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Review

The role of insulin and IGF-1 signaling in longevity

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Abstract. There are many theories of aging and parameters that influence lifespan, including genetic instability, telomerase activity and oxidative stress. The role of caloric restriction, metabolism and insulin and insulin-like growth factor-1 signaling in the process of aging is especially well conserved throughout evolution. These latter factors interact with each other, the former factors and histone deacetylases of the SIR family in a complex interaction to influence lifespan.

Key words. Aging; lifespan; genetic instability; telomerase; oxidative stress; superoxide dismutase; oxidants; antioxidants; reactive oxygen species; gluthatione; thioredoxin metabolism; calorie restriction; insulin; IGF-1; growth hormone; signaling; Sir; FOXO; p66; klotho; animal models; *S. cerevisiae*; *C. elegans*; *D. melanogaster*; mouse; knockout; human; syndrome; Ames Dwarf; Snell Dwarf; FIRKO.

Introduction

What is aging? Why do we age? Why do some species live longer than the others? Do genes determine lifespan? What is the role of metabolism on longevity? These are some of the questions that have intrigued biologists for ages.

Social scientists have raised other considerations: Do we want to live longer? And if so, how much longer? Is increasing longevity good for survival of the species, since natural/energy resources (water, food etc.) are limited? Will artificially prolonged lifespan alter natural evolutionary processes? How do we balance quality of life with quantity of life?

These two perspectives of aging and longevity are certainly connected, but are also distinct. One is the biology of aging and lifespan and the other is the social and evolutionary forces that may interact with the biology. In this review, we will focus on the biology of aging, and try to answer some of the first group of questions. We will focus especially on the role of metabolism and insulin and insulin-like growth factor-1 (IGF-1) signaling in this process.

What is aging?

Aging is a progressive loss of physiological functions that increases the probability of death. This decline in function occurs both within individual cells and within the organism as a whole. Life expectancy (or average lifespan) depends highly on both the biology of aging and the life circumstances of the organism. Evolutionarily speaking, very few organisms or animals were allowed to age, since mortality from starvation, predators, infection, diseases or environmental stresses often resulted in death before the biology of aging could play a role. Even human aging has become common in only the past few centuries. Two hundred years ago average lifespan was about 24 years due to high infant mortality, poor hygiene and inability to treat infectious disease [1, 2]. Now, with the development of good principles of hygiene, a wide range of effective

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medicines and relatively abundant food, the average lifespan in many developed countries is 80 or more years [3, 4]. With this increase in lifespan, causes of death have also changed, with infectious diseases and trauma being replaced by cardiovascular disease, cancer, diabetes mellitus and diseases of the elderly such as Parkinson's and Alzheimer's diseases. Interestingly, it appears that maximum lifespan (longevity) has not changed dramatically and seems to rest at about 120 years [5]. Thus, although the number of centenarians has increased, maximum human lifespan has not.

Theories of aging

There are several theories of aging [6] that point to four broad physiological processes important for longevity: genetic stability, telomere shortening, stress resistance and metabolic control.

Genetic stability: accumulation of genetic errors

One of the classic theories of aging is based on the role of accumulation of genetic errors. It has been recognized for many years that ionizing radiation causes DNA damage that can lead to early aging in mice [7–9]. Conversely, resistance to ultraviolet radiation is associated with increased lifespan in yeast [10]. Somatic mutations and chromosomal abnormalities, such as translocations and aneuploidy, are increased in cells isolated from old individuals compared to young individuals, presumably as a result of such DNA damage [11, 12].

In addition, dysregulation of DNA repair, cell cycle and integrity of extracellular matrix are found in cells isolated from old people and individuals with some premature aging syndromes [13–16]. Patients with Werner's syndrome who have mutations in the *WRN* gene, which encodes a helicase needed for DNA repair, show early signs of aging and have decreased lifespan [17, 18]. Patients with Cockayne syndrome have mutations in genes responsible for DNA repair and reduced lifespan [19]. Patients with ataxia telangiectasia who lack the ATM gene, a gene product required for detecting DNA damage and initiating repair response, also die young [20]. *ATM* kinase is activated by insulin and has a wide role in signal transduction and cell growth as well as in sensing redox homeostasis [21]. Thus, individuals with ATM mutations are also predisposed to cancer [22]. Children with Hutchinson-Gilford progeria syndrome show signs of aging very early and often die in their teens [23]. This disease is caused by mutations in the gene for lamin (*LMNA*). Although not proven to be directly involved in genetic stability, this is an intermediate filament protein that stabilizes the inner membrane of the nuclear envelope [24, 25]. Interestingly, mutations in this same gene are also associated with lipoatrophic diabetes [26–30]. DNA repair rates appear to correlate positively with lifespan among mammals. Furthermore, the DNA repair rate positively correlates with body size, which itself correlates with lifespan [31]. Accumulation of genetic errors also causes cancer, so it is not surprising that incidence of cancer increases with age.

In addition to mutations of nuclear DNA, point mutations and deletions of mitochondrial DNA (mtDNA) accumulate in a variety of tissues during aging in humans [32], monkeys [33] and rodents [34]. They cause a mosaic pattern of respiratory chain deficiency in tissues such as heart [35], skeletal muscle [36] and brain [37]. Mice that express a proofreading-deficient version of the nucleus-encoded catalytic subunit of mtDNA polymerase develop mtDNA mutator phenotype with an increased level of point mutations and deletions [38]. This is associated with reduced lifespan and premature onset of aging-related phenotypes, such as weight loss, reduced subcutaneous fat, alopecia (hair loss), kyphosis (curvature of the spine), osteoporosis, anaemia, reduced fertility and heart enlargement [38]. This provides a causative link between mtDNA mutations and aging phenotypes in mammals.

Telomere shortening: a 'biological clock'

Telomeres are the termini of linear eukaryotic chromosomes. They are involved in stabilizing the integrity of the ends of chromosomes [39], inhibiting the aberrant fusions and rearrangements that occur on broken chromosomes, and aiding the completion of duplication. During each cell cycle telomeric repeats (TTAGGG) are lost because DNA polymerase is unable to replicate the 3′ end of linear DNA completely, leaving a G-strand overhang. In germ cells and carcinomas, telomeric repeats are maintained by ribonucleoprotein telomerase that is capable of elongating telomeres de *novo* [40]. However, in the absence of telomerase activity, telomeres shorten with each cell division, reflecting the age of the cell lineage [41–43]. Many studies in a variety of tissues from mammals and other vertebrates have shown a gradual decrease of telomere length with age [43–46] and with doubling time in cell culture [41]. In addition, a positive correlation between telomere shortening and maximum lifespan has been shown in birds and mammals [47–49], suggesting that regulation of telomere length is not only associated with cellular replicative lifespan, but also with lifespan of the whole organism.

Genome size (DNA quantity per cell) is also positively correlated with longevity in birds [50] and in fishes [51]. However, how genome size can affect an organism's phenotype and longevity remains unclear.

In yeast, one of the manifestations of aging is a loss of transcriptional silencing of genes located in the heterochromatic region near telomeres and at the silent mat-

ing loci [52, 53]. This genetic dysregulation has been suggested to be a possible cause of aging in yeast. It has been shown that the histone deacetylase genes *SIR2*, *RPD3* and *HDA1* determine yeast longevity [54]. Aging in yeast is associated with an increase in ribosomal RNA coupled to a reduction in the efficiency of protein synthesis [55]. In fact, the effects of these genes on longevity are associated with enhanced silencing of ribosomal DNA.

Recent results suggest that overexpression of the type-three histone deacetylase SIRT1, the human orthologue of Sir2, in HeLa cells activates telomerase by increasing the expression of a telomerase protein component (hTERT) [56]. In contrast, infecton of the cells with a retrovirus expressing a dominant negative SIRT1 reduced hTERT messenger RNA (mRNA) levels [56]. Since telomerase promotes cell longevity in cultured cells, it is possible that SIRT1 functions as a global promoter of longevity in eukaryotes.

Free-radical theory of aging

Almost 50 years ago, Denham Harman noted parallels between effects of aging and of ionizing radiation. He suggested that free radicals produced during aerobic respiration cause cumulative oxidative damage, resulting in aging and ultimately death [57]. The theory gained credibility with discovery of enzyme superoxide dismutase (SOD) [58], evidence of in vivo generation of superoxide anion (O_2^- •) and elucidation of elaborate antioxidant defenses [59].

Mitochondria are the main source of endogenous free radicals [60]. It has been known for almost 100 years that, in general, species with higher metabolic rates have shorter maximum lifespan, i.e. they age faster. This is in agreement with an old 'rate-of-living' hypothesis that proposed that energy consumption per se was responsible for senescence. Realization that energy consumption by mitochondria may result in O_2^- • production, which causes damage in cells, provided a mechanism for the rate-of-living theory, and the two concepts eventually merged. Basically, the concept is that a faster rate of respiration is associated with a greater generation of oxygen radicals and thus hastens aging. Recently, this theory has also been extended to explain how diabetes may also produce long-term complications that affect shorter lifespan [61, 62].

