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Review Article

Trends in oxidative aging theories

Florian L. Muller [a], Michael S. Lustgarten [b], Youngmok Jang [a], Arlan Richardson [a,b,c,d], Holly Van Remmen [a,b,c,d,*]

[a] *Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA*
[b] *Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA*
[c] *South Texas Veterans Health Care System, San Antonio, TX 78284-7762, USA*
[d] *Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA*

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Abstract

The early observations on the rate-of-living theory by Max Rubner and the report by Gershman that oxygen free radicals exist in vivo culminated in the seminal proposal in the 1950s by Denham Harman that reactive oxygen species are a cause of aging (free radical theory of aging). The goal of this review is to analyze recent findings relevant in evaluating Harman's theory using experimental results as grouped by model organisms (i.e., invertebrate models and mice). In this regard, we have focused primarily on recent work involving genetic manipulations. Because the free radical theory of aging is not the only theorem proposed to explain the mechanism(s) involved in aging at the molecular level, we also discuss how this theory is related to other areas of research in biogerontology, specifically, telomere/cell senescence, genomic instability, and the mitochondrial hypothesis of aging. We also discuss where we think the free radical theory is headed. It is now possible to give at least a partial answer to the question *whether oxidative stress determines life span* as Harman posed so long ago. Based on studies to date, we argue that a tentative case for oxidative stress as a life-span determinant can be made in *Drosophila melanogaster*. Studies in mice argue for a role of oxidative stress in age-related disease, especially cancer; however, with regard to aging per se, the data either do not support or remain inconclusive on whether oxidative stress determines life span.

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Abbreviations: ApoD, apolipoprotein D; CAT, catalase; GCL, glutamate-cysteine ligase; Gpx1, glutathione peroxidase 1; MsrA, methionine-*S*-sulfoxide reductase; ROS, reactive oxygen species; SOD, superoxide dismutase.

* Corresponding author. Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, MSC 7762, San Antonio, TX 78229-3900, USA. Fax: +1 210 562 6110.
E-mail address: vanremmen@uthscsa.edu (H. Van Remmen).

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Introduction and historical background

Max Rubner, in his exploration of the relationship of metabolic rate (oxygen consumption) and body mass, first noted the now well-known inverse correlation between the rate of oxygen consumption and the longevity in eutherian mammals [1,2]. This observation was expanded further by Pearl, in the so-called rate-of-living hypothesis, which states that lifetime metabolic (energy) expenditure is finite and that life span is determined by how fast it is expended—in other words—that life span is strictly an inverse function of oxygen consumption or metabolic rate [3]. Although the rate-of-living hypothesis is no longer accepted [2,4,5], it directed the attention of aging research onto oxygen metabolism. The rate-of-living theory fits quite well with the observation that oxygen tension, in excess of that normally present in the atmosphere (21%), is toxic to almost all animals (fatal to mammals at 100% O_2 [6]). In the mid 1930s it was observed that metabolic rate and hyperoxic death are closely interrelated: high metabolic rate (brought about by hyperthyroidism) accelerated and a low metabolic rate (hypothyroidism) delayed death by hyperoxia in rats [7].

The origins of the free radical theory of aging go back to the mid 20th century, when it was discovered that oxygen free radicals, traditionally thought to be too reactive to exist in biological systems, are formed in situ in response to radiation and oxygen poisoning and are responsible for the associated toxicities [8,9] (Fig. 1). Noting that radiation "induces mutation, cancer and aging" (citing [10]) and, drawing on the rate-of-living hypothesis [3], Denham Harman proposed that oxygen free radicals (specifically hydroxyl, $OH^{\bullet}$, and hydroperoxyl, $HO_2^{\bullet}$, radicals) are formed endogenously from normal oxygen-utilizing metabolic processes and play an essential role in the aging process [11]. Interest in the free radical theory was at first very limited because of the persistent doubt about the existence of oxygen free radicals in biological systems, despite the reports by Gerschman et al. [8] and the detection of radicals by Commoner and co-workers [12,13]. The discovery of superoxide dismutase by McCord and Fridovich [14], and the demonstration of the existence of H_2O_2 in vivo by Chance [15] gave credibility and raised the profile of the hypothesis. In 1972, Harman proposed a modification of the free radical theory, giving a central role to mitochondria [16], because these organelles generate a disproportionately large amount of reactive oxygen species (ROS) in cells [15]. In subsequent years, much correlative evidence supporting the theory was published. The majority of studies verified the first aspect of Harman's hypothesis: that oxidative damage increases during aging (e.g., [17–19], reviewed in [19–21]). In agreement with Harman's modified proposal, that mitochondria are central to aging, it was discovered that mtDNA deletions (and, more recently, mtDNA point mutations) are induced by oxidative stress and dramatically accumulate with age in organisms ranging from worms to humans [22–24].

In the last 50 years, Harman's hypothesis has also been refined to encompass not only free radicals, but also other forms of activated oxygen. Many reactive oxygen species such as peroxides and aldehydes (which are not technically free radicals) also play a role in oxidative damage in cells. This realization led to a modification of the free radical theory, i.e., the oxidative stress theory of aging [25]. This modification of the free radical theory is based on the fact that a chronic state of oxidative stress exists in cells of aerobic organisms even under normal physiological conditions because of an imbalance between prooxidants and antioxidants. The imbalance leads to a steady-state accumulation of oxidative damage in a variety of macromolecules that increases during aging, resulting in a progressive loss in the functional efficiency of various cellular processes. In a recent review, Beckman and Ames made a useful addition to this debate by dividing the hypothesis into "strong" and "weak" versions [26]. The strong

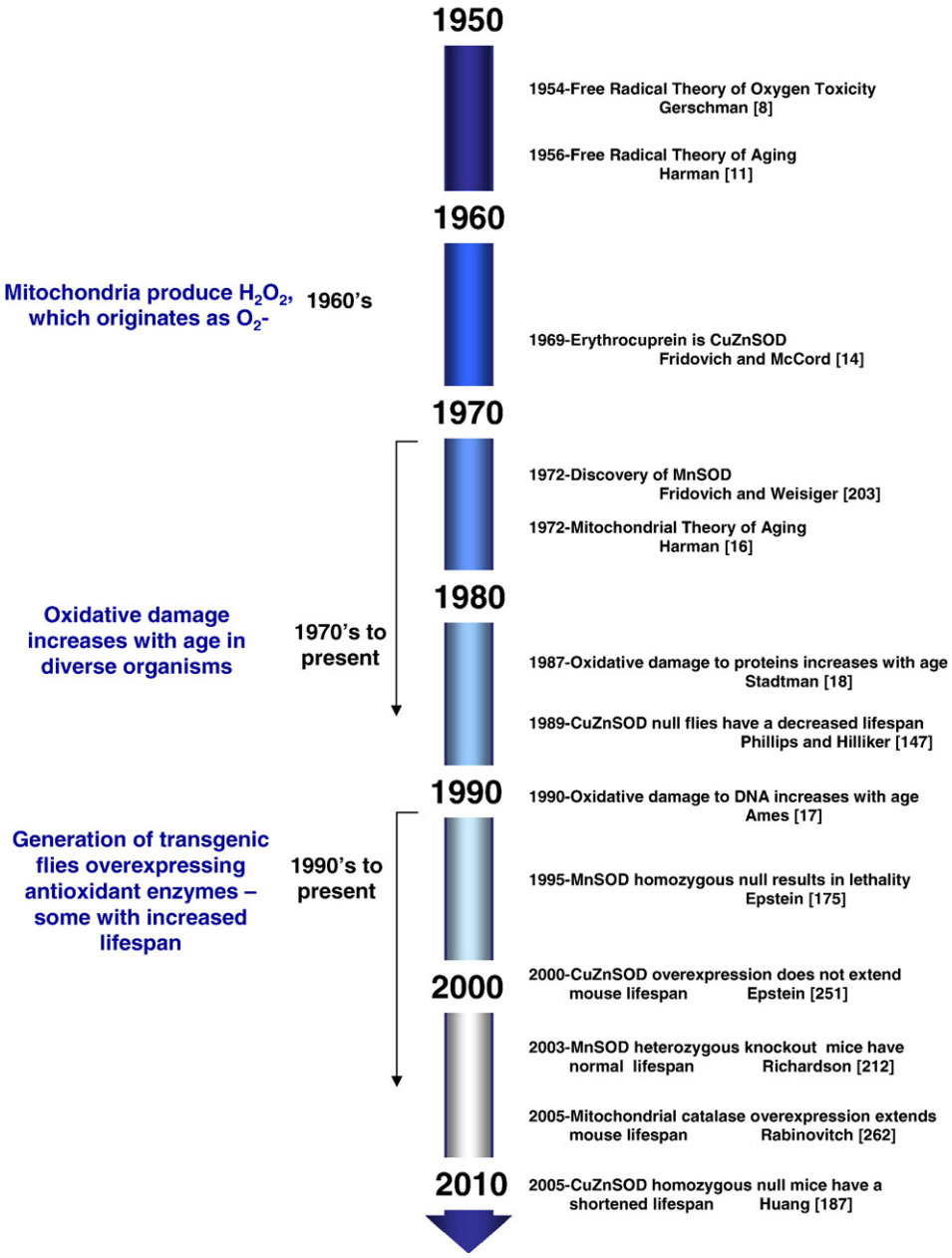

Fig. 1. Specific events and trends in the free radical theory of aging.

version of the theory states that oxidative damage determines life span, while the weaker version postulates that oxidative damage is "associated" with age-related disease. Obviously there also exists a continuum between these two extreme hypotheses. We suggest that the weak version of the oxidative theory of aging is already well established in that there are multiple studies showing an association between elevated oxidative damage and age and age-related disorders [26]. However, in this review we have evaluated the evidence for and against the "strongest" version of the oxidative stress hypothesis of aging. We have placed special emphasis on "interventionist" (as opposed to correlative) studies, specifically those involving genetic modifications leading to elevated or reduced oxidative stress (and evaluating the resulting effect on life span). Traditionally, this has consisted of overexpressing antioxidant enzymes. However, the biggest misconception surrounding these experiments is that increasing the levels of an antioxidant enzyme will, by the virtue of their name, necessarily reduce oxidative stress (in some cases it may do so, in others it may not [27]). For example, extensive work has focused on answering the question of whether increasing the levels of the enzyme superoxide dismutase [28,29] will reduce oxidative stress both in vivo and in vitro. While a full summary of this literature is beyond the scope of this review, the answer is far from unambiguous [30]. Indeed, to properly interpret many experiments aimed at testing the free radical theory, a thorough understanding of redox and oxygen biochemistry is helpful (for review, see [31,32]). As a guide,

