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Oxidative Stress and Aging in *Caenorhabditis Elegans*

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Much attention has been focused on the hypothesis that oxidative damage plays in cellular and organismal aging. A *mev-1* (*kn1*) mutant of *Caenorhabditis elegans*, isolated on the basis of its methyl viologen (paraquat) hypersensitivity, is also hypersensitive to elevated oxygen levels. Unlike the wild type, its life span decreases dramatically as oxygen concentrations are increased from 1% to 60%. Strains, which bear this mutation, accumulate fluorescent materials and protein carbonyl groups, markers of aging, at faster rates than the wild type. We have cloned *mev-1* gene by transformation rescue and found that it is, in fact, the previously sequenced gene (*cyt-1*) that encodes succinate dehydrogenase cytochrome b. A missense mutation abolishes complex II activity in the mitochondrial membrane but not succinate dehydrogenase enzyme activity per se. These data suggest that CYT-1 directly participates in electron transport from $FADH_2$ to coenzyme Q. Moreover, mutational inactivation of this process renders animals susceptible to oxidative stress and, as a result, leads to premature aging.

INTRODUCTION

It has been proposed that free radicals, especially those derived from molecular oxygen, may accelerate aging in animals [1]. Oxygen is indispensable for aerobic organisms, serving as a terminal electron acceptor. Paradoxically, however, oxygen can also damage cells via its reduction to highly toxic compounds, such as superoxide radicals, peroxide radicals and hydrogen peroxide [2]. Free radicals are also produced by ionizing radiation, near UV light and redox-active compounds as well as by oxygenating enzymes such as xanthin oxidase. These reaction species inactivate biological materials such as DNA, proteins and lipids in organisms, thereby accelerating cellular aging. A number of genes act to modulate this process. Some may act to accelerate aging by increasing free radical production. Conversely, others prolong aging by producing antioxidant defenses and repair systems.

Given the above, a genetic approach is useful for elucidating the involvement of free radicals in aging. In particular, *Caenorhabditis elegans* (*C. elegans*) has proven to be a valuable organism for the study of aging. This free-living nematode can be grown using simple microbiological techniques and has a short generation time of 3.5 days. The maximum life-span is about 25 days. In addition, both molecular and Mendelian genetics has been exploited. To investigate the possible role of oxygen free radicals in aging, mutants of *C. elegans*, which are hypersensitive to methyl viologen (paraquat) were isolated. The

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toxic effects of this herbicidal drug on cells and animals are believed to be mediated by superoxide anions [3]. The focus of the present study is on the characterization of one short-lived mutant, *mev-1*, that is hypersensitive to oxygen

MUTATION ISOLATION, SENSITIVITY TO METHYL VIOLOGEN AND OXYGEN

Two methyl viologen (paraquat)-sensitive mutants were isolated from ethyl methane sulfonate-mutagenized wild-type animals [4]. L1 larvae of these mutants were cultured on plates containing various concentrations of methyl viologen. At the highest concentrations of methyl viologen examined (0.2mM), most wild-type animals developed into L4 larvae or adults within 4 days. However, these mutants usually arrested as L1 or L2 larvae [4]. (Figure 1a)

One of these mutants, *mev-1* (*kn1*), is even more hypersensitive to oxygen gas than to methyl viologen. Whereas wild-type animals are capable of developing nearly normally under 90% oxygen, few *mev-1* larvae survive a 3-day exposure. The growth and movement of *mev-1* larvae were nearly normal for the first day of exposure, but arrested thereafter [4]. (Figure 1b).

Life span

The mean and maximum life spans of both the wild type and *mev-1* increased and decreased under conditions of low and high concentrations of oxygen, respectively. The mean and maximum life spans of the wild type under 21% oxygen were 26 days and 33 days, respectively. Their life spans under 1% oxygen were extended significantly (mean, 30 days; maximum, 41 days), while those under 60% oxygen were shortened considerably (mean, 23 days; maximum, 28 days). Those under oxygen concentrations within a range between 2 and 40% remained unchanged. In addition, the mean and maximum life spans of the mutant under 21% oxygen were 21 and 26 days, respectively. Their life spans under 1% oxygen were also longer (mean, 26 days; maximum, 35 days), while those under 60% oxygen were much shorter (mean, 8 days; maximum, 10 days). The life spans were observed to vary over a wide range of oxygen concentration [4,5]. (Figure 2)

