Advances in Experimental Medicine and Biology 847

Gil Atzmon, PhD *Editor*

Longevity Genes

A Blueprint for Aging

Springer

Advances in Experimental Medicine and Biology

Volume 847

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Editor
Gil Atzmon, PhD
Albert Einstein College of Medicine
Bronx
New York
USA

University of Haifa
Mount Carmel, Haifa
Israel

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-1-4939-2403-5 ISBN 978-1-4939-2404-2 (eBook)
DOI 10.1007/978-1-4939-2404-2

Library of Congress Control Number: 2015933511

Springer New York Heidelberg Dordrecht London

Printed on acid-free paper

Springer New York is part of Springer Science+Business Media (www.springer.com)

Preface

Aging, involving a complex array of systemic changes over time, has entered the spotlight of biological research, mainly due to the dramatic increase in life expectancy worldwide. The trends of population aging, accompanied by the rapid improvements in research tools and capabilities, have promoted the research of aging to the prominent position it occupies today. Nonetheless, until recently the major efforts in research of aging have been dedicated to sociological, behavioral, clinical and nursing aspects of aging, as those aspects were perceived as having immediate significance for healthcare and its costs. Less effort and resources were allocated to studies of biological aging processes, in particular the genetics of aging, despite their tremendous importance for the development of age-related disabilities and diseases and thus causing the healthcare burden in the first place.

Despite the diminished focus compared to other fields of aging research, the genetic research of aging is a rapidly growing field with a tremendous potential for developing future treatments. There are strong grounds to expect that highlighting aging-related gene function may suggest interventions to delay or even prevent the development of aging-associated diseases, disability and mortality. Thus, this research will not only further our understanding of the complexity of aging processes, but will have far-reaching, long-term practical significance for global healthcare and economy. In this book, we have reviewed some of the major and most current efforts in the research of genetics of aging.

Gil Atzmon, PhD

Contents

Contributors

Yair Argon Division of Cell Pathology, Department of Pathology and Lab Medicine, The Children's Hospital of Philadelphia and the University of Pennsylvania, Philadelphia, PA, USA

Diana Athonvarangkul Department of Medicine and Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, USA

Marian Beekman Department of Medical Statistics and Bioinformatics, Section of Molecular Epidemiology, Leiden University Medical Center, Leiden, Netherlands

IDEAL-consortium European Union FP7 framework, Leiden, Netherlands

Dan Ben-Avraham Departments of Genetics and Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

Aviv Bergman Albert Einstein College of Medicine, Bronx, NY, USA

Kendra Bolt Albert Einstein College of Medicine, Bronx, NY, USA

Linda Broer Department of Epidemiology, Erasmus University Medical Center, Rotterdam, Netherlands

Joris Deelen Department of Medical Statistics and Bioinformatics, Section of Molecular Epidemiology, Leiden University Medical Center, Leiden, Netherlands

IDEAL-consortium European Union FP7 framework, Leiden, Netherlands

Tali Gidalevitz Department of Biology, Drexel University, Philadelphia, PA, USA

Diddahally R. Govindaraju Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

David Karasik HSL Institute for Aging Research, Boston, MA, USA

Liron Levin Department of Life Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel

Nuria Martinez-Lopez Department of Medicine and Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, USA

Dan Mishmar Department of Life Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel

Anne Newman Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA, USA

Rajat Singh Department of Medicine (Endocrinology), and Molecular Pharmacology, Diabetes Research Center, Albert Einstein College of Medicine, Bronx, NY, USA

P. Eline Slagboom Department of Medical Statistics and Bioinformatics, Section of Molecular Epidemiology, Leiden University Medical Center, Leiden, Netherlands

IDEAL-consortium European Union FP7 framework, Leiden, Netherlands

Erik B. van den Akker Department of Medical Statistics and Bioinformatics, Section of Molecular Epidemiology, Leiden University Medical Center, Leiden, Netherlands

Department of Intelligent Systems, The Delft Bioinformatics Lab, Delft University of Technology, CD, Delft, Netherlands

IDEAL-consortium European Union FP7 framework, Leiden, Netherlands

Cornelia M. van Duijn Department of Epidemiology, Erasmus University Medical Center, Rotterdam, Netherlands

Acknowledgements

I would like to thanks Dr. Lital Sharvit and Mrs. Danielle Gutman for critical and insightful comments

Chapter 1
Evolutionary Genetic Bases of Longevity and Senescence

Diddahally R. Govindaraju

"Some few grow old, most suffer and fall sick,
But all must die."
- Edwin Arnold (1906)

"So Nature deals with us and takes away
Our playthings one by one…"
- H. W. Longfellow (2004)

"Deaths from old age are due to the breakdown of one organ or another…"
- J. B. S. Haldane (1949)

Introduction

The question of why organisms live as long as they do; closely tracks the other audacious question in biology—why is there diversity of life? Darwin wrote, "From so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved" [42]. Needless to say, each one of those forms also has its own characteristic life span, molded by both proximate (immediate) and ultimate (evolutionary) causes [160]. It is widely held that recent discoveries on the biological basis of life span among organisms with diverse life-histories would open up new avenues to improve healthy life span in humans [14, 35, 38, 217]. A vast body of theoretical [29, 30] and empirical studies [10, 26, 65, 66, 209] have already addressed many aspects of proximate and ultimate biological mechanisms underlying the diversity of life span among organisms, including humans. Therefore, one would wonder if there is a need for another review on the topic. However, with the recent availability of rich and diverse data on genomic and phenomic (extragenomic;

D. R. Govindaraju (✉)
Beth Israel Deaconess Medical Center, Harvard Medical School,
330 Brookline Ave., Boston, MA 02215, USA
e-mail: drgrajugis@gmail.com

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2_1

sensu, [44]) factors that affect the aging process in model organisms and in humans, as well as our ability to integrate these data using systems approaches [191], are pointing to the contrary. These new developments offer exciting opportunities to understand the antecedents and attendant consequences of genomic variation in relation to developmental and physiological (epigenetic) processes that influence life span and senescence, which could be extended to improve the healthy life span in humans [14, 153, 191].

Life Span as An Aspect of the Darwinian Process: Life span defined as the average length of life from birth to death, is an evolved species specific life-history property of organisms [26]. It encompasses the two universally invariant properties of life—birth and death. In a prosaic sense, birth and death represent demographic (Malthusian) processes only; life history, on the other hand, is a product of all aspects of evolutionary processes. In this chapter, we will take an integrated "Darwinian population" approach [82], to describe the evolutionary genetic underpinnings of longevity and senescence. This approach is useful because it spans over Malthusian (demographic— enumeration of age and stage specific mortalities), Mendelian (inheritance and variation) and Darwinian fitness (viability and reproductive) aspects underlying the diversity of life span. Further, because life span is an integral part of life history [26, 138], Darwinian approach allows us to examine the demographic genetic base of longevity in relation to the other covariates of fitness: size at birth, growth rate, age of maturation, size at maturity age-specific reproductive investment, size and number of offspring, age specific survival, and the associated trade-off mechanisms [69, 206, 227]. These traits show genetic and developmental relationships and covary with life span. Although the aging process or senescence is commonly analyzed from the perspective of an individual's overall health and life span [14], it operates at all levels of biological hierarchy: gametes, zygotes, cells, somatic and reproductive (oogonia and spermatogonia) tissues, organs, individuals and populations, as well as provides an opportunity for selection at those levels - or simply, multilevel selection [39, 86, 145]. Hence, the concept of multilevel selection could be extended to analyze longevity and senescence as a part of the Darwinian process.

Integration of Genotype and Phenotype through Life History: Demographic processes are commonly studied using the familiar Gompertz –Makeham growth model [65]. From a developmental and evolutionary perspective, however, it is useful to analyze longevity and senescence from the time of zygote formation (fertilization), through intermediate stages till the time-of-death as sequential events, because strength of selection varies across life history (see below). Selection is influenced by mutually dependent genetic, developmental, demographic and ecological factors operating throughout the life-history of individuals, in relation to populations. The dynamic aging profiles of specific individuals, could be studied within families/populations in terms of norms of reaction [9, 149], which reflects the proximate aspects of evolutionary process. Norms of reaction includes both phenotypic and developmental plasticity as well as genetic and developmental mechanisms associated with the survival and reproduction in an individual's life time

[108, 171]. Population perspectives, on the other hand, provide an opportunity to estimate genetic variation for environmental sensitivity among individuals within and among specific populations [179]; hence useful to gain insights on the broader evolutionary mechanisms. Following Lewontin [147], Flatt and Hayland [69] have argued that "our understanding of evolutionary processes and dynamics will remain incomplete until we integrate information on phenotypes with information on genotypes and the intermediate mechanisms that connect them." By extension, both individual and population centered approaches that incorporate genetic-physiological/developmental-phenotypic approaches are necessary to elucidate the mechanisms that influence the maintenance and evolution of life span in humans. Therefore, we will use the genotype– phenotype (G-P) map approach as a framework [111, 147] to examine the evolutionary genetic basis of life span diversity and aging process in humans. The need for a broader application of the G-P map for defining new paradigms in biology, medicine and production sciences has been recently emphasized [36, 111, 184].

For clarity, we will define six commonly used terms in aging research: life span, longevity, aging, aging process, senescence and health span. Life span refers to the average life *expectancy* at birth for individuals between birth and death [26]; thus the term carries a predictive outlook. Longevity, on the other hand, is a more elusive concept, and may be defined as an individual's ability to reach longer life span under ideal and proximal conditions. It is influenced by a combination of genetic, epigenetic and environmental factors during development, growth, maturity and older stages. Thus, to a limited extent, longevity is a modifiable feature of specific individuals; hence plastic. Aging is the process of growing old, regardless of chronological age [26]. Health span is defined as the period of life during which an individual is free of chronic illness and substantial functional decrements [158]. Senescence or the process of aging (also termed biological aging), on the other hand, is considered as an assemblage of deteriorative physiological and developmental processes, that are cumulative with the passage of time [26, 242]. Hence, senescence is broadly defined as a time dependent developmental process accompanied by declining vitality, fecundity and increased vulnerability to diseases [66, 185]. From this view point, numerous Mendelian and complex genetic disorders, including cancers that are known to adversely affect both health span and life span from zygote to older ages [80, 120, 164, 215] could be potentially included while discussing the aging process. *It is important to note, however, that although the incidence of certain diseases increases with age (due to genetic or other reasons), aging is not a disease; but some or all diseases may affect the aging process. Instead, senescence is the individual or population specific phenotypic manifestation of evolutionary and biochemical genetic changes with the passage of time.* In accordance with the Darwinian (multilevel) and developmental model, the aging process is initiated, in principle, shortly after the formation of zygote ((i.e., cellular senescence followed by death of individual cells or apoptosis within the embryo, or the death of the entire embryo (selection)) and progresses into older ages, culminating in death. From a gerontological perspective, however, the aging phenotype is initiated at 30 years [181]. For convenience, we ignore the subtle differences between the

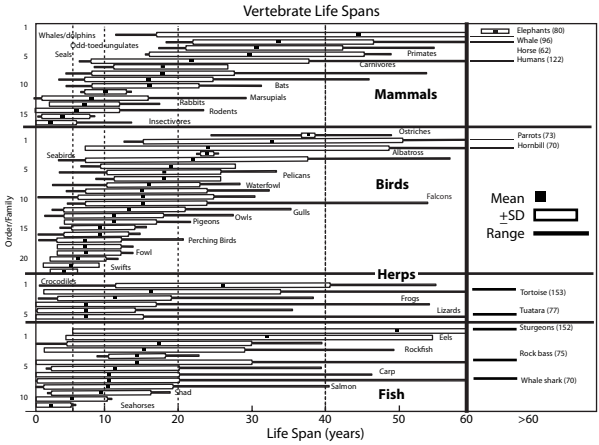

Fig. 1.1 A comparative view of life span across selected taxa [26]

terms life span and longevity, and use them interchangeably. We also assume that: (a) the life-course concept [137] used in public health is analogous to, or a subset of, life-history theory [206, 227], and (b) the term genome is a subset of genotype, and phenotype is the ultimate product of the interactions between all extra-genomic components or phenome and the environment.

We have four objectives: (a) to present a broad comparative perspective of life span among various taxa, including humans; (b) to analyze the evolution of life span and senescence from the genotype-phenotype (G-P) map and life history (Darwinian population) perspective; (c) to examine some evolutionary bases for exceptional longevity in humans; (d) to speculate on the possibilities of extending healthy life span by using evolutionary approaches for developing predictive and interventional approaches in order to manage age-related disorders.

Life Span: Its Descent and Diversity among Taxonomic Units

Organisms display a tremendous diversity for life span, varying from only a few minutes to thousands of years, and the patterns (i.e., the shape of trajectories) through which maximum life span is achieved also differ across "the tree of life" [119]. A useful comparison of Life span diversity among various animal taxa is provided in Fig. 1.1 [26]. Note the large standard errors associated with each of these bars; they correspond to difficulties encountered with accurately measuring the exact terminal point of life, due to diverse causes and artifacts associated with determining death, and small samples sizes [26]. Thus, life span in most species is considered "indeterminate" (*sensu,* hard to determine) but not limitless [26]. While primates are long-lived species among mammals, humans enjoy the longest life

span among primates. Interestingly, in many organisms, including humans, mortality tends to flatten at later ages—a feature often referred to as late-life plateau [167, 244]. This observation, however, remains controversial [79].

In accordance with numerous studies that show a Darwinian basis for the evolution of life span [178], it is reasonable to assume that genomes and genomic regions that influence life span are also subjected to birth and death (demographic) processes [183]. Correspondingly, genomic regions associated with life span may also have evolved from less complexity to greater complexity in lower and higher organisms, respectively over millions of years. In other words, the entire system of G-P space of life span itself is subjected to Darwinian processes. In principle, genetic processes associated with the observed life span diversity could be traced back by "looking backward in time' or applying the coalescent, or "gene-genealogy" models [59, 127]. Indeed, phylogenetic analyses of life span among diverse taxa serve as a general representation of this process [68, 204].

Variation of Life Span among Contemporary Human Populations: Opportunity for Selection

Life span varies greatly among contemporary human societies. For instance, life expectancy ranges from 47.5, 79.8 and 87.2 years, in Sierra Leone, United States and Monaco, respectively, with a median of 71.3 for global populations [3]. However, the average age of living super-centenarians is 112.5 (WWW.grg.org). Even if we assume that lower life expectancy among the developing countries is largely influenced by environmental factors and diseases, genetic variation for the trait among specific individuals within populations [146] and some degree of adaptation of these populations to local environments is expected [113]. Individuals who live beyond 100 years, commonly referred to as centenarians have been called the "privileged group" [43]. Such individuals occur at a very low frequency, but their numbers are increasing globally [169]. By convention, centenarians live for 100–110 years and super-centenarians survive beyond 110. The highest recorded life-span in humans ranges from 115—to 122.3 with an average of 118 [26]. Therefore the level of longevity reached by Lady Calment (122.3 years) may be considered as the uppermost bound for phenotypic diversity of human life span in the recent human history. Upon plotting, centenarians and super-centenarians occupy the farthest right-hand tail of the normal distribution, relative to the life expectancy patterns of the entire global populations (Fig. 1.2).

Longevity as a Composite Trait: A wide range of phenotypic variation for life span among populations suggests that it has all the features of a classic quantitative trait, and exceptional longevity may be considered as a threshold trait [63]; because these are expressed among a limited number of individuals after a certain cut-off point in the distribution range. These traits, like any other quantitative traits, have an evolutionary genetic basis [206]. Furthermore, individuals within populations express considerable genetic variation for their environmental sensitivity [102], as well as

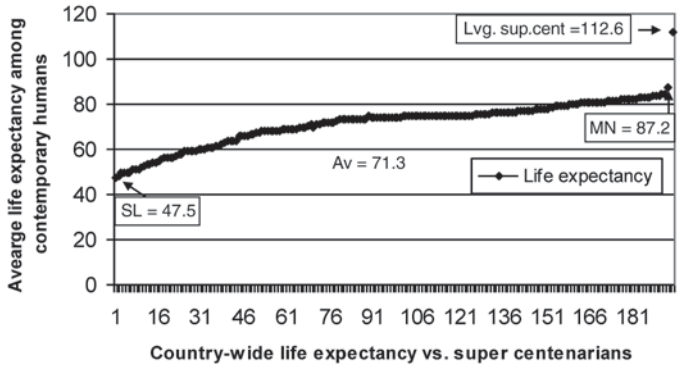

Fig. 1.2 Distribution of life expectancy expectancy among contemporary human populations representing 193 countries. These vary from 47.5 to 87.2 yrs in Sierra Leone (*SL*) and in Monaco (*MN*), respectively, with a global average (*AV*) of 71.3 yrs. Mean age of living super centenarians is 112.5 years [3, 26]

experience "common environmental effects" [63]. All life history traits, including longevity, by definition, vary individually and exert direct, indirect, compensatory and opposite influence, as well as covary with others in different directions (also called trade-off; [206, 227]), over all the phases of growth, and ultimately influence longevity [198]. In the sense of Riska [205], life span may be treated as an ensemble of life-history and other complex traits or simply a composite trait. A wide range of variation for life span across global populations is indicative of the underlying genetic variation for longevity, because often tracks genotypic variation [32]. Heritability of life span, an index of genetic variation, described broadly as a ratio between genetic to phenotypic variance is about 0.30 [67], which also implies that around 70 % of the variation in longevity may be attributable to environmental causes, which is approximately similar to other life-history traits [94]. Because longevity co-varies with other life history traits, its co-heritability [63] with them is also expected. Clearly, both phenotypic variability, and moderate levels of heritability for life span offer opportunity for natural selection to act on longevity and its correlated traits [39, 134, 228]. Hence, the importance of genetic variation for life span among individuals within as well as among global populations and their potential for evolutionary responses may not be overlooked.

Age and Stage Structure in Human Populations: Demographic Selection

From a Darwinian perspective, it helps to understand the life span and the aging process of individuals in relation to populations, because evolution operates at the level of populations. Similar to most long-lived organisms, in any given human population, individuals that belong to different age groups live together, in which

new ones are recruited, reproductive adults mate with individuals of the same or different age groups, reproduce at different times and older individuals die [28, 29]. Diverse mating systems in human societies [25, 143], further increase variation among individuals within families and populations, which in turn could affect fitness and its components, at those levels. In other words, information on both age and stage could also provide insights on variation in the developmental timing of individuals carrying germ line and somatic mutations, epimutations and the lag-time required for the expression of these mutations during major transitions in individual's life history. In a strict sense, because age and stage dependent development of individuals is accompanied by genomic plasticity or instability [219, 243], genotypes of any given parents at a specific age/stage would be different at another age/stage in their life history. These individuals would carry specific genetic changes both in their reproductive (germline) and in somatic tissues, as well as mosaics specific to those demographic and developmental stages. Hence, age/stage specific genetic contribution of parents would also influence offspring fitness resulting from the union of gametes produced at that age/stage of parents. Despite their central importance, these demographic genetic features are almost always ignored in human genetics.

Darwinian processes by definition, include bio-demographic processes such as age-specific survival, fertility and fecundity which mould the distribution of phenotypes in (age structured) populations [93, 139, 244]. In an evolutionary context, the individual phenotype (in any age- and stage-structured population) is exposed to natural selection as an integrated unit—simply, individual is the target of selection [161, 266]. Natural selection on individuals not only purges deleterious mutations and alters the age distributions of individuals in populations, but also affects the associated gene frequencies in subsequent generations [74]. This general process of age and stage specific selection is also called demographic selection [26, 196].

Inheritance and Diversity of Longevity and Senescence

A Genotype– Phenotype (G-P or Genome - Phenome) Map Perspective: All inherited genetic variation (inherited epigenetic variation is viewed as a special case of inheritance) is generated by mutations both in the nuclear and in the mitochondrial genomes (simply genome). Novel mutations along with the inherited variation upon interaction with the environment influence longevity and its components, through developmental processes. The selection process among individuals in any age-structured populations, from the time of fertilization to death and across generations, may be represented using the G-P map [147]. Hence, it serves as a useful metaphor to describe the Darwinian processes [111] (Fig. 1.3).

The G-P map consists of three spaces or planes: genotypic, epigenetic and phenotypic. Briefly, the genotype space encompasses genes and genome specific processes, such as mutation, recombination, deletions, duplications, translocations, inversions, etc. The epigenetic space, on the other hand, is an embedded system of

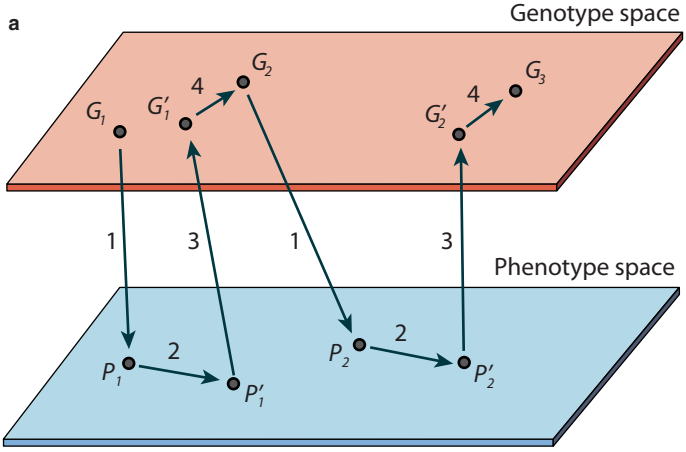

Fig. 1.3 A map of the genotype-epigenetic-phenotype (G-E-P)spaces across generations [111, 147]. Phenotypic variation (P_1) results from the transformation of genotypic variation (G_1), across the Wright -Waddington developmental (epigenetic) space (1, 3) in relation to environment. Note that genotypes and phenotypes also change within their own spaces (2, 4)

genes, gene products, biochemical networks and physiological processes that are associated with development, differentiation and growth [246, 266]. Phenotype is the ultimate product of the interaction between genes and developmental processes in relation to environments. From a systems perspective, however, genotype and epigenetic spaces may be viewed as embedded systems within the phenotype space which interfaces environments. The latter includes, among others, age, stage, gender, nutrition, medicine, microbial, and physical environments.

Selection and Life-stages- a Demographic Perspective: According to the G-P map, the Darwinian selection process is initiated at least from the time of fertilization (upon ignoring gametic selection) and the formation of zygotes. In a broader sense, longevity involves a succession of developmental transformations from the time of fertilization (zygote formation) to death. Hence the influence of development is pervasive in the maintenance and evolution of life history traits [226], of these life span is one. This scenario is also compatible with the biogerontology view, in the sense that the aging process includes "all possible changes in an organism between conception to death" [242]. Yet, as a general rule, in any human population, mortality attributable to genomic or other factors is greater in pre-natal [259] and pre-reproductive stages than it is among adults. Briefly, beginning roughly at late adolescence and continuing until late-life, age-specific mortality increases linearly on a natural log scale in accordance with the Gompertz-Makeham model of mortality. In the very old, however, the increase in mortality with age seems to plateau in the mid-90's, at annual death rates of approximately 50%, suggesting physiological deceleration process [239].

Levitis [144] reviewed several evolutionary hypotheses for greater mortality during juvenile stages, including genetic heterogeneity, growth-related phenotypic trade-offs, reductions in external hazards with age and kin selection. He noted

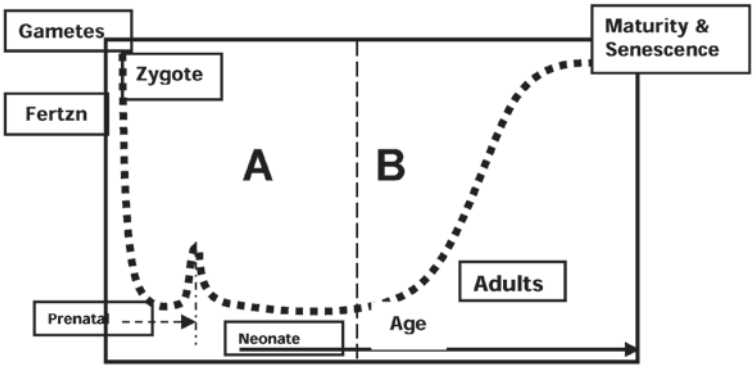

Fig. 1.4 A generalized scheme of mortality patterns in relation to life history. Death of pre-reproductive and reproductive individuals (including embryonic stages) in zone A, and past reproductive stages in zone B represent hard and soft selection, respectively. Note that hard selection is commonly associated with Mendelian and chromosomal disorders, and soft selection with genes with small effects (complex disorders) that escape selection (modified from [33, 144])

that pre-natal mortality changes with age in a manner similar to post-natal period; zygotic death rates are highest a week after fertilization (accurate estimation of embryonic death is difficult to estimate) and then monotonically decreases until just before birth, at which time mortality increases. Ontogenetic, demographic and cultural factors such as consanguinity and inbreeding among parents could affect genetic variation, quality of gametes, zygotes and fitness of individuals [99]. Genetic variation among individuals (zygotes/embryos) almost certainly contributes to the early decline in mortality rates as this is the age at which most chromosomally aberrant individuals are spontaneously aborted. Numerous examples of prenatal and neonatal mortality due to genes and structural variation are found in medical genetics texts [120]. The genetic causes for the declining leg of the prenatal survivorship "U" are very similar to the declining leg of the postnatal "U", at the age when postnatal mortality eliminates frail infants. Joining the pre- and post-natal mortality curves yields an approximately "flat-bottomed W"- shaped mortality trajectory for the entirety of the human life span from conception to death (Fig. 1.4). This model is consistent with the emerging views in demography [113], clinical aspects of human ontology [33, 174], and life-history evolution in the classical evolutionary schema [69].

