Aging, Bench to Bedside: A Collection in *PLoS Genetics*

This collection focuses on a rapidly evolving field in which the study of both species-specific and ubiquitous aging mechanisms informs the biological process of aging. Yet the field is not without substantial controversy. Differing views arise as we come to understand aging across model systems – from bacteria to humans. *Image: modified from a photograph kindly provided by the Keane family, PLoS Biology 4[4]: e119.*

Editorials

**An Age-Old Problem**

"The advent of molecular biology and genetics has offered a unique opportunity to help us understand why organisms age, which in turn might offer clues as to how one might decelerate, stop, or even reverse this process." Reviews Editors Nicholas Katsanis and Susan Rosenberg introduce this interdisciplinary series.

*Katsanis N, Rosenberg SM*  
doi:10.1371/journal.pgen.0030037

**Entropy Explains Aging, Genetic Determinism Explains Longevity, and Undefined Terminology Explains Misunderstanding Both**

In this closing editorial, Leonard Hayflick explains that "Communication in the field of biogerontology is a minefield." He highlights that the lack of universally accepted definitions "also produces erroneous interpretations of research results; illogical allocation of research funds; and misdirected scientific, economic, social, and political policy decisions."

*Hayflick L*  
doi:10.1371/journal.pgen.0030220

Reviews

**The Role of Mitochondrial DNA Mutations in Mammalian Aging**

The authors examine the evidence supporting a causative role for mtDNA mutations in mammalian aging.

*Kujoth GC, Bradshaw PC, Haroon S, Prolla TA*  
doi:10.1371/journal.pgen.0030024

**Dietary Restriction in *Drosophila*: Delayed Aging or Experimental Artefact?**

The authors review the recent literature on dietary restriction in *Drosophila* to point out some methodological issues that can obscure mechanistic interpretations.

*Piper MDW, Partridge L*  
doi:10.1371/journal.pgen.0030057
**Recent Developments in Yeast Aging**
The authors briefly outline aging in yeast and describe recent findings that continue to keep this "simple" eukaryote at the forefront of aging research.  
*Kaeberlein M, Burtner CR, Kennedy BK*  
doi:10.1371/journal.pgen.0030084

**Genetic Determinants of Human Health Span and Life Span: Progress and New Opportunities**  
The authors conclude that there are great opportunities for research on the genetics of human aging, particularly given the huge fund of information on human biology and pathobiology, and the rapidly developing knowledge of the human genome.  
*Martin GM, Bergman A, Barzilai N*  
doi:10.1371/journal.pgen.0030125

**Genetics of Aging in Caenorhabditis elegans**  
A dissection of longevity in *Caenorhabditis elegans* reveals that animal life span is influenced by genes, environment, and stochastic factors.  
*Antebi A*  
doi:10.1371/journal.pgen.0030129

**Perspective**

**The Role of Mitochondrial DNA Mutations in Mammalian Aging**  
"One of the most exciting features of the discovery of a mandatory aging phenomenon in bacteria and eukaryotes dividing by binary fission is that, by virtue of being exquisitely tractable systems for genetic and biochemical analysis, there is a good chance of identifying the true aging agents in these systems."  
*Nyström T*  
doi:10.1371/journal.pgen.0030224
An Age-Old Problem
Nicholas Katsanis*, Susan M. Rosenberg

Contra vim mortis non crescit herba in hortis. “There is no herb in the gardens against the power of death.” —King Sigismund III (1566–1632) on his deathbed

Rapid advances in the basic and medical sciences, while rapidly expanding our knowledge base on a host of physiological processes (and their cellular and organismal consequences when disrupted), are also posing a significant burden. The sheer volume of information amassed from a host of experimental systems and model organisms can represent a daunting assimilation task to both the neophyte and the experienced investigator, a problem often compounded by contradictory and/or conflicting data that are inherent to the constant flux of scientific discovery.

With these challenges in mind, we are pleased to introduce a new series initiative by *PLoS* Genetics. In keeping with the mission statement of our journal to present interdisciplinary research in the broadest possible context, we have commissioned a series of Review and Opinion articles bound thematically to a discrete set of topics of inherent complexity, as well as broad interest. Each component of the series will examine a facet of the chosen problem and we hope that the amalgam of each series, which will be available electronically as a unified entity, will transmit to our readership an appreciation of the progress made and the future trends in each field.

Our inaugural series focuses on aging, a field under both rapid evolution and substantial controversy. Humans are arguably unique among sentient species in that we are cognizant of our own mortality even at moments when it is not imminent—a fact that has constituted a major force in shaping our civilization (in all its iterations). The advent of molecular biology and genetics has offered a unique opportunity to help us understand why organisms age, which in turn might offer clues as to how one might decelerate, stop, or even reverse this process. When examining the field of aging research, we can identify three major spheres: cellular aging (senescence/quotescence), organismal aging, and age-related disorders. In this series, we will focus primarily of the first two areas, because the study of age-related disorders does not necessarily inform the basic questions, namely: (a) what are the basic determinants of lifespan, and (b) what are the fundamental cellular and molecular mechanisms that underscore aging processes?

Like most disciplines, aging research has benefited from the availability of a host of prokaryotic and eukaryotic model organisms. However, although some aging mechanisms seem to hold true in diverse species, such as the benefits of caloric restriction in prolonging lifespan, there are also multiple aging mechanisms, and their possible clade specificity has generated potential for controversy. This highlights an important feature of the aging process; aging occurs in many species in nature, and is particularly obvious in captivity where natural hazard is removed. The aging process is in itself, however, nonadaptive and it evolves because natural selection is relatively powerless to act on the traits of older individuals. Nonetheless, the study of both species-specific and ubiquitous aging mechanisms will inform the biological process of aging and will ultimately be better reflected in the management of aging in humans.

We are pleased to introduce the first element of our series in this month’s issue, in which Tom Prolla and colleagues will discuss the effect of...
mitochondrial mutations on the aging process in the mouse [1]. As our discussion progresses, we will bring perspectives that encapsulate work in other species and cross-reference those electronically, accompanied by selected original research papers from the field. Linda Partridge will summarize recent progress in understanding the aging process in *Drosophila*, and a separate review will address the current state of affairs in our understanding of the genetic and epigenetic modulation of healthspan and lifespan in humans (including allied premature aging disorders). In parallel, papers will focus on the aging process in yeast, recent work in the field of *C. elegans* aging, and recent exciting work in bacterial aging.

We very much hope that our readership will enjoy these articles, appreciate both the depth and breadth of the topic, and find this electronically bound series a useful intellectual and educational tool.

References

Communication in the field of biogerontology is a minefield because all of the commonly used terms have no universally accepted definitions. In a series of five annual meetings that I chaired recently in an attempt to define common terms, the dozen or more experts who attended could not agree on the definition of almost all of them, including “aging.” The committee was disbanded and the communications dilemma remains.

“Aging, Bench to Bedside,” the collection of mini reviews published as a series in this journal, is representative of this unsolved problem. Not only does the problem result in communication failures, it also produces erroneous interpretations of research results; illogical allocation of research funds; and misdirected scientific, economic, social, and political policy decisions [1–3]. There is no other field of science in which a similar bleak situation exists.

As a consequence of the terminology dilemma, I will define for use in this editorial the four aspects of the finitude of life: aging, the determinants of longevity, age-associated diseases, and death. I will not discuss the latter, although even this word defies a universally accepted definition.

The Aging Process

Age changes can occur in only two fundamental ways: by a purposeful program driven by genes or by random, accidental events.

It is a cornerstone of modern biology that a purposeful genetic program drives all biological processes that occur from the beginning of life to reproductive maturation. However, once reproductive maturation is reached, thought is divided with respect to whether the emerging aging process is a continuation of the genetic program or whether it is the result of the accumulation of random, irreparable losses in molecular fidelity.

The deterministic dream of 19th century physicists was torpedoed in the 20th century with Heisenberg’s discovery of the uncertainty principle. In fact, the fundamental laws of physics can only be expressed as probabilities. The most compelling evidence for the belief that biological aging is also a random process is that everything in the universe changes or ages in space-time without being driven by a purposeful program. Although there is no direct evidence that genes drive age changes, their critical role in longevity determination is indisputable.

There is a huge body of knowledge supporting the belief that age changes are characterized by increasing entropy, which results in the random loss of molecular fidelity, and accumulates to slowly overwhelm maintenance systems [1–4].

Both biological systems and inanimate objects incur change over time. Living systems, however, are, among other properties, distinguishable from inanimate objects, because a purposeful genetic program governs the changes that occur from their beginning until reproductive maturation. In inanimate objects, change is not programmed. It is continuous and never ending. Whether the changes that occur in inanimate objects are called age changes or not occurs because of the tendency for humans to view the physical world in anthropomorphic terms.

The common denominator that underlies all modern theories of biological aging is change in molecular structure and, hence, function. These changes are the result of entropic changes, which is now supported by the recent reinterpretation of the Second Law of Thermodynamics, where the belief that it only applies to closed systems has been overturned [3].

Entropy is the tendency for concentrated energy to disperse when unhindered regardless of whether the system is open or closed. The hindrance of entropic change is the relative strength of chemical bonds. The prevention of chemical bond breakage, among other structural changes, is absolutely essential for life. Through evolution, natural selection has favored energy states capable of maintaining fidelity in most molecules until reproductive maturation, after which there is no species survival value for those energy states to be maintained indefinitely.

The dispersal of energy may result in a biologically inactive or malfunctioning molecule. Energy dispersal is never entirely eliminated but can be circumvented for varying time periods by repair or replacement processes. The internal presence of
these repair or replacement processes represents a major difference between living and inanimate forms.

From the standpoint of a physicist, a lowered energy state is not necessarily disorder, because it simply results in the identical molecule with a lowered energy state. The fact that such a molecule might be biologically inactive may not concern the physicist, but it definitely does concern the biologist and, especially, the biogerontologist.

The aging process occurs because the changed energy states of biomolecules renders them inactive or malfunctioning. Identical events also occur before the aging phenotype appears, but repair and replacement processes are capable of maintaining the balance in favor of functioning molecules; otherwise, the species would vanish. After reproductive maturation, this balance slowly shifts to one in which molecules that lose their biologically active energy states are less likely to be replaced or repaired. The diminution of repair and replacement capability is further exacerbated, because the enormously complex biomolecules that compose the repair and replacement systems also suffer the same fate as their substrate biomolecules.

When the escalating loss of molecular fidelity ultimately exceeds repair and turnover capacity, vulnerability to pathology or age-associated diseases increases [1,3,6]. Immortal biological systems cannot exist, if for no other reason than molecular turnover (or dilution) insures that the molecules present at the beginning of a biological lineage are unlikely to be present in that lineage when it reaches Avogadro’s Number of about $6 \times 10^{25}$ cells. The only biological property that is long lasting is the weakest link. When a bacterium like Escherichia coli divides by fission, one of the two daughter lineages is “damaged enriched” and the other has “low damage” [9]. The former are “non-culturable or genetically dead” while the latter are “reproductively competent.” In Caulobacter crescentus, replicative senescence has been observed [10], a phenomenon that we first described in normal human cells more than 45 years ago [11]. The phenomenon has also been reported to occur in E. coli and in Saccharomyces cerevisiae [12]. The occurrence of replicative senescence in normal cells appears to be a universal biological phenomenon.

**The Determinants of Longevity**

The second aspect of the finitude of life is longevity determination—a process that is completely different from aging.

Unlike aging, the genome governs the processes that determine longevity. These are the systems that synthesize molecules and repair or replace them. When the repair or replacement systems are unable to maintain the positive balance that existed prior to reproductive success, a tipping point is reached where the aging phenotype slowly becomes manifest.

Aging must occur in molecules that previously existed with no age changes. It is this prior functional state of molecules and the subsequent efficiency of their maintenance that governs longevity determination.

Unlike the stochastic process that characterizes aging, longevity determination is not a random process. It is governed by the level of physiological reserve reached at the time of reproductive maturation that, through natural selection, was achieved to better guarantee survival to that age. The determination of longevity is incidental to the main goal of the genome, which is to govern events until reproductive maturity occurs. Thus, the genome only indirectly governs longevity.

The variations in excess physiological capacity, repair, and turnover account for the differences found in longevity both within and among species. One might think of longevity determination as the energy state of molecules before they incur age changes, and aging as the state of molecules after energy dissipation results in an irreparable state of functional loss. Longevity determination is a genome-driven anabolic process that addresses the question: “Why do we live as long as we do?” Aging is a chance-driven catabolic process that addresses the question: “Why do things finally go wrong?” Studies on “dietary restriction” (DR) [13–15] would be better interpreted to have contributed to our understanding of longevity determination than to our...
understanding of aging. The increase in longevity found by DR does not provide proof that it directly affects the aging process, because longevity is commonly used as the endpoint in these studies, and not age changes. Increased longevity also could occur if DR eliminated or delayed the appearance of pathology, because biomarkers for aging in most animals are either unknown or not evaluated. Furthermore, because controls are either fed ad lib, or some arbitrary number of calories, it would be just as logical to conclude that overfed animals have a reduced longevity as it would to conclude that DR increases longevity. Indeed, alternating periods of feast and famine is the usual lifestyle for most animals and this is much more likely to mimic the effects of DR. Indeed, DR research might be telling us more about the actual longevity of feral animals absent causes of death attributable to predation, disease, or accidents.

The many studies on gene mutations in C. elegans, drosophila, and other invertebrates [13–15] that have led to the view that genes involved in aging have not demonstrated that gene manipulation has slowed, stopped, or reversed biomarkers of aging. When all-cause mortality is used as the end point, as is done in experiments with these animals, it cannot be assumed that age changes are being affected. These studies are more accurately interpreted to have an impact on our understanding of longevity determination. Furthermore, genes that govern the aging process are unnecessary for it to occur. Just as blueprints are vital to construct a complex machine, but contain no information describing a system to cause its aging, the genome is necessary to govern biological development and maintenance, but it contains no instructions to cause the animal to age. Automobiles know how to age without requiring instructions. Both ultimately fail because of changes in molecular fidelity driven by increasing entropy.

In unicellular organisms like yeast, aging has been defined either as the length of time that a yeast cell can survive in a nondividing state, or by the number of daughter cells produced by a mother cell before senescence [13]. In higher animals, chronological time is generally recognized as a poor measure of the rate of aging because of the enormous variations in the aging phenotype among individuals. And, the number of progeny produced before senescence occurs has never been considered to be related to aging. It is more likely that what is being studied are longevity determinants for reasons already given. It has been known for more than a century that longevity determinants in invertebrates are, unlike aging, capable of manipulation.

**Age-Associated Diseases**

The third aspect of the finitude of life is age-associated disease. The distinction between the aging process and age-associated disease is not only based on the definition of aging described above, but it is also rooted in several practical observations. Unlike any disease, age changes:

1. Occur in every multicellular animal that reaches a fixed size at reproductive maturity.
2. Cross virtually all species barriers.
3. Occur in all members of a species only after the age of reproductive maturation.
4. Occur in all animals removed from the wild and protected by humans even when that species probably has not experienced aging for thousands or even millions of years.
5. Occur in virtually all animate and inanimate matter.
6. Have the same universal molecular etiology, that is, thermodynamic instability.

Unlike aging, there is no disease or pathology that shares these six qualities. The inexorable loss of molecular fidelity that defines aging can either lead to changes that may be nonpathological affronts to vanity, inconveniences, or simply uncomfortable. When the same kind of molecular mischief occurs in the cells of vital organs, leading to an increase in vulnerability to disease or pathology, treatment is required because life may be threatened.

The fundamental aging process is not a disease but it increases vulnerability to disease. Because this critical distinction is generally unappreciated, there is a continuing belief that the resolution of age-associated diseases will advance our understanding of the fundamental aging process [16]. It will not. This is analogous to believing that the successful resolution of childhood pathologies, such as poliomyelitis, Wilms’ tumors, and iron deficiency anemia advanced our understanding of childhood development. It did not.

It is often observed that, “The classical evolutionary biological theory of aging tells us that senescence occurs in age-structured populations because of the decline in the force of natural selection with age” [17]. And, a less common belief that, “…the force of natural selection could conceivably increase with age” [17]. These beliefs belie the fact that the forces of natural selection are constant and that large changes usually occur only on an evolutionary time scale. What changes with age is an animal’s ability to adapt to the constant forces of natural selection.

The failure to distinguish the fundamental biology of aging (biogerontology) from age-associated pathology (geriatric medicine), and both from longevity determinants, is the most serious impediment to our understanding of the aging process. This failure is exemplified best by realizing that under the rubric “Aging Research,” misled policy makers have appropriated most available funds to research on age-associated diseases. Yet no advance in geriatric medicine will add to our knowledge of the fundamental biology of aging [1–3].

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**References**

The Role of Mitochondrial DNA Mutations in Mammalian Aging

Gregory C. Kujoth, Patrick C. Bradshaw, Suraiya Haroon, Tomas A. Prolla*

ABSTRACT

Mitochondrial DNA (mtDNA) accumulates both base-substitution mutations and deletions with aging in several tissues in mammals. Here, we examine the evidence supporting a causative role for mtDNA mutations in mammalian aging. We describe and compare human diseases and mouse models associated with mitochondrial genome instability. We also discuss potential mechanisms for the generation of these mutations and the means by which they may mediate their pathological consequences. Strategies for slowing the accumulation and attenuating the effects of mtDNA mutations are discussed.

Introduction

The mitochondrial theory of aging is based on the premise that reactive oxygen species (ROS), generated throughout the lifespan of an organism, damage mitochondrial macromolecules, including proteins, lipids, and mtDNA. Although most molecular damage is reversible through repair or molecular turnover mechanisms, unrepaired DNA damage may lead to mutations that accumulate as a function of age. The accumulation of mutations ultimately leads to permanent age-related mitochondrial dysfunction, which contributes to the aging phenotype. The mammalian mitochondrial genome is compact (~16 kbp), encoding 13 essential subunits of the respiratory chain and multiple tRNAs and rRNAs. Because cells may have hundreds of mitochondria, and each carries multiple copies of mtDNA, the contribution of mtDNA mutations and deletions to normal aging remains a controversial issue.

Evidence for a Causal Role of mtDNA Mutations in Aging

Because the most obvious consequence of mtDNA mutations is an impairment of energy metabolism, most studies addressing aging effects have focused on tissues that are postmitotic and display high energetic demands, such as the heart, skeletal muscle, and the brain. Indeed, several studies have unambiguously demonstrated that mtDNA base-substitution mutations accumulate as a result of aging in a variety of tissues and species, including rodents, rhesus monkeys, and humans. In humans, initial studies focused on quantification of individual base-substitution mutations in mtDNA that were shown previously to be pathological in human inherited mitochondrial diseases. For example, the A3243G mtDNA mutation, which results in maternally inherited mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS), increases with age in the skeletal muscle of normal humans [1], but only a small fraction of mtDNA molecules in phenotypically normal humans is likely to carry these disease-associated mutations. Thus, it is unlikely that these mutations have deleterious consequences in normal aging. Studies performed in the Attardi laboratory have established that some specific base-substitution mutations can reach high levels in fibroblast cells derived from aged individuals [2] and also in skeletal muscle [3]. The reason why these specific mutations accumulate in mtDNA is unclear, but they are tissue-specific and occur in mtDNA control sites for replication. Interestingly, the same group has found a C150T transition mutation that occurs in most or all mtDNA molecules (i.e., a homoplasmic mutation) is present in leukocytes from approximately 17% of individuals aged 99–106 years old. This mutation is associated with a new replication origin position, suggesting that it may confer a survival advantage in humans [4].