Aging marker

Fluorescent materials (repofuscin) and protein carbonyl derivatives are formed in vitro as a result of metal-catalyzed oxidation and accumulate during aging in disparate model systems [6–10]. These results indicate that fluorescent materials and protein carbonyl modifications can be a specific indicator of oxidized lipid and protein.

a) fluorescent materials

As observed using fluorescence microscopy, the intestinal cells of *C. elegans* contain blue autofluorescent granules and other materials. These granules and materials accumulated in the wild type in an age-dependent fashion. On day 5 after hatching, the amount of fluorescence appeared to be approximately the same in wild type and *mev-1* mutants. Conversely, on day 10, the substances in the *mev-1* mutant had accumulated to a much higher level than in wild type.

In addition, the fluorescent material in methanol/water extracts of both wild type and *mev-1* accumulated with increasing age. The fluorescent material in *mev-1* accumulated to a greater extent than in the wild type. The amount of *mev-1* extracts on day 10 was approximately two times greater than that observed in the wild type on the same days.

When incubated under 90% oxygen, the fluorescent materials in the *mev-1* but not wild-type accumulated more rapidly, compared with incubation under atmospheric conditions. Conversely, the materials did not accumulate in either wild type or *mev-1* under 2% oxygen [11]. (Figure 3a)

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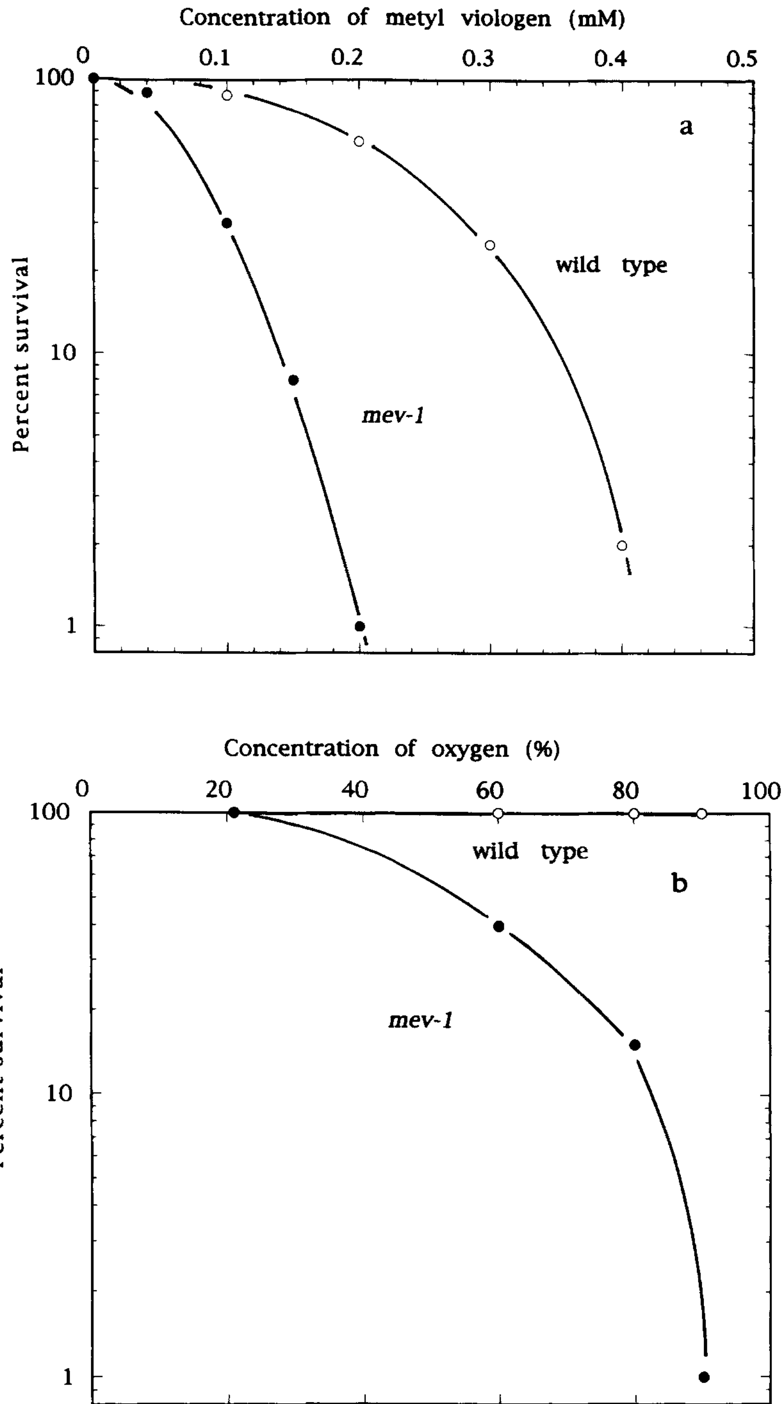