Reactive oxygen species generation

Ground-state oxygen (O_2) is not reactive, because it possesses two unpaired electrons with parallel spins. However, O_2 can become reactive by adsorption of energy (e.g. from ultraviolet light), and this results in production of energetically excited singlet oxygen that have their two unpaired electrons with opposite spins, enabling a greater reactivity. In vivo O_2 is activated by coordinated, serial, enzyme-catalyzed one-electron reductions by enzymes that possess active-site radical species, such as iron. One- and two-electron reduction of O_2 generates O_2^- • and hydrogen peroxide (H_2O_2), respectively. In the presence of iron or copper, O_2^- • and H_2O_2 generate the extremely reactive hydroxyl radical (•OH), which is assumed to be responsible for initiating oxidative destruction of biomolecules. All of these reactive species of oxygen are collectively called free radicals or reactive oxygen species (ROS), or sometime just oxidants. They are produced in vivo and in excess can cause significant harm [59, 60, 63–66].

Four main sites of ROS generation in cells are mitochondrial electron transport, peroxisomal fatty acid metabolism, cytochrome P450 reactions and the 'respiratory burst' in phagocytic cells. The three main classes of biological macromolecules (lipids, nucleic acids and proteins) are targets of endogenous ROS and suffer oxidative damage in vivo. Free radicals cause lipid peroxidation [67] leading to production of unsaturated aldehydes, which are reactive and may act as mutagens [68], inactivate enzymes [69, 70] or react with proteins and nucleic acids to form heterogenous cross-links [71]. The ultimate result of lipid peroxidation is altered membrane properties and disruption of membrane-bound proteins [72]. Oxidative damage to nucleic acids may induce adducts of base and sugar groups, single- or double-stranded breaks in the backbone and cross-links to other molecules. Oxidative damage of proteins includes oxidation of sulfhydryl groups, oxidative adduction of nucleic acid residues close to metal binding sites, reactions with aldehydes, protein-protein cross-linking and peptide fragmentation [73, 74], all of which may occur as a result of increased ROS.

Antioxidant defense mechanisms

To combat oxidative damage, cells possess a very wide repertoire of defense mechanisms.

1) Antioxidant enzymes, such as SOD, hasten conversion of O_2^- • to H_2O_2, and catalase, peroxidase and glutathione-peroxidase convert this H_2O_2 to water. Glutathione-reductase promotes reduction of oxidized forms of small molecular antioxidants, and thioredoxin-reductase helps to maintain protein thiols.

2) Small antioxidant molecular scavengers (such as urate, glutathione, thioredoxin), and those derived from dietary fruits and vegetables [59, 75] (such as ascorbate, tocopherols, flavonoids and carotenoids) can scavange free radicals.

3) Cellular metabolism maintains a reducing environment, e.g. glucose-6-phosphate dehydrogenase regulates NADPH production.

There are also some mechanisms of repair of oxidative damage, such as phospholipase A_2 that cleaves lipid per-

oxides from phospholipids [76], or glycosylases that recognize and excise oxidized bases from double-stranded DNA (dsDNA) [77–80] and oxidized proteins [81–84]. This balance between ROS generation, antioxidant protection and repair of oxidative damage is very important (fig. 1) [85]. Thus, it is not surprising that these processes have been examined both as a function of age in individuals of the same species, as well as between species of differing maximal lifespan.

Animal models that support the 'free-radical' theory

Studies in several animal species, including worms, flies and mice support this free-radical theory of aging.

Caenorhabditis elegans

Several long-lived mutant strains of *Caenorhabditis elegans* have been identified. One involves the *clk* genes. Representative of this group is *clk*-1, a gene involved in control of respiratory rate in *C. elegans* [86]. Clk-1 mutants have slower metabolic rate, slower rates of oxidant generation and longer lifespans [87]. The *clk-1* homologue in yeast is *CAT5/COO7* [88], a gene involved in control of the shift from anaerobic to aerobic metabolism [89], as well as the biosynthesis of the mitochondrial electron transport component, ubiquinone (UQ) [90].

A mutant with the opposite effect of clk-1 is *mev-1*. This is a mutation in the cytochrome b subunit of the respiratory chain, and presumably prevents the efficient passage of electrons from complex II to complex III via UQ [91–93]. The activity of SOD in these animals is roughly one-half of wild type, and the average lifespan is reduced by ~35% [91]. Lipofuscin-like fluorescence accumulated more rapidly in mev-1 mutants than in wild type nematodes [94], and these worms were hypersensitive to both hyperoxia and paraquat (a compound widely used to apply oxidative stress to cells and organisms by leading to continuous intracellular generation of O_2^- • radicals).

Finally, augmentation of natural antioxidant systems in *C. elegans* with small synthetic SOD/catalase mimetics increased their lifespan by a mean of 44%, while treatment of prematurely aging worms resulted in normalization of their lifespan (by a 67% increase) [95]. These results confirm importance of oxidative stress in determination of lifespan in this species.

Drosophila melanogaster

In *Drosophila melanogaster*, some long-lived mutants have been shown to have greater metabolic capacity [96, 97], resistance to heat, desiccation and ethanol, and have a higher activity of several antioxidant enzymes, including SOD [98]. Direct evidence for the significance of antioxidative defense for longevity in the fly has been obtained through generation of transgenic animals expressing copper/zinc SOD, catalase or both. These transgenic flies live longer and suffer less oxidative damage [99–102]. Interestingly, expression of SOD in motor neurons alone is sufficient to increase lifespan in *Drosophila* [103].

JNK (jun N-terminal kinase) and p38 are stress-activated protein kinases (SAPKs) that are involved in one of sev-

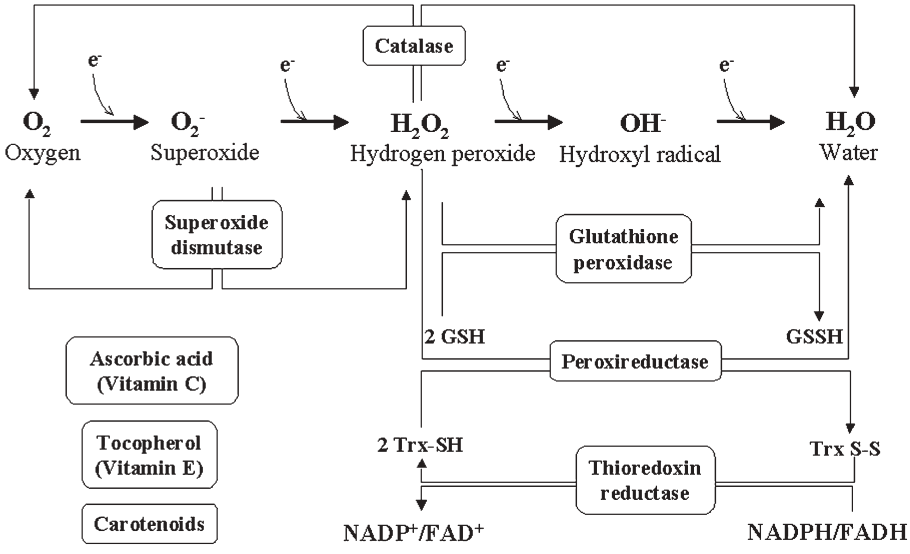

Figure 1. Cellular oxidative stress defence mechanisms. Ground-state oxygen is activated by successive one-electron reductions producing superoxide, hydrogen peroxide and hydroxyl radical, collectively called free radicals or reactive oxygen species (ROS). However, cells possess antioxidant defence mechanisms that include superoxide dismutase (SOD), catalase, gluthatione peroxidase, peroxireductase and thioredoxin reductase. SOD hastens conversion of superoxide to hydrogen peroxide, which is then converted by catalase, peroxireductase and gluthatione-peroxidase to water. Ascorbic acid, tocopherol and carotenoids are some of natural antioxidants, derived from fruits and vegetables.

eral stress-sensing mitogen-activated protein (MAP) kinase-signaling pathways [104–106]. The JNK signaling cascade is triggered by a variety of insults, including ultraviolet (UV) radiation and oxidative stress [107]. In vitro, JNK signaling may have either a protective function [108] or promote apoptosis [109]. Dramatically prolonged life expectancy and increased resistance to oxidative stress induced by paraquat are found in flies with mutations that augment JNK signaling compared to wild type animals [107]. Conversely, flies with decreased JNK signaling are more sensitive to moderate doses of paraquat. As with SOD, it has been shown that increased JNK activity in neurons alone is sufficient to reduce paraquat toxicity [107]. Overexpression of Hsp70 and its JNK-inducible relative Hsp68 also extends lifespan in *Drosophila* [110]. These chaperones have been implicated in resistance to oxidative stress [111] and may have repair functions downstream of JNK signaling. The G-protein-coupled transmembrane receptor methuselah has also been identified in mutants showing increased longevity [112], and enhanced resistance to heat, starvation and oxidative stress.