With the development of high-throughput sequencing methods, an unbiased large-scale examination of either selected regions or the entire mtDNA sequence has become feasible. Using a PCR-based amplification and subsequent cloning and sequencing of individual mtDNA fragments, Lin et al. reported that the brains of elderly human subjects had a high aggregate of mtDNA base-substitution mutations, reaching $2 \times 10^{-4}$ mutations/bp [5]. Several studies in rodent and primate tissues are in agreement with this estimate of mtDNA mutational burden, but a study using direct cloning of mtDNA reported much lower levels [6]. This suggests that technical issues remain a problem in determining mtDNA mutation frequencies. Deletions, which can be readily detected by PCR but are not easily quantified, also increase with aging in multiple tissues in rodents [7] and humans [8,9] and can be clonal, as determined by analysis of individual cardiomicyocytes from aged humans [10]. In agreement with the hypothesis that mtDNA deletions contribute to mammalian aging, it has been shown that they accumulate exponentially in several tissues, and do so much faster in short-lived mice as compared to long-lived humans [11].

* To whom correspondence should be addressed. E-mail: tprolla@wisc.edu.
An ongoing debate in the field relates to the issue of causality: are mtDNA mutations merely markers of biological age, or do they lead to a decline in physiological function that contributes to the aging process? Two important age-related phenotypes have helped to address this issue. A common feature of aging in multiple species, including humans, is the age-related loss of muscle mass, termed sarcopenia. Studies using laser capture microdissection to study single muscle fibers in skeletal muscle from sarcopenic rats have shown that mtDNA deletions colocalize with electron transport system abnormalities, fiber atrophy, and splitting [12]. Interestingly, the mutations are largely clonal and absent from phenotypically normal regions within individual muscle fibers [13]. In a similar study of aged (69–82 years old) human muscle biopsies, an association between a deficiency in the mitochondrially encoded cytochrome c oxidase (COX) and clonally expanded base-substitution mutations and deletions was shown [14]. Perhaps the strongest evidence that clonally expanded mtDNA mutations can be causal in both age-related dysfunction and disease comes from recent studies of neurons present in the substantia nigra region of the human brain. These dopamine-rich, pigmented neurons contain very high levels of mtDNA deletions. Deleted mtDNA molecules are clonal in each neuron, and are associated with respiratory chain deficiency [15]. The level of mtDNA deletions increases with normal aging, and is higher in Parkinson’s disease [16]. Cytochrome c oxidase–deficient cells have also been shown to increase with age in both hippocampal pyramidal neurons and choroid plexus epithelial cells [17]. Although these studies do not prove causality, they provide strong evidence in support of the hypothesis that mtDNA deletions contribute to aging in mammals.

A significant gap in our knowledge concerns the mechanisms of age-related clonal expansion of mtDNA base-substitution mutations. Using single-cell sequence analysis, Nekhaeva et al. [10] first reported that a high proportion of human buccal epithelial cells and cardiomyocytes carry clonally expanded mtDNA base-substitution mutations. These clonally expanded mtDNA mutations are abundant in cells of aged individuals and result in very different mtDNA mutational spectra in these two cell types. Specifically, epithelial cells display a mutational hotspot in a homopolymeric C7–8 tract, whereas almost all cardiomyocyte mutations were observed within a 30-bp sequence in the control region. This sequence was postulated to represent either a binding site for a mitochondrial protein or a secondary structure of functional importance to mitochondria [10]. Because only a small fraction (~5%) of the mtDNA genome was sequenced in this study, it appears very likely that most human cells carry clonally expanded mtDNA base-substitution mutations.

A recently described observation, the accumulation of mtDNA mutations in human crypt stem cells, has also provided insights on the mechanisms of clonal mtDNA mutation accumulation. Taylor et al. described the high incidence of COX-negative cells in intestinal crypts of aged humans [18], and a more recent study strongly suggests that intestinal crypts carrying mtDNA mutations clonally expand by fission [19]. Interestingly, the pattern of distribution of these cells in individual crypts is not random, suggesting that mutations arising in adult stem cells result in the accumulation of such mutations in the tissue. But how do mtDNA mutations become clonal within a cell in the first place? Because the spectrum of expanded mutations is very different between cardiomyocytes and epithelial cells, different mechanisms of expansion, namely random segregation or positive selection, have been proposed for these cell types [10]. Interestingly, modeling of mtDNA replication in human cells suggests that genetic drift and expansion of mutations that occur in early adult life may account for the abundance of specific mtDNA mutations within individual cells [20]. The finding that the clonal expansion of mtDNA base-substitution mutations is a widespread process in human somatic cells may have profound implications for both aging and age-related diseases.

**Human Disorders Associated with Instability of the Mitochondrial Genome**

Normal human aging is a gradual, cumulative process that spans decades and most likely involves multiple mechanisms. Information on the specific contribution of mtDNA instability to human aging can be inferred through the analysis of disorders associated with increased mtDNA mutation or deletion frequency. Tissues most affected by disorders associated with inherited mtDNA mutations are the same tissues markedly affected by normal aging; these include the brain, heart, skeletal muscle, kidney and the endocrine system [21]. Disorders associated with increased levels of mtDNA mutations generally fall into two classes: those associated with specific, maternally-inherited mtDNA mutations; and, those associated with mutations in nucleus-encoded genes important for maintaining the fidelity of mtDNA replication and mtDNA stability. Because disorders in the latter category result in random accumulation of many different mtDNA mutations and deletions, they may better represent the potential consequences of age-related mtDNA mutation accumulation in humans.

Nucleus-encoded DNA polymerase γ (POLG) is the only known DNA polymerase in animal cell mitochondria. It has conserved polymerase and exonuclease domains, the combined action of which results in high-fidelity mtDNA replication with human POLG displaying an average error frequency of ~1 error/500,000 bp in vitro [22,23]. Mutations in the human POLG gene are associated with progressive external ophthalmoplegia (PEO), Alpers syndrome, and ataxia (see Figure 1). Disease onset typically occurs after the mid-twenties and can be associated with a variety of symptoms, including ophthalmoplegia, cataracts, progressive muscle weakness, parkinsonism, premature ovarian failure, male infertility, hearing loss (presbycusis), and cardiac dysfunction [24–30]. There are over 80 pathogenic mutations in POLG in humans (Figure 1). Most reported mutations are recessive and are commonly found in combination with other mutations in POLG, or with mutations in genes encoding other proteins that function in mtDNA replication (such as TWINKLE and ANTI). At the molecular level, these mutations are often associated with the accumulation of mtDNA deletions in multiple tissues. The few dominant POLG mutations reported in PEO occur within the polymerase domain and tend to disrupt the interaction between the polymerase and the incoming nucleotide; this can cause misincorporation of nucleotides and may also lead to large...
deletions between direct repeats [31,32]. Interestingly, sequencing of mtDNA deletions from patients suggests that replication stalling may be the major mechanism of deletion formation [33].

PEO can also result from mutations in the gene encoding TWINKLE [34], a mitochondrial helicase and putative primase that functions as a hexamer. Mutations in TWINKLE are thought to be the cause of 35% of autosomal dominant PEO cases [35]. These mutations seem to enhance dNTPase activity and thus may lower the pool of nucleotides available for mtDNA replication. There are recessive TWINKLE mutations that cosegregate with POLG mutations, resulting in derepression of ANT1 and is associated with facioscapulohumeral muscular dystrophy [168].

Mouse Models of Disease Associated with mtDNA Mutations

A number of transgenic and knock-in mouse models have been developed to test the in vivo effects of increased mtDNA mutation accumulation. Two groups have independently generated knock-in mice expressing an exonuclease-deficient version of the mitochondrial DNA polymerase γ (PolγΔ257A) [47,48]. The lack of proofreading activity in PolγΔ257A mice results in mitochondrial mutation frequencies that are increased by at least 3- to 11-fold in multiple tissues, with accumulation of mtDNA base-substitution mutations beginning in development. Deletions of mtDNA can also be detected in these mice [48]. The two models have very similar phenotypes resembling aspects of premature aging; these include hair graying and loss, reduced bone density and increased incidence of kyphosis, reduced muscle mass, severe reduction in body fat, early loss of fertility, dilated cardiac hypertrophy, accelerated thymic atrophy, presbycusis, and reduced survival. Anemia and intestinal dysplasia are also seen. A progressive decline in respiratory function of

Figure 1. Human Disease-Associated Mutations in Genes Involved in mtDNA Replication and Maintenance

Mutations reported in POLG [36,151–162], TWINKLE (gene also known as PEO1) [34,38,39,163–167], and ANT1 (gene also known as SLC25A4) [35,40] proteins associated with human diseases. Mutations in black are associated with PEO, those in blue are associated with Alpers syndrome, red indicates mutations present in both PEO and Alpers, and green indicates mutations associated with other disorders, italics indicate changes in DNA sequence.

A) POLG. The light green and light blue segments represent the exonuclease and polymerase domains, respectively. Highly conserved motifs within each are shown as red segments. The POLG mutation figure is adapted from the Human DNA Polymerase Gamma Mutation Database maintained by the Mitochondrial Replication Group at the National Institute of Environmental Health Sciences (http://dir-apps.niehs.nih.gov/polg).

B) TWINKLE. The pink domain is the primase-helicase linker region, as identified by homology to T7 phage protein [34].

C) ANT1. In addition to the pathogenic mutations shown within the protein, a 3.3-kb deletion upstream of ANT1 results in derepression of ANT1 and is associated with cataracts, presbycusis, Parkinson's disease, early menopause, and decreased cardiac and skeletal muscle function suggests that these aging phenotypes are most likely to be influenced by the age-related accumulation of mtDNA base-substitution mutations and deletions.
mitochondrially encoded complexes was evident as early as 12 weeks, resulting in decreased oxygen consumption and ATP production [48,49]. No increase in DNA, RNA, protein, or lipid markers of oxidative stress was observed in these mice and antioxidant defense systems were likewise not upregulated [47,49]. Instead, mtDNA mutation accumulation was associated with the activation of apoptosis in multiple tissues as measured by TUNEL and cleaved caspase-3 assays [47].

Additional mitochondrial mutator mouse models have employed tissue-specific Polg<sup>DISA</sup> exonuclease-deficient transgenes, expressed primarily in the heart [6] or in the brain [50]. Both base-substitution mutations and mtDNA deletions accumulated in these models. Elevated levels of mtDNA mutations in the heart, beginning after birth, resulted in dilated cardiomyopathy by 4 wk of age, with mutant mice dying of congestive heart failure by ~6 mo [6]. Respiratory function remained comparable to controls [51]. Similarly to the Polg knock-in mice, the transgenic hearts did not display increased oxidative damage to proteins (including oxidation-sensitive aconitate enzyme activity) or mtDNA, nor elevated antioxidant defenses [52]. Cytosolic fractions from transgenic hearts contained cytochrome c [51], mitochondrial release of which is a hallmark of apoptosis. Interestingly, although apoptotic (TUNEL-positive and morphologically dying) cells in the transgenic hearts exceeded controls by ~3 wk of age [51], a subsequent protective antiapoptotic response involving upregulation of Bel-2, Bel-xL, Bfl-1, XIAP, and Hsp27 transcript or protein levels was noted in nearly all transgenic myocytes [52,53]. This survival response was of functional consequence, in that it could protect the transgenic hearts from further apoptotic stress induced by doxorubicin [53]. This suggests that mtDNA base-substitution mutations and/or deletions in the heart may trigger a retrograde signaling system from the mitochondria to the nucleus. Alternatively, those cells with the highest mutational burden could release cell-autonomous factors that induce widespread gene expression changes throughout the heart. Cell death seems to be a key element driving the pathology of mtDNA mutations in the heart because cyclosporin A, a cell-death inhibitor that blocks the opening of the mitochondrial permeability transition pore, prevents the cardiomyopathy of the transgenic mice [54].

As discussed earlier, mutations in human POLG are associated with chronic PEO, with some patients exhibiting mood disorders [29,30]. Furthermore, mitochondrial dysfunction and altered energy metabolism have been implicated in the etiology of bipolar disorder by magnetic resonance spectroscopy, mtDNA polymorphism association, and detection of mtDNA deletions in bipolar patient brains (for reviews, see [55–57]). In mice with neuronal expression of proofreading-deficient Polg<sup>DISA</sup> (under control of the Ca<sup>2+</sup>/calmodulin-activated protein kinase Iβ promoter, CaMKIIβ), behavioral phenotypes resembling mood disorder were observed, including reduced wheel-running and altered day-night activity patterns [50]. These behaviors were worsened by treatment with amitriptyline hydrochloride, an antidepressant that can induce mania in individuals with bipolar disorder. Although total wheel-running activity decreased, a 5-d pattern of peak activity coinciding with the estrus cycle was observed in female transgenic mice; treatment with lithium, commonly used as a mood stabilizer in the treatment of bipolar disorder, diminished this periodicity. No measurements of respiratory function, apoptosis, or oxidative stress were reported for this model.

In addition to Polg, mtDNA replication and maintenance involves the activities of other nuclear genes such as T<sub>fam</sub> (mitochondrial transcription factor A) [58,59], Twinkle (also known as Peo1 or C100orf2) [60], Tfb1m (mitochondrial transcription factor B1, previously called mtTFB) [61], R<sub>N</sub> H<sub>1</sub> [62], and S<sub>shp1</sub> (mitochondrial single-strand binding protein; also called mtSSB) [63]. Mutations in these genes result in reduction or loss of mtDNA content and mice deficient for some of these genes die during development [58,62]. For example, several mouse models with general [58] or tissue-specific [64–67] deficiencies in T<sub>fam</sub> have been generated, all based on a <i>loxP</i>-flanked <i>Tfam</i> allele (T<i>fam</i><sup>loxp</sup>), and are associated with mtDNA depletion (see Table 1). All of these <i>Tfam</i> mouse models exhibit a delay between onset of <i>cre</i> expression and the occurrence of respiratory dysfunction, which can be attributed to the time needed to turn over Tfam, mtDNA, and respiratory enzyme subunits.

Pathogenic mutations in <i>TWINKLE</i> have been identified in human PEO families [34]. Transgenic mice expressing mutant <i>Twinkle</i> isoforms modeled after those mutations seen in human disease display progressive localized mitochondrial respiratory deficiencies, particularly in individual muscle fibers and neuronal subpopulations (transgene expression was noted in heart, muscle, and brain), and mild myopathy at about 1 y of age [68]. These mice acquire multiple mtDNA deletions but do not show increased mtDNA base-substitution mutations. Premature aging does not appear to be a feature of these <i>Twinkle</i> transgenic mice, although it is unclear if transgene expression was achieved in most tissues or cell types.

Similarly, mutations in the heart- and muscle-specific isoform of the adenine nucleotide transporter 1 (<i>ANT1</i>) gene are present in human PEO families [41]. Disruption of <i>Ant1</i> inhibits oxidative phosphorylation by compromising exchange of ADP and ATP across the inner mitochondrial membrane. <i>Ant1</i><sup>−/−</sup> mice display cardiomyopathy and peripheral myopathy with ragged red fibers (a histological marker of mitochondrial proliferation), severe respiratory defects (although electron transport enzyme activities <i>per se</i> are intact), elevated serum lactate levels, and exercise intolerance [69]. Increased H<sub>2</sub>O<sub>2</sub> production in heart, muscle, and brain was observed in <i>Ant1</i><sup>−/−</sup> mice (in addition to high levels in heart and muscle, <i>Ant1</i> is expressed at lower levels in the brain and a few other tissues [70]), and was accompanied by varying levels of augmented antioxidant enzymes, depending upon the tissue [71]. Accumulation of mtDNA deletions or rearrangements was observed, with levels in line with the extent of induced antioxidant defenses. In the heart, which showed maximal H<sub>2</sub>O<sub>2</sub> production (i.e., antimycin A treatment did not further increase ROS levels in the <i>Ant1</i><sup>−/−</sup> tissue) and minimal induction of Sod2 and Gpx1 defense enzymes, mtDNA deletions increased to considerably higher levels than in skeletal muscle, where antioxidant defenses were more robust.

An additional mouse model carrying mtDNA deletions has been generated via a methodology distinct from gene targeting in mouse embryonic stem cells. The so-called “mito-mouse” was generated when synaptosomes (presynaptic terminals isolated after subcellular fractionation) containing...
Table 1. Mouse Models with mtDNA Mutations, Deletions, or Depletion

| Mouse model | Tissue | Onseta | mtDNA Mutationsb | mtDNA Deletionsb | mtDNA Depletion | Phenotypes | | Oxid. Stress | | Apop. References |
|-------------|--------|--------|-----------------|-----------------|----------------|------------|----------|----------|------------------|
| PolgD257Aknock-in | Ubiquitous | Embryonic | $2.5 \times 10^{-4}$–$1.5 \times 10^{-3}$ (at 2–6 mo); (wt: $5 \times 10^{-5}$–6 $10^{-4}$) | Yes | No | Premature aging | No | Yes | [47–49] |
| PolgD181ATg | Heart | $\leq 1$ week | $1.4-2 \times 10^{-4}$ (at 4–12 wk); (non-Tg: $5.9 \times 10^{-5}$) | Yes | No | Dilated cardiomyopathy; antiapoptotic survival response | No | Yes | [6] |
| CaMKII-PolyD181ATg | Brain | Embryonic | $1.2 \times 10^{-4}$ (at 68 wk); (non-Tg $\leq 10^{-5}$) | Yes (17+ wk) | No | Behavioral disorders | N/D | N/D | [50] |
| TwinleGFP Tg (β-actin promoter) | Ubiquitous | Embryonic | $2.5-4.5 \times 10^{-3}$ (at 18 mo) | Yes (18+ mo) | $54-63\%$ | Late onset (>12 mo) localized progressive respiratory deficiency; altered mitochondrial morphology; no premature aging | N/D | N/D | [68] |
| TwinleA359T Tg | Ubiquitous | Embryonic | N/D | N/D | N/D | Occasional Cox- muscle fibers | N/D | N/D | [68] |
| Tlam−/− | Ubiquitous | Embryonic | N/D | N/D | N/D | Embryonic lethal; severe respiratory deficiency; massive apoptosis at E9.5 | N/D | N/D | [38,147] |
| Tlam−/−+/+ /Ckmm-cre | Ubiquitous | Embryonic (post-natal mtDNA depletion) | N/D | N/D | $34\%$ | Reduced respiration; viable | N/D | N/D | [58] |
| Tlam−/−+/+ /Myhca-cre | Heart; muscle | Embryonic | N/D | N/D | $69\%$ | Mosaic progressive respiratory deficiency (heart not muscle); dilated cardiomyopathy/conduction blocks; death at 2–4 weeks | N/D | N/D | [64,147] |
| Tlam−/−+/+ /Mlc1f-cre | Muscle | Embryonic | N/D | N/D | $69\%$ | Progressive respiratory deficiency; ragged red fibers; reduced force in fast-twitch muscle; reduced physical activity at 3–4 mo; weight loss | N/D | N/D | [148] |
| Tlam−/−+/+ /CaMKII-cre | Brain; testis | 2+ mo (deficiency) | N/D | N/D | $40\%$ | Respiratory deficiency at 4 mo; neurodegeneration at 5 mo; death 1–2 wk thereafter | Not likely | Yes | [67] |
| Tlam−/−+/+ /RIP-cre | β-cells; brain | 7+ wk (deficiency) | N/D | N/D | Yes | Respiratory deficiency at 7 wk; diabetes; early- insulin release; late loss of β-cells | N/D | No | (7 wk) | [66] |
| Art1−/− | Heart; muscle; brain | Embryonic | N/D | Yes (16+ mo) | No | Cardiac hypertrophy; myopathy with ragged red fibers; exercise intolerance | Yes | N/D | ([Mt11] | [69,71] |
| Mito-mouse (ΔmtDNA4696) | Ubiquitous | Embryonic | N/D | Yes | No | Mosaic respiratory deficiency in muscle, heart & kidney; lactic acidosis; ↓ body weight; atrioventricular block w/o cardiac dilation; anemia; death from renal failure at ~200 days | N/D | Yes | [72,75] |
| Mito-mouse (ΔmtDNA) | Ubiquitous | Embryonic | N/D | Yes (>$80\%$) | No | Hearing loss | N/D | N/D | [76] |
| Mito-mouse (T6589CmtDNA) | Ubiquitous | Embryonic | Homoplasmic T6589C | N/D | No | Respiratory deficiency; ↓ serum lactate; ↓ body weight | N/D | N/D | [77] |
| CAP4 (mt 16S rRNA) | Ubiquitous | Embryonic | T2413C | N/D | No | Growth retardation; myopathy; dilated cardiomyopathy; embryonic or perinatal lethality | N/D | N/D | [78] |

a Onset of mutations is determined by expression pattern and timing of locus or promoter used to drive the mutant allele.
b Age at which mutations or deletions was measured is given in parentheses. Mutation frequency is given in mutations/bp.
c Mutation sequencing done by PCR amplification and subcloning of mtDNA fragments.
d Mutation sequencing done by restriction digestion and subcloning of mtDNA fragments.
e In-frame duplication of amino acids 353–365 in mouse Twinkle gene.
f Mouse TwinkleGFP corresponds to TwinleGFP mutation in human PEO patients.
N/D, not determined; Tg, transgenic; wt, wild type