FIGURE 1a,b Sensitivities of wild type and *mev-1* (*kn-1*) to methyl viologen (a) and oxygen (b). (from Reference 4)

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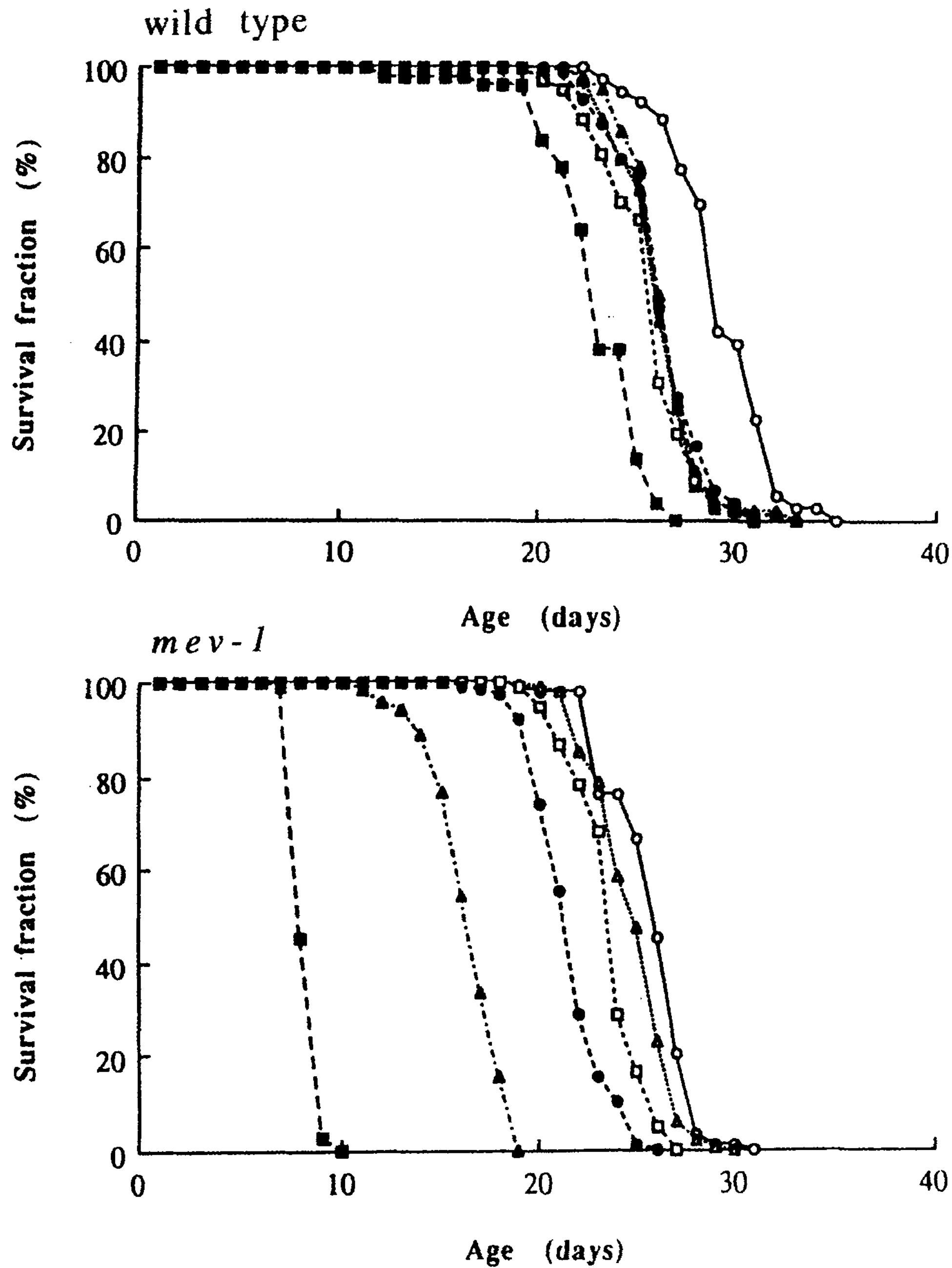