To generalize, intense selection occurs in the pre-natal, neo-natal and pre-reproductive stages (Fig. 1.4A) mainly due to genetic reasons; hence individual and familial risks for some genetic disorders could be predicted [270]. In contrast, selection gets weaker in post reproductive stages (Fig. 1.4B); consequently, predictions made on the occurrence of genetic disorders associated with this stage will be clouded with uncertainties. In other words, there is a general conformity between evolutionary and demographic predictions on the one hand, and the origins of hereditary diseases that affect the life span of individuals, on the other. Such predic-

tions would help devise preventative and management measures to deal with diseases that affect healthy life span in relation to developmental stages. These patterns may also be interpreted in terms of hard and soft selection concepts, respectively, that are commonly employed in population biology [142]. Hard selection and soft selection are features typical of juvenile and adult stages, respectively [107].

Some Evolutionary Theories of Aging

Why do humans (and all other organisms) full of vitality in their youth become frail in their old age, succumb to diseases and eventually die? Numerous explanations have been offered to address this enduring question - even six decades after Medawar's conjecture [166]. Over 300 theories have been advanced to identify causal factors underlying the phenomenon of aging [168]. Recently, Lopez-Otin et al. [153] have identified nine major causes of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteolysis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication. In accordance with our G-E-P map, all of the nine causes may be collapsed into four categories: genotypic (genomic), physiological (epigenetic), phenotypic and environmental [111]. Below we present three widely discussed theories.

Generation cycles: Evolutionary theories of aging or senescence may be traced back to Weismann [252] and Haldane [90], who respectively proposed opposite approaches to explain the aging phenomenon - organismic (top-down) and gene centric (bottom –up). Weismann extended Malthus' theory on population growth to explain the diversity of life span, and suggested that organisms with early reproduction and maturity will have shorter life span and vice versa. This idea has been further elaborated in terms of *r* and *K* strategies in the broader context of evolution of life span [155]. Later, Kirkwood [131] reasoned that because reproduction is essential for the continuity of any species, (hence, in theory germ line is immortal), greatest fidelity and precision is required till the completion of reproductive period, but dwindle slowly past reproduction. Consequently, organisms channel substantial amounts of energy toward preserving the germline rather than somatic tissues. They exhaust a great deal of energy by the time they complete their reproductive period, followed by disposing off assemblages of somatic tissue through species specific evolved mechanisms; also termed, "disposable soma," theory. To a large extent, Kirkwood's view resonates with that of Weismanns's germ theory. The more recent "extended phenotype" concept [45], shares many similarities with Kirkwood's theory. Genes influence both internal and external aspects of phenotypes. Unless useful to progeny, individuals would die soon after their last reproduction, as seen in organisms including humans that are generally large and invest their resources for protecting and raising their progeny. This post-reproductive period varies among species, however. For instance, gorilla lives only for 2 years after giving birth to its

last baby; but humans live for several decades after completing their reproductive period [66]. In humans, however, with the exception of individuals with Mendelian and infectious diseases, most maintain good health up to 45–50; thus post-reproductive period constitutes a significant stretch of life span. Centenarians have the most protracted post-reproductive health span relative to individuals with normal life span.

Mutation accumulation: Both beneficial and harmful mutations arise spontaneously and sequentially among genomes and serve as substrates for evolutionary change. Most lethal mutations are purged during pre-natal and neo-natal stages (Fig. 1.4a) due to infantile mortality. A few, however, escape and accumulate past reproduction. Haldane [90] suggested that some lethal diseases such as Huntington's disease are expressed past reproductive age, after their carriers have already contributed to reproduction. A decade later, Medawar [166] extended Haldane's idea further to include the origin and accumulation of numerous deleterious mutations across the genome and their cumulative effects on aging; which has come to be known as "mutation accumulation" theory. Although not common in the literature on aging, Muller [180] also presented a more general and comprehensive account of the deleterious effects of accumulated mutations on human health termed, "our load of mutations." Williams [256] on the other hand, interpreted the process of aging, using one of the universal properties of genes - pleiotropy and their contextual effects [60, 266]. He reasoned that because most genes exert pleiotropic effects, these effects may be relatively more beneficial prior to and during the reproductive phase; but the same genes could exert opposite (antagonistic) effects past reproductive stages, also termed, "antagonistic pleiotropy." Parsons [190], as opposed to Williams, has argued that individuals in age and stage structured populations, pass through many stressful periods during their long life. Under these conditions, "positive pleiotropy" may be expected for genes that influence fitness traits to maximize or maintain metabolic efficiency across varying ages and stages. Hence, antagonistic pleiotropy concept may not be applicable under all conditions; but it might operate only under benign stressful conditions [190]. Recently Kimber and Chippendale [125], tested the idea of positive pleiotropy on Drosophila by allowing mutations to accumulate for 35 generations. They indeed found a positive relationship between early-life fitness and post reproductive longevity and interpreted that "extended post-reproductive longevity is actively maintained by selection for early-life fitness via positive pleiotropy…" This work suggests that at least certain components of the genome (e.g., APOE; FOXO1; IGF signaling) in specific pathways could maintain positive pleiotropy (possibly) from embryonic stages through old age. Perhaps such genes, in the sense of Waddington [246] are "canalized," and maintain orthologous relationship and consistently influence overall fitness across diverse taxa [238]. Mutations in genes that exert positive pleiotropy could have a relatively greater adverse effects on longevity.

Crow [40] has reviewed the role of mutation accumulation in both somatic and reproductive tissues in relation to human health, and concluded that novel mutations may contribute not only to age related diseases, due to an increased frequency of

somatic mutation but also increases the incidence of various inherited diseases. In general, Haldane - Medawar-Muller-Crow's view suggests that mutations occur at low frequencies, and accumulate in reproductive and somatic tissues of individuals at different rates over time (both within and across generations), and contribute directly or indirectly to senescence. A wide array of mutations among individuals that contribute to genetic heterogeneity, may be found both within and among genes distributed across biochemical networks [140]. Recent molecular studies have shed light on mutation accumulation. For instance, Akey's group [71] sequenced 15,336 genes in 6515 individuals of European American and African American ancestry and found that approximately 86% of all deleterious single nucleotide variants (SNVs) in these genes arose in the last 5000–10,000 years. They emphasized the role of these rare variants in inducing heritable phenotypic variation and disease susceptibility, especially the burden of Mendelian disorders in contemporary populations. Similarly, Ezawa and Innan [62] modeled the role of somatic mutations in human health and suggested that they have substantially large effect on fitness, particularly when rare. Obviously, such a "deluge" of rare variants of functional importance both within and across generations could have important consequences on health and life span [71, 244].

Physiological: Among others, the physiological theory of aging has been widely recognized. This theory posits that aging cells progressively accumulate highly reactive species of free radicals which in turn could damage cells also called oxidative damage [95, 269], and initiate cellular senescence. This idea is complimentary to accumulation of biochemical waste products, due to mutations among genes located in many biochemical pathways, leading to metabolic diseases, commonly encountered in medical genetics [76, 77, 215]. Nonetheless, the relative importance of cellular damage caused by natural mutations in the genome and that of free radicals is largely unknown and the role of the latter in aging process has been recently questioned [269].

Genotype—phenotype Architecture of Life Span and Senescence

Recall that interaction among genetic, development and phenotype phases (in the G-P map) and the influence of environment on the latter pervades the entire life span of individuals and across generations. Below are some particulars of the three phases in relation to the aging process.

Genotype space: The Haldane-Medawar-Williams-Muller-Crow (H-M-W-M-C) model also indicate that causal connections among genotypic/genomic variation, the aging process and health. From the G-P map perspective, we assume that genome includes both mitochondrial and nuclear genomes and the interaction between them, also termed cyto-nuclear disequilibria [37] influence human health and longevity [258]. Although genomic space represents all genes in a single zygote or in

a population of genotypes (zygotes), it is assumed that the health of these zygotes (and their developmental trajectories) is also influenced by both genetic (allelic) constitution of the gametes and the demographic history of their donors. Variation in the genomic material of zygotes could be attributed to genomic aberrations such as substitutions, insertions, deletions, duplications, recombination, translocations and inversions of individual nucleotide sites (also called single nucleotide polymorphism—SNPs) or segments of the DNA molecule (structural variation), as well as variation caused by transposable elements. These genomic "units" influence phenomic "targets" of selection through individuals [161, 232, 266] and are not mutually exclusive. Structural variation by definition includes all the DNA variation above the SNP level measuring 2bp to large segments of chromosomes to whole chromosomes [214]. Of these, copy number variants (CNVs) whose size range vary from 5 Kb–5 MB are the most abundant ones. All genomic variations can be traced ultimately to mutations caused by biological and physical factors, respectively called DNA damage and lesion. While DNA damage could be repaired, lesions cannot be repaired; but both could influence the development of age related functional decline and disease [243]. As far back as 1925, Haldane and Crew [92] reported a decrease in the strength of linkage with age, and postulated that the observed variation in linkage relationships during aging "might be due to diminished rigidity of the chromosomes, increase of the forces tending to break them ….pointing to presenile changes in the behaviour of the dividing nucleus, and as being …a change with age of the 'germ-plasm' of an individual."

How might DNA damage occur? One major explanation is that every single cell division is accompanied by transcriptional errors (mutations) in the DNA replication process. Since the number of cell divisions in humans from zygote to full maturity has an upper limit of about 60 bounds from 0 to ~ 60 [97, 216], there is abundant scope for age and stage dependent accumulation of transcriptional errors among cells, tissues and individual genomes [62]. Mutation accumulation through this well-known recurrent "Hayflick process" among nucleotide and structural variants in the genomes of somatic and reproductive tissues might be augmented by mutations from other sources, including the environment [70, 213]. For instance, the rate of accumulation of recurrent mutations could be influenced by demographic factors such as parental age, inbreeding in the population and range expansions, etc., [40, 195]. These *de novo* recurrent mutations coupled with the inherited mutations will have manifold effects on various components of the G-P landscape, including disease phenotypes [124]. More specifically, chromosomal mutations could potentially break up the *cis* and *trans* configuration of individual haplotypes within and among spatially organized networks of genes and their aggregate co-evolved gene complexes (e.g., MHC). Breaks in these complex "haplotype networks" could progressively retard or even shut down repair mechanisms [57] leading to diverse patho-physiologies at various levels of biological organization [229]. For instance, mutations in the LMNA gene affect transcriptional control, nuclear and cellular integrity, which ultimately bring about cascading changes in cellular organelles, endoplasmic reticulum, cellular physiology, cell-cell connections and communication. These changes may lead to muscular and nerve cell diseases, as well as initiate

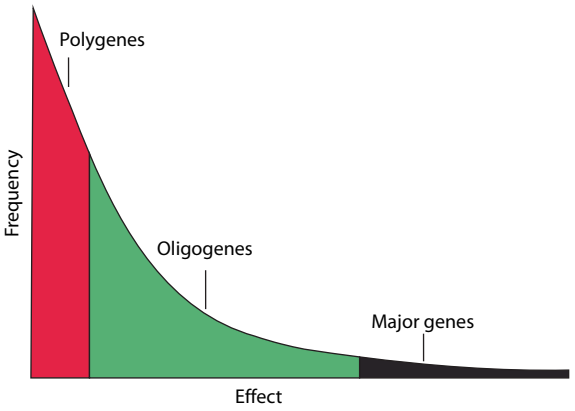

Fig. 1.5 A general view of the frequency distribution of genes and genomic regions underlying quantitative traits, in relation to their effect sizes [175]. Note the numerical excess of polygenes and oligogenes relative to major genes

many changes in morphological traits in both childhood and adult progeria [22]. These are comparable to normal aging process.

Anywhere between 300 to slightly over 750 genes have been shown to influence longevity in humans [23], including some of the familiar ones: APOE, AKT-1, AR, APP, ATM, BCL2, BRCA1, CETP, CAT, FOXO, GRB, IRS, KLOTHO, LMNA, NR3, TERC,TERT, etc., [230]. Although all of these genes in this fairly comprehensive list are known to show variable influence on longevity, either in the form of penetrance or population specificity, the list does not include much of the structural genomic variants [156] nor does it include rare alleles [71]. Nonetheless, a qualitative depiction of the distribution of these genes and genomic regions in relation to longevity or any other quantitative trait would follow a leptokurtic distribution (Fig. 1.5; [175]).

Clearly a great deal of genetic variation underlying any complex trait may be attributed to oligogenes and polygenes, out of hundreds, only a minor fraction of the genes and genomic regions would exert a disproportionately large effect on longevity (Fig. 1.5), but a majority of them, including numerous rare alleles [71, 233, 244] would have a range (strong, medium and null) of effects on the longevity phenotype. Ultimately, a differential but cumulative influence of many of these variants could potentially contribute to senescence. For instance, polymorphism in certain genes and genomic regions with large effects such as APOE, FOXO1, GRB, IGF-1, KL and LMNA, not only influence longevity, but also other traits that are associated with it throughout the life span of individuals. These genes could also maintain both positive pleiotropy and impart certain amount of longevity assurance [7, 8, 158, 220]. Because they are also essential to carry out metabolic functions throughout the life span, it is reasonable to suggest that such genes are developmentally canalized [246]; in order to facilitate robustness required to cope with demographic and environmental uncertainties [1, 200].

Both point mutations and structural variations induce genomic instability, which is well-known to affect health, diseases and senescence [243, 271]. Conversely, some point mutations, and the addition of genomic material in the form of gene

duplications may be advantageous. Gene duplications could influence flexibility, vitality and longevity (e.g., SIR2; FOXO, telomere and subtelomeric regions) as well as robustness to protect the organism against deleterious mutations through neo-functionalization and functional complementation [88]. Differential accumulation of DNA damage and lesions among individuals in different age classes may lead to age specific mortality of individuals and groups as postulated by the mutation accumulation theory. Note that mutation accumulation also creates a burden on individuals and populations that harbor them, also called genetic load; which ultimately affects their fitness [218]. Although numerous studies have attributed aging process to mutation accumulation, a clear example of the relationship between MA and aging has been recently provided by [54] on Drosophila.

In short, the genetic architecture of longevity in humans appears to resemble other major anthropometric traits such as height and body mass index (BMI), fairly closely. Unlike anthropometric traits, such as height and weight, the genomic architecture of longevity as a composite trait is perhaps more complicated, as discussed earlier. Like longevity, both height and BMI are influenced by hundreds of common and rare variants (including structural variants) that show varying degrees of phenotypic effects, hence governed by polygenic inheritance [98]. In an evolutionary sense, these new findings on the nature of molecular genetic architecture of complex traits are congruent with the infinite allele model [111, 126]. As a general rule, in polygenic systems, the advantage or disadvantage of any given allele depends upon the alleles present at the other loci in the system, and some variability may even remain hidden under the facade of phenotypic uniformity [159].

Epigenetic (developmental) space: The G-E-P map scheme suggests that genomic information passes (filters) through the developmental or epigenetic space and transforms the phenotype space in relation to environment throughout the life span of individuals. Waddington [245] defined epigenetics as "concatenations of processes linked together in a network, so that a disturbance at an early stage may gradually cause more far reaching abnormalities in many different organs and tissues." Although not explicit in the G-E-P map scheme, in accordance with the recent discoveries, we will assume that some epigenetic information is inherited [202, 225] along with the genetic information. In a fundamental sense, transcription of DNA into mRNA followed by translation into proteins occurs largely in the epigenetic space representing networks of causal sources, direction and distribution of their effects [266]. The Wright-Waddington fields of networks are common features of all biochemical pathways, cellular processes and metabolic disorders [215], and hence, longevity.

Briefly, epigenetic processes constitute differential distribution or dissipation of primary and secondary products of DNA as well as post- translational modifications of histones (the primary components of chromatin) into various components of the phenome. Enzymes modulate gene expression by attaching methyl, acetyl or phosphate groups to histone tails, in response to epigenetic epigenetic, demographic (age, stage, gender) and environmental factors. At least three mechanisms are involved in this process: methylation, chromatin remodeling and micro-RNAs. Methylation, the addition of a methyl group to cytosine which results in methyl cytosine is gener-

ally associated with decreased expression of specific genes. Chromatin represents the DNA-histone complex, and the transcriptional ability of genes is inversely related to compactness of this complex. Chromatin remodeling occurs among many genes in the genome, and has been shown to be an integral part of the aging process. Micro-RNAs, on the other hand, are non-coding RNA molecules which inhibit or degrade total mRNA [162]. Out of these three, methylation patterns are frequently employed to understand the epigenetic processes [83] For instance, Richardson [203] studied patterns of methylation among over 200 loci in lymphocytes among newborn, middle aged, and elderly and found locus and age specific signatures of methylation, Further, gene expression patterns have been shown to vary among nested hierarchies: within individuals and among individuals within families, in an Icelandic cohort [18, 192]. More recently, Day et al. [46] reported tissue specific gene expression among nearly 26, 500 methylation sites spanning 14,000 human genes across tissues such as, brain, blood, kidney and skeletal muscle in relation to age. They found age associated changes in genes involved with developmental processes, transcription and morphogenesis; while some these changes were common across tissues, others were tissue specific. Passtoors et al. [192] studied expression of 244 genes implicated in aging, using whole blood between nonagenarians and middle aged controls. They reported 21 genes to differentiate between nonagenarians and the control group; and found "longevity signature" in two genes - ASF1 and IL7R. The expression level of these two genes in nonagenarians was about 1.3 fold less compared to middle aged families. Nonagenarians also showed a favorable metabolic profile. Recently, Horvath [109] conducted a comprehensive analysis of methylation patterns among cell types and tissues in relation to age using the publicly available gene expression data sets. He found that methylation levels were close to zero for embryonic and induced pluripotent cells. Methylation levels also correlated well with number of cell passages and contributed to a heritable measure of age acceleration. These results could be extended even to chimpanzee tissues. Based on this study, he concluded that levels of methylation could be used as predictive indices of the aging process (similar to using telomere, an important genomic marker) across a broad spectrum of cell types, tissues and even across species. Gene expression studies are generally affected by large variation both within and among taxa. In order to find commonalties in age related gene expression across mice, rats and humans, de Magalhaes et al. [49] conducted a meta-analysis using 27 data sets. Their analysis revealed overall changes in the expression patters of collagen and energy metabolism cell cycle, cellular senescence reflecting degenerative aging processes. Epigenetic mechanisms, including imprinting, have been shown to be associated with many developmental and complex diseases, such as Type 2 diabetes and obesity [80] which directly affect healthy life-span. Recently, Bernal and Jirtle [16] have provided a detailed account of how commonly used chemicals could disrupt epigenetic programming and lead to developmental disorders in the exposed populations. On an encouraging note, recent studies suggest that some epigenetic changes associated with aging could be reversed through behavioral, drug or nutritional interventions [201], and thus improve the health span of specific individuals.

Epigenetic approaches have also been extended to detect and measure direct and indirect interactions among genes that reflect pleiotropy and epistasis, termed expression QTL (eQTL) analysis [31]. These interactions could occur among regions within or closer to a gene (*cis;* promoters and enhancer regions) and among genes and genomic regions of the entire genome (*trans*; transcription factors); all of which will have subtle to significant effects on the gene expression phenotype as well as down stream phenotypic consequences. In general, *cis*—regulation appears to be more predominant than *trans* regulation [117, 260]. From a quantitative genetic perspective *cis* and *trans* interactions have been equated to classical additive genetic variance and non-additive (dominance, epistatic and pleiotropy) variances, respectively [197, 249]. The role of epigenetic variation in the maintenance and evolution of quantitative traits has been recently discussed [19, 72].

Phenotype space: Phenotype is the ultimate product of the interaction among genes, epigenetic processes and the environment acting directly upon it. According to Medawar and Medawar [167], "Genetics proposes; epigenetics disposes," phenotypes in relation to environment. While phenotypic variation is partitioned into genetic and environmental components, genetic variation (architecture) underlying phenotypic traits is further partitioned into, additive, dominance and epistatic components [63]. Much of the variation underlying quantitative traits is predominantly additive, and non-additive variance is relatively low [197], Although, this observation is congruent with the conclusions drawn from classical quantitative genetic analysis on various organisms, including humans [104], there are many examples of exceptions to this rule [250, 257]. Individuals, families and populations show differential sensitivity to environmental variations and are routinely estimated using genotype x environment interaction models [63]. Such interactions could alter the phenotypic expansion of quantitative traits at those levels.

As a departure from the conventional way of partitioning phenotypic variation into heredity (H) and environmental (E) components, Wright [262] suggested that the latter includes "irregularities in development" (D) component. Accordingly, he reported that in outbred guinea pigs, variances due to heredity (h^2), environment (e^2) and irregularities in development (d^2) were 42, 0.003 and 58 percent, respectively. In the inbred line, however, these variances were 3 (h^2), 5 (e^2) and 92 (d^2), percent, respectively. In other words, it is likely that the magnitude of epigenetic variation could occasionally exceed variation due to either hereditary or environmental factors or both.

If mutation accumulation (genetic load) has phenotypic effects, as predicted by the late acting mutation accumulation and antagonistic pleiotropy concepts, and the effect size of individual mutations vary among phenomic components; then one should be able to measure the modular effects of such mutations on the expression of the above phenotypic domains and their components, by measuring the variance and covariances among constituent traits. Numerous age related phenotypic changes during growth and development among all tissues and organ systems occur during the aging process, which are commonly described as "frailty" in the elderly [65, 66]. In general, the aging phenotype in humans and in other organisms consists

of loss of integrity and function in four phenotypic domains—body composition, energetic imbalance between availability and demand, homeostatic dysregulation, and neurodegeneration [64]. Assuming causal effects of mutation accumulation on the phenotype and its components, several investigators have attempted to measure the phenotypic manifestation of mutations and their cumulative effects in terms of age specific genetic variances and covariances. For instance, Hughes and Charlesworth [115] reported increased genetic variance over time, but it was greatly affected by environmental effects, as predicted by the mutation accumulation theory. Conversely, Snoke and Promislow [221] found a decrease of additive genetic variance for mortality, and concluded that both patterns may commonly occur in nature depending upon the traits measured. These results also emphasize the need for estimating age and stage specific modulation of heritabilities, co-heritabilities and covariances, because mutation accumulation reflects age-specific effects [54]. The same arguments could be extended toward measuring the strength of correlations (hence covariances) among longevity on the one hand, and the traits that influence it, on the other [110]. The magnitude of such effects could increase, decrease, or remain the same or even become negative [82], of these, the latter represents antagonistic pleiotropy [207]. Clearly, it seems critical to measure variance attributable to developmental factors , in order to obtain better estimates of penotypic variation of longevity as suggested by Wright [262].

Environmental Dimensions: Relatively moderate levels of heritability for longevity (~0.3) suggests that all things being equal, the influence of environmental factors on longevity are as important or perhaps more important (under certain conditions), than genetic factors. The definition of environment for humans, however, gets more complicated than it is for any other organisms. Environmental aspects could include, ecological, social and cultural factors, and show environment (niche, sensu Hutchinson [112]) specific adaptations. Unlike other organisms, humans also actively and consciously "construct" their own extensive and elaborate environments, these efforts which could exert both advantageous and adverse effects on longevity at all levels of biological organization: cells, tissues, organisms, families, and populations within and across generations. The latter has been termed "niche construction" [148, 188], and has been shown to influence evolutionary processes (even some undesirable effects) in a wide ranging organisms including humans [27]. The ever increasing life span among global populations over the last few centuries [240] could be attributed in part, to certain aspects of niche construction (see below).

Modularity and Plasticity of Longevity

An organism is the ultimate product of sequential and hierarchical differentiation and development as well as a conjugated multilevel system of traits. These traits, which include longevity, show developmental and functional integration, interaction and modification throughout life [247, 248]. This view purports that longevity as an emergent trait, is associated not only with several other life history traits

preceding it, but also consistently influenced by traits such as size at birth, growth rate, age-specific reproduction, and number of offspring [26]. However, during the course of development, the influence of one quantitative trait on other latent traits is partitioned and differentially distributed according to development and environmental contexts (see below); hence low heritability associated with fitness traits including longevity is unsurprising [198].

The phenomic components of longevity (viz., gene expression, proteins, cell through organ systems) show: (a) direct, indirect and even opposite effects as expected by the age-specific action of genes [109, 207]; (b) covary in relation to the age and stage of development in order to optimize the available resources through trade-off mechanisms, which are central to evolution of life-history traits [26, 206]; (c) in accordance with multilevel organization and selection, these are also influenced by the well-known population biological principles. The "5Cs" - connectivity, cooperation, competition, coevolution and colonization [145, 186], and (d) in a much broader sense, all of these components operate as "mutualistic networks," and represent both initiation and cessation of multilevel processes, in the, genotype-epigenetic-phenotypic spaces, through time and space [21].