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Mitochondria from aged C57Bl/6J mouse brain were fused to rho− cells devoid of mtDNA. The resulting cytoplasmic hybrid cells (cybrids) were screened by PCR to identify those containing a high proportion of mtDNA deletions. Such cybrid clones were enucleated and fused to donor embryos to create heteroplasmic founder females that could transmit the mtDNA deletion–containing mitochondria through their germline [72]. Germline transmission of mtDNA deletions in humans is rare [73], and partially duplicated mtDNA intermediates were postulated to allow for such transmission in the mice [72]. Mito-mice carry a 4,696 bp deletion in mtDNA that removes six tRNA and seven structural genes (for complexes I, IV, and V) from the mitochondrial genome. F1 and F2 generations of mice contained varying proportions of the ΔmtDNA4696 deletion and exhibited COX-negative muscle fibers when deletion levels rose to >85%; classic ragged red fibers were not observed, however. Similar mosaic respiratory deficits were noted in heart and kidney. An atrioventricular conduction block was reported, but in the absence of cardiac dilation [74]. Mito-mice were anemic and died from renal failure by 200 days of age. No phenotypes traditionally present in mitochondrial disease or aging were reported. An increase in TUNEL staining was seen in the kidneys of mito-mice, implicating apoptosis as an important mechanism of pathology [75]. A second mito-mouse model with >80% deleted mtDNA exhibited age-related hearing loss with onset between 3 mo and 6 mo [76]. The molecular nature of the mtDNA deletions was not characterized.

Generation of transmitochondrial mice has also been extended to include mtDNA base-substitution mutations. Mito-mice with homoplasmic T6589C-mutated mtDNA, encoding a V421A substitution in the COI gene (a COX subunit), show specific loss of COX activity, increased serum lactate, and lower body weight [77]. No further characterization of aging phenotypes is available yet.

Transmitochondrial mice bearing T2433C 16S rRNA– mutant mtDNAs (denoted CAPR mice, because the mutation confers resistance to chloramphenicol) displayed growth retardation, myopathy, dilated cardiomyopathy, and embryonic or perinatal lethality [78]. These models of homoplasmic mtDNA base-substitution mutations are more reflective of the inherited mitochondrial disease situation in humans, as opposed to the more random accumulation of mutations and deletions that occurs in normal aging.

Although direct comparisons of mouse models derived through gene targeting, insertional transgenesis, and cybrid approaches is complicated by differences in gene dosage and tissue-specific expression patterns, it is curious to note that multi-system aging-like phenotypes are much more obvious in models bearing increased base-substitution mutations and deletions such as PolgD257A mice, as opposed to those with only increased deletions (see Table 1). Whether this is biologically meaningful or reflects technical differences in the methodology of mouse generation remains to be determined. Two issues are in particular need of clarification because they could explain the observed differences: First, do the mouse models showing aging phenotypes result in more extensive accumulation of mutations/deletions? And second, do the “ubiquitously expressed” transgenic models truly result in widespread transgenic expression in multiple tissues and cell types?

**Mechanisms of mtDNA Mutation Generation**

**Base-substitution mutations caused by polymerase infidelity.**

The mitochondrial polymerase γ holoenzyme consists of two separate proteins, POLG and POLG2. POLG, the catalytic subunit, contains the polymerase domain, an editing exonuclease domain, as well as a deoxyribose phosphate lyase activity necessary for DNA repair. POLG2, the accessory subunit, increases the affinity of the complex for DNA, elevating polymerase processivity [79] and repair [80]. The human holoenzyme consists of a heterotrimer of two accessory subunits attached to one catalytic subunit [81]. Detailed kinetics experiments with and without the accessory subunit and exonuclease domain have yielded important insights into the mechanism of polymerase fidelity [23,82,83]. It is important to note that polymerase infidelity has been hypothesized to be the major cause of mutation in human mtDNA and may be responsible for many of the mutational hotspots that appear across individuals [84].

Although in vitro the POLG exonuclease domain plays only a small role in the overall fidelity of the enzyme as compared to the discrimination between incoming dNTPs by the catalytic domain [82], this proofreading activity has been demonstrated to be essential in preventing the accumulation of mutations with age in mice [6,47,48,50] and human cells in culture [85]. Overexpression of the exonuclease-deficient protein in human cells had a dominant negative effect, resulting in the accumulation of mtDNA base-substitution mutations over time. After 3 mo in culture, one mutation was found for every 1,700 bp of mtDNA [85]. These results demonstrate the importance of proper proofreading to prevent mtDNA base-substitution mutations that cause cell and tissue dysfunction with age.

The vast majority of DNA polymorphisms and disease-causing base-substitution mutations that have been detected in human mtDNA are transition mutations [86]. Transition mutations are also the predominant type of mutation in both wild-type and PolgD257A mice [47,48]. This can be partly explained by the slight infidelity of the POLG enzyme, which allows G:T mismatches to occur as a relatively frequent event [83]. These particular misincorporation events can be exacerbated by dNTP pool imbalances. As shown in rats, dGTP is present at a much higher concentration than dATP in mitochondria from many postmitotic tissues, including heart and skeletal muscle, possibly increasing the frequency of G:T mismatches [87]. In contrast, dTTP is present at the lowest concentration of the four deoxynucleotides in mitochondria from these tissues. These pool imbalances do not differ between young and old animals. At this time, it is unknown what role dNTP pool imbalances play in the generation of the other specific types of mtDNA mutations that occur with age, such as transversion or deletion mutations.

**Oxidative damage to mitochondrial DNA.**

tDNA has been shown to replicate by two distinct mechanisms. In the traditional strand-asynchronous model, replication begins at the heavy (guanine-rich) strand origin and proceeds approximately two-thirds of the way around the mitochondrial genome before initiation of light (cytosine-rich) strand synthesis begins [88]. There is a positive correlation between the rate of accumulation of base-
substitution mutations in mammalian mitochondrial genomes and the distance from the origin of light strand replication, relating to the amount of time mtDNA is single stranded during replication [89]. This suggests that mtDNA may be particularly susceptible to oxidative damage when single stranded. Pathogenic mitochondrial base-substitution mutations are found at a disproportionately high level in mitochondrial tRNA genes and it has been hypothesized that this high frequency is due to a stem-loop structure formed when these regions are single stranded during mtDNA replication [90]. However, further evidence is needed to support or refute this suggestion. The spectrum of base-substitution mutations that accumulate in aged individuals differs across tissues [3]. This may be due to variations in the mechanism of replication in different tissues. Specifically, evidence for coupled leading and lagging strand mtDNA synthesis has emerged in recent years [91]. If coupled-strand replication differs from the strand-asynchronous mechanism in its susceptibility to mutation generation, then differential reliance on the two modes of replication among tissue types or under different cellular conditions might contribute to tissue-specific mutation patterns. Alternately, even when replication proceeds primarily via the strand-asynchronous model, utilization of alternate origins of light strand replication may influence mutation specificity by variations in proximity to the heavy strand replication origin and, thus, differences in the time that mtDNA is present in single-stranded form [88].

Mitochondria do not have the enzymes necessary for nucleotide excision repair of DNA. They do, however, possess base excision repair enzymes that are capable of repairing oxidatively damaged bases in mtDNA, and many of these repair enzymes are alternatively spliced variants of nucleus-targeted proteins [92]. Mitochondrial base excision repair activity declines in the aging mouse brain [93]; if applicable to tissues in general, this may contribute to the accumulation of mtDNA mutations with age. Base-substitution mutations may occur as a result of POLG replicating across these lesions. In mitochondria, 8-oxoguanine is the most abundant oxidative lesion and can cause transversion mutations if unpaired [94]. Mitochondria contain an 8-oxoguanine DNA glycosylase (OGG1) that repairs the vast majority of this damage. Mice lacking Ogg1 had 20-fold higher levels of 8-oxoguanine in mtDNA isolated from liver [95] but this did not lead to respiratory defects [96]. Mitochondria also possess an 8-oxoGTPase (MTH1) to prevent oxidized dGTP from being incorporated into DNA [97]. mtDNA in brains from mice lacking Mth1 accumulate more 8-oxoguanine than controls [98,99]. No accelerated aging phenotypes were observed in either Ogg1−/− [95,100] or Mth1−/− mice, although both are slightly more prone to certain types of tumors [101,102]. Unexpectedly, Ogg1−/−Mth1−/− mice have a decreased incidence of tumorigenesis [101]. Both OGG1 and MTH1 also function outside mitochondria to protect nuclear DNA, so it is unclear if the phenotypes of these mice are related to mtDNA or nuclear DNA mutations.

Mitochondrial DNA deletions.

mtDNA deletions may play a contributing role in age-related tissue dysfunction in human postmitotic tissues. Deleted mtDNA molecules can accumulate, reaching up to 60% of the total mtDNA and cause oxidative phosphorylation defects and COX-negative staining in specific cells of aged postmitotic tissues [14,15]. The mechanism of deletion formation is unknown. However, many deletions are thought to involve base pairing by direct repeat sequences [103] and this occurs more frequently during oxidative stress, perhaps due to polymerase stalling, slipping, and mispairing during replication (Table 2). Topoisomerase II cleavage and other DNA double strand breaks have also been proposed as possible mechanisms of deletion formation [103,104]. A study analyzing deletions in human mtDNA suggests that most deletion formation may be linked to two 13-bp repeats in mtDNA [105].

**Mechanisms of Pathology Induced by mtDNA Mutations**

Data from mitochondrial mutator mouse models support the hypothesis that mtDNA mutations can promote tissue dysfunction through the loss of critical irreplacable cells due to activation of apoptosis. In support of this hypothesis, human cells bearing mutations causing Leber’s hereditary optic neuropathy, an inherited mtDNA disease, are sensitized to Fas-induced apoptosis [106]. Is apoptosis required for development of mtDNA-induced phenotypes, and how might mtDNA mutations trigger the apoptotic process? Loss of respiratory function is associated with activation of apoptosis (e.g., see mouse models of mtDNA depletion in Table 1), and mitochondrial bioenergetics are compromised in mitochondrial mutator mice [48,49]. Release of apoptotic factors, such as cytochrome c, Smac/diablo, apoptosis-inducing factor (AIF), Omi/Htra2, and endonuclease G from the mitochondrial intermembrane space can occur through two mechanisms [107,108]. In the first, channels in the outer mitochondrial membrane can open in a process regulated by Bcl-2 family members without the involvement of inner mitochondrial membrane components. In the second, opening of a permeability transition (PT) pore, involving components of the outer mitochondrial membrane (VDAC, Bax, and Bcl-2), inner mitochondrial membrane (ANT), and matrix (Cyp D) results in osmotic mitochondrial swelling, outer mitochondrial membrane rupture, and release of apoptogenic factors. The observation that cyclosporin A–mediated inhibition of PT pore opening was successful in preventing cardiomyopathy in the heart-specific mitochondrial mutator model [54] implicates a central role for the PT pore generally, and cyclophilin D (Cyp D) in particular, in mtDNA mutation-mediated cell-death signaling in the heart, because Cyp D is the main mitochondrial binding target of cyclosporin A [109]. However, mitochondria from the PolgD181A transgenic hearts are purportedly more

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**Table 2. Mechanisms for mtDNA Mutation**

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<tr>
<th>Mutation Type</th>
<th>Mechanism</th>
<th>References</th>
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<tr>
<td>Base substitutions</td>
<td>Polymerase infidelity</td>
<td>[84]</td>
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<tr>
<td>Deletions</td>
<td>Replicating across damaged bases</td>
<td>[84]</td>
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<td></td>
<td>DNA strand slippage and mispairing</td>
<td>[149]</td>
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<tr>
<td></td>
<td>Illegitimate recombination</td>
<td>[103]</td>
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<td></td>
<td>DNA double strand breaks</td>
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"Ogg1–/–Mth1–/– mice have a decreased incidence of tumorigenesis [101]. Both OGG1 and MTH1 also function outside mitochondria to protect nuclear DNA, so it is unclear if the phenotypes of these mice are related to mtDNA or nuclear DNA mutations."
resistant to calcium-induced PT pore opening than those from control hearts [110], an effect attributed to the protective actions of induced Bcl-2 in the pro-survival response. Thus, other functions of Cyp D aside from its role in PT pore opening (such as its chaperone activity) may be important. Mouse models deficient for many of the genes involved with apoptotic regulation (e.g., Bax, Bak, and Cyp D) are available. Examining the effects of these apoptotic modulators on the aging phenotypes of mitochondrial mutator mice should help to establish whether apoptosis is required for the downstream effects of mitochondrial mutations.

Recently, Zassenhaus and colleagues proposed an intriguing mechanism whereby mtDNA mutations would generate a pool of misfolded mitochondrial proteins, some small proportion of which might have the conformation necessary to bind to Bax or Bak and thereby activate apoptosis or perhaps bind to Cyp D and inhibit its chaperone function [110]. This hypothesis could explain how heteroplasmic mtDNA mutations could elicit a cell-death response in the presence of many wild-type copies of mtDNA.

A long-standing tenet of the mitochondrial free radical theory of aging is the expectation of increased ROS production in mitochondria compromised by respiration-inactivating mtDNA mutations (i.e., “the vicious cycle”). However, we [47] and others [49,52] have clearly demonstrated that mitochondrial mutator mice do not have increased levels of oxidative stress. Mitochondria treated with specific chemical electron transport chain (ETC) inhibitors can indeed produce increased ROS levels [111]. Similarly, mouse models such as the Ant1−/− mice also exhibit elevated levels of ROS production [71]. However, inhibition of ETC function in Ant1−/− mice or by chemical inhibitors may generate ROS because all mitochondria show the same defect (e.g., lack of available ADP or blockage of electron flow at a specific point in the ETC). Upstream complexes can still function, resulting in electron stalling and transfer to O2 to generate the superoxide anion. By contrast, in the mitochondrial mutator mice, a variety of mutations is present and multiple upstream complexes could be nonfunctional or be lacking subunits if mitochondrial rRNA or tRNA mutations are numerous. Thus, electron flow through all the complexes (except nucleus-encoded complex II) may be impaired and reduced intermediates may not be accumulating. In the case where mtDNA mutation levels are much lower, the presence of many wild-type copies of mtDNA will mask the effects of specific respiratory mutations.

If mtDNA mutations do not lead to increased ROS damage in mitochondrial mutator mice, how does this finding fit into the field of oxidative stress and aging? Certainly, oxidative stress could be playing a role in the generation of mtDNA mutations in wild-type animals. The rate of mitochondrial ROS production, extent of mtDNA (but not nuclear DNA) oxidative damage, and degree of membrane fatty acid unsaturation (a determinant of vulnerability to lipid peroxidation) are all inversely correlated with longevity across species [112–115]. Most of these parameters are reversed by caloric restriction (CR) [116]. Mice expressing mitochondrion-targeted catalase show reduced total DNA oxidative damage (in skeletal muscle), fewer mtDNA deletions, and extended mean and maximal lifespan by 17%–21% [117], suggesting that mitochondrial accumulation of oxidative damage can limit rodent lifespan. However, mice with reduced levels of the mitochondrial MnSOD enzyme (Sod2−/−) do not appear to age any faster than their wild-type counterparts, despite harboring increased levels of oxidative damage to both nuclear and mtDNA [118]. Similarly, mice deficient for Oggl1 or Mth1 do not exhibit accelerated aging features [95,100,102]. Thus, increased mitochondrial oxidative damage is not sufficient for accelerated aging. It is unclear, however, whether the increased oxidative damage to mtDNA observed in the Sod2−/− mouse model actually leads to increased base substitutions or deletions, and, if so, to what extent the mutation levels compare to those of the Polg mutator mice or natural aging.

The mitochondrial mutator mice suggest that activation of apoptotic pathways is important for the induction of an aging phenotype. Indeed, we speculate that activation of apoptosis may be a common underlying mechanism in many accelerated aging models. For example, mice that are both deficient in Werner’s (Wn) helicase and possess shortened telomeres display a phenotype strikingly similar to PolgD257A mice and exhibit elevated levels of apoptosis [119]. Livers from old, but not young or middle-aged, Sod2−/− mice have 3-fold more TUNEL-positive cells [120]. Therefore, the long delay before activation of apoptosis in the Sod2−/− mice might account for the failure to see early aging phenotypes in these animals.

The phenotypes of multiple mouse models of mtDNA base-substitution or deletion mutation accumulation that have been generated in recent years have lent support to the notion that mtDNA mutations can play a causative role in aging-related degenerative processes. This interpretation is not without controversy, however, with opponents arguing that the levels of mtDNA mutations present in the mitochondrial mutator mice are catastrophically high and exceed those associated with human aging [121–123]. The manifestation of clinical phenotypes of classic mitochondrial diseases are dependent upon mtDNA mutations or deletions rising above a critical threshold, so one might also expect that aging phenotypes require accumulation of mutations exceeding a threshold. High mtDNA mutational loads accompanied by severe respiratory deficiency have been observed in muscle fibers, intestinal crypts, and substantia nigra neurons [2,3,5,15,16,18,19]. Ultimately, testing the effect of reduced mtDNA mutation accumulation on lifespan and aging phenotypes will provide the strongest support of a causal relationship between mtDNA mutations and aging.

It is important to note that aging is a complex process that is likely to have multifactorial causes (Figure 2). Mitochondrial DNA mutations can arise directly from errors during DNA replication. Oxidative stress may also generate mtDNA mutations as well as damaged proteins that might be able to directly signal apoptosis through a misfolded protein response. Respiratory deficiency could contribute to apoptotic signaling or be directly responsible for some aspects of tissue dysfunction. The importance of cell loss versus metabolic dysfunction to aging phenotypes might vary depending upon the tissue type.