FIGURE 2 Survival curves of wild type and *mev-1* (*kn-1*) under various concentrations of oxygen. At 4 days after hatching, the wild type and *mev-1* (*kn-1*) mutant (B) were exposed to 1% (○), 2% (△), 8% (□), 21% (●), 40% (▲), or 60% oxygen (■). (from Reference 5)

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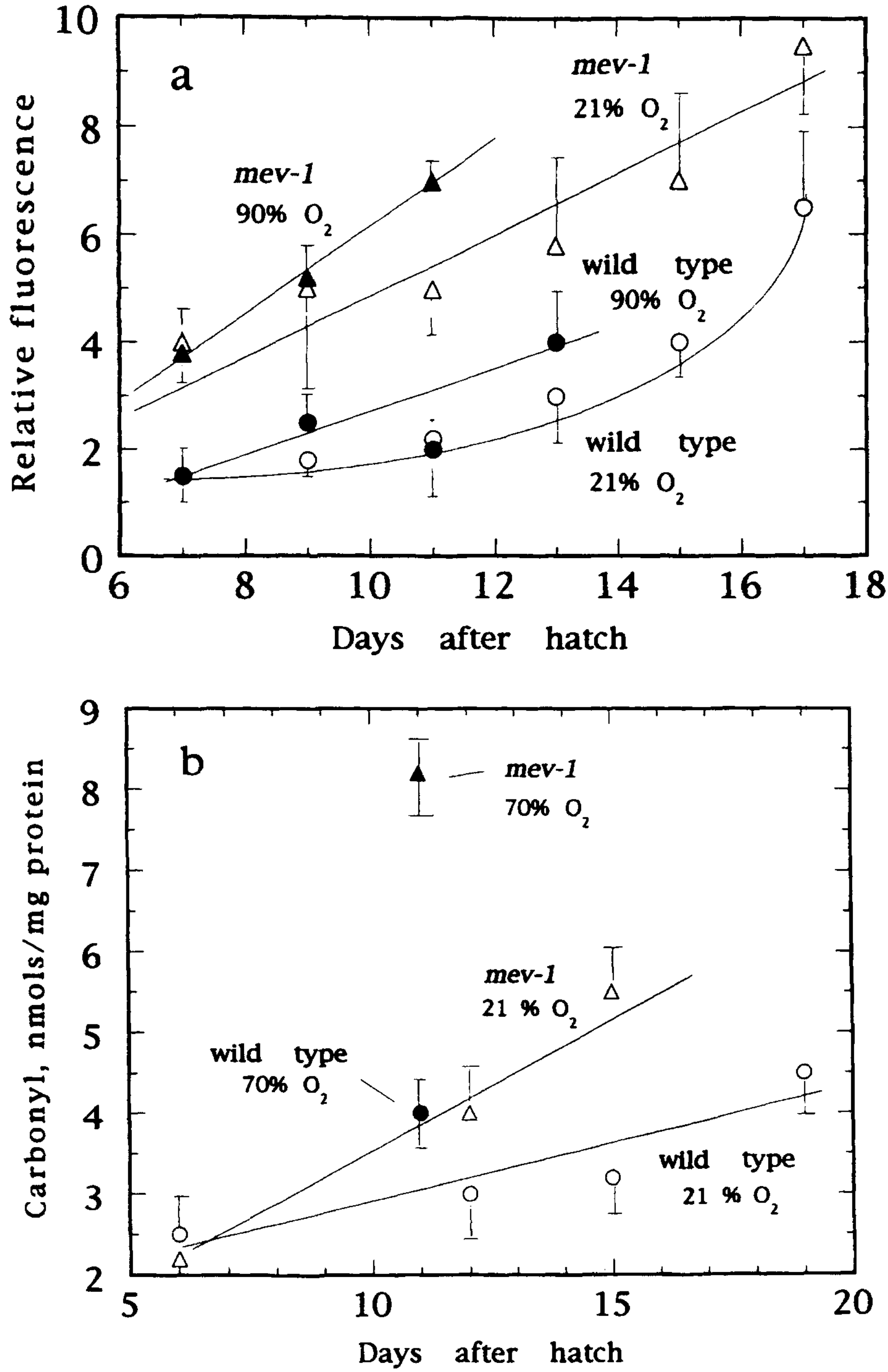