Mice

Models have been found that support the role of oxidative stress in mammalian survival. The senescence accelerated mouse (SAM), specifically SAM-prone (SAM-P) and SAM-resistant (SAM-R) strains, are very important models in gerontology [113]. SAM-P strains have a mean lifespan of 9 months compared to 13 months of the SAM-R strain. Mitochondrial SOD activity in the liver of these mice is reduced about 50% compared to the SAM-R strain [113]. SAM-P mice also have increased lipid peroxidation [113] and enhanced damage in response to oxidative stress [114]. Another very useful model is the S strain of Wistar rats, which has been selected for sensitivity to galactose. These rats have an inherited increase in cellular hexose uptake associated with increased intracellular oxidant generation and increased endogenous lipid peroxidation. Wistar S rats also have mitochondrial dysfunction and an increased age-specific incidence of degenerative diseases [115, 116]. SOD and catalase activities in the blood of S rats are decreased compared to R rats, and this may explain increased oxidative damage in that strain [117].

Very useful models for understanding the role of oxidative stress in aging and longevity are mice in which the genes encoding antioxidant proteins are either overexpressed or knocked out. For example, transgenic mice overexpressing thioredoxin (Trx), a small multifunctional protein that acts together with glutathione (GSG) for the maintenance of intracellular redox status, have prolonged median and maximum lifespan [118, 119]. These mice have higher GSSG/GSG ratios in peritoneal macrophages compared to wild type controls, probably reflecting the importance of glutathione in alleviation of oxidative stress [120]. In addition, they show elevation of interferon-γ (IFN-γ) and reduction of interleukin (IL)-10 with a moderate change in IL-4 produced by $CD4^+$ cells, while wild type mice show inverse changes of IFN-γ/IL-4 and IFN-γ/IL-10 ratios during aging [120]. Thioredoxin negatively regulates p38 MAP kinase activation and IL-6 production by tumor necrosis factor-α (TNF-α) [121]. Also, bone marrow cells from these mice are more resistant to UV C-induced cytocide, and telomerase activity in the spleen of transgenic mice is higher than in controls [118]. The thioredoxin superfamily plays a crucial role in biological responses to oxidative stress [122].

Generation of glutathione-peroxidase (*gpx-1*) knockout mice proved that the contribution of Gpx-1 under normal animal development and physiological conditions is very limited [123]. However, Gpx-1 is a major selenoenzyme that protects mice against acute, lethal oxidative stress induced by the ROS generators paraquat or diquat [124–126]. Mice deficient in Gpx-1 are susceptible to ischemia/reperfusion injury [127], virus-induced myocarditis [128] and neurotoxicity [129]. In addition, fibroblasts derived from these mice have reduced proliferative capacity, reduced DNA synthesis, reduced responsiveness to epithelial growth factor (EGF) and serum, and increased levels of Cip1 and nuclear factor kappa B (NF-κB) activation [130]. Fibroblasts from these mice also have morphological features of senescent cells, as well as dose-dependent susceptibility to H_2O_2-induced apoptosis [130].

While mice deficient in cytosolic SOD1 or extracellular SOD3 have a very benign phenotype [131, 132], mitochondrial SOD2 knockout mice have a mean lifespan of only 8 days, and at death present with dilated cardiomyopathy, liver dysfunction, metabolic acidosis, many different mitochondrial enzymatic abnormalities and oxidative DNA damage [133, 134]. SOD2 heterozygous mice also show evidence of increased proton leak, inhibition of respiration, early and rapid accumulation of mitochondrial oxidative damage [135], and increased DNA damage that is the cause of the increased incidence of cancer seen in these mice [136]. Furthermore, chronic oxidative stress in these mice results in an increased sensitization of the mitochondrial permeability transition pore (regulates apoptosis by cytochrome c release after swelling of the mitochondria because of diffusion of molecules between the matrix and cytosol) and the premature induction of apoptosis [137]. In addition, SOD2 mutant mice have increased pulmonary sensitivity to oxygen toxicity [138]. Wild type mice with normal SOD2 levels show the same age-related mitochondrial decline as the heterozygotes, but it occurs much later in life.

Metabolism and insulin-receptor and IGF 1-receptor signaling

Since the discovery of insulin in 1921, most studies have focused on the role of this hormone in metabolism and glucose homeostasis [139, 140]. However, both obesity and diabetes are associated with shortened life expectancy [141–144]. Both of these disorders are also on the rise in industrialized countries due to more sedentary lifestyles and higher caloric intake throughout the civilized world.

Studies over the last several years have revealed a central role of insulin signaling in lifespan and aging in diverse organisms, ranging from yeast to rodents. These discoveries indicate that aging is a programmed and well-controlled process regulated by the same pathways that affect growth, development and metabolism in these organisms. This supports the hypothesis that the impact of these genes on longevity of different species is an evolutionarily conserved process (fig. 2).

Calorie restriction

The most striking and the most consistent model of extended lifespan, and one which dramatically demonstrates the role of metabolism in this process, is calorie restriction. Indeed, calorie restriction retards aging and extends median and maximal lifespan in yeast, worms, fish, flies, mice, rats and monkeys [145–148], and recent data suggest even in humans [148].

Some of the common and consistent effects of calorie restriction in rodents and nonhuman primates include lower fat mass, particularly visceral fat, lower circulating insulin and IGF-1 concentrations, increased insulin sensitivity, lower body temperature, lower fat-free mass, lower sedentary energy expenditure (adjusted for fat-free mass), decreased levels of thyroid hormones and decreased oxidative stress (table 1) [149]. In addition, calorie restriction in young animals delays sexual maturation, but this seems unrelated to the increased lifespan, since calorie restriction has the same effect when initiated in older animals [150, 151]. Reduced metabolism and the consequent reduction of free-radical production is another possible explanation for the anti-aging effects of calorie restriction. However, other effects of calorie restriction, such as lower body temperature, increased insulin sensitivity, decreased insulin/IGF-1 levels, sympathetic nervous system activity, and altered gene expression in muscle, heart and brain of calorie-restricted animals, have all been suggested to play a role in the effects of calorie restriction on longevity. Calorie restricted monkeys also have increased dehydroepiandrosterone sulfate [145], and this has been suggested as a possible marker of longevity in humans [152, 153].

Because of the difficulties involved in conducting long-term calorie restriction studies, including ethical and methodological considerations, there is little information on the effects of calorie restriction in humans on actual lifespan. There have been some naturally occurring episodes of calorie restriction in human populations, although these usually involve exposure to diets lacking protein and micronutrients [149]. In these cases, calorie restriction is often associated with short stature, late reproductive maturation, lower gonadal steroid production in adults, suppressed ovarian function [154], impaired lactation performance [155], impaired fecundity and im-

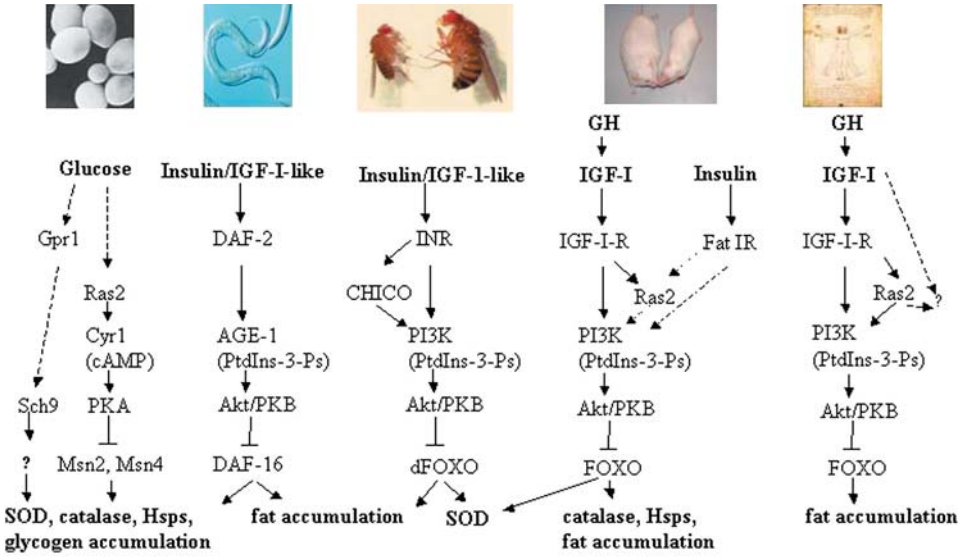

Figure 2. Conserved regulation of longevity. In different organisms, the conserved glucose or insulin/IGF-1-like pathways downregulate antioxidant enzymes and heat shock proteins, reduce the accumulation of glycogen or fat and increase growth and mortality. Decreased activity of these pathways extends longevity by simulating calorie restriction. Modified from [336].

Table 1. The effects of chronic calorie restriction on markers of aging in rodents and non-human primates and predicted outcomes in non-obese humans.