Interventions to Retard mtDNA Mutations and Its Consequences

Because mtDNA mutations cause dysfunction in cells, it is of interest to determine if preventing these mutations could
Several strategies have the potential to retard age-related accumulation of mtDNA mutations (Table 3). Decreasing the generation of ROS by caloric restriction. The only known intervention that has consistently delayed aging in multiple species is CR [124]. Decreased levels of mtDNA deletions are detected in calorically restricted animals [125]. The mechanism of protection by CR may involve decreased production of ROS from Complex I of the mitochondrial ETC [126]. Although this involves altering the degree of reduction of Complex I, the exact mechanism as to how this occurs is unknown. One hypothesis is that decreased levels of methionine ingested during CR lead to a higher reduced/oxidized cellular glutathione ratio, which decreases Complex I free radical generation [127]. Another hypothesis is that mitochondria from calorically restricted animals undergo increased biogenesis, and are more efficient than normal at generating equal amounts of ATP with a lower membrane potential, oxygen consumption, and free radical production [128].

Decreasing oxidative damage with antioxidants. The use of nutritional and genetically encoded antioxidants can prevent mtDNA mutations. Expression of mitochondrion-targeted catalase, which decreases hydrogen peroxide levels, prevented mtDNA deletions and extended the lifespan of mice [117]. Nutritional antioxidants may function, in part, by maintaining mitochondrial glutathione in the reduced state, which can prevent the increase in free radical generation from the ETC that occurs with age and damages mtDNA [129]. Examples of dietary antioxidants that decrease the accumulation of potentially mutagenic 8-oxoguanosine levels in mtDNA include carnitine [130], alpha tocopherol (Vitamin
Table 3. Strategies to Slow the Age-Related Increase in mtDNA Mutations

<table>
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<tr>
<td>CR mimetics to decrease mitochondrial ROS production</td>
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<td>Mitochondrial-targeted antioxidants</td>
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<tr>
<td>Increasing the fidelity of mitochondrial DNA polymerase gamma</td>
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<td>Mitochondrial targeting of DNA repair enzymes</td>
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doi:10.1371/journal.pgen.0030024.t003

E) [131], Vitamins C and E in combination [129], thiazolidine carboxylate [129], and Ginkgo biloba extract [132]. Oxidative stress also leads to deletions in mtDNA that can be prevented by dietary antioxidants. Beta-carotene and creatine protected against mtDNA deletions in skin human fibroblasts exposed to UVA radiation [133,134], while coenzyme Q lowered the level of mtDNA deletions in a mouse model of oxidative stress [135]. Interestingly, dihydrolipoic acid partially rescued the phenotype of yeast cells expressing a POLG variant carrying a mutation found in PEO patients [136]. However, none of these antioxidant compounds has yet consistently been shown to be beneficial in the treatment of human mitochondrial disease [137], and likewise none has been shown to retard mammalian aging.

Increasing mitochondrial DNA repair. There is evidence in cell culture that expression and mitochondrial targeting of base excision repair enzymes protects against oxidant-induced cell death. Proteins found to be protective include human 8-oxoguanine DNA glycosylase/apurinic lyase (OGG1) [138] and E. coli endonucleases III and VIII [139]. The MTH1 protein, a free 8-oxo-GTPase present in both the nucleus and mitochondria, also protects cells from oxidative stress [140]. Extensive studies examining the roles of these proteins in aging have yet to be performed and may yield insight into possible connections between mtDNA damage and aging.

It has been reported that liver mitochondria contain a DNA mismatch repair activity [141], a pathway that corrects DNA polymerase errors and inhibits other kinds of genome instability. Consistent with this observation, the DNA mismatch repair enzyme Mlh1 has been localized to mouse mitochondria [142,143]. There have also been mixed reports on the localization of the mismatch repair protein Msh2 in mitochondria [141,143]. Bioinformatic analysis implicates Msh5 as a candidate mtDNA repair enzyme [142]. Whether or not these enzymes actually constitute a functional mitochondrial mismatch repair system awaits further verification. Yeast and plant mitochondria utilize the MSH1 protein in mitochondrial mismatch repair, whereas no MSH1 homolog is present in mammalian mitochondria [144]. Increased expression of one or more of these proteins in mitochondria might have the potential to delay the accumulation of mtDNA mutations with age.

Conclusion

The hypothesis that aging is due in part to mtDNA damage and associated mutations [145,146] was based on the observations that mtDNA is located in the organelle that generates most cellular ROS, that mtDNA is relatively unprotected from ROS damage due to a lack of histones, and also that mtDNA repair may be limited. Although provocative, this hypothesis is only viable as a major aging mechanism if three conditions are met for any given tissue: 1) mutations must accumulate with age; 2) due to the high copy number of mtDNA, most mutations should reach near or complete homoplasmy; and 3) such mutations must be of functional consequence. The first two conditions have clearly been satisfied for several cell types examined in humans. Future developments in the field are likely to focus on identifying the functional consequences of specific mtDNA mutations found in aged human tissues, mechanisms of clonal expansion, and the dissection of pathways that mediate the deleterious effects of mtDNA base-substitution mutations and deletions using animal models. These studies should help uncover the relevance of mtDNA mutations to animal aging, and allow the rational design of therapeutic interventions.

Accession Numbers

The National Center for Biotechnology Information Entrez Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene) accession numbers for the genes and gene products discussed in this paper are POLG (5428); TWINKLE, or PEO1 (6652); and ANT1, or SLC25A4 (291).

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Competing interests. GCK and TAP were awarded a United States patent (7,126,040) for the Poly(ADP) ribose mouse model described here. TAP is a partial owner and scientific consultant for LifeGen Technologies, specializing in the application of DNA microarray analysis to analyzing nutraceutical interventions in aging.

References

abnormalities, muscle fiber atrophy, fiber splitting, and oxidative damage in sarcopenia. Faseb J 15: 322–332.


and higher incidence of cancer but does not accelerate aging. Physiol Genomics 16: 29–37.


Dietary Restriction in *Drosophila*: Delayed Aging or Experimental Artefact?

Matthew D. W. Piper, Linda Partridge*

ABSTRACT

Lifespan can be extended by reduction of dietary intake. This practice is referred to as dietary restriction (DR), and extension of lifespan by DR is evolutionarily conserved in taxonomically diverse organisms including yeast, invertebrates, and mammals. Although these two often-stated facts carry the implication that the mechanisms of DR are also evolutionarily conserved, extension of lifespan could be a case of evolutionary convergence, with different underlying mechanisms in different taxa. Furthermore, extension of lifespan by different methods of DR in the same organism may operate through different mechanisms. These topics remain unresolved because of the very fact that the mechanisms of DR are unknown. Given these uncertainties, it is essential that work on the mechanisms of DR is not clouded by imprecise descriptions of methods or by technical problems. Here we review the recent literature on DR in *Drosophila* to point out some methodological issues that can obscure mechanistic interpretations. We also indicate some experiments that could be performed to determine if DR in *Drosophila* operates through similar mechanisms to the process in rodents.

Introduction

At the beginning of the 20th century, the first experiments studying the effects of environmental interventions on lifespan were undertaken using the fruit fly *Drosophila melanogaster* [1]. Of particular note is the work of Loeb and Northrop [2], which found that *Drosophila* maintained at lower temperatures were longer lived. Since metabolic rate in poikilotherms is determined by ambient temperature, it was proposed by Raymond Pearl that organisms have a genetically predetermined amount of energy to expend in a lifetime and, therefore, that the rate of its expenditure would determine lifespan [3,4]. The central mechanism of this “rate of living” hypothesis is that aging and subsequent death are brought about by an accumulation of the detrimental effects of the products of normal metabolism that cannot be adequately cleared.

At about the same time, Stefan Kopć [5], working on the causes of death by starvation, reached the conclusion that it was brought about by “auto-intoxication” from products of hunger metabolism. In addition, he proposed that starvation provided “antitoxins” against those detrimental effects of normal metabolism referred to by Pearl above and, likewise, that normal feeding provided “antitoxins” against starvation. He thus reasoned that balancing these effects might benefit the organism and lead to prolonged lifespan. He tested this hypothesis experimentally in 1928 using *Drosophila* maintained on a regime of alternating periods of feeding and fasting [5]. Interestingly, Kopć reported that the “…results pointed undoubtedly to the conclusion that periods of intermittent starvation for six hours out of every 24 increases longevity of the flies …” [5]. However, examination of the data in the paper shows the effect was less than convincing, ranging from 16% shortening of mean lifespan to 17% extension, with an average effect over ten trials of 2% extension.

Seven years later, Clive McCay reported the effects of an intermittent feeding regime on the lifespan of white rats [6]. This resulted in males achieving almost double the lifespan of those fed *ad libitum*, an effect that has since been repeated in rodents many times using a variety of different methods, and now termed caloric restriction or dietary restriction (DR) [7]. We use the more general term “DR” in this review, because it covers both cases where total caloric intake correlated with extended lifespan and those where specific nutrients are of importance, leaving open which of these mechanisms is operative. Despite the initial lack of success that Kopć [5] in fact had with the intermittent feeding DR protocol in flies, it has since been shown that it is possible, with appropriate techniques, to extend the lifespan of many organisms, including *Drosophila*, by reducing food intake (see Table S1 for a summary of DR experiments with flies and the range of techniques implemented). This has opened the way for the use of short-lived, invertebrate model organisms such as yeast, nematodes, and flies to investigate the mechanisms of DR that could also be operative in mammals.

A potential problem of comparative DR studies using distantly related species is that the mechanisms underlying the prolongation of life may not be conserved between different lineages during evolution, because the mechanisms by which DR extends lifespan within each species are unknown. To make progress with resolving the issue of evolutionary conservation, it is essential that studies of DR are reported in sufficient detail for the procedures to be replicated and that any intervention described as “DR” does...
in fact operate through a reduction in the supply of nutrients, rather than through the removal of some other detrimental effect. Despite the simplicity of this point, it is overlooked surprisingly frequently. Here, we consider how such technical issues could have affected conclusions of studies of DR in *Drosophila*. Resolving these issues are of central importance for uncovering the mechanisms of DR through comparative model organism studies.

**Restricting Access to Nutrients**

The most practical method for implementing DR in *Drosophila* is by dilution of the food medium to which the flies have *ad libitum* access [8]. In addition to the work by Kopeć [5] cited above, several other laboratories have unsuccessfully attempted to implement DR in a variety of fly species using intermittent feeding regimes [9–12] (Table S1). This lack of success has been interpreted by some to indicate that DR does not work in flies [9,12,13]. An alternative explanation is that there is a detrimental effect of daily periods of extended starvation for flies that is not found for rodents when fed intermittently. This detrimental effect could counter-act any beneficial effects of underfeeding and so fail to extend lifespan. This could occur if the intermittently fed animals have specific nutritional needs during the starvation period that must be met for lifespan to be extended by intermittent feeding. Interestingly, Partridge et al. [14] found that when they intermittently fed yeast to flies (every sixth day) that had constant access to sugar-water, lifespan was extended by some 30% compared with flies fed yeast and sugar *ad libitum*. This contrasts with the negative results from other intermittent feeding studies, where the flies had constant access to water that did not contain sucrose [5,12]. These data indicate that a carbohydrate-enriched diet may be necessary to rescue a detrimental effect of starvation periods for *Drosophila*. The result also points to the specific nutritional effects of yeast being important for lifespan extension by DR in flies, which has recently been confirmed [15].

**Food Dilution—Toxicity versus Nutrient-Dependent Lifespan Extension**

As mentioned above, DR in flies can be achieved by diluting the concentration of nutrients in their food medium, which is always present in excess (Table S1). One food type for *Drosophila* consists of an agar-gelled diet of dried autolysed yeast and sucrose [16,17]. The effect of changing the concentrations of these nutrients ranges from outright starvation at the lowest food levels, through a lifespan peak (DR), to a decrease in lifespan as nutrient concentration becomes “high” (Figure 1). The increase in lifespan seen from “high” levels of nutrition to DR is generally interpreted as a consequence of a positive effect of withdrawal of nutrients on the systems that ensure longer life, perhaps via a reduction in activities such as reproduction that may cause somatic damage [18]. A risk of using food dilution to implement DR is the possibility that flies compensate for lowered nutrient levels by increasing their feeding behaviour. At this stage, both evidence for [19] and against [15,20] compensatory feeding has been published. This discrepancy needs to be addressed with further work and may simply be due to the technical difficulties in determining the feeding rate of such a small organism (see Video S1 for an example of *Drosophila* feeding). It is estimated that *Drosophila* only consume between one and two microlitres of food per 24 hour period [19,21], 40-fold less than a blowfly for the same period of time [22]. Despite these difficulties, it is clear that egg-laying output, which is known to be under nutrient control [23–25], increases as food concentration increases [17,26]. Thus, if compensatory feeding does occur, it is not sufficient to overcome the degree of nutrient dilution in the diet, and the lifespan increase seen under DR correlates with a decrease in acquisition of biologically useful nutrients. However, this correlation does not reveal causation and an alternative explanation is that reduced food supply simply relieves “high”-food flies from a non-nutritional, toxic effect of the diet.

Dietary toxicity could occur from either the presence of a poisonous element in the food, or via an indirect effect of the food being nutritionally inadequate or unbalanced, or physically dangerous, or a source of infection. Whatever the precise mechanism, if toxicity increases as the food concentration increases, the effect is indistinguishable from DR, since lifespan is shortened as food concentration increases. For *Drosophila*, where the food is the nutrient and water supply as well as a large part of the physical environment, increasing the nutritional value of the food also results in an increase in the ratio of dissolved nutrients to water. This could therefore cause flies to suffer shortened lifespan due to increased food hardness or water stress. We have recently tested this possibility and shown that when experiments are carefully performed, neither food hardness nor water shortage account for the life-shortening effects of high-nutrient food [27]. It has also been suggested that increased nutrient content may enhance microbial growth, which could shorten fly lifespan by making the food more sticky or by encouraging a greater chance of lethal infection. Where these two factors have been tested, they have been shown not to affect lifespan [15,26]. For *C. elegans*, recent work has indicated that its commonly used laboratory feedstock, *E. coli*, has a toxic effect that shortens worm lifespan. In addition to worms surviving longer on killed *E. coli* media or in its complete absence [28–31], when worms were fed the soil bacterium *B. subtilis* instead of *E. coli* they were much longer lived [32,33]. This means that studies of “DR” in worms that rely on dilution of an *E. coli* food supply report the combined effects of reduced toxicity as well as reduced nutrient supply. In a more general sense, any natural food supply could contain toxins as well as nutrients, and therefore it is important to establish that any “DR” effect is in fact due to reduced intake of nutrients and not any direct or indirect toxicity effect of the food.

For flies, the problem of food toxicity is to some extent countered by using female egg-laying as a biologically relevant read-out of nutritional quality. If egg-laying increases with food supply, then it is reasonable to deduce that nutrient intake is increased. In combination with lifespan, egg-laying can indicate if food toxicity might be the cause of lifespan shortening, the argument being that if a fly does not increase its egg-laying for nutrient level increases that decrease lifespan, the food may be having a general toxic effect. For DR therefore, each increase in nutrient concentration that leads to a reduction in lifespan should be accompanied by an elevation in daily and lifetime fecundity (Figure 1). At the very least, this ensures that dietary types
that are used for DR do result in increased nutrition over the range tested. A further test for food toxicity could be made using behaviour assays such as negative geotaxis [34] on young flies. Since the quality of industrially produced yeasts is dependent on the production method and seasonal quality of the feedstock, it is important that laboratories empirically determine whether they are working with yeast that is not toxic to flies. Unfortunately, not all studies have taken this precaution (Table S1). For instance, some work on the effects of dietary lipids on lifespan was performed without any simultaneous measure of egg laying or activity, thus making it impossible to know if increased food supply was in fact associated with increased nutrition, or if the short lifespans associated with elevated lipid supply were due to a nutritional effect or instead due to toxicity of the lipid sources added [35–37]. In some cases, such as experiments with males or sterile females, egg laying cannot be used to indicate the food quality. In these instances, informed choices can be made using data from fertile females of the same genetic stock or, as mentioned above, by the use of behavioural assays such as climbing ability. A biological indicator of nutrient quality other than lifespan is essential, because inter-species comparisons of the mechanisms of DR rely on the fact that DR is actually being studied in each organism.

One important line of work to uncover the mechanisms of DR has focussed on isolating the specific nutritional requirements for the effect on lifespan. The community working on DR in rodents has generally taken the view that reduced calorie intake is critical for the lifespan-extension associated with food reduction and has therefore adopted the term caloric restriction [7,38]. However, it is also acknowledged that other types of food restriction that do not affect calorie intake can also extend lifespan [7,39–46]. Each of these interventions may or may not lead to lifespan-extension through the same mechanisms. For instance, dietary restriction for a particular nutrient such as methionine [42,45] may in fact affect the utilization of other dietary components such that the organism ultimately
experiences physiological energy restriction. Studies of the effect of exposure to diets of varying composition on food intake, and of the effects diet composition has on absorption of specific nutrients are needed to resolve these issues. In addition to these effects, there is evidence in worms and flies that ollaction of food, as well as food intake, can shorten lifespan [47,48]. One clear fact to emerge from the body of work on rodents is that, in addition to the data implicating reduced caloric intake as sufficient for lifespan extension, changes to either the quality or quantity of the protein component of the diet has the capacity to alter lifespan, even when caloric intake is equivalent between cohorts [39,40,44,45,49].

Recently, work on Drosophila has reached a similar conclusion, by showing that specific reduction of the yeast component in an otherwise isocaloric diet can extend lifespan to a similar degree as whole-food (yeast and sucrose) reduction, which also has lower caloric content [15]. Even more recently, an attempt has been made to use semi-defined media to study DR in flies [50]. This paper concluded that the protein component of the diet was critical for the effect. However, the flies on the semi-defined diet had dramatically reduced daily and lifetime fecundity and were not longer-lived than fully fed controls, indicating a toxic effect of the protein source (casein) being used. Thus it is important that future work focuses on developing appropriate semi- and fully-defined diets that avoid complications from toxicity.

An attractive candidate mechanism for the prolongation of life in response to food reduction, and more specifically protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53]. This process turns over subcellular material in response to low protein shortage, is the activation of autophagy [51–53].

The Consequences of Sex during DR

One of the long-known features of DR animals is that they have compromised reproductive capacity. Indeed, this feature of DR has been used to form hypotheses about the evolutionary significance of the response to DR, based on the concept of allocation of limiting resources between somatic maintenance and reproduction [59–62]. For many organisms, it could be beneficial during lean times to increase allocation of nutritional resources into maintaining the adult, to increase the chance of survival to a time when the food supply is restored. In Drosophila, high levels of nutrition increase female egg production. This leads them to use up their supplies of stored sperm more rapidly [63] and to re-mate more frequently than those with poorer nourishment [17]. Sexual activity has been shown to shorten the lifespan of both male and female flies [14,64–66]. Thus, flies that are allowed to mate freely throughout life maintained on high-nutrient food will be shorter lived than those on low-nutrient food, due to differences in sexual activity, as well as differences caused by direct nutritional effects of DR. For this reason, it is essential for any study investigating the effects of DR on fly lifespan to control mating status; for instance, by using single-sex cohorts. At this stage it is unknown whether male–male or female–female interactions such as fighting [67] also affect lifespan in a nutrient-level-dependent manner. While the effect of adult density on lifespan is already known [68], the difference in longevity between individually and group-housed flies at different food levels remains untested.