FIGURE 3a,b Accumulation of fluorescent compounds (a) and carbonyl compounds (b) in wild type and *mev-1* (*kn-1*) grown in either 21 or 90% oxygen. (modified from Reference 11 and 12)

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b) protein carbonyl groups

The protein carbonyl contents in young wild-type and *mev-1* adults at the age of 4–8 days were similar. Afterwards, differences in the accumulations of carbonyl groups were observed, as a function of the genotype and increasing age. In the wild type, an age-dependent accumulation in carbonyl content was observed up to the end of the life span, around 20 days reaching 4.7 nmol/mg protein, whereas in *mev-1* it occurred at a faster rate to reach 5.7 nmol/mg protein at the end of life span (age 15–16 days).

To determine if the antioxidant defense potential in *mev-1* operated under higher levels of oxidative stress, protein carbonyl contents were compared after exposure to 70% oxygen between age 4 and 11 days. At the age of 11 days, this hyperoxia caused 100% and 31 % increases in carbonyl in *mev-1* and wild type over the respective basal levels in an ambient atmosphere of 21% oxygen, respectively [12]. (Figure 3b)

Molecular cloning

Three-factor crosses using visible genetic markers indicated that *mev-1* was located between *unc-50* (*e306*) and *unc-49* (*e382*) of chromosome III. We tested cosmids from this region for their abilities to rescue *mev-1* mutants from oxygen-hypersensitivity after germline transformation. Only cosmid T07C4 was able to rescue the *mev-1* mutant phenotype. By testing various subclones from this cosmid, we identified a 5.6kb fragment that also restored wild-type resistance. This fragment includes a putative gene, named *cyt-1*, which is homologous to bovine succinate dehydrogenase (SDH) cytochrome b_{560} (GenBank accession number L26545). We found the *mev-1* strain contained a missense mutation resulting in a glycine to glutamic acid substitution in *cyt-1* [13]

Electron transport is mediated by five multimeric complexes (complex I-V) that are embedded in the inner membrane of the mitochondrion. Mitochondrial succinate-ubiquinone reductase (complex II), which catalyzes electron transport from succinate to ubiquinone, is composed of succinate dehydrogenase (SDH) (flavin protein: Fp and iron-sulfur protein: Ip) and two other subunits which contain cytochrome b_{560}. In vivo, SDH is anchored to the inner membrane with the cytochrome b560 and is the catalytic component of complex II. Using separate assays, it is possible to specifically quantify both SDH activity and complex II activity. This was accomplished after the wild-type and *mev-1* extracts were subjected to differential centrifugation to separate mitochondria and mitochondrial membranes from the cytosol. The SDH activity in the *mev-1* mitochondrial fraction was experimentally identical to that of the wild type. Conversely, complex II activity in the *mev-1* membrane fraction was reduced over 80% relative to wild type. As would be expected of a mitochondrial enzyme, no SDH activity was observed in the cytosol. Thus, the *mev-1* mutation affects neither SDH anchoring to the membrane nor SDH activity per se. However, it dramatically compromises the ability of complex II to participate in electron transport.

