

The Cell Aging Regulation System (CARS)

Gustavo Barja

Department of Animal Physiology-II, Faculty of Biological Sciences, Complutense University of Madrid (UCM), Madrid, Spain

Correspondence: gbarja@bio.ucm.es (G.B.)

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ABSTRACT | The updated mitochondrial free radical theory of aging (MFRTA) is reviewed as part of the cell aging regulatory system (CARS). Only two known parameters correlate with species longevity in the right sense: the mitochondrial rate of reactive oxygen species production (mitROSp) and the degree of fatty acid unsaturation of tissue membranes (the double bond index, DBI). Both are lower in long-lived animal species. The life-extending manipulation dietary restriction (DR) also decreases mitROSp and the percent free radical leak (FRL) at complex I and oxidative damage to mtDNA. This seems to increase longevity by decreasing mtDNA fragment accumulation inside nuclear DNA, offering a new end point mechanism for MFRTA. Decreased mitROSp and FRL at complex I also occur during protein or methionine restriction and rapamycin treatment, manipulations which also increase longevity. The decreases in mitROSp during these life-extending manipulations occur at the matrix domain of complex I. The updated MFRTA focuses on low mitROSp and low sensitivity of membranes to oxidation in long-lived animals. The three best known aging effectors of the genetic aging program of aerobic tissues are mitROSp, DBI, and autophagy. This program responds to signaling cytoplasmic proteins, which are influenced in turn by nutrients, drugs, and hormones, and by changing the activity of the mitROSp and macroautophagy aging effectors. An analogous program, although with additional gene clusters of aging involved, and stronger effector activity, can determine longevity in different animal species.

KEYWORDS | Aging; Cell aging regulation system; Dietary restriction; Free radicals; H₂O₂ production; Methionine restriction; Mitochondria; Reactive oxygen species production

ABBREVIATIONS | CARS, cellular aging regulation system; DBI, double bond index; DR, dietary restriction; DRs, dietary restrictions in general (referring to DR, PR, or MetR); ETC, electron transport chain; FRL, %free radical leak in the respiratory chain (mitROSp as % of total electron flow at the respiratory chain); IF, intermittent fasting; MetR, (isocaloric) methionine restriction; MFRTA, mitochondrial free radical theory of aging; mitROSp, mitochondrial reactive oxygen species production; mtDNA, mitochondrial DNA; mTOR, mammalian target of rapamycin; nDNA, nuclear DNA; 8-oxo-dG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; PAP, pro-aging program; PI, peroxidizability index; PR, (isocaloric) protein restriction

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1. INTRODUCTION

Aging is the main cause of death in humans today. It causes 100,000 deaths per day worldwide. It is responsible for 70% of all human deaths (90% in developed countries). Degenerative diseases including most cancers and cardiovascular diseases, senile dementias like Alzheimer disease, Parkinson’s disease, osteoporosis, and type 2 diabetes have a common basic cause: aging. Even if some can survive one of these, another illness will come soon because the body is old and heavily damaged. Most human cancers will never be eliminated unless aging is defeated. Treating all the degenerative diseases one by one will never eliminate them. However, defeating aging, all of them will be eliminated with a single manipulation. This will restart evolution of increasing longevity, which has been among the main causes of the great success of our species on the planet, from our last common primate ancestors, the early small plesiadapiforms-like *Purgatorius* which lived only around 10 years, to the 122 years that we can live now at best, a 10-fold (1,000%) life extension. In order to accomplish this major goal, that will restart our evo-

lutionary path towards an even higher complexity, we must first understand the fundamental basic mechanisms of aging down to the cell level. Once we know them, the large capacity of modern molecular biology and pharmacological techniques will finally allow accomplishing the old dream of decreasing aging rate of the human race.

When the above goal is accomplished, it will be possible for human beings to reach an age of 100 years with a biological age equivalent to that of a young person of today of 25 years of age (around four-fold lifespan extension). That would be “healthy aging”, which is clearly not the case for most human centenarians nowadays. The previous approach implemented in the past, simply to protect most people from death but not from aging, increased the percentage of people that reached old age. They survived to a longer age but they are biologically old and therefore weak and with an ever increasing exponential chance of suffering sooner or later degenerative diseases and death. The result is the ever increasing percentage of aged people in our societies, generating a huge load for social security and health care systems, strongly increasing the proportion of

fragile, disabled, and longtime suffering old individuals in the population. This constitutes the socio-economic “aging problem” of our aged societies. This can be solved by progressively attaining negligible senescence with the help of gerontological science.

What causes aging? Many different theories of aging have been proposed. Among them, the mitochondrial free radical theory of aging (MFRTA) is one of the best supported current explanations of aging and longevity in mammals, birds, and multicellular animals in general. Any theory of aging must explain why maximum longevity (referred here throughout as “longevity”) varies so widely in animals: 30-fold from mice to men, 200-fold from shrews to the longest-living whales, or more than 5,000-fold from perhaps a single day of life in some invertebrates to *Arctica islandica* mussels (longevity around 500 years). Such large differences indicate that longevity is markedly regulated and flexible during species evolution. Imitating only a small part of this huge natural capacity will make possible in the future to reach negligible senescence in humans. Animals closely related by phylogeny, like *Mus musculus* (longevity 4 years) and *Peromyscus leucopus* (longevity 8 years) mice have very different longevities, indicating that evolution of longevity is also a relatively easy and fast process. Substantially decreasing the aging rate in mammals including humans will be relatively easy once the underlying basic mechanisms controlling longevity at physiological, cellular, and molecular levels are clarified.

The life expectancy at birth of the individuals of a population (and mean longevity) depends more on the environment than on the genes. On the contrary, (maximum) longevity, and its inverse—the species aging rate, depends mostly on the genotype, as it is also the case for the other species-specific traits. Longevity and aging rate are the parameters that matter concerning the endogenous process of aging. Only two known factors correlate in the right sense with animal longevity in vertebrates including mammals and birds: (1) the rate of mitROSp (mitochondrial reactive oxygen species production) [1–4]; and (2) the degree of fatty acid unsaturation (calculated as the double bond index, DBI) of tissue cellular membranes including the mitochondrial ones [5, 6]. The longer the longevity of a species, the smaller the value corresponding to these two parameters. The low mitROSp of long-lived animal species decreases their rate of generation of endogenous (free radical)

damage at mitochondria. The low fatty acid DBI and peroxidizability index (PI) decrease the sensitivity of the cellular and mitochondrial membranes to free radical attack and lipid peroxidation. No other theory of aging focuses on parameters like these two, which correlate in the right sense with longevity across animal species, and offer plausible mechanistic explanations for the final accumulation of damage from endogenous origin leading to aging.

2. ANTIOXIDANTS AND LONGEVITY

Early investigations concerning aging and oxidative stress mainly studied antioxidants. In 1993, it was found in the author’s laboratory that both enzymatic and non-enzymatic endogenous tissue antioxidants, including superoxide dismutase (SOD), catalase, glutathione peroxidases, glutathione reductases, reduced form of glutathione (GSH), or ascorbate strongly correlated with longevity across vertebrates. However, surprisingly, such correlation was negative, instead of positive as it was until that time widely believed. Our review on the relationship between endogenous antioxidants and vertebrate longevity [7] included all the available published data on the subject obtained in vertebrates including mammals by us as well as other laboratories. All those data, coming from various different sources consistently agreed that the longer the longevity, the lower were the levels of most endogenous tissue antioxidants and antioxidant enzymes. A later consideration of the subject [8] confirmed the existence of a generally negative correlation between tissue antioxidants and longevity in all kinds of vertebrate animals. It was interesting that long-lived animals had lower instead of higher antioxidant levels. Among 27 studied correlations, 21 negatively correlated with longevity, six did not show significant differences, and not a single positive correlation with longevity was found [7]. SOD was among the antioxidants tending to show no association with longevity. The previous belief that this enzymatic activity was positively associated with longevity was due to referring the SOD activity values (total SOD) to the oxygen consumption (VO_2) of the whole animal (to the weight-specific metabolic rate). Since weight-specific metabolic rate strongly decreases as body size increases, the larger SOD/ VO_2 of humans compared to rats was due to the lower value of the denominator in the humans (lower

weight-specific metabolic rate than rats) instead of to a higher value of the numerator (SOD). In fact, when the tissue SOD values (total SOD without dividing by VO_2) were plotted against longevity across species they showed no significant correlation with mammalian longevity in mammals in the original publication [9]. In the brain and lung of vertebrate species, but not in the liver, the correlation between SOD (total SOD) and longevity was again negative like for the other antioxidants. Further studies in different mammals including long-lived naked mole-rats, as well as ants, honey bees, and marine bivalves also found a negative correlation with longevity for this antioxidant enzyme—SOD [8]. In a more recent and comprehensive review on the subject, among a total of 78 correlations between endogenous tissue antioxidants and longevity, 72 were negative, six did not show significant differences, and only a single one was positive [8], corroborating global studies performed almost two decades ago [7]. Therefore, it must be concluded that the endogenous antioxidant levels do not determine animal longevity.

3. MITOCHONDRIAL ROS GENERATION, mtDNA OXIDATIVE DAMAGE, AND LONGEVITY

3.1. Comparative Studies of mitROSp

Antioxidants do not determine longevity. But, why long-lived animals need less antioxidant levels in their vital organs? We have proposed that the rate of mitROSp could be negatively correlated with longevity and that this would be the critical factor for aging, instead of the antioxidant levels [10]. Long-lived animals would not need to maintain high antioxidant enzyme levels, which is energetically expensive, because they would produce mtROS slowly, and they could transiently induce the antioxidants if needed. The low mitROSp of long-lived animal species was indeed experimentally corroborated comparing mammals with different longevities [3] as well as comparing short-lived rodents (rats and mice) with 5–8-fold longer lived birds (pigeons, parakeets, and canaries) of similar body size and weight-specific metabolic rate [11, 12]. A posterior investigation studying up to 12 different mammalian species confirmed these findings even after correcting for body size [4].

The investigations in birds are especially important because the studies performed in mammals had used mostly species following the Pearl's rate of living law of aging: "the lower the weight-specific metabolic rate the longer the longevity". Thus, one could not discard, in principle, the possibility that the species with longer longevity included in the comparisons between different mammals could show low rates of mitROSp simply because their rates of oxygen consumption were also lower than those of the short-lived ones. In fact, mitROSp was positively correlated with mitochondrial O_2 consumption and with global metabolic rate in those studies [3]. It was then important to study the problem in some of the many species that strongly deviate from the Pearl's rate of living law of aging. Three groups of warm-blooded vertebrates have much higher longevity than expected for their body size or weight-specific metabolic rate compared to most mammals: birds, bats, and primates. Birds have both a high rate of oxygen consumption per gram of tissue and a high longevity. This makes them ideal to solve the problem mentioned above. The lower mitROSp of pigeons, canaries, and parakeets, when compared to rats in the first case and to mice in the second and third case, strongly reinforces the MFRTA since it indicates that the low mitROSp of long-lived animals occurs both in comparisons between animals following Pearl's law as well as in those not following it. A high longevity is not a simple consequence of a slow rate of living. It can be obtained—as the bird case shows—together with high rates of oxygen consumption and animal aerobic activity. High longevity in the studied birds is associated with a low rate of mitROSp both in absolute terms, and also as percentage of mitochondrial oxygen consumption and thus of electron flow at the electron transport chain. Birds have a low percent free radical leak (FRL), and the same seems to be true also in the bats and humans studied so far.

3.2. The mitROSp Site at the ETC Important for Longevity

It has been widely believed during decades that complex III was the respiratory complex responsible for most ROS production in the ETC (the mitochondrial electron transport chain) [13]. However later studies using freshly isolated and well coupled functional mitochondria showed that complex I also produces ROS in the heart or brain mitochondria

isolated from rats, mice, pigeons, canaries, and parakeets [12, 14], which was soon confirmed in rats by many other laboratories [15, 16] and soon became established knowledge. A key experiment to detect complex I ROS production was to measure mitROSp with succinate alone as well as with succinate plus rotenone. In the second situation, the rate of mitROSp acutely decreases because rotenone does not allow the electrons to flow back to complex I from succinate-complex II through reverse electron flow [17]. But the habitual procedure of adding succinate alone, followed or not followed by antimycin A, and rarely using complex I-linked substrates, contributed to the erroneous belief over decades that mitROS came mainly from complex III-semiquinone.

