	no ch.	hyperoxia	BJ foreskin HDFs	(Saretzki et al. 1998)
fibronectin / osteonectin / SM22	mRNA	up	up	H_2O_2	IMR-90 lung HDFs	(Dumont et al. 2000b)
		up	up	t-BHP	WI-38 lung HDFs	(Dumont et al. 2000b)
		up	up	UVB	AG04431 skin HDFs	(Chainiaux et al. 2002a)
α1(I)-procollagen / apolipoprotein J / SS9 / GTP binding protein-α subunit	mRNA	up	up	t-BHP	WI-38 lung HDFs	(Dumont et al. 2000b)
		up	up	H_2O_2	IMR-90 lung HDFs	(Dumont et al. 2000b)
pyruvate kinase M	prot.	up	up	t-BHP	WI-38 lung HDFs	(Dierick et al. 2002c)
				ethanol		(Dierick et al. 2002c)
14-3-3 tau protein / actin isoform (±35 kDa)	prot.	up	no ch.	t-BHP	WI-38 lung HDFs	(Dierick et al. 2002c)
				ethanol		(Dierick et al. 2002c)

Table 1. continued

Gene/protein	level	SIPS	RS	stress	cell type	reference(s)
esterase D / guanidinoacetate N methyltransferase	prot.	up	no ch.	ethanol	WI-38 lung HDFs	(Dierick et al. 2002c)
HSP90	prot.	up	no ch.	ethanol	WI-38 lung HDFs	(Dierick et al. 2002c)
glucosidase II precursor	prot.	up	no ch.	t-BHP	WI-38 lung HDFs	(Dierick et al. 2002c)
$p14/15^{Ink-4b}$ / $p16^{Ink-4a}$	mRNA	up	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2000)
$p19^{Ink-4d}$ / $p27^{Kip-1}$ / p107 / CdK2	mRNA	down	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2000)
CdK2	prot.	down	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2003)
TGF-ß1	mRNA	up	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2001)
	prot.	up	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2001)
	mRNA	up	n.t.	UVB	AG04431 skin HDFs	(Chainiaux et al., 2002b)
phosphorylated p38	prot.	up	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2002
phosphorylated ATF-2	prot.	up	n.t.	H_2O_2	IMR-90 lung HDFs	(Frippiat et al. 2002

SIPS can also be induced when cellular homeostasis is perturbated by inhibition of enzymes like phosphatidylinositol 3-kinase (Collado et al. 2000, Tresini et al. 1998), Cu/Zn superoxide dismutase, glucose-6-phosphate dehydrogenase (Siwik et al. 2001), histone deacetylase (Ogryzko et al. 1996) or DNA topoisomerase (Michishita et al. 1998). The adjunction of molecules like ceramides (Venable et al. 1995) can trigger a similar response. The expression of a mutant of ß-catenin can also trigger SIPS in murine fibroblasts (Damalas et al. 2001). Overexpression of proto-oncogenes such as Ras or Raf-1 can also lead to SIPS (Ravi et al. 1999, Serrano et al. 1997).

10.8 Conclusion: Could SIPS participate in tissular ageing?

We have seen that biomarkers of replicative senescence appear in vivo. On the other hand, these 'biomarkers' are also observed in SIPS. Theoretically, starting from the two first telomerase-negative fibroblasts that appear during embryogenesis, i.e. fibroblasts with telomere loss due to DNA duplication, 2^{80} fibroblasts ($>10^{24}$ cells) must be produced before the first telomere-dependent replicatively senescent HDFs appear, after 80 population doublings under physiological low O_2 concentrations. This represents cubic kilometric volumes of cells. Of course, tissular turnover and asymmetric division processes must be considered, which decreases these figures. However, there are more than two telomerase-negative fibroblasts and many other proliferative cell types than fibroblasts in a mammalian embryo. Therefore, the chances are very high that the irreversibly growth arrested HDFs found in vivo represent cells in SIPS rather than cells in telomere-dependent

replicative senescence. In vivo, cells are often exposed to abnormal oxidative stress which nature depends on their location in the body, on particular conditions such a local asymptomatic inflammation or microinflammation, or pathologies such as age-related neurodegenerative diseases, diabetes, atherosclerosis, Down syndrome, etc. (for a review: Toussaint et al. 2002b). Molecular scars might form in vivo at several days or weeks after exposure to different stressors like to H_2O_2, UV, inflammation, ischaemia-reperfusion, ethanol abuse, etc. Finding out of some molecular scars appearing in these conditions could help in understanding the pathophysiological consequences of such conditions.

Several studies already investigated the occurrence of prematurely senescent cells in vivo. Increases of SA ß-gal activity were observed in cells from endoscopic biopsies in the upper gastrointestinal tract from Barret's mucosa and intestinal metaplasia from the stomach (Going et al. 2002). Fibroblasts cultured from venous ulcers display reduced growth rates, larger cell morphology, increased proportion of SA ß-gal activity-positive cells and fibronectin overexpression (Mendez et al. 1998a). These biomarkers of replicative senescence were also found in fibroblasts cultured from distal lower extremities in patients with venous reflux, which precedes the development of venous ulcers (Mendez et al. 1998b). These investigators exposed neonatal skin fibroblasts to venous ulcer wound fluid, which contains high levels of Tumor Necrosis Factor-α, in order to investigate the appearance of senescence-like cells in venous ulcer (Mendez et al. 1999). As we have seen above, Tumor Necrosis Factor-α can induce SIPS in vitro. These data suggest that Tumor Necrosis Factor-α might be involved in the development of venous ulcer and in the appearance of senescence-like cells. SA ß-gal positive cells are also found in arteries subjected to balloon angioplasty (Fenton et al. 2001), tissues surrounding hepatocellular carcinoma (Paradis et al. 2001) and prostatic hyperplasia (Choi et al. 2000).

Endothelial cells also undergo premature senescence in atherosclerotic lesions of the coronary arteries from patients who died from ischaemic heart diseases (Minamino et al. 2002) and in aorta of Zuker diabetic rats (Chen et al. 2002). SIPS is also observed in fibrosis since skin fibroblasts cultured from cystic fibrosis patients display several characteristics of replicative senescence (Shapiro et al. 1979). Lastly, human ageing is characterised by an increase in Tumor Necrosis Factor-α (Franceschi et al. 1996), favouring the installation of a pro-inflammatory state and most likely SIPS.

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Abbreviations

ATF-2: transcription factor ATF-2
cDNA: complementary DNA
CPD: cumulative population doubling
HDF: human diploid fibroblast
mRNA: messenger RNA
$p38^{MAPK}$: mitogen-activated protein kinase p38
SA ß-gal: senescence-associated ß-galactosidase
SIPS: stress-induced premature senescence
t-BHP: *tert*-butylhydroperoxide
TGF-ß1: transforming growth factor-ß1
UV: ultraviolet

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