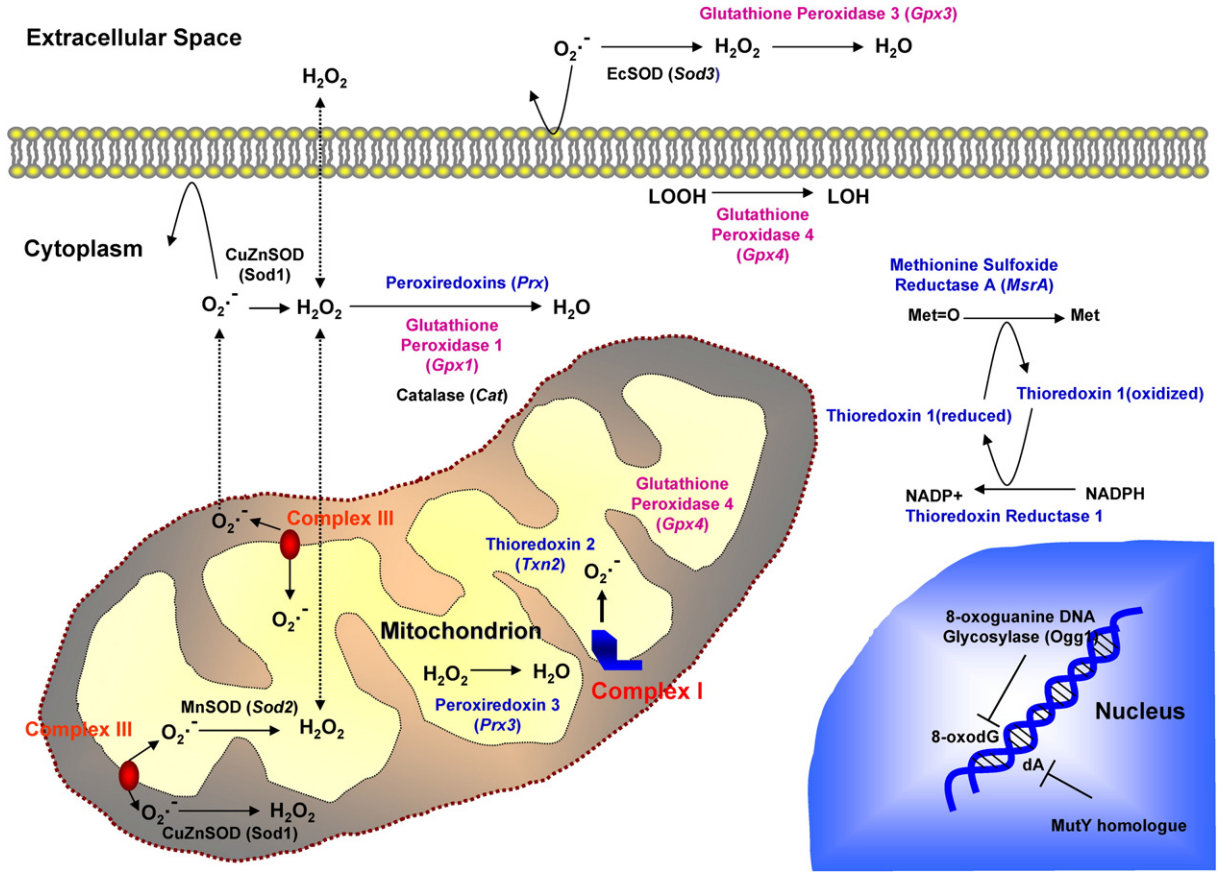

Fig. 2. Reactive oxygen species, their sites of production and antioxidant scavengers. The diagram represents a typical mammalian cell, with the localization of the major antioxidant enzymes and the sites of ROS production. While there are several cytosolic enzymes capable of generating superoxide (and many more capable of generating H_2O_2), the mitochondrion is emphasized here, as it is generally acknowledged as the most important ROS production locus. Superoxide ($O_2^{\bullet -}$) is not membrane permeable while H_2O_2 is readily diffusible. Complex I (CI in light blue) releases superoxide exclusively into the mitochondrial matrix while Complex III (CIII in red) can release superoxide to both sides of the inner mitochondrial membrane [184], with that being released into the intermembrane space reaching the cytosol via the VDAC channels [368]. Superoxide is very labile, either dismutating (spontaneously or enzymatically by SOD) or being reduced to H_2O_2. While it is agreed that SODs are the main superoxide scavenging system in the cell, there is some debate about the relative importance of catalase, glutathione peroxidase, and peroxiredoxins in the removal of H_2O_2. Based on strong deleterious phenotype of *Prdx1* and *Prdx2* knockout mice and the lack of phenotype of *Gpx1* and Catalase knockout (Table 1), it would appear that peroxiredoxins are more important in scavenging endogenously produced H_2O_2. H_2O_2 can form the highly deleterious $OH^{\bullet}$ radical, which can damage a wide variety of macromolecules, including proteins, lipid, and DNA. Lipid hydroperoxides are reduced to alcohols by glutathione peroxidase 4 [361]. One of the major DNA oxidative products is 8-oxo-dG, which is repaired by the enzyme Ogg1 [250]. Ogg1 works in conjunction with Myh, which removes dA mispaired to 8-oxo-dG [250]. Working in conjunction, these two enzymes prevent 8-oxo-dG from forming transverse G to T mutations. While neither $Ogg1^{-/-}$ nor $Myh^{-/-}$ mice have a deleterious phenotype, when combined, the mice suffer from a dramatic reduction in life span due to massive carcinogenesis [250]. The two major suppliers of reducing equivalent to the antioxidant system are the thioredoxin and glutathione. MsrA and peroxiredoxins require thioredoxin (emphasized in blue), while the glutathione peroxidases require glutathione (emphasized in pink). Ablation of either system is extremely deleterious, resulting in early embryonic lethality.

some key points related to reactive oxygen species, their sites of production, and antioxidant enzymes are summarized in Fig. 2.

Oxidative stress and aging in select model organisms

Podospora anserine

The role of mitochondria and oxidative stress in aging is most solidly supported in *Podospora anserina*. *P. anserina* is a fungus of the ascomycete family that has been used in aging research since the 1960s (reviewed in [33]). In this organism, hyphae growth is not indeterminate, but once reaching a certain length, growth arrests and the hyphae eventually wither and die. Senescence has been observed in every *P. anserina* strain so far isolated and the length of hyphal growth before senescence is highly reproducible [34–36]. Work in the 1970s pointed to a central involvement of mitochondria in determining the timing of senescence, i.e., life span [35]. In recent work, Dufour et al. [34] demonstrated that mitochondrial ROS are, in fact, the central player in life-span limitation. It was shown that elimination of the mitochondrial electron transport chain (rho 0) extended the hyphal life span of *Podospora* by at least 3-fold. The mechanism by which mitochondrial ROS trigger senescence involves oxidative damage to mitochondrial DNA, which promotes the excision of an intron which accumulates to very high concentrations in long ("old") hyphae [36,37]. Unfortunately, mitochondrial DNA from metazoans does not have

introns. Thus, this particular aging mechanism is probably specific to *Podospora*, but nevertheless suggests that mitochondrial DNA instability (brought about by oxidative stress) could be a life-span-limiting factor, even in higher organisms.

Saccharomyces cerevisiae

S. cerevisiae, or baker's yeast, is a unicellular fungus of the ascomycete family. A unicellular organism would seem a poor choice as a model system for aging, since mitosis, or cell division, leaves two identical daughter cells, which would result in either population immortality or extinction. But *S. cerevisiae* undergoes asymmetric cell division (contrary to prokaryotes and the closely related fungus *Schizosaccharomyces pombe*); i.e., there is a distinct mother and daughter cell. A mother cell gives rise to a smaller daughter cell in a process called budding [38,39]. A single mother cell can only bud a finite number of times, after which it becomes sterile, a phenomenon termed "clonal" or "replicative" senescence [38,39]. It should be noted that in contrast to animal aging, mother cells that cannot bud anymore do not actually die; instead they remain metabolically active for an indeterminate period of time. "Life span" (clonal life span) in *S. cerevisiae* can be defined by the number of buddings. Clonal life span is probably an unsuitable model for studying energy metabolism and aging as it applies to man, because under these experimental conditions, *S. cerevisiae* derives almost all its energy from anaerobic glycolysis, and mitochondrial respiration is actively repressed [40]. Besides the clonal type of senescence, *S. cerevisiae* also undergo chronological senescence. When grown to saturation and glucose is exhausted, yeast stop dividing and the culture gradually loses viability. Measurement of chronological senescence consists of examining the progressive viability loss during stationary phase (somewhat similar to the G_0 phase of eukaryotic cells [41,42]).

There is evidence of oxidative damage accumulation during clonal life span. Oxidatively damaged proteins have been measured to segregate preferentially in mother cells during budding and have been found to accumulate during clonal aging [43]. Some evidence suggests that this oxidative damage could be life span limiting. Knocking out *Sod1*, which encodes CuZnSOD—the cytosolic and mitochondrial intermembrane space isoform of superoxide dismutase—shortens life span [44]. Furthermore, overexpression of the methionine oxidation repair enzyme, MsrB, increases life span under low glucose conditions [45]. However, a critical experiment strongly argues against ROS being life span limiting for clonal senescence. Growing *S. cerevisiae* under complete anaerobic conditions decreases, rather than increases, clonal life span [45]. Because the vast majority of ROS are ultimately derived from oxygen, this experiment strongly indicates that ROS *do not* limit yeast clonal life span.

On the other hand, substantial evidence exists that oxidative stress limits chronological life span in yeast. Protein carbonyls have been shown to accumulate during chronological aging, in a manner dependent on the rate of mitochondrial ROS production [42,46]. In addition, chronological life span can be dramatically increased by (indirectly) blocking mitochondrial free radical production at Complex III using potassium cyanide [41]. Conversely, chronological life span can be significantly shortened by the addition of antimycin A [47,48], which stimulates superoxide production by Complex III (reviewed in [49,50]). This effect can be reversed by the concomitant addition of potassium cyanide [47], which indirectly prevents antimycin A-induced superoxide production by Complex III by locking the cytochrome *c* pool in the reduced state, thus preventing ubisemiquinone formation [47,51]. Genetic manipulations have also provided strong evidence for oxidative stress being limiting during chronological life span. Deletions of either CuZnSOD and/or MnSOD in yeast dramatically accelerate chronological aging, and this can be partially reversed by decreasing the oxygen tension (i.e., decreasing mitochondrial superoxide production [41,52]). Overexpression of antioxidants enzymes conversely increases life span. Longo and others have shown that overexpression of MnSOD (the mitochondrial matrix isoform of superoxide dismutase, *Sod2*) unambiguously increases chronological life span [53,54]. Collectively, these data suggest that reactive oxygen species do limit life span during chronological aging in *S. cerevisiae*. In summary, current evidence suggests that oxidative stress is a critical determinant in chronological life span, but either has no role or a rather limited role in clonal life span.

What then is the relationship between chronological and clonal life span? The two must be somehow related as evidenced by the fact that chronologically aged *S. cerevisiae* also have a much shorter clonal life span [55]. But, some genetic manipulations, such as overexpression of *Sod2*, have the exact opposite effect on chronological vs clonal aging [54]. In our opinion, the critical difference between the two states is the differential reliance of mitochondrial respiration: under clonal conditions, mitochondrial electron flux is small and the consequent production of ROS is not physiologically significant—*Sod2* knockout yeast grow just as well as its wild-type counterpart in logarithmic phase on glucose medium [41,52,56] (the conditions used in clonal life-span experiments) but die much more rapidly than wild-type counterparts in the post-diauxic phase[41,52] (the conditions used in chronological life-span experiments). Considering the high rates of flux through glycolysis in yeast, ensuing production of reactive aldehydes such as methyglyoxal (leaked from triosephosphate isomerase [57,58]) and the clonal aging-related increase in protein carbonyls [43], we suggest that advanced glycation end products [59,60] may play an important role in replicative yeast life span. Such a scenario would explain the age-related accumulation of protein carbonyls (which can be formed by Michael addition of advanced glycation end products; see ([60,61] for a review of the chemistry involved) and the beneficial effect of MrsB overexpression, even though anaerobic conditions do not extend life span.

Caenorhabditis elegans

C. elegans is a pseudo-coelomate nematode mainly used as a model organism in developmental biology. The same qualities

that have made this organism so attractive to developmental biologists, such as large number of offspring, short generation time (life span of around 20 days), and the ability to be stored frozen have also brought it to the attention of biogerontologists. But the most outstanding feature of *C. elegans*, its deterministic development and defined number of adult cells, could also be problematic for its use as a model of mammalian aging. A further important difference in comparison to mammals is that the key determinant of life span in *C. elegans* (as well as *Drosophila*) is the temperature at which it is grown. The life span of either species can be doubled by lowering ambient temperature by ~6°C. The study of aging in *C. elegans* has centered around the *dauer* (an alternative life stage induced by food shortage and characterized by very long life span) mutants [62,63], that are thought to operate by a similar mechanism to the life-span-extending Ames and Snell-dwarf mutation, in that insulin-like growth factor signaling is ablated [64–66]. Data from the *dauer* mutants (the best known being mutant *daf-2*) are generally supportive with the oxidative hypothesis, in that resistance to oxidative stress is consistently elevated in *dauer* mutants and antioxidant enzymes (most dramatically, MnSOD) are upregulated [67–69]. In fact, some antioxidant genes appear to be necessary for life span extension by *daf-2* [70,71].