Recent investigations using computational biology approaches to understand organismal complexities have shown that modularity transcends across genomic space through epigenetic space into phenotype space [170]. Using these approaches, any shifts in the causal relationships among a system of mutually influencing variables at critical points during development, could be measured and changes in specific developmental/biochemical paths and nodes may be monitored. For instance, major shifts in physiological pathways, following important developmental transitions such as puberty, child bearing, and menopause [56] could be monitored and perhaps prudently managed through medical/nutritional interventions. Sewall Wright [261, 263, 265] pioneered approaches for the causal analysis of changes in physiological pathways through the development of path analysis in order to understand the direct and indirect 'causal sources, direction of their flow, and their effects' on co-varying traits as well as their importance as independent variables. Because aging affects the G-E-P spaces of all traits in the system, and covariation among them, several authors have advocated the use of causal and systems approaches in longevity studies and for developing interventions that decelerate the aging process [82, 130, 163, 222, 253].

Links between obesity on the one hand, and copious versus constrained calorie consumption, on the other, serve as useful examples of plasticity of modular organ systems, and their cumulative influence on longevity. Neel [182], in order to explain the prevalence of metabolic disorders such as type 2 diabetes, hypothesized that during the course of evolution, humans occasionally consumed excessive amount of food in order to compensate for periods of starvation—also known as "thrifty gene hypothesis." Excessive food consumption allows rapid secretion of insulin and promotes assimilation of fat in the adipose tissue. This initiates a cascade of both beneficial and harmful metabolic changes in the body, and could also lead to multi-organ dysfunction, collectively called metabolic syndrome. Both reproductive and viability components of fitness (earlier puberty, infertility and age related complex diseases, cancer, etc.) are affected by the metabolic syndrome, which could elicit

early morbidity and mortality [17]. These are just the components of demographic selection as discussed earlier. Clearly, certain traits such as obesity could influence both the direction and magnitude of plasticity associated with longevity and its covariates. Systematic calorie restriction, on the contrary, also induces numerous metabolic changes, which has been shown to have both beneficial [20] and harmful [251] effects on health and longevity in a wide range of model organisms. Unfortunately, however, more recent studies on the relationship between calorie restriction and longevity have shown inconsistent relationships in model organisms [11]. Hence, its applicability to humans remains an open question.

Senescence as Sequential Slippage of Co-regulated Units in the G-E-P Space: If the aging process involves a progressive decline of physiological functions, accompanied by an increase in the age-specific mortality rate, then, signatures of these processes should be seen in all the three phases of the G-E-P map. Indeed, gene expression studies designed to explore the links between the genotype-epigenetic domains, have revealed that *cis*-regulatory (local regulation) elements among genes direct cell-specific expression in response to signal transduction networks [117, 157]. It is also known that breaks in the DNA molecule and other structural variations, including position effects, at *cis* or *trans* regulatory (distant regulation) regions of individual genes or haplotype complexes, collectively induce genome instability [243]. Such "*cis-ruption"* processes could potentially destabilize the co-regulated *cis* and *trans* relationships in signal transduction networks [117, 132]. Interruptions in cell signaling processes are known to affect the robustness of gene networks [210]. These could also lead to age dependent deterioration of nuclear pore complexes and loss of nuclear integrity in post-mitotic cells [95]. Disregulation of *cis-trans* complexes have been implicated in many Mendelian and complex disorders, including cancer [117, 157, 241]. Then, it is conceivable that mutation accumulation (both germ line and somatic) could induce various degrees of *cis-ruption* in the genome (nuclear-mitochondrial complex), which would naturally have destabilizing effects on gene networks and coevolved haplotype complexes. These genomic *cis-ruptive* processes may also lead to dysregulation of cell and cell/tissue specific methylation and expression patterns or "epigenetic disruption," during the aging process analagous to cancer progression [109, 212, 234]. Such a synergistic tripartite process (i.e., DNA breaks, cis-ruption and epigenetic dysregulation) might bring about cumulative, cascading and disintegrating effects in the conjugated system of cells, tissues and organs, resulting in the observed frailty among physiological, reproductive and viability components over the life time of an individual, collectively termed senescence. These changes could involve perturbations in subtle cell-cell connections, cooperation, coevolution and competition among cells and the components of intra-and inter-cellular organization, which are the hallmarks of multilevel organization in all biological systems [186]. Phenotypic expression of such causal and latent cellular processes (transcriptional errors, *cis-ruptive* and *epigenetic disruptive* changes) represents a step-wise and stalled action. The delayed phenomic expression of these combined processes during senescence may be best represented by the "phenomic lag" concept [44]. In

short, it seems that potentially at every cycle of cell division, *cis-ruption* coupled with the dysregulation of epigenetic programming leaves a subtle, yet indelible signatures of systematic deterioration of the genome, symmetry of cell division, integrity of cells, their communication networks, tissues, and various organs, their inter-relationships. In short, senescence may be interpreted as a series of time-dependent sequential slippage events in the G-E-P space. It is also likely that during this process, cells harboring new mutations in various tissues could even form genetically different lineages (mosaics) which could become benign or malignant tissues (neoplasia), mimicking evolutionary processes. Aging process is known to promote mosaicism both in reproductive and somatic tissues [50]. In this regard, both cancer and the aging process may share a few common developmental patterns and pathways as proposed by Hoeijmakers [105]. These senescent processes may become accelerated and also increase exponentially past reproductive age. This agrees with the view that developmental instabilities driven by either genetic or epigenetic instabilities or both could lead to mortality of individuals attributable to developmental process, or developmental selection; which is known to alter the opportunity for selection of individuals in populations [196]. From a quantitative genetic perspective, these combined genetic and epigenetic processes may be reflected in the observed levels of additive and non-additive genetic variance and covariance components among the observed and latent phenomic traits as well as in the emergent trait—longevity.

In contrast, relative stability of the entire system to internal and environmental perturbations as a whole may indicate its "robustness" [247]; which is defined as the constancy of phenotype in the face of genetic and environmental perturbations [51]- an idea similar to genetic and physiological homeostasis [141]. Accordingly, natural selection will favor genotypes that resist environmental fluctuations through robustness [176] during development. This concept naturally leads us to think, at least from the perspective of senescence, deceleration or acceleration of aging processes could be attributed, in part, to evolutionary genetic processes. Individuals and families that show a natural tendency to resist harmful effects of mutations and other sources of perturbations are expected to maintain stable phenotypes and slower pace of senescence [136].

Exceptional Longevity in Humans: An Enigma or An Evolutionary Rarity?

As noted earlier, the average life expectancy among countries varies from about 47.5 years in Sierra Leone to 87.2 in Monaco. However, individuals with exceptionally long life span of 100 or more are increasing globally. Exceptional life span is a relative term, however. For countries with low life expectancy (e.g., 47.5 in Sierra Leone) an average life span of 80 in the U.S. may be exceptional, and people living until 100 or more (centenarians) are exceptionally rare even in the developed countries. Considering approximately 25 years as the average generation time in humans [2, 61], centenarians are "bestowed with" about an extra human generation

time, compared to individuals with normal life span of about 80 years in the U. S. Thus, centenarians occupy a distinct region of the distribution of life span among human populations (Fig. 1.2). Occurrence of centenarians is clearly rare in any human populations, as they are found at a frequency of just 1.73 and 3.43 per every 10,000 in the U.S. and in Japan, respectively [169]. Rare individuals in any species, often possess superior physiological and evolutionary properties [91, 106]. Besides their extraordinary life span, centenarians have many desirable health attributes. For instance, the Ashkenazi and Okinawan centenarians remain generally healthier, and preserve "youthful" qualities better, relative to individuals with normal life span, till advanced ages, and this trait appears to run in families [8, 255, 257]. They also have favorable lipid profiles (high HDL, large LDL, Apo-A1 levels [7]; which are some of the common indices of cardiac health [85]. Below we discuss a few ideas on the origin and maintenance of centenarians and their unique features.

Neoteny: Following Haldane [89] and others, Montagu [172] has argued that "neoteny"—a wide spread and evolutionary conserved process by which juvenile traits are preserved till later stages of individual development, could help decelerate the degenerative processes associated with senescence. He provided numerous examples in support of this concept. Recent work on mice by Kamran et. al [121] also lend some support to this concept. They exposed older mice to "young blood" by injecting plasma isolated from younger animals into older ones (this is a modified version of a classical technique known as parabiosis in which whole blood is transfused between young and older animals by sowing the skin of young and older animals together). This process improved many aspects of cognitive memory in older animals. Rubin and coworkers [122] went a step further and showed that among others, GDF-11 protein, a key component of embryogenesis found in young mice and plays a pivotal role in vascular remodeling and cognitive function in aging mice. These studies appear to support the concept of neoteny as a factor in promoting exceptional longevity.

Superior Genomic Integrity: From the genomic perspective, centenarians appear to be unique as well. For instance, Bergman et al. [15] suggested that centenarians may carry certain favorable allelic combinations, and these alleles might exert attenuated and compensatory buffering effects against harmful genotypes either independently or perhaps through other mechanisms such as positive pleiotropy. Erceg et al. [58] studied structural variation among individuals whose age ranged from 65 to 95, and found a greater level of variation among 70 year olds relative to the 95 years olds. This led them to suggest that "the relatively low level of chromosomal aberrations in the 'oldest old' people is likely to be both a consequence of their genetic stability and a contributing factor to their attainment of advanced age. We can assume that very old people had the slower rate of accumulation of genetic damage throughout their lives. Thus, the relatively low level of chromosomal aberrations in the "oldest old" people is likely to be both a consequence of their genetic stability and a contributing factor to their attainment of advanced age. They are practically in the same position as the normal middle aged population and represent the special subgroup of elderly—the privileged one." Similarly, in nematodes, while wild strains accumu-

lated massive amounts of copy numbers in their genome, the long-lived strain (daf-2) rarely did [84]. Accordingly, perhaps it is reasonable to suggest that genome integrity may be one of the factors that promote deceleration of aging process in humans.

Negative Frequency Dependent Selection: From an evolutionary perspective, exceptional longevity as a threshold quantitative trait shows non-linear and distinct phenotypic variation relative to individuals with normal life span (Fig. 1.2). By definition, threshold traits, show "two phenotypically distinct morphs that are assumed to be genetically determined by a continuously distributed underlying trait called the liability…Individuals which have liabilities above a particular threshold develop into one morph while individuals below the threshold develop into the alternative" [208]. Threshold traits are maintained by negative frequency dependent selection [173], and their occurrence in natural populations follow a Poisson distribution [114]. This form of selection facilitates the maintenance of genetic variation among traits associated with fitness. Haldane [91] speculated that rare genotypes arise randomly in natural populations, at very low frequencies (hence fit the Poisson process); they often show unusual physiological features. He commented that "It is an advantage to the individual to possess a rare biochemical phenotype" and "… to a species to be biochemically diverse, and even to be mutable as regards genes concerned in diseases resistance. For the biochemically diverse species will contain at least some members capable of resisting any particular pestilence… Selection of rare biochemical genotypes has been an important agent …in keeping species variable…" Considering that immune system that protects individuals from infections gets progressively weaker in relation to age [66], superior physiological functions and resistance to diseases among centenarians, relative to the majority of individuals with normal life span is not surprising. Note that rare genotypes with superior physiological attributes could confer some fitness advantages to the population to which they belong; but such populations may also offer some advantages to the rare genotypes, in return. Hence, there may be some degree of reciprocity between the rare genotypes imbedded among the common ones.

Exceptional Longevity as An Intrinsic Natural Phenomenon: At this stage we could extend two interesting ideas that are gaining popularity in the natural and social sciences, to explain the origin and maintenance of rare events/traits such as exceptional longevity in human populations – black swans[1] and dragon-kings[2]. Taleb [231] introduced black swan metaphor to explain the occurrence of unusual and unpredictable events mentioned in the physical, natural, economics and social sciences.

[1] White and black swans are different species; hence occupy distinct ecological niches in Northern Europe and Southern Australia, respectively. For this reason, black swans are rare in Europe, so are the white ones in Australia. Rarity of black swans in the sixteenth century England was associated with the adage "rare as a black swan." This expression was extended by Taleb [231] to explain the occurrence of rare and unpredictable events primarily in social contexts.

[2] The term "dragon" represents "the mythical animal that belongs to a different animal kingdom beyond the normal, with extraordinary characteristics. The term "king" … emphasize(s) the importance of those events, which are beyond the Parteo law distribution of wealth of their subjects." Sornette and Ouillion [224].

This concept suggests that, "small, large, and extreme events belong to the same population, the same distribution, and reflect the same underlying mechanisms(s)," and by their unpredictable nature, future events cannot be forecasted in advance [224]. Indeed, Vacante et al. [237] have extended this concept to examine the ever-increasing number of centenarians and super centenarians among global populations. In principle, the black swan concept shows qualitative similarities with frequency dependent selection. On the other hand, others [189, 223, 224], have also recognized the ubiquity of unpredictable events such as extraordinary concentration of wealth and other natural phenomena ranging from earth quakes to seizures, associated with the tail of frequency distributions. They argued that such events should be predictable *ex ante*, "directly from specific transient structures developing in the systems," and advanced the "dragon-king" $(DK)^2$ hypothesis to counter Taleb's conjecture. According to the DK hypothesis, the great wealth of dragon kings is a product of having both independent means of acquiring their own wealth, and also deriving a part of their wealth from those with ordinary levels of wealth. This concept has been extended to predict very rare events such as the origin of great cities, earth quakes, tsunamis, forest fires and even unpredictable health events such as epilepsies [189]. Considering the unique genomic, phenomic and demographic attributes of centenarians and super-centenarians, and leaving mathematical details aside, we could equate their distinctiveness with the "transient structure," described in the DK model, and designate these unique individuals (i.e., centenarians) as "dragon-kings," (see; Figs. 1.3 and 1.4 in Sachs et al. [211]). Upon extending the DK hypothesis to predict the occurrence of centenarians, and keeping other factors that influence aging among normal aging populations constant, we could speculate that a large number of centenarians may arise in countries that are already harboring a large number of octogenarians and nonagenarians and also foster an environment conducive for the emergence of future centenarians (see Fig. 1.1.).

Grand Parents as Promoters of Longevity: Another interesting view that supports the evolution and maintenance of longer life span among species that have parental care and overlapping generations is that, allocation of resources from older to younger generations, also called intergenerational transfers, would favor greater reproductive value of younger individuals and evolution of longevity [26]. Contributions from grandparents toward caring and passing their experience to the successive generations, which in turn may contribute the over all Darwinian fitness of grand children, has been advanced to the "grand-mother hypothesis" [96]. Various aspects of this interesting hypothesis that impacts the maintenance and evolution of longevity, and its applications in contemporary human society are discussed by Danielsbacka et al. [41]. Clearly, patterns of cultural inheritance in relation to Darwinian fitness needs to be examined among families and populations that harbor most centenarians. In addition to genetic factors, Promislow et al. [199] have explored the role of parasites (microbiome) and sexual selection (conflicts and female choice) in the maintenance and evolution of aging.

Perhaps a few or all of the above factors could explain the superior health and longer life span that is unique to most centenarians. Also, among the exceptionally long lived humans, genomic robustness maintained negative-frequency dependent

selection and other compensatory mechanisms may provide system–wide robustness across the G-E-P landscape map against DNA damage and the associated processes described above. As an extension, it is reasonable to speculate that other interesting but rare traits (not necessarily associated with longevity) such as exceptional cognitive, sensory, musical, athletic ability, etc., found in human populations, may be governed by similar evolutionary mechanisms. For instance, Lykken et. al. [154] have described the origin of exceptionally gifted individuals by the term, "emergenesis." Perhaps the same concept could be extended to describe the origin of centenarians.

Need for Integrating Other Approaches in Longevity Studies

Genetics: Although Haldane [90] used Huntington disease (HD) as an example to explain the role of mutation accumulation in the aging process, he was unaware of the details of the genomic processes associated with the expression of the disease. More specifically the expression of HD depends on the number of trinucleotide repeats. Healthy individuals carry between 10–26 repeats; individuals with intermediate levels (27–36) remain unaffected, but variable penetrance of the disease is common among individuals carrying 36–41 repeats. Similarly, the role of numerous chromosomal and Mendelian disorders on longevity and senescence is not explicitly discussed in the aging literature, with the exception of early and adult onset progeria, some chronic diseases and overall physical condition of frailty (e.g., [66, 242]). Molecular bases of at least 3995 phenotypes of Mendelian disorders (e.g., disorders of carbohydrate and organic acid metabolism; amino acid, lipid, lysosomal, peroxisomal, purine and pyrimidine disorders) that result in the accumulation of toxic product(s), and also affect healthy aging are known [162]. In a Darwinian sense, metabolic disorders affect both reproductive and viability fitness. The influence of genetic disorders on life span, however, remains largely unaccounted for by the Gompertz-Mackeham model; but needs to be explored as recognized by Garrod [78] decades ago. If the overall goal of aging research is to increase the healthy life span, then any form interventions (e.g., gene-editing, tissue engineering using stem cells, drug/nutritional interventions used in metabolic disorders) at various levels of biological organization and stages in life history that contribute directly to improve health (survival), development, longevity and reproduction, which in turn affect micro-evolutionary processes, should ideally become a part of demographic predictions.

Epigenetics: It is well-known that genetic effects radiate through a large field of biochemical networks embedded in the epigenetic space [83, 245, 266], and mutations in these networks lead to a general deterioration, of most physiological functions over time, including the aging phenotype [177]. Gene expression studies further suggest that mutations affect both *cis* and *trans* regulation of genes in the bio-

chemical networks [73, 75] and their effects could be measured in terms of additive and non-additive variance and covariance components of the phenotype. Moreover, these studies also indicate that the innate robustness (which includes both genetic and physiological homeostasis [141] to cope with external and internal stresses, could be a function of the integrity of the biochemical/physiological system. Yet, the role of epigenetics (development/physiology/developmental homeostasis) is conspicuously missing in most, in not all of the classical evolutionary theories of aging. This trend appears to continue as most evolutionary biologists still focus mainly on quantitative aspects of age and stage specific fitness and mortality patterns. It is almost imperative to estimate "Wright's d^2" (variance due to 'irregularities in development' [262] in addition to calculating variance due to genetic (h^2) and environmental (e^2) factors in relation to population specific age and stage in order to fully comprehend the biological bases of longevity.

Phenotypic: At the phenotypic level, evolutionary approaches could provide useful avenues to make predictions not only on longevity, but also perhaps to identify important "inflection points" (prenatal, neonatal, pre-reproductive, etc), at which dramatic metabolic changes occur [56, 254], as opposed to smooth developmental transitions, in the life history of individuals. Because longevity is a composite and a plastic trait, in which many traits interact and determine the ultimate health of individuals, it can be modulated by environmental factors as a system. For instance, Hamilton's theory predicts late life survival, based on early fitness related traits [177, 178], because genetic correlations between fitness traits are maintained by pleiotropic effects of genes. A substantial proportion of increased life span in developed countries may be attributed to the influence of superior environmental conditions available to people throughout their life history across generations [14, 34]. Similarly, from a developmental perspective, diseases that have *in utero* origins may have long-lasting effects on the longevity of an individual [78, 194]. Perhaps a detailed investigation on the nature of these inflection points, using the G-E-P approach could provide opportunities for medical or other interventions in order to modulate healthy aging. Administration of folic acid to malnourished pregnant mothers has greatly reduced the occurrence of many developmental disorders such as neural tube defects, cleft-lip, cardiac septal defects and eye defects in the new born [4], serves as a case in point. Similarly, management of a common metabolic disorder, Phenylketonuria (PKU) by administering phenylalanine free diets has greatly increased healthy life span of these patients. Examples of this nature are commonly encountered in treating many metabolic disorders [24, 215]. Similarly, life style modifications such as diet and exercise could reduce the severity of metabolic syndrome and thus impact the life span of affected individuals. Such issues of public health importance are rarely discussed in human demography. Recent "phenotype ontology" project consists of various phenotype data on human diseases represents a useful resource in this regard [133].

Systems thinking: As noted, phenotype and its components, genes, gene expression and fitness traits are hierarchically organized. These are also interconnected (auto-correlated to various degrees) and work as sub—systems within the large net-

work of biological system. Various paths in the network, show direct, indirect, reticulate and even opposite "causal effects" with each other during growth and development of organisms—a view Sewall Wright maintained throughout his long career [261, 267]. Accordingly, it is perhaps practical to monitor the developmental pattern in relation to age and stage of specific individuals using norms of reaction approach [128] employing systems approaches. Clearly, while classical evolutionary theories have provided a general framework to examine the evolution of senescence, these insights need to be amended with the newly emerging details terms of G-E-P map and life history theories. It is becoming clearer that evolutionary insights could be used as guideposts for developing predictive and preventive medicines to improve human health and longevity [86, 150].

Could life span in humans evolve? Many lively debates revolve around this topic. We suggest, that there are opportunities for evolution of life span in humans, for the following reasons: first, there is a tremendous amount of phenotypic diversity for life span in the human species. Phenotypic diversity is a reasonable surrogate of genetic diversity [32]. Natural selection operates on individual phenotypes and variation in family size. Second, heritability of life span is about 30 % [67]. Although this is a moderate amount, typical to many life history traits [94, 198], much of the variation in quantitative traits is additive, and selection operates on additive genetic variation [39, 104, 134]. Third, since exceptional longevity is a quantitative trait, and is known to run in families. In accordance with the principles of quantitative genetic inheritance, the mean longevity of progeny resulting from the union of members from families with normal life span would be increased (or shifted slightly and positively) relative to individuals in general populations. Fourth, negative frequency dependent selection may be maintaining exceptional longevity. Assortative mating among members of exceptionally long-lived individuals could maintain longevity in their progeny, but opportunities for such unions in general populations are rare and impractical for various reasons. With the novel assisted reproductive technologies, however, feasibilities to achieve such objectives are definitely in place. On the other hand, should individuals with a family history of exceptional longevity marry individuals from families with normal life span (similar to cosmopolitan population) or vice versa, the mean expected life span in such unions may be even reduced because of partial disassociations among gene combinations due to mixing effects [151]. As a general rule, in out-breeding species such as humans, "recombination breaks up adaptive gene combinations, even if the do arise at low frequency, and again prevents establishment of all but immediately favorable variant [13]. Over time, in accordance with the law of regression to the mean, this process would move the mean of the mixed population back to the mean that would closely resemble cosmopolitan populations. Despite some of these limitations, life history traits, of which life span is one, "really do tend to have a high potential for rapid evolution, and this is most likely due to them being functions of many components through which many genes can induce variation" [94]. Rearing environment could also modulate the expression of additive genetic variance of fitness traits at specific developmental stages [53]. Accordingly, life span being a composite trait has the potential to evolve in contemporary human populations and may show context-

specific responses to biological and physical environments, similar to other life history traits.

How might this happen? It is well-known that individual genotypes respond differently to macro (temperature, diet, etc.) and micro (subtle and unknown) environmental factors. Differential sensitivity of individual genotypes to both micro- and macro-environmental variation have been measured and interpreted in terms of genetic variance for environmental sensitivity in quantitative genetics and evolutionary biology [47, 102]. Recently, Mulder et al. [179] reported environmental sensitivity of milk yield in dairy cattle. The same reasoning could be extended to composite traits such as body weight and longevity in humans. Perhaps it is reasonable to suggest that at least some fraction of increased life expectancy over the past 170 years in countries with increased availability of nutrition and improved living conditions could be attributed to improved environmental conditions and life style changes [66]. These could potentially influence "phenotypic heritability" through epigenetic mechanisms among individuals within families, and families within populations [72]. Perhaps increased long-term life expectancy which has proceeded at a pace of 2–5 years per decade [240] may be partially attributed to such epigenetic mechanisms; which also suggests a potential for increased life expectancy in some populations in the coming decades. Indeed, Christiansen and colleagues [35] predict that "most babies born since 2000 in France, Germany, Italy, UK, the USA, Canada, Japan…will celebrate their hundredth birthdays." This optimism, however, also portends the corresponding public health burdens on future generations.