On the other hand, although both complexes I and III produce ROS, we found that the lower mtROS generation rate observed in birds compared to mammals of similar body size and weight-specific metabolic rate occurred only at complex I [12, 14, 17], not at complex III. This is interesting because we found the same afterwards in dietary restriction (DR) rat models (see Section 5). Three possible complex I ROS generators have been suggested, the flavin at the beginning of the electron path within the complex, the FeS clusters of the hydrophilic matrix domain, and the ubiquinone located in the membrane domain. Various researchers have supported the role of the flavin based on experiments with the inhibitor diphenyliodonium, which strongly decreases mitROSp. However, the site of action of diphenyliodonium, at the beginning of the electron path, also avoids electrons to reach the other two possible generators, the various FeS clusters and the ubiquinone, which therefore cannot be discarded as sources of ROS. In other investigations, it was concluded that electron leak to oxygen occurred between the ferricyanide reduction site and the rotenone binding site of complex I both in intact mitochondria [12, 14, 17] and in submitochondrial particles [18]. FeS clusters with a higher midpoint potential than FeSN1a, which could be situated in the electron path after the ferricyanide reduction site [15, 18], or the unstable semiquinone known to be present in the membrane domain of complex I and possibly functioning in H⁺ pumping coupled to electron transport [19], could be the complex I oxygen radical generators. However, many different complex I FeS clusters could be responsible for complex I mitROSp because, under physiological conditions: (a) their reduced and oxi-

dized states will not be present in equal concentrations; (b) interactions with many different factors and surrounding macromolecules could modify the final redox potential of the carriers *in vivo*; and (c) the exact position of many FeS clusters in the complex I electron path is still unknown. Thus, the important aging-related question—whether flavin, FeS clusters, or ubisemiquinone, or a combination of them are responsible for the complex I ROS generation relevant for aging—is still unanswered.

3.3. Oxidative Damage to mtDNA and Longevity

Mitochondrial DNA (mtDNA) is situated very close to the site of mtROS generation, the ETC at the inner mitochondrial membrane. ROS production also occurs at other cellular sites like endoplasmic reticulum, peroxisomes or membrane-bound NADPH oxidases, and the rate of ROS generation at these sites can substantially exceed in various situations that coming from mitochondria. However, the ROS produced at mitochondria is still most important for longevity due to the presence of mtDNA within the mitochondria and not at the other organelles or parts of the cell. Since long-lived animal species have low rates of mtROS generation, it was logical to expect that this should have an effect on the steady-state level of oxidative damage in their mtDNA. Therefore, we measured the level of 8-oxo-dG (8-oxo-7,8-dihydro-2'-deoxyguanosine) in the heart and brain mitochondrial and nuclear DNA of eight different mammalian species differing by up to 13-fold in longevity. The results showed that the level of 8-oxo-dG in the mtDNA of both organs is negatively correlated with longevity [20]. The longer the longevity of a species, the smaller is its mtDNA oxidative damage degree. In contrast, the 8-oxo-dG level in nuclear DNA (nDNA) did not correlate with longevity in any organ even though mitochondrial and nuclear DNA was measured in the same samples taken from the same individual animals [20]. Therefore, the different mitROSp rates of the different species seem to have a direct impact on oxidative damage to mtDNA, and not to nDNA. This makes sense since the site of ROS generation at mitochondria is situated far away from nDNA, whereas it is very close to mtDNA.

The variations in the steady-state levels of 8-oxo-dG in mtDNA closely reflect the variations in the rate of mitROSp, both in comparative and in dietary restriction (DR; see Section 5) studies. This suggests

that the mitROSp in vitro measurements are closely indicative of the situation in vivo. In addition, the level of 8-oxo-dG in mtDNA was generally lower in the heart and brain of three long-lived birds when compared to two short-lived mammals of similar body size and specific metabolic rate, in agreement with the superior longevity of the birds, whereas again this was not the case for nDNA [21]. These investigations also showed that the intensity of oxidative damage is several fold higher in mtDNA than in nDNA in the heart and brain of all the 11 different species of mammals and birds studied [20, 21], which is again consistent with the close proximity between mtDNA and the sites of mtROS generation. Studies on the longest-lived known metazoan, the bivalve *Arctica islandica* (longevity 500 years) have also shown lower DNA oxidative damage than in other bivalve species with much shorter longevity [22].

3.4. Increasing Antioxidants Does not Change Longevity

Many investigations on the effect of adding dietary antioxidants to the diet were performed during the 1970s and 1980s. The general result was that antioxidants did not increase (maximum) longevity [23, 24]. In some experiments they increased only mean longevity [23–25]. Interestingly, this tended to occur when the (maximum) longevity of the control rodents was short, usually less than 3 years. This suggests that antioxidants, when the husbandry conditions are suboptimal, could protect from causes of early death, and thus they can make more rectangular the survival curve. This is what happened in humans during the twentieth century in many developing western countries when mean life expectancy increased from around 40 to 80 years without decreasing the aging rate. That is why now old people are so abundant in western populations. Antioxidants, in the animal studies above, were bringing back towards optimum the diminished survival of the controls reared under suboptimum environmental conditions, which is interesting but not the goal of gerontology. Ironically, the poorer the survival curve of the controls, the larger is the chance of obtaining a positive result in terms of mean longevity. On contrary, the better one performs the experiment, the smaller are the chances of obtaining an increase in mean longevity. In summary, like in the comparative

inter-specific studies described above, antioxidants cannot be longevity determinants. Long-lived animals have low, instead of high, levels of endogenous antioxidants. And increasing antioxidants through the diet does not increase longevity.

Posterior studies created transgenic or knockout mice with increased or lack of expression of genes codifying for antioxidant enzymes like SODs, catalase, or glutathione peroxidases. But the results were similarly disappointing [26, 27]. The antioxidant enzyme activities increased through modification of gene expression, like the non-enzymatic dietary antioxidants added to the diet, did not slow aging. Independently of the way in which the antioxidants were manipulated, dietary or genetic, the result was the same: a lack of effect of antioxidants on mammalian longevity. This has been interpreted by some [27], but not other [28], authors as the “death” of the MFRTA [27]. But such a conclusion [27] does not take into account that what correlates with longevity in the right sense is not the level of the antioxidants, but the mitROSp rate and the DBI of the cellular membranes [23, 29].

Investigations in simpler organisms like the fungus *Podospora anserina* have also provided strong evidence for a role of mitROSp in aging including DR-effects, because strains of this fungus deprived of mitROSp are converted to “eternal” non-aging organisms [30]. Most interestingly, recent experiments in 29 different *C. elegans* longevity mutants, and 26 different environmental situations and drugs that increase (DR, PR, MetR, rapamycin, starvation, and others) or decrease longevity used a new fluorescent probe claimed to estimate mitROSp in vivo [31]. In all the 55 cases lower “mitROSp” (mitoflash) was always observed in long-lived animals [31].

3.5. The Contact Hypothesis of Aging—mitROSp in Contact with mtDNA can Control Aging

Investigations performed in complex animals including mammals strongly suggest that the rate of mitROS generation importantly contributes to determining longevity, whereas antioxidants do not [23, 24, 29, 32]. This is counterintuitive only if we erroneously consider the cell as a homogeneous system without compartmentation. But cells are not designed in such a way. The global cellular level of oxidative stress should depend both on the rates of ROS production and ROS elimination. Both contrib-

ute to determining cell survival or cell death according to the general “balance” between them, and likely affect mean lifespan. However, the ROS concentration in particular compartments like mitochondria, and most especially very near to the places of mitROSp generation like complex I, should be much more dependent on mitROSp than on antioxidants as the free radical generation site relevant for aging is approached at micro level. At such places, it is mitROSp that would mainly determine the local ROS concentration. This is especially important because the main target for aging, mtDNA, is located very close in the vicinity, perhaps even in contact with the free radical generation source. This would explain why lowering the rate of mitROSp instead of increasing antioxidants was selected during the evolution of long-lived mammals, birds, and other species. This would be consistent with the view that mitochondria are causal players in the aging process. The existence of contact between mtDNA and specific mitochondrial proteins agrees with classic electron microscopic studies [33]. Mitochondrial nucleoid structure has been long debated, with an estimation of about 2–10 mtDNA molecules coated by an unspecified number of proteins [34]. Using high-resolution microscopy, the structure of the nucleoids has been further clarified as single mtDNA circular molecules compacted by mitochondrial transcription factor A (TFAM) [35], which forces mtDNA to undergo a U-turn, thus collapsing the mtDNA molecule. And images of strong attachment of a protein structure of probable membrane derivation to mtDNA have been obtained [33].

3.6. Low mitROSp, and Low Endogenous Antioxidant Defenses and DNA Repair in Long-Lived Animals

To obtain a low level of damage at mtDNA it is much more efficient to lower the rate of generation of damage than to have a high rate of damage generation and, afterwards, try to intercept the generated ROS using antioxidants, or try to repair the damaged already inflicted on mtDNA. This last approach would not make sense if generation of damage can be controlled in the first place. This is why long-lived species have not used high (antioxidant) defense [7, 8, 10, 11, 23, 24, 29, 32] or high repair of endogenous DNA damage to increase longevity. Base excision repair tends to be, on the contrary, low

in long-lived animals, both in interspecies comparisons and in DR animals (reviewed in [29]). In addition, it would be very costly to continuously maintain high levels of antioxidant or repair enzymes in long-lived mammals to counteract a high rate of damage generation. Instead, lowering mitROSp: (1) is much easier; (2) is 100% efficient; and (3) avoids much damage at around zero cost. In summary, to obtain a low level of damage in mtDNA it is both much more efficient and less costly to lower the rate of mitROSp than to increase antioxidants or DNA repair. This is most likely why long-lived animals have chosen to lower mitROSp during evolution to increase their longevity.

3.7. FRL Is not a Constant Percentage, and mitROS Are not Simple “Byproducts” of the ETC

It is well known that the rate of mitROSp is low in long-lived species irrespective of the value of their specific metabolic rate and their rates of mitochondrial oxygen consumption. This is clearly against the common belief that mitROS production is an unavoidable “byproduct” of the respiratory chain. Mitochondria can vary the percentage free radical leak (FRL = %mitROSp/mitO₂ consumption) in the respiratory chain. They decrease such percentage (the FRL) in especially long-lived animals, like birds or dietary restricted rats. They lower the FRL to decrease mitROSp (to age slowly), while their oxygen consumption is not depressed so as to be able to continue mitochondrial ATP generation at the rates needed for the normal activity level of the animal. If ROS leakage were an “unavoidable byproduct” of the mitochondrial ETC, the FRL would be a rather constant percentage of the rate of electron flux at the ETC. Instead, the FRL is decreased in especially long-lived animals like birds that live 2–3-fold longer than mammals of the same body size and specific metabolic rate. Contrary to the unproven “byproduct” assumption, mitROSp is finely tuned (regulated) in each animal, and if needed it varies independently of the rate of mitochondrial oxygen consumption and the total rate of electron flow at the ETC. Thus, the FRL varies in many cases to contribute to determining the aging rate and the longevity of the species (birds), or the individual (during the DRs). The unproven assumption currently stated in many scientific articles that mitROS are “unavoidable byproducts of the ETC” should be avoided because

the measurements on functional isolated mitochondria data have demonstrated it to be false on too many occasions. The use of this “byproduct” concept should be exclusively limited to particular situations on which it is known to be correct, but not as a generalization for every situation. This is important because this wrong concept is making a large damage to the scientific advance concerning MFRTA, and therefore towards approaching negligible senescence.

4. LONGEVITY AND MEMBRANE FATTY ACID UNSATURATION

The fatty acid unsaturation degree of cellular (including mitochondrial) membranes, like mitROSp, also correlates with longevity in the right sense, because it is low in long-lived animal species. This has been studied many times in more than 20 well controlled different investigations and concordant results were always obtained. The degree of fatty acid unsaturation can be summarized as the double bond index (DBI), or alternatively, as the peroxidizability index (PI). The longer the longevity of the species, the smaller the total number of tissue fatty acid double bonds (the smaller the DBI and PI). A constitutively low DBI strongly decreases the sensitivity of the cellular and mitochondrial membranes to lipid peroxidation, a highly destructive process. Lipid peroxidation, in addition to membrane damage, produces mutagenic and toxic metabolites. Peroxidation of lipids quantitatively is the most intense destructive process produced in cells by ROS. Fatty acids containing a high number of double bonds (like 20:4n-6 and especially 22:6n-3) are the cellular molecules most sensitive to lipid peroxidation, and their sensitivity to lipid peroxidation increases exponentially as the number of double bonds per fatty acid molecule increases. The low DBI and PI of long-lived animals was first described in 1996 in rat compared to pigeon and human mitochondria [36] followed by many studies in mammals and birds (reviewed in [5, 6]). A total of 23 studies extended the first seminal observation [36] to many different mammals, various bird species, and some invertebrates, without finding a single exception: low tissue DBI in long-lived animals [6]. The low degree of fatty acid unsaturation occurs both in mitochondrial as well as in total cellular membranes in tissues of long-lived animals. It can therefore strongly diminish lipid oxidation-derived

damage in various cellular compartments, and especially in the mitochondria. At those organelles there is strong abundance of membranes together with a nearby and rather constant source of ROS during mitochondrial respiration throughout life.