In a study highly supportive of the free radical theory of aging, Lithgow's group published that the catalytic antioxidants (SOD/catalase mimetics) markedly extend life span of *C. elegans* [72]. However, other investigators have not been able to demonstrate increased life span, observing dose-dependent toxicity instead [73,74]. Regardless of these data, other studies have provided evidence for oxidative stress as a life-span determinant in *C. elegans*. As in *Drosophila* (discussed below), oxygen tension can modulate life span [75,76]. Point mutations in Complex I (*gas-1*) and Complex II (*mev-1*) of the mitochondrial electron transfer chain increase superoxide production [77] and have been shown to reduce life span [78–80]. *mev*-1 mutants have been demonstrated to have elevated levels of oxidative damage in chromosomal DNA, and, an increased mutation frequency under hypoxia, relative to the wild-type strain [78]. When *gas-1* and *mev-1* are crossed, the phenotype is lethal [79]. However, these point mutations are the exception rather than the rule. Through RNAi screens, it has been discovered that knocking down almost any protein in the electron transfer chain will result in a ~30% increase in *C. elegans* life span [81,82]. This manipulation is only effective when applied during development; when applied to adult worms, no life-span extension is observed. Furthermore, life-span extension is still observed when the RNAi is removed after development, indicating that some kind of metabolic reprogramming might have taken place. Mutations in *isp-1* (Rieske iron-sulfur protein of Complex III [83]) and *clk-1*[84] (defective in ubiquinone biosynthesis [85]), presumed to have decreased mitochondrial superoxide production, also show extended life span [84,86,87]. Going along with this trend, combining the *gas-1* mutant with a deletion mutation of a close paralogue, creating a mutant with very little Complex I activity, results in a 3-fold extension of life span [88]. These data all fit the general paradigm that reducing mitochondrial electron transfer extends, rather than reduces, life span. Even treatment with the Complex III inhibitor, antimycin A (which is extremely toxic to mammals), also extends life span [81]. This would seem to argue against the oxidative hypothesis, because antimycin A treatment drastically increases superoxide production [15,50,89]. However, it has been observed that a near-complete inhibition of Complex III must be attained before antimycin A stimulates superoxide production [90,91].

The consensus of the data in *C. elegans*—that inhibiting mitochondrial electron transport increases life span—is in exact opposition to what one observes in mammals (and even in *Drosophila*), where partial inhibition of the mitochondrial electron transport chain (either by mutations or pharmacology) leads to a host of severe pathologies including myopathy and encephalopathy [92,93] and Parkinsonism [94–96]. Further illustrating this point, suppression of the frataxin homolog gene (*frh-1*) by RNAi significantly extends worm life span ([97] though another study reported a reduced life span, [98]). In almost exact opposition, recessive mutations in frataxin cause the devastating human neurodegenerative disease, Friedreich's ataxia, [99] and frataxin ablation in mice causes a decreased life span [100].

Thus, there appears to be a fundamental difference between *C. elegans* and mammals, as to how energy metabolism and oxidative stress affect pathogenesis. While mammals rely overwhelmingly on aerobic metabolism to remain alive (i.e., the mitochondrial electron transport chain), the anaerobic energy producing capacity (glycolysis, Krebs, and glyoxylate cycles) of *C. elegans* is so great that it can survive for extended periods of time in partial as well as complete anaerobiosis (~48 h for wild type, considerably longer for *dauer* larve and *dauer* mutants [101–103]). This anaerotolerance is probably an ecological adaptation to living in low-oxygen environments like moist topsoil. Physiologically, this is enabled by an anaerobic energy-generating pathway, not found in mammals, involving reverse electron transfer via fumarate reductase and malate dismutation [63,104]. Indeed, the long-lived *dauer* larva's metabolism appears to be shifted even more toward anaerobic pathways [104], a fact supported by the increased anaerotoleance, which is dependent on glycolysis [105]. The reliance on nonmitochondrial anaerobic metabolism may also explain *C. elegans*' extreme tolerance to hyperoxia. It is quite distinct from higher metazoans by its ability to grow, live, and reproduce at 100% O_2 [106]. Rea and Johnson have presented a detailed hypothesis integrating the above findings, arguing that upregulation of the malate dismutation pathway and the ensuing decrease in mitochondrial superoxide production are responsible for the extended life span observed in the electron transport chain Complex knockdowns [63].

Drosophila melanogaster

The fruit fly *Drosophila* has served as a work-horse model organism for genetics as well as developmental biology. The free radical theory has been more extensively tested in this organism than in any other. Indeed, the strongest evidence supporting the role of oxygen radicals in aging metazoans has

come from studies in *Drosophila*. Many antioxidant-supplementation studies have been conducted in this organism, some with positive results. These antioxidant supplementation studies have been reviewed rather extensively [26,107] and will not be discussed further in this review, except to say that the literature is inconsistent: in some laboratories a given antioxidant may clearly extend life span, while in others it may have no effect [107]. A series of point mutations with increased life span (e.g., *methusela* [108] and its ligand, *stunted* [109]) have been identified; studies with these mutants are generally consistent with the free radical theory in that these exhibit increased resistance to a variety of oxidative stresses, but as in *C. elegans*, they offer correlative, not interventionist, evidence for the free radical theory and will therefore not be discussed at length in this review.

Atmospheric oxygen tension modulates life span of Drosophila

The oldest method of modulating in situ oxidative damage is through the manipulation of oxygen tension. Indeed, the oxygen "poisoning" experiments of Gerschman et al. were an important foundation to Harman's free radical theory of aging [8]. The essence of these experiments is that in situ oxidative damage can be modulated by changing the oxygen tension (atmospheric O_2). Because O_2 is the substrate for superoxide ($O_2^{\bullet -}$) production, an increased O_2 tension results in increased superoxide formation, including at mitochondrial sites [110–112]. Of course, this manipulation will only work in an organism that minimally regulates its in situ oxygen tension, such as *Drosophila* [113]. Early experiments have shown that there is a quasi-linear, inverse relationship between life span and oxygen tension in *Drosophila* [114–116]. Thus, if oxygen tension is increased above 21%, life span is correspondingly shortened (reviewed in Ref. [114]). Even at 30% O_2, there is a statistically significant decrease in life span and at 40% O_2, life span is reduced by about one-third. Despite the potentially increased ROS production [paradoxically] caused by hypoxia (summarized in Ref. [117]), one report indicates that decreasing oxygen tension below 21% actually increases life span [116].

Traditionally, these experiments have been interpreted strictly in support of the rate-of-living theory of aging [115]. This is now an unlikely explanation. First, there is now substantial evidence that there is no direct relationship between metabolic rate and life span in *Drosophila* [118–121]. Second, empirical data show that metabolic rate is not increased at 40% O_2 [113]. In *C. elegans*, respiration saturates below 2% oxygen [106] and metabolic rate at 40% O_2 is no larger than at 21%, in agreement with observations in several insect species [122]. This is only to be expected because the K_m of cytochrome *c* oxidase for O_2 is very low (μM range); thus the rate of respiration (i.e., metabolic rate) saturates at very low oxygen concentration and is not increased by increasing O_2 above normal atmospheric levels [123]. Increased ROS formation, not increased metabolic rate, is thus the most likely reason for the oxygen-dependent life-span reduction.

Still, we do not know whether the life-span shortening in response to increased oxygen tension is due to "oxygen poisoning" or accelerated aging. Oxygen poisoning seems unlikely given that survival at critical stages in young flies (such as at eclosion) is unaffected by 40% O_2 [113]. A recent microarray study compared gene expression patterns in old and young flies and in young flies treated with 100% oxygen [124]. It was discovered that young flies treated with 100% oxygen exhibit many of the gene expression changes seen in old flies [124]. This indicates that oxidative injury plays a prominent role in normal fly aging and suggests that the life-span shortening under mild hyperoxia (40% O_2) may be true accelerated aging. Further supporting this point is a recent histopathological study by Walker and Benzer: 100% O_2 induces severe mitochondrial malformations, "swirls," in *Drosophila* flight muscle [112]. Strikingly, these same abnormalities are also observed in old flies reared under normal (21%) O_2 tension. A *Drosophila* mutant, "Hyperswirl," which exhibits accelerated formation of these swirls also exhibits a greatly reduced life span. On the whole, these oxygen tension manipulation experiments unambiguously demonstrate that oxygen free radicals *can be* a life-span-limiting factor but do not prove that they actually *are,* at normal atmospheric O_2 tension.

Genetic manipulation of SOD in Drosophila

Early studies showed that artificial selection for increasing longevity (~60% increase in both mean and maximal life span, [125]) results in the correlated response of increased CuZnSOD content and activity (~2-fold) elevated mRNA content of CuZnSOD (as high as ~3-fold), MnSOD (as high as ~4-fold), and CAT (~2- to 3-fold). Also measured were decreases in protein carbonyls and lipid peroxidation in the long-lived strain (derived via artificial selection, [125,126]). Data from these studies further indicate that [127] the phenotype of an elevated paraquat (a free radical generator) resistance always accompanies the long-lived strain. In addition, reversed selection applied to this long-lived strain results in the simultaneous decrease of both life span and paraquat resistance, thus making the direct association between oxidative stress and life span. These data would suggest that elevated antioxidant defense is necessary for extended life span (though one cannot say for sure, since this selection regime altered many variables). Nevertheless, these results provide a strong rationale for manipulating individual antioxidant genes.

A large amount of contradictory work has been published on the effect of genetically upregulating antioxidant enzymes on life span. Taken at face value, several papers would suggest that the strong version of the oxidative theory has been disproved. However, other works point to exactly the opposite conclusion. Most of this work has focused on the superoxide dismutase enzymes. Superoxide dismutase, the scavenger of the superoxide anion, occurs in both cytoplasmic and mitochondrial isoforms (encoded by two different genes, *Sod1* and *Sod2*, respectively) in *Drosophila*. Increasing the levels of superoxide dismutase seems like an obvious way to decrease oxidative damage, and, to test the free radical theory of aging The first such experiments were conducted in the early 1990s [128–130]. Generally, a constitutive promoter (such as β-actin) was used to overexpress *Sod1*. The results were contradictory: some studies

reported an increase in average life span [130], others found no effect, and others found a decrease in life span [128]. Very high overexpression of superoxide dismutase was found to be lethal [130]. The most well known of these experiments was the work of Sohal, Orr, and co-workers, in which concomitant overexpression of superoxide dismutase and catalase increased both average and maximum life span [131]. However, transgenic studies in *Drosophila* using P-element-mediated transformation are problematic—the control and experimental lines have different genetic backgrounds, a factor that has been shown to alter life span independently from any transgenic manipulation [132]. To minimize these background issues, Parkes et al. targeted the overexpression of CuZnSOD in *Drosophila* to motorneurons using a yeast UAS element that was regulated by a GAL4 activator and found that overexpression of CuZnSOD in motorneurons resulted in an increase in life span as well as an increase in resistance to paraquat and γ-irradiation [133]. The Phillips group also reported that MnSOD overexpression increased life span, albeit to a somewhat smaller extent [134]. Although this seems unlikely, there remains a small chance that genetic background might have played a role in the life-span extension independent of the *Sod1* transgene. To address this question, Tower's group used a conditional promoter to overexpress *Sod1* [135] (as well as *Sod2* [136,137]), allowing for isogeny in both the experimental and the control groups. Using this approach, a statistically significant extension of mean life span was obtained in several independent insertion lines, but maximum life span remained essentially unchanged. Combined overexpression of *Sod1* and *Sod2* was shown to have an additive effect—the increase in life span in independent insertion lines was proportional to the level of SOD overexpression [135–137]. Although the magnitude of the life span increase is relatively low, this work remains among the best evidence that superoxide, and by extension oxidative damage, is a life-span-limiting factor in *Drosophila* [135]. While the results of Phillips's and Tower's lab are supportive of the oxidative theory, Sohal's group demonstrated that constitutive (as opposed to inducible in the studies above) overexpression of CuZnSOD, MnSOD, catalase, or thioredoxin reductase in long-lived *Drosophila* strains had no effect on life span [138–140], though an increase in acute oxidative stress resistance was present in some of these strains. Similarly, Sohal's group has also demonstrated that simultaneous overexpression of MnSOD and catalase (ectopically targeted to the mitochondrial matrix of transgenic *Drosophila*) decreased extramitochondrial H_2O_2 release and had an enhanced resistance to experimental oxidative stress (as induced by dietary H_2O_2 administration or by exposure to 100% ambient oxygen), but decreased life span, by up to 43% [141].