Phenotypic Evolution: stasis and spurts: The above optimistic inference is primarily based on relatively short term evolutionary responses of longevity to environmental changes in the last few centuries. This view needs to be evaluated further from the perspective of global and long term behavior of similar traits. First, most quantitative traits have moderate amounts of genetic variation and respond readily to both artificial and natural selection, as demonstrated on numerous domesticated plants and animals [63, 128]. A number of these traits also exhibit differential sensitivity to common environments [102], and to various dimensions of constructed environments (niche construction, NC [188]). Individual traits, however, cannot respond to selective forces forever, as they eventually reach selection limits due to exhaustion of genetic variance of the trait, and constraints imposed by other covariates or "trait combinations" [13]. For instance, the two composite traits, body size and longevity show an allometric relationship, and thus covary [48, 204]. The component traits of these two traits also covary. Therefore, evolutionary genetic influence of one trait on others cannot be easily decoupled without imposing substantial costs on dependent traits and their respective components. Domestication of dogs serves as a good example, in this regard. Artificial selection to change body size in dogs (for fancy and other reasons), has also increased longevity in some breeds [135], but only at the cost of overall health among these pedigreed breeds [116]. Second, as a rule, any quantitative trait such as body size or longevity of a given species, could only evolve within the "space of possible phenotypes" of that species [13], as natural selection operates only on the available phenotypic variation [129]. A great deal of phenotypic variation of any quantitative trait, however, is dispersed

in a space that resembles the "long barrel of a blunderbuss [5]." Only a minor fraction of the variation is concentrated in the flared muzzle region of the model. This model implies that phenotypic variation and evolution of any quantitative trait in any species is circumscribed by the "blunderbuss space," unless "rare bursts of evolution carry lineages out of established adaptive zones" [5]. Such events, however, occur at a rate of about one per million years [5, 236]. These two scenarios need further consideration. The first scenario is commonly encountered in the domestication of plants and animals. Recent studies suggest that continued selection, in many of these systems, has reached plateaus [87]. Despite its appeal, this approach cannot be extended to humans because of ethical concerns as well as moral and societal responsibilities. The latter (i.e., sudden bursts of events) could only happen as a part of a speciation event due to origin of new set of genes, which may occur at a rate of "three to five new genes per genome per million years" [52]. Hence, in terms of longevity, it is unlikely that humans would surpass the "Calment bound" to any significant extent in the near future. Paradoxically, older people do not contribute to reproductive fitness, and therefore, any decision on longevity extension must be based on their progeny, in accordance with the well-known parent-offspring relationships, frequently employed in quantitative genetics [63]. Although longevity is known to run in families [6], it is difficult to precisely predict the longevity of any given individual *a priori*, during their development and reproductive stages, because longevity is influenced by the contextual effects of biological and environmental factors. Even if this could be achieved, in the coming years, recombination due to peculiarities of human mating systems that have evolved to avoid the ill-effects of inbreeding, and to provide homeostatic mechanisms would break up adaptive gene combinations, preventing the establishment of all but immediately favorable variants [13]. These processes would gradually push back the average life span in the progeny of long-lived individuals close to population specific distributions. Moreover, even if some men at older ages contribute to the gene pool, their gametes carry greater number of mutations (mutational load), relative to their younger counterparts, and the resulting progeny would be generally less fit compared to cosmopolitan populations. These scenarios leave us with the possibility of improving the environment and various dimensions of niche construction as the only two major avenues to improve human longevity within the bounds of the available phenotypic variation commonly encountered among human populations.

Conclusions, Prospects and Challenges

> "Hence if man goes on selecting, and thus augmenting, any peculiarity, we will almost certainly unconsciously modify other parts of the structure, owing to the mysterious laws of correlation of growth." [42]

This chapter has discussed some evolutionary aspects of longevity and senescence from a G-E-P map and life history perspective. The G-E-P approach represents both Malthusian and Mendelian processes, collectively called Darwinian processes. Darwinian approaches provide unique opportunities to study senescence as an integral

part of the multilevel selection process consisting of units and targets of selection [145, 187] that include cells, tissues, organs and organism, throughout the life span of an individual. As a corollary, longevity phenotype represents a temporal trajectory of interacting and integrated system of genes and epigenetic processes of variable resilience within individuals and individuals nested in families and populations—all influenced by Darwinian processes. The following conclusions are drawn.

1. The classic Gompertz—Makeham model has been useful for predicting the universal properties of population growth from birth to death. But, it provides no idea on mortality patterns in pre or post and neonatal stages due to genetic and epigenetic factors, which represent periods of intense selection. Further, many diseases associated with early childhood mortality and morbidity have *in utero* origins, and often exert lasting effects on adult health and longevity [80]. Hence from an evolutionary, development and health perspective, it may be useful to consider aging process from the time of conception to death, and to include ancestral information on demographic and cultural aspects such as inbreeding. How would these factors influence the G-M curves in different populations in relation to congenital factors is largely unknown. Detailed genetic, epigenetic and demographic analysis of these processes would provide an opportunity to devise general and specific preventative and interventional measures as well as managing disorders that have genetic or physiological origins.
2. Longevity is a terminal and a telescopic life-history trait; it has ontogenetic relationships with other complex traits that precede it, and also covaries with them during the course of development. Hence, it is a composite and an emergent trait. Although recent molecular studies have shown that a few genes and genomic regions exert disproportionally large effect on longevity, it is still influenced by numerous genes and genomic regions. These findings are congruent with the infinite allele model of Kimura and Crow [126] that is universally applicable to most quantitative traits. Genes that influence longevity also operate through other genes in the network that affect various life-history traits throughout the life span of individuals. Hence, in the Wrightian sense, even the strongest effect of any given gene or a genomic region or a set of these regions on longevity may be viewed as the latent and the emergent properties of those genes in the entire gene complex.
3. Among others, mutation accumulation and antagonistic pleiotropy concepts advanced over 70 years ago still remain as the dominant theories to explain longevity and senescence. The more recent, “positive pleiotropy” idea provides another variation on this theme. In support of this view, certain “canalized” genes such as APOE, IGF1, FOXO1, distributed across diverse taxa appear to exert pleiotropic influence throughout the life span of individuals. Under certain situations, extending the concept of antagonistic pleiotropy without paying attention to age, stage and gender specific aspects of gene networks could be misleading. For instance, recent studies using network analysis have revealed that almost all genes in the longevity and other physiological pathways exhibit pleiotropic properties; which are generally inferred by establishing statistical associations. The sign of such statistical associations (i.e., positive or negative) between genes (or traits), however, could reverse upon conditioning on a third gene or a trait,

a phenomenon known as "Simpson's paradox" [193]. Although some important individual genes (e.g. APOE, FOXO1, ApoE, IGF-1) appear to show specific effects on the aging process, it is often difficult to uncouple their influence from other genes, as well as their contextual and transient effects as they are parts of mutualistic networks. Hence, both normal and exceptional aging are maintained and evolve as systems and must be understood as such. Greater integrity of the system may contribute to superior homeostasis, and to healthy aging. Such integrated approaches would provide insights for developing reliable indices for predicting the trajectories of health span; they would also compliment demographic projections.

Recent molecular evolutionary genetic studies indicate that contemporary populations carry tremendous load of rare mutations and they also increase with age. Hence, detailed analysis of their influence on physiological (epigenetic) mechanisms, age and stage specific modulation of variances, covariances, (sensu Ref. 262) and robustness/homeostasis, in relation to at all phases of development, and growth are sorely needed. As a cautionary note, and in view of hundreds of genes, gender, age, stage, anatomical and physiological systems that are known to influence longevity and senescence, uncritical interpretation of associations between individual genes and longevity (or any other phenotype and phenotypic components), without paying attention to context, could lead to untenable conclusions. In this regard, a critical examination of antagonistic pleiotropy concept is needed.

4. Longevity as a composite developmental trait shares similarities with multilevel features of natural selection. Recent advances in gene expression and epigenetic analysis combined with systems thinking would provide a useful way to examine the consequences of mutation accumulation, and the nature of gene regulation in aging process, in relation to multilevel organization and selection from intracellular level to the level of individual and beyond. In this respect, the Hayflick process, c*is-ruption* and epigenetic dysregulation mechanisms may represent a tripartite process. The components of which, may act either independently or synergistically, and in relation to environment, to bring about cryptic, cascading and destabilizing effects on cellular transduction mechanisms, connectivity and cooperation among cells and higher levels of organization over time (phenomic lag). These are essential for the integrity of multilevel organization ranging from genome architecture within a cell to organ systems in an individual. In accordance with the Gompertz-Makeham law, the senescent processes may increase exponentially past reproductive ages and force of selection gets weaker. Thus, senescence may be viewed as a sustained sequential slippage in the G-E-P space from fertilization to death; but the modulation of which depends on the stage of development. Individuals, who carry innate decelerating mechanisms against sequential slippage may be expected to have greater longevity. The use of classical variance-covariance and causal models along with longitudinal (representing both age and stage) studies would only compliment the insights gained from molecular mechanisms underlying longevity.
5. At the phenotypic level, longevity is linked to other life-history traits; hence covaries with them. Although heritability for longevity in general population is

low (0.30), it still has the "evolvability" potential. Both genetic and epigenetic factors associated with each of the life-history traits might act synergistically toward modulating longevity—ordinary or extraordinary. Accumulating evidence suggests that human life expectancy is increasing globally, along with exceptionally long-lived individuals in almost all global populations, especially in developed countries. This could be attributed to the extraordinary abilities of humans for niche construction. If we extend the DK hypothesis to examine low levels of heritability for most life-history traits, including longevity [198], they appear to conform to Pareto law[3].

The exceptional life span and health span among centenarians suggest that negative frequency dependent selection may be maintaining this aspect of fitness, and occurrence of exceptional longevity at population level could be predicted using the D-K hypothesis. Perhaps contingency measures could be put in place to deal with the elderly population. Clearly, longevity phenotype, when defined by just the number years lived provides a useful statistic; but it would only represent the "tip of an iceberg" of its biological complexity. Hence it is critical to understand its ontogenetic relationship, specifically covariation with other traits, in the context of the individual and in relation to the population and the environment it represents.

6. Individual quantitative traits are known to respond to selection, but their response is constrained by their covariates, which is common to most complex quantitative traits, including traits such as body weight and longevity. Although general life expectancy in humans appears to be increasing globally in the last few centuries, a comprehensive analysis of phenotypic evolution over millions of years, based on a large number of phenotypes, suggests that the distribution of most quantitative traits generally fit into the blunderbuss model of evolution, and individual traits may have an inherent and impending ceiling on this trend [5]. Hence, it is reasonable to suggest that like all quantitative traits, human longevity also evolves only within the available phenotype space unless a major revolution occurs in the entire genomic space due to the origin of a new set of genes that would radically change the genetic architecture of longevity and its covariates that contribute to Darwinian fitness. Such genomic revolutions may require at least a million years. Consider the following: (a) anatomically modern humans evolved only 150–195,000 years B.P. [235], (b) the upper bounds for human longevity or maximum life span (~100–115 years) may not have changed in the last few centuries [26], and c) despite medical advances, evolution in modern humans is occurring at a pace comparable to most higher organisms [228]. Therefore it is highly unlikely that *major revolutions* in the human genetic architecture would

[3] Pareto Law, popularly known as "80–20 rule" suggests that in any self-organized systems, 80% of effects emanate from about 20% causes [12]Evolution is indeed a prime example of self-organizing system [123, 264]. Extending Wright's principle of causality [261, 263, 265] which provided a basis for developing the concept of heritability [101], it may be suggested that low heritability (around 30%) associated with life history traits, including longevity, among diverse species [198], could be explained through the universal self-organizing principle that spans across the entire tree of life [12, 123].

occur in the near future, such that longevity will be pushed into greater levels exceeding that of the Calment bound. Evolution of longevity would most likely be confined to the phenotypic space encountered in contemporary human populations for a long time. Hence, the outlook for long-term evolution of human longevity may be best represented by the myth of "Scylla and Charybdis."

7. Application of the G-E-P map approach to examine longevity as a composite life-history trait subsumes Darwinian processes. Hence it provides a useful way of identifying the genetic, epigenetic and phenotypic topography of critical inflection points associated with physiological transitions and some anatomical transformations during the growth and development. Identification of such points coupled with an understanding of the initiation of *cis-ruptive* process and phenomic lag span may help us discover optimal timing in order to devise interventional approaches for enhancing healthy life span. Such approaches may not necessarily contribute to reproductive fitness, but would improve the health span of specific individuals, as the ultimate goal of all aging research is to improve the overall human health, including reproductive health. Many useful examples of this nature are found in medical genetics literature.

In summary, from a Darwinian perspective, the G-E-P map serves as an excellent metaphor to study longevity and senescence as integrated modular components of life history. It emphasizes the need for incorporating genetic, epigenetic, demographic and evolutionary-ecological insights in order to achieve a longer and healthier life span, and also underscores the fact that many fundamental constituents of longevity and senescence have ancestral and developmental origins. Correspondingly, longevity and senescence are distinctively individual-specific and so are their trajectories from conception to old age. In accordance with the Darwinian process, individual is the target of selection as well as of medical intervention [86]. Natural selection operates on the entire G-E-P system of individuals, hence deploying a combination of genomic and phenomic markers along with their distribution patterns associated with their ancestry and inheritance would provide a useful way to predict the lifespan of individuals. Evolutionary genetic approaches would be helpful not only for predicting and preventing genetic disorders associated with the aging process, but also for greatly advancing our understanding of healthy lifespan as a component of multilevel process. Recent advances in genomic and computational technologies in combination with the *ex ante* predictive power of D-K hypothesis may enable us toward charting the dynamic nature of self-organizing systems, of which life span is one. These approaches may provide unparalleled opportunities to elucidate the universal and particular properties of life span at the genomic, developmental, individual, family and population levels simultaneously, which seemed impossible even only a few years ago. In a broader sense, research into the evolutionary genetic bases of longevity and senescence would help explain the relationships among complex traits and genetic disorders during development and growth. It would also pave the way for designing effective interventions in order to extend healthy life span as well as illuminate our understanding of the organizing principles of life itself!

Acknowledgements I am grateful to Drs. Gil Atzmon for inspiring me to write this chapter, Nir Barzilai for the enduring encouragement, and Konstantin Khrapko for logistical support. My special thanks are due also to Dr. Sri Raj, for insightful comments. I express my profound gratitude to my family for their enduring patience and moral support.

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Chapter 2
Candidate Genes That Affect Aging Through Protein Homeostasis

Yair Argon and Tali Gidalevitz

Introduction

Aging is a multifactorial and pleiotropic process with many interacting mechanisms contributing to the decline. Because of this complexity, the candidate gene approach to understanding aging often relies on identifying human genes whose function during aging can be predicted based on animal models of aging. Then, association to longevity in humans is determined statistically if a variant of the candidate gene is prevalent among people who live healthy, long lives compared to people with average health span and lifespan. Many of the longevity genes identified thus far influence one of the following pathways: the insulin/insulin-like growth factor pathway, TOR (target of rapamycin) signaling, DNA repair, lipid metabolism, mitochondrial activity and nutrient intake. Understanding how these genetically distinct mechanisms interact at the molecular level to control longevity is a fundamental and fascinating problem, which has been subject to many reviews, e.g. [1–3]. In some cases, as for telomerase, the role in longevity is rather clear—maintaining telomere length [4, 5]. In others, such as mitochondrial activity, the connection is vague, because redox state and reactive oxygen species (ROS) encompass so many aspects of biology. In the case of other candidate genes, for example apolipoprotein E alleles that are prominently identified by genomic association studies [6], their mechanistic contribution to longevity is related to their different biochemical activities and in particular to their differential binding to damaged protein (reviewed recently by

Y. Argon (✉)
Division of Cell Pathology, Department of Pathology and Lab Medicine,
The Children's Hospital of Philadelphia and the University of Pennsylvania,
3615 Civic Center Blvd., Philadelphia, PA 19104, USA
e-mail: yargon@mail.med.upenn.edu

T. Gidalevitz
Department of Biology, Drexel University, 418 Papadakis Integrated Science Bldg.,
3245 Chestnut Street, Philadelphia, PA 19104, USA
e-mail: TG443@drexel.edu

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine
and Biology 847, DOI 10.1007/978-1-4939-2404-2_2

[7, 8]). Common to all these pathways is their role in cytoprotection against various cellular stresses. Clearly, maximal lifespan extension demands the co-activity of multiple cytoprotective pathways, as emphasized below. In this chapter we focus on the processes that guard the proteome during aging, an aspect that had been considered less than DNA repair or lipid metabolism.

Even at a simple-minded level, it is clear that the various types of stress impact or damage the proteome. Loss of enzymatic activity and translational fidelity, accumulation of somatic mutations, altered post-translational modification, are all ways in which the protein homeostasis network is functionally eroded upon alteration the conditions in either the cytosol, the mitochondria or the endoplasmic reticulum (ER) (Fig. 2.1). Damage to the proteome is intimately linked to the body's stress responses, which deal with altered or damaged proteins by either attempting to repair the damage, dispose of the damaged molecules and/or compensate by increasing expression of the active one.

Cumulative Protein Damage and Aging

Cumulative damage is an often-discussed concept in the context of the genome, but it is clearly also important in the context of the proteome. Sustained exposure to environmental factors like UV radiation or free radicals causes incre-

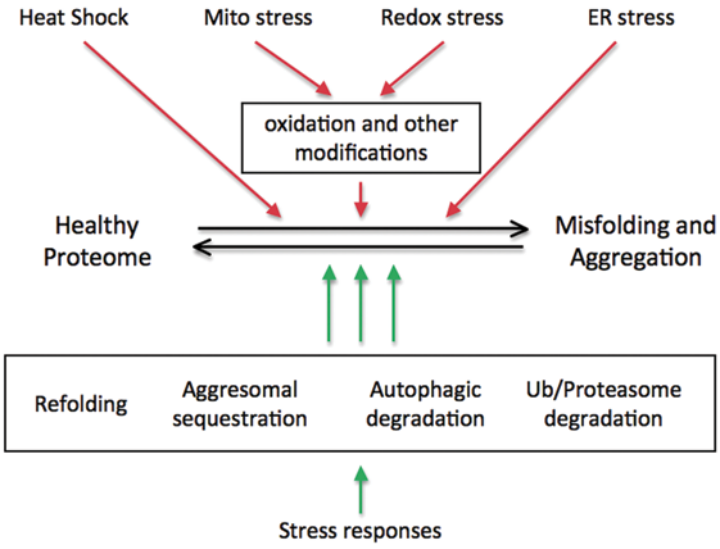

Fig. 2.1 Proteotoxic stresses and stress responses in maintaining a healthy proteome. While heat shock and ER stress largely cause protein misfolding, mitochondrial and other redox stresses mostly cause damaged proteins indirectly, through oxidation and other post-translational modification (*red arrows*). Stress responses, located at the various cellular compartments, control protein misfolding either by refolding, sequestration or degradation (*green arrows*)

mental damage that is proportional to age and is problematic in particular with long-lived proteins. Even normal metabolism associated with daily activities, such as respiration and signaling, interface with stress responses and can cause cumulative protein damage (Fig. 2.1). The late onset of many degenerative diseases is commonly interpreted as reflecting cumulative damage, for example by accumulation of misfolded or aggregated proteins. In addition, proteome analysis by global methods often report on progressively damaged proteins through post-translational modifications that increase with age [9–11]. A remarkable example was provided by two species of bats, which generally live far longer than predicted by their size, relative to other organisms. The proteomes of these bats exhibit significantly lower protein carbonylation, resistance to protein oxidation under conditions of acute oxidative stress, low levels of ubiquitination and reduced proteasome activity [12]. Thus, diminished protein damage and removal are related to longevity, yet much more needs to be learned about the process of cumulative protein damage and how the thresholds are set so as to initiate disease late in life.

The accumulation of damaged proteins with aging is coupled to a progressively decreased ability of the normal quality control mechanisms, as discussed further in subsequent sections. The ability of quality control systems to detect and deal with damaged proteins is important at all times, but becomes particular critical when the quality control capacity is reduced. For example, the level of expression of molecular chaperones, which assist other proteins to attain or maintain a folded conformation, itself changes with age.

Perhaps the most consistently identified ‘aging gene’ in humans that affects both long lifespan and susceptibility to certain age-related diseases is the apolipoprotein E gene (ApoE) [7, 8]. The inheritance of the three alleles of ApoE correlates with protection or susceptibility to aspects of aging [7, 8, 13]. This correlation has been appreciated since the early 1990s, when a case-control studies found the ε4 allele, a previously known risk factor for cardio-vascular diseases and Alzheimer’s disease, to be significantly less frequent in centenarians than in controls. The ApoE allele most frequent in centenarians was ε2, suggesting that it was protective [14]. The nature of the protective effect of the ε2 allele is not entirely clear, as it has been associated with risk for certain types of ischemic heart disease and hyperlipoproteinemia [15], or found to have no association, positive or negative, with heart disease or stroke [16, 17].

Multiple mechanisms have been proposed to explain the effects of the ε4 isoform of the protein, which differs by 2 amino acids from the ε2 isoform (arginines instead of cysteines at positions 112 and 158). First, hyperlipidemia and hypercholesterolemia resulting from lipid transport abnormalities can directly contribute to heart disease, atherosclerosis, and stroke (reviewed in [7, 8]. In Alzheimer’s disease, ApoE is thought to be involved in binding and clearance of amyloid β(Aβ). ApoE ε4 has decreased interaction with Aβ [7, 8] and its deposition into senile plaques is higher in patients carrying the ε4 allele. Its direct role in developing the disease is still not clear, though, as increased deposition of amyloid is also seen in cognitively healthy ε4 carriers [18]. Soluble Aβ is another potential target of ApoE: in a mouse

model, clearance of soluble Aβ from the brain depended on the isoform of human ApoE expressed [19]. Alternatively, ApoE ε4 may directly promote Aβ oligomer formation, more so than ε3 or ε2 [20]. Another hypothesis suggests a negative effect of ApoE ε4 on recycling of the ApoE receptor and thus on neural function [21]. Other possibilities include disruption of neural connectivity, independent of Aβ; increased tau hyperphosphorylation, cytoskeletal disruption and mitochondrial dysfunction by the proteolytic fragments of ApoE ε4, decreased protection from excitotoxicity, decreased synaptic function, and other deficiencies [22]. How all these phenotypic changes are caused by the two cys/arg substitutions is not known. One molecular consequence of the substitutions is decreased interaction of N-terminal and C-terminal domains of the ApoE protein, leading to decreased binding to the receptor and possibly to other interacting proteins [7].

It is worth noting that the association of specific alleles of ApoE with the detrimental cognitive phenotypes appears to be age-dependent [23]. For example, the presence of ε4 allele has been associated with better, not worse, memory and neural efficiency in young healthy people [24]. The differential effect of ε4 allele on memory performance depending on age was suggested to indicate antagonistic pleiotropy, a theme common for longevity genes: ApoE ε4 may be beneficial during development and early adulthood, at the expense of accelerated decline in cognitive function with ageing [25].

The action of ApoE exemplifies one type of coping with protein damage, systemic mechanisms that react to extracellular damaged proteins. The main focus of this chapter addresses a different type—intracellular mechanisms that monitor aging-dependent protein damage.

Proteostasis

Proteostasis, or protein homeostasis, is the state of a healthy proteome that is maintained by many sets of interactions among proteins and between proteins and metabolites, which enable the proteome to react to perturbations [26]. Like aging itself, proteostasis is highly complex and involves many hundreds of genes whose products engage in diverse protein complexes and function in many distinct metabolic pathways [26]. The interacting networks include protein synthesis, machineries of post-translational modifications, chaperone machines that assist in proper folding and various disposal systems. These protein networks are usually thought of as ‘stress responses’ (Fig. 2.1), but it is important to emphasize that they operate during normal physiology, even without external stress. Of course, since proteins are made and fold in different cellular compartments that present distinct environments, proteostasis networks in the cytosol, the endoplasmic reticulum and in other organelles have distinct functions and components, which evolved to address the distinct environmental conditions [27].

Also like aging, proteostasis is by definition tissue-specific, and while it has been studied in single cells for a long time, the molecular tools for studying it in whole

organisms are still evolving. Here, we focus on some concepts and challenges linking these longevity pathways to the homeostasis of proteins.

Caloric Restriction

One of the most basic and general factors in determining lifespan is the diet. Much experimental work in animal models has shown that caloric restriction (CR) enables extension of lifespan, and studies in human populations often emphasize the inverse relationship between caloric intake and healthy aging [28]. Much of our understanding of the genetic factors that favor long lifespan also relates the function of these genes to their effects on the metabolism. A simplistic view of caloric restriction is that increased food intake leads to metabolic demands, which over time stress the organism and thus translates to shorter maximal lifespan or to poorer quality of aging. Clearly, sensing the intracellular nutrient and energy status involves many of the arms of the proteostasis signaling pathways in the cytosol, the secretory apparatus and the mitochondria. Caloric restriction has been shown to affect many genes involved in preserving cellular homeostasis during stressful conditions. Such genes, sometimes termed 'vitagenes' [29], encompass heat shock proteins (HSP), the thioredoxin and sirtuin protein systems, nutrient sensing systems like mTOR and redox systems. As discussed below, all these systems affect protein homeostasis.

Protein Misfolding During Aging

Many diseases whose molecular basis is misfolding of proteins are associated with aging and affect almost every tissue. A typical feature of diseases such as Huntington disease, ALS, Alzheimer's disease or systemic amyloidosis is their onset late in the life of adults. An illustrative example of such diseases is cataract, where most commonly progressive loss of function of the crystallins in the lens leads to visual degeneration. Lens cells lack the machinery for folding and degradation of proteins and are therefore thought to depend on "holdase" chaperone function to prevent protein aggregation. α-crystallin, one of the small heat shock proteins, is the built-in lens chaperone. It is present throughout life in the protected environment of the lens and over time it accumulates damage from oxidation, non-enzymatic modifications and proteolysis. This damage partially unfolds the crystallins and creates aggregation-prone intermediates. In the young lens, α-crystallin sequesters such intermediates effectively, but in an old lens α-crystallin's capacity to bind polypeptides is saturated, and lens proteins are able to aggregate, promoting light scatter and loss of visual acuity [30].