	Rodents	Non-human primates	Expected outcomes in non-obese humans
Insulin	↓	↓	↓
Body temperature	↓	↓	↓
Energy expenditure			
Total[2]	↔[3]↓	↔↓	↓
Sedentary[2]	↓	↓	↓
Voluntary activity	↑	↔	↓
Oxidative stress	↓	↓	↓
Fat mass and fat-free mass	↓	↓	↓
Visceral fat	↓	↓	↓
Intramyocellular lipids	?	?	↓
Insulin sensitivity	↑	↑	↑
IGF-1	↓	↓	↓
Thyroid axis	↓	↓	↓
HPA axis	↑	?	↓
DHEAS	?[1]	↑	↓
Gonadotropic axis	↓	↔	↑
Somatotropic axis	↓	?	↓
Symphatetic axis	↓	?	↓

[1] Effect not known.
[2] Adjusted for fat-free mass.
[3] No effect.
DHEAS, dehydroepiandrosterone sulfate; HPA, hypothalamus-pituitary-adrenal.

paired immune function [149]. Observational studies of prolonged calorie restriction in the context of a high-quality diet have been done on the island of Okinawa, Japan [156]. These individuals have lower total energy consumption than the average intake for the Japanese population as whole, and this is accompanied by lower rates of death due to cerebrovascular disease, malignancy and heart disease, and a high prevalence of centenarians. However, the importance of other environmental factors in this extended longevity cannot be excluded, since Okinawans also have a distinctive lifestyle (sleeping, exercising and eating habits) [157].

Similar observations have been made in the Biosphere 2 experiment, in which eight healthy nonobese humans (men and women) spent 2 years in an enclosed glass and steel structure that was constructed as a self-contained ecologic 'miniworld' and prototype planetary habitat. Due to problems in growing vegetables, there was a smaller amount of food available than originally predicted, and all individuals experienced a marked weight loss. Numerous changes in physiologic, haematologic, hormonal and biochemical variables were observed [158], including improved insulin sensitivity, decrease fat mass and reduced tumor incidence. Recent results show that long-term calorie restriction is also highly effective in reducing the risk for atherosclerosis in humans [148].

The connection between calorie restriction, metabolism and chromatin structure in yeast has pointed to a role of a group of proteins called sirtuins (Sir). Sirtuins are histone deacetylases, i.e. enzymes that remove acetyl groups from lysines of histones H3 and H4 [159]. Sir activity decreases transcription due to chromatin silencing and also influences chromatin stability [160, 161]. Increasing the level of Sir-2 in yeast [162] and *C. elegans* [163] prolongs lifespan, while decreasing Sir activity decreases lifespan in yeast. In *S. cerevisiae*, the increased Sir-2 activity is coupled with a change in yeast metabolism, due to low glucose availability, which changes from anaerobic (fermentation) to aerobic metabolism (TCA cycle), thus, producing more energy (ATP) from each glucose molecule metabolized [164]. Sir activity is NAD^+-dependent and inhibited by NADH or nicotinamide [165]. Therefore, the NAD/NADH ratio (one end product of metabolism) regulates Sir activity [166]. Indeed, calorie restriction extends yeast lifespan by lowering the level of NADH [166, 167]. It has been shown that Pnc1, an enzyme that converts NADH to nicotinic acid, enhances Sir-2 activity by increasing the NAD/NADH ratio in cells. In addition, yeast that overexpress the pnc-1 gene show slower aging and prolonged lifespan [168]. The prolongation of lifespan in yeast by glucose deprivation requires Pnc1 [168]. Pnc1 levels are also elevated under other conditions

known to extend yeast lifespan, including amino acid restriction, salt stress and heat stress [168]. All these results show a strong connection between maintenance of chromatin structure/silencing, metabolism and longevity in yeast.

In mammals, it has been shown recently that SIRT1 activates a critical component of calorie restriction: fat mobilization in white adipocytes [169]. Upon food withdrawal SIRT1 protein binds to and represses genes controlled by the fat regulator PPAR-γ (peroxisome proliferator-activated receptor-γ), including genes that mediate fat storage, by docking with its cofactors NCoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors). Mobilization of fatty acids from white adipocytes upon fasting is compromised in Sirt1$^{+/-}$ mice [169].

In addition, overexpression of SIRT1 in 3T3-L1 adipocytes attenutates adipogenesis, while RNA interference of SIRT1 enhances it. Also, upregulation of SIRT1 in differentiated fat cells triggers lipolysis and loss of fat [169].

Insulin/IGF-1 signaling

Insulin and the insulin-like growth factors (IGF-1 and IGF-2) represent a family of hormones/growth factors that regulate metabolism, growth, cell differentiation and survival of most tissues in mammals [170]. These effects are mediated by two closely related receptors, the insulin and type 1 IGF receptors, both of which are expressed on the surface of most mammalian cells. Although classically, skeletal muscle, liver and adipose tissue were considered the only insulin responsive tissues [171], recently it has been shown that insulin (and IGF-1) receptors, as well as most of their signaling partners, are functional in non-classical tissues, including pancreatic islets [172–178], vascular endothelial cells [179] and the central nervous system [180, 181].

Insulin and IGF-1 initiate their action via highly homologous signaling systems. The insulin and IGF-1 receptors are members of the tyrosine kinase family of receptors. Both receptors consist of two α- and two β-subunits. Insulin and IGF-1 bind to the α-subunits of these receptors, activating the kinase activity of the β-subunits. This results in autophosphorylation of the receptor and further activation of the receptor kinase toward intracellular substrates. At least 10 intracellular substrates for the insulin and IGF-1 receptors have been identified. The best characterized are the insulin receptor substrate proteins (IRS) IRS-1, -2, -3 and -4 [182–186]. Following tyrosine phosphorylation, each of these substrates associates with one or more intracellular molecules through interaction of the tyrosine phosphorylation sites in the substrates with SH2 domains of intracellular proteins to generate downstream signals. The two most important SH2 molecules with respect to insulin action are the enzyme phosphatidylinositol 3-kinase (PI 3-kinase) and the adaptor molecule Grb2 [187, 188]. Grb2 links insulin action to the Ras-MAP kinase pathway, and plays a role in the ability of insulin to stimulate cell growth and differentiation [189]. PI 3-kinase, on the other hand, is the critical link between the insulin receptor and all of the metabolic actions of the hormone [190]. PI 3-kinase activates Akt/protein-kinase B (PKB) and protein-kinase C (PKC), which subsequently leads to activation of p70 S6K and glycogen-synthase kinase 3 (GSK3). This results in stimulation of glycogen, lipid and protein synthesis, as well as in glucose transporter translocation to the plasma membrane with an increase in glucose transport [191, 192]. Akt/PKB also phosphorylates forkhead transcription factors of the FOXO subfamily (previously known as FKHR [193]), and this leads to their inactivation and retention in cytoplasm [194–197]. Under conditions of insulin or serum withdrawal, FOXO proteins are not phosphorylated and reside into the nucleus, where they are active and regulate gene expression [195, 196]. Insulin, IGF-1 and serum stimulate Akt, resulting in phosphorylation of FOXO transcription factors, their retention in the cytoplasm and reduced transcriptional activity [198–201]. Depending on the nature of the activation signal, FOXO can regulate apoptosis [202, 203], cell cycle [204–207], differentiation [199, 200], or the expression of genes involved in DNA repair [208] and oxidative stress resistance [209]. Negative regulators of the insulin signaling pathway are protein tyrosine-phosphatases (PTPs), particularly PTP-1B (PTP1B) and the lipid phosphatases PTEN [210, 211] and SHIP2 [212, 213], which can dephosphorylate the products of PI 3-kinase.

Genetically modified animal models

Over the past few years, a variety of studies have provided insights into involvement of the insulin/IGF-1 pathway in the control of aging and longevity in yeast, worms, flies and rodents.

Invertebrates

Saccharomyces cerevisiae. The fact that age-associated alterations in energy metabolism can be analyzed more easily in a unicellular eukaryote with a short natural lifespan than in a multicellular organism with specialized cell types has made *S. cerevisiae* an attractive model for studying how glucose and energy metabolism are linked to aging. Although yeast do not have an insulin-signaling pathway, they appear to have precursors of such pathways that function in a glucose/nutrient-signaling cascade. These pathways include Sch9 and Cyr1 signaling cascades, homologues of the serine/threonine kinase Akt/PKB and the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathways, respectively. Indeed, the *cyr1* gene encodes yeast adenylate cyclase, which stimulates

the PKA activity required for cell cycle progression and growth. Furthermore, the carboxy-terminal region of Sch9 is highly homologous to the Akt/PKB in *C. elegans*, *D. melanogaster* and mammals.

Screening for long-lived mutants in *S. cerevisiae* has demonstrated that mutations in *cyr1/PKA* and *sch9* genes can extend the longevity of non-dividing cells up to threefold [214]. Both pathways mediate glucose-dependent signaling, stimulate growth and glycolysis and decrease stress resistance, glycogen formation and gluconeogenesis [214, 215]. In yeast, downregulation of glucose signaling increases resistance to thermal stress by activating the transcription factors Msn2 and Msn4. These induce the expression of genes encoding for several heat shock proteins (Hsps), catalase (Ctt1), DNA damage-inducible genes and superoxide dismutase 2 (SOD2) [216]. Moreover, expression of mitochondrial SOD2 is required for the extension of lifespan in yeast caused by mutations decreasing the activity of Ras/Cyr1/PKA and Sch9 pathways, confirming that superoxide toxicity plays an important role in yeast aging and death [214, 217].