While these results do not support the oxidative theory, they cannot be used to disprove it either, considering that no actual measurements of oxidative damage were reported, which is an issue because high overexpression of SOD can be deleterious and can lead to more, not less, oxidative damage [27,29,30,130,142,143]. In addition to these contradictory data, Orr et al. [144] take issue with the small magnitude of life-span extension in Tower's SOD overexpression studies [135–137], pointing out that the largest increases in life span were obtained in the lines with the shortest life span. This point is somewhat muted by the fact that even in the very long-lived wild-caught backgrounds, Phillip's UAS-GAL-driven *Sod1* overexpressor still extends life span [145]. However, more disconcerting is the fact that even 5% of wild-type CuZnSOD levels are sufficient to restore normal life span in *Sod1*-null mutants [146].

Before discussing this point further it is worth commenting on the phenotypes of both *Sod1*- and *Sod2*-null flies. Phillips' group first isolated a null point mutant of *Sod1* in *Drosophila* [147]. As expected, the phenotype was quite deleterious, resulting in life-span shortening of around 80% [148]. While this study elegantly demonstrates the biological toxicity of superoxide, is there any relation to aging? Rogina and Helfand have demonstrated that *Sod1*-null flies exhibit an acceleration of the temporal-age pattern of expression of the *wingless* gene; in so far as this is an aging-related marker, these results suggest that *Sod1*-null flies do exhibit accelerated aging [149]. If the shortened life span of *Sod1*-null flies is indeed accelerated aging, one would predict (extrapolating from the correlation of *Sod1* overexpression and increased life span in John Tower's experiments [135–137]) that a fly with *Sod1* levels intermediate between those of the wild-type and the *Sod1* null should exhibit a life span that is somewhere in between these extremes. While one study found that ectopic expression of *Sod1* at levels 30% of wild type only partially rescued (as expected) the reduced life span of the *Sod1* null [148], another found that expression of *Sod1* as low as 5% of wild type fully restored normal life span [146]. The reason for this difference remains unclear; but it would be difficult to explain how 5% of normal *Sod1* activity (i.e., a 95% knockdown) is sufficient for completely normal life span [146] yet *Sod1* levels 150% of normal (in the *Sod1* overexpression studies) actually result in an extension of life span [136].

While it was already known for some years that ablation of *Sod1* in *Drosophila* led to a drastic shortening of life span, only very recently were the phenotypical results of an *Sod2* ablation published [150,151]. Phillips' group used an RNAi knockdown strategy to repress expression of the *Drosophila* *Sod2* gene. This resulted in postnatal lethality (about 10 days after birth), only slightly worse than the *Sod1*-null mutant [146]. Duttaroy et al. [152] used the excision of a P-insertional element proximal to the *Sod2* locus to delete this gene. This is a true knockout, one whose phenotype was even more deleterious than that observed by Phillip's group using RNAi. Duttaroy's *Sod2* knockout was found to be postnatal lethal—by 36 h after birth (hatching), all *Sod2*-null flies were dead. This result establishes that mitochondrial superoxide dismutase is indispensable; however, as with the *Sod1* null, is it more than just superoxide toxicity; is it accelerated aging? If the latter is the case, then one would expect a fly that is partially lacking *Sod2* to have a life span somewhere in between that of the null and the wild type. The published data suggests that *Sod2* heterozygous knockouts have a reduced survival as compared to wild type (the survival of the *Sod2* $n^{283/+}$ appears lower than the Canton-S in Fig. 3 of Ref. [152]). Further experiments have shown that the life span of the *Sod2* $n^{283/+}$ is reduced by 25% compared to the wild type (A. Duttaroy, personal communication). Thus,

contrary to the *Sod1* overexpression studies, the interpretation of the *Sod2* overexpression experiments is not complicated by the seeming restoration of normal life span by even low levels of *Sod1* [146]. The data from Tower [136] and Duttaroy [152] together suggest that a dose–response relationship exists between MnSOD and life span, *exactly as the free radical theory would predict*.

Other genetic manipulations in Drosophila

MsrA overexpression. Very recent transgenic studies provide more evidence for the oxidative stress theory of aging in *Drosophila*. UAS/GAL4-driven overexpression of the protein-methionine oxidative damage repair enzyme, peptide-*S*-methionine sulfoxide reductase (MsrA), was found to increase average life span in several independent insertion lines, up to 85% [153]; maximum life span was also increased. As expected, resistance to paraquat was also increased. Great pains were taken in this study to establish that the changes in life span were not due to genetic background issues as previously discussed. In addition, bearing Orr and Sohal's criticisms in mind regarding the use of short-lived strains in life-span studies [144], it is worth noting that even the wild-type parent strains used in that study had a very long life span. As with the GAL-UAS overexpression study of Phillips and co-workers [133], the greatest increase in life span was observed when MsrA was overexpressed specifically in the motorneurons. In addition to life-span extension, MsrA overexpression also delayed the age-related decrease in spontaneous activity and fertility. One minor criticism of this study is that no measurements of oxidative damage were conducted, meaning that one cannot absolutely conclude that the extended life span was due to decreased methionine sulfoxide (or other oxidative damage). Nevertheless, the increased resistance to paraquat would suggest so. As such, this experiment is a convincing piece of evidence in favor of the free radical theory. Concerning the dose–response argument presented in the case of *Sod1* and *Sod2* overexpression, it would be of interest to know the effect of a deletion of MsrA on life span, especially considering that it has been reported to shorten life span in mice [154].

Glutamate-cysteine ligase (GCL) overexpression. Glutathione is a primary antioxidant in both *Drosophila* and mammals, synthesized in a pathway whose rate-limiting step is catalyzed by glutamate-cysteine ligase. Global overexpression of the regulatory subunit of GCL (resulting in 60% increase in GCL enzymatic activity and up to 50% increase in total glutathione content) extended the mean life span of *Drosophila* up to 24%. More interestingly, neuronal overexpression of the catalytic subunit of GCL extended mean and maximum life span up to 50%, without affecting the rate of oxygen consumption, i.e., not decreasing metabolic rate [155]. Resistance to paraquat and H_2O_2 was concomitantly increased (though as with the MsrA study noted above, no measurements of oxidative damage were reported). These results are even more remarkable considering that the life span of the control strains was very long [155]. In fact, in absolute terms, it appears likely that this is the longest-lived *Drosophila* strain made to date. This result is strongly supportive of the free radical theory. Again, we are unaware of any information on the life span of the GCL knockout flies: in mice, knockout of the catalytic subunit of GCL is lethal, while that of regulatory subunit of GCL is viable.

Neuronal overexpression of human UCP2. In vitro studies on isolated mitochondria have demonstrated that ROS production is strongly dependent on proton motive force (Δp), again in vitro, and can be decreased considerably by chemical uncouplers (reviewed in [49,50]). A family of proteins, termed "uncoupling proteins," serves to dissipate Δp, possibly to minimize ROS production [156]. Expressing human *UCP2* in mitochondria of adult fly neurons results in increased state 4 (basal) respiration, decreased ROS production, and oxidative damage, a heightened resistance to the free radical generator paraquat, and an extension of *Drosophila* life span [157]. Average life span was significantly extended—28 and 11% in females and males, respectively; but the effect on maximal life span was relatively small. The larger life-span extension in females could partly be explained by a 90% greater expression of *hUCP2* in females, when compared to males [157]. Similar to Tower's experiments with SOD [136,137], the influence of genetic background was negated via an inducible expression system. It should also be noted that targeted overexpression of *hUCP2* to *Drosophila* muscle has no effect on life span, recapitulating what had been observed with SOD [133,134].

Apolipoprotein D overexpression. During an overexpression screen of genes for resistance to hyperoxia (100% O_2), Walker et al. [158] identified *Glial Lazarillo*, homologue of apolipoprotein D (ApoD). Overexpression of ApoD thus conferred extended life span under hyperoxia and remarkably, this also resulted in a 29% extension of life span at normoxia; although other studies have shown that resistance to hyperoxia does not always associate with extended life span [138], Walker et al's study demonstrates that the two phenomena are not independent and that screens under hyperoxia can be used as a successful strategy to identify aging-related genes. In further support of this finding, an independent group of investigators has conversely reported that knocking out ApoD leads to a 20% reduction in median life span and increased oxidative damage [159]. Although the exact biological function of ApoD is unknown, it has been reported to be upregulated in a number of neurological disorders, including Alzheimer's, schizophrenia, stroke, and, in the aging brain. ApoD belongs to the lipocalin family, which bind and transport small hydrophobic molecules [160]. Evidently, it plays an important role in protection from oxidative stress (though as with several other studies noted before, no measurements of oxidative damage were reported), and as such, its overexpression is supportive of the free radical theory.

Other invertebrates

A variety of other invertebrate species have been used to study oxidative stress and aging. In a study of considerable importance that is seldom cited, Georges Reversat reported a

truly amazing result [161]. In an anaerotolerant helminth species, *Heterodera oryzae* (Tylenchida; *Heteroderidae*), Reversat determined life span under both aerobic and anaerobic conditions. He discovered that while under aerobic conditions (21% O_2) around 90% of *H. oryzae* were dead after 27 days (comparable to the life span of *C. elegans*), but under anaerobic conditions only 10% of the helminths had died. As judged from the references cited within his paper, Reversat seems to have been completely unaware of the free radical theory of aging and the relevance of his results (reducing investigator bias). Obviously there are certain caveats to this study, but the simplest interpretation of his data is that total lack of oxygen, and therefore total lack of reactive oxygen species, extends life span, and quite dramatically so.

Oxidative stress and aging in vertebrates

Vertebrate animals, the metazoan phylum to which mammals (and humans) belong, are quite distinct in development and life history from the species discussed previously. An often raised argument regarding the evolutionary conservation of molecular biological processes (e.g., [162]) is that "if it is observed in such divergent organisms as *C. elegans* and is also observed in *Drosophila*, then it must also be true in *Mus musculus* (and by extension, all mammals)." We now know that this reasoning is flawed since *Drosophila* and *C. elegans* are much closer evolutionarily to each other than to mammals [163,164]. To give a more familiar analogy, it would be similar to arguing that a molecular pathway conserved between rats and mice must also be conserved in humans. Therefore, one cannot simply assume that if the free radical theory (or any other theory of aging) is true in flies and worms, it will necessarily hold true for mammals. With this in mind, we will consider experiments aimed at testing the free radical theory of aging in vertebrates, as we get a step closer to the true goal of validating the theory in Man.

There are only two interventions consistently reported to increase life span in laboratory mice: caloric restriction [165] and modulation of the anabolic GH/IGF-1 axis [64,65,166,167]. The CR effect is highly supportive (albeit correlatively) of the free radical theory, in that rates of mitochondrial ROS production and levels of oxidative damage are consistently decreased and resistance to oxidative stress is increased in CR animals (Extensively reviewed in [165,168]). In the case of the GH/IGF-1 modulation of life span, scattered data would suggest that this is the case as well. The situation is further complicated in that Ames and Snell dwarf mice, which exhibit the greatest increase in longevity (up to 55%), also have a compounded deficiency in several hormones, including GH and TSH [65]. TSH deficiency by itself (hypothyroidism) is known to decrease metabolic rate, mitochondrial ROS production, and steady-state levels of oxidative damage [169–171]. There is even an isolated report showing that hypothyroidism increases, and hyperthyroidism decreases, life span in rats [172]. Although the study of CR- and GH-deficient mice has provided evidence consistent with the free radical theory, these treatments affect a large number of physiological and biochemical processes, meaning that it is impossible to say whether changes in oxidative stress parameters were responsible or even necessary for the extended life span. For this reason, these will not be discussed further, instead we will focus on genetic changes of single antioxidant genes. In the following section we will discuss recent studies in mice documenting the effect of altered antioxidant defense systems on life span.