The presence of insoluble proteins is a common feature of most aging-associated protein misfolding diseases (Fig. 2.1). Alzheimer's and Parkinson's diseases, type II diabetes and a host of lesser-known, but often equally serious conditions

such as fatal familial insomnia are characterized by insoluble protein aggregates. These aggregates are found either in the cytoplasm, the nucleus or the endoplasmic reticulum (ER) and are often used in the diagnosis of the diseases [31]. Nonetheless, all too often the data only correlate the aggregates with aging; relatively few studies address whether the misfolded protein itself causes accelerated aging through cellular dysfunction, or whether its misfolding and aggregation are consequences of the progressive decline of proteostasis [32–34]. The increased lifespan of *C. elegans* when aggregation-prone proteins are knocked down [35], or when proteostasis-stimulating compounds are used [36–38], supports the causative role of misfolding/aggregation. However, these approaches do not distinguish between the formation of protein aggregates themselves and accumulation of earlier misfolded intermediates. They also do not distinguish between genetic factors that change the aggregation behavior of the misfolded protein, and factors that change the organismal response to the misfolded protein; these can modify the lifespan independently of each other [39] and further work is needed to distinguish between these mechanisms. Furthermore, more data on the relation between protein aggregation and longevity in mammals is still required to demonstrate evolutionary conservation of the mechanisms that operate in the model genetic organisms [40].

Oxidative Damage to Proteins

Many candidate aging genes involved in the control of redox states and the response to oxidative stress have been highlighted and explored, no doubt because of the impact of the long-standing free radical theory of aging [41]. Oxidative damage to proteins, like damage to nucleic acids and lipids, is mediated by reactive oxygen species (ROS) and increases with age. ROS formation is a highly regulated process controlled by a complex network of intracellular signaling pathways. The intracellular nutrient and energy status of the cell, the functional state of mitochondria, and the concentration of ROS produced in mitochondria, in the cytosol and the ER are all sensed during normal physiology. CR appears to prolong life by reducing reactive oxygen species (ROS)-mediated oxidative damage. However, the correlation between ROS tolerance and lifespan is complex, which at the minimum contradict the existence of a simple relationship between ROS and aging [41].

Many long-lived mutants are resistant to treatment with ROS, and mutants selected for resistance to ROS are also long lived, but an exhaustive study on the effect of under- or over-expressing a wide variety of mouse genes coding for antioxidant enzymes showed that only the deletion of the *Sod-1* gene affects lifespan [42]. Consistent with this, *C. elegans* devoid of all 5 SOD enzymes have normal lifespan under non-stressful conditions [43].

Numerous antioxidative enzymes (catalases, superoxide dismutases, glutathione enzymes, metal-binding proteins) have provided promising avenues for mechanis-

tic analyses of ROS defenses, but inactivation of such candidate genes has shown at best a modest effect on lifespan. The current opinion is that ROS are not the sole determinant, but only a contributor to aging [41, 44].

Age-Related Decline in the Proteostasis Network

The proteostasis network is designed to react to the stresses that lead to misfolding, to the misfolded protein species, and to the protein aggregates that form [26]. Each of these processes can be countered by different arms of the network: Transcription and synthesis of anti-stress proteins such as folding enzymes or molecular chaperones are activated through the heat shock response (HSR), the unfolded protein response (UPR) in the ER and the mitochondrial UPR; Activities of chaperones are involved in recognition of misfolded molecules; Disposal systems such the proteasome or autophagy are activated to rid the tissues of the toxic misfolded species; Toxic proteins can be segregated to prevent cytotoxic effects.

The cytosolic stress-responsive machinery Since misfolded proteins usually accumulate in the cytosol, even if they originate from the secreted proteome [31] they occupy and titrate cytosolic HSPs, and thereby activate HSF1, the main regulator of the HSR [45]. The activation of HSF1 involves hyper-phosphorylation and deacetylation, allowing HSF1 to trimerize, translocate into the nucleus, and bind to heat-shock elements in the promoter regions of the many HSR target genes. During aging, there is clearly a decline in the capacity of the HSR to counter stress. Up-regulation of the stress-inducible HSP70 by heat shock is reduced approximately 50% in hepatocytes from old rats [46] and this decrease in HSP70 expression is also seen in other cells, such as human mononuclear cells and lymphocytes [47]. Similarly, expression of the other major cytosolic chaperone, HSP90, is reduced in fibroblasts from old rats [48]. The ability of HSF1 to bind the promoters of heat-shock genes in response to increased temperature was absent in the brains of 36 month old rats, while its levels were not affected [49]. In *C. elegans*, the inducibility of HSR becomes impaired in early adulthood [33], and this sharp transition seems to be mediated by the germline stem cell signaling at the onset of reproduction [50].

Expression of many HSPs differs significantly between tissues of ‘normal’ and long-lived Pit1(dw/dw) Snell dwarf mice. The magnitude and direction of the expression changes are tissue-specific and where HSP expression declines, it is often related to GH and/or IGF-I signaling [51]. These observations in mice parallel the role of IGF/insulin signaling in determining worm lifespan. On the other hand, forced overexpression of molecular chaperones is deleterious to growth of normal mammalian cells [52, 53], while the transformed phenotype of cancer cells depends on HSF1 and on an increased expression of chaperones [54–56]. A related example is the expression of the XBP-1 UPR transcription factor. Ectopic expression of the active form of XBP-1 specifically in *C. elegans* muscle reduced lifespan while expression in neurons prolonged longevity [57]. It appears then that the precise

regulation of proteostasis networks is essential for the health of the organism, while accumulating evidence points to its dysregulation during aging as one of the main causes of cellular dysfunction and disease onset (Fig. 2.2).

Molecular chaperones work in transient complexes with co-chaperones and catalytic factors and diminished chaperone capacity is brought about by down-regulation of these components, not only HSP70 and HSP90. For example, HSP110, which suppresses the important pro-apoptotic p38 MAPK stress signaling [58], is down-regulated in livers of three longevity mouse models (Pit1(dw/dw), Prop1(df/df), $Ghr^{-/-}$), and in both kidney and heart of Pit1(dw/dw) and $Ghr^{-/-}$ [51]. Hsph1 expression is likewise reduced in many tissues of mice exposed to the anti-aging effects of caloric restriction, including liver, heart, white adipose tissue, hypothalamus and colon [59].

Perhaps more importantly than the level of expression, the functionality of HSP70 from old rat livers was half that of HSP70 from young livers, as measured by *in vitro* chaperone assays [60]. The age-associated lower activity is important, because even if there is reduced level of HSP70 or HSP90, they are still highly abundant, accounting for more than 1 % of total cell protein even in aged tissues. Thus, reduced activity attests to reduced chaperone capacity, which may prevent repair of protein damage, leading to degeneration and cell death.

The ER stress-responsive machinery Reduced inducibility of stress responses and reduced levels of chaperones with age are not restricted to the cytosolic proteostasis network. Several studies had documented age-related decline in UPR function, another important arm of the proteostatic network that reacts to stress in the ER. In *C. elegans*, the capacity to activate UPR signaling is strikingly reduced during early adulthood [50, 57] coinciding with the decline of HSR [33], and when the ability to respond is diminished, the extension of lifespan is also diminished.

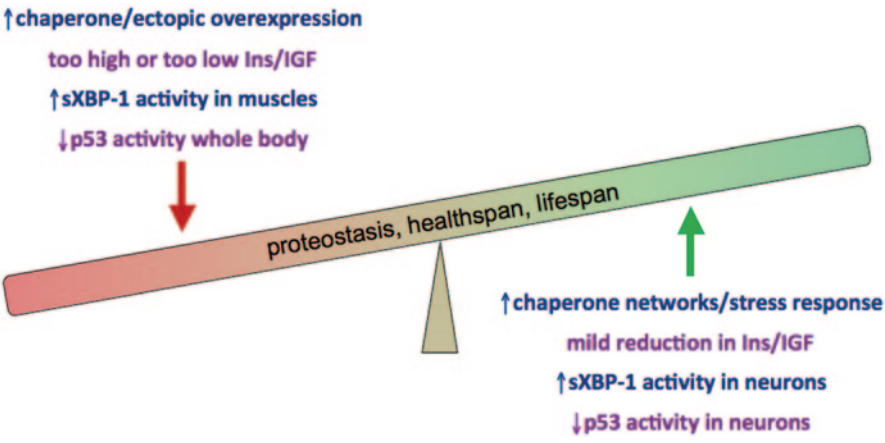

Fig. 2.2 Complexity of the effects of longevity pathways on aging. Examples of how intervention/regulation of transcription factors and chaperones either increase or decrease longevity and its quality

Constitutive expression of ER chaperones and folding enzymes also declines with age. Hepatic expression of BiP, calnexin, ERp57 and ERp72 declined 30−50% in aged rats [61]. Liver expression of the ER gene dnajc3 (encoding p58IPK) was diminished by all three of the above murine longevity models and was also lower in kidney, muscle, and heart of the Pit1(dw/dw) mice. Dnajc3 is a stress-inducible co-chaperone for the UPR chaperone BiP critical for survival in response to ER stress [62], and there is evidence that this role of Dnajc3 in cellular stress response can have widespread physiological effects. For instance, mice that lack p58IPK have severely reduced body fat levels, low body weight and are diabetic, most likely due to apoptosis of pancreatic β cells [63]. Since 1/4−1/3 of the proteome is secretory and originates in the ER, and considering the preponderance of membrane and secreted protein among aging-related protein misfolding diseases, these data support the concept that diminished chaperone capacity contributes to the physiological decline associated with aging.

The UPR is implicated in many of these neurodegenerative and aging-related protein folding diseases. Activation of the UPR signaling by accumulation of misfolded proteins (not necessarily aggregates) is well established [64], but the outcomes of this activation are varied. For example, response to most of the misfolded mutants of α1 anti-trypsin results in induction of ER-associated proteasomal degradation (ERAD), but in response to accumulation of the PiZ mutant the UPR induces mostly the autophagy mechanism of disposal [65]. In addition, the effects of individual UPR pathways are quite complex and can be disease-specific. For example, deletion of XBP1, the central mediator of the IRE1 pathway, did not influence prion disease progression in animal models [66], but did delay onset and progression of ALS and HD in respective mouse models by stimulating autophagy [67].

Proteasomes The proteasome is a multi-catalytic enzyme complex that mediates the degradation of both normal and damaged proteins. An age-related decline in proteasomal activity has been implicated in various age-related pathologies. Tomaru *et al*. created a transgenic mouse model with decreased proteasomal chymotrypsin-like activity, by over-expression of a thymus-specific subunit β5 that has weak chymotryptic activity [68]. These mice exhibited a shortened life span and developed age-related phenotypes. Poly-ubiquitinated and oxidized proteins accumulated in these mice, and the expression levels of cellular proteins such as Bcl-xL and RNase L were altered. When fed a high-fat diet, the β5 transgenic mice developed more pronounced obesity and hepatic steatosis than did wild-type mice. These results provide *in vivo* evidence that decreased proteasomal chymotrypsin-like activity affects longevity and aggravates age-related metabolic disorders [68]. In *C. elegans*, adaptation of peptide accessibility to the proteasome's active site in response to environmental stress, through modifying its 19 S regulatory particle, is necessary for both resistance to proteotoxic insults and maintenance of animal life span under normal conditions [69].

Although proteasome activity is diminished when poly-ubiquitinated substrates accumulate, Hipp *et al* showed that an aggregated ubiquitinated substrate is not a direct inhibitor of proteasomes, but rather impairs degradation by saturating some as-yet-unidentified component [70].

At least one signaling pathway for proteasome dysfunction is through reactive oxygen species that originate from malfunctioning mitochondria and trigger an Nrf2-dependent up-regulation of the proteasomal subunits. This is a tissue- and age-specific regulatory circuit that adjusts the cellular proteasome activity according to temporal and/or spatial demands [71].

Proteasomes and aggresomes are likely complementary quality control pathways, since aggresome formation/growth is promoted when proteasomes are inhibited [72]. Each of these arms of the proteostasis network declines with age, thus diminishing the response to metabolic stress or to accumulating misfolded proteins and reducing the chances of return to homeostasis.

Autophagy An independent way to dispose damaged proteins is the process of autophagy, where whole organelles can be engulfed by membrane, the contents of which then becomes an acidic degradative compartment. The formation of autophagosome requires a dedicated machinery that includes chaperones, and their relevance to aging is demonstrated by mutational inactivation of autophagy genes, which accelerates tissues aging in *C. elegans* and *Drosophila*. [73]. Inactivation of autophagy also suppresses life span extension by CR, aberrant insulin/IGF-1 or mTOR signaling, and lowers mitochondrial respiration. These findings suggest that the different longevity pathways in converge on the autophagy pathway to slow down aging and increase life span. Thus, autophagy is one central effector mechanism of animal aging [74].

Aggresomes One way to cope with accumulated misfolded proteins is to segregate them in cytosolic protein inclusions, termed ‘aggresomes’, that are usually perinuclear, near the microtubule organizing center and whose formation and maintenance depends on microtubule-based protein traffic [75]. Aggresomes often contain aging-relevant misfolded proteins and were suggested as a coping mechanism to protect cells from the cytotoxic effects of misfolded proteins [31]. One line of evidence supporting this notion is the enhanced proteo-toxicity in a yeast poly-Q model when aggresome formation is inhibited [76]. Targeting misfolded proteins to aggresomes seem to depend on recognition signals, like proline-rich domains, present on some aggregation-prone proteins. Another signal that is likely involved in the formation of aggresomes is the ankyrin-like repeat in synphilin 1, a protein related to Parkinson Disease [77]. It is not currently known how the aggresome formation machinery changes with age.

Proteostasis in Somatic Tissues Vs. the Gonads

Hallmarks of somatic tissues of aging flies are gradual accumulation of ubiquitinated and carbonylated proteins and reduction of proteasome expression and activity. In contrast, gonads of aging flies were relatively free of proteome oxidative damage and maintained substantial and highly active proteasomes. Exposure of flies to oxidants induced higher proteasome activities in the gonads, which were,

independently of age, more resistant than soma to oxidative challenge, based on reporter transgenes. Finally, inducible Nrf2 activation in transgenic flies promoted youthful proteasome expression levels in the aged soma, suggesting that age-dependent Nrf2 dysfunction leads to decreased somatic proteasome expression during aging [78]. The higher investment in maintenance of proteostasis in the gonads perhaps facilitates proteome stability across generations [78].

Like in *Drosophila*, *C. elegans* gonadal cells also display transcriptional and proteomic programs that endow them with stress-resistance, which renders them immortal and capable of regeneration on a scale not shared by somatic tissues. *C. elegans* mutants with increased longevity exhibit gene expression programs normally limited to the germ line. Decreased insulin-like signaling causes the somatic mis-expression of the germline-limited *pie-1* and *pgl* family of genes in intestinal and ectodermal tissues. The FOXO transcription factor DAF-16, the major transcriptional effector of insulin-like signaling, directly regulates *pie-1* [79]. Furthermore, Ruvkun *et al.* tested whether any of the other genes identified in screens for worm lifespan extension also displayed expression of germline genetic markers in somatic tissues, by tracking the expression of endogenous PGL-1. PGL-1 is normally restricted to the germline where it forms perinuclear punctate structures around mitotic and meiotic germ cells. However, RNAi inactivation of the *cct-4* and *cct-6* subunits of the cytosolic chaperonin complex, known to increase longevity, caused somatic pattern of expression of PGL-1—punctate perinuclear rings in hypodermal cells and cytoplasmic granules in intestinal cells, the same tissues where misexpression of *pie-1* in the insulin-like signaling mutants was observed [79].

The ability of *C. elegans* to maintain proteostasis declines sharply after the onset of oogenesis [50]. Arrest of germline stem cell rescued protein quality control, resulting in maintenance of robust proteostasis in different somatic tissues of adult animals. Germline stem cell-dependent modulation of proteostasis requires several different signaling pathways, each affecting different aspects of proteostasis and unable to functionally complement the other pathways. Shemesh *et al*. propose that the decline in the maintenance of proteostasis in somatic tissues is linked in this fashion to reproduction [50].

Together, these disparate observations support a model where longevity-promoting mutations such as decreased insulin-like signaling or reduction of chaperone capacity causes mis-expression of germline specific transcription factors in somatic tissues and this change contributes to the increased survival and health of these animals.

Hormesis, Proteostasis and Aging

One of the well-known phenomena regarding stress responses is that low doses of stress are protective. Such phenomena, termed “pre-conditioning” have been well-known from studying heat shock response in flies (e.g. [80]) and are related to the concept of ‘hormesis’ that emerged from toxicology [81] and applied to aging

research [29]. Many *C. elegans* longevity genes are associated with antagonistically pleiotropic effects on development, fertility and/or behavior [82]. One unifying principle is that life extension is generally associated with increased resistance to stress, but does not imply extension of good quality of life.

The *C. elegans* response to heat shock and mitochondrial dysfunction illustrates the role of hormesis in lifespan extension. Treatment of *C. elegans* with a brief but intense heat shock provides longevity increase, whereas sustained exposure is lethal, and subsequent treatments increasingly extend lifespan [83, 84]. This pattern is recapitulated in the response to inactivation of the mitochondrial ATPase *atp-3* [85]. Both studies highlight the importance of low dose of stress for aging. They also underscore why functional inactivation of genes in the same pathway, e.g. RNAi, do not necessarily yield comparable longevity. Because knockdown experiments differ in the amount of residual protein activity, the longevity effects are either not seen or are found to contradict those of other studies.

An important and little understood feature of hormesis is that mild exposure to one stress improves tolerance to other deleterious treatments, like reduced mitochondrial function, oxidative stress, radiation and toxic compounds [86]. Together with increased stress tolerance, hormesis also increases lifespan [86]. Thus, maintenance of physiological processes and repair of damage are improved by low stress and increase the ability to buffer the damage when higher level of stress is encountered. It will be important to define in molecular terms to what extent the hormetic cross-protection is due to activation of multiple cytoprotective mechanisms or to unknown protection effects conferred by individual pathways. For example, the combination of mild ER stress and apoptotic signals triggers an autophagic response, which is necessary for the hormetic protection from neurodegeneration. This activation of autophagy inhibits caspase activation and apoptosis [87]. This is an example of how effects of different longevity promoting pathways can be integrated by autophagy to promote longevity.

Relations of Candidate Pathways of Longevity Genes to Proteostasis

The Insulin-like Pathway

Tom Johnson and colleagues discovered in the 1980s the first gene shown to limit lifespan in the *C. elegans*, named *age-1* [88, 89], which was later shown to encode a PI3K protein [90]. The discovery and subsequent cloning of the *daf-2* gene [91, 92], encoding a *C. elegans* insulin-IGF receptor, brought the insulin-like signaling pathway (ILS) into focus as the major lifespan regulator in worms. When *daf-2* or *age-1* expression was “silenced” the activity of the insulin/IGF-1 pathway also decreased and the worms lived longer. Since then, many other genes associated with the insulin-like pathway have been found to affect the lifespan of fruit flies and mice. Reducing the activity of the insulin-like signaling cascade through mutant receptors

protects worms from proteotoxicity of various aggregative proteins, including the HD-linked peptide, polyQ40 [93] and the AD-associated peptide Aβ [94]. Inactivation of *daf-16* or *hsf-1*, encoding a FOXO transcription factor in the ILS pathway and a heat-shock transcription factor, respectively, and of genes for small heat shock proteins (HSPs), accelerated the aggregation of polyQ40, supporting the idea that the ILS coordinately influences aging and protein aggregation through the action of DAF-16 and HSF1 [95, 96]. Thus, the hypothesis that the insulin-like signaling pathway plays an important role in the aging process is well supported by studies of aging in model organisms.

Echoing these studies in model genetic organisms, the ILS pathway is clearly important for aging in mammals. Reduction of IGF1 signaling (as in animals heterozygous for the IGF1 receptor) was reported to protect mice from oxidative stress [97] and from Aβ toxicity, and to extend their lifespan [98]. In humans, Suh *et al.* demonstrated a gender-specific over-representation of heterozygous mutations in the IGF1R gene among female centenarians, associated with reduced activity of the IGF1R, high serum IGF1 levels and smaller stature [99]. Variants of other genes involved in the ILS pathway predominate among long-lived individuals, like the FOXO3a transcription factor, which is linked to extreme longevity in several, ethnically-disparate populations [100–103]. The extreme example of decreased ILS signaling in humans is presented by Laron syndrome—IGF1 deficiency due to inactive GH receptor that results in dwarfism and obesity on the one hand, but apparently complete protection from cancer and relatively long life on the other [104]. The emerging picture is of evolutionary conservation for the action of the ILS pathway in conferring both protection from aggregation diseases and increased longevity [34, 105].

Why and how does the ILS pathway impact longevity? The answers are complex and not entirely coherent. In part, the answers are complicated because candidate proteins (e.g. FOXO3a, IGF1R) regulate multiple processes that are not necessarily linked mechanistically. This leads to pleiotropy, which cannot be simply related to longevity. For example, $IGF1R^{+/-}$ mice [106] and humans [107] displayed growth retardation and other skeletal defects not seen in some of the other studies. At least some of these phenotypic differences are due to the genetic background [108], but clearly the conclusion is not as simple as reducing IGF signaling increases lifespan in all cases, and the multiple effects on other morbidities should be taken into account. All these effects, due not only to amount of signaling but also to its timing and its tissue location, are integrated to yield the seemingly opposite effects of the ILS pathway on longevity (Fig. 2.2).

Another complicating aspect is that serum IGF-I levels peak during human puberty and declines thereafter. This is an important aspect of IGF signaling during aging that has been linked to cerebrovascular and brain aging and cognitive decline [109]. The congenital animal deficiency models do not mimic this age-related decline. A recent model where murine IGF-I production is ablated late in adulthood shows that an intact GH/IGF-1 axis is essential to maintain health span. Its disruption, even late in life, causes increased liver pathology, oxidative stress in liver and muscle and accelerated bone loss [110].

The observations that IGF-deficient humans have lower risk of developing diabetes or cancer [104, 111], provide an evolutionary driving force for maintaining mutations in ILS pathway components, even if they come at a cost to development. This realization spurred manipulation of aging as an emerging strategy aimed at postponing the manifestation of late-onset neurodegenerative disorders such as Alzheimer's (AD) and Huntington's diseases (HD) and slowing their progression once emerged. Indeed, a novel ILS inhibitor, NT219, mediates a long-lasting, inhibition of this signaling cascade by a dual mechanism; it reduces the auto- phosphorylation of IGF1R and directs the insulin receptor substrates 1 and 2 (IRS-1 and -2) for degradation [38]. While NT219 treatment provides a proof-of-concept that ILS inhibitors can be useful against aging-onset diseases, it also highlights the complexity of the problem, because while decreased signaling is beneficial for an ALS worm model [112], increased IGF-I administration at disease onset slows the progression ALS in human subjects [113].

The ILS Pathway and Stress Responses

Insulin-like signaling and the stress responses in the cytosol (HSR), the ER (UPR) and the mitochondria are related mechanistically by proteostatic networks (Fig. 2.3a). One connection is through the *C. elegans* FOXO transcription factor *daf-16,* which appears to regulate HSP expression by interacting with HSF [95]. Unlike mammals, which have four FOXO genes with overlapping and different functions, *C. elegans* has to account for the same functions with a single FOXO-like protein, DAF-16. How then is its specificity achieved in biological regulation? Distinct isoforms are used to fine-tune the ILS-mediated processes in the context of a whole organism, and are expressed in distinct tissue patterns. One isoform, DAF-16a, is known to regulate longevity, stress response and dauer diapause while DAF-16b has only a minor role. A recently identified isoform, DAF-16d/f, is an additional isoform that is important for regulating longevity. DAF-16a and DAF-16d/f functionally cooperate to modulate IIS-mediated processes through differential tissue enrichment, preferential modulation by upstream kinases, and regulating distinct and overlapping target genes [114]. Another possible connection between HSR and ILS in *C. elegans* was recently proposed, by demonstration that ILS can directly control HSF activity by regulating two HSF-inhibitory proteins, DDL-1 and DDL-2. While both these proteins are evolutionarily conserved, it is not yet known whether this regulatory connection is also conserved [115].