In addition, Snf1p, the yeast homologue of AMP kinase (AMPK), is required for normal cellular response to glucose starvation [218]. Snf1p is incorporated into a complex that contains Snf4p, Sip1p, Sip2p and Gal83p. Under limiting glucose concentrations Snf4p activates Snf1p kinase, which phosphorylates a number of target genes, including transcriptional regulators of genes involved in alternative carbon source utilization, gluconeogenesis and respiration [218]. While loss of Snf4p produces a 20% increase in lifespan, increased expression of Snf1 causes rapid aging [219]. In mammals, AMPK is activated in response to various stresses, including glucose deprivation, heat shock, hypoxia and exercise [218]. Once activated, AMPK phosphorylates some of the proteins homologous to the downstream targets of Snf1p in yeast, including acetyl-coenzyme A (CoA) carboxylase and glycogen synthase [218, 219].

Caenorhabditis elegans. Because of its small size, relatively short lifespan (~20 days), rapid reproductive rate, well-characterized genetics [220], defined cell lineage and ability to respond to environmental changes (such as food deprivation) by entering into a quiescent state of diapause called dauer, *C. elegans* has proven to be a powerful genetic model for discovery of genes controlling lifespan ('gerontogenes') [221]. Furthermore, the importance of the insulin/IGF-1 signaling pathway in the evolutionarily conserved mechanisms that control aging and lifespan was first suggested by results obtained in these invertebrates [222, 223].

The insulin-like signaling cascade in *C. elegans* consists of proteins encoded by genes for insulin-like proteins (*ins-7* and multiple other insulin-like ligands), *daf-2* (the insulin/IGF-1 receptor homologue), *age-1* (protein similar to the mammalian p110 catalytic subunit of PI 3-kinase in mammals), *akt-2* (the homologue of Akt/PKB), *daf-16* (the homologue of the forkhead family of transcription factors in mammals [224–226]) and *daf-18* (the homologue of PTEN). This pathway has been shown to regulate the transition to dauer (an alternative life form of the worm when the food is scarce, which appears not to age) and reproduction, as well as to influence adult lifespan of nematodes. Mutation of these genes has revealed the important role of insulin/IGF-1 signal transduction as a central regulator of aging in *C. elegans*.

Daf-2 is believed to be a common ancestor of human insulin and IGF-1 receptors genes. Like the insulin and IGF-1 receptors, decreased Daf-2 signaling induces important metabolic changes in *C. elegans* [227, 228]. Thus, null mutations in *daf-2* cause constitutive arrest at the dauer larval stage [227, 229], which has slower metabolic rates, stores large amounts of fat and lives longer than reproductive adults. Indeed, mutations of *daf-2* can double the lifespan of *C. elegans* [230]. When coupled with removal of germline precursor cells, which independently extends lifespan by ~60%, *daf-2* mutant worms can live four times longer than controls [231]. This additional extension of lifespan seems not to be a result of sterility, but of altered endocrine signaling [231, 232]. By combining *daf-2* mutations, the use of RNA interference (RNAi) and gonad ablation, it is possible to increase lifespan sixfold [233].

Mutation of the downstream gene *age-1* also leads to a 65% increase in mean lifespan [229]. This effect and the effect of *daf-2* have been shown to depend on dauer formation protein-16 (Daf-16) expression. Thus, a null mutation in *daf-16* can suppress both *daf-2* and age-1 phenotypes. The eventual activation of this forkhead-like transcription factor indicates that, at least in worms, insulin/IGF-1 signal pathways regulate aging by modulating gene expression [224, 225]. In addition, inactivation of the *daf-18* gene, the homologue of mammalian PTEN tumor suppressor protein (dual-specificity phosphatase, which inhibits integrin-mediated signaling and regulates levels of phosphatidylinositol 3,4,5-triphosphate) [234] suppresses life extension of *C. elegans* and constitutive dauer formation associated with *daf-2* or *age-1* mutants [235–237]. Thus, PTEN/Daf-18 antagonizes the Daf-2/Age-1 pathway.

Interestingly, the same signals in different tissues do not influence aging equally. The *C. elegans* genome contains 37 'insulin-like' ligands [238]. These are expressed mainly in neurons, but have been also found in intestine, muscle, epidermis and gonad [239, 240]. Decreased Daf-2 signaling in germline and somatic gonads significantly increased the lifespan of the worm [241]. However, some studies have shown that Daf-2 signaling in the nervous system is a critical regulator of longevity [242]. For example, restoration of the PI 3-kinase signaling pathway in

muscle of *daf-2* mutants rescues metabolic defects but not longevity, whereas restoration of the pathway in neurons rescues both defects [242]. In addition, expression of *daf-2* under control of neural promoters shortens the lifespan of *daf-2* mutants. Interestingly, in contrast to expectation, reconstitution of Daf-16 activity specifically in neurons of *daf-16* (–); *daf-2* (–) animals produces only a very modest (5–20%) extension of lifespan, while expression of Daf-16 in the intestine is sufficient to extend lifespans of these animals by 50–60%, and can completely rescue the longevity of *daf-16* (–) germline-defective mutants [243]. These studies indicate a network of tissue interactions and feedback regulation in the insulin/IGF signaling system in the control of aging in *C. elegans*.

While it is clear that insulin/IGF-1R signaling have important roles in metabolism and lifespan of *C. elegans*, the question remains as to what mechanisms link these pathways to aging and longevity. The answer appears to be that in nematodes the Daf-2 pathways are linked to increased resistance to oxidative stress. Indeed, *daf-2* mutants express high levels of antioxidative enzymes, such as catalase and SOD [244, 245]. Likewise, the *age-1* mutation partially prevents the age-associated decrease of catalase in adult worms [242, 244, 245]. Indeed, the lower level of free radicals in *daf-2* signaling mutants has been shown to be essential for the lifespan extension [246, 247]. The *ctl-1* gene, which encodes a cytosolic catalase which has been shown to be a downstream target of Daf signaling, is required for the extension of adult lifespan by *daf-2* [248]. Since Daf-16 has been shown to have a key role in extension of lifespan of *daf/age* mutants, a number efforts have been made to find the genes, which act downstream of Daf-16. Microarray analysis of gene expression in *daf-2* (–) compared to *daf-16* (–) *C. elegans* reveals two clusters of genes of interest [249, 250]. The class 1 genes are those induced in daf-2 mutants and repressed in *daf-16* mutants, and represent candidate genes for lifespan extension. The second cluster, class 2, has the opposite profile of expression and consists of the genes that shorten lifespan. Using this approach, genes previously thought to be regulated by Daf-16 and also influence aging have been identified, including the metallothionein homologue *mtl-1* [251] and the SOD gene *sod-3* [245], both of which are involved in stress response. In addition, catalase genes *ctl-1* and *ctl-2*, the gluthatione-S-transferase gene *gst-4* and small heat shock protein genes were all increased in animals with reduced Daf-2 activity and decreased in animals with decreased Daf-16 activity.

Several other studies have shown that reduced expression of heat shock factor-1 (Hsf-1), which activates transcription of genes encoding the molecular chaperones, heat shock proteins (HSPs) and proteases in response to stress, causes a rapid aging phenotype and shortens lifespan [252]. Moreover, since it has been shown that *daf-2* mutation does not extend the lifespan of *hsf-1* mutants and *daf-16* is required for *hsf-1* overexpression to extend lifespan, it has been concluded that Hsf-1 may act together with Daf-16 to promote longevity [253]. By contrast, expression of *ins-7* (which behaves as a Daf-2 agonist) is repressed in animals with reduced Daf-2 activity and elevated in animals with reduced Daf-16 activity. In addition to *ins-7*, a number of other genes that encode potential signaling molecules are regulated by Daf-2 and Daf-16. One is the *scl-1* gene, which encodes a putative secreted protein that promotes longevity [254].

A large number of class 1 genes encode proteins that might potentially participate in synthesis of steroid or lipid-soluble hormones, including four cytochrome P450s, two estradiol-17-β-dehydrogenases, two alcohol/short-chain dehydrogenases, several esterases, two UDP-glucoronosyltransferases and several fat genes known to function in fatty acid desaturation. In addition, *gcy-6* and *gcy-18*, guanylate cyclases that are expressed in neurons, are repressed under *daf-2* (–) conditions.

C. elegans feeds on bacteria. Wild type animals exhibit pharyngeal and intestinal bacterial packing as they age, and are ultimately killed by proliferating bacteria [252]. Daf-2 mutants display reduced bacterial packing when compared with wild type worms of the same age [252]. In addition, several genes encoding antibacterial lysosymes, such as *lys-7* and *lys-8*, are induced in *daf-2* mutants, and saposin-like gene *spp-1*, which has demonstrated antibacterial activity [255], is also upregulated in *daf* (–) animals. In addition, several other genes in the class 2 cluster are found to be *daf-2*/daf-16 regulated with a substantial effect on lifespan, such as vitellogenin genes *vit-2* and *vit-5*, several proteases and metabolic genes, including some peptidases, amino-oxidase, aminoacylase, oligopeptide transporters, and several proteins involved in ubiquitin-mediated protein degradation. Finally, the expression of Rbp-2, which has been implicated in transcriptional regulation and chromatin remodeling, is down-regulated in *daf-2* mutants in a *daf-16* dependent manner [256]. This finding is consistent with the finding that Sir2, which is also involved in chromatin remodeling, also modulates longevity in *C. elegans*, in the same way as it did in *S. cerevisiae* [160, 163].