The life span of antioxidant and oxidative damage repair knockout mice

As in most areas of biology, gene-targeting "knockout" technology [173] has revolutionized the study of oxygen free radicals and oxidative damage in aging and disease [174,175]. In this section, we will review the phenotypes of antioxidant/oxidative damage repair enzyme knockout mice, with the goal of understanding the role of reactive oxygen species in aging. The strongest version of the free radical theory makes the broad prediction that life span is determined by oxidative damage and, thus, that increased oxidative damage will shorten life span. Although as Miller argues [176], a shortened life span does not prove accelerated aging, determining the life span of antioxidant knockout mice can be used to test the hypothesis: it follows that if an animal has increased oxidative damage but exhibits no change in life span, the result falsifies the hypothesis. A shortened life span cannot be taken as proof positive that oxidative stress causes aging but clearly fails to disprove the hypothesis. Further, it indicates that oxidative stress can be life span limiting. In this type of experiment, the outcome (life span, oxidative stress) in knockout is potentially even more informative than in transgenic mice (overexpression) because a negative result in overexpression studies leaves open the possibility that reduction of oxidative stress is necessary but not sufficient for extended life span. In the ideal case scenario for the free radical theory, there ought to be a dose–response relationship between a given antioxidant enzyme and life span. Thus if a homozygous knockout of a given antioxidant enzyme results in a dramatically reduced life span, the heterozygote knockout ought to have a life span intermediate between it and wild type. The one big caveat in this logic is that it assumes no compensatory antioxidant upregulation in antioxidant knockout mice. Finally, it is important to realize that our understanding of the antioxidant system is still far from complete. New antioxidant genes are still being discovered, for example, heme oxygenase 1 and sulfiredoxin 1 [177,178]. An overview of the phenotypes of antioxidant knockout mice is given in Table 1. Note that for most of these knockout mouse models, no definite information is published on life span. Below, we have summarized the studies in mice with alterations in expression of proteins responsible for antioxidant defense for which life span data are actually available. Although measuring life span would seem to be straightforward, the quality of life span experiments varies widely. Several factors can influence the quality of life span experiments. Very importantly, mouse husbandry conditions should be such that extrinsic mortality (infections, falls, flooding) does not limit survival (extrinsic mortality is evident when the survival curve is linear rather than Gompertzian,

Table 1
Phenotype of antioxidant and oxidative damage repair knockout mice

Ref.	Gene symbol	Gene name (localization)	Phenotype (life span)	Main antioxidant function
[235]	*Cat*$^{-/-}$	Catalase (C)[b]	"Normal," life span not reported[a]	Scavenges H_2O_2
[359]	*Gclc*$^{-/-}$	Glutamate cysteine ligase, Catalytic subunit (C)	Embryonic lethal	Glutathione biosynthesis
[360]	*Gclm*$^{-/-}$	Glutamate cysteine ligase, Regulatory subunit (C)	"Normal," life span not reported	Glutathione biosynthesis
[219]	*Gpx1*$^{-/-}$	Glutathione peroxidase 1 (C)	"Normal," life span not reported	Scavenges H_2O_2
[247]	*Gpx2*$^{-/-}$	Glutathione peroxidase 2 (E)	"Normal," life span not reported	Scavenges H_2O_2
[361]	*Gpx4*$^{-/-}$	Phospholipid glutathione peroxidase (M,C)	Embryonic lethal	Scavenges LOOH
[154]	*MrsA*$^{-/-}$	Methionine *R* sufloxide reductase (M, C)	~40% shortened life span	Repairs oxidized methionine
[264]	*Mt1*$^{-/-}$*Mt2*$^{-/-}$	Metallothionein 1 and 2 (C)	Viable, life span not reported >52 weeks	Metal and $OH^{\bullet}$ scavenger
[362]	*Mt3*$^{-/-}$	Metallothionein 3 (C)	Viable, life span not reported	Same as above
[229]	*Prdx1*$^{-/-}$	Peroxiredoxin 1 (C)	Hemolytic anemia, increased cancer, shortened life span	Scavenges peroxide[c]
[228]	*Prdx2*$^{-/-}$	Peroxiredoxin 2 (C)	Hemolytic anemia, life span not reported	Scavenges peroxide[c]
[234]	*Prdx6*$^{-/-}$	Peroxiredoxin 6 (C)	"Normal," life span not reported	Scavenges peroxide[c]
[187]	*Sod1*$^{-/-}$	CuZn superoxide dismutase (C, M)	Multiple pathologies, ~30% shortened life span	Scavenger of $O_2^{\bullet -}$
[175]	*Sod2*$^{-/-}$	Mn superoxide dismutase (M)	Neonatal lethal	Scavenger of $O_2^{\bullet -}$
[201]	*Sod3*$^{-/-}$	Extracellular superoxide dismutase (E)	"Normal," no reduction in life span	Scavenger of $O_2^{\bullet -}$
[363]	*Txn1*$^{-/-}$	Thioredoxin 1 (C)	Embryonic lethal	Reducing substrate to Msr and peroxiredoxns
[364]	*Txn2*$^{-/-}$	Thioredoxin 2 (M)	Embryonic lethal	Same as above
[365]	*Txnrd1*$^{-/-}$	Thioredoxin reductase 1 (C)	Embryonic lethal	Reduces thioredoxin by NADPH
[366]	*Txnrd2*$^{-/-}$	Thioredoxin reductase 2 (M)	Embryonic lethal	Same as above
[367]	*Ttpa*$^{-/-}$	Tocopherol transfer protein (C)	Neurodegeneration, life span not reported,	Loading of vitamin E on LDL
[250]	*Ogg1*$^{-/-}$	8-oxo-dG glycosylase (N)	"Normal," life span not reported	Repairs 8-oxo-dG DNA damage product
[250]	*Myh*$^{-/-}$	MutY homologue (N)	"Normal," life span not reported	Removes adenine mispaired to 8-oxo-dG
[250]	*Myh*$^{-/-}$*xOgg1*$^{-/-}$		~50% Shortened life span, massive tumor incidence	

While quite a few antioxidant/oxidative damage repair knockout mice have been generated, true life span data are available for only a small number. For the animals labeled as "Normal", the viability did not differ noticeably from wild type at 6 to 12 months of age. Because all heterozygous knockouts are described as "Normal" they were omitted from the table. With the exception of the *Sod2*$^{+/-}$ mice [212], no data on life span of any heterozygotes are available. Also indicated are the combination knockouts which resulted in a noteworthy deleterious phenotype.

[a] "Normal" indicates that no gross obvious major pathology has been described.

[b] Subcellular localization (C, cytosol; M, mitochondrion; E, extracellular; N, nuclear).

[c] Peroxiredoxins scavenge H_2O_2, short chain organic peroxides, fatty acid alkyl hydroperoxides, and in the case of *Prdx6*, also lipid hydroperoxides.

[179]). The wild-type control mice should show characteristic length of life for their particular strain. In addition, the study should include a large enough sample size to reach statistical power and a defined, though not necessarily inbred, genetic background.

CuZnSOD knockout

CuZnSOD is the major cytoplasmic superoxide scavenger [180] and is also found in the mitochondrial intermembrane space [181,182]. This latter localization is likely of considerable biological importance because the mitochondrial electron transport chain can release superoxide into that compartment [50,183,184]. Despite the assumed importance of CuZnSOD, *Sod1*$^{-/-}$ mice appear healthy at birth and were at first thought not to be under oxidative stress and to be phenotypically normal [174]. However, more recent studies indicate that *Sod1*$^{-/-}$ mice have very high levels of oxidative stress and an acceleration of selected age-related pathologies. Plasma F_2-isoprostanes, a measure of tissue-wide lipid peroxidation, are elevated 2- to 3-fold in *Sod1*$^{-/-}$ mice [185]. This is the highest level of noninduced plasma isoprostanes we have observed in any antioxidant knockout animal in our laboratory (Muller, Van Remmen, et al, unpublished). In the liver, there is an increase in DNA mutation frequency [186], and oxidative damage to DNA, protein carbonyls, and lipid peroxidation are all considerably elevated [187]. Interestingly, fibroblasts from *Sod1*$^{-/-}$ mice grow very poorly in culture [188]). *Sod1*$^{-/-}$ females have very low fertility [189,190], or more accurately, *Sod1*$^{-/-}$ females exhibit a rapid, age-dependent decline in fertility (See Table III in [190]). *Sod1*$^{-/-}$ mice exhibit acceleration of age-related hearing loss [191–193] and macular degeneration [194], as well as an early incidence of cataracts [195]. *Sod1*$^{-/-}$ mice also exhibit vascular hypertrophy and decreased endothelial relaxation [196]. Vascular relaxation is even decreased in aged *Sod1*$^{+/-}$ mice [197]. In a recent study from our laboratory, we reported that *Sod1*$^{-/-}$ mice exhibit a dramatic acceleration of age-related muscle atrophy (hind-leg muscle mass

being 50% lower in *Sod1*$^{-/-}$ compared to wild-type litter mates, by 20 months of age [198]), likely the result of earlier reported neuromuscular-junction defects [174,199,200]. Finally, both average and maximum life span are decreased by ~30% in *Sod1*$^{-/-}$ mice (both in C57B6/J [187] and mixed backgrounds [201]; also see Fig. 3).

Does this mean that *Sod1*$^{-/-}$ mice exhibit accelerated aging? Although several age-related pathologies are clearly accelerated in these mice, the apparent cause of death in 50% of females and 80% of males is hepatocellular carcinoma (which is not a typical age-related pathology in the C57B6/J genetic background). Nevertheless, the remaining mice that show no evidence of hepatocellular carcinoma still have a reduced life span and it is striking to note that carbonic anhydrase III, an age-related marker that decreases with age in both mice and rats [202], was found to be decreased earlier in the life span of *Sod1*$^{-/-}$ mice compared to wild-type mice [187]. Intriguingly, *Sod1*$^{+/-}$ heterozygous knockout mice also appeared to have a small increase in hepatocellular carcinoma and a decrease in average life span; however, the difference was not statistically significant (probably due to the small number of animals studied, $n=12$). No information on the oxidative stress status of the *Sod1*$^{+/-}$ is currently available.

MnSOD knockout

MnSOD (*Sod2*) is the main scavenger of superoxide in the mitochondrial matrix [180,203]. Genetic ablation of this enzyme in mice (*Sod2*$^{-/-}$ mice) results in neonatal lethality between 1 to 24 days after birth, depending on the genetic background [175,204,205]. The cause of death in the longest-lived genetic background (at ~24 days after birth) is neurodegeneration [204]. These mice have ~4-fold increase in DNA oxidative damage as measured by 8-OH-guanine, 8-OH-adenine, and 5-OH-cytosine [206], and severe enzymatic deficits consistent with massive oxidative stress [206]. *Sod2*$^{-/-}$ mice are also extremely sensitive to hyperoxia [207,208]. Although the neonatal lethality of *Sod2*$^{-/-}$ indicates that maintaining low mitochondrial superoxide levels is necessary for life, (i.e., that elevated superoxide is toxic), this does not prove that mitochondrial superoxide (and by extension oxidative stress) causes aging. However, if increased superoxide levels were responsible for accelerated aging, one would expect that a modest decrease of *Sod2* should lead to an increase in endogenous oxidative damage and a diminution of life span. To address this question, our laboratory measured oxidative damage and life span in *Sod2*$^{+/-}$ mice, i.e., mice with one wild-type and one deleted allele of *Sod2* [209–211]. In all tissues assayed, the activity of MnSOD was decreased by ~50% and, no compensatory increase in the activity of Gpx1 or CuZnSOD was measured [210–212]. In addition, *Sod2*$^{+/-}$ mice were more sensitive to paraquat, though not to 10 Gy of gamma irradiation and hyperoxia [207,208,213]. Both nuclear and mitochondrial DNA oxidative damage (8-oxo-dG) were increased by approximately 30 to 80%) [210–212]. Thus, in *Sod2*$^{+/-}$ mice, the increase in oxidative damage would predict a shortened life span if the free radical theory of aging is correct. In contrast, the natural life span of *Sod2*$^{+/-}$ mice in a large cohort study in pure C57B6/J under SPF conditions was statistically indistinguishable from that of wild-type mice ($n=69$ for wild type, $n=70$ for *Sod2*$^{+/-}$ mice). The median and maximum were 30.2 and 40.7 months in the wild-type and 29.4 and 39.1 months in the *Sod2*$^{+/-}$ mice. In addition, various aging biomarkers (cataracts, immune response, advanced glyco-oxidation end products) also failed to show any differences between *Sod2*$^{+/-}$ and wild-type mice. Notably, the tumor burden at 26 months of age was significantly higher in *Sod2*$^{+/-}$ mice, but this did not result in a difference in survival [212]. We believe that this result may have considerable implications for our understanding of the relationship between aging and cancer and we will discuss this later. Nevertheless, in its strongest form the free radical theory makes the simple prediction that more oxidative damage will shorten life span. *Therefore, the simplest interpretation of this study is that the strongest version of the free radical theory is incorrect.* However, this conclusion must be tempered by the fact that not all markers of oxidative damage are actually increased in *Sod2*$^{+/-}$ mice. For example, mtDNA deletions [214], F_2-isoprostanes, protein carbonyls (Van Remmen et al., unpublished data), and methionine sulfoxide levels [215] were not significantly elevated in *Sod2*$^{+/-}$ mice when compared to wild-type mice. Thus, with the exception of increased DNA oxidative damage, there is not much evidence for a significant increase in oxidative damage in vivo in *Sod2*$^{+/-}$ mice. If the *Sod2*$^{+/-}$ mice are not exposed to elevated oxidative stress in vivo, the lack of life-span shortening in the *Sod2*$^{+/-}$ does not directly challenge the free radical theory of aging.