Many different fatty acids composing the cellular membranes are responsible for the strong decrease in DBI (and PI) as longevity increases among species. But the most important ones, both due to their content in double bonds (low or high) as well as for their larger quantitative presence and variation among species, are 18:2n-6, 18:3n-3 and 22:6n-3 in mammals, and in some phylogenetic groups also 18:1n-9 (at least in the studied birds) and 20:4n-6. As longevity increases across mammalian species tissue 18:1n-9, 18:2n-6 and 18:3n-3 significantly increase, and 20:4n-6 and especially 22:6n-3 significantly decrease in the skeletal muscle, liver, and heart cellular membranes. Among them, the decrease in 22:6n-3 in long-lived animals usually is the most important to quantitatively explain their low DBI and PI values. Interestingly, the final result is that the total percentage of unsaturated and saturated fatty acids does not change among species with different longevities. Instead it is the unsaturation degree of the polyunsaturated fatty acids present that decreases from short- to long-lived animals. Long-lived animals have fatty acids with a lower degree of unsaturation, with less double bonds per fatty acid molecule. With this kind of fatty acid redistribution long-lived animals obtain a strong decrease in the sensitivity of their cellular membranes to the destructive and mutagenic process of lipid peroxidation, while likely avoiding strong changes in the fluidity of their membranes, the so called homeoviscous-longevity adaptation [5]. In addition, it has been recently shown that feeding 18:1n-9 reverses the increases in 20:4n-6 and 22:6n-3 and the decreases in 18:1n-9 and 18:2n-6 and complexes I and IV activities observed in the skeletal muscle of old rats [37]. Recent confirmation of the low tissue fatty acid unsaturation of long-lived animals has been obtained through shotgun lipidomic analysis of mitochondrial phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in the skeletal muscle, liver and brain of mammals with widely different longevities, including mice, pigs and humans [38].

The low degree of fatty acid unsaturation of long-lived animals likely protects not only the lipids but also other kinds of cellular components. Since lipid

TABLE 1. Summary of changes in free radical-related parameters induced by all manipulations known to consistently increase, or have no effect on, longevity in laboratory rodents

Experimental Manipulation	mtROSp (at Cx I)	mtVO ₂	FRL (at Cx I)	8-oxo-dG in mtDNA	8-oxo-dG in nDNA	mtDNA Frags in nDNA	Longevity (MLSP)
DR	↓	↔	↓	↓	↔	nd	↑
PR	↓	↔	↓	↓	↔	nd	↑
LR	↔	↔	↔	↔	↔	nd	↔
CHR	↔	↔	↔	↔	↔	nd	↔
MetR	↓	↔	↓	↓	↔	nd	↑
Rapamycin	↓	↔	↓	↓ (ns)	nd	↓	↑

Note: DR, dietary restriction; PR, protein restriction; LR, lipid restriction; CHR, carbohydrate restriction; MetR, methionine restriction; rapamycin (14 mg/kg diet). The effects of DR, PR, and MetR on oxidative stress related parameters were obtained at a level of 40% restriction, and also at 80% MetR. The effect of MetR on longevity has been studied always restricting methionine by 80% in the diet [56, 57, 59]. The rapamycin effect of mitochondrial oxidative stress was studied in B6D2F1 mice [139]; the rest of the experiments were performed in Wistar rats. While DR, PR, and MetR decreased mtROSp and 8-oxo-dG in mtDNA in all the vital organs studied (liver, heart, and brain), rapamycin (at 14 mg/kg diet) did it in the liver but not in the heart of mice; mtDNA fragments (Frag) inside nDNA were studied in the liver. See text for other references. mtVO₂, mitochondrial oxygen consumption; FRL, %free radical leak from complex (Cx) I of functional mitochondria; nDNA, nuclear DNA; MLSP, maximum life span potential. DR, PR, MetR, and rapamycin all increase both mean lifespan, and maximum longevity (by up to 40% in DR, by 20% in PR and DR, and by 11% with rapamycin) in rats and mice. ↔, no change; nd, not determined; ns, statistically not significant.

peroxidation is a relatively massive process compared to oxidative damage to other kinds of macromolecules, long-lived animals, due to their low DBI, will produce far smaller amounts per unit time of highly toxic and mutagenic lipid peroxidation products like hydroxynonenal, malondialdehyde, and many others. These, having carbonyl groups, can modify free amino groups in proteins and DNA. Lipid peroxidation-derived protein modification seems to be involved also in aging. Comparisons among different mammalian species have found that the amount of malondialdehyde-lysine adducts in heart proteins is lower in long-lived animals [39].

What is the mechanistic cause of the low fatty acid unsaturation degree of long-lived animals? A role for acylation/deacylation of the constitutive membrane fatty acids cannot be discarded. However, since the more unsaturated 20:4n-6 and 22:6n-3 are essential fatty acids synthesized from their dietary precursors 18:2n-6 and 18:3n-3 respectively, the enzymatic processes that control the n-3 and n-6 biosynthetic pathways seem to be involved. In this respect, in var-

ious comparative studies relating the degree of fatty acid unsaturation to longevity, the results suggest that desaturase and elongase enzymatic activities in the n-3 and n-6 series (which are rate limiting for those biosynthetic pathways) are constitutively low in long-lived animals. In some cases, decreases in peroxisomal beta-oxidation could also be involved. It is now considered that this last process is responsible for the last steps in the synthesis of the highly unsaturated 22:6n-3 in the n-3 pathway. The low delta-5 and delta-6 desaturase activities (which are rate limiting enzymes in the n-3 and n-6 fatty acid synthesis pathways) of long-lived animals will decrease the conversion of the less unsaturated 18:2n-6 and 18:3n-3 diet-derived precursors to the highly unsaturated 20:4n-6 and 22:6n-3 products. Thus, 18:2n-6 and 18:3n-3 would accumulate and 20:4n-6 and 22:6n-3 will diminish, which is just the general kind of fatty acid profile observed in long-lived animal species. In summary, the membrane fatty acid unsaturation degree is low in tissues of long-lived animals. This is the only other known factor, in addition to

mitROSp, which correlates with longevity in the right sense. Importantly, this is true concerning the MFRTA as well as any other theory of aging.

5. DIETARY RESTRICTION

Dietary calorie restriction (DR) increases not only mean but also maximum longevity (up to 40%) and decreases and delays the incidence of degenerative diseases in most animal species including rotifers, flies, spiders, worms, fish, laboratory rodents, and many other mammals (Table 1) [40]. In a life-long study in rhesus monkeys, 30% DR strongly decreased age-related mortality (from 37% to 13%), and the incidence of many age-related diseases including diabetes, cancer, cardiovascular disease, and brain atrophy [41]. Many effects of DR have been discovered involving lowered growth hormone (GH) and insulin/IGF-1-like signaling, modifications in nutrition and amino acid-sensing pathways, changes in sirtuins [42], apoptosis, and signaling proteins and transcription factors like mTOR, S6K, AKT, PKA, or FOXO and tissue-specific changes in gene expression profiles. Many of these changes and others are interrelated and seem to be part of an integrated cellular aging regulation system (see Section 9) which includes mitochondrial oxidative stress-related damage and sensitivity to lipid peroxidation as two of its main aging effectors [42].

Long-lived animals have lower rates of mitROSp and lower mtDNA oxidative damage than short-lived ones [29]. But what occurs with these parameters in DR? If the MFRTA is correct also within species, those two parameters should also decrease during DR. Initial studies, like in the case of the comparison between different species, focused mainly on antioxidants. They showed that DR in rodents does not lead to a generalized increase in antioxidants. Instead of increases, decreases or lack of changes, depending on the particular antioxidant measured, has been reported even within the same study [43]. Therefore during DR, similar to what happens in the interspecies case the key to longevity again is not based on the antioxidant levels.

In the case of the mitochondrial ROS generation, the situation is different. The effect of DR on the rate of mitROSp has been investigated in mice and rats in many different laboratories. The results of these investigations consistently agreed that long-term

standard (40%) DR, as well as short-term (e.g., 7 weeks) DR, significantly decrease the rate of mtROS generation in rat organs including the skeletal muscle, kidney, liver, heart and brain (Table 1) [44]. This agrees again with the concept that lowering mitROSp contributes to increasing longevity. The decrease in mitROSp during DR was found in freshly isolated functional mitochondria exposed to similar incubation conditions in the ad libitum fed and DR groups. Thus, DR mitochondria are qualitatively different from those obtained from ad libitum fed animals, and this difference (due to DR) is responsible for the lowered mitROSp detected in vitro. In addition to this, classical data suggest that complex I substrates like pyruvate decrease during DR in tissues [45], which has been recently confirmed by comprehensive metabolome analysis [46]. The decrease in pyruvate and other mitochondrial substrates during DR would decrease the matrix NADH level thus lowering electron input to complex I. This would in turn decrease the degree of electronic reduction of the complex I ROS generator, and therefore its rate of oxygen radical production. Indeed, DR also decreases the NADH concentration [45, 46], a change that is known to strongly decrease the rate of mitROSp [16]. This will lead to a further decrease in the rate of mitROSp in vivo which would add to that due to the lowered capacity of DR mitochondria to generate ROS detected in vitro. On the other hand, the decrease in mitROSp in DR rats specifically occurred at complex I in all the organs studied [47–49]. Thus, a low rate of mitROSp at complex I is a trait of both long-lived species and DR mammals. A recent study showed that a single nucleotide mutation in complex I suppresses mouse fibroblast aging [50], and inhibition of this complex by the antihyperglycemic and proposed antiaging modulators biguanides lowers its rate of mitROSp [51]. Many investigations have shown that mitochondrial functionality and even morphology are detrimentally altered in tissues of old animals [52]. Recently, proteomic analysis of 57 out of the 96 known mouse ETC proteins (and 67% of complex I proteins), showed that low abundance of the “matrix domain-only” of complex I lowers mitROSp and is related to increased longevity in both DR and longer-lived mouse strains, while it increases during mouse aging [53]. This is interesting because various studies have located the ROS generator relevant for aging precisely inside that matrix domain of complex I [15, 18, 47].

In addition to lowering mitROSp, DR also decreases the FRL (Table 1). This indicates that the efficiency of the mitochondrial respiratory chain in avoiding ROS generation increases in DR animals. Birds (pigeons, canaries, and parakeets), which are especially long-lived homeothermic animals for their body size and metabolic rate, also show lower FRL values than the much shorter-lived rats or mice [12, 17]. This suggests that lowering the FRL is a conserved mechanism of life span extension both between and within species that can be obtained without the need to decrease mitochondrial oxygen consumption. This is relevant when it is necessary to increase longevity without decreasing the general level of animal activity and thus competitiveness in the ecological niche. A decrease in FRL could be obtained through qualitative changes in the redox midpoint potential of the complex I ROS generator related to aging. This would lead to a decrease in the degree of electronic reduction of that generator and thus in complex I mitROSp without decreasing electron flow in the ETC and thus mitochondrial oxygen consumption. Both a low mitROSp and a low FRL at complex I have been reported in many different investigations in long-lived animals [11, 12, 14, 17, 18, 29, 47] (Table 1). Interestingly, extensive proteomic analysis of the ETC in mice showed that partial assembly of the “matrix domain-only” of complex I occurs during ad libitum feeding (in addition to the whole complex I protein) to a much higher extent than during DR [53]. This can help to explain both the decrease in mitROSp and the decrease in FRL at complex I in DR. Moreover, rapamycin, the only known drug that consistently increases mouse longevity (see Section 7), also lowers mitROSp and FRL at complex I and decreases the amount of its “matrix domain-only” in the mitochondria of mouse liver [53] (Table 1). It is this matrix domain which seems to contain the subunits responsible for the low mitROSp at complex I occurring in long-lived species [15, 16, 18, 29] as well as in DR [44, 47] and rapamycin-treated animals.