D. melanogaster. Until recently, the major impact of genetics in aging research in the fruit fly *D. melanogaster* has been the selection of lines displaying extended longevity [97]. Fruit flies thus selected show greater metabolic capacity; are resistant to heat, desiccation and ethanol; and have higher activities of several antioxidant enzymes. In addition, they are more efficient in their utilization of nutrients and have enhanced storage of lipid and glycogen – many of the features found in yeast and worms with extended lifespan [257].

In *Drosophila*, the insulin/IGF receptor, the insulin receptor substrate Chico, the PI 3-kinase Dp110/p60 and PKB (Akt1) form a signaling pathway that regulates metabolism, growth, size and lifespan [258]. The *Drosophila* insulin-like receptor (InR) is homologous to mammalian insulin and IGF-1 receptors [259, 260], as well as to Daf-2. As in *C. elegans*, mutation of InR in the fly significantly extends adult longevity [261]. Females with an insulin receptor heteroallelic hypomorphic genotype are small, infertile and live 85% longer than wild type controls. The long-lived mutant flies share some important characteristics with wild type adults that are in reproductive diapause, including increased triglycerides and SOD, as well as reduced synthesis of juvenile hormone, a neurohormone that influences reproduction and exhibits some functional homology to vertebrate thyroid hormones [261].

The lifespan of female *D. melanogaster* is also extended by mutation of the IRS homologue *chico* [262–264]. Heterozygous flies of both sexes have slightly increased longevity; however, life extension by 48% is shown only in homozygous knockout female flies, while homozygous males are actually short-lived. It has been shown that flies homozygous for chico deficiency have reduced body sizes, increased levels of SOD and greatly reduced fecundity. Experiments involving genetic rescue of the effects of chico on somatic growth in transgenic flies and crosses with flies heterozygous for a dominant mutation causing female sterility have provided evidence that neither the dwarf phenotype nor infertility is required for the increased longevity of *chico* minus flies [264].

Recently, the *D. melanogaster* homologue of mammalian FOXO and *C. elegans* Daf-16 (dFOXO) has been identified [265–267]. Expression of dFOXO during early larval development causes inhibition of larval growth and alterations in feeding behavior. In addition, expression of dFOXO during certain larval or developmental stages leads to generation of adults that are reduced in size due to decreases in both cell size and cell number. This phenotype can be rescued by co-expression of upstream insulin signaling components dPI 3-kinase and dAkt, although it is not the case when FOXO is mutated to a constitutively active form. The alterations in larval development seen upon overexpression of dFOXO closely mimic the phenotypic effects of starvation, suggesting a role for dFOXO in the response to nutritional changes seen also in yeast and worm.

The most recent results have shown that activation of dFOXO in the adult pericerebral fat body of *D. melanogaster* is sufficient to increase both male and female lifespan, to increase resistance to oxidative challenge and to alter whole-animal lipid metabolism [268]. It reduces expression of the fly insulin-like peptide, *dilp-2*, that is synthesized in neurons, and represses endogenous insulin-dependent signaling in the peripheral fat body [268]. These findings suggest that, as in *C. elegans*, autonomous and non-autonomous roles of insulin signaling combine to control aging.

Vertebrates

Mice. Various naturally occurring transgenic and knockout rodent models have provided a unique opportunity to evaluate the relationship between the insulin/IGF-1 signaling pathway and longevity. Mice with mutations of Prop-1 (Ames Dwarf mouse) and Pit-1 (Snell Dwarf mouse), genes encoding transcription factors that control pituitary development, have reduced growth rate and body size, and also have 40–65% extended lifespan compared to wild type counterparts [269, 270]. These mice are deficient in serum growth hormone (GH), thyroid-stimulating hormone (TSH) and prolactin, as well as IGF-1, which is normally secreted by the liver upon stimulation with GH and mediates much of its activity. Since mice that cannot release GH in response to growth hormone-releasing hormone also live longer, it appeared that GH and IGF-1 deficiency mediated by the effects of Prop-1 and Pit-1 mutations may be the most important in the effect on longevity. The fact that mice with a knockout of the GH receptor/binding protein (GHR/BP-KO), which have high plasma levels of GH, but a 90% reduction in IGF-1 levels, also live longer than wild type mice suggests that the reduction in plasma IGF-1 is actually responsible for a major portion of the lifespan increase in dwarf and GH-deficient mice.

Although IGF-1 knockout mice are not viable [271] and insulin receptor knockout mice die within 8 days after birth [272], a moderate decrease in insulin and IGF-1 signaling has been shown to extend longevity in mice. Thus, loss of the single copy of *Igf1r* gene results in an average 26% increase in lifespan (females live 33% longer that wild type animals, males only 16%) [273]. This occurs with only a minimal reduction in growth. In addition, in contrast to naturally occurring long-lived Ames and Snell mice, *Igf1r* heterozygous mice have no alteration in the age of sexual maturation and fertility. Not surprisingly, serum IGF-1 levels are upregulated in both female and male heterozygous knockout mice. Males also tend to have higher fed glucose levels and impaired glucose tolerance, while females have lower fed glucose levels and are more insulin sensitive, although insulin levels are the same in all groups. In addition, metabolism in both the fed and resting state, body temperature, physical activity, food intake, fertility and reproduction do not differ between mutant and controls. Adult *Igf1r* heterozygous mice subjected to oxidative stress by injection of paraquat are more resistant to this challenge than controls, suggesting that the prolonged lifespan of mutant mice could be due to resistance to oxidative stress. This result is supported by the fact that the proportion of surviving mouse embryonic fibroblasts (MEFs) isolated from mutant mice is signifi-

cantly higher than controls after treatment with low doses of H_2O_2. Besides reduction of IGF-1R levels and IGF-1-induced tyrosine phosphorylation of IGF-1R and IRS1, IGF-1-stimulated tyrosine phosphorylation of the p52 and p66 isoforms of Shc is also reduced by 50%, indicating a link between Shc activation and mechanism by which IGF-1 regulates oxidative stress resistance in the mice.

It has been shown previously that mice with a targeted disruption of the *p66Shc* gene have increased stress resistance and prolonged lifespan by ~30% [274]. In addition, deletion of the *p66Shc* gene reduces systemic and tissue oxidative stress, vascular cell apoptosis and early atherogenesis in mice fed with high-fat diet [275]. It has been shown that a fraction of cytosolic p66Shc localizes within mitochondria where it forms a complex with mitochondrial heat shock protein 70 (Hsp 70) and regulates transmembrane potential and apoptosis [276]. Since it is suggested that mitochondria regulate lifespan through their effects on the energetic metabolism, p66Shc could contribute to lifespan determination by mitochondrial regulation of apoptosis.

Interestingly, a relation between p66Shc and FKHR-L1 (FOXO3a), a member of the forkhead (FOXO) family of transcription factors, has been also established. By comparison with the Daf2/Daf16 pathway in *C. elegans*, one would expect that greater activity of Daf-16-related FOXO transcription factor should result in an extension of mammalian lifespan. p66Shc is activated by Ser36 phosphorylation after UV irradiation or oxidant stimulation [274], and this is associated with an inactivation of FOXO transcription factors via phosphorylation, probably mediated by Akt [277]. FOXO3a tends to stimulate survival and resistance responses under oxidative stress, and its inactivation induces apoptosis [277]. Lack of p66Shc, therefore, could contribute to the increased longevity by this mechanism. In addition, FOXO3a stimulates the DNA repair pathway through Gadd45a protein, which could be the possible mechanism influencing lifespan [208].

There are three mammalian homologues of Daf-16 [197, 278, 279]: FOXO1 (or FKHR), FOXO3a (or FKHR-L1) and FOXO4 (AFX). Following stimulation by growth factors or cytokines, FOXOs are inhibited in most mammalian cells and tissues [197, 278, 280]. Although activation of this family of proteins has a clear effect on cellular lifespan, the specific roles of FOXO isoforms are still not fully understood, and mice lacking the three main isoforms display remarkably different phenotypes [281]. *Foxo1* homozygous knockout mice die before birth, whereas heterozygous *Foxo1* mice are healthy and protected against the development of diabetes induced by heterozygous deletion of the insulin receptor and insulin receptor substrate gene [282, 283]. In contrast, FOXO3a null mice have relatively few defects [284], and FOXO4 null mice have no obvious phenotype [281].