The study of murine HSPs by [51] suggests important differences between the effects of GH/IGF-I signaling on HSP expression in mice, and the effects of ILS signaling on HSP expression in worms. The inhibition of ILS in the *daf-2* mutant increases expression of HSP70 and HSP90 family members [116, 117] as well as small heat shock proteins [95, 118, 119], all outcomes that affect cellular protein homeostasis. All these effects of systemic ILS mutations in *C. elegans* on heat shock gene expression, still do not provide much insight into the cells and tissues that express HSP differentially. The effects of diminished GH and/or IGF-I signals on

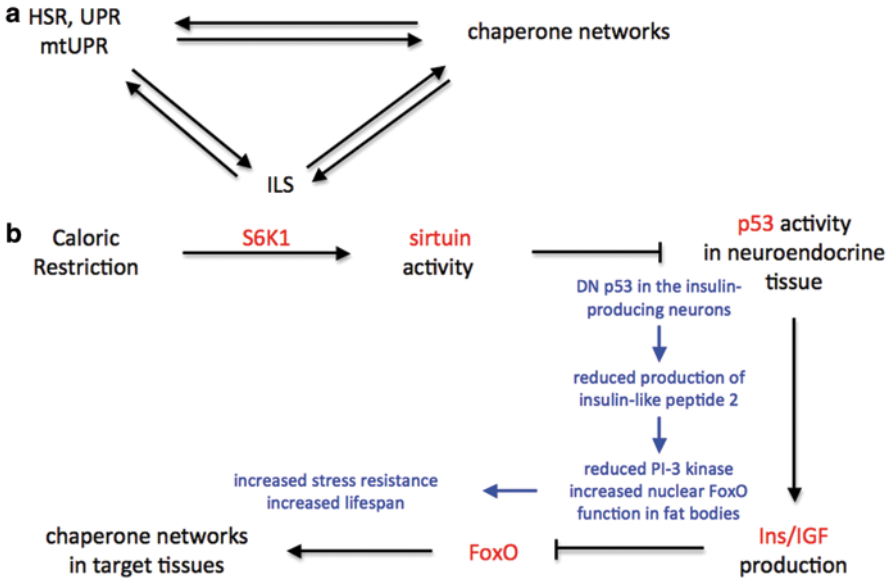

Fig. 2.3 Connections among proteostasis pathways affecting longevity. **a** Heat shock response (HSR) and the ER or mitochondrial unfolded protein responses (UPR, mtUPR respectively) up-regulate distinct chaperone networks. ILS can directly regulate chaperones through the FOXO transcription factor and it also interacts with the stress responses, via mechanisms that are still not fully elucidated. Chaperones, in turn, also impact ILS, for example by regulating IGF production. **b** Caloric Restriction increases sirtuin activity that, among other things, inhibits p53 activity in neuroendocrine cells with a consequent effect on insulin signaling. *Red*, representative components that have been defined as candidate longevity genes. *Blue*, a specific example from *Drosophila* shows how a dominant negative p53 (DN p53) reduces production of of insulin-like peptide 2 and thereby reduces downstream effectors and increases both stress resistance and lifespan [140]

HSP gene expression in mice are more complicated than those described in *C. elegans*. When growth hormone receptor-deficient mice are compared to long-lived dwarf mice, the beneficial phenotypes like increased lifespan and lower incidence of kidney disease, cataracts, and joint disease [120] are not related in a simple manner to the level or type of HSP expression [51]. In humans, genetic association studies have correlated polymorphisms of HSPs genes with longevity and age-related disease [121], but the mechanisms underlying such associations are not clear. While HSP expression appears to enhance overall proteome stability [26], and may counteract age-related conditions resulting from accumulation of poly-Q or prion proteins [122, 123], CR in mice promotes widespread diminution of HSP expression [59]. HSP expression may therefore have both positive and negative effects, depending upon the tissues or cell types, or upon the developmental or physiological state of the organism.

Even though growth occurs in every tissue and IGF-1 receptors are found throughout the body, there is considerable difference in the contribution of distinct cells and tissues to the ILS-related effects on aging [124, 125]. In retrospect, this

is not surprising given the hormonal nature of ILS, but nonetheless was only fully appreciated reasonably recently. The non-autonomous nature of ILS in *C. elegans* was proposed on the basis of both genetic [126] and microarray analyses [125] and followed by direct experimental demonstration that highlighted the role of neurons in secretion of insulin-like hormones [127].

The neuroendocrine nature of ILS signaling is also evident in extension of mouse lifespan through tissue-specific knockout of insulin receptor substrates [128, 129] and the IGF-1R+/− mice (see above). The reduced ILS as a result of targeting the brain, rather than in peripheral tissues, is particularly important as this sets the pattern for growth as well as lifespan [130]. IGF-1 feedback onto the hypothalamus during development plays a key role in determining the set point of the somatotropic axis throughout life. Thus, in both worms and mice neurons play a central role in ILS. Remarkably, as discussed below, the hypothalamus is also a central tissue for lifespan extension *via* another pathway, the sirtuins.

Another important connection between heat shock proteins and ILS is that during ER stress, IGF-I stimulates translational recovery and induces expression of the key molecular chaperone of the UPR, BiP/GRP78, thereby enhancing the folding capacity of the ER and promoting recovery from ER stress [131]. Conversely, BiP expression in CR mouse livers is reduced by 40% and IGF1R signaling regulates BiP expression *via* the PI3K/AKT/mTORC1 axis, independent of the canonical UPR and FOXO1 [132]. The stress response and chaperone systems also impact ILS by controlling the production of hormones (Fig. 2.3a). An example is the dependence of IGF-I and –II themselves on GRP94. As shown in both cell culture and a mouse model, the level of IGFs produced is proportional to the activity of GRP94 [133–135]. Therefore, it is possible that the decline in IGF levels in humans with advanced age reflects a decline in chaperone activity. Indeed, preliminary indications suggest that hypomorphic alleles of GRP94 exist in human populations and affect ILS signaling (Argon et al. unpublished).

In a *Drosophila* model of muscle mitochondrial injury, mild muscle mitochondrial distress preserves mitochondrial function, impedes the age-dependent deterioration of muscle function and architecture, and prolongs lifespan. Strikingly, this effect is mediated by at least two pro-longevity signaling pathways: muscle-restricted, redox-dependent induction of genes that regulate the mitochondrial UPR and the transcriptional induction of the *Drosophila* ortholog of insulin-like growth factor-binding protein 7, which systemically antagonizes insulin signaling and facilitates mitophagy (autophagic degradation of mitochondria). Given that several secreted IGF-binding proteins (IGFBPs) exist in mammals, this work raises the possibility that muscle mitochondrial injury in humans may similarly result in the secretion of IGFBPs, with important ramifications for insulin-like signaling [136].

The P53 Pathway

p53 is often studied as either a tumor suppressor or as a guardian of genomic integrity in response to cellular damage and stress. Yet p53 plays a third role—a longevity

regulator. Mouse models that expressed 2 truncated forms or a temperature-sensitive mutant of p53 [137, 138] were resistant to cancer, yet had significantly shortened longevity and a number of premature aging phenotypes. This effect on mouse longevity led Helfand *et al.* to investigate the role of the *Drosophila* p53 homologue, Dmp53, in longevity [139]. They found that null Dmp53 flies, while viable, are sick and have reduced lifespan, probably due to early negative effects on embryonic development. However, when two dominant-negative (DN) mutants of p53 that inhibit wild type p53 transactivation activity were expressed in neuronal cells of *Drosophila*, but not in other tissues, longevity was increased. Even more specifically, expression of the DN Dmp53 transgene in the 14 neurons of the brain that produce insulin-like peptides extends lifespan to a similar extent as pan-neuronal expression [140] (Fig. 2.2). The primary effect of DN Dmp53 in the insulin-producing neurons was on reducing production of insulin-like peptide 2 (dILP2), while other dILPs remained unaffected. This was sufficient to inhibit downstream insulin signaling, as evidenced by reduced PI-3 kinase function in fat bodies of both larval and adult flies (Fig. 2.3b). Moreover, increased dFoxO nuclear accumulation was observed in fat body cells, a key downstream readout for attenuated insulin signaling, as increased nuclear FoxO is associated with increased stress resistance [141]. Turning on expression of the DN Dmp53 construct in adult *Drosophila* brains increased lifespan while turning the expression off reduced lifespan extension, both in proportion to the age of turn-on/off [140].

Thus, alterations in p53 signaling in a specific subset of secretory neurons were shown to cause major effects on insulin signaling pathways in critical target organs such as the fat body. CR did not provide any additional lifespan extension beyond that observed in non-restricted neuronal DN Dmp53 flies [139], arguing that p53 signaling and CR operate in the same pathway (Fig. 2.3b). It is likely that this model applies also to mammalian systems, although with a more complicated network of interactors, whose unraveling should provide profound new insights into the genetics and biology of aging and longevity.

The Sirtuin Pathway

Sirtuins are evolutionarily conserved enzymes with histone deacetylases activity, which affect multiple pathways that are important for metabolic regulation and the overall health of organisms [142]. NAD(+) is a rate-limiting substrate for sirtuin deacylases and therefore an important cofactor regulating metabolic homeostasis. In the context of aging, an extra copy of the yeast *Sir2* sirtuin gene was found in the 1990s to increase the lifespan of yeast and similar extension of lifespan has been replicated in other organisms, including flies and worms [143]. However, the role of sirtuins in promoting mammalian longevity has engendered a fierce controversy. Some studies suggested that sirtuins mediate the lifespan-extending effects of CR. Phenotypically, resveratrol supplementation of obese humans for 30 days induced metabolic changes that mimic the effects of CR on energy metabolism and behavior [144]; this resveratrol effect is thought to be mediated by activation of SIRT1.

However, whole-body expression of *Sirt1* (the homologue of yeast *Sir2*) was not sufficient to extend longevity in mice as it does in invertebrates, although it improved general health [145]. In fact, even the effects in worms and flies were called into question because longevity co-segregated with a second-site mutation affecting sensory neurons in the background of the experimental strains [146].

It has since been shown that a restricted diet boosts *Sirt1* and neuronal activity in certain hypothalamic nuclei that control central autonomic nervous system functions in mice [147]. Like caloric restricted wild-type mice, BRASTO mice, which overexpress Sirt1 only in the brain, lived 11 % longer than their wild-type counterparts even on a normal diet [147, 148]. The adults were also more active physically and metabolically, just like young mice, suggesting that physiological aging slows down in BRASTO mice compared to controls. The benefit is at least partly due to more orderly sarcomeres and more normal mitochondria in their skeletal muscle [148]. Curiously, another BRASTO mouse with more uniform overexpression of *Sirt1* throughout all nuclei in the hypothalamus exhibited none of these signs of youth, suggesting that *Sirt1* expression specifically in the dorsomedial and lateral hypothalamus is important for the anti-aging effect [148]. This finding could help settle the controversy about the role of *Sirt1* aging in mice as high *Sirt1* expression throughout the body might suppress neural activity in the dorsomedial and lateral hypothalamus. It seems that in mammals, like in model organisms, the effects on longevity are due to the activity of certain neurons affecting certain target tissues, rather than to whole body, multi-tissue effects.

Another focus of controversy about sirtuins and aging has been whether the red-wine ingredient resveratrol exerts similar longevity effects to those of CR. Sinclair and colleagues discovered in 2003 that resveratrol sped up the normal deacetylase activity of SIR2 *in vitro* [149]. That discovery was challenged as pertaining only to *in vitro* assays and not to living cells. However, two new studies support that the fluorescent molecules used *in vitro* may have mimicked hydrophobic amino acids found in certain natural SIRT1 substrates. In fact, sirtuin activators appear to speed up SIRT1 only when it interacts with substrates that contain such hydrophobic residues [150, 151]. Importantly, the number of such substrates is limited and includes ones thought to help induce some of calorie restriction's key health-promoting effects, such as FOXO3a. The SIRT1 protein includes a single amino acid, Glu320, which is not essential for its normal deacetylase activity, but is critical for boosting the enzyme's activity and the mitochondrial effects by sirtuin activating compounds [151]. This implies that SIRT1 serves as a key channel for inducing the mitochondrial aspects of longevity.

Regarding sirtuin substrates, estrogen receptor (ER) signaling has a variety of neuroprotective effects, and several proteins involved in ERα signaling are acetylated, including ERα itself and Hsp90, a key chaperone in the functional regulation of ERα. The acetylation is important to the functions of these proteins, but deacetylases other than SIRT2 appear to be involved [152].

dSir2 overexpression in *Drosophila* and CR longevity extension were shown to operate in the same pathway [153]. Moreover, longevity extension via the sirtuins

pathway is related mechanistically to the effects of the p53 pathway, because *dSir2* overexpressing DN Dmp53 flies showed the same longevity as Sir2 overexpressing flies, indicating no additive effects. This suggests that CR, *dSir2* and DN Dmp53 all act through similar pathways of longevity extension, through the inhibition of p53's transcriptional activity by sirtuins, as discussed above (Fig. 2.3b).

A different pro-longevity mechanism for sirtuins was recently pointed out by Schmeisser *et al.*, who found that sirtuin-mediated lifespan extension depends on methylation of nicotinamide [154]. This is an unexpected activity of sirtuins beyond histone deacetylation. All sirtuins convert NAD(+) into nicotinamide, and provision of either nicotinamide or its metabolite, 1-methylnicotinamide, extend *C. elegans* lifespan even in the absence of *sir-2.1*. A previously unknown *C. elegans* nicotinamide-N-methyltransferase, encoded by a gene now named *anmt-1*, generates 1-methylnicotinamide from nicotinamide. Disruption and overexpression of *anmt-1* have opposing effects on lifespan independent of sirtuins, with loss of *anmt-1* fully inhibiting *sir-2.1*-mediated lifespan extension. 1-methylnicotinamide is in turn a substrate for an aldehyde oxidase, GAD-3, that generates hydrogen peroxide, which acts as a mitohormetic reactive oxygen species signal promoting *C. elegans* longevity [154]. NAD(+) levels are reduced in aged mice and worms and decreasing NAD(+) levels results in a further reduction in worm lifespan. Conversely, genetic or pharmacological restoration of NAD(+) prevents age-associated metabolic decline and promotes longevity in worms [155]. These effects are dependent upon the protein deacetylase SIR-2.1 and involve the activation of stress signaling via the mitochondrial unfolded protein response (mtUPR) and the nuclear translocation and activation of FOXO transcription factor DAF-16. The implication is that augmenting mitochondrial stress signaling by modulating NAD(+) levels may be a way to prevent or treat age-related decline [155]. Acetylation by SIRT1 was also shown to directly regulate the DNA-binding activity of human HSF1, providing a mechanistic connection for the requirement of HSF1 in regulating life span [156].

The mitochondrial deacetylase *Sirt3* is essential for antioxidant defense system [157] but its pro-aging effects are also becoming clearer at a biochemical level. In fasting mice, *Sirt3* expression is decreased in skeletal muscle, resulting in increased mitochondrial protein acetylation. Deletion of *Sirt3* led to impaired glucose oxidation in muscle, which was associated with decreased pyruvate dehydrogenase (PDH) activity, accumulation of pyruvate and lactate metabolites, and an inability of insulin to suppress fatty acid oxidation. Proteomic analysis showed that a major target of *Sirt3* deacetylation is the E1α subunit of PDH (PDH E1α) and *Sirt3* knockout or knockdown in myoblasts induced hyperacetylation of the PDH E1α, altering its phosphorylation and leading to lower PDH enzymatic activity. This inhibition of PDH activity switched skeletal muscle substrate utilization from carbohydrate oxidation toward lactate production and fatty acid utilization even in the fed state, contributing to a loss of metabolic flexibility. Thus, *Sirt3* plays an important role in part by regulating PDH function through deacetylation, thus affecting mitochondrial substrate choice and metabolic flexibility [158].

The Mtor Pathway

Much like sirtuins link genome quality control to a wide range of metabolic processes that impact aging, so do the mammalian (or mechanistic) target of rapamycin (mTOR) complexes. While the enzymatic activity of sirtuins is deacetylation, the activity of the TOR complexes is phosphorylation. Each is a common post-translation modification of enzymes, structural proteins or signaling proteins, and therefore affects either the level of activity or the stability of the protein and pathway.

The importance of mTOR for lifespan was first demonstrated by RNAi knockdown in *C. elegans* [159] and then extended by modulating the TOR homologues in flies, yeast and recently in mice. Lamming *et al.* demonstrated that female $Mtor^{+/-}Mlst8^{+/-}$ mice have reduced mTORC1 activity and increased longevity [160], similar to the phenotype reported by Selman *et al.* for mice that lack S6K1, one of the principal substrates of mTOR [161]. Thus, the linkage between mTOR and lifespan is conserved in evolution.

Most commonly, the tool used to invoke mTOR complexes in longevity is the inhibitor rapamycin. Rapamycin treatment was shown to extend lifespan at blood levels that were 3X those of the typical therapeutic range for human immunosuppression [162]. However, the mechanism(s) accounting for the anti-aging effects of rapamycin is not yet clear. The effect on aging maybe through the anti-cancer effect, or by reducing protein synthesis, or by inducing autophagy, or by affecting stem cell, as it favors the retention of "stemness" and a more youthful phenotype in the adult stem cells types that have been studied (for details see review by [163]).

In vivo, the mTOR pathway receives inputs through a wide variety of signaling mechanisms and has roles in many aspects of physiology, which have been reviewed in depth [164]. These inputs are integrated by two mTOR complexes: mTORC1 responds to signals that include amino acids, glucose, WNT ligands, oxygen, cAMP, and insulin/IGF-1, namely to changes in metabolites or hormones that often signify metabolic stress. When activated, mTORC1 regulates protein synthesis and cell growth through phosphorylation of substrates that include S6 kinase (S6K) and eukaryotic initiation factor eIF4E binding protein (4E-BP). Thus, mTORC1 shapes the proteome in part through new protein synthesis [165]. mTORC2 (consisting of mTOR, rictor, mLST8/GβL, mSIN1, protor, DEPTOR) responds to less clear signals but may be activated by interaction with ribosomes [166], and thus affects the proteome at least at the level of protein synthesis. Nonetheless, mTORC2 activity regulates diverse kinases, including AKT, serum/glucocorticoid regulated kinase, and PKC-α, and thus indirectly regulates activity and or stability of many kinase substrates [166].

Acute treatment with rapamycin inhibits mTORC1 signaling, restricting growth and promoting longevity without reducing insulin sensitivity. On the other hand, chronic rapamycin treatment inhibits mTORC2 as well, impairing growth and insulin signaling, and perhaps promoting longevity [167, 168]. Interestingly, lifespan extension by disruption of mTORC1 in worms requires the worm's homologues of NRF1/2 and FOXO, both transcription factors that control genes involved in stress defenses. Lifespan extension by rapamycin or by disruption of mTORC2, however,

requires only the NRF1/2 homologue. Consistent with a role for general stress defenses in the benefits of rapamycin, both worms and flies with impaired TOR function are stress resistant, and induction of NRF1/2 and FOXO target genes has been detected in the livers of rapamycin-treated mice [167, 169].

Despite the large number of studies with rapamycin, only recently was there genetic evidence that variation in any of the mTOR pathway genes plays a role in human longevity. Recently, using the Leiden Longevity cohort, seven of 40 mTOR pathway genes, and in particular Raptor, exhibited significant differential expression associated with longevity. This association was not explained by variation between the groups in the prevalence of type 2 diabetes, glucose levels or cancer [170]. It will be fascinating to discover which activities of mTORC1 are changed by these allelic variants.

It is important to emphasize that pathway cross-talks are important also to understanding mTOR activity towards longevity. There is an extensive cross talk between p53 and mTOR, one manifestation of which is that the activity of mTOR is increased in some but not all tissues of $p53^{-/-}$ mice, associated with the tendency to increased insulin and IGF-1 levels [171] (Fig. 2.3). At the same time, some of the endocrine and metabolic changes seen in diet-restricted mice were not seen in mice exposed to rapamycin, and the pattern of expression of hepatic genes involved in xenobiotic metabolism is also quite distinct in rapamycin-treated and diet-restricted mice [172]. These observations suggest that CR and mTOR inhibition extend mouse lifespan in different ways.

Conclusions

Many of the candidate longevity genes and so-called ‘vitagens’ affect longevity by impacting the health of the proteome. The major pathways of candidate longevity genes discussed here, meant only as examples, are all intimately connected with protein homeostasis: the mTOR pathway—though protein synthesis and activation of phosphorylation cascades; sirtuins—through changes in gene expression and protein modifications; ILS—through control of chaperone and anti-oxidant systems. These effects on the proteome lead to changes in the function and regulation of tissue and whole body metabolism. Even in the few major pathways used here as examples, aging is accompanied by reduced quantity of important components, or more importantly—reduced activity. These changes invariably degrade the ability of the protein networks to respond to stress, and even to normal life’s activities, and consequently, to restore the homeostasis. Understanding the impact of these and other pathways on the proteome is already beginning to yield chemical genetics approaches to improve protein homeostasis during aging.

A theme that emerges from multiple longevity pathways and several organisms is that the source of signals that matter for longevity is often neuroendocrine, either specific head neurons in the worm and fly or nuclei in the mammalian hypothalamus. This feature is shared between the sirtuin and ILS pathways, stress responses including HSR and UPR, and perhaps many other longevity genes.

Another non-surprising theme to many longevity genes is the cross-talk between their action in one longevity pathway and other pathways. The intercalation of the longevity pathways is simply a mechanistic reiteration of the complexity of the aging process. As a consequence of the extensive cross-talk, another expected theme is that "there is no free lunch". Almost any extension to lifespan or improvement of healthspan through manipulation of proteostasis genes comes at a cost of causing some morbidity. These considerations highlight the need for multiple specific readouts of manipulating longevity genes, beyond the simple measure of lifespan.

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Chapter 3
Autophagy and Aging

Nuria Martinez-Lopez, Diana Athonvarangkul and Rajat Singh

An Introduction to Autophagy

Autophagy is an evolutionarily conserved recycling pathway that maintains protein and organelle quality control in systems ranging from unicellular organisms such as yeast to complex multicellular systems i.e., flies, worms, and mammals [28]. In essence, autophagic pathways entail the recognition, sequestration, and delivery of cytosolic cargo to lysosomes for degradation [28]. In mammalian systems, three distinct forms of autophagy have been described, macroautophagy [28], microautophagy [61], and chaperone-mediated autophagy (CMA) [1, 14], that differ from each other in mechanism of delivery and the type of cargo delivered to the lysosome. Macroautophagy is an in-bulk degradative pathway that turns over redundant or damaged organelles and protein aggregates as well as soluble proteins (Fig. 3.1). Macroautophagy requires the *de novo* formation of double membrane structures termed autophagosomes, which sequester cargo and then fuse with lysosomes or late endosomes to form autophago-lysosomes and amphisomes, respectively. These fusion events result in the exposure of sequestered cargo to lysosomal acid-sensitive hydrolases that lead to cargo degradation (Fig. 3.1). In contrast, CMA selectively degrades single soluble proteins containing a specific amino acid signature, the KFERQ motif, which is recognized by cytosolic Hsc70 followed by substrate delivery to lysosomes by the CMA receptor, lysosome-associated membrane protein (LAMP)-2A (Fig. 3.2) [1]. In essence, autophagy pathways are considered as protective pathways, and a significant body of

R. Singh (✉)
Department of Medicine (Endocrinology), and Molecular Pharmacology, Diabetes Research Center, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Forchheimer Building, Room 505D, Bronx, NY 10461, USA
e-mail: rajat.singh@einstein.yu.edu

N. Martinez-Lopez · D. Athonvarangkul
Department of Medicine and Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2_3

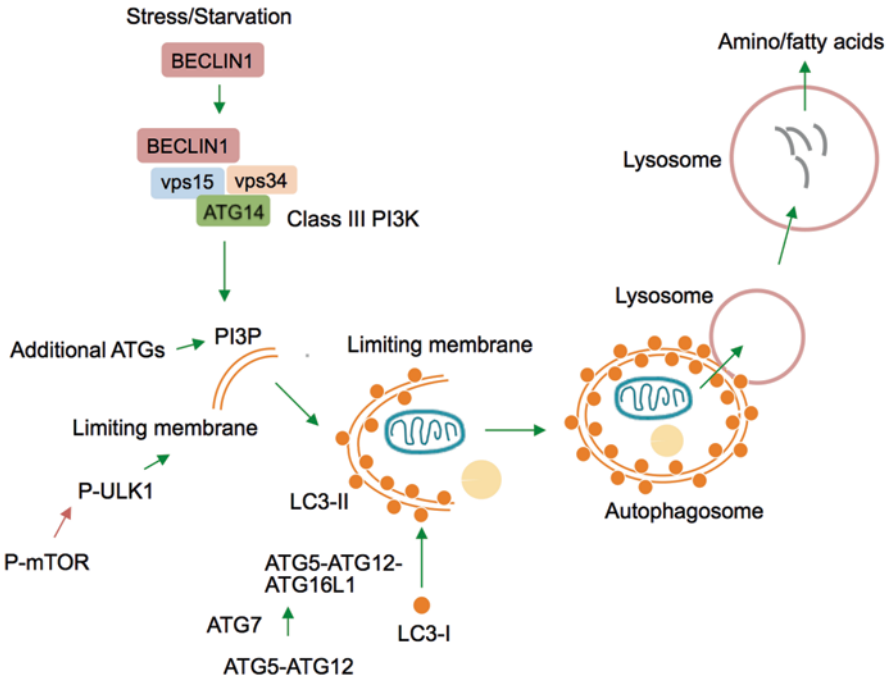

Fig. 3.1 Steps in the macroautophagy pathway. Macroautophagy is a cellular quality control mechanism that requires greater than 30 *Atg* gene products to deliver cytosolic cargo to lysosomes for their degradation. Starvation or stressors activate macroautophagy by increasing the interaction of Beclin1 with additional proteins to generate the Class III phosphoinositide 3-kinase (PI3K) complex, which gives rise to PI3Ps to recruit ATG proteins (e.g., ULK1) for autophagosome formation. Activation of ATG7 (E1-like ligase) forms the ATG5-ATG12 conjugate, which binds to ATG16L1 to form the ATG5-ATG12-ATG16L1 complex. ATG5-ATG12-ATG16L1 facilitates lipidation of cytosolic LC3-I into autophagosome membrane-bound LC3-II. LC3-II-positive autophagosomes engulf cargo and target it to lysosomes, wherein a battery of hydrolases degrades cargo into amino- and fatty acids. A tight regulatory crosstalk between the nutrient sensor mTOR and ULK1 orchestrates autophagy. *Green arrows* indicate activating steps and *red arrows* indicate inhibitory steps

evidence now supports this notion wherein cell/tissue-specific loss of autophagy has been shown to give rise to neurodegenerative disorders, metabolic defects, and cancers, to mention just a few. Macroautophagy and CMA have also been shown to decrease with age [9, 12], underscoring the possibility that compromised autophagy activity with age contributes to development of age-related diseases, for instance, neurodegeneration [39] and metabolic defects [66]. Macroautophagy and CMA are better characterized out of the autophagy pathways, and thus the discussion on the roles of these autophagy pathways on development of age-associated conditions will remain the focus of this chapter.