During ad libitum feeding, the extra copies of the “matrix domain-only” complex I would take electrons from NADH but could not pass them to ubiquinone and the following complexes of the ETC. This would strongly fill up that domain with electrons and would thus strongly reduce with electrons the complex I ROS generator(s). This would increase mitROSp at this “matrix domain-only” complex. The

result would be a strong increase in mitROSp without any ATP generation, because electrons in “matrix domain only” complex I cannot reach the other separated complexes. In this way, the ad libitum fed animal would have a high FRL because it has both complete complex I copies and “matrix domain-only” complex I copies that increase mitROSp but not oxygen consumption or ETC electron flow. In contrast, the DR and the rapamycin-treated animals have complete fully assembled complex I copies and reduced abundance (20–50% decrease) of “matrix domain-only” complex I copies. The DR mice would thus exhibit decreases in mitROSp without decreases in mitochondrial oxygen consumption (lower FRL) with a normal ATP production. Therefore, a quantitative decrease in the amount of the “matrix domain-only” of complex I can lead to a qualitative change (decreased FRL) in DR and rapamycin-treated rodents. Lowering of the midpoint redox potential of the complex I ROS generator would be another (qualitative in this case) mechanism that can also contribute to decreasing mitROSp in long-lived animals [14, 17, 18, 29, 47].

At variance with mitROSp, which is low both in long-lived species and in DR rodents, a low DBI occurs in long-lived species (see the previous section) and in 80% methionine restricted (MetR) rats, but not in 40% MetR (see Section 6) or 40% DR rats. Therefore, a low DBI, like a low mitROSp, can contribute to decreasing aging rate during evolution of long-lived animal species. But, in a single species, the membrane needs to decrease its sensitivity to oxidative damage by lowering fatty acid unsaturation only when the level of protein restriction in the available food is very strong, whereas the decrease in mitROSp is a response already recruited at milder (40%) levels of DR. This makes sense, in principle, since dietary protein availability is essential for growing of the offspring. At 80% MetR there is not enough protein for adequate growing, and the competitiveness of the offspring in the wild would be very low. Therefore, it is even more important than in 40% DR to postpone aging to reproduce only afterwards, when the levels of protein availability increase again in the ecological niche. This is why at 80% protein (methionine) restriction both a low mitROSp and a low DBI response are recruited. In relation to this, a recent comparison among DR diets containing fish oil, soybean oil, or lard as lipid source, showed that the lard containing diet (with

less unsaturated fatty acids) was the one maximizing the beneficial effects of DR on mitROSp, proton leak, ETC, lipid peroxidation, mitochondrial structure, or mitochondrial apoptotic signaling, likely due, in part, to increases in monounsaturated fatty acid content in the tissues [54]. This improvement in mitochondrial functionality of diets promoting some decrease in tissue DBI is consistent again with a healthy and pro-longevity effect of membranes containing a low degree of fatty acid unsaturation.

On the other hand, since mitROSp is lower in DR than in the ad libitum-fed control animals, oxidative damage should also be lower in the nearby situated mtDNA of the restricted animals. In agreement with this, it was found that the level of 8-oxo-dG in mtDNA was significantly lower in the liver, heart, and brain of the long-term DR rats in which mitochondrial ROS production was also diminished (reviewed in [55]). Such a decrease in 8-oxo-dG occurred only in mtDNA, or in both mtDNA and nDNA, depending on the organ studied (Table 1).

6. PROTEIN AND METHIONINE RESTRICTION

6.1. Effect on Longevity

It has been thought for a long time that calorie intake per se would be exclusively responsible for the increase in longevity induced by DR in rodents. However, now many studies question this classical consensus. The results of many investigations indicate that part of the life-extending effects of DR are due to the decreased intake of specific components of the diet, such as proteins, particularly the amino acid methionine [56–60]. The few available studies do not support the possibility that either life-long isocaloric carbohydrate or lipid restriction increases rodent life span. Two investigations of carbohydrate restriction or supplementation reported opposite and minor changes in rat longevity [61, 62], and it was found that the longevity of Fisher 344 rats does not change after life-long lipid restriction [63]. In contrast, the large majority of the investigations on the effects of isocaloric protein restriction (PR) in rats and mice found increases in longevity (Table 1). Ten out of eleven PR investigations in rats or mice (16 out of 18 different life-long survival experiments) reported increases in longevity [58], although the

mean magnitude of this increase (around 19%) was lower than that usually found in 40% DR (up to 40% increase). Thus, PR would be responsible for around half of the life-extension effect of DR.

Which is the amino acid responsible for the increase in longevity induced by PR? It is known that isocaloric 80% methionine restriction (MetR) increases longevity in F344 rats [56] and mice [57–59] to a similar extent than PR. The mean increase in (maximum) longevity in the three available MetR life-long experiments taken together was around 18% increase. This occurred even when MetR was started as late as at 12 months of age in C6BF1 mice [59]. Studies performed in *D. melanogaster* have also shown that casein restriction [64] and methionine restriction [65] extend longevity independently of the caloric intake. Moreover, other recent studies link essential amino acids, and again especially methionine, with the positive effect of DR on longevity in yeast [66] and *D. melanogaster* [67]. Interestingly, PR results in profound changes in methionine and serine metabolism [68].

MetR (80%), in addition to extending longevity, also decreases disease-associated markers and the incidence of age-related degenerative diseases [69, 70]. The beneficial effects of this intervention in rodents include decreases in serum glucose, insulin, IGF-1, cholesterol, triglycerides, and leptin. Moreover, MetR protects against age-related changes in immunity, slows cataract development [57], improves colon tight junction barrier function [71] and improves metabolic flexibility, and increasing respiratory uncoupling [72]. MetR may be also used in the future to inhibit tumor growth, particularly in many cancers that exhibit the known phenomenon of “methionine dependence”. These include bladder, breast, colon, glioma, kidney, melanoma, prostate, and other cancers in which tumor cells have a much greater reliance on methionine than normal cells [73–75]. MetR (80%) also decreases total adipose tissue mass and lowers visceral fat in association with an improvement in insulin sensitivity [76]. In addition, MetR decreases leptin and increases adiponectin in rodents in agreement with the decrease in visceral adiposity and the size of white adipose tissue depots. These beneficial effects seem to be mediated by tissue-specific responses that favor increased mitochondrial function and biogenesis, fatty acid oxidation, and total energy expenditure possibly mediated by β -adrenergic receptor signaling and chang-

es in lipid homeostasis [77]. Metabolomic and genomic MetR studies found changes in the expression of a large number of genes and proteins that led the authors to conclude that MetR increases lipid metabolism in adipose tissue and muscle whereas it decreases lipid synthesis in the liver [78]. These changes in lipid metabolism seem to be involved in the strong decrease in adiposity and increased insulin sensitivity observed in isocaloric restriction of dietary methionine. MetR also alters the levels of sulfur-containing amino acids. Serum concentrations of methionine, cysteine, cystathionine, and taurine decrease in MetR rats, whereas levels of homocysteine [79] and glutathione [56] increase. Interestingly, adding cysteine to the MetR diet reverses most of the studied beneficial changes on adiposity and insulin resistance [79] and increases the transcription of various genes associated with inflammation and carcinogenesis [78]. Therefore, the beneficial changes of the MetR diet have been attributed to the decrease of cysteine in the serum [80] or liver [78].

While many beneficial effects of MetR have been described, excessive intake of dietary methionine is toxic. This toxicity far exceeds that produced by any other amino acid [81], leading to damage in some vital organs and increases in tissue oxidative stress [81, 82] with similar negative effects to those observed in rats fed diets with a high protein content. Chronic and excessive methionine supplementation increases plasma hydroperoxides and LDL-cholesterol [83], induces vascular [84] and kidney damage with tubular hypertrophy [85], raises iron accumulation and lipid peroxidation, and leads to liver dysfunction [86], besides other alterations in other organs. In addition, methionine supplementation increases methionine and its two more closely derived methionine cycle metabolites, S-adenosylmethionine and S-adenosylhomocysteine in the rat liver and kidney [87]. Some of the harmful effects of methionine supplementation have been attributed to methionine-related metabolites like S-adenosylmethionine, S-adenosylhomocysteine, or homocysteine, rather than to methionine itself, although in other investigations a direct methionine toxic effect has been suggested [80, 84]. This fits with the observation that direct addition of methionine to isolated mitochondria in vitro increases their rate of mitROSp [87].

Oxidative modification of methionine residues in proteins generates methionine sulfoxide, depriving

them of their function as methyl donors, and may lead to loss of their biological activity [88]. This can be repaired by methionine sulfoxide reductase in a thioredoxin-dependent reaction. In this context, it is interesting that overexpression of methionine sulfoxide reductase increases lifespan in *D. melanogaster* [89] and the opposite manipulation, knocking out the same enzyme, increases protein carbonyls and decreases longevity [90]. There is evidence that this enzyme plays an important role in protection against oxidative, cold, and heat stress, and seems to be involved in the regulation of aging in *D. melanogaster* [91]. Also in agreement with a methionine role in aging, it has been reported that long-lived Ames dwarf mice have an altered methionine metabolism showing a marked increase in the transsulfuration pathway compared to their wild-type siblings [92]. In summary, part of the longevity extension effect of DR seems to be due to restriction of a single dietary substance: methionine.

6.2. Role of mtROS Generation and Oxidative Damage

Dietary restriction lowers mitochondrial oxidative stress. But, what is the dietary component responsible for the decreases in mtROSp and oxidative damage to mtDNA during DR? In agreement with their lack of effect on longevity [61–63], neither isocaloric 40% lipid restriction [93] nor isocaloric 40% carbohydrate restriction [94] modifies mitROSp or 8-oxo-dG in mtDNA (Table 1). However, isocaloric 40% PR decreases mitROSp and oxidative damage to mtDNA in the rat liver [95] in a similar way, quantitatively and qualitatively to 40% DR (Table 1). The effect of PR was investigated in the rat liver without changing the amount eaten per day of the other dietary components. It was found, like in 40% DR, that 40% PR decreases liver mitROSp and FRL specifically at complex I, lowers 8-oxo-dG in mtDNA [95] (Table 1), and decreases five specific markers of protein oxidative, glycoxidative, and lipoxidative modification as well as the amount of complex I protein in rat liver mitochondria and tissue [96]. Strikingly, the direction of change, the magnitude, mechanisms, and site of action exerted by PR on mitROSp and 8-oxo-dG in mtDNA are almost identical to those found in 40% DR [58]. Taken together, those studies suggest that proteins are the dietary components responsible for most or all of the de-

crease in mitROSp and oxidative damage to mtDNA, and for part of the increase in longevity that takes place in DR.

Since it was already known that MetR, independently of energy restriction, increases rat (maximum) longevity [56], while such effect had not been described for any of the other dietary amino acids, it was logical to suspect that dietary methionine could be involved in those PR and DR effects. This is why the effects of MetR on mitROSp and oxidative stress were studied (Table 1). The results showed that isocaloric MetR (40% and 80%), applied to young rats during 7 weeks, lowers mitROSp (mainly at complex I), the FRL, the complex I content, 8-oxo-dG in mtDNA (Table 1), and specific markers of protein oxidative, glycoxidative, and lipoxidative modification in the rat heart (at 40% and 80%MetR) [97, 98] or liver (at 40% MetR) [99, 100] mitochondria, similarly to what occurs after 7 weeks of 40% MetR in the rat kidney and brain mitochondria [101, 102]. In order to obtain these decreases, it was enough to restrict methionine by 40% (Table 1). These decreases in mitochondrial ROSp (at complex I) and oxidative stress have recently been reproduced in liver mitochondria of pigs subjected to MetR [103].

Consistently with an important role of methionine in the DR beneficial effects, when all the dietary amino acids, except methionine, were restricted (also by 40%) during 7 weeks, neither the rate of mitROSp nor the level of 8-oxo-dG in mtDNA changed [104]. In addition, it was found that 40%MetR also decreases mitROSp, FRL, and 8-oxo-dG in mtDNA and reverses aging-related increases in protein modification when implemented during only 7 weeks in 24 months old rats [99]. All these results, taken together, indicate that the lowered ingestion of methionine during MetR (and PR and DR) is responsible for all or most of the decreases in mitROSp and oxidative stress observed during these three longevity extending manipulations. Such lowered methionine ingestion is most likely also responsible for all (during PR and MetR) or part (during DR) of the life-extension effects observed during these dietary manipulations.

The decrease in mtROSp during MetR can occur through different mechanisms. A simple one would be based on a decrease in the content of the complex I protein in MetR that would directly lead to a decreased rate of mitROSp. This has been reported un-

der 40% MetR in the majority of tissues studied (Table 1), and also during DR and PR, as well as in long-lived birds (pigeons, canaries, and parakeets) compared to the much short-lived mammals (rats and mice) of similar body size and weight-specific metabolic rate [105, 106]. But, in principle, this would not be the full explanation because MetR also decreases FRL indicating that it also induces qualitative mitochondrial changes. Such reduction could be due to a decrease in the midpoint potential—and thus the degree of electronic reduction—of the complex I ROS generator, because the decrease in mitROSp during MetR is observed, like in DR, with partial complex I electronic reduction (with complex I-linked substrates alone) but not with full electronic reduction (complex I-linked substrates plus rotenone). The result is that MetR mitochondria (from both young and old animals) are more efficient in avoiding mtROS generation than those of ad libitum fed rats. MetR mitochondria leak less radicals per unit of electron flow in the respiratory chain, similarly to what has been found in especially long-lived compared to short-lived animal species (birds), as well as in DR and PR rats compared to ad libitum fed ones [55]. These quantitative and qualitative changes could be due to: (i) direct interaction of methionine, or more likely, of a more chemically reactive methionine metabolite, with the matrix domain complex I polypeptide(s) involved in ROS generation; (ii) changes in cellular signaling molecules and the ensuing modification of specific gene expression of mitochondrial proteins; and (iii) decreases in the concentration of mitochondrial complex I substrates like pyruvate which would decrease matrix NADH and are known to occur at least during DR (see Section 5).