While the roles of insulin signaling in glucose metabolism and development of diabetes mellitus in mice have been studied in detail [285–287], examination of the effects of insulin on longevity and aging in mammals has been relatively recent. The hypothesis that insulin is involved in mammalian aging has evolved from several studies. In the Snell Dwarf mice, GH deficiency leads to reduced insulin release and alterations in insulin signaling, including decreased IRS-2, and reduced PI 3-kinase activity [288]. By contrast, in liver of Ames Dwarf mice insulin sensitivity is increased with concomitant insulin receptor, IRS-1 and IRS-2 upregulation and lower levels of insulin [289]. This is similar to the improved insulin sensitivity in calorie-restricted animals that have increased lifespan.

In the mouse, genetic disruption of the insulin receptor or proteins involved in insulin signaling, either in the whole body or specific organs usually leads to insulin resistance and the tendency to develop diabetes [288]. In most cases, the effect on longevity is unknown. On the other hand, mice with fat-specific disruption of the insulin receptor gene (FIRKO) are born with the expected frequency, survive well after weaning, are fertile and do not develop diabetes [290]. While insulin receptor expression is preserved in other organs, its expression is markedly reduced in brown and white adipose tissue, as is insulin-stimulated glucose uptake in white adipocytes. In addition, fat pads of FIRKO mice have a polarization of adipocyte cell size into large or small fat cells, but very few intermediate sized cells. The cells also show decreased expression of fatty acid synthase (FAS) and the adipogenic transcription factors SREBP-1 and C/EBPα, and increased expression of the adipokine ACRP30 [290].

Although growth curves of both male and female FIRKO mice from birth to 4 weeks of age are normal, by 4 months of age FIRKO mice have gained less weight than controls. At this age FIRKO mice have a 50–60% decrease in perigonadal fat pad mass, intrascupular brown fat pad mass and a ~30% decrease in whole body triglyceride content despite normal food intake. Because FIRKO mice are leaner, and eat the same as a normal mouse, the food intake of FIRKO mice expressed per gram of body weight exceeds that of controls by 50–55%. Fasted and fed glucose concentrations are indistinguishable between FIRKO and control mice. However, although there is no significant difference in plasma-fed insulin concentrations, FIRKO mice have significantly lower fasted insulin concentrations compared to controls. While serum triglyceride levels are significantly reduced in FIRKO mice, they also have normal serum free fatty acids, cholesterol, lactate levels and IGF-1 concetrations in serum. Interestingly, despite the decreased whole-body fat mass, FIRKO mice of both genders have about 25% higher plasma leptin levels than

control mice (~3-fold elevated when expressed per milligram of fat pad mass), and adipocyte complement-related protein (ACRP30) in serum is also significantly increased in FIRKO mice by the age of 10 months.

One striking aspect of the phenotype is protection from diabetes. Thus, FIRKO mice do not develop impaired glucose tolerance that occurs with normal aging in control mice. In addition, they are resistant to weight gain with aging or following induction of hyperphagia induced by gold thioglucose (GTG) treatment. Moreover, because they remain lean, FIRKO mice, unlike controls, do not develop impaired glucose tolerance 12 weeks after GTG treatment.

Most important, in the context of this review, the lifespan of both male and female FIRKO mice is increased (fig. 3). Thus, the median lifespan of all three control genotypes is 30 months, while about 80% of FIRKO mice remain alive at that age [291]. Indeed, the mean lifespan of FIRKO mice is increased by 134 days (18%), and the median lifespan is increased by 3.5 months, while maximum lifespan is extended by ~5 months. At 36 months all mice in the control group died, whereas at that time, 25% of FIRKO mice were still alive, and the longest-lived FIRKO mouse died at the age of 41 months [291].

The FIRKO mouse model clearly shows that reduced adiposity, even in the presence of normal or increased food intake, can extend lifespan. It also suggests a special role for the insulin signaling pathway in fat in the longevity process. Reduced adiposity also tends to result in lower insulin levels and protection from diabetes. Thus, in some ways, the FIRKO mouse mimics some of the effects of calorie restriction without caloric restriction. Whereas calorie restriction in *C. elegans* and *Drosophila* appears to extend life expectancy by slowing mitochondrial metabolism and metabolic rate, calorie restriction of rodents appears to extend lifespan without decreasing metabolic rate, and in the FIRKO mice, increased food intake relative to body weight suggests that metabolic rate is actually increased. FIRKO mice also exhibit increased UCP-1 expression per milligram of brown adipose tissue [290]. That is in agreement with Fisher 344 rats and outbred mice that have a higher metabolic rate than in long-lived animals [292–294]. If free-radical damage is an important factor for extended lifespan, then in the FIRKO mouse this must be derived directly or indirectly from the decreased fat mass rather than the diet.

Little is known about the effect of other signaling mutations on longevity. Thus far, no studies have been performed on the IRS, PI 3-kinase or Akt knockout with regard to lifespan. GLUT4 is the insulin-sensitive glucose transporter in muscle and adipose tissue, the major sites for glucose disposal. It has been demonstrated that functional GLUT4 protein is essential for sustained growth, normal cellular glucose and fat metabolism and longevity. In fact, GLUT4 knockout mice are growth retarded and exhibit decreased longevity associated with cardiac hypertrophy and severely reduced adipose tissue deposits [295].

Another interesting short-lived mouse model, which may be linked to the insulin/IGF-1 signaling system, is the *klotho* mouse [296]. In the *klotho* mouse a transgene was inserted in the 5′ promoter region of the previously un-

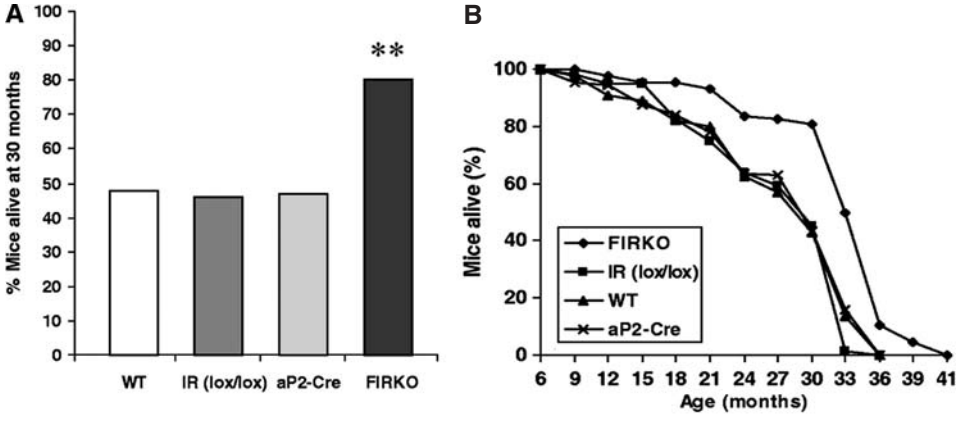

Figure 3. Extended lifespan in FIRKO mice. (**A**) Percentage of mice alive at 30 months of age. The median lifespan of the three control genotypes [WT, IR (lox/lox), aP2-Cre] was ~30 months. The fraction of mice alive at 30 months from founder line 1 [WT (n = 34), IR (lox/lox) (n = 35), aP2-Cre (n = 30), and FIRKO (n = 32)] and from line 2 [WT (n = 33), IR (lox/lox) (n = 31), aP2-Cre (n = 27), and FIRKO (n = 28)] are pooled. Data for males and females are also pooled, because they were similar in each of the groups. **$p < 0.05$. (**B**) Pooled survival curves for FIRKO mice derived from two different aP2-Cre founder lines. Pairwise comparison among genotypes for age-specific survival by log-rank test with significance corrected for multiple tests. Median lifespan in line 1 (n = 131) did not differ among control groups [WT, IR (lox/lox), and aP2-Cre] ($p = 0.31$), whereas median lifespan of FIRKO mice was 33.5 months and was significantly increased compared to controls ($p < 0.001$). Median lifespan in line 2 (n = 119) was 33.4 months for FIRKO mice. The maximum longevity (average lifespan of the 10% longest lived mice) was significantly increased from 34.7 months in the controls to 39.5 months in FIRKO mice ($p < 0.001$). Among the control groups WT, IR (lox/lox) and aP2-Cre, maximum lifespan did not differ ($p = 0.62$). The curves shown represent the pooled data from both of these lines. The figure is taken from [291].

recognized *klotho* gene, generating a strong hypomorphic *klotho* allele. *Klotho* mice (KL –/–), homozygous for the mutated allele, develop normally for 3–4 weeks, but then begin to manifest multiple age-related disorders, including infertility, ectopic calcification, lipodystrophy, skin and muscle atrophy, osteoporosis, arteriosclerosis and pulmonary emphysema. *Klotho* mice die around 2 months of age.

Recent studies indicate that the *Klotho* protein is a novel single-pass transmembrane protein, which is expressed in distal convoluted tubules of the kidney and the choroid plexus of the brain. The molecular mechanism by which the *klotho* gene product suppresses the aging phenotype is not clear. Single nucleotide polymorphisms (SNPs) in the human klotho gene have been associated with altered lifespan [297], and associations between SNPs in the human klotho gene and coronary artery disease [298] and senile osteoporosis [299, 300], common age-related disorders in humans, have been recently demonstrated.