Mechanism of cell damage caused by the mitochondrial dysfunction

How, then, does the *mev-1* mutation exert its effects on the mitochondria and, ultimately, the nematode? The premature aging and free-radical hypersensitivity of *mev-1* could result from two distinct mechanisms. (Figure 4) First, the mutation lead to the degradation of electron transport to the extent that oxygen uptake into the mitochondria is higher than in wild type. This, in turn, could result in the increased production of free radicals, primarily the superoxide anion. In the case of complex I (NADH-ubiquinone reductase) and complex III (ubiquinone-cytochrome c reductase), ubiquinone is generally thought to be the main source of superoxide anion [14–16]. In particular, in coenzyme Q cycle,

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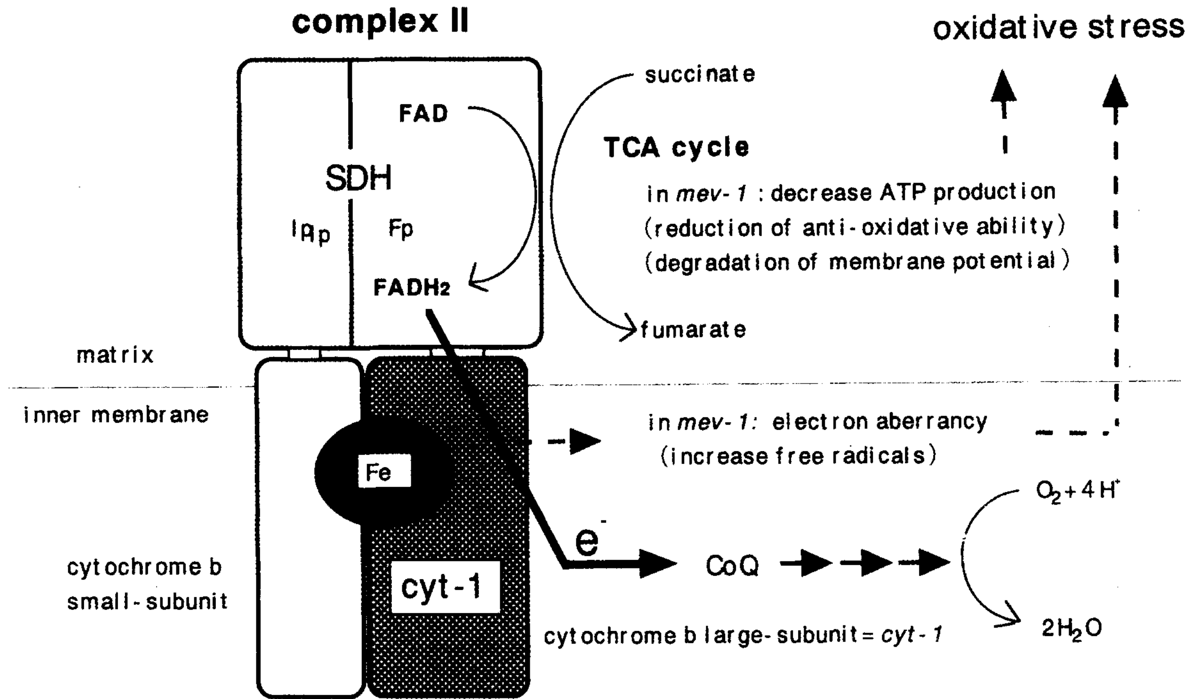

FIGURE 4 Schematic representation of complex II, which is situated in the mitochondrial membrane. The *mev-1* (*kn-1*) mutation could confer hypersensitivity to oxidative stress through either decreased ATP production or on increase in free radical generation

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ubisemiquinone can be spontaneously oxidized to quinone with concomitant production of superoxide anion [15]. In the case of complex III, the function of cytochrome b is to remove the semiquinone by effectively acting as a dismutase in the Q cycle. It is possible that similar events occur in complex II. The *mev-1* mutation would then compromise the ability of Q semiquinone to dismutate, resulting in elevated the production of superoxide anions.

Second, mutational perturbation of respiration could compromise ATP production and, as a result, lead to premature aging. (Figure 4) Given the fact that complex II plays a role in, not only electron transport, but also the Krebs cycle, a deficiency in complex II may affect energy metabolism by reducing the extent of ATP production. It has been postulated that such reductions could have major impacts on the fidelity of cellular defenses and repair processes [17]. In fact, Lieberthal and colleagues showed that cells, which had been subjected to a depletion of ATP to levels below 15% of the controls died uniformly of necrosis [18]. Moreover, cells, which are subjected to ATP depletion of between 25 and 70% of the controls, died by apoptosis. Milder depletions may lead to human myopathies and neurological diseases that include the development of premature aging [19].

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