Many studies that have used Drosophila to investigate the mechanisms of DR have done so using mixed-sex groups [20,26,50,69–77] (Table S1). From this body of work, it has been concluded that p53, SIR2, and resveratrol function in the same pathway, which is essential for DR’s lifespan-extending effects [73–75]. However, the use of mixed-sex groups without controlling for mating status in these experiments means it is impossible to discern the interaction between genotype or drug treatment and diet on lifespan, because sexual activity will act as a confounding variable. Thus, it is important for future work on flies, as well as comparative mechanistic studies of DR that flies are housed in single-sex cohorts, or even singly, for experiments.

Aging, Diseases, and Death

The long history of DR studies in rodents has provided us with a great deal of data that show that, with few exceptions, DR extends the lifespan of a variety of lab-maintained strains of rats and mice [7]. Many of these are inbred strains that are theoretically genetically homogenous and homozygous at almost all loci. Inevitably, different inbred lines become prone to different aging-related disorders [78], and yet they generally respond to DR with extended lifespan and a delay in a host of aging-related diseases [7,39,40,79–81]. What is more, many models of specific diseases that are associated with aging, such as Alzheimer disease, appear to respond favourably to DR, with a delay in disease [82,83]. These observations and DR’s ability to slow a wide array of aging-related disorders, as well as the evolutionary conservation of its lifespan-extending effect, can be interpreted to indicate that DR affects the root of aging itself and is not just reducing a specific disease by diet interaction [7]. If a similarly deep-rooted mechanism is at work in flies, a direct comparison of a
variety of inbred laboratory lines of Drosophila should reveal that most respond to DR. This should also be true of flies affected by specific aging-related disorders, such as the fly model of Alzheimer disease [84]. As a by-product of this work, any mutants or inbred lines that do not respond to DR will be important tools in future work to uncover the mechanisms of lifespan extension.

Conclusions

Despite many years of work, the mechanisms that underlie the effect of DR on lifespan remain unknown. Although historically much of the work has been performed with rodents, large-scale lifespan experiments under many conditions and genetic analysis are better suited to shorter-lived, and more easily housed, model organisms such as the invertebrates. However, if work in invertebrates is to be of any relevance to the study of aging in higher organisms, it is important to establish techniques that eliminate the confounding effects of nonaging-related causes of death, such as food toxicity and altered sexual activity. Only then can the mechanistic relationship between diet and death be established, providing modes of action to be tested in the longer-lived models. To test whether the mechanisms of DR are likely to be conserved from flies to mice, it will be interesting to see if Drosophila of different inbred lines or disease models respond to DR in a manner similar to their rodent counterparts. These studies are likely to be further refined by dietary interventions that focus on altering specific nutrients and on discovering mutations that block or alter the response to DR. What is exciting about studying DR in Drosophila is that each of the tools to perform these investigations are currently available or within close reach, meaning that characterisation of DR in Drosophila is likely to continue to be a fertile ground for research.

Supporting Information

Table S1. Summary of Various DR Experiments Performed with Flies Found at doi:10.1371/journal.pgen.0030057.s001 (96 KB DOC).

Video S1. Female Drosophila Feeding on DR and Control Media Found at doi:10.1371/journal.pgen.0030057.s001 (9.2 MB WMV).

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References


Recent Developments in Yeast Aging
Matt Kaeberlein*, Christopher R. Burtner, Brian K. Kennedy*

ABSTRACT
In the last decade, research into the molecular determinants of aging has progressed rapidly and much of this progress can be attributed to studies in invertebrate eukaryotic model organisms. Of these, single-celled yeast is the least complicated and most amenable to genetic and molecular manipulations. Supporting the use of this organism for aging research, increasing evidence has accumulated that a subset of pathways influencing longevity in yeast are conserved in other eukaryotes, including mammals. Here we briefly outline aging in yeast and describe recent findings that continue to keep this “simple” eukaryote at the forefront of aging research.

Introduction
The budding yeast Saccharomyces cerevisiae is a widely used model of cellular and organismal aging [1–4]. The first studies of yeast aging were published over 50 years ago, in which yeast cells were shown to have a finite replicative capacity [5]. Replicative life span was thus defined as the number of daughter cells produced by a mother cell before senescence. A second model of aging has more recently been developed in yeast, termed chronological aging. In contrast to replicative life span (RLS), chronological life span (CLS) is defined as the length of time a yeast cell can survive in a nondividing state [6]. These two models for aging in yeast (Figure 1) provide a unique opportunity to compare and contrast the aging processes of both proliferating and nonproliferating cells in a simple single-celled organism [3].

An interesting parallel has emerged from studies in both yeast aging models linking environmental nutrients to longevity. In the lab, yeast cells are typically grown in media containing high levels of glucose (2%) and abundant amino acids. Independent studies have determined that reducing either the glucose or amino acid concentrations of the media (or both) can increase replicative and chronological life span [7–12]. These different nutrient restriction paradigms have all been referred to as calorie restriction. Calorie restriction is known to increase life span in a variety of organisms other than yeast, including worms, flies, and rodents [13,14]. Given that there is some debate about whether the life-span benefits of these interventions are a direct result of reduced caloric input [15–17], we have chosen to use term dietary restriction (DR) hereafter. There is much interest in determining whether the mechanism(s) by which DR increases longevity in yeast are evolutionarily conserved. A major focus of yeast aging research recently has been directed at understanding the mechanisms that underlie life span extension by DR in yeast.

Dietary Restriction and Sir2: Still Looking for Consensus
Much of the popular interest in yeast aging over the past several years has developed from studies of the silent information regulator 2 (Sir2) family of protein deacetylases (sirtuins). A role for sirtuins in longevity determination was first suggested from work showing that deletion of SIR2 shortens replicative life span [18], while overexpression increases replicative life span [19]. Sir2 orthologs have since been reported to play a similar role in determining the longevity of both worms and flies [20, 21].

In yeast, both overexpression of SIR2 and deletion of FOB1 repress homologous recombination at rDNA repeats. Recombination of rDNA results in the accumulation of extrachromosomal rDNA circles, which can lead to replicative senescence [22]. While it was initially proposed that DR increases RLS in yeast by activating the Sir2 enzyme [11], this model has been challenged by a series of recent studies demonstrating that DR can increase RLS by a SIR2-independent mechanism [23–25]. Although DR fails to increase RLS in a sir2Δ mutant, DR robustly increases the RLS of sir2Δ fob1Δ double mutant cells, demonstrating that Sir2 is not required for life span extension by DR [24]. It remains controversial whether the Sir2 ortholog Hst2 could mediate RLS extension by DR in yeast under specific DR conditions when Sir2 is absent [26,27]; however, recent findings indicate that DR can increase RLS through a mechanism that is independent of all yeast sirtuins [28]. Arguments regarding the relevance of Sir2 in DR have been covered in greater detail in recent reviews and commentaries, and we refer the interested reader to these sources [29–31].

In the chronological aging paradigm Sir2 does not promote longevity and appears to play an antagonistic role in the response to DR [8]. Unlike RLS, deletion of SIR2 does not shorten CLS under normal growth conditions [8]. When cells are subjected to DR, deletion of Sir2 significantly increases CLS [8]. One mechanism that has been proposed for this antilongevity function of Sir2 involves regulating the expression of alcohol dehydrogenase, which is important for metabolism of ethanol late in stationary phase [8]. Whether additional functions of Sir2 are involved as well, such as its...
This results in an increase in chronological longevity, but also suggests that apoptosis exerts an effect on longevity only after the majority of cells have already undergone senescence. Thus, for most cells in the population, activation of the apoptosis-like pathway may be a response to the damage leading to senescence. The observation that apoptotic markers are present in both replicative and chronologically senescent cells may be an indication that the ultimate cause of senescence is similar in both dividing and nondividing yeast cells. This would be consistent with the finding that chronologically aged cells have a reduced RLS [41] and that some interventions (e.g., DR or reduced target-of-rapamycin (TOR) signaling) increase both RLS and CLS (see below).

It has also been suggested that apoptosis-like events in chronologically aged cells provide an opportunity for a few individual cells within the population to resume cell division. This argument is based on observations that, at a low frequency, vegetative growth will occasionally be observed late in stationary-phase cultures [42]. It has recently been speculated that this “gaping” effect is an altruistic phenomenon, whereby the majority of cells in an aging population die through a process resembling apoptosis in order to facilitate the outgrowth of a few remaining viable cells [42]. This hypothesis, although controversial, provides an interesting link between replicative and chronological longevity and also suggests a potential mechanism for how an apoptosis-like pathway might evolve in a single-celled eukaryote.

Genome-Wide Screens Elaborate the Importance of Nutrient-Responsive Kinases

An important advance in the field of yeast aging over the last two years has been the development and application of genomic methods for assaying longevity [12,25]. Previously, studies of aging in yeast had been limited to a relatively small number of genes and relied on biased approaches: testing of candidate genes based on prior knowledge or assumptions and screening for secondary phenotypes, such as stress resistance [18,43] or age-associated changes in gene expression that may correlate with longevity [44].

Two reports from genome-wide studies of yeast aging, carried out using a collection of ~4,900 isogenic single-gene deletion strains [45], have identified the nutrient-responsive TOR signaling pathway as an important mediator of both replicative and chronological life span [12,25]. Mutations that decrease TOR activity were found to increase longevity of both dividing and nondividing yeast cells [12,25]. Interestingly, decreased TOR activity also increases life span in both worms and flies [46–48], suggesting an evolutionarily conserved role for TOR as a conduit linking nutrient status to longevity.

In yeast, TOR acts in concert with other nutrient-responsive kinases, Sch9 and protein kinase A (PKA), to coordinate the cellular response to altered glucose and nitrogen levels [49,50]. Prior studies had implicated roles for both Sch9 and PKA in yeast aging. Similar to inhibition of TOR, deletion of Sch9 increases both replicative and chronological life span [25,43,51]. Likewise, a temperature-sensitive allele of yeast adenylyl cyclase (cyr1–1) that decreases PKA activity increases both replicative and chronological life span [9,43]. Surprisingly, deletion of small G proteins that activate the PKA pathway, Ras1 and Ras2,
results in opposite effects on the chronological and replicative life spans; deletion of RAS1 increases RLS while slightly decreasing CLS, while deletion of RAS2 decreases RLS, but dramatically extends CLS [9,51,52]. Thus, multiple studies have independently uncovered an important role for these nutrient-responsive signaling pathways in determining yeast longevity.

How might decreased activity of nutrient-responsive kinases lead to increased life span? TOR, Sch9, and PKA play overlapping regulatory roles in several cellular processes that could be of relevance for longevity (Figure 2). In the remainder of this review, we consider which downstream functions are most likely to determine longevity in yeast and, potentially, other organisms.

**Stress response.** One function of TOR, Sch9, and PKA is to repress a general stress response by regulating localization of the transcription factors Msn2 and Msn4 [53–56]. Under conditions of high nutrient availability Msn2/4 are retained in the cytoplasm, where they are unable to activate transcription of starvation-induced stress proteins [53]. Under starvation conditions, or upon treatment with the TOR-inhibitor rapamycin, Msn2/4 relocalize to the nucleus, resulting in enhanced resistance to oxidative and temperature stress. Extension of CLS by the RAS2 deletion appears to be due in part to Msn2/4 activation [9]; however, the chronological life span extension imparted by deletion of Sch9 is independent of Msn2/4; instead, it partly involves activation of RIM15 [43], which mediates entry into stationary phase and activation of stress-responsive genes under those conditions [57].

Although it has not been demonstrated that activation of Msn2/4 is sufficient to either increase replicative or chronological life span, there is indirect evidence supporting the idea that Msn2/4 are involved in chronological life span extension from TOR inhibition. For instance, overexpression of the Msn2/4 target genes SOD1 and SOD2 is sufficient to increase chronological life span [9], suggesting that decreased TOR activity results in increased chronological life span, at least partially, through upregulation of superoxide dismutase activity. Thus, one mechanism by which decreased nutrient availability might slow chronological aging is through an upregulation of stress resistance via activation of Msn2/4 and other pathways.

Interestingly, replicative life span extension from SCH9 deletion, mutations reducing PKA activity, or DR is not dependent on Msn2/4 [11,58]. Thus, given the current available data, the Msn2/4-mediated stress response appears to play an important role in nutrient-mediated chronological, but perhaps not replicative, life-span extension in yeast.

**Retrograde response.** The retrograde response has been defined as a mitochondrion-to-nucleus signaling pathway that is activated in response to mitochondrial dysfunction [59,60]. This process is mediated by the transcription factors Rtg1 and Rtg3, which coordinate expression of enzymes involved in anapleurotic production of α-ketoglutarate. The retrograde response has been previously implicated in yeast replicative longevity, with the observation that deletion of mitochondrial DNA (rho<sup>−</sup>) can increase life span in a retrograde-dependent manner [61]. The relevance of this finding has been difficult to determine, however, because deletion of mitochondrial DNA increases replicative life span in only one out of the six yeast strains in which it has been studied [23,52,61].

In addition to mitochondrial dysfunction, however, retrograde gene expression is also regulated by TOR activity, and treatment of cells with rapamycin induces Rtg1/3-target genes [62,63]. Thus, it is reasonable to speculate that one mechanism by which TOR inhibition could influence replicative longevity is by altering retrograde gene expression. It will be of interest to determine whether Rtg1 and Rtg3 are required for replicative or chronological life span extension from TOR inhibition. Interestingly, deletion of the retrograde target gene IDH2, coding for isocitrate dehydrogenase, also increases yeast replicative life span [25] and two different isocitrate dehydrogenase enzymes are reported to similarly affect longevity in *C. elegans* [64]. Thus, there is evidence that altering the expression of TOR-regulated retrograde target genes can influence longevity.

**Autophagy.** Yet another important function of TOR proteins is to repress autophagy [65,66]. Autophagy is a starvation response in which cellular macromolecules are recycled through vesicular transport and degradation in lysosomal or vacuolar compartments [67]. Autophagy has been implicated in age-associated disease, and autophagy decreases as a function of age in rodents [68–71]. More recently, it was shown that increased autophagy is required for full life-span extension in *C. elegans* in a long-lived *daf-2* mutant [72].

Although direct experimental data is lacking, autophagy could be an important mediator of yeast longevity, particularly chronological life span. It is known that yeast cells upregulate autophagy during entry into stationary phase, presumably as an adaptive response to starvation [66]. Consistent with this, several mutants defective for autophagy are short-lived in the chronological aging assay [12].
Treatment of yeast cells with rapamycin or growth under nitrogen starvation induces autophagy [66] and also increases chronological life span [12]. Enhanced autophagy could have several beneficial properties in aging post-mitotic cells, including degradation of oxidatively damaged proteins, inhibition of protein aggregation, and recycling of damaged mitochondria. It will therefore be of interest to determine whether increased autophagy is important for life-span extension from DR or TOR inhibition in either or both of the yeast aging models.

Changes in carbon metabolism. Yeast cells have evolved to undergo a variety of metabolic changes in response to fluctuating nutrient levels in the environment, many of which are coordinated by TOR, Sch9, and PKA. In particular, yeast respond robustly to decreasing glucose levels by shifting their metabolic state from one that favors fermentation to one that favors respiration. It has been proposed that this shift in carbon metabolism may account for the increase in RLS observed in response to DR [73]. The mechanism postulated by this model was that enhanced respiratory activity would activate Sir2, thus increasing life span. Contrary to this hypothesis, DR increases the RLS of respiratory-deficient cells [23]. This is true, even in cells completely lacking mitochondrial DNA. Similar to the case with Sir2 and DR, there continues to be disagreement about the requirement of respiration for life-span extension by DR, and it has been recently reported that deletion of LAT1, which encodes a component of the mitochondrial pyruvate dehydrogenase complex, also is required for life span extension by DR [74].

There is additional evidence that changes associated with respiratory metabolism can influence both RLS and CLS. For example, overexpression of the glucose-repressible gene \( HAP4 \) is sufficient to increase both RLS [73] and CLS [4], even when glucose levels are high. \( HAP4 \) is a regulatory subunit required for optimal transcriptional activation by the Hap2/3/5 complex, which induces respiratory genes in response to the available carbon source. Putative Hap2/3/5 binding domains have also been identified in the \( TSA2 \) (thiol-specific antioxidant) promoter, which responds to increased oxidative and nitrosative stress [75]. In that report, overexpression of \( HAP4 \) was demonstrated to induce \( TSA2 \) expression. Thus, in addition to inducing respiration, \( HAP4 \) is important for promoting cellular stress resistance. It remains to be determined whether the effects of \( HAP4 \) overexpression on replicative and chronological longevity are related to its effects on respiratory metabolism or a different function.

Decreased ribosome biogenesis and translation. One of the primary functions of TOR, Sch9, and PKA is to modulate protein translation in response to environmental cues [49,76,77]. In yeast, one mechanism by which these kinases regulate translation is by promoting transcription of ribosomal proteins (RPs) and rRNA processing factors. Under conditions of glucose or nitrogen starvation, or upon inhibition of TOR with rapamycin, RP transcription is dramatically reduced and translation in general is impaired [49,63,76,77].

A link between TOR, RPs, and longevity was suggested from the initial results of a genome-wide screen for replictively long-lived mutants. Replicative life span was determined for 564 single-gene deletion strains randomly chosen from the yeast ORF deletion collection, resulting in the identification of 15 long-lived mutants [25]. In addition to \( TOR1 \), the deleted genes from these 13 long-lived strains included two TOR-regulated RP genes, \( RPL31A \) and \( RPL6B \). We have since determined that several other RP and rRNA processing factor deletion mutants are also long-lived (our unpublished data), and Chiocetti et al. [78] recently reported that \( RPS6B \) and \( RPL10 \) similarly regulate replicative longevity. These findings suggest the possibility that one mechanism by which decreased TOR activity can increase replicative life span is by decreasing ribosome function and translation. In this regard, it is noteworthy that mutations in S6 kinase, a downstream target of TOR involved in ribosome maturation, have been reported to increase life span in flies [47], and several recent reports have implicated mRNA translation as a critical determinant of longevity in worms [79–81].

From Yeast to Mammals

It remains an open question how much of the aging process will be conserved from yeast into higher organisms. Clearly, some aspects of aging in yeast are specific to yeast. Others, however, appear to be highly conserved. Life span extension from Sir2-overexpression, TOR-inhibition, Sch9/Akt or DR, for example, has been observed in yeast, worms, and flies. It is likely that several additional conserved longevity factors will be identified from ongoing genome-wide screens in yeast and worms [12,25,52,64,82–85] and studies in mammalian models. If a given gene functions similarly to regulate longevity in yeast, worms, and mice, there is a good chance this function will be conserved in humans. In this way, yeast may serve as a foundation for identifying potential targets for intervening in human longevity and age-associated disease.