ECSOD knockout

Besides the mitochondrial and cytosolic SOD enzymes, the third and least abundant SOD enzyme is ECSOD, encoded by the *Sod3* gene. ECSOD is located in the extracellular space being present in plasma and most abundantly in the lung [216]. *Sod3*$^{-/-}$ mice are viable and fertile and do not show a decreased life span [201,217]; however, they have been shown to be more sensitive than wild-type mice to hyperoxia. While the lack of life-span shortening would seem like evidence against the free radical theory, it is unclear whether these mice actually have increased levels of oxidative stress: young *Sod3*$^{-/-}$ mice (3 months) do not show an increase in urinary F_2-isoprostanes; in contrast, at 3 months of age mice lacking CuZnSOD (*Sod1*$^{-/-}$ mice) show increased levels of urinary F_2-isoprostanes [201].

Glutathione peroxidase 1 knockout

Glutathione peroxidase 1 is abundant in almost all mammalian tissues and is traditionally thought to be the main cellular scavenger of H_2O_2 (e.g., [218]). It is surprising then that a knockout of this enzyme fails to generate a dramatic phenotype [219]. The only major pathological finding to date is that *Gpx1*$^{-/-}$ mice develop cataracts at a considerably younger age than wild-type mice [220,221]. There was an initial report that *Gpx1*$^{-/-}$ mice had reduced body mass [222], but we have been unable to confirm this in our colony even in *Gpx1*$^{-/-}$*Sod2*$^{+/-}$ double knockout mice [213]. *Gpx1*$^{-/-}$ mice are exquisitely sensitive to paraquat and diquat [223], though not to hyperoxia (100% O_2) [224]. There is currrently no published information on life span of these mice;

however, preliminary data from our laboratory suggest that the lack of Gpx1 does not alter life span.

Peroxiredoxin knockout

While most experimental attention has focused on the major antioxidant enzymes (catalase, superoxide dismutases, and glutathione peroxidases) there is now increasing evidence that these enzymes are only a part of a larger redox-balance maintenance system. The antioxidant defense system is highly complex and contains multiple components. In fact, as late as 2003, a new antioxidant protein, sulfiredoxin, was discovered [178] in yeast and mammals. Also among the recently discovered members of the antioxidant defense are the peroxiredoxins, most of which are thioredoxin peroxidases [225]. The substrates for these enzymes include (but are not limited to) hydrogen peroxide, organic peroxides, and peroxynitrite [225,226]. Six peroxiredoxins have been identified in the mammalian genome [225]. Knockout mouse models lacking peroxiredoxin 1, 2 ,and 6 have been generated and the phenotypes are of considerable interest to the free radical theory of aging [227–229]. Peroxiredoxin 1 is a thioredoxin peroxidase found in most tissues, and it is especially abundant in erythrocytes. Mice null for peroxiredoxn 1 (*Prxd1*$^{-/-}$) have hemolytic anemia, an increase in cancer incidence, and a reduced life span [229]. The extent of life-span reduction is not known, since the survival curve presented in [229] was not complete. The demographics of the survival curve were also somewhat unusual, so that it is only possible to qualitatively assess the effect of *Prdx1*$^{-/-}$ on life span (it would appear to be a ~20% reduction in life span). An increase in oxidative damage to both DNA and protein has been demonstrated in *Prxd1*$^{-/-}$ mice [229,230]. Interestingly, the *Prxd1*$^{+/-}$ heterozygous knockout mice show an increased tumor burden but no decrease in life span compared to wild-type mice [229], a result similar to the life span and pathology study in *Sod2*$^{+/-}$ mice [212]. Peroxiredoxin 2 is also found in most tissues, and is especially abundant in erythrocytes [228]. Mice null for *Prxd2* also suffer from hemolytic anemia [228], but apparently in a milder form than that observed in the *Prxd1*$^{-/-}$ mice. *Prxd2*$^{-/-}$ mice also show decreased circulating erythrocyte half-life. In addition, senescence in mouse embryonic fibroblasts has been shown to be accelerated in *Prx2*$^{-/-}$ mice [231]. Mice lacking peroxiredoxn 6 (*Prx6*$^{-/-}$ mice) are sensitive to hyperoxia (85–100% O_2) [232] and paraquat administration [227,233] and are also more vulnerable to heart ischemia–reperfusion injury [234] In response to hyperoxia, survival of the heterozygous *Prdx6*$^{+/-}$ mice was intermediate to that of the nulls, when compared to wild-type mice. No data on life span are yet available for *Prxd2*$^{-/-}$ or *Prxd6*$^{-/-}$ mice [228]. Mice deficient in catalase or glutathione peroxidase, the other major hydrogen peroxide scavengers in the cell, do not develop anemia or any other pathologies evident in the *Prdx1*$^{-/-}$ and *Prdx2*$^{-/-}$ mice [219,228,229,235], suggesting that peroxiredoxins are potentially more critical in peroxide protection than previously realized. This is even more puzzling considering that the rate constant of glutathione peroxidase reacting with hydrogen peroxide is higher than the rate of reaction of hydrogen peroxide with peroxiredoxins [225]. Cellular localization and the high abundance of peroxiredoxins might explain in part why their absence is more deleterious than lack of glutathione peroxidase 1 [225,228,229]. Phenotype and life span data for the other peroxiredoxins, especially the mitochondrial peroxiredoxin *Prdx3*, will be of considerable importance to the free radical theory.

MsrA knockout

Another component of the antioxidant system that has been studied using a knockout mouse model is methionine sulfoxide reductase. This enzyme selectively reduces (using thioredoxin as an electron source) the sulfoxide of methionine back to its thioether. It is found in the cytoplasm as well as in mitochondria [236]. As discussed above, overexpression of one isoform of this enzyme (methionine-*S*-sulfoxide reductase, *MsrA*) [237]) leads to an increase in life span in flies [153] and yeast [45]. In mice, deletion of *MsrA* shortens life span by ~40% [154]. The average life span of wild-type animals was 680±71 days (n=14), that of *MsrA*$^{+/-}$ was 672±80 days (n=8), and that of *MsrA*$^{-/-}$ was 409±33 days (n=17). These animals also showed increased sensitivity to oxidative stress as judged by their decreased survival under hyperoxia. The number of animals used in this study is very small, and a further cause of concern is the very short life span of the wild-type control. Protein carbonyls were increased in some, but not all, tissues. The cause of death was not analyzed in this study, nor was general pathology reported. However, the heterozygous knockouts did not have a shortened life span. It will be especially interesting to determine whether oxidative damage is elevated in the heterozygote, and if tumor burden is increased as in the *Prxd1*$^{+/-}$ and *Sod2*$^{+/-}$ mice [212,229].

Coq7 heterozygous knockout mice

Ubiquinone/ubiquinol is the electron carrier between Complex I and Complex II to Complex III; it is thought that a ubisemiquinone is responsible for the superoxide produced by Complex III and perhaps also by Complex I (reviewed in [50]). In yeast *S. cerevisiae*, knockout of ubiquinone biosynthesis can partially rescue SOD knockouts [52,238,239] and mutants lacking ubiquinone demonstrate decreased mitochondrial ROS production [240]. As already noted, inactivation of the *C. elegans* gene *clk-1* (required for ubiquinone biosynthesis) leads to an increased life span. While completely knocking out *Coq7* (the mouse orthologue of *clk1*) is lethal in mice [241,242], *Coq7*$^{+/-}$ mice display a substantial increase in mean life span in three different genetic backgrounds (29Sv/J, a 15% increase, n=10 and 12; 129Sv/J x Balb/c, a 31% increase, n=9 and 5; C57BL/6J, a 20% increase, n=5 and 8 [242]). In addition, *Coq7*$^{-/-}$ embryonic stem cells demonstrate reduced ROS levels, decreased ROS sensitivity, and decreased DNA damage [242]. These data would provide exceptionally strong evidence in favor of the free radical theory of aging, if not for the fact that the life-span data are weak. The life-span studies were conducted with exceptionally small numbers (as few as 5 animals per group) and it is interesting to note that the smallest life-span extension was observed in the experiment with the

largest *n* size (the 129 × Blab/c). Further, the survival study also consisted of mixed sexes, which do differ in the C57B6/J background with respect to longevity. Finally, the life span of control animals was exceptionally short (e.g., the wild-type C57B6/J median life span was 686 days [242] vs 930 days in our colony), indicating the potential of infectious disease in the colony.

p66shc knockout mice

p66shc is a splice variant of p52shc/p46shc [243], a cytoplasmic signal transducer involved in the transmission of mitogenic signals from activated receptors to Ras [244]. Mice null for p66shc exhibit a 22% in median and a 30% increase in maximal life span [245] and show an increased resistance to the oxidative stressor, paraquat. p66Shc has been suggested to regulate intracellular levels of ROS and to mediate ROS upregulation during p53-induced apoptosis. Furthermore, the p53-p66Shc pathway has been found to act as a sensor of the levels of intracellular oxidative signals and as a regulator of intracellular levels of oxidants and of oxidative damage [246]. This study would seem to be consistent with the oxidative stress theory; however, the very short life span of the wild-type control, the small number of animals, and the lack of information on genetic background, sex, and the pathological status of the mice confound a direct interpretation of this study.

Combinations of antioxidant knockouts

The question of redundancy and complementation in the antioxidant system is often raised. For example, crossing *Gpx1*$^{-/-}$ and *Gpx2*$^{-/-}$ (neither of which have a strong deleterious phenotype on their own) results in a lethal intestinal inflammation [247] (though interestingly, this does not happen when performed on a pure C57B6/J background [248]). The glycosylases Ogg1 and Myh work in conjunction to prevent mutagensis by 8-oxo-dG, Ogg1 by removing 8-oxo-dG from DNA and Myh by removing adenine misincorproated with 8-oxo-dG. While *Ogg1*$^{-/-}$ and *Myh*$^{-/-}$ mice alone are described as "Normal" (in the case of *Ogg1*$^{-/-}$, initial reports of lung tumors [249] have not been confirmed [250]), the *Ogg1*$^{-/-}$x*Myh*$^{-/-}$ double knockout mice exhibit a dramatic (40 to 60%) reduction in life span brought about by massive carcinogenesis [250]. This result seems to be the exception rather than the rule. For example, crossing *Sod3*$^{-/-}$ and *Sod1*$^{-/-}$ mice does not yield higher oxidative stress or a shorter life span than observed in *Sod1*$^{-/-}$ mice alone [201]. Similarly, we find that *Sod1*$^{-/-}$x*Sod2*$^{+/-}$ mice or *Sod1*$^{-/-}$x*Gpx1*$^{-/-}$ or *Sod1*$^{-/-}$x*Sod2*$^{+/-}$x*Gpx1*$^{-/-}$ also do not have a shorter life span than *Sod1*$^{-/-}$ mice alone (Fig. 3). The lack of "interaction" of various SOD knockouts is somewhat expected considering that anionic superoxide is highly membrane impermeable (reviewed in [184]), and reaffirms that the pools of superoxide are highly segregated. However, that *Gpx1*$^{-/-}$x*Sod1*$^{-/-}$ mice are no different from *Sod1*$^{-/-}$ alone is somewhat surprising. We performed a more in-depth study on *Sod2*$^{+/-}$*Gpx1*$^{-/-}$ (heterozygous–homozygous knockout) mice characterizing oxidative biochemistry and stress resistance. These animals are phenotypically normal with respect to gross physiology but are considerably more sensitive to exogenous oxidative stress (paraquat, diquat) than either knockout alone [213]. In addition, *Sod2*$^{+/-}$*Gpx1*$^{-/-}$ mice have increased sensitivity to exogenous oxidative stressors to which the single knockouts exhibit the same sensitivity as wild type (e.g., gamma radiation). The increased incidence of cataracts observed in *Gpx1*$^{-/-}$ mice was not further aggravated in *Sod2*$^{+/-}$ *Gpx1*$^{-/-}$ mice [220].