In relation to the possible direct interaction of methionine or its metabolites with mitochondria without the information passing through the nucleus (mechanism “i”), it is known that direct in vitro addition of methionine to isolated functional rat mitochondria increases their rates of mitROSp [87]. Therefore, a rather direct and rapid effect of methionine on complex I seems to occur. A methionine metabolite could be responsible for this effect because in the case of methionine, differing from homocysteine or cysteine, the potentially reactive sulfur is located inside the molecule and is therefore not available for direct covalent chemical reaction with protein thiols. Interestingly, the reaction of methionine with hydroxyl radicals generates methionine radicals (carbon-, ni-

trogen- and sulfur-centered radicals) as intermediates in the formation of the methanethiol product, as detected by EPR spin trap techniques and GC-FID and GC-MS techniques [107]. These radicals or methanethiol (CH_3SH) itself could react with complex I or some of its subunits leading to increases in mtROS generation. Since it is known that GSSG thiolization of isolated complex I increases its rate of ROS production [108] a similar reaction of methanethiol, or cysteine (which also has a free thiol group available for direct reaction) with complex I thiol groups could be involved in the decrease in mitROSp in MetR. MetR decreases hepatic methionine and cysteine [78] and likely methanethiol levels, which can decrease thiolization of complex I subunits and consequently their rates of mitROSp. Alternatively, cysteine could also interact with the protein cysteines of some of the FeS clusters of the hydrophilic matrix domain of complex I. Interestingly, those FeS clusters have been pointed out as the ROS generator relevant for aging [15, 18, 47]. Their reaction with cysteine would lead to iron release or availability for reaction and then to ROS generation. Therefore, through this kind of mechanism, the lowered cysteine levels in MetR could also decrease mitROSp.

The MetR effects can also involve changes in gene expression (mechanism “ii”). MetR studies found changes in the expression of a large number of genes and proteins involved in lipid metabolism [78]. In addition, modifications of DNA methylation could be also involved [109, 110]. Methionine is an essential amino acid with many key roles in mammalian metabolism including protein synthesis and function, as well as protein and DNA methylation [111]. Since aging seems to be associated with site-specific changes in DNA methylation [112–116], MetR diets could also extend longevity in rodents through modulation of DNA methylation patterns, specific changes in gene expression, and changes in translation rates, whose final effects could include decreases in mtROS generation and oxidative damage and increases in autophagy (see Section 9). In agreement with this notion, we have recently detected that MetR induces a small but statistically significant decrease in global genomic DNA methylation in the heart of young immature rats [98], which did not reach statistical significance in the case of old rat liver [99].

In relation to mechanism “iii”, decreased NADH, which is more likely in DR than in MetR, due to the decreased ingestion of a large number of metabolites

in DR than in (isocaloric) MetR. Substrates like pyruvate, malate, and succinate, as well as NADH and the NADH/NAD⁺ ratio are indeed decreased in the tissues of rodents subjected to DR [45, 46]. This will decrease matrix NADH and NADH/NAD⁺ ratio, and therefore the degree of electronic reduction of the complex I ROS generator and its rate of mitROSp (see Section 5). Treatment with a precursor of the oxidized form of the coenzyme, NAD⁺, also rejuvenates the skeletal muscle, improves mitochondrial and stem cell function in aged mice, and increases their lifespan [117]. This could be due to a sirtuin-dependent effect or to a decrease in complex I electronic reduction and thus in mitROS generation.

To summarize, DR, PR, and MetR are nutritional interventions that increase longevity in rodents, although the magnitude of the longevity extension of MetR and PR is around 50% that of DR. This lower, but significant, life extension effect in MetR than in DR would agree with the notion that various different environmental signals target the cell to modify its aging rate. Restriction of methionine intake can be responsible for part of the aging-delaying effects of DR by decreasing mitROSp at complex I and oxidative damage to mtDNA and macromolecules acting, at least in this sense, as a “DR-mimic”. The information available strongly indicates that methionine is the single dietary substance responsible for the beneficial changes of DR on mitochondrial oxidative stress. The remaining effects of DR on aging rate could be due to decreases in other dietary components, or in the calories themselves, acting through different additional signaling mechanisms (**Figure 1**) that could recruit different gene clusters of the aging program in the cell nucleus, changing their gene expression levels with different intensities (see Section 9). Among these additional longevity-extending mechanisms during DR, increased autophagy is emerging as the most important one. In any case, it is interesting that not only 80% MetR, but also 40% MetR and 40% PR decrease mitochondrial oxidative stress, because PR does not involve the stronger behavioral and nutritional stress of caloric restriction and, therefore, seems to be a much more feasible option for wide application to human populations. Negative effects such as delays in puberty and decreases in growth rate and final body size are shared by (40%) DR and 80% MetR, but do not occur at 40% MetR. 40% MetR (implemented through PR) could be the best kind of dietary restriction for humans be-

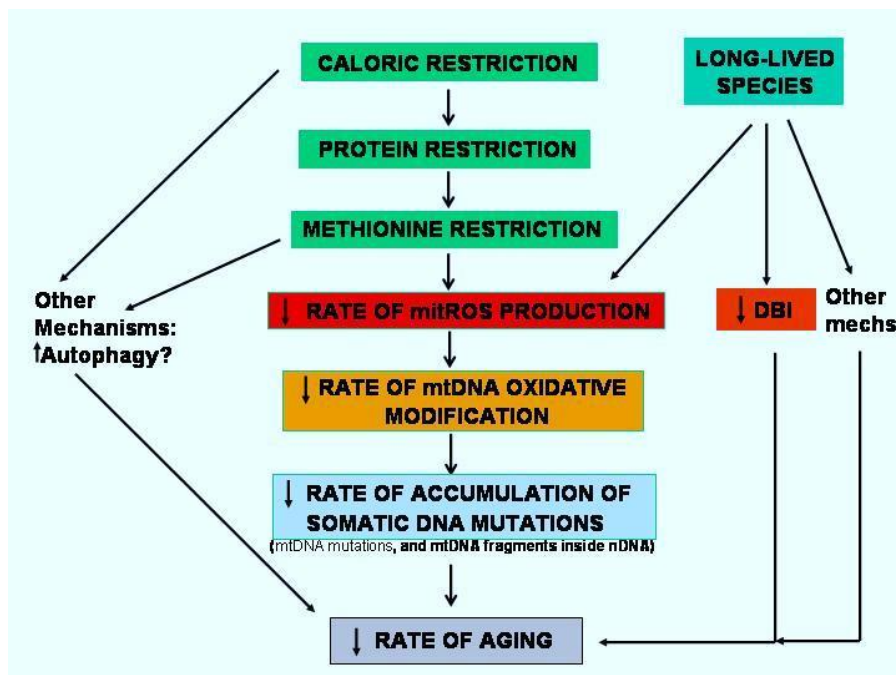


FIGURE 1. Low mitROS_p in both long-lived species and in calorie dietary restriction (DR) as a contributor to a low aging rate. Low mitROS_p decreases oxidative damage to mtDNA. Oxidation of DNA bases like 8-oxo-dG are repaired also in mitochondria, and 8-oxo-dG is useful as a steady-state marker of rate of mtDNA oxidative modification (which is also lower in long-lived animals) [20]. The final forms of damage responsible for aging (here represented as “somatic DNA mutations”) should be due to mitROS_p also causing DNA strand breaks leading to large mtDNA deletions plus the remaining mtDNA fragments which insert into nuclear DNA and accumulate during aging [139; 147–149]. The low mitROS_p of long-lived animals during DR is due to restriction of a single amino acid, methionine, and not to the other amino acids or carbohydrates or fats present in the diet [29, 93–95, 97, 104]. Low fatty acid unsaturation (low double bond index [DBI]) also occurs in long-lived animals but not in 40% DR or 40% MetR (methionine restriction). A decrease in DBI has been observed only at 80% MetR (which also increases rodent longevity). Since the effect of protein restriction or MetR on longevity (~20%) is around half that of DR (up to 40%), other mechanisms, in addition to a low mitROS_p, must be involved in the decrease in aging rate induced by DR. A strong candidate is an increased autophagy (see also Section 9 and Figure 3).

cause it lowers mitROS_p and 8-oxo-dG in mtDNA to a similar extent compared to 80% MetR, without decreasing at all body and organ weight, growth rate, maturation, and likely final body size, at variance with what occurs in 80% MetR and 40% DR.

Human beings can obtain health benefits by consuming “prudent” diets based on the intake of vegetables containing proteins rich in essential amino acids but low in the sulfur-containing amino acids methionine and cysteine (like pulses), or almost totally lacking methionine and cysteine (like many

fruits and other vegetables), and avoiding the presently excessive intake of animal proteins and fats typical of western diets. The results already available after many years of PR intervention in humans seem to be positive for human health and of similar character to those found in MetR and DR rats [118]. These studies also suggest that DR and PR can protect from obesity, mortality, and degenerative diseases including at least cardiovascular disorders, diabetes, and cancer, and can potentially increase human longevity.

7. RAPAMYCIN, MITOCHONDRIAL OXIDATIVE STRESS, AND LONGEVITY

DR increases longevity in many different animals including mammals, and seemingly also in monkeys [41]. Nevertheless, DR is difficult to apply to large human populations due to subnutrition risks especially in children, very old people, individuals with limited education, and those with chronic pathologies. Because of this, it would be desirable to develop drugs that can increase longevity without the need to restrict the human diet [119, 120]. The United States National Institute of Aging Interventions Testing Program (ITP) evaluates the effects of different molecules to be candidate antiaging drugs in mice (<http://www.nia.nih.gov/research/dab/interventions-testing-program-ityp/compounds-testing>). These include known drugs, antidiabetics, antibiotics, and others, like aspirin, resveratrol, simvastatin, or metformin. While the rest of the studied compounds have not shown reproducible positive effects on longevity [119], rapamycin significantly increased both mean and maximum lifespan (mean of the NIA ITP performed at three sites) by 9% in males and by 14% in females in heterogeneous strains of mice [121]. Posterior studies have confirmed these results and have shown that dietary rapamycin extends lifespan when initiated in young [119] or middle aged mice [122], or in mixed age mice [123]. Therefore, rapamycin is widely recognized as the first drug known to consistently increase longevity in mammals. Rapamycin also increases longevity in yeast [124], *C. elegans* [125] and *D. melanogaster* [126] through inhibition of the TOR protein complex, equivalent to mTOR (mammalian target of rapamycin). This indicates that this longevity pathway is highly conserved in evolution. The mTOR complex also shows signs of rapid evolution in amniotes and signs of positive selection [127].

While rapamycin increases mammalian longevity, the final effector mechanisms involved remains elusive, although decreases in mitochondrial damage, increases in autophagy, or modifications in cell growth/proliferation could be implicated. Rapamycin also inhibits the mTOR signaling pathway [128]. Interestingly, the longevity extending nutritional intervention DR also decreases mTOR function [129]. This indicates the existence of overlapping mechanisms of action for rapamycin and DR. Rapamycin treatment decreases body weight and food intake in

mice [130], but the increase in longevity induced by the drug is independent of the decrease in food intake per se. However, since the longevity extension effect of DR (up to 40%) is much larger than that of rapamycin (around 11%), rapamycin controls only part of the final aging effector mechanisms increasing longevity during DR, affects them with smaller intensity than DR, or does not affect similarly all mammalian organs. Most likely, DR targets also other cytosolic pre-nuclear longevity signaling molecules (see Section 9) different from mTOR that are not targeted by rapamycin. Alternatively, there is evidence that DR directly modifies some of the most likely effectors of aging like mitROSp, bypassing the nuclear aging program (see Section 9), e.g., by decreasing mitochondrial matrix NADH (see Sections 5 and 6).