The observation that TOR, Sch9/Akt, and PKA could be regulating longevity differently in replicative and chronologically aging yeast cells is noteworthy, given that DR appears to retard a variety of age-associated diseases in tissues of higher animals [14]. The beneficial effects of reduced nutrient signaling may be dependent on the proliferative state of the tissue in question in mammals. Mice subjected to DR are resistant to carcinogenesis and display reduced age-associated pathologies in brain, liver, heart, muscle, and other tissues. How is it that DR has such a broad spectrum of beneficial effects in complex organisms? Based on the studies in yeast described above, we speculate that a few key nutrient-responsive proteins (such as TOR) may serve as evolutionarily conserved gatekeepers to synthesize inputs from the environment into appropriate tissue-specific outputs. For example, in neuronal cells, enhanced degradation of aggregated proteins through increased autophagy might be of particular relevance, whereas in fibroblasts increased resistance to stress or appropriate modulation of ribosome function could be most important. Future studies of the differential responses of different cell types to DR and TOR inhibition will be important for testing this idea.

A growing body of evidence clearly suggests that aging is determined, at least in part, by ancestral evolutionary origins. Due to this conservation, yeast remains a powerful tool for dissecting the genetic and biochemical factors that modulate longevity. As large-scale screens for long-lived yeast deletion mutants draw closer to completion, new and unexpected pathways are being uncovered, bringing a global picture of cellular aging into sharper focus. The knowledge gained from the molecular biology of aging in yeast yields a foundation on...
which to approach the puzzle of multicellular aging in tissues and in higher organisms. ■

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References

dependent gene expression to regulate growth, stress response and
glycogen accumulation. EMBO J 17: 3556–3564.
and the crossroads of nutrient signalling pathways in Saccharomyces cerevisiae.
Cell Div 1: 3
Chronological aging-independent replicative life span regulation by Msn2/3
Genomic instability and aging-like phenotype in the absence of mammalian SIRT6.
60. Liao X, Butow RA (1995) RTG1 and RTG2: Two yeast genes required for
a novel path of communication from mitochondrion to the nucleus. Cell 72:
61–71.
negatively regulates RTG target gene expression in S. cerevisiae. Curr Biol
mechanism for metabolic regulation. Curr Top Microbiol Immunol 279:
39–51.
65. Noda T, Ohsuni Y (1998) Tor, a phosphatidylinositol kinase homologue,
67. Yoshida T, Klonosky DJ (2005) Autophagy: Molecular machinery for self-
68. Cuervo AM, Dice JF (2000) Age-related decline in chaperone-mediated
dehydrolipoamide acetyltransferase is a novel metabolic longevity factor
and is required for calorie restriction-mediated life span extension. J Biol
70. Martinez-Vicente M, Sovak G, Cuervo AM (2005) Protein degradation and
dehydrolipoamide acetyltransferase is a novel metabolic longevity factor
and is required for calorie restriction-mediated life span extension. J Biol
dehydrolipoamide acetyltransferase is a novel metabolic longevity factor
and is required for calorie restriction-mediated life span extension.
Caloric restriction extends Saccharomyces cerevisiae lifespan by increasing
dehydrolipoamide acetyltransferase is a novel metabolic longevity factor
and is required for calorie restriction-mediated life span extension. J Biol
Genetic Determinants of Human Health Span and Life Span: Progress and New Opportunities

George M. Martin*, Aviv Bergman, Nir Barzilai

ABSTRACT

We review three approaches to the genetic analysis of the biology and pathobiology of human aging. The first and so far the best-developed is the search for the biochemical genetic basis of varying susceptibilities to major geriatric disorders. These include a range of progeroid syndromes. Collectively, they tell us much about the genetics of health span. Given that the major risk factor for virtually all geriatric disorders is biological aging, they may also serve as markers for the study of intrinsic biological aging. The second approach seeks to identify allelic contributions to exceptionally long life spans. While linkage to a locus on Chromosome 4 has not been confirmed, association studies have revealed a number of significant polymorphisms that impact upon late-life diseases and life span. The third approach remains theoretical. It would require longitudinal studies of large numbers of middle-aged sib-pairs who are extremely discordant or concordant for their rates of decline in various physiological functions. We can conclude that there are great opportunities for research on the genetics of human aging, particularly given the huge fund of information on human biology and pathobiology, and the rapidly developing knowledge of the human genome.

Introduction

Other articles in PLoS Genetics and elsewhere have documented remarkable progress in genetic aspects of aging in model organisms. These studies have revealed what can be regarded as the first “public” mechanism of aging, that is to say, a biochemical genetic pathway, modulation of which can alter the life spans of diverse species [1–3]. We applaud the remarkable achievements of our colleagues working with these models. One of us was in fact an early champion of this approach [4]. These model systems have several limitations, however. First, the life spans of these models are dramatically shorter than those of humans. The mutations that extend life span result in increases of a few weeks or months in invertebrates and a year or so in rodents. These species are “r” selected species, characterized, in part, by rapid rates of development, high degrees of fecundity, and short life spans [5]. Human beings are “K” selected organisms, characterized, in part, by long periods of development, comparatively few progeny and long life spans [5]. It is therefore possible that the biochemical genetic results from model organisms may not be relevant for humans, whose life history strategies are quite different. It should be noted, for example, that a polymorphic locus (CETP) that modulates susceptibility to several major diseases of aging does not exist in invertebrates or rodents.

Second, while we have learned a great deal about developmental biology from worms, flies, and mice, there is a paucity of detailed information on the pathophysiology of aging and of their variations among genetically heterogeneous wild-type populations, particularly in worms and flies. In contrast, there is a vast literature on these and all other aspects of human biology, including remarkable progress in human genetics (Table 1). Moreover, physicians have provided detailed characterizations of late-life disabilities and diseases in human populations (Table 2). Third, additional and unique DNA sequences have evolved in Homo sapiens, including rapidly evolving functionally significant intronic sequences that distinguish us from our nearest relative, the common chimpanzee (Pan troglodytes), whose life span is approximately half that of humans) [6,7] (see, however, evidence of a single outlier who lived to age 74, http://genomics.senescence.info/species/biblio.php?id=0505). Fourth, most gerontological investigations of model organisms have utilized highly inbred organisms typically examined in a single environment. By contrast, human geneticists, particularly medical geneticists, see the results of a huge range of gene–gene and gene–environmental interactions. They therefore have considerable opportunities to contribute to our understanding of why individual patterns of aging exhibit such substantial variations. Some of this understanding should prove to be unique to our species, as they will include “private” biochemical genetic mechanisms—that is to say, mechanisms that are characteristic of only particular subsets of individuals [1–3]. These different patterns of aging certainly include...
various degrees of susceptibility to both common and rare late-life diseases and disabilities. As we shall see below, these are all part of a spectrum of phenotypes that escape the force of natural selection and are thus, in our view, part and parcel of complex aging processes. We can refer to them generically as “senescent phenotypes” [8]. Moreover, given the likelihood that intrinsic physiological declines in structure and function are early precursors of such geriatric disorders, investigators have the opportunity to work backwards from the disease to elucidate underlying mechanisms of aging, mechanisms that set the stage for the emergence of these clinically important disorders. Finally, studies of genetic contributions to late-life disorders can elucidate variations in health span, which can be defined as the period of life during which an individual is free of chronic illness and substantial functional decrements. Genetic and epigenetic factors that limit health span are certainly legitimate aspects of biogerontological research, particularly from the point of view of medical economics. A cogent example of why long life span cannot be equated with the population. The evolutionary biological theory of aging predicts a polygenic basis for the control of rates of aging. During transient environmental challenges. As such, they will eventually be trumped by a variety of gene actions that are predicted by the evolutionary biological theory of aging [21]. As discussed below, evolutionary ideas can also be borrowed and applied to one or two overlapping generations as an aid to the discovery of genotypes modulating longevity and age-related diseases.

An Evolutionary Biological Approach to Understanding Gene Action in Human Aging

The classical evolutionary biological theory of aging tells us that senescence occurs in age-structured populations because of the decline in the force of natural selection with age [11–13]. That generalization has been challenged recently [14]. Certain species of fish, for example, continue to grow throughout their life spans; for such species, rates of aging may be “negligible” [15,16] and the force of natural selection could conceivably increase with age [17]. No human being has ever exhibited such a life history, however. All physiological studies have confirmed gradual functional declines in multiple body systems beginning at middle age, even for cohorts of exceptional athletes [18]. A wide variety of diseases and disabilities accompany these physiological declines.

It is not generally appreciated that the evolutionary theory of why we age provides clues to how we age [19–21]. Classes of such gene actions include: (1) “longevity assurance,” genes that enhance structure and function of the organism throughout the life span; (2) “antagonistic pleiotropy,” alleles selected because of enhanced reproductive fitness early in the life span, but with negative effects late in the life span, when those effects will have escaped the force of natural selection; and (3) “mutation accumulation,” constitutional mutations that do not reach a level of phenotypic expression until late in the life span—once again, when they will have escaped the force of natural selection, and thus could not be purged from the population. The evolutionary biological theory of aging predicts a polygenic basis for the control of rates of aging. While single gene variations can indeed enhance the life spans of model organisms, these involve the tweaking of diapausa [22,23].


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The Search for Polymorphisms and Mutations Modulating Susceptibility to Late-Life Disorders

Table 2 lists 87 disease and disability phenotypes that commonly emerge in geriatric subjects. These result in a substantial proportion of the overall morbidity and mortality in the developed societies and are responsible for a major proportion of the huge costs of Medicare in the United States. These costs are likely to increase substantially, given demographic trends [24]. A very large number of genetic variants that advance the ages of onset and/or the rates of progression of these phenotypes can be found by online searches of Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM). These have the potential to reveal pathogenetic mechanisms. They can be divided into two major categories. One subset consists of mutations that impact upon a number of such phenotypes; they have been referred to as “segmental progeroid syndromes” [25]. A second group consists of allelic variants that impact predominantly on only a single tissue or organ system; these have been referred to as “unimodal progeroid syndromes” [26]. We should be alert, however, to the possibility that putative unimodal syndromes may in fact have more widespread, systemic effects; a striking example has been uncovered for the case of a mouse model of Huntington disease, in which a metabolic abnormality of brown fat and a defective regulation of body temperature were found to be associated with the triplet repeat sequences previously thought to have an exclusive impact upon the central nervous system [27]. Striking examples of segmental and unimodal progeroid syndromes have been recently reviewed [8,28]; these have indeed provided important insights into mechanisms of aging—for example, the role of genomic instability for the case of many segmental disorders and the role of abnormal protein aggregates for the case of various unimodal dementing disorders. No single locus has ever been discovered, however, that appears to accelerate the rates of onset and/or the rates of progression of all senescent phenotypes [25]. Patients with Werner syndrome (WS), caused by mutation at a member (WRN) of the RecQ family of helicases [29], exhibit accelerating rates of development of all forms of arteriosclerosis, type 2 diabetes mellitus, gonadal atrophy, skin atrophy, hair loss and hair greying, regional loss of subcutaneous tissue, osteoporosis, ocular cataracts, and neoplasia [25]. While deserving of greater study, there is so far no convincing evidence of an acceleration in the rates of development of synaptic loss, beta amyloidosis of blood vessels or parenchyma, granulovacuolar degenerations, or neurofibrillary lesions [30], markers that accumulate, to varying degrees, in the aging brains of many aging human subjects, with or without a clinical or neuropathological diagnosis of Alzheimer disease. Those lesions are very abundant in that disorder, which exhibits exponential increases after the age of 65, with prevalence rates 25%–48% for persons over age 85 [31]. There are additional interesting discords between the phenotype of WS subjects and what is commonly found in “usual” or “normative” aging. For example, the osteoporosis of WS is disproportionately severe in the bones of the lower limbs rather than in the vertebral bodies [25] and the patterns of neoplasia are quite unusual, as WS patients exhibit a high prevalence of sarcomas and rare neoplasms [32]. These are among the reasons for referring to such syndromes as “progeroid” (“like” premature aging). On the other hand, as noted above, the biochemical genetic findings are consistent with a growing body of evidence implicating genomic instability as a common basic mechanism of aging [33,34]. Somatic cells from subjects with WS exhibit marked accelerations in the rates of replicative senescence of several somatic cell types [35]. The discovery that G quartet motifs at telomeres are among the favored substrates for the WRN locus [36,37] is consistent with the important role of telomere loss as a mechanism leading to the replicative senescence of somatic cells [38]. WS has also pointed to the importance of aberrations in DNA transactions within the lens epithelial cells as an important mechanism of cataractogenesis, as opposed to post-translational alterations of lens crystallins, an alternative pathogenetic mechanism [39]. Patients with WS and the Hutchinson-Gilford Progeria Syndrome (HGPS), as well as a number of other progeroid syndromes, exhibit an accelerated loss of somatic cells, consistent with the widespread atrophy one observes in senescent human subjects. Finally, an important argument for a commonality of mechanisms of aging in HGPS and usual aging is that the splice variant caused by the common HGPS mutation, which functions as a dominant negative, thus impairing lamin A structure and function [40], also appears in the aging tissues of normal humans [41]. This may explain the appearance of comparable nuclear morphological abnormalities in the aging somatic cells of normal individuals, albeit at lower frequencies than that observed in the cells of HGPS patients [41].

A recent example of encouraging progress in the discovery of unimodal progeroid syndromes was the discovery of two polymorphic loci, complement factor H and a predicted gene on Chromosome 10q, LOC387715, that modulate susceptibility to age-related macular degeneration [42–44]. A prospective study of US nurses and health professionals has revealed a ~50-fold increase (95% CI: 10.8–237) in the risk of age-related macular degeneration for subjects who are homozygous for both risk alleles [45]. Smoking and obesity increased the risks associated with these variants [45]. Of interest is the lack of evidence for an association with complement factor H in a Japanese population [46], but the presence of an association in this population with the polymorphism at the 10q locus [47]. Enormous efforts have also been directed to families with both early- and late-onset dementias of the Alzheimer type. All three autosomal dominant genes responsible for the comparatively early-onset variety impact upon the processing of the beta amyloid precursor protein, the result being increased proportions of the highly amyloidogenic amyloid beta 1–42 peptide (for a brief and selective review of this huge literature, see [48]). These findings are consistent with a major etiological role of amyloid beta peptides in the vastly more common late onset cases, where the neuropathological diagnosis is made on the basis of amyloid plaques and neurofibrillary tangles. With the exception of the important risk factor of the epsilon 4 allele at the APOE locus (reviewed by [49]), numerous candidate loci remain to be fully validated as contributors to the much more common late onset forms of late-life dementia. For a comprehensive list of these candidate loci and their polymorphic alleles, see http://www.alzforum.org/res/com/gen/alzgene/default.asp.
Linkage and Association Studies of Exceptional Human Longevities

Centenarians have outlived any exceptionally long-lived invertebrate model by ~100 years and any comparable rodent model by ~30-fold. Human subjects in general and centenarians in particular outlive their nearest relatives, the common chimpanzee, by many decades [50]. This happy state of affairs is clearly the result of evolutionary changes in our constitutional genomes [51]. Since the structure of our proteins and those of chimpanzees is very nearly identical [52], our enhanced life spans are probably related primarily to regulatory RNA species, an area of scholarship that has just begun [6,7] and has not yet been applied to the study of the evolution of varying longevities.

Our task here, however, is to review progress towards the elucidation of genetic factors that contribute to exceptional longevities of individual members of Homo sapiens. The life expectancies of centenarians at birth are nearly double that of most members of their birth cohort and, on average, have surpassed current life expectancy by 22 years. Environmental and stochastic contributions to human life span likely play important roles in the determination of such exceptionally long survivals, as inferred from the twin studies discussed below. Familial aggregates of exceptional longevity do not rule out major environmental factors that are the result of cultural inheritance (e.g., lifestyles, nutrition); such factors could explain, in part, why the progeny of long-lived members of the Framingham study exhibit advantageous cardiovascular risk profiles in middle age [53]. Nevertheless, evidence consistent with a significant heritable component of exceptional longevity is impressive. Parents of centenarians (born in ~1870) were shown to have approximately nine times the odds of living to the tenth decade as compared to controls [54]. Siblings of centenarians were shown to have up to an ~18-fold increase in the chance of achieving a similar age [55]. Such data have raised the possibility that some specific genetic modulators of aging in humans can be identified using such populations, and that conserved pathways for exceptional longevity might thus be validated. Exceptional longevity is obviously coupled with exceptional resistance to diseases that lead to earlier mortalities. We do not have the required biomarkers, however, to clearly disentangle the two phenomena. The research suggested in the last section of this essay may eventually lead to such markers, however.

During the last decade, centenarian populations (New England American, Mormon, Ashkenazi Jewish, Islandic, Okinawan, Japanese, Italian, Irish, and Dutch, among others [54,56-60] have been used for association studies to search for candidate longevity genes or pathways. Particularly striking examples have included PON1 [61–67] IGF-1 [68–71], PPAR-1 [72,73], cytokines, enzymatic antioxidants such as superoxide dismutases [74,75], and elements of lipid metabolism [76,77]. Some significant differences have been noted between younger cohorts and centenarians in the prevalence of specific genotypes and sometimes in their associated protein activities. These interesting observations, however, have suffered and will continue to suffer from several limitations. In addition to the usual problems and pitfalls of association studies, particularly as we enter the new age of whole genome scans [78], there is the special problem of the identification of appropriate controls for a cohort of exceptionally long-lived individuals. One innovative approach has been an experimental design based upon a genetic analysis of the progeny of centenarians, giving the opportunity for matched spousal controls [79].

The New England Centenarian Study recruited long-lived sb-ships for a genome-wide scan of linkage to exceptional longevity. A region on Chromosome 4 was implicated [80]. By high density SNP analysis an exonic genotype in microsomal transfer protein was thought to be the locus associated with the exceptional longevity [81]. The original finding could not be replicated in independent populations [82]. Such validation is crucial because of the considerable rates of false positives. While it is possible that the role for this gene in longevity may only be significant in certain populations, the most likely explanation for the original linkage was population stratification. The ethnic mix within the long-lived and younger control populations was likely to have differed [83]. Nevertheless, it would be helpful to evaluate other allelic variants in the same gene or in other related genes. In any case, this early study emphasizes the need to establish additional phenotypes associated with the polymorphism. Although microsomal transfer protein cannot be directly measured, evidence for a role in lipoprotein characteristics or a relationship to age-related diseases would have been helpful in support of a protective role. The population stratification problem can be ameliorated by the selection of better-defined populations, as was done for the case of the Dutch study cited above [83].

Studies performed at the Albert Einstein College of Medicine were based upon populations of Ashkenazi Jews and the following considerations [84]. First, exceptional longevity is obviously a rare phenotype (~1/10,000 individuals live to the age of 94–110). Second, it is also apparent that, for any given cohort, genotypes associated with comparatively early mortality are “weeded out,” while a subset of genotypes are associated with survival. Given large cohorts representing each decade of the life span, one can examine whether those who continue to survive exhibit biologically distinctive phenotypes and genotypes as compared to those of younger cohorts. Thus, the relative prevalence of favorable “longevity” genotypes within the population can be expected to rise monotonically rather than abruptly or intermittently over the life course. Because the genotypes of survivors are “selected,” the greater the attribution of a genotype to longevity, the greater is the divergence from Hardy–Weinberg equilibrium among the elderly. Using this strategy, the Einstein group recruited significant numbers of Ashkenazi Jews of all ages, including ~400 individuals between ages 95–110. Significant increases within aging cohorts were observed for three genotypes from among hundreds of candidate genotypes (selected because of their relevance for lipoprotein phenotypes) that were tested in unrelated populations consisting of individuals between ages 50–110 years (Figure 1) [79,85]. These genotypes were: (1) the CETP gene codon 405 isoleucine to valine variant (CETP VV); (2) the apolipoprotein C-3 (APOC-3) gene codon A (~641) C variant (APOC-3 CC); and (3) a deletion at t+2019 in the adiponectin (ADIPOQ) gene. The enrichment of the CETP genotype is supported by evidence from two independent populations [77,86].