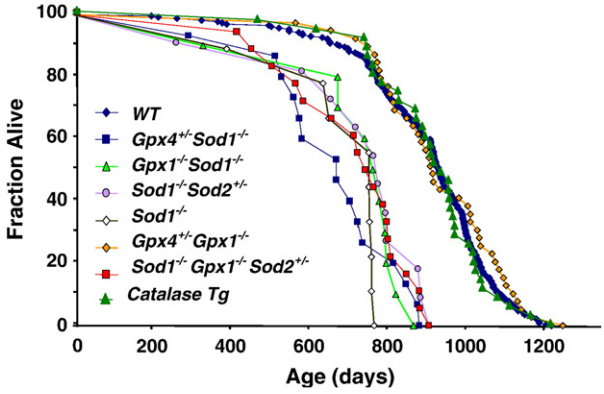

Fig. 3. Life span of combined antioxidant knockout mice. Survival studies were conducted at the Nathan Shock Center of the University of Texas Health Science Center under specific pathogen-free conditions. Mice lacking *Sod1*$^{-/-}$ show a roughly 30% reduction in life span; however, combined deficiency *Sod1*$^{-/-}$ and other antioxidant enzymes (*Sod2*$^{+/-}$, *Gpx4*$^{+/-}$, *Gpx1*$^{-/-}$) did not further reduce life span, even (surprisingly) for a mouse completely lacking Sod1, Gpx1, and partially deficient in Sod2 (*Sod1*$^{-/-}$ *Gpx1*$^{-/-}$ *Sod2*$^{+/-}$ mice). Actual absence of Sod1, Sod2, and Gpx1 was verified by enzymatic activity (data not shown).

Overexpression of antioxidant enzymes in mice

While the study of antioxidant knockouts can be used to test the strong oxidative hypothesis (that is, demonstrate if oxidative stress is limiting to life span), the alternative approach is to overexpress antioxidant enzymes with the intention of reducing in vivo oxidative stress and extending life span. Extension of life span in a transgenic would demonstrate that oxidative stress is not only limiting to life span, but in fact must be the main life-span-determining factor. As previously noted, the overexpression approach has provided some positive results in *D. melanogaster*. In mice, only a handful of studies examining life span in response to overexpression of antioxidant enzymes have been published. A large cohort study showed that overexpression of CuZnSOD does not increase life span [251]. A key question in studies using overexpression of antioxidant enzymes is whether increased activity of antioxidant enzymes actually reduces endogenous oxidative stress [27,252,253]. In fact, in some instances it may even increase oxidative damage. Indeed, high overexpression of either CuZnSOD and MnSOD leads to very deleterious phenotypes [27,252–254]. While overexpression of antioxidant enzymes generally provides protection against oxidative stress in cell culture models [255], in the whole organism, this is not at all clear. Thus we found that overexpression of catalase in mice actually leads to an increased sensitivity to gamma irradiation [256]. Further, cross-species comparisons reveal that although life span negatively correlates with oxidative damage (as the oxidative theory would predict),

the levels of antioxidant enzymes are consistently lower in longer lived ones [50,168,257].

Thioredoxin 1 transgenic

Yodoi's group reported an increase in life span of mice with a ~3-fold overexpression of human thioredoxin 1 (*hTrx1*) in kidney, liver, brain, skin, and lung. In addition, greater than a 6-fold increase was measured in the heart [258]. An increased resistance to cerebral ischemia and to UV-induced oxidative stress was also demonstrated in *hTrx1* mice. *hTrx1* mice lived ~35% longer than littermate controls. In addition, a 22% increase in maximal life span was observed [259]. This result would be supportive of the free radical theory of aging, though while the sample size (n=53) was appropriate, the life span of the wild-type control was very short (median life span of ~500 days in the WT C57B6 control mice in their study).

Metallothionein transgenic mice

Metallothionein is a cysteine rich (about one-third of its constituent amino acids are cysteine) low molecular weight protein with toxic heavy metal (Hg and Cd) detoxification and free radical scavenging abilities [260]. Metallothionein is also highly induced by oxidative stress [261]. Transgenic mice overexpressing metallothionein (MT-TG) were shown to have an increased mean life span of ~15%, relative to control mice. Cardiomyocytes from MT-TG mice have attenuated age-related increases in superoxide generation, cytochrome *c* release, $p47^{phox}$ expression, and higher aconitase activity [262]. These data are consistent with the free radical theory; unfortunately, life-span data from MT knockout mice have not been reported [263,264].

Mitochondrial-targeted catalase transgenic mice

Transgenic mice that overexpress human catalase in peroxisomes, the nucleus, or mitochondria were generated to test the effect of catalase overexpression on life span [265]. The life-span study was conducted with large (>30 animals per genotype) numbers of animals and the life span of the wild-type control animals was in the expected range (median: ~27 months). There was a trend of increased life span observed in all catalase overexpressing lines; however, only the mitochondrial overexpression of catalase (MCAT mice) induced a statistically significant extension of median and maximum life span of 5 and 5.5 months (~21% extension). Concomitant overexpression of MnSOD further (though modestly) increased life span. Age-related changes in oxidative damage (8-oxo-dG and mitochondrial DNA deletions in skeletal muscle), hydrogen peroxide production (heart mitochondria), and hydrogen peroxide-induced aconitase inactivation (in heart) were attenuated in MCAT animals. It should be noted that the highest levels of MCAT were found in heart and skeletal muscle, suggesting a potential role for tissue-specific antioxidant upregulation in life-span extension. A significant delay in the development of age-related cataracts was also discovered in these mice [220]. This is an important finding because the development of age-related cataracts has been shown to be inversely related to life span in humans [266], and its incidence is reduced in mice that exhibit extended longevity [220]. This study supports the free radical theory rather strongly; however, the authors state that while the survival studies were conducted in mice backcrossed to C57B6 for 2 to 4 generations, life span extension in mice backcrossed at least 9 generations extension appears to be less evident. This issue must be resolved before the impact of this study on life span can be rigorously evaluated. We have generated mice overexpressing catalase 2- to 4-fold in its normal cellular location [256], the peroxisome, and did not observe a significant extension of maximal life span, although median life span was modestly increased in one founder line (by up to 13%; Fig. 3).

Polymorphisms in antioxidant genes and human life span

Although it is not possible to study the knockout of antioxidant enzymes in humans, natural variation, measurable by genetic polymorphisms, is a substitute. While few studies have directly investigated the effect of antioxidant enzyme polymorphisms on life span, an abundant literature exists on whether certain polymorphisms present risk factors for disease in which oxidative damage is suspected. A dedicated review would be necessary to cover this vast and contradictory literature. For the sake of brevity, we focus on one of the most studied polymorphism, the V16A polymorphism of MnSOD. A polymorphism in the mitochondrial localization sequence of MnSOD (the Val/Ala polymorphism at position -9 from the mature protein or 16 from the full-length precursor) has been evaluated as a risk factor for a number of pathologies (although there are suggestions that the Val allele leads to less MnSOD import [267], we are unaware of a clear demonstration of decreased MnSOD activity as a result of this polymorphism and, if so, that this actually results in changes in oxidative stress).

For some pathologies, such as diabetic nephropathy, studies in diverse populations agree that the Val allele (presumably yielding lower MnSOD activity) constitutes an independent (though modest) risk factor [268–270]. However, in other pathologies, the literature is highly contradictory. To illustrate this, consider the case of the role of MnSOD and breast cancer, a question on which a large number of (large scale) studies have been published. Paradoxically, the Ala allele (presumably yielding high activity) has been found by some studies to increase the risk of breast cancer [271–273] though others find no association [274–279]. Another study reported no association between the Ala polymorphism of MnSOD and breast cancer, but reported a positive association between a combination of the Ala allele in MnSOD and the Pro198Leu allele in Gpx1 [280], though another study using a similar approach did not observe this [281]. Yet another study reported that the Val allele is a risk factor for early incidence of breast cancer [282].

Regarding life span, the literature is sparse. Three, small-scale studies have reported that the presence of the polymorphism does not correlate with mortality [283–285]. Assuming that the polymorphism actually decreases MnSOD activity in vivo (which remains to be conclusively shown), these results would mirror what we discovered in $Sod2^{+/-}$ mice. However, even assuming that enzymatic activity is decreased, it remains to be seen whether this actually affects the levels of oxidative stress

(which is not clear even in our *Sod2*$^{+/-}$ mice). We suggest that at present, this area is not sufficiently mature to reach definite conclusions concerning the role of oxidative stress in human life span.

Interaction of the free radical theory with other aging theories

The free radical theory does not exist in a vacuum and not even its most ardent proponents would seriously argue that oxidative stress is the *only* factor limiting animal life span. If aging is a consequence of decreased selective pressures at a later cohort age due to high extrinsic mortality [286], then it follows that any metabolic process that can accumulate deleterious changes over time will eventually become limiting to life span. It is thus unnecessary to argue that various aging theories are mutually exclusive; we would suggest that the free radical theory can "fit" with various other mechanisms of aging.

Cell senescence, telomere shortening, and oxidative stress

Attempts at synthesizing two major areas of focus in aging research, cell senescence [287,288] and free radicals, have been made since the 1970s (for a recent review see [289,290]). Early results by Packer and Smith suggested that vitamin E treatment could completely prevent cell senescence [291]; however, this result proved to be irreproducible [292]. Nevertheless, it was observed that decreasing oxygen tension, from the customary 21% O_2 to more physiological levels (3% O_2, as would be found in vivo) led to an increase in cell doublings before senescence (i.e., an increase in the Hayflick limit or replicative life span [293–296]). Similar effects were also reported using antioxidants [296–298]. In the 1990s, von Zglinicki et al. reported that a mild increase in oxygen tension (~40%) triggered senescence within 3 cell divisions in human fibroblasts [299]. von Zglinicki and co-workers proposed that oxidative damage to telomeres was responsible for the rapid triggering of senescence [299–301] and recent studies show that telomeric DNA may be particularly sensitive to oxidative damage [302]. Following von Zglinicki et al.'s report, other investigators, using different oxidative stressors and different cell types, have reported very similar results. Mild oxidative stress reduces clonal life span and conversely, reduction of oxidative stress extends clonal life span [303–307]. Guarente's lab has provided additional evidence in this general direction, with the demonstration that RNAi knockdown of *Sod1* triggered early senescence in human fibroblasts [308]. This result is consistent with the earlier report by Epstein's laboratory that fibroblasts derived from *Sod1*$^{-/-}$ mice failed to grow at all in culture [188]. A great breakthrough in this area occurred when Campisi's lab demonstrated that senescence could be prevented completely in primary mouse cells when the cells were grown at 3% oxygen, instead of the customary 21% [309]. This also resulted in a dramatic reduction of oxidative damage-signature mutations [310]. In other words, these investigators demonstrated that in vitro senescence in mice cells was directly related to oxygen toxicity, i.e., oxidative damage.

Wright, Shay, and co-workers reexamined the relationship between oxygen tension and clonal life span in human adult and fetal cells; these authors concluded that low oxygen tension (2 to 5%) increased clonal life span of both adult and embryonic cells as compared to 21% O_2 [303]. These increases in clonal life span at low oxygen were accompanied by a reduced rate of telomere loss. Intriguingly, while adult cells could be immortalized by telomerase overexpression at 21% O_2 tension, embryonic cells (WI38) could only be telomerase-immortalized at low O_2. The inability of WI38 to be telomerase-immortalized at 21% O_2 was found to be due to oxidative stress-driven telomere shortening, which was greatly diminished at low O_2. These results cement the conclusion that oxidative stress plays a key role in telomere shortening and cell senescence [303].