As mentioned before, sirtuins extend lifespan in yeast and worms, and it seems that Sir2p in yeast plays an essential regulatory role during calorie restriction. Extra copies of the *C. elegans* homologue Sir-2.1 extend lifespan of wild type, but not Daf-16 worms, indicating that in worms Sir-2 requires Daf-16 for longevity.

In mammals, Sir2 is represented by seven homologues [301, 302]. The ortholog of Sir2, SIRT1, is an NAD-dependent deacetylase that, in addition to histones, also deacetylates other proteins, including MyoD and the tumour suppressor p53 [303–309]. Increased SIRT1 activity in cultured cells reduces p53-mediated apoptosis in response to radiation or oxidative stress. SIRT1 has been also shown to repress terminal differentiation in dividing myocytes [308]. *SIRT1* knockout mice have a severe phenotype with a high degree of embryonic and postnatal lethality [309]. However, on outcrossed backgrounds, some *SIRT1* homozygous knockout mice survive to adulthood. These mice are also smaller than wild type, sterile, display hyperacetylated p53 and show developmental defects [309].

Recently it was shown that SIRT1 deacetylates and represses the activity of the forkhead transcription factor FOXO3a and other mammalian forkhead factors, repressing their activity [209, 310], opposite to the effect found in *C. elegans*. SIRT1 also reduces forkhead-dependent apoptosis, and this parallels the effect of this deacetylase on p53. Some investigators have speculated that downregulation of these two classes of damage-responsive mammalian factors may lead to reduced cancer incidence and favor a long lifespan under calorie restriction. Finally, regulation of FOXO by SIRT1 may link this sirtuin with forkhead-mediated metabolic changes in mammals, such as gluconeogenesis, insulin secretion/action, lipid usage and ketogenesis during calorie restriction.

Recent results have shown that mammalian SIRT1 is induced in many tissues of calorie-restricted rats, including brain, visceral fat pads, kidney and liver [311]. It is also increased in FaO rat hepatoma and human 293 cells treated with serum from calorie restricted rats. Insulin and IGF-1 attenuate this response. SIRT1 inhibits stress-induced apoptotic cell death by deacetylating the DNA repair factor Ku70, causing it to sequester the proapoptotic factor Bax away from mitochondria [311, 312]. Thus, calorie restriction could extend lifespan by promoting the long-term survival of irreplaceable cells.

Humans. Evidence for involvement of the insulin/IGF-1 pathway in the control of aging and longevity in humans is suggestive, but somewhat conflicting, because of the severe metabolic consequences and development of diabetes that accompanies deficiency of insulin or the insulin receptor. In humans, insulin sensitivity normally decreases during aging, and insulin resistance is an important risk factor [313, 314] for a variety of illnesses that affect morbidity and mortality among the elderly [315, 316], including hypertension, atherosclerosis, obesity and diabetes.

Interestingly, one of the striking physiological characteristics recently identified in centenarians is their greatly increased insulin sensitivity compared with younger subjects [317–319]. The results of the initial study on a limited number of centenarians living in southern Italy showed that this group have a preserved glucose tolerance and insulin action and lower plasma IGF-1 levels compared with aged subjects [319, 320]. More recently, data from 466 healthy subjects with an age range from 28 to 110 years demonstrated a significant reduction of insulin resistance in subjects from 90 to 100 years old, even after adjustment for body mass index ($p < 0.010$) [321]. These data indicate that an efficient insulin response may have an impact on human longevity, but could also indicate selection of a protected population.

In addition, it has been demonstrated that polymorphic variants of the IGF-1 receptor and phosphatidylinositol PI 3-kinase genes can affect IGF-1 plasma levels and may impact on human longevity [322], in particular an A/G polymorphism at position 3174 (codon 1013) in the IGF-1 receptor locus. It has been found that individuals bearing at least one A allele at the IGF-1R locus have lower plasma IGF-1 levels, and this variant is found at an increased proportion in long-lived individuals. Furthermore, an interaction between the A allele of the IGF-1R locus and the T allele at the P I3-kinase (PI3KCB) locus have been shown to have the lowest IGF-1 plasma levels and have been also found among long-lived individuals. On the other hand, IGF-1R mutations have been shown to result in intrauterine and postnatal growth retardation [323].

As mentioned before, findings in different organisms are consistent with the hypothesis that mutations in genes of

the insulin-like signaling network confer oxidative stress resistance as well as lifespan extension, suggesting that diminished oxidative damage of macromolecules might be the final common pathway of the effects of the insulin/IGF-1 signaling mutation on longevity. Studies in humans have shown that free radicals play a key role in the pathogenesis of a wide variety of diseases, including cardiovascular diseases, atherosclerosis and diabetes mellitus [324, 325]. In accordance with that hypothesis, healthy centenarians have been shown to have a low degree of oxidative stress and high antioxidant defenses [326, 327].

In addition, it has been shown that insulin and IGF-1 signaling plays a role in cellular senescence, which is the limited ability of human cells to divide when cultured in vitro. Senesence is accompanied by a specific set of changes in cell morphology, gene expression and function, which have been implicated in human aging [328]. The cellular hypothesis of aging was established about 30 years ago [329] and is supported by evidence that the replicative potential of primary cultured human cells is dependent on donor age.

Although Akt/PKB has been reported to promote proliferation and survival of mammalian cells, recent results have shown that PKB/Akt activity increases along with cellular senescence and its inhibition extends the lifespan of primary cultured endothelial cells [330, 331]. It has been demonstrated that constitutive activation of Akt/PKB promotes senescence-like arrest of cell growth via a p53/p21-dependent pathway. Akt/PKB inhibition of FOXO3a, which influences p53 activity by regulating the level of reactive oxygen species, is essential for this growth arrest to occur, because Akt/PKB-induced growth arrest can be inhibited by a mutated forkhead transcription factor. Moreover, insulin increased p53 activity via the Akt/PKB-dependent mechanism and reduced the lifespan of endothelial cells [330]. Thus, except for its role in atherosclerosis [331–333], Akt/PKB-induced senescence-like phenotype may be also involved in diabetic vasculopathy, since hyperinsulinemia could constitutively activate Akt/PKB in endothelial cells.

All the aforementioned results suggest that a genetic link between histone deacetylation, insulin-like signaling, oxidative stress and longevity, originally discovered in invertebrates and mice, may also exist in humans (fig. 4). However, the exact interrelation among the insulin/IGF-1 signaling pathway, oxidative stress and longevity still needs to be revealed. Recently, a sequence poly-morphism G477T in the human sirtuin 3 (SIRT3) gene, encoding a putative mitochondrial NAD-dependent deacetylase SIRT3, has been studied for its relationship to longevity [334]. The study found that in males a TT genotype increases, while the GT genotype decreases survival in elderly, suggesting that SIRT3 itself or a gene strictly linked to SIRT3 may have a role in human longevity.

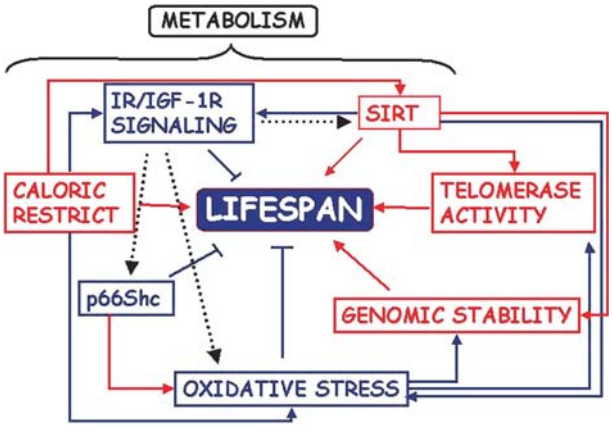

Figure 4. Interrelation of parameters that influence lifespan. Calorie restriction, telomerase activity, genomic stability and overexpression of SIRT proteins extend, while oxidative stress, p66Shc and insulin/IGF-I signalling shorten lifespan. There is, however, interrelation of all these parameters.

Conclusions

In conclusion, strong similarities exist between insulin and IGF-1 signaling systems in yeast, worms, flies, mammals and humans. These may be linked to oxidative stress resistance, metabolic regulation, food utilization and lifespan in each of these organisms. Such similarities suggest that the insulin/IGF-1 system arose early in evolution and that it is a central component of an anti-aging system, which is conserved from yeast to humans. However, there are differences that have to be taken into account. Thus, in *C. elegans* and *Drosophila* defects in the insulin-like signaling system cause diapause (inactive, non-feeding stage of life), which is not case in mammals. Furthermore, the complexity of the insulin/IGF-1 pathways has increased greatly during evolution [335]. Worms and flies have only one receptor for both hormones, whereas vertebrates have at least three closely related receptors. In mammals, insulin is primarily involved in metabolism and glucose homeostasis, while the primary role of IGF-1 is mediating effects of GH on somatic growth. Therefore, distinct aspects of the physiology and metabolism controlled by different parts of the insulin/IGF-1 signaling network and their relationship to longevity still need to be elucidated.

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