While a significant overrepresentation of a single genotype...
Figure 1. Visual Presentation of the Frequency Trends of Favorable Genotypes with Exceptional Longevity

This trend was obtained in ~400 Ashkenazi Jewish subjects over age 95 and ~600 subjects between ages 60–95 [76,87]. While these genotypes were assessed cross-sectionally in groups between ages 60–110, it is important to realize that marked selection occurs during the life course. One also should be aware of the fact that very few subjects achieve centenarian status. Of many polymorphic candidate loci, only subjects homozygous for CETP VV, APOC-3 CC, and ADIPOQ del/del genotypes are markedly and significantly enriched among centenarians (see details in text). To be considered a favorable longevity genotype, a monotonic increase should be observed among age groups. This criterion helps to exclude false-positive associations that occur only in one age group but that do not exhibit trends among sequential age groups. Genotypes with unchanged frequencies among age groups serve as partial controls for genotypic distribution and stratification tests. The analysis of such patterns is useful for the identification of candidate “longevity genes.”

among nonagenarians and centenarians operationally defines a candidate gene, several other criteria should be fulfilled before considering it to be an important longevity assurance gene (Figure 2).

The first step by which the functionality of the genotype can be studied is by determining the serum and plasma levels of the coded protein, if it is secreted and circulating. For example, for each of the genotypes in Figure 1 (CETP, APOC3, and ADIPOQ), appropriate alterations in plasma levels have been demonstrated [76,85–89]. Detailed information on the approach to the choice of controls typically used for these studies is given in [87]. They included spouses or other nonrelated age-matched pairs for the progeny of centenarians. The genomes of the latter can be expected to be enriched with alleles for unusual longevity. Indeed, these offspring were shown to be healthier than age-matched controls and had more favorable lipid profiles.

A second step in establishing functionality is the identification of an intermediate phenotype. For example, alleles at CETP and APOC3 differentially modulate lipoprotein characteristics. These effects may vary with age and should therefore be examined in cohorts of varying ages. The functional value of a genotype can also be assessed directly by functional studies of the mutant in a cellular system in vitro. Such studies may underestimate or overestimate the real physiological importance of the relevant gene action in vivo, however. If the gene encodes a protease, then studies of its specificity, tissue distribution, and regulation are called for. If the gene encodes a cell-surface receptor, then studies of the biochemistry of the receptor should be done. For example, the Einstein group recently identified novel mutations in the IGF-1 receptor of three centenarians. AKT phosphorylation was assessed in lymphoblastoid cell lines from these subjects and controls, both basal and induced levels (stimulating with IGF-1). A marked decrease in phosphorylation was observed in cells from the centenarians with the mutant IGF-1 receptors [89,90].

As noted in the Introduction, the enhancement of life spans in model organisms via single gene mutations raises the question of whether allelic variations at this pathway in human subjects might impact intrinsic biological aging within all tissues and thus lead to substantial increases in life span. Among the many billions of human beings who have lived since the time of recorded history, it is unlikely that a spontaneous mutant of this type, say leading to a doubling of the usual human life span, would have been missed. One could not rule out gene actions of this type, however, that contribute to the generation of rare, relatively healthy centenarians. This possibility is supported by at least three lines of evidence. First, as in the case of CETP VV, the protection provided by certain genotypes can extend beyond a known role in a disease entity associated with its ascertainment (in this example, cardiovascular disease). The CETP VV genotype is also associated with enhanced insulin sensitivity and lower risk for hypertension, the metabolic syndrome, and diabetes [87]. Moreover, the CETP VV genotype protects against age-related cognitive decline and Alzheimer’s disease [91], although the role of particular haplotypes at that region may interact with polymorphic alleles at the APOE locus [92].

A second example is a 2.5-fold increase, among centenarians, in the prevalence of an apoC III promoter variant. This variant is associated with significant declines in plasma levels of apoC III and a phenotype of large
lipoprotein particles. There is also significantly less hypertension among subjects homozygous for this variant. The most striking data, however, were obtained from a retrospective study of a cohort of subjects bearing this variant. They live significantly longer; in fact, subjects <95 years old with this genotype lived on average over four years longer than those who were not homozygous for the variant. This is indeed a very large impact upon life span when one considers the conclusions of demographers, who have noted that the elimination of ischemic heart disease, a disorder that was responsible for 25.73% of all deaths in 1985, would increase life expectancy at birth by only 3.0 years for females and 3.55 years for males [93].

A third argument suggesting that some longevity genes are not merely disease specific is the marked conservation of some of these loci. For example, apoC III is under the control of FOXO-1, a transcription factor homologous to the DAF16 gene of Caenorhabditis elegans. DAF16 is a key regulator of a downstream suite of genes that are thought to protect the organism from macromolecular damage and thus enhance life span [94]. Some centenarians have novel functional mutations in the IGF-1 receptor, as noted above. There is evidence that the homologue in mice regulates life span and resistance to oxidative stress [95]. Thus, while the impact of variants at the CETP locus upon age-related diseases and longevity may be a special feature of the biology of humans, there is also evidence that the fruits of research on the genetic modulation of the life spans of worms, flies, and mice may in fact be applicable to our species.

Some favorable “longevity genotypes” may act to buffer the deleterious effects of genes that lead to age-related diseases. As a result, the frequencies of deleterious genotypes may, paradoxically, be increased among individuals with extreme life spans. This may explain why the cholesteryl ester transfer protein (CETP-VV) genotype appears to exhibit an additional advantageous effect—the neutralization of the deleterious effects of the lipoprotein(a) (Lp(a)) gene [84]. Such buffering effects cannot be ascribed to genetic linkage. For the example just cited, those loci are in fact on separate chromosomes. More generally, however, it is clear that one can define two distinct populations, each bearing the disease susceptibility allele in question, but only one of which exhibits the putative buffering effect.

The Role of Stochastic Events in the Modulations of Health Span and Life Span

A study of uncensored pairs of Danish human twins has indicated that only about one quarter of the heritability of life span can be attributed to the constitutional genotype [96]. There are indications from twin studies of very old individuals, however, that more robust genetic contributions to superior health and superior cognitive functioning can be identified [97,98]. In any case, it is quite clear that there are substantial impacts of both environmental and stochastic influences upon both life span and health span [99]. Discussion of environmental factors is beyond the scope of this minireview. Suffice it to say that there are likely to be a host of “gerontogens” [100] with the potential to modulate segmental and unimodal aspects of the pathobiology of aging. Cigarette smoking is a prime example [101]. With regard to stochastic factors, we must look to the work of colleagues who have demonstrated, in numerous publications over many decades, remarkable variations in life spans among highly inbred worms, flies, mice, hamsters, and rats, despite every effort to control the environments in which such organisms are aged. The most cogent example involved studies of C. elegans grown in liquid cultures with axenic medium [102]. One can imagine several distinct types of stochastic events to explain such observations. First, one can imagine stochastic variations in the epigenetic control of gene expression. Such a mechanism might explain, in part, recent experiments demonstrating correlations of the expression of a transgene for an inducible heat shock promoter/reporter with the longevity of cohorts of C. elegans [103]. There is also evidence of substantial “epigenetic drift” of gene expression within aging pairs of human identical twins [104,105]. The siruIn family of histone deacetylases represents a potential causal link between epigenetic regulation, caloric restriction, and longevity in a number of organisms, including fruit flies [106]; moreover, inactivation of a member of the sirtuin family in mice causes phenotypes consistent with premature aging [107]. A second obvious candidate is somatic mutation, within both the nuclear [108] and mitochondrial [109] genomes. The latter is a particularly attractive idea, as the stochastic events could involve both the timing and specificity of the mutations and the events leading from the heteroplasmic to the homoplastic state. In that respect, certain classes of mitochondrial rearrangements leading to multiple replication origins might be more likely to evolve towards a homoplastic state [110]. In contrast to point mutations, which appear not to be major contributors to senescence [111], such rearranged mitochondrial DNA molecules might enjoy a selective replicative advantage over wild-type mitochondrial DNA. A third possibility could be related to what has been referred to in the microbial literature as “noise”—random fluctuations in gene expression; see, for example, [112]. Whatever the mechanism, these stochastic events, and the heritability studies mentioned above, diminish the power of genetic analysis to discover loci at which allelic variation modulates health span and life span. We already know a great deal about how the constitutional genome modulates the initiation and accumulation of somatic mutations, particularly nuclear mutations [34], but we know very little about how DNA sequences might set the stage for differing degrees of epigenetic variation and transcriptional or post-transcriptional noise.

Opportunities for Discovering Genetic Contributions to Differential Rates of Physiological Declines in Middle-Aged Subjects

Human geneticists, most of whom are practicing medical geneticists, suffer from a biased ascertainment of their subjects. With the notable exception of studies of nonagenarians and centenarians discussed above, their subjects present themselves because of dysfunctions and never because of remarkably robust function. Centenarians, however, often have a variety of comorbidities. Moreover, given their extreme old age, it is not feasible to carry out longitudinal studies of the rates of change of specific physiological functions. An argument has been developed for a different approach to the discovery of allelic variants that are associated with unusual degrees of maintenance of
structure and function during aging [113]. The suggested experimental design included a focus upon subjects in their early middle age, when early functional declines unfold, as predicted by the evolutionary biological theory of aging. Such subjects are typically free of comorbidities. In contrast to centenarians, there are vast numbers of such individuals and they are typically more compliant. They can be followed longitudinally for many years. Moreover, they are members of nuclear families, permitting sib-pair analysis and the use of multiple generations for the establishment of phase relationships of genetic markers. For many populations, there is a rich association of relevant clinical and pedigree information. Given sufficiently large cohorts of such individuals, one has the potential to detect individuals who are at the extreme ends of a statistical distribution of assays for a range of physiological functions. Those physiological assays must be highly sensitive, in order to identify individuals at the statistical extreme of exceptionally superior functioning. The assays should measure very specific physiological functions, functions that are not likely to be under highly polygenic controls. Ideally, they should also be relatively noninnvasive, relatively inexpensive, relatively rapid, and not subject to major motivational influences that could impact peak performance. Multiple body systems should be interrogated, since an important null hypothesis to be tested, as noted in our Introduction, is the lack of tight coupling of rates of functional change among the various body systems. Given the availability of such assays, one would be in a position to carry out a genetic analysis on sib-pairs, starting with index cases from the extreme tails of the distributions. As argued by Risch and Zhang, the statistical power of such sib-pair studies would be enhanced by the selection of sibs showing extreme discordances [114]. The rapid technical advances that are being made in whole genome scans (see, e.g., http://www.perlegen.com/index.html?whatweoffer/whyy_whole_genome.html) and statistical methodologies [115] should greatly facilitate the genetic analysis of exceptionally slow rates of human aging in various organ systems.

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Substantial genetic influence on cognitive abilities in twins 80 or more years old. Science 276: 1560–1563.


Genetics of Aging in *Caenorhabditis elegans*

Adam Antebi

**ABSTRACT**

A dissection of longevity in *Caenorhabditis elegans* reveals that animal life span is influenced by genes, environment, and stochastic factors. From molecules to physiology, a remarkable degree of evolutionary conservation is seen.

**Introduction**

Over the last 20 years, fundamental insights into the biology of aging have emerged through the study of model genetic organisms, where undoubtedly the tiny nematode *C. elegans* has led the way. Importantly, the discovery that the single gene mutants *age-1* and *daf-2* could extend the short three-week life span 1-2-fold revealed that longevity is under genetic control [1,2]. Long life was shown to depend on another locus, *daf-16*, defining an epistasis pathway for this process. Moreover, the molecular identification of all three as components of insulin/IGF-I signaling (IIS) [3–6] eventually led to the striking realization that a modest downregulation of IIS promotes stress resistance and longevity across taxa [7–10].

Molecular genetic studies suggest that in response to insulin-like peptides (ILPs) [11,12], activation of the DAF-2/insulin/IGF-1 receptor tyrosine kinase triggers a PI3/AKT/SGK kinase cascade that phosphorylates the DAF-16/FOXO transcription factor [13–16] (Figure 1). Consequently, DAF-16/FOXO is retained cytoplasmically and animals live normal life spans. In response to increased DAF-18/PTEN activity [14,17], stress (heat, oxidative, starvation) or reduced IIS, DAF-16/FOXO enters the nucleus [18–20], where it turns on survival genes, including those that manage oxidative stress, heat shock, innate immunity, metabolism, autophagy, and xenobiotic response, among others [21–25]. Indeed, over- or underexpression of such targets often impacts stress resistance and longevity [22,26]. Many of these points and more have been reviewed elsewhere [27,28].

Importantly, IIS is best understood as a signaling pathway selected in evolution to regulate organismal survival, not aging per se. Indeed, during larval development, DAF-16/FOXO specifies the dauer diapause, a long-lived stress-resistant larval stage specialized for survival, but can independently trigger longevity in adults [29]. Evidently, the primary role of IIS is to invest in somatic endurance to outlast hard times, and conversely specify growth and reproduction in good times, with secondary consequences on adult life span. Consistent with this, many longevity mutants have reduced Darwinian fitness under conditions favoring short-term rapid reproductive output [30].

Since the elucidation of this core pathway, scores of longevity genes have emerged from candidate approaches, unbiased genome-wide RNAI screens, and transcriptional profiles, some working through IIS, others not [22,31–35]. Because they cannot all be enumerated here, we have taken an admittedly “transduction-centric” view to highlight new developments. We start with upstream signaling events of sensory perception, walk through new signaling inputs, focus on transcriptional factors, and then discuss broad physiological processes (respiration, translation, checkpoint control, diet restriction), which may impact longevity through hormesis, the induction of enhanced robustness and survival by exposure to low levels of stress [36].

**Sensory Input: The Senseless Liveth**

Critical for making preemptive decisions that prepare the animal for times ahead, sensory perception can also dramatically influence life span. Mutants deficient in sensory neuron structure and function have extended longevity, which is largely but not entirely *daf-16* dependent [37]. Moreover, such mutants cause DAF-16/FOXO to relocate to the nucleus [19]. Components of sensory cell signal transduction, including G-protein coupled receptors, G-proteins, cGMP channel subunits, ciliary proteins, Tubby, and proteins implicated in synaptic transmission [37–42] are thought to regulate release of ILPs from sensory cells, thereby influencing organismal physiology. Cell ablation studies show that both gustatory and olfactory neurons contribute, with specific neurons promoting longevity and others suppressing it [38]. The exact sensory cues governing ILP synthesis and release are likely to include nutrients, and various repellants or attractants, but this area remains largely unexplored. Related findings have recently emerged in *Drosophila*, in which *or83b* mutants, broadly deficient in olfaction, live long [43]. Moreover, odorants from yeast paste reduce longevity induced under conditions of dietary restriction. Thus, perception as well as ingestion may impact animal life span in diverse taxa.

In addition to sensory cues, serotonergic inputs may work upstream of IIS. Notably, mutants in the serotonergic receptor *ser-1* are longer lived, in a *daf-16/FOXO*-dependent manner [44]. Surprisingly, mutants in *ser-4*, another serotonin receptor, are somewhat short lived, suggesting the receptors could act antagonistically, similar to the cells described above. Moreover, mutants deficient in serotonin production, such as *tph-1*, are stress resistant, but not long lived [44–46]; opposing effects of the receptors might explain this phenomenon.
Because serotonergic signaling is phyletically ancient, it may have a conserved physiologic role in life span regulation, and be amenable to pharmacologic intervention.

**Signal Transduction: Wiring Longevity**

Aside from IIS, several other transduction pathways appear to impinge upon DAF-16/FOXO. Whereas the IIS kinase inputs described above inhibit DAF-16/FOXO, several kinases described below stimulate it (Figure 1). JUN kinase, a mediator of the stress response, results in life span extension, increased resistance to oxidative stress, and DAF-16/FOXO nuclear localization upon overexpression [47]. Longevity is daf-16/FOXO dependent, and FOXO is a JNK substrate. Because JNK overexpression further increases daf-2/IIR mutant longevity, it may work in parallel to PI3/AKT. Importantly, this role is evolutionarily conserved. In *D. melanogaster*, JNK activation specifically within insulin-producing neurons results in nuclear localization of dFOXO, suppression of insulin production, and extension of life span [48,49]. Intriguingly, this suggests that the organismal stress response can be coordinated centrally. MST is a ste20-like kinase implicated in growth control. Like JNK-1, overexpression of the *C. elegans* homolog slows aging, extends life span in a daf-16-dependent manner, and further extends longevity of daf-2/RNAi treated animals [50]. Moreover, loss of function reduces daf-2 longevity. Although JNK and MST phosphorylate different residues, it is unclear if they identify parallel pathways or the same pathway. The mammalian MST1 also activates FOXO, but instead stimulates apoptosis in primary neurons in response to oxidative stress, i.e., decreasing cellular survival [50]. Conceivably, the mammalian response may be tissue specific. The p38 MAP kinase pathway, implicated in the *C. elegans* stress response and innate immunity as well as RAS signaling, is required in part for daf-2 longevity [51,52]. Finally, mutants of AMP-activated protein kinase (AMPK), a fuel sensor sensitive to AMP/ATP ratios, suppress daf-2 longevity, while overexpression modestly extends life [53]. How these and other kinases interact to control FOXO activity is not well understood, and it will be fascinating to deduce the kinase inputs, decipher the signaling specificity, and elucidate the covalent code modulating FOXO activity.

**Figure 1.** A Model for Insulin/IGF-1 Signal Transduction, Showing Associated Kinase Inputs and Nuclear Factors (See Text)
Aside from phosphorylation, FOXO is also covalently modified by ubiquitination and acetylation (below). Recently, an evolutionarily conserved E3 ubiquitin ligase, called RLE-1, has been shown to ubiquitinate DAF-16/FOXO, leading to its destabilization [54]. Conversely, RLE-1 loss results in increased levels of FOXO protein, sustained nuclear localization, stress resistance, and longevity. As expected, the longevity of rle-1 mutants is daf-16 dependent. RLE-1 may be one of several ubiquitin ligases that controls FOXO stability.

Transcriptional Control: The Master Regulators of Survival

As the discussion above reveals, DAF-16/FOXO is a master regulator of survival that integrates multiple inputs. Likewise, several different transcription factors/cofactors work in concert with DAF-16/FOXO to modulate its output (Figure 1). The conserved nuclear factor, SMK-1, is responsible for mediating specific aspects of FOXO biology, namely IIS and germline longevity and pathogen, UV, and oxidative-stress resistance, but not heat resistance and dauer formation [55]. Conceivably, SMK-1 works as a coregulator or cofactor for DAF-16/FOXO, since it modulates its transcriptional output. Moreover, SMK-1 harbors LXXLL motifs typical of transcriptional coactivators, but its exact molecular activity remains to be determined. In response to thermal stress, HSF-1, a heat shock transcription factor, induces various heat shock chaperones, which clear misfolded proteins and protect cells. Loss of activity accelerates proteotoxicity and tissue aging, while overexpression increases heat resistance and extends life span in a daf-16(+)-dependent manner. Moreover, hsf-1 is required for daf-2 longevity [56,57] as well as the innate immune response, broadening the spectrum of its age-related functions [58].