Integration of oxidative stress and cell senescence (and, by extension, telomere shortening and in vitro senescence in general) is thus very well established in vitro. The only question remaining is whether this relationship (and the underling phenomenon) is also true, and biologically significant, in vivo. Certain correlative data would point in that direction. Short telomeres have been identified as markers in human disease in which oxidative stress is also thought to be involved (for example, [311], reviewed in Ref. [312]). Even more interesting is the finding that telomere length is a statistical predictor of survival in humans over 60 [313] and mortality and morbidity in several age-related diseases [314]. It is tempting to speculate that this may be the result of an underlying oxidative stress, though further work will be needed to prove this point.

Oxidative stress and genomic instability

Genomic instability, that is, the time-dependent loss or corruption of information in DNA, has been suggested as a mechanism of aging as early as the 1950s. In some respects, the genomic instability theory of aging and the free radical theory sprang from the same source. Studies in which mice exposed to radiation showed a reduction in life span [10], and it was the same experiments that led Leo Szilard to propose the mutation theory of aging [315]. While the primary focus of Szilard's theory was point mutations, the emphasis has now shifted to not only point mutations but small deletions, insertions, large translocations, and illegitimate recombination, i.e., chromosomal instability [316,317]. The strongest evidence in favor of the genomic instability hypothesis of aging is that many of the segmental progeroid syndromes summarized by Martin (e.g., Werner and Cockayne syndrome, ataxia-telangictasia [318]) have now been identified as mutations in DNA repair genes [319]. By contrast, there are no segmental progeroid syndromes that can be unambiguously attributed to an oxidative stress-related gene. Nevertheless, what is the initial cause of the endogenous genomic damage and instability? Clearly, reactive oxygen species are an obvious potential candidate [143,319,320]. Much work has gone into exploring the hypothesis that oxidative damage to DNA causes mutations and cancer. At least ~100 different types of oxidative DNA lesions have been reported, including base modifications (for example, 8-oxo-dG, thymidine

glycol, and 8-hydroxycytosine), single- and double-strand breaks, and interstrand cross-links [321]. Several studies in invertebrates show that reactive oxygen species cause point mutations (both nuclear and mitochondrial) and large-scale genomic rearrangements [78,322–325].

Many of the progeroid mice models also exhibit increased sensitivity to oxidative stress [319]. Senescence of cells from Werner syndrome patients occurs at much earlier passage number than in normal cells and this early senescence can be greatly delayed by growing cells at 3% O_2 rather than the usual 21% O_2 [294]. A mouse strain overexpressing high levels of CuZnSOD (high enough to cause increased, rather than decreased oxidative stress) was found to exhibit an increased number of double-strand breaks at the cellular level, which could be modulated by oxygen tension [143]. *Ku86*$^{-/-}$ embryonic fibroblasts also exhibit increased double-strand breaks in culture: as with the effect of *Sod1* overexpression, this can be decreased by growing cells at 3% O_2 instead of 21% [143]. Strikingly, crossing this strain with *Ku86*$^{-/-}$ (defective in double-strand break repair and exhibiting features of accelerated senescence [326]) with *Sod1* tg mice (the same line as in the studies above) results in synthetic lethality [142]. The phenotype of *Ku86*$^{-/-}$ mice in a pure C57B6/J background is considerably worse (the above results were conducted in mixed backgrounds), with very few surviving longer than a month after birth. Treatment with the thiol antioxidant *N*-acetylcysteine to dams increased the percentage of *Ku86*$^{-/-}$ pups alive at 1 month after birth from 1.5% in untreated to 7.7% after supplementation, a result that further strengthens the role of endogenous oxidative stress in genomic instability [327]. Similarly, cancer incidence can be reduced and life span extended by *N*-acetylcysteine supplementation in *Atm*$^{-/-}$ and *p53*$^{-/-}$ mice, both suffering from genomic instability due to defective DNA damage checkpoint signaling [328,329]. While more data of this nature need to be available to make a stronger conclusion, it would seem that endogenous oxidative damage is indeed a significant driver of genomic instability.

Oxidative stress and mitochondrial DNA

Not long after it was discovered that mitochondria have their own genetic apparatus, Harman proposed that mitochondria play a central role in the free radical theory of aging [16]. This idea was developed further by Miquel et al. [330], and the notion that mtDNA mutagenesis played a role in aging took hold. The phenotypical importance of mutations in mtDNA was demonstrated by Wallace et al. [331] and Holt et al. [332], who first showed that Leber's hereditary optic neuropathy and mitochondrial myopathies were caused by mtDNA mutations (reviewed in [333]). Because mtDNA is so close to the site of mitochondrial ROS production, it is exposed to considerably higher oxidative stress, resulting in 3-fold higher levels of DNA oxidative damage (the previously quoted 20-fold figure is apparently due to an isolation artifact [334,335]). In the 1990s a series of papers reported that the frequency of mitochondrial DNA deletions increases dramatically with age, being essentially undetectable in young individuals and reaching levels as high as 2% of mtDNA in old individuals. This age-related increase in mtDNA deletions was found in organisms as diverse as worms, mice, and humans (reviewed in [24,336]). The same is also true with mtDNA point mutations [337,338]. Certain mtDNA polymorphisms have been found in increased frequency in centenarians, implying a protective effect during aging [339–341]. Similar protective effects of mtDNA polymorphisms have been reported for the age-related neurodegenerative condition, Parkinson's disease [342].

Although the mitochondrial theory of aging clearly arose out of the free radical theory, in some respects, the focus on mtDNA instability has forced a split between these two hypotheses. Although reactive oxygen species clearly are capable of promoting mtDNA instability [322,343–345] there are potentially many other causes of mtDNA mutagenesis (for example, misincorporation of nucleotides during DNA replication). Indeed, mutations in proteins involved in mtDNA repair and replication, such as the DNA helicase twinkle and DNA polymerase gamma (PolG), have since been identified as causing "mitochondrial" diseases in human patients, i.e., syndromes resembling the classical mitochondrial myopathies and accumulating mtDNA deletions and mutations [346,347]. The best evidence to date that mtDNA point mutations are involved in aging comes from two studies in mice involving the manipulation of DNA polymerase gamma (PolgA). PolgA is the nucleus-encoded catalytic subunit of mtDNA polymerase that is responsible for proofreading newly synthesized mtDNA, via its 3′-5′-exonuclease activity. Zassenhaus's lab reported that heart-specific overexpression PolG lacking the proofreading function caused a massive cardiomyopathy leading to death within weeks after birth [348,349]. Using a knock-in, rather than an overexpression approach, Trifunovic et al. generated mice lacking the proofreading activity of PolgA throughout the organism [350]. These mice displayed a milder phenotype. Still, both mean and maximal life spans were reduced to 48 and 61 weeks, respectively (a 70% reduction relative to wild-type mice). mtDNA point mutation levels were increased by ~3- to 5-fold; amounts of deleted mtDNA were also increased. It is tempting to speculate that these mitochondrial point mutations lead to an increase in mitochondrial ROS production (as has been documented for certain mutations causing human disease [351,352] and accelerated aging in *C. elegans* and *Drosophila* [79,353]). However, Kujoth et al. [354] reported no changes in lipid peroxidation (F_2-isprostanes) in liver, skeletal muscle, and heart in a similar model of PolgA mutant mice. Further, liver DNA did not show increased oxidative damage, as 8-hydroxy-2′-deoxyguanosine was unchanged in these mice relative to controls. Intriguingly, mutations in human polG have also been found to be profoundly pathogenic. In addition to causing progressive external ophthalmoplegia, mutations in polG also elevated the risk for Parkinson's disease and decreased the age of menopause to below 35 years of age [347]. There are still many questions regarding the role of mtDNA mutations in aging [355] and while these studies do not prove that mtDNA instability causes normal aging, they do certainly show that mtDNA

mutations *can* be a life-span-limiting factor (even when only moderately elevated) and in the absence of elevated oxidative stress/damage. But the question remains: in wild-type animals, is the dramatic age-related increase in mitochondrial DNA mutagenesis due to misincorporations by PolG or due to oxidative DNA damage? At present, we see no evidence that the latter does not contribute.

Conclusions and future directions

The free radical theory has come a long way since it was first proposed. It is now clear that oxygen radicals are produced in vivo under normal conditions and that if left unchecked, are incompatible with animal life. Yet, multiple layers of antioxidant defenses exist and the question remains whether under normal conditions oxidative damage (although clearly present) is high enough to be limiting to life span. In some, but not all, invertebrate model organisms this seems to be the case. In our judgment, the weight of evidence currently does not support that oxidative stress is limiting to life span in *S. cerevisiae* clonal aging. In *C. elegans* this remains inconclusive. However, oxidative stress is unambiguously limiting to hyphae senescence in *P. anserina* and chronological aging of *S. cerevisiae*. In *D. melanogaster*, considering the strong dose–response between life span and O_2 tension, the life-span shortening in *Sod1* and *Sod2* knockout, the life span extension by *MsrA*, *Gclc*, *ApoD,* and *Sod2* overexpression, a tentative case can be made (despite some contradictory data) that oxidative stress appears to be one of the main limiting factors for life span, even in wild-type flies under normal atmospheric oxygen tension.

However, is oxidative stress sufficiently high under normal conditions to limit life span in mice (and by extension, in mammals in general)? While we have reviewed that ablation of *Sod1*,[1] *Prxd1*, and *Sod2* shortens life span, mice with only 50% of *Sod2*, *Prdx1, and Sod1* have a normal (or near normal) life span. The life-span and pathology data from $Sod2^{+/-}$, $Prxd1^{+/-}$, and $Sod1^{+/-}$ mice demonstrate that although antioxidant enzyme deficiency causes an elevated tumor burden (and in the case of $Sod2^{+/-}$ increases oxidative stress, although this depends on the marker in question, see below), this does not actually decrease survival (life span) nor does it accelerate aging biomarkers. Lack of life-span decrease despite elevated oxidative stress is in direct contradiction with the strong version of the free radical theory. In fact, this result is more damaging to the theory than lack of life-span extension by antioxidant overexpression, because the latter experiment would only indicate that oxidative stress is not the *only* life-span-limiting factor, whereas the former would indicate that it is not life span limiting, *period*. However, the interpretation of the $Sod2^{+/-}$ result must be tempered somewhat by the fact that the only marker of oxidative damage to be increased is 8-oxo-dG. Other markers of oxidative stress, such as F_2-isoprostanes, mtDNA deletions, and methionine sulfoxide are not altered. We should emphasize though, that the actual life span of few antioxidant knockout mice is known for sure (see Table 1). Further, the in vivo workings of the antioxidant system are still incompletely understood (see, for example, the discussion on the role of Gpx1 vs peroxiredoxns in scavenging H_2O_2). Thus, despite tremendous recent progress, 50 years or so after Harman's initial proposal, the status of the free radical theory remains inconclusive.

What can we expect in the next few years for the free radical theory? An ongoing trend in the field is that the recognition of ROS can be important signaling molecules [356]. Conversely, there is increasing evidence for signaling networks that coordinately "manage" the levels of ROS within the cell. Potential examples includes FOXO3a [357], p66SHC [245], Nrf2-ARE [358], and most recently, p53 [329]. It was recently discovered that p53 regulates the expression of multiple antioxidant genes and that $p53^{-/-}$ mice have elevated oxidative stress [329]. The importance of this oxidative stress is emphasized by the fact the life span and carcinogenesis in $p53^{-/-}$ mice can be rescued by pharmacological doses of the antioxidant *N*-acetylcysteine [329]. Thus, rather than altering individual antioxidant enzymes, perhaps a better way of testing the free radical theory is to understand, and eventually to manipulate, the signaling pathways that control intracellular levels of ROS.

[1] Incidentally, knockout of CuZnSOD causes shortened life span in all aging systems in which it was tested, *S. cerevisiae* for both clonal and chronological lifespan, *Drosophila* and *Mus.*

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