Rapamycin also attenuates age-associated declines in various measures of cardiac, immune, muscular, and cognitive function [122, 131]. The increase in lifespan induced by rapamycin was initially unexpected, because rapamycin was used, together with other drugs, in post-transplant therapy [132]. However, increases (instead of decreases) in various immune functions have been observed at low rapamycin doses [129, 133]. It is now known that mTOR inhibition in mammals has positive functional effects in most physiological systems [129, 131] and protects from degenerative diseases, solid tumors, cardiovascular disorders, metabolic diseases and obesity in humans. Rapamycin also prevented degenerative brain changes in an Alzheimer disease mouse model and improved anxiety and depression in normal mice [134]. Although some detrimental effects of the drug have been described, the positive effects are much more diverse and extensive. In any case, the global effect of rapamycin is positive because this drug increases longevity.

DR lowers mTOR function [59], mitROSp, FRL, and oxidative stress [29], and increases longevity. And intermittent rapamycin administration, like intermittent DR, also extends lifespan in mice [135]. Decreased mTOR downstream activity could constitute one of the various signaling mechanisms through which DR decreases aging rate [129, 136, 137]. In agreement with this, knocking out the gene coding the ribosome protein kinase S6K (a main target of mTOR signaling) slowed aging of bone, immune, and motor functions, and led to a larger than normal longevity [138]. In many cases, the nuclear gene re-

sponse to these signals (see Section 9) slows the aging rate. Among them, the decrease in mitROSp produced by both DR and rapamycin can be important. In agreement with this concept, rapamycin dietary treatment, at the same dose that increased longevity [121], decreased hepatic mitROSp and FRL at complex I both in mice subjected to a diet that induced fatty liver [53] and in middle aged mice receiving a standard diet [139] (Table 1). In addition, this drug completely reversed age-related increases in mitROSp, and the age-related increase in the insertion of mtDNA fragments inside nDNA (Fig. 5B), in the liver of middle aged mice [139]. In the same investigation, it also completely reversed age-related decreases in the autophagy marker ATG13, and partially reversed the accumulation of the best tissue marker of aging, lipofuscin, in the liver. Rapamycin also increases mouse longevity when the treatment is started at middle age, with an effect similar to that observed starting at 9 months [119, 121], agreeing again with the decreases observed in mitROSp also in middle aged mice [139].

To summarize, all the (four) experimental manipulations which consistently and reproducibly increase both mean and maximum longevity in mammals, DR, PR, MetR and rapamycin, decrease mitROSp and FRL at complex I and mtDNA damage (Table 1). This strongly supports the antioxidant-independent version of MFRTA exposed in this review that focuses on three aging effectors: (1) the mitochondrial rate of ROS production; (2) the degree of fatty acid unsaturation (DBI); and (3) autophagy.

8. MTDNA FRAGMENTS INSIDE NUCLEAR DNA AND AGING

In addition to oxidative damage to mtDNA, it is possible that mitochondrial ROS-derived damage affects aging genes back in the nucleus through the insertion of mtDNA fragments inside nDNA (Figure 2). Oxidative damage to mtDNA bases, like 8-oxo-dG, is also repaired in the mitochondria. But mitROS, in addition to causing DNA base and sugar oxidative modifications, have the capacity to produce double strand breaks in DNA in general, and particularly in the very nearby situated mtDNA. Fragmentation of mtDNA through double strand breaks by the nearby generated mitROS can be one cause of the well-known accumulation of mtDNA mutations, including

large mtDNA deletions, with age [140]. It has been proposed that mtDNA mutations can also be due to errors during DNA replication and repair, rather than to mitROSp. However, while these random errors can contribute to accumulated damage during the lifespan of a single individual, they cannot be responsible for the strongly different longevity of the different species or the change in longevity induced by the different kinds of DRs, since these longevities are genetically, instead of randomly, controlled. In other words, there are no plausible mechanisms that would lead rats to commit 30-fold more errors than humans during mtDNA replication or repair. Replication and repair as a source of mtDNA mutations suffers the same limitation to many other proposals based on random processes. Instead, the longevity of a species and fine tuning of longevity to a new level in DR are genetically determined. Then, they must be due to the existence of genetically programmed processes residing in the cell nucleus which can respond to environmental nutrient availability (DRs) with appropriate increases in lifespan.

It has been proposed that large mtDNA deletions, which increase with age in mammalian tissues, are among the main final detrimental effects causing aging. Since mtDNA is highly compacted, without introns, the large deletions detected in old tissues would lead to the lack of various genes coding for electron transport chain or mitochondrial ribosome subunits in a single mtDNA circle molecule. However it is now clear that, with the exception of a few tissues, the level of these deletions does not reach the threshold needed, in homoplasmy, to be of negative functional consequences in most tissues of old animals. The high level of heteroplasmy of mtDNA, due to the presence of thousands of mitochondria per cell, and various mtDNA copies per mitochondrion, strongly protects against direct functional effects of mtDNA mutations. Many copies of mtDNA are present in each cell. Only if a large majority of these mtDNA copies are mutated, mitochondrial ATP production would be compromised. Cells essentially homoplasmic for deleted mtDNA are abundant (up to 60% of cells) in a few areas like substantia nigra in humans [141, 142], but in the brain in general and in other vital tissues of old individuals, their percentage is too low (less than 2%) to cause decreases in tissue functionality.

However, double strand breaks in mtDNA not only generate deletions, but also produce mtDNA frag-

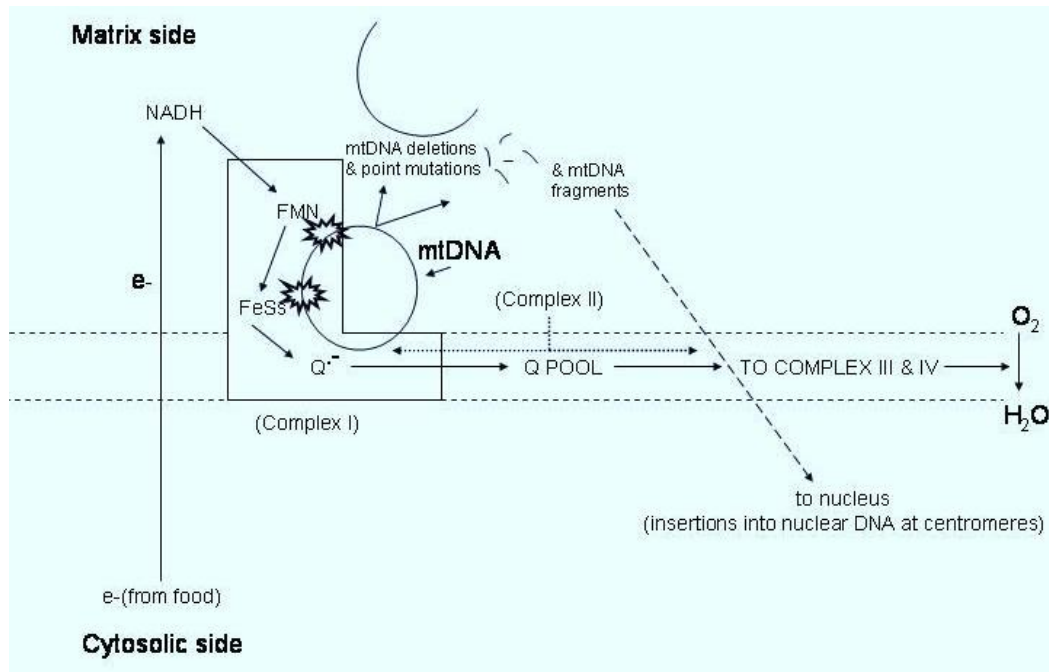


FIGURE 2. mtROSp and mtDNA fragments inside nuclear DNA. mtROS produced at complex I generators (stars) cause damage in the mtDNA situated nearby or even in contact with the complex I site of ROS production. This causes, in addition to oxidized bases, double strand breaks leading to large deletions and, most importantly, also mtDNA fragments. These fragments exit the mitochondria and insert into nuclear DNA at the centromeres during aging in yeast, rats, and mice, and contribute to aging. This is reminiscent of what happened during evolution after the symbiogenesis of the eukaryotic cell from α -proteobacteria and Archaea around 2 billion years ago. Rapamycin treatment decreases such mtDNA fragment accumulation in the liver of middle aged mice [139, 143–149].

ments, the segments of deleted mtDNA. These fragments can escape from mitochondria [143–146] and are present inside the nucleus (Figure 2). It has been hypothesized that this could randomly change nuclear gene information and thereby contribute to causing cancer and aging [143]. It has now been demonstrated that these mtDNA fragments accumulate inside nDNA with age both in yeast [147] and in rat liver and brain [148], and that such accumulation causes damage and contributes to yeast aging [149]. Mouse liver also accumulates mtDNA fragments inserted inside nDNA with age [139]. And the dietary treatment with rapamycin, in addition to decreasing mitROSp, totally reversed mtDNA fragment accumulation in nDNA [139] and decreased lipofuscin (non-autophagocytosed materials) in middle-aged mice. This last change can be due to a decrease in the

presence of damaged mitochondria, and/or to the increase in autophagy induced by the drug [139]. The changes observed for mitROSp and for mtDNA fragments inserted in nDNA are strikingly similar since full (100%) reversion of age related increases occurred in both cases. This could be due to a cause-effect relationship between these two parameters, since ROS have strong capacity to produce double strand breaks and then DNA fragmentation. The increase in autophagy and the decrease in lipofuscin could also represent a cause-effect relationship although in the case of lipofuscin, reversion to young levels was only partial. Part of the increase in longevity of rapamycin-treated mice can be due to: (1) a decrease in mitROSp; (2) a decrease in the insertion of mtDNA fragments inside nDNA; and (3) an increase in autophagy. Interestingly, in a parallel ex-

periment, we did not detect significant changes in mitROSp in the heart after the rapamycin treatment. Therefore, the reason why rapamycin increases life span to a much lower extent (11% increase in maximum lifespan) than DR (up to 40% increase) can be due to the impact of DR on more longevity signaling pathways than those involving mTOR (Figure 3). But it can also be due to the possibility that rapamycin decreases mitROSp, or increases autophagy, in some but not in all organs.

Rapamycin treatment in mouse liver decreased the amount of complex I, which contains the ROS generator relevant for aging. This was associated with an increase (instead of decrease) in the mitochondrial biogenesis marker PGC1 α [139]. A possible explanation of this apparently paradoxical result is that rapamycin could selectively induce mitochondrial biogenesis from the more youthful pool of liver mitochondria. These are expected to show lowered FRL than the more damaged ones. This selection could be another reason why rapamycin decreased mitROSp. It has been observed that rapamycin increases autophagy and mitochondrial biogenesis in mouse heart suggesting that damaged mitochondria are replaced by newly synthesized ones to rejuvenate mitochondrial homeostasis. Likely related to this possibility, it has been observed that DR (which also inhibits mTOR) and rapamycin both lower mitROSp and FRL and decrease the amount of the “matrix domain-only” of complex I in the mitochondria of mouse liver [53]. This matrix domain contains the mitROS generator responsible for the decrease in mitROSp during rapamycin treatment and [139] during the DRs [15–18, 47, 53].

The mtDNA fragments exit from the mitochondria towards the nucleus during the lifetime of the individual and they insert into nDNA. They are visualized to be heavily concentrated at the centromeres [139]. Thus, the centromeres seem to be the “entry doors” for the access of mtDNA fragments into chromosomes, perhaps to be distributed afterwards to other specific chromosome locations at regulatory regions of the master genes controlling the “gene clusters of aging” (constituting the pro-aging program) likely lying in the cell nucleus (see Section 9). The final result would be to promote aging and degenerative diseases including cancer. If that phenomenon finally were a regulated one, the initial proposal [143] would only be wrong concerning the suggested randomness of the process. Random insertion of

mtDNA fragments inside nDNA, even if it were restricted to the structural genes, a very small percentage of total nDNA, could cause cancer (e.g., inserting in, and randomly inactivating tumor suppressor genes) but not aging, because longevity is a tightly regulated species-specific property. Further studies are needed to ascertain the random or regulated character of mtDNA fragment insertion into nDNA starting at the centromeres.

The transfer of mtDNA fragments from mitochondria to the nucleus is strongly reminiscent of what happened during millions of years of evolution after the symbiogenesis event that created the eukaryotic cell around 2 billion years ago. During such evolution, most genes of the initially free living Rickettsia-like α -proteobacteria were transferred to what is now the nuclear genome of the eukaryotic cell. This would constitute a further example of the old observation that in various cases “ontogeny recapitulates phylogeny”, and in this case, it applies to programmed aging as a continuation of development.