Best known for its developmental role in wnt signal transduction, β-catenin also guards cells against oxidative stress. Mutants in the C. elegans homolog, bar-1, are more susceptible to oxidative stress and are short lived [59]. Interestingly, BAR-1 physically associates with DAF-16/FOXO, as do the mammalian counterparts. Moreover, β-catenin enhances superoxide dismutase expression in both systems. However, whether bar-1 or other components of wnt signaling mediate daf-2 longevity has not been directly tested.

Sirtuins are NAD+-dependent protein deacetylases whose increased dose augments longevity in yeast, worms, and flies [60–62]. In mammals, sirtuins deacetylate a number of targets, including p53, PGC-1alpha, and FOXO, to name a few [63–69]. Recently, the steroid pregnenolone has been reportedly found in higher concentrations in germline-less animals compared to wild type [84]. Moreover, exogenous pregnenolone enhances longevity in a daf-12(+)-dependent fashion. However, pregnenolone is not a DAF-12/NHR ligand, suggesting an indirect effect. DAF-12 is also required for longevity in the dauer pathways. Biosynthetic mutants devoid of dafachronic acids, such as daf-9/cholesterol P450, manifest adult longevity and stress resistance that is daf-12(+)-dependent [79,85,86]. Moreover, hormone supplementation restores normal life span and stress resistance [80]. Altogether, these findings provide crucial evidence for bile acid-like steroids modulating animal life span.

MicroRNAs: Timing Long Life?

The heterochronic loci control C. elegans larval developmental timing, specifying stage specific programs and the life plan, but curiously, a handful also impact adult life span. lin-4 encodes an evolutionarily conserved microRNA, which downregulates the nuclear factor LIN-14 via translational inhibition of the 3’ UTR [87,88]. These genes work in tandem in a timing switch that advances early larval stage programs. With respect to adult longevity, lin-4 mutants are short lived, while lin-14 mutants are long lived, suggesting that lin-4(+/-) retards aging through downregulation of lin-14(+/-) [89]. Interestingly, longevity is also daf-16/FOXO dependent. Conceivably, long life arises from perturbations of a timer in
the adult. Alternatively, *lin-4/lin-14* may regulate IIS directly, since the insulin-like gene, *ins-33*, is a bona fide LIN-14 transcriptional target [90]. Intriguingly, numerous microRNAs undergo distinct changes in expression pattern during adult life, including those predicted to target known regulators of survival [91]. Perhaps they too function in adult aging.

### Checkpoint Control

A critical facet of cellular function is the response to DNA damage, genotoxic stress, and other insults. Aging in higher animals may be influenced by the balance of cell survival versus death, a decision often governed by checkpoint proteins in dividing cells. However, adult *C. elegans* animals lack dividing cells except for the germline. Surprisingly, then, deficiencies in checkpoint control—including *cd-1* poly(A⁺) polymerase, *chk-1* kinase, *cdc-25* phosphatase, as well as *clk-2*! RAD5—result in longer adult life but reduced broods [92,93]. Moreover, checkpoint-deficient animals are typically thermotolerant and upregulate *sod-3/MnSOD* (in intestine) and the ER resident *hsp-4/BiP* (in vulva), showing molecular induction of a stress response. However, *chk-1* RNAi longevity is *daf-16/Foxo* independent, and therefore likely works through another transcriptional regulator. A good candidate is *cep-1*, whose mammalian counterpart, p53, works downstream of *chk1*. In mammals, p53 loss increases tumorigenesis, while specific gain-of-function alleles reduce tumor incidence but accelerate aging, suggesting a trade-off between tumor surveillance and stem cell maintenance [94]. Intriguingly then, disabling checkpoint function in *C. elegans* mitotic germline cells or postmitotic somatic cells triggers states of enhanced organismal survival. Modulating checkpoints so that benefits are not outweighed by detriments remains a future challenge.

### Mitochondrial-Induced Longevity

Mitochondrial function is central to cellular metabolism and apoptosis, while dysfunction causes numerous age-associated diseases, including diabetes, cardiomyopathy, and neurodegeneration. Surprisingly, then, a major class of *C. elegans* longevity mutants are deficient in mitochondrial function. The first described include *clk-1*, which is defective in ubiquinone biosynthesis, and *isp-1*, which lacks a Rieske FeS protein of complex III [93,95,96]. Genome-wide RNAi screens identified many other mitochondrial components, all of which extend life independent of *daf-16* and *daf-2* [33,34]. These mutants display increased developmental times, slowed behavior, and smaller broods. As expected, most diminish respiration, although it is controversial whether *clk-1* does or not [97,98]. Evidently, levels of mitochondrial gene activity must be optimized, since severe loss of function results in lethality or shortevity [99]. How might disabling mitochondria provoke extended survival? Conceivably, it produces a lower rate of living, and consequently decreased production of reactive oxygen species (ROS) [96]. Alternately, perturbation of electron transport may actually increase ROS, provoking a hormetic adaptive response [99]. Or it may stimulate mitochondrial turnover and thrifty metabolism. In any case, longevity likely depends in part on signaling, since molecules such as *aak-2/iAMPK* are required [100]. Can this example illuminate aging in higher organisms? Perhaps yes, since heterozygous knockouts of the mouse *clk-1* are longer lived, suggesting that partial loss of function can confer benefits [101].

### Dietary Restriction–Induced Longevity

Dietary restriction (DR), the reduction of dietary intake without malnutrition, extends life span from yeast to rodents, and likely involves evolutionarily conserved mechanisms. In *C. elegans*, DR is induced by various regimens, including limiting the amount of bacterial food in liquid culture [102], liquid growth in a defined synthetic medium [103], or limiting dietary intake with feeding mutants such as *eat-2* [104]. Even shifting adults onto agar plates without bacterial food, but with residual nutrients from peptone and agar, robustly extends life [105,106]. Whether these regimens all pinpoint the same process is still unclear, and each one has its own merits and caveats.

Recently, some exciting progress has been made in identifying genes mediating DR. Namely, PHA-4/Foxa1 and SKN-1/Nrf transcription factors have been shown to be required for DR induced longevity [107,108]. When these genes are inactivated in the adult, animals are no longer long lived under reduced dietary intake. The effect is specific, since these mutants have little effect on *daf-2* longevity; moreover, *daf-16/Foxo* mutants are still susceptible to DR. SKN-1 has an early role in pharynx and gut specification, and a later role in the response to oxidative stress in the gut [109,110]. Interestingly, however, SKN-1 activity specifically in two neurosensory cells, called the ASIs, mediates DR-induced longevity [108], implying sensory inputs into DR. Nonetheless, mutants deficient in sensory transduction still respond to DR. By inference, neuronal SKN-1 must regulate the organismal response to DR through a hormonal mechanism. Accordingly, DR globally stimulates respiration, presumably to maximize organismal energy efficiency. Similarly, PHA-4/Foxa1 has an early role in endomesoderm specification in worms and mammals, but also regulates mammalian glucose homeostasis later in life [111,112]. *C. elegans* PHA-4 is expressed in the adult gut, gonad, and nervous system, yet influences life span of the entire organism, also implying endocrine control. Consistent with this, the mammalian FOXA regulates pancreatic glucagon production [112]. Dissecting their respective signaling pathways promises to further open up DR to a molecular analysis.

Sirtuins and related molecules have been implicated as potential mediators of DR in yeast and flies, although this remains controversial [62,113,114]. Conflicting reports also leave this issue unresolved in worms [115,116]. Discrepancies might arise because of unknown differences in culture conditions. Evidence also suggests that TOR (target of rapamycin) kinase mediates DR in flies and yeast [117,118]. TOR kinase promotes growth and protein synthesis, while its reduction dampens translation and increases recycling of cellular components through autophagy. In worms, a reduction of TOR, the downstream S6 kinase, ribosomal initiation factors, as well as ribosomal protein subunits themselves, reduce translation and extend adult life span [115,119–123]. Consistent with a role in DR, TOR longevity is not further extended in *eat-2* mutants. Surprisingly, however, downstream components do extend life span beyond *eat-2*, suggesting that TOR outputs independent of translation could be critical for DR. Interestingly, deletion of ribosomal
subunits in *S. cerevisiae* also extends replicative life span [118]. It will be critical to understand whether longevity arises from benefits due to globally reduced translation itself or from the regulation of specific factors.

**Aging and Age-Related Disease**

With all these longevity genes, a critical question is how do worms age, and from what do they die? With age, there is a progressive decline in body movement and pharyngeal pumping [124]. Most striking, muscle integrity deteriorates dramatically [125], with derangement of muscle fibers and overt changes in nuclear morphology—phenotypes reminiscent of sarcopenia, a major contributor of age-related decline in people. Surprisingly, the worm nervous system appears resilient, with little obvious change in structure or reporter expression, although function has not been critically tested. Among other things, aging worms show an increase in the appearance of necrotic cells, crosslinked cuticle, lipid droplets [126], endomitotic DNA synthesis in the germline [127], AMP/ATP ratios [53], oxidized protein [128], and lipofuscin [129]. Worms are described to die of enteric bacterial infection, and antibiotics extend life [126]. Conceivably, infection may be secondary to a decline in enteric muscle function, which is necessary to expel bacteria.

Although genotype determines the mean life span of a population, individual longevity has a large stochastic component, with several-fold differences observed even with an isogenic population in a uniform environment. Nonetheless, specific markers serve as good predictors of individual life expectancy. For example, stochastic induction of a hsp-16::gfp reporter predicts survival, while premature appearance of lipofuscin predicts early death [129,130]. Significantly, long-lived genotypes also delay the onset of aging and age-related disease, e.g., *daf-2* mutants curtail many of the age-dependent changes described above, and ameliorate models of polyQ repeat protein disease, Aβ-proteotoxicity, and germline tumorigenesis [56,131,132]. Conversely, *daf-16 or hsf-1* mutants often accelerate pathology. Conceivably, multiple age-related diseases could be mitigated at once by targeting IIS or other longevity pathways.

**Conclusions**

Animal life span is unexpectedly plastic, reflecting regulatory pathways responsive to environmental signals such as nutrients and stress. The future challenge will be to determine how these different pathways map onto and interact with each other, and decipher their molecular mechanisms. For some, basic questions such as where and when are they required, and whether they work cell autonomously or nonautonomously need to be addressed. Another major challenge will be to clarify how global processes (e.g., DR, mitochondrial longevity, checkpoint control, translation, and others) impact aging. What tradeoffs do they entail? Do they invoke signaling events or do they passively divert resources to somatic maintenance? Finally, what are the fundamental causes of aging and how can they be offset? Is it altered protein metabolism, organelar turnover, immune function, metabolic efficiency, ROS or xenobiotic detoxification, genome stability, or all of the above? Answers to these and other questions will be key to understanding broadly conserved aspects of life span determination.

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**References**


 gene required to specify the fate of ventral blastomeres in the early C. elegans embryo.

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A Bacterial Kind of Aging

Thomas Nyström

Bacteria are sometimes honored with a few lines in books and reviews on aging as an example of organisms that do not age. This is because binary fission of bacteria has been assumed to proceed with a nonconservative dispersion of both undamaged and damaged constituents, such that there are no adult forms of bacterial cells and the bacterial population is not age structured. However, some authors have expressed different views; for example, Partridge and Barton [1] consider asymmetry in simple unicellular systems and how this might develop into aging, and Tom Kirkwood [2] argues, on theoretical grounds, that damage segregation could be selected for in simple unicellular systems dividing by binary fission, and that sibling-specific deterioration may confer a selective advantage. Indeed, recent reports lend experimental support to this notion and point to mandatory aging also being a part of the life history of bacteria.

These reports include the demonstration that cells of an Escherichia coli population exhibit markedly different loads of damaged proteins [3]. This damage heterogeneity does not follow a simple normal distribution but rather indicates that the population consists of two discrete populations with respect to damage; a damage-enriched and a low-damage population [3]. Moreover, the low-damage cells remain reproductively competent, whereas damage-enriched cells become genetically dead (non-culturable) [3]. In addition, and most importantly, bacterial cells have been shown to exhibit signs of replicative aging, or loss of fitness, in a sibling-specific manner during exponential growth; i.e., a cumulative loss of fitness in one sibling lineage that could be argued to serve as a “mother-type” lineage, similar to that of the budding yeast Saccharomyces cerevisiae [4,5].

Evidence of Mandatory Bacterial Aging

Caulobacter crescentus, in which cytokinesis is intrinsically asymmetrical, was the first bacterium reported to exhibit replicative aging [4]. In this bacterium, a stalked cell generates a motile swarmer cell, which, after differentiation into a stalked cell, can itself give rise to progeny. However, with each division, the stalked cell requires progressively longer times to produce a swarmer cell, a manifestation of replicative aging [4]. The second bacterium reported to show signs of replicative aging was E. coli, an organism that divides by binary fission, and, as far as we know, lacks a sibling-specific differentiation. By tracking the poles of E. coli cells and measuring the cells’ increases in length during growth, it was possible to calculate the generation time of individual cells [5]. By doing so, the authors found that the growth rate decreases in cells inheriting old poles, suggesting that E. coli cells, like C. crescentus and S. cerevisiae, are subjected to lineage-specific replicative aging [5]. Prior to the study on E. coli, Barker and Walmsley [6] demonstrated that a eukaryotic organism, Schizosaccharomyces pombe, dividing by symmetrical binary fission, also shows signs of replicative aging.

Thus, the accumulated data from different unicellular models suggest that a sibling-specific reduction in fitness (growth rate) may be more common than previously anticipated and that cytokinesis during binary fission is inherently asymmetrical. But can we extrapolate the data and assume that the reduction in sibling-specific growth rate will eventually cause the cell to die? Woldridge [7] argued against such a conclusion, because taking the variabilities of E. coli cell length and age at division into account, the sibling-specific decreases in growth rate fall within the expected variation, and are sufficiently different from the catastrophe-like cell death arrived at through replicative aging. However, the growth rate of old-pole E. coli cells becomes successively slower during the divisions studied [5,8], and it would be almost impossible to carry the experiment out long enough to get statistically significant data on sibling-specific cell death in the system employed. Regardless of whether the system eventually reaches a catastrophe or a steady state, the progressive reduction in sibling-specific growth rate is highly intriguing because it raises questions regarding the ultimate and proximate causation of fitness asymmetry in a unicellular system.

Ultimate Causation for Asymmetry

Is there an advantage to producing daughter cells of unequal reproductive potential or is asymmetry caused by accidental, physical, or metabolic constraints that have no obvious bearing on fitness? In an attempt to elucidate the pros and cons of symmetrical and asymmetrical bacterial division, Wawer et al. [9] modeled growth and the propagation of growth-limiting components of a unicellular system using a modified Leslie matrix framework. As developed, the model points to asymmetrical division favoring rapid growth, whereas symmetry results in slow growth but higher efficiency; i.e., a higher growth yield [9]. Similarly, using an individual-based simulation approach, Ackermann et al. [10] found that a differentiation between an aging parental cell and a rejuvenated progeny readily evolves to cope with self-inflicted damage. Johnson and Mangel [11], using the Euler-Lotkas equation, came to a similar conclusion. In addition, asymmetrical segregation of damage that cannot be repaired may be beneficial at high cell densities and slow rates of replication [12]. Also, upon transient external stresses reaching lethal levels, an asymmetrical segregation of...
Old pole  New pole

Figure 1. Schematic Representation of Possible Aging Factors in a System Dividing by Binary Fission

During cytokinesis, one daughter cell will inherit a pole that is older than the pole inherited by its sibling. Intriguingly, the “old pole” cells of E. coli display a progressive increase in their generation time [5]. There are several potential reasons for this decline in physiological fitness: (1) Inheritance of older cell-surface material may reduce the ability of the cell to insulate itself against the environment. (2) Segregation of differently damaged, and potentially cytotoxic, DNA strands [15] could provide one daughter with a noncorrupt message akin to the “immortal DNA strand” cosegregation mechanism originally proposed by Cairns for preserving the integrity of stem cell genomes [27]. (3) Segregation of cytotoxic molecules, such as extragenomic episomes or oxidatively damaged and aggregated proteins, may result in sibling-specific deterioration. (4) Segregation of damage could cause a reduction in fitness even in the absence of cytotoxicity, since the sibling inheriting damage more so than the other may, as a consequence, upregulate maintenance (M) (damage defense) systems. In view of the fact that the transcriptional power of cells like E. coli is limiting, such an elevation in maintenance activities could be traded for a reduction of growth-related activities (G) [28,29].

irreparable damage may permit survival of the clone at the expense of the “mother-type” cells, in which the damage is retained [13].

Thus, very different types of models and simulations suggest that sibling-specific asymmetry may provide the system with a fitness advantage and that replicative aging evolved early in the history of life [10]. However, at present, the models and simulations are hampered by the fact that we know very little about the nature of the critical components (aging factors) reducing cellular fitness and the mechanisms establishing their asymmetrical distribution. Elucidating these features will be critical in estimating the energetic costs for damage segregation versus damage removal (assuming that damage is, at least partly, responsible for bacterial aging) and why segregation might, in some cases, be selected over damage repair/removal.

**Proximate Causation for Asymmetry**

One common assumption in the reports modeling potential benefits of asymmetry is that the establishment of age asymmetry is linked to damage segregation [9–13]. The question that arises is, what kind of damaged, or toxic, molecules are critical in affecting sibling-specific fitness? In E. coli, is it the old pole itself, the parental DNA strand segregating to the old pole [14], damaged and cytotoxic DNA molecules predominantly inherited by the old pole cell [15], or some deteriorated and potentially cytotoxic molecules, such as protein aggregates, in the cytoplasm (Figure 1)?

In budding yeast, cytotoxic extrachromosomal rDNA circles and oxidatively damaged proteins are segregated such that the mother cell retains most of these molecules during cytokinesis [16,17]. The yeast anti-aging protein Sir2p governs the management of both extrachromosomal rDNA circles and oxidatively damaged proteins [16,17], and a model for the Sir2p-dependent retention of oxidatively damaged proteins was recently presented, involving the aggregation-remodeling factor Hsp104p in concert with the actin cytoskeleton [18]. Interestingly, damage segregation in budding yeast becomes more pronounced following increased oxidative stress [17], suggesting that the efficiency of damage segregation is not fixed in this species but can be adjusted with changing environmental demands. This raises the question of whether replicative aging in the bacterial systems studied becomes more or less pronounced depending on growth conditions; for example, during growth at different oxygen tensions or on plates containing antioxidants.

Stationary-phase die-off of S. cerevisiae cells (sometimes referred to as chronological aging) has been firmly linked to oxidative damage and genetic alterations affecting reactive oxygen species production and scavenging are effective in retarding stationary phase death in this model system [19–22]. Likewise, self-inflicted oxidative damage has been implicated in cellular degeneration of stationary-phase bacteria [23–25], and a recent report showed that three different classes of bactericidal antibiotics, regardless of their drug–target interactions, cause bacterial cell death by stimulating the production of highly deleterious reactive oxygen species [26]. Thus, it would be of great interest to learn whether oxidatively damaged (aggregated) molecules are segregated during bacterial cytokinesis, and if they, indeed, act as bona fide aging factors.

However, one should not put all of one’s eggs in the same basket; indeed, one of the most exciting features of the discovery of a mandatory aging phenomenon in bacteria and eukaryotes dividing by binary fission is that, by virtue of being exquisitely tractable systems for genetic and biochemical analysis, there is a good chance of identifying the true aging agents in these systems. Such knowledge may have an enormous impact on the aging field as a whole.

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**References**