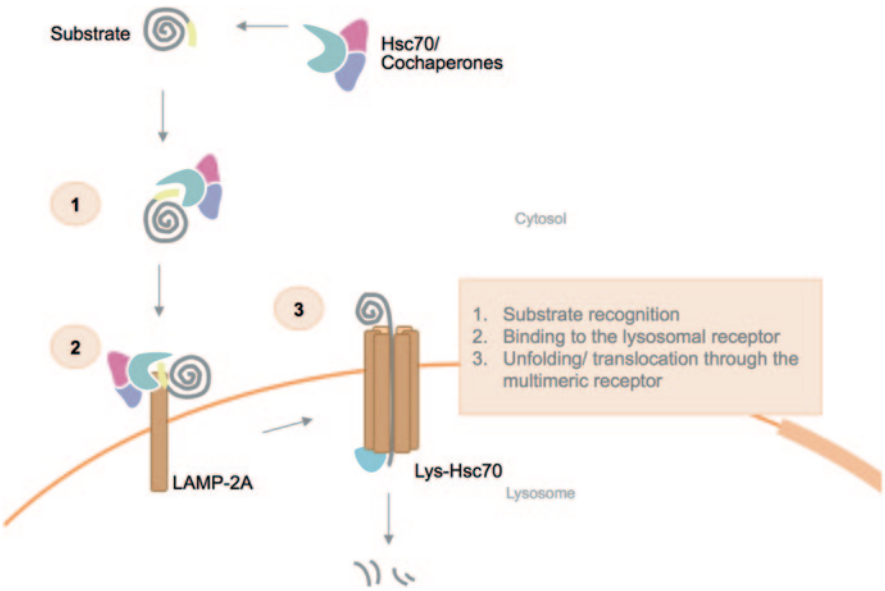

Fig. 3.2 Regulation of chaperone-mediated autophagy. Chaperone-mediated autophagy ensues by (*1*) recognition of substrate by the Hsc70/Co-chaperone complex followed by (*2*) binding of the substrate to the cytosolic portion of the LAMP-2A receptor. (*3*) Binding is followed by substrate unfolding, and along with additional regulatory proteins and lysosomal resident Hsc70 (Lys-Hsc70) the unfolded substrate is translocated across the lysosomal membrane substrate via LAMP-2A multimers for their degradation

The Macroautophagy Apparatus

Yeast genetic studies have identified over 30 autophagy gene (ATG) proteins that regulate tight orchestration of macroautophagy during basal conditions and its activation during stress or starvation [26, 62, 73]. In mammals, induction of macroautophagy requires Beclin-1 to interact with additional ATG proteins and vacuole protein sorting (vps) proteins that together constitute class III PI3K activity (Fig. 3.1) [74]. Activation of class III PI3K generates PI3Ps that recruit additional ATG proteins for autophagic limiting membrane formation [28]. Two ubiquitin-like conjugation cascades allow the expansion of the limiting membranes into autophagosomes [22]. An identity of some of the critical ATG proteins involved in autophagosome biogenesis is as follows: ATG7 displays a unique ubiquitin E1-like ligase activity that initiates binding of ATG12 with ATG5 (Fig. 3.1) [54, 70]. In subsequent steps, ATG12-ATG5 interacts with ATG16L1 to give rise to ATG12-ATG5-ATG16L1 [59]. ATG12-ATG5-ATG16L1 trimers, in turn, catalyze the conjugation microtubule-associated protein 1 light chain 3 (LC3) to phosphatidylethanolamine on the autophagic membrane (Fig. 3.1) resulting in membrane formation [23]. Lipidated LC3, i.e. LC3-II, is a molecular signature of the autophagosome [71], and is structurally and functionally essential for autophagosome formation and cargo

recognition due to its ability to bind to a cargo adaptor p62 [52]. Autophagosomes traffic along microtubules to fuse with lysosomes in a SNARE protein-dependent manner resulting in cargo degradation [30].

Signaling Pathways and Regulatory Steps of Macroautophagy

Induction of macroautophagy is tightly regulated by multiple signaling pathways that allow appropriate fine-tuning of this cascade in response to diverse environmental cues [28]. An important negative regulator of macroautophagy is the key nutrient sensor, mammalian target of rapamycin (mTOR) [56]. Nutrient availability and growth factors activate mTOR, which decreases macroautophagy activity by phosphorylating and inhibiting a key upstream kinase that activates macroautophagy, Ulk1 [36]. In contrast, nutrient depletion decreases cellular ATP and activates adenosine mono-phosphate-activated protein kinase (AMPK), a kinase known to initiate a cellular response following depletion of nutrients [25]. Activated AMPK induces macroautophagy by phosphorylating Ulk1 at residues [20] distinct from those phosphorylated by mTOR. The activation of Ulk1 facilitates trafficking of select ATG proteins to sites of *de novo* autophagic membranogenesis [78] through direct phosphorylation of these ATGs [53]. It had long been considered that macroautophagy activity did not require induction of ATG or lysosomal gene expression; however, recent studies from Ballabio and colleagues have identified a gene regulatory network that enhances macroautophagy by increasing lysosomal biogenesis [63]. Indeed, using systems biology, Ballabio and colleagues identified that a basic HLH-leucine zipper, transcription factor EB (TFEB), is a master regulator that positively controls expression of lysosomal and *Atg* genes by binding to their promoters [63]. In consistency with the known role of macroautophagy in quality control, follow up studies have now demonstrated that TFEB-induced activation of macroautophagy protects against α-synuclein toxicity in midbrain dopaminergic neurons, and in this way prevents progression of Parkinson's disease [16]. Similarly, overexpression of TFEB alleviates glycogen buildup in muscles as a result of Pompe disease [68], a severe form of metabolic myopathy occurring as a consequence of an absence of acid α-glucosidase. Increasing TFEB availability led to activation of macroautophagy followed by the exocytosis of autophagolysosomes containing glycogen cargo.

TFEB is regulated by its nuclear exclusion, and studies exploring regulation of TFEB activity identified two kinases—extracellular signal-regulated kinase (ERK2) and mTOR that control macroautophagy by modulating TFEB phosphorylation and hence its cellular localization [63, 64]. It has been shown that nutrient/growth factor-stimulated ERK2 activation results in TFEB phosphorylation, its cytoplasmic accumulation and macroautophagy blockage [63]. Work from the same group has also elucidated a novel lysosome-to-nucleus signaling by TFEB that involves mTOR [64]. In this regulatory axis, the availability of nutrients recruits

mTOR to the lysosomal surface [64]. At the lysosomal surface, active mTOR interacts and phosphorylates TFEB at Serine-142, which retains it in the cytosol and reduces macroautophagy. In contrast, fasting releases mTOR from lysosomes and decreases TFEB phosphorylation, which allows it to traverse to the nucleus and express macroautophagy genes. Recent work has also elucidated a master repressor of macroautophagy genes, ZKSCAN3, a zinc finger DNA-binding protein [6]. Indeed, knocking down ZKSCAN3 increased lysosomal biogenesis and activated macroautophagy [6]. Whether TFEB and ZKSCAN3 regulatory cascades are perturbed with aging remain unclear. Given the intricate control of macroautophagy at several steps by a plethora of regulatory proteins, it is conceivable that disruptions in any of the regulatory steps would contribute to age-related decline sin macroautophagy and cell function.

Molecular Players in Chaperone-Mediated Autophagy

A second form of autophagy, chaperone-mediated autophagy (CMA), functions in a manner quite distinct from macroautophagy. CMA utilizes a cytosolic chaperone, heat shock-cognate protein of 70 KDa (hsc70) for recognizing substrates, and the lysosomal-associated membrane protein 2A (LAMP-2A) receptor for internalizing these substrates into the lysosome [1]. The steps in the CMA pathway include (i) substrate recognition and delivery to lysosomes, (ii) protein binding and unfolding, and (iii) protein translocation into the lysosome followed by (iv) degradation in the lysosomal lumen (Fig. 3.2) [1]. The recognition motif for proteins selectively targeted for degradation via CMA lies in the primary structure of the protein itself, i.e., in the amino acid sequence KFERQ of the substrate protein (Fig. 3.2) [1]. The interaction of the substrate with cytosolic hsc70 allows its targeting to the lysosomal surface where it binds to the CMA receptor, lysosome membrane-spanning protein LAMP-2A (Fig. 3.2) [10]. The binding of substrate to monomeric form of LAMP-2A is followed by substrate unfolding possibly assisted by hsc70 and its co-chaperones [10]. It has also been shown that LAMP-2A multimerization at the lysosomal surface plays a critical role in substrate internalization [2]. The complete internalization of a substrate protein across the lysosome membrane also requires the lysosome-resident form of hsc70 (lys-hsc70) [1]. Lys-hsc70 together with lysosomal hsp90 also contributes to the assembly and disassembly of LAMP-2A at the lysosome membrane [2]. In addition, two interacting proteins, GFAP and EF1α, have been described to regulate CMA activity by modulating LAMP-2A multimerization, and its mobilization to lipid microdomains [3]. It is thus possible that changes in activity or levels of any of these proteins can compromise CMA activity with age.

Basal CMA activity has been described in multiple cell types as a quality control system to maintain functionality of the proteome [1]. Macroautophagy is the first line of defense during cellular stress, for instance, during starvation or oxidative stress; however, when the insult persists beyond six to eight hours, the induction of

CMA ensures cellular homeostasis through selective removal of damaged proteins. [1]. However the response to starvation appears to occur in a cell- and tissue-specific manner. Starvation-induced CMA is accompanied by changes in the distribution and properties of the lysosomal compartment [13]. The percentage of CMA-active lysosomes amplifies to accommodate cellular requirements for enhanced degradation. Such an expansion of the CMA-competent lysosomal system is paralleled by an increase in the levels of LAMP-2A present at the lysosomal membrane [13]. The elevated amount of LAMP-2A in these conditions is attained not by *de novo* synthesis but by decreased degradation of this CMA receptor at lysosomes [15]. The intrinsic selectivity of CMA is also well suited for the removal of proteins damaged during stress without perturbing nearby normally functioning forms of the same protein, by way of differential accessibility to the Hsc70 chaperone, of the CMA-targeting motif in its folded and unfolded state.

Macroautophagy and Aging

Decreased macroautophagy with age has been reported extensively in a variety of systems [18, 60]. The earliest studies to identify macroautophagy genes in the aging process were conducted in model organisms. An unbiased screen for chronological aging factors in yeast *Saccharomyces cerevisiae* identified multiple short-lived mutants with defects in macroautophagy [49]. Decreased lifespan in the nematode *Caenorhabditis elegans* ensued in mutants with loss-of-function of *Atg1* (Unc-51), *Atg7*, *Atg18* and *Beclin-1* [72]. Similar findings in the fruit fly *Drosophila melanogaster* also revealed reduced lifespan in mutants with reduced expression of *Atg1* and *Atg8* [65]. The generation of conditional mice models to selectively delete *Atg7* [38], *Atg5* [24], *Beclin* [44] or *Atg16l1* [5] was a significant step in our understanding of the consequences of loss of each of these genes in a tissue-specific manner. Indeed, whole body knockout of essential *Atg* genes leads to early postnatal death in murine models underscoring the crucial role of these genes in quality control, organogenesis, and differentiation. In contrast, conditional tissue-specific knockouts of *Atg7* or *Atg5* revealed milder phenotypes whereby younger knockout mice mimicked multiple age-associated phenomena such as aggregation of intracellular inclusion bodies and neurodegeneration [39], accumulation of lysosomes filled with the age-related pigment lipofuscin [69], disordered and defective mitochondria [35], accumulation of lipid droplets [67], increased protein oxidation [51], decreased muscle mass [48] as observed during sarcopenia of aging, and reduced differentiation of myogenic progenitors into muscle or fat [46]. A unifying theme emerging from all of these studies was the central role of macroautophagy in maintenance of quality control, and these studies suggest that a major determinant of the aging process is progressive loss of quality control. Consequently, it is not difficult to conceive that accumulation of toxic insults over a period of time as macroautophagy function wanes off results in age-associated pathologies, which include

reduced muscle mass, neurodegeneration, cardiac malfunction, lipid accumulation and insulin insensitivity.

In addition to changes in quality control, secondary factors may come into play to determine loss of tissue function with age. For instance, a recent study reveals that acutely depleting *Atg7* during adulthood was sufficient to dedifferentiate brown adipose tissue (BAT) and promote lipid accumulation [46]. Indeed, *Atg7*-depleted BAT displayed reduced expression of typical BAT markers, for instance the mitochondrial uncoupling protein-1. It is thus possible that lack of macroautophagy with age may allow highly specialized tissues, for instance muscle or brown fat, to dedifferentiate and lose their molecular and morphological signatures, which in turn would disrupt tissue function. A third layer of compromise added onto aged tissues is defective genome maintenance occurring as a result of the age-associated loss of macroautophagy. While it is well established that generation of oxidative stress and cumulative toxic insults with age associate with DNA damage, whether macroautophagy plays a role in genome maintenance remained unclear. To that end, two recent studies have now identified roles of macroautophagy gene products in regulation of genomic maintenance. In the first study, authors revealed that absence of macroautophagy results in a persistent delay in cell cycle progression that associates with aneuploidy due to insufficient cell growth and defective nuclear division [50]. In the second study, the authors highlight that UVRAG, a protein associated with macroautophagy, interacts with DNA repair enzymes to promote DNA double-strand break repairs, and that the absence of UVRAG results in heightened sensitivity of cells to genomic instability due to exposure to irradiation. In addition, the authors reveal that UVRAG interacts with centrosomes to enhance their stability, and interfering with this function gives rise to aneuploidy [80]. Given these recent developments, it is easy to place ATG proteins on a functional framework to consider how their loss could accelerate aging through effects on quality control, tissue differentiation and aberrant genomic stability.

How macroautophagy decreases with age remains unclear. Given the complexities in the orchestration of macroautophagy, and the stochastic nature of the aging process, it is likely that the mechanisms contributing to inhibition of macroautophagy are complex and multifactorial. Transcriptional down-regulation of key macroautophagy genes such as *Atg5* and *Atg7* was detected by genome-wide analysis in human brain during normal aging [45]. Age-associated decreases in autophagic proteolysis have been detected *in vivo* in rodent models and also *in vitro* in isolated hepatocytes from these animals [4, 19]. Our work also revealed age-associated decreases in ATG7 protein levels and macroautophagy activity in hypothalamic proopiomelanocortin (POMC) neurons, which regulate food intake and energy balance [33]. The precise mechanisms leading to decreases in ATG levels remain unclear, and whether reduced ATG protein levels with age are due to a general failure to maintain *Atg* gene expression or reduced upstream signaling remains to be seen.

To that end, mechanisms disrupting signaling pathways that converge on the macroautophagy pathway could also disrupt quality control and accelerate aging. The aging process also associates with differential effects on the regulation of macroautophagy activity by hormones; for instance, during aging, glucagon's stimula-

tory effect on macroautophagy is blunted while insulin's inhibitory effect remains largely intact. Thus, it is possible that early changes in the regulation of macroautophagy may be secondary to age-related perturbations in metabolism and aberrant hormonal responses to starvation [17–19]. Perhaps the best-described regulatory mechanism contributing to suppression of macroautophagy with age is mTOR signaling. As detailed in the previous sections, the nutrient sensor mTOR exerts an inhibitory effect on macroautophagy at multiple steps [56, 64], and aging and obesity are both associated with hyperphosphorylation, and hence hyperactivation, of mTOR [8, 31, 75]. It is thus plausible that increased mTOR signaling plays a predominant role in age-associated suppression of macroautophagy and in many of the previously reported metabolic and neurodegenerative disorders occurring with age. In support of this notion, studies have demonstrated that dietary incorporation of rapamycin, an agent that activates macroautophagy by suppressing mTOR, increases longevity in male and female rodents [27], although the direct contribution of macroautophagy activation to longevity in this model has remained untested. Furthermore, a recent study has revealed that intracerebroventricular administration of rapamycin reduced age-related defects in energy balance and obesity, at least in part, from restoring the function of hypothalamic POMC neurons [77].

Studies have identified several steps in macroautophagy that could be affected so as to disrupt cargo degradation. For instance, "cargo recognition failure", wherein the macroautophagy apparatus fails to recognize typical degradation signatures associated with aged or damaged organelles result in accumulation of unwanted substrates [47]. Amongst others, these mechanisms contribute to the development of neurological disorders, for example, Huntington's disease and Familial Parkinson's disease. In the latter condition, mutations in proteins, *parkin* and *PINK1* that give rise to the "eat me" signal to activate mitophagy, result in accumulation of leaky, reactive oxygen species (ROS)-generating mitochondria [21], which in turn compromises cellular function. A crucial protein p62 serves as an adaptor that allows cargo to be recognized and sequestered by autophagosomes [52]. It is thus expected that mutations in *p62/SQSTM1* would result in the failure to recognize cargo. Indeed, p62 mutations associate with Paget's disease of the bone and the severely debilitating neurological condition, amyotrophic lateral sclerosis [57], while mutations in Huntingtin, which alters its binding to p62, has been shown to give rise to a generic cargo recognition failure [47]. In addition, post-translational changes to p62, for instance phosphorylation of p62, have been linked to an antioxidant response via its ability to regulate the Keap1-Nrf2 antioxidant response pathway [29], and thus in principle, age-associated changes to p62 phosphorylation could modify macroautophagy and resistance to oxidative stress. Mutations disrupting early events in macroautophagy, for example autophagosome formation and membrane elongation, have also been linked to a number of disorders. While loss of a single allele of *Beclin1*/*Atg6* (Class III PI3K component) disrupts macroautophagy and results in increased incidence of cancers [44], mutations in *Atg16l1*, which determines autophagosome membrane elongation, has been associated with Crohn's disease and inflammation of the bowel [5]. Autophagosome-lysosome fusion events are also amenable to disruptions that would lead to accumulation of cargo-laden

autophagosomes, which in turn would compromise quality control. Recent work from the Cuervo laboratory [37] has identified that the previously reported inhibition of macroautophagy in rodent obesity models [67] occurs, at least in part, due to changes in the lipid composition of autophagosome and lysosomal membranes [37], which decreases their abilities to fuse with each other. It is thus conceivable that age-associated alterations in lipid metabolism and lipid accumulation could trigger a vicious cycle, which would further increase lipid accumulation and promote metabolic compromise by blocking autophago-lysosomal fusion.

Since it appears from the above observations that aging is associated with insufficient macroautophagy, the question arose whether increasing macroautophagy would delay aging and extend lifespan. In most animals studied to date, caloric restriction, which is reduced food intake without malnutrition, is the key anti-aging intervention [7] and is the most potent physiological inducer of macroautophagy [40, 43]. Caloric restriction not only extends lifespan but reduces the incidence of diabetes, cardiovascular disease, cancer, and brain-related diseases [7]. The effects of caloric restriction on macroautophagy induction are believed to be mediated by the activation of either of two energy sensors, AMPK and Sirtuin 1 (SIRT1) or by the inhibition of the insulin/insulin-like growth factor signaling pathway that ultimately leads to inhibition of mTOR signaling.

While inhibition of mTOR, either pharmacologically with rapamycin or genetically, extends lifespan in yeast, *C. elegans*, and *D. melanogaster*, the addition of caloric restriction does not further promote longevity. This suggests that there is a common mechanism that mediates the anti-aging effects of both these interventions, and macroautophagy may be the mediator as it has been shown that knockdown of essential *Atg* genes abrogates all life-extending effects of rapamycin in all species investigated. While rapamycin potently induces macroautophagy, it may also promote anti-aging effects via its anti-inflammatory properties. Likewise, inhibition of mTOR has major effects on protein translation and thus it is unclear whether mTOR inhibition extends lifespan primarily through induction of macroautophagy or whether the effects are mediated by macroautophagy-independent changes to the proteome. While transgenic overexpression of SIRT1 clearly delays aging and promotes lifespan, how SIRT1 triggers macroautophagy remains incompletely understood. Although it has been shown that SIRT1 forms complexes with essential macroautophagy proteins, ATG7, ATG5 and ATG8 and maintains them in the deacetylated state [42], further studies would be necessary to reveal the precise mechanism that is behind the requirement of SIRT1 in activation of macroautophagy.

A direct role of macroautophagy in promoting life-span, or perhaps more critically increasing healthspan, comes from studies showing that liver-specific overexpression of ATG7 prevents hepatic endoplasmic reticulum stress, increases hepatic insulin action, and improves glucose tolerance in a model of diet-induced obesity [76]. Studies in *C. elegans* have revealed that overexpression of the TFEB orthologue *hlh-30* extends lifespan that associates with increased nuclear translocation of this transcription factor indicating that longevity was a result of increased TFEB-driven expression of macroautophagy-related genes [41]. Interestingly, livers of dietary-restricted mice also revealed increased nuclear TFEB content [41]. On

similar lines, overexpression of *Atg5* in mice has been shown to extend lifespan by ~17%, increase leanness, and improve glucose clearance and motor function [55]. Furthermore, fibroblasts from transgenic ATG5 overexpressors have been shown to resist oxidative stress-induced cell death [55]. These restorative studies are a testament of the important role played by macroautophagy in extending lifespan and improving healthspan across multiple model systems. Given the function of macroautophagy in maintaining "clean" cells, it is likely that upholding macroautophagy function during aging will reduce the amount of toxic protein aggregates, reduce cell death, and improve cell function, all of which would, in principle, improve cellular function. Although, the effects of aging on the second form of autophagy, microautophagy [61], remain unknown, it is likely that microautophagy activity will decrease with age. However, further studies would be necessary to elucidate the consequences of aging on microautophagy.

Chaperone-Mediated Autophagy and Aging

In parallel to the inhibition of macroautophagy with age, CMA has also been reported to decrease with age [11]. Studies in livers from young and old rats showed a reduction in levels of the CMA receptor LAMP-2A in lysosomal membranes from aged livers [11]. These decreases in LAMP-2A levels did not associate with any changes in levels of cytosolic Hsc70, in fact a compensatory increase in lysosomal Hsc70 levels was observed in livers from aged rats [11]. Age-associated reduction in LAMP-2A levels correlated with decreased substrate binding to lysosomes and reduced substrate uptake by lysosomes, which would in all likelihood impact proteolysis rates and cellular quality control. Substrate binding/uptake assays in aged livers revealed a greater compromise in substrate uptake by lysosomes than in the ability of substrates to bind to lysosomes. These functional studies provided the first direct evidence that CMA decreases with age [11]. Subsequent work from the same group revealed that decreased LAMP-2A levels in aged livers were not due to a decrease in transcriptional regulation of LAMP-2A but due to increases in its degradation rates [34]. Kiffin et. al. used early passage fibroblasts and senescent late passage fibroblasts that display decreased LAMP-2A levels and CMA activity as observed in aged animals, revealed reduced half-life and faster degradation rates of LAMP-2A in late passage fibroblasts [34]. Indeed metabolic labeling and immunoprecipitation of LAMP-2A followed by quantification of radioactivity revealed a significant reduction in the half-life of LAMP-2A from 38 h (h) in early passage fibroblasts to 26 h in late passage senescent fibroblasts under basal conditions [34]. Although the mechanism for decreased lysosomal LAMP-2A levels with age remains unclear, it has been suggested that alteration in lysosomal membrane cholesterol content with age could contribute to changes in levels of LAMP-2A in cholesterol-rich lysosomal lipid microdomains [34] that are typically enriched in LAMP-2A [32]. Interestingly, feeding rodents a high fat diet or a diet enriched in cholesterol resulted in decreased lysosomal LAMP-2A levels [58]. This reduc-

tion in LAMP-2A mimicked those observed in livers from aged mice [11] and occurred due to mobilization of LAMP-2A to specific lipid regions on the lysosomal membrane where the receptor was unstable and underwent rapid degradation. Furthermore, changes in lysosomal membrane lipid composition following dietary challenges were similar to those observed in aged mice levels [58]. These results demonstrate that as observed with macroautophagy, chronic overnutrition plays a suppressive role of autophagy pathways. It thus remains possible that chronic dietary stress could contribute, in part, to decreased macroautophagy and CMA observed with age.

The final evidence of the benefit of increasing LAMP-2A levels to upregulate CMA and combat age-related conditions comes from a study demonstrating that supplying an extra copy of this receptor in the liver reduces damaged protein content [79]. Transgenic LAMP-2A overexpressors demonstrate improved cellular homeostasis, reduced oxidized protein load, and improved liver function. Employing zoxazolamine-induced paralysis time assay wherein recovery times are noted as a measure of hepatic function, it was shown that LAMP-2A-overexpressing aged mice were able to metabolize zoxazolamine in their livers at comparable rates to young wild-type controls [79]. This work demonstrates a direct role of restoring CMA-mediated quality control on improving organ function and builds a case for the activation of autophagy as an attractive strategy in the fight against age-related disorders.