The lack of increase to phenotypic threshold (minimum of around 60% deleted) of mtDNA deletions due to the very high copy number of mtDNA in heteroplasmy per cell was a strong problem for the validity of MFRTA, similarly to the main origin of mtDNA point mutations from replication/repair instead of from mitROSp as deduced from the relative frequency of base transversions versus transitions. However, now there is evidence that mtDNA fragments accumulate inside nDNA with age in yeast and mammals, and that this promotes aging [147]. Furthermore, the longevity-increasing drug rapamycin reverses such age-related accumulation in mouse liver strongly paralleling what happens for complex I mitROSp [139]. All this means that MFRTA cannot be considered “dead”. On the contrary, the mtDNA fragment accumulation in the nucleus as an end point mechanism for aging maintains MFRTA alive. Perhaps the failure to detect mtDNA mutations relevant for aging has been due to looking at the wrong place. The ROS related to aging should be looked at in mitochondria. However, what could be more important for aging would not be the (remaining) deleted mtDNA, but what is lacking at mtDNA after the deletion: the mtDNA fragments. The consequences likely relevant for aging, the mtDNA fragments, have been found inserted into nDNA inside the nucleus. Therefore, we should look both at the mitochondria and to the nucleus to understand aging.

9. THE CELLULAR AGING REGULATION SYSTEM

9.1. Signaling, the Nuclear Aging Program, and Aging Effectors

The integrated cellular aging regulation system (CARS) is composed of three main parts (**Figure 3**): (1) cytoplasmic pre-nuclear signaling; (2) the nuclear genetic pro-aging program (PAP); and (3) post-nuclear effectors (executors) of aging.

The CARS occurs in aerobic tissues and in those containing post-mitotic or mitotic cells. Mitotic cells could harbor other additional hypothetical aging effectors specific for such kinds of tissues, like telomere shortening or perhaps apoptosis, but these are not expected to be relevant in cells that do not divide. If the cell does not divide, the telomeres do not shorten. And the most important tissues for mammalian aging are these mainly composed of post-mitotic cells.

Aging research has clarified a series of important facts concerning the CARS:

- (1) Studies in mice have shown that inactivation of many single genes leads to important increases in longevity (up to 40%). Importantly, these genes are highly conserved in evolution which agrees with the concept that aging is adaptive. Many of these genes are homologous in organisms that are so different, including yeast, nematodes (e.g., *C. elegans*), insects (e.g., *D. Melanogaster*), and many vertebrates (e.g., mammals and humans). Therefore, aging is a very old adaptation of most multicellular animals and even of some unicellular eukaryotic life.
- (2) Although both DR and MetR can control aging in part by directly modifying the aging effectors, they also bring about changes in expression of a large number of nuclear genes [78, 150, 151]. The modified expression of those genes, in turn, modifies the activity of the three known aging effectors.
- (3) The different kinds of DRs increase mouse longevity by modifying the expression levels of hundreds of genes, as shown by many microarray-based studies. These changes are species-

and tissue-specific. This, together with the increase in longevity after inactivation of single genes codifying for pre-nuclear hormones, their receptors, cytoplasmic signaling proteins, or transcription factors (part A of the CARS in **Figure 3**), constitutes robust evidence that a PAP exists in the nucleus.

- (4) The DRs signal the abundance of food or proteins available for feeding in the external world to the cells inside the body using humoral factors like insulin, GH/IGF-1, or blood amino acids like methionine. These in turn modify the activity of many cytoplasmic signaling proteins like mTOR, S6K, AMPK, AKT, PI3K, FGF21, ULK1, NF- κ B, HIF, and many others. Many of these signals enter the nucleus, where, through the action of transcription factors like FOXOs or TFEB [152], modify the expression of PAP genes involved in the control of longevity. These have been proposed to be organized in different but interrelated gene clusters of aging, analogous to the Hox genes involved in the control of development from embryo to adult [153].
- (5) Long-lived mutant mice can live up to 40% longer than the wild-type animals. Interestingly, this coincides with the amount of longevity extension elicited by DR (also up to 40%). Many of these known genes mainly codify for proteins involved in GH or insulin/IGF-1-like pre-nuclear signaling, cytoplasmic pre-PAP cytosolic signaling proteins, or nuclear transcription factors.
- (6) Pre-nuclear cytosolic signaling of longevity works in many cases by modifying nuclear PAP activity. The induced changes in gene expression modify the levels of specific proteins (PAP output). The final result is the variation in activity level of the systems finally controlling the rate of aging, the cellular post-nuclear aging effectors. Among them, there is evidence concerning three of them: (a) the mitochondrial rate of mitROSp at complex I; (b) the degree of fatty acid unsaturation (DBI); and (d) the autophagy system.

Increased longevity during DR is obtained through decreased mitROSp at complex I, and increased autophagy. Decreases in mitROSp and cell membrane DBI are also involved in increasing longevity across

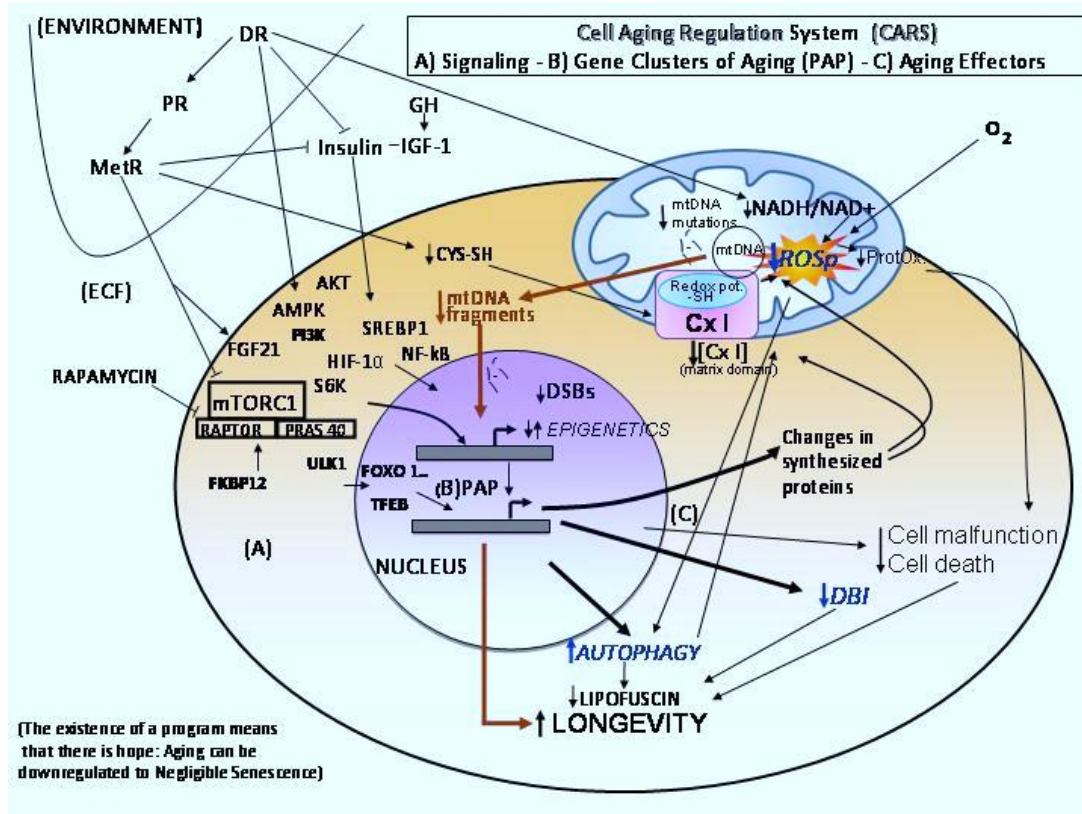


FIGURE 3. Cell aging regulation system (CARS). The working model of the CARS (including the pro-aging program-PAP lying inside the nucleus) broadly depicts known mechanisms of control of longevity at the cellular level. Different kinds of dietary restrictions (DR, PR, MetR) and rapamycin (signals coming from the environment) alter humoral, endocrine, and finally cytosolic pre-PAP signaling proteins like mTOR, AMPK, and many others (area A: left of the figure). In many cases this modifies the expression (through transcription factors like FOXOs, TEFB, and others) of nuclear genes likely organized as gene clusters of aging [153] of the PAP (area B: center of the figure). PAP output (solid arrows leaving the nucleus on the right of the figure), in turn, modifies the activity of at least three aging effectors (shown in italics; area C: right): (a) mitochondria (including mitROSp), (b) fatty acid double bond index (DBI), and (c) and likely autophagy. The integrated responses of the aging effectors to the DRs and rapamycin or to knocking out GH or IGF-1/insulin-like signaling genes, increase longevity. The same (plus additional) genes forming the PAP are constitutively active at different levels in species with different longevities. In some cases, the signals can reach the mitochondria directly bypassing the nuclear PAP, as it is the case for MetR possibly lowering cysteine thiol groups at complex I [159], mtDNA deletions, and mtDNA fragments inserted in the nucleus increase with age [139, 147-49] and can contribute to aging [139, 149]. Mitochondrial ROSp (and updated MFRTA) are not alternative to the humoral (e.g., insulin-IGF-1 like) or cytosolic proteins (e.g., mTOR or S6K) part of CARS and signaling to PAP. Instead, the three main kinds of cellular processes (A, B, and C) work together and are integrated into a single CARS to control longevity in each species and to finely adjust changes in longevity in response to DRs or pro-longevity drugs like rapamycin. During 40% DR, lowered mitROSp and increased autophagy execute the PAP response. At 80% (but not 40%) MetR, decreasing the DBI is also a recruited response. The three aging effectors can be involved in the change in longevity between species. Epigenetics can be also involved in changing the PAP gene expression level. DBI (double bond index) indicates the number of double bonds of membrane fatty acids (DBI). ECF, extracellular fluids; MFRTA, mitochondrial free radical theory of aging.

species. The first two aging effectors (mitROSp and DBI) were identified in the 1990s. Concerning these two, the key to long life is to decrease the rate of generation of (oxidative) damage at mitochondria and to have cellular membranes, including those of mitochondria, composed of fatty acid constituents more resistant (with less double bonds) to ROS-induced damage. Now autophagy is emerging as a third possible aging effector. Macro-autophagy [154] can eliminate heavily cross-linked, oxidized, and aggregated mixtures of peroxidized lipids and proteins [155] and even sometimes whole heavily damaged mitochondria [156]. Thus, animals like us seem to age slowly at least because they “purposely” (in evolutionary sense) produce less toxic substances (ROS) per unit time, their membrane fatty acids are more resistant to ROS damage, and most of the still remaining molecular damage is eliminated by the macro-autophagocytosis machinery. This shows the strong interrelationship between the three aging effectors. The materials that cannot be digested and eliminated by autophagy accumulate in the cytosol as lipofuscin granules.

Mutant mice with single autophagy gene knocked out have a decreased life span (reviewed in [154]). However, we still lack rigorous evidence that overexpression of macro-autophagy genes increases longevity. The same problem affects DBI except for successful knocking out of desaturase/elongase genes that increased longevity in *C. elegans* nematodes. It is known, however, that mitROSp at complex I, consistently decreases in all of the four known experimental manipulations that increase mammalian longevity: DR, PR, MetR, and rapamycin feeding. Some positive evidence suggesting that increasing autophagy increases longevity has been published, although it was limited to a short-lived mice strain in which overexpression of the essential autophagosomal protein ATG5 increased maximum lifespan from only 781 to 900 days [157]. In contrast, in the first three experiments taken together in which rapamycin increased longevity, the rapamycin-treated mice reached 1,212 days of age at 90% mortality [121]. And MetR and DR lead to even longer life-spans. Any theory of aging must explain why different animals age at so different rates. It is still unknown whether autophagy is higher or not in tissues of long-lived animals compared to those of short-lived ones. Consistent and reproducible evidence concerning these aspects is urgently needed before definitively

considering macro-autophagy a third aging effector. Although correlation does not necessarily imply causality, the central parameters of any valid theory of aging must correlate with the longevity of different species in the right sense. Thus, mitROSp is low in tissues of long-lived mammals and birds, which fully fits with the modern version of MFRTA. On the contrary, antioxidant enzymes also correlate with longevity but in the wrong sense: they are up to more than one order of magnitude lower (instead of higher) in long-lived than in short-lived mammals or vertebrates [7]. The same is true for the repair of DNA damage of endogenous origin (reviewed in [29]). Therefore, both defense (antioxidants) and DNA repair are discarded as causes of aging. High “maintenance” of the animal (leading to high longevity) is not due to defense plus repair contrary to what the three mainstream evolutionary theories of aging (mutation accumulation, antagonist pleiotropy, and disposable soma theory) wrongly predicted. On the contrary, high maintenance is due to a low rate of generation of endogenous damage (low mitROSp and low DBI) and, likely, also to a high rate of elimination of damaged components (high autophagy). MitROSp and autophagy are strongly complementary mechanisms, helped also by a third factor: high resistance of tissue cellular membranes to lipid peroxidation obtained through a low DBI and PI. In short, longevity is obtained through a low rate of production of “damage” (mitROS), a high rate of cleaning its consequences (high autophagy), and a high constitutive resistance to oxidative modification (e.g., low DBI). The identification of the rate of mitROSp with the concept of “rate of generation of endogenous damage” comes from the fact that, after more than one century of intensive gerontological research, ROS continue to be the only known highly damaging substances endogenously produced by the healthy animal organism throughout their lives that have the capacity to break covalent bonds. If any other different family of substances having that property is discovered in the future, it could be tested to be added or not to the list of aging effectors. Meanwhile, mitROS continue to be the only toxic and highly damaging substances that the body “purposely” produces to cause and increase its aging rate.

The pre-nuclear signals, the PAP, and the aging effectors, are integrated working together to constitute the CARS. Therefore, it is illogical to consider MFRTA incorrect while believing at the same time

that proteins like mTOR, AMPK, or S6K, et cetera, control longevity. Looking at Figure 3, it is evident that the three parts of the CARS work together in a highly integrated manner to determine species longevity. On the other hand, it is logical to suspect that the same gene clusters of aging central to the PAP and reacting to the DRs or rapamycin, plus others additional ones, are also involved in the control of longevity between different species. It is already known that different genes can change their expression in different tissues in response to the different kinds of dietary restrictions (DR, PR, MetR, or intermittent fasting [IF]) or pro-longevity drugs. These responses include both quantitative and qualitative changes concerning the genes involved in each case as well as their levels of gene expression. The same is expected between species with much bigger differences in longevity than in the DRs. Between species, the same genes involved in response to the DRs would be involved, plus additional inter-species genes. The degree of expression of the longevity genes involved is expected to vary more intensely in different species than in the DRs. Since the number of genes involved in the response to DR is already large (up to 1,000) in relation to the total number of structural genes (around 20,000 genes), it would be a prohibited luxury for evolution to use totally different genes to control aging rate in the case of the different species than in the DRs. Instead, the same genes would be used, plus additional inter-specific ones. One well known example is the case of oxidative stress. While both mitROSp and cell membrane DBI (global fatty acid unsaturation) vary between species, in the case of (40%) DR and (40%) MetR, mitROSp at complex I is decreased while the DBI does not change (see Sections 5 and 6). However, at 80% MetR, the DBI is also lowered [97]. Thus, the implication of a given effector in a response depends on whether: (1) the longevity difference occurs at the individual or at the species level; and (2) the intensity of the signal reaching the PAP. In addition, there can be also inter-individual differences involved. Transcription factors like FOXO1 reacting to DR show polymorphisms in relation to differences in longevity in human populations [158].

Although a large part of the change in aging rate is controlled by the flow of information through the PAP in the nucleus, at least in the case of mitochondria, part of the control can directly flow from the dietary substances and derived substrates to modify

mitROSp bypassing the PAP. This occurs in DR that decreases metabolites like pyruvate and others, which in turn lower matrix NADH and then mitROSp at complex I (Figure 3). Analogously, in the case of MetR, direct control of mitROSp by post-translational modification of complex I could occur. Reaction with complex I thiol cysteine groups could decrease the degree of electronic reduction of the complex I ROS generator, like the lowered NADH in DR, and then its rate of mitROSp [159]. On the other hand, components of CARS are interrelated in more complex ways than delineated in Figure 3. Thus, the mitochondrial uncoupler 2,4-dinitrophenol lowers mitROSp, which is expected since it accelerates electron flow at ETC, like during the state 4 (without ADP) to state 3 (with ADP) energy transition. However, it also lowers mTOR activity and insulin-PI3K-MAPK signaling, and increases autophagy [160]. Another example is the recent description that lowering mitROSp decreases the amount of damaged mitochondria and the cellular level of lipofuscin [161], again relating two main cellular effectors of longevity of post-mitotic cells, mitochondria and autophagy. Mitochondria have been repeatedly observed half-digested inside secondary lysosomes under the electron microscope, these vesicles contributing afterwards to forming lipofuscin granules. Strikingly, many genes controlling autophagy are also involved in the increase in longevity induced by decreasing mitochondrial oxygen consumption [154].

9.2. Intermittent Food Restriction: Is the Aging Program Functioning Gradual or not?

In principle, the PAP can respond to DR in a graded form or like an on-off switch. This last possibility is supported by the fact that DR can also be performed by intermittent fasting (IF) instead of decreasing the calorie or methionine intake. It is known that IF increases longevity even in mouse strains that gorge the days that they receive food. In this last case, even though the total number of calories eaten per day is roughly the same in the IF animals and the controls, longevity still increases in IF. This apparently paradoxical result would be explained if the PAP genes react to DR (Figure 3), at least to a certain extent, in an on-off fashion, instead of responding in strict proportion to the amount of calories eaten. If this were the case, when the animal eats a certain amount of food and a threshold of calories is reached, the PAP

would increase its activity output (increase its aging effector activity) from a low (“off”) to a higher (“on”) level, irrespective of the amount of calories eaten above the threshold. The PAP will never be at zero activity in mammals because a minimum activity (here called “off”) is needed to give a PAP output that generates the mean rate of aging of each species. This “off” aging rate would be maintained when there is no food available. Shortly after eating, food is digested and absorbed and tissue PAP activity would jump in a qualitative leap to the “on” higher output level. When the animal reaches the postprandial state and the substrates ingested have been fully metabolized, PAP activity will go back to the lower “off” resting level.

It is important to clarify whether the PAP output is graded or “all-or-none”, because this could have implications concerning which is the healthiest way of eating for human beings. If PAP works qualitatively (on/off) rather than gradually, the classical recommendation of the last decade, eating 5 times per day, would not be the best. On the contrary, putting all the food in a single early meal, or perhaps, in two big meals one at around 15:00 and the other at around 20:00, together with a very frugal breakfast, as it is typical in Mediterranean countries which are among the most long-lived worldwide, would be an adequate option. In this way, people would expend most of the day (19 hours) fasting (the PAP working in “off” position), and their PAP will be turned on for a few hours only once or twice per day. If this were correct, the habitude of eating small amounts of food, so typical of some western countries, but with high frequency at almost all time during the day should be avoided. Food that strongly signals to PAP, like simple carbohydrates (sugar) or sweetened beverages generating high insulin peaks should be specially avoided, while complex carbohydrates that are slowly absorbed would lead to much lower insulin peaks. High amounts of methionine, an amino acid that specifically signals the PAP, could be prevented avoiding excessive consumption of meat and restricting the intake of total protein to around 0.6–0.8 g of protein/kg of body weight per day.

9.3. Epigenetics, Aging, and PAP

Many recent studies point to an involvement of epigenetics in aging. Interactions within the cell nucleus functionally affecting the nuclear gene clusters of

aging PAP genes could occur. In addition to varying their expression in response to cytoplasmic, hormonal, and environmental signals, there can be nuclear feed-back among these genes due to epigenetic modifications (**Figure 3**). Epigenetic changes like DNA methylation, acetylation or phosphorylation, and histone modifications seem to be important factors in aging. Epigenetic marks establish changes in gene expression in response to environmental stimuli (like the different DRs) and drugs (like rapamycin) and can be even part of the PAP itself [162]. Some authors even support the existence of an “epigenetic clock” [163, 164]. This would be essentially different from the “epigenetic drift” during aging which would correspond to stochastic changes that will be of small or no interest for the control of species longevity [164]. There is a decrease in global DNA methylation during aging, whereas there is an increase with aging in local methylation at CpG islands and specific gene regions [164–166].

Human body epigenome analysis has found widespread tissue-specific differential CG methylation, allele-specific methylation and transcription, and the unexpected presence of non-CG methylation in almost all human tissues which correlates with tissue-specific functions [167]. Changes in specific histones have been also described during aging, including global increases in H4K20 trimethylation and H3S10 phosphorylation, and decreases in H3K9 and H3K27 trimethylation and H3K9 acetylation [168], which would contribute to modulating aging rate. Such DNA modifications modulate gene expression through regulation of chromatin structure, which is known to change during aging. Many age-dependent histone methylations are reversed by both DR and rapamycin treatments in mouse brain [169]. Histone deacetylases like SIRT1 and the mitochondria-specific SIRT3 are involved in the decrease in oxidative damage and antiaging effects induced by DR in mice [42]. Interestingly, SIRT3 deacetylates various complex I subunits [170] which could modulate the nearby placed sites of mitROSp. Epigenetics seems to influence two main effectors of PAP, the autophagy system [154], and the mitochondria [171–173].

10. CONCLUSIONS

Mitochondrial ROS generation rates at complex I, levels of mtDNA oxidative damage, and fatty acid

unsaturation degrees in cellular and mitochondrial membranes are all low in long-lived mammalian and bird species. The rate of mitROSp and the fatty acid unsaturation of cellular membranes are the only two known traits correlating with animal longevity in the right sense and offering a plausible mechanism to cause aging. This is true not only concerning MFRTA but also all theories of aging in general so far proposed. The close vicinity or even contact between the site of ROS generation and mtDNA avoids antioxidants to interfere with the ROS produced at the complex I mitROS generator relevant for aging. This is likely why antioxidants do not modify longevity. The ROS-dependent final forms of mtDNA damage likely most relevant for aging—mtDNA fragments inside nDNA—seem to be controlled by the rate of mitROSp, significantly contributing to determining the aging rate.

Dietary restriction also decreases mitROSp and FRL at complex I and oxidative damage to mtDNA. This is exclusively due to the lower methionine intake (MetR) of the animals subjected to DR. Around 50% of the longevity extension effect of DR and PR is due to MetR, and seems to increase longevity in part through decreases in mitROSp at complex I; the other 50% effect of DR on longevity would act through other mechanisms like increased autophagy. Rapamycin, the only known drug that consistently increases longevity in mammals also decreases mitROSp at complex I and FRL. All the four known experimental manipulations that have proven to increase mammalian longevity (DR, PR, MetR, and rapamycin) decrease mitROSp at complex I, FRL, and oxidatively derived forms of mtDNA damage.

mitROS generate oxidative damage in mtDNA (e.g., 8-oxo-dG) which is repairable and can also lead to point mutations in the process. Notably, mitROS also generate single and double strand breaks in mtDNA. This leads to irreversible forms of damage (mutations) like mtDNA deletions, and mtDNA fragments which enter the chromosomes through the centromeres and accumulate in nDNA during aging. The steady-state level of 8-oxo-dG in mtDNA is a marker of the flow of ROS-dependent damage generation and repair through mtDNA. Its measurement is a useful marker of the rate of generation of mtDNA deletions and mtDNA fragments. Mutations can also arise due to processes unrelated to oxidative stress like mtDNA replication and repair. However, it is highly unlikely that these last mechanisms of

damage generation are related to longevity, because their random nature cannot explain the determination of longevity during DRs and in different animal species. It has been argued that the types of base mutations (transitions or transversions) mainly present in mtDNA indicate that they mainly come from mtDNA replication and repair. This has been taken as evidence against MFRTA. But, this applies only to base substitutions and does not concern mitROS-induced DNA strand breaks leading to mtDNA large deletions, and to mtDNA fragments insertion inside nDNA. It is now known that mtDNA fragments accumulate during aging inside nDNA in yeast, rat liver and brain, and mouse liver, causing an increase in chronological aging in yeast [147]. Such accumulation is reversed by rapamycin. The mtDNA fragments enter nDNA through the centromeres. From there, they can potentially disseminate to other chromosome regions, potentially altering genes or their regulatory regions, thus contributing to aging and cancer. The pericentromeric mtDNA fragments, due to their location, can also generate aneuploidy or chromosome rearrangements, causing cell death or malfunction during aging.

The low fatty acid unsaturation degree of cellular and mitochondrial membranes of long-lived animals leads to relatively low rates of endogenous lipid peroxidation *in vivo*, decreasing membrane damage and the generation of highly toxic and mutagenic lipid peroxidation products like malondialdehyde, hydroxynonenal, and many others. Some of these products have the potential to modify DNA. This would add secondary DNA damage to that primarily coming from the complex I mitROS generator relevant for aging.

Many different kinds of evidence indicate that there is a pro-aging program (PAP) lying inside the cell nucleus. The PAP is a central part of the cellular aging regulatory system (CARS). In this CARS, the PAP reacts to environmental, humoral, and cytoplasmic signals by modifying the activity of different PAP genes. This modifies the synthesis of specific proteins which change the activity level of the different aging effectors.

Three candidate aging effectors emerge at present: (1) mitochondria (including mitROSp); (2) the degree of fatty acid unsaturation (DBI) of cellular membranes; and (3) autophagy. Decreases in mitROSp and DBI and increases in autophagy contribute to increase longevity.

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