Concluding Remarks

Autophagic pathways play crucial roles in cellular quality control, and a plethora of evidence has linked compromised autophagy to development of age-related disorders. While accumulation of damaged proteins, organelles, and aggregates were major considerations for age-associated loss of cellular function; the recently elucidated roles for autophagy in mobilization of lipid droplets and glycogen granules, tumor development, and microbial pathogenesis has expanded the list of conditions likely to be associated with loss of autophagy during aging. Although multiple mechanisms have been attributed to the loss of autophagy during aging, restoration studies with rapamycin or with upregulation of critical regulatory genes have demonstrated that longevity and health-span can potentially be improved by activating these pathways. Consequently, understanding the mechanisms contributing to reduction of autophagy with age, and exploring new ways to activate autophagy remain critical in fighting age-related disorders and improving healthspan in the aging population.

Acknowledgements We thankfully acknowledge NIH grants, DK087776 and AG043517, and an Ellison Medical Foundation award to RS. DA is supported by training grants 5T32GM728837 and T32GM007288. We thank Dr. Susmita Kaushik for assisting with designs of the models in Figs. 3.1 and 3.2.

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Chapter 4
A Genetic View of the Mitochondrial Role in Ageing: Killing Us Softly

Liron Levin and Dan Mishmar

Introduction

Ageing, ironic as it might sound, is the most common mortal condition in humans. The major difference among people is the way they age, namely their health status during the course of life, their ability to maintain their life style and their increased dependence on family members. Modern medicine has improved health care, increased awareness of the public to the contribution of life style to the propensity to develop complex disorders and in many cases provides medical treatments that increase life expectancy in the presence of diagnosed medical conditions. Despite that, we all get older, age differently and die.

Searching for the genetic basis of ageing has led to the discovery of multiple genes which are involved in increased maximum life span (Reviewed in:[1]). Another group of studies employed both family-based and hypothesis-free genome wide scans in centenarians to identify risk and protective factors against various types of age-related disorders and ageing itself [1]. A third type of studies identified certain changes in life style, such as dietary restriction, that alter life span (reviewed in: [2]). All these suggest, as intuitively might be expected, that ageing is not only a common phenotype but also highly complex, and therefore is influenced by the interplay of many genetic and environmental players.

Similarly to other common complex phenotypes, the genes associated with either extreme longevity or with susceptibility to age-related disorders explain only part of the genetic basis of these phenotypes, thus underlining the 'missing heritability' problem [3, 4]. Additionally, in many cases, genome-wide association studies identify only the residual effect of single genes on phenotypes. Since hypothesis-free studies that assess disease-association of combination of variants have computational and statistical limitations, alternative approaches should be sought. Hypothesis-driven approaches might overcome these limitations. Accordingly, one could

D. Mishmar (✉) · L. Levin
Department of Life Sciences, Ben Gurion University of the Negev, 84105 Beer Sheva, Israel
e-mail: dmishmar@bgu.ac.il

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2_4

assess the association of longevity with combinations of variants within factors that are known to interact in the frame of well-studied biochemical pathways. Here, we shall discuss pieces of evidence that support applying such a combinatorial approach to a central biochemical pathway in cellular metabolism and ageing—mitochondrial biogenesis.

The Mitochondrial Energy Metabolism and Age-Related Phenotypes

Mitochondria harbor the most efficient energy producing cellular machinery, the oxidative phosphorylation (OXPHOS). This machinery is operated by five multisubunit protein complexes harboring ~80 polypeptides: NADH unibiquinone oxidoreductase (complex I), Succinate unibiquinone oxidoreductase (complex II), Ubiquinol cytochrome c oxidoreductase (complex III), Cytochrome c oxidase (complex IV), and ATP synthase (complex V). Four of these complexes (complexes I, III-V) harbor subunits encoded both by the mitochondrial DNA (mtDNA) and the nuclear genome with the former evolving an order of magnitude faster than the latter (see detailed discussion below) [5]. It has been shown that the interactions between mtDNA and nuclear DNA-encoded factors are essential for mitochondrial activity and for the health status of the organism [6–8]. Hence, it is not surprising that many studies, including research performed by our group, revealed association of common genetic variants within genes encoded by both the mitochondrial and nuclear genomes with altered susceptibility to age-related disorders [9]. Accordingly, nuclear DNA-encoded modifying factors altered the penetrance of mtDNA disease causing mutations [6, 7, 10]. Taken together, the interactions between mtDNA and nuclear DNA-encoded factors are important for mitochondrial activity and for the life of the organism; additionally it is plausible that some mitochondrial-nucleus genotype combinations are more beneficial than others (see below detailed discussion).

Since the 1950's mitochondrial involvement in the ageing process has been suspected to occur mainly via the so-called 'vicious cycle' [11, 12]. This suggestion is based on the by-products of the energy producing activity of the mitochondria—the reactive oxygen species (ROS), which was thoroughly discussed elsewhere [13] and will not be the focus of this chapter. However, with this in mind, it is noteworthy that fast aging mice, overexpressing a mutant DNA polymerase gamma (Polgamma), the main mtDNA polymerase, had elevated mtDNA mutation rate with no excess of ROS, thus questioning the role of ROS in the so-called 'vicious cycle' of ageing [14]. This is in contrast to directed delivery of catalase, a player in the antioxidant stress response machinery, to the mitochondria in mice which extended their maximum life span [12]). Therefore, the accumulation of mtDNA damage over the lifetime of the individual and its role in ageing, still nourish a lively debate [13, 15]. Nevertheless the involvement of mtDNA variability in ageing has compelling evidence that will be discussed here.

The MtDNA: Its Higher Mutation Rate and the Functional Potential of MtDNA Genetic Variants

The essentiality of the maternally inherited circular mitochondrial genome (mtDNA) was already shown over 20 years ago when the first mtDNA disease-causing mutations were discovered [16, 17]. Since then, more than a hundred different mtDNA disease-causing mutations were described thus further fortifying the functional importance of the genetic information encoded by this genome [18, 19]. Hence, it is strange that the mtDNA in vertebrates is subjected to an order of magnitude higher mutation rate than the nuclear genome, mainly because of the reduced efficiency of mtDNA repair mechanisms and, possibly, due to the multiple replication cycles it undergoes [20]. This higher mutation rate is especially odd, since mitochondrial activities are operated by nearly 1500 factors of which most are encoded by the nuclear genome and interact with only 37 encoded by the mtDNA [21, 22]. Human mtDNA (~16.5 kb in length) encodes for 13 polypeptides of the OXPHOS system, 2 rRNAs and 22 tRNAs. The 13 mtDNA-encoded polypeptides include seven complex I subunits (ND1-ND6, ND4L), one complex III subunit (CYTB), three complex IV subunits (COXI-III) and two complex V subunits (ATP6, ATP8). Replication and transcription of the mtDNA are modulated solely by nuclear DNA-encoded factors which bind regulatory sites mainly located within the major mtDNA non-coding region, the D-loop.

Given the above, why did animals retain a genome that evolves so fast and hence, intuitively speaking, increases the odds of deleterious mutations to occur? Partial reply comes from a previous interpretation of the highly variable mtDNA as enabling adaptive flexibility to a large repertoire of environments that differ in nutrient resources and climate [22]. Another outcome of the higher mtDNA mutation rate is the accumulation of recurrent mutations in unrelated phylogenetic branches within a population of a given species (homoplasy) [23, 24]. By rigorous analysis of multiple human whole mtDNA sequences from ~9800 individuals, sampled from all major global populations, we found a large number of recurrent mutations that lie in the base of two or more unrelated phylogenetic nodes [23]. Among those, there were mutations with comparable functional potential to disease-causing mutations. Since these functional changes were retained in the population for thousands of years and were not removed by negative selection they are not likely to be neutral, neither are they deleterious. Hence, we hypothesized that these recurrent nodal mutations (RNMs) are the best candidates to carry adaptive properties. Alternatively, these RNMs were retained because their functionality was compensated by another mutation. As the recurrent 'functional' mutations did not occur in combinations within the mtDNA it is more likely that their candidate compensatory mutations hide somewhere in the nuclear genome. In consistence with this thought, because of the order of magnitude higher mutation rate of the mtDNA as compared to that of the nuclear genome in animals, mito-nuclear co-evolution is required to maintain proper mitochondrial function (see detailed discussion below [25–27]). The screen for such putative compensatory mutations should be performed in disease conditions suspected to have interfered with mito-nuclear interactions as compared

to controls. It could start by focusing first on nuclear DNA-encoded factors that directly interact with mtDNA-encoded factors (i.e. within the OXPHOS and the mitochondrial translation system); this screen could be eventually extended into all factors with known mitochondrial localization, such as those listed in Mitocarta [21, 28]. This thought directly applies to ageing: although the interactions between the nuclear DNA and mtDNA-encoded factors occur in all individuals and are essential to life, certain mito-nuclear genotype combinations could be less compatible than others [27, 29, 30]. Such reduced compatibility will be mild and un-noticed during most of the individual's life, but it might increase the susceptibility to either develop age-related disorders or undergo accelerated ageing. We elaborate on this thought in our 'Hypothesis' section.

One requirement for the above-described hypothesis is that at least some of the mtDNA variants should have functional consequences. Most of the mtDNA variants have little or no functional potential (neutral) but quite a few have occurred in highly conserved positions, thus implying a functional potential and adaptive properties during human evolution [23, 24, 31, 32]. Since mtDNA is maternally inherited it has been proposed that mtDNA mutations affecting male specific traits will not be selected against and hence will be retained in the population in contrast to mutations affecting traits expressed in both genders that will be removed by negative selection [33]. Indeed, it has been shown in Drosophila that mtDNA harbors a different mutational pattern in males as compared to females and hence mutations affecting male specific traits (such as sperm motility) were not selected against [33]. Maternal inheritance also results in the mtDNA being a single locus with negligible paternal contribution due to selective removal of sperm mitochondria [34–36]; accordingly, mutations accumulate over time to create mtDNA haplotypes in linkage disequilibrium. These haplotypes could be classified according to phylogenetic considerations and genetic distance into haplogroups. This phenomenon puts an obstacle in front of efforts to assess the functionality of specific mtDNA mutations independent of their linked genetic backgrounds (haplotypes, haplogroups).

Nevertheless, several findings support the functionality of mtDNA variants. First, mitochondrial depletion and re-population experiments in cell culture generated cytoplasmic hybrids (cybrids) that shared the same nuclear genetic background and differed in their mtDNAs. When mitochondrial function was compared between cybrids harboring mtDNAs of different mouse strains, rates of ROS production differed [37]. Secondly, cybrids harboring the M and N human mtDNA macro-haplogroups differed in calcium uptake [38]. Third, mutations defining mtDNA haplogroups altered the phenotypic penetrance of mtDNA disease-causing mutations such as Leber's hereditary Optic Neuropathy (LHON) [39, 40] or NARP (neuropathy, ataxia, and retinitis pigmentosa) [41]. Moreover, mtDNA haplogroups were associated with altered susceptibility to various disorders including schizophrenia [42, 43], age related macular degeneration [44], Parkinson's disease [45, 46], type II diabetes [8, 47–50] and asthenozoospermia [51], to name a few. Fourth, mtDNA haplogroups were associated with successful longevity in some populations [52–56] but not in others [57]. Fifth, cybrids harboring the human M9 haplogroup, which was over-represented in Tibetans and was interpreted as a player in

adaptation to high altitude, had higher complex I activity than cybrids with the B4c and F1 haplogroups [58]. Sixth, rat lines carrying different mtDNA backgrounds but constant nuclear genetic landscape differed in mitochondrial activity [59]. Similarly, experiments in introgression lines showed that certain mito-nuclear combinations altered lifespan in drosophila [27]. Finally, ancient mtDNA variants that occur within the mtDNA promoter region affected in vitro transcription, transcription factor binding capacity, and mtDNA copy number [60]. Hence common mtDNA variants could be functional, and since they were retained in the human population for a long time without being removed they could have adaptive properties [31]. It is therefore only logical that since the environmental conditions changed dramatically from ancient to modern times as well as life span and life style, and since adaptation is context dependent, some of the mtDNA haplogroups which carry those ancient mutations associate today with altered susceptibility to diseases. A good example is the mtDNA T3394C mutation that recurred in two mtDNA genetic backgrounds (haplogroups J1C1 and M9a) and altered the penetrance of LHON in these haplogroups [61–63]. However, such disease-association is not only influenced by the ever changing interplay of genes and environment but it is also modulated by the inherent interaction of mtDNA and nuclear DNA encoded factors.

Co-Evolution with the Nuclear Genome Plays a Role in Human Diseases

Since mtDNA mutations in animals accumulate an order of magnitude faster than loci within the nuclear genome, interacting factors encoded by the two genomes have to co-evolve in order to maintain mitochondrial function. Indeed, co-evolution was detected while analyzing the sequences of mtDNA and nuclear DNA members of the OXPHOS system in primates and other vertebrates [64–66]. Accelerated rate of evolution was also observed in nuclear DNA-encoded protein components of the mitochondrial ribosome as compared to cytoplasmic ribosomal proteins, thus probably adjusting for the high mutation rate of the mtDNA-encoded rRNA genes [67]. Similarly, in the case of mtDNA transcriptional regulation, while human mitochondrial RNA polymerase (POLRMT) could promote transcription of human mtDNA it could not transcribe the mouse mitochondrial genome [68]. Thus, three systems exhibiting mito-nuclear interactions, i.e. protein-protein interactions within OXPHOS, protein-RNA interactions within the mitochondrial ribosome, and protein-DNA interactions within mtDNA transcriptional machinery show evidence for mito-nuclear co-evolution.

The creation of cells harboring a human nucleus but mitochondrial DNAs from either chimpanzee or gorilla resulted in specific 40% reduction in the activity of OXPHOS complex I, thus exemplifying the functional importance of the mito-nuclear co-evolution [69]. We have already reviewed the importance of mito-nuclear co-evolution for the creation of reproductive barriers, an important step towards the emergence of new species [70]. Therefore, these topics will not be elaborated in the

current chapter. However, we do underline one aspect of the co-evolution between the mtDNA and nuclear DNA-encoded factors—its role in disease phenotypes. It has been shown that nuclear DNA genetic backgrounds could alter the penetrance of mtDNA disease-causing mutations [6, 7, 10]. Genetic variants of genotype combination of mtDNA and nuclear DNA were shown to synergistically increase the susceptibility to complex disorders such as type 2 diabetes [71]. Accordingly, we found that mtDNA haplogroup J altered the susceptibility to develop type 2 diabetes depending on the health status of the parents, implying the existence of a nuclear DNA-encoded modifier for the phenotypic impact of this mtDNA haplogroup [8]. Whereas certain mtDNA or nuclear DNA-encoded variants alone did not alter susceptibility to type 2 diabetes, their combination did in Ashkenazi Jews [72]. Finally, it has been shown in *C. elegans* (and to a certain degree in mice) that interfering with one of the mitochondrial ribosome proteins (MRPS5) triggered mito-nuclear protein imbalance, reduced mitochondrial respiration, and activated the mitochondrial unfolded protein response which directly affected longevity [73]. Taken together, these findings underline the importance of mito-nuclear interactions for the proper activity of the mitochondria. Affecting mito-nuclear interaction could lead to the emergence of complex disorders and may imply its role in ageing [74].

All the above-described examples focused on functional consequences of inherited mito-nuclear combinations of genotypes. Since multiple mtDNAs reside in each cell of our body (excluding erythrocytes) mutations in some of these mtDNA molecules could create intra-cellular mtDNA variation of which some mutants could, in principle, interfere with the proper mito-nuclear interaction and play a role in diseases involving mitochondrial function. In the next section this possibility is discussed.

Heteroplasmy: MtDNA Variation at the Organism and Intracellular Levels and Its Phenotypic Impact on Ageing and Age-Related Disorders

Unlike the nuclear genome, mitochondria reside in multiple copies per cell (a mean of a ~ 1000 in somatic cells), with each organelle containing an estimate of 2–10 mtDNA molecules that may differ in sequence due to point mutations or In-Dels (heteroplasmy). Since mitochondria are strictly maternally inherited, heteroplasmy does not stem from the mixture of maternal and paternal mtDNAs, apart from rare paternal leakage [75, 76]. Moreover, studies in mice, drosophila and *C. elegans* showed that autophagy-related mechanisms are involved in specific removal of sperm mitochondria [34–36] probably to avoid the adverse consequences of such mixtures [77]. Therefore, heteroplasmy in a given individual is most commonly comprised of mtDNA molecules sharing the same haplotype yet differing in only few mutations. Since intracellular heteroplasmy is based on a single haplotype, intra-cellular recombination events between mtDNA molecules, which is a mechanism not fully understood and demonstrated in mammals [61, 78], will likely not contribute much to the patterns of heteroplasmy.

Many mitochondrial disorders are caused by mtDNA mutations, and their level of heteroplasmy differs among tissues and determines the severity of the disease and even its penetrance [79]. Moreover, level of heteroplasmic mutations and deletions was shown to increase during the aging process [80–111], in the age-related Parkinson's disease [112–114], and may be correlated with age-related reduction in mitochondrial activity [89, 115] [and see thorough review by [74, 116]]. Does this mean that heteroplasmy is necessarily a 'bad thing' that the cells should protect against? Heteroplasmy was identified in many mammals and hence it is a normal phenomenon, not inevitably associated with disease [117–121]. If that is the case, what might be the contribution of this phenomenon to ageing and age-related disorders? By massive parallel sequencing and analysis of the population of mtDNA in blood and skeletal muscle of monozygotic twins we identified a strong tendency of heteroplasmic mutations to occur in non-coding mtDNA sequences thus reflecting the signature of negative selection [122]. Moreover, such negative selection acted on heteroplasmic mutations regardless of their prevalence in the studied samples, i.e. both in low abundance (<5%) or high abundance (>10%) heteroplasmy, thus reflecting a highly sensitive mitochondrial selective machinery. Similar selective sieve was observed while analyzing heteroplasmy in samples of the 1000 genomes project [123]. Since mtDNA disease-causing mutations will affect the phenotype of the individual only at high levels of heteroplasmy (~80% for point mutations, ~60% for large mtDNA deletions), the negative selection likely operates at the mitochondrial level rather than at the cellular or organism levels. Mitophagy is a mechanism that selectively removes dysfunctional mitochondria, and had been shown to be impaired only in certain types of Parkinsonism, which carry PARK2 mutations [124]. It is therefore uncertain whether the action of selective mechanisms such as mitophagy notably deteriorates with age thus leading to impaired selective removal of dysfunctional mitochondria and accumulation of deleterious mtDNA mutations. Since cellular mitochondria are usually interconnected via a network in which mitochondrial fusion plays a central role, nutrients and mitochondrial matrix components are shared among mitochondria thus allowing complementation of the reduced activity in some mitochondria by the more active ones. Since the efficiency of fusion is reduced during the ageing of the individual [125], the accumulation of heteroplasmic mutations will be less likely to be compensated, thus increasing the involvement of low abundance heteroplasmic mutations in reduced mitochondrial activity in particular, and in the ageing process in general (Fig. 4.1). This attractive hypothesis should be tested experimentally.

Are Mechanisms of Intracellular Selection of Functional Mitochondria Involved in Ageing and Age-Related Disorders?

Studies of the repertoire of heteroplasmic mtDNA mutations in the Pol-gamma mutated mice and in elderly (>60 years of age) human monozygotic twins revealed a repertoire of negatively selected mutations [118, 122, 126]. This implies that selective removal of dysfunctional mitochondria by a mechanism such as mitophagy

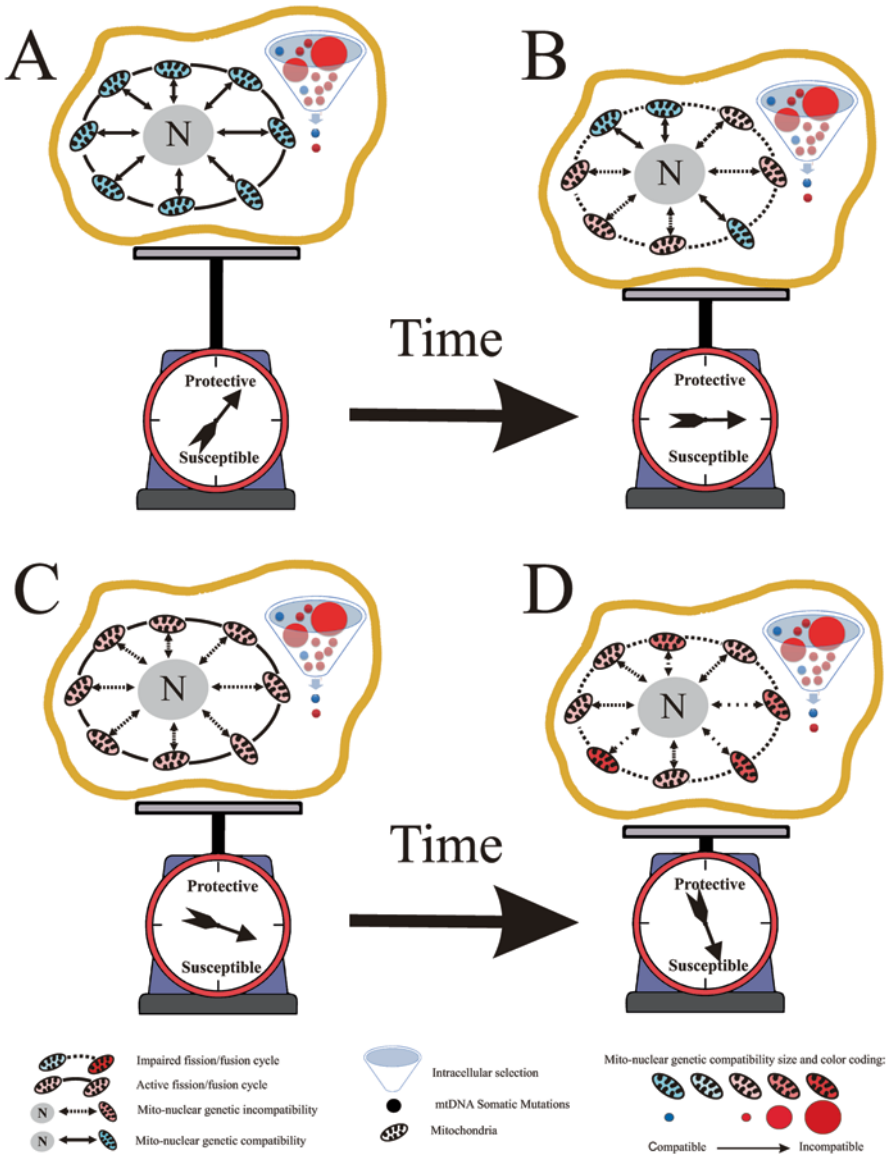

Fig. 4.1 An integrative model of mitochondrial genetic involvement in ageing**.** *Upper panel*: **a** Mito-nuclear inherited compatibility along with active fission/fusion cycles provides a protective starting point against chronic health conditions and increase the odds for life expectancy. **b** Accumulation of somatic (mildly) functional mtDNA mutations, which are overlooked by intracellular selection, impairs mito-nuclear inherited compatibility in a cumulative manner during the course of life. This, together with the deterioration of the efficiency of fission/fusion cycles, increases the susceptibility to chronic health conditions. *Lower panel*: **c** Inherited mild mito-nuclear incompatibility increases the susceptibility to age-related disorders and decreases the odds of longevity. **d** The accumulation of mtDNA somatic mutations with mild functionality during the course of life [which were overlooked by intracellular selection] further increases mito-nuclear genetic incompatibility

[127], is active both in early and in old age. Importantly, altered levels of Parkin affected heteroplasmic mutational levels (only of mutations that impaired mitochondrial membrane potential) in human cells [128]. In contrast to this assumption, members of the mitophagy mechanism, such as Parkin and PINK1, were shown to be mutated in certain types of the age-related Parkinson's disease. Additionally, Parkin overexpression in drosophila increased lifespan, reduced the level of a major mitochondrial fusion protein, mitofusin, in ageing flies, and increased mitochondrial activity [129]. Moreover, reduced activity of fusion-fission cycles in aged versus young human endothelial cells was observed [130], and reduced mitochondrial fission in a fungal system of ageing via mutated Dnm1 led to increased lifespan [130]. A computation model that took the latter finding into account showed that age-related deceleration of fusion–fission cycles reduces the odds of 'infecting' active mitochondria by compounds transferred from impaired organelles thus resulting in expansion of impaired mitochondria [125]. Undoubtedly, the involvement of mitophagy and fusion-fission cycles in ageing requires further investigation.

It was shown that age-related accumulation of mtDNA damage is mostly characterized by mutations with little phenotypic impact, as found in the mtDNA 'mutator' mice [126]. Therefore, the deceleration of fusion-fission cycles might reduce the ability of functional complementation between mitochondria within the cellular network, thus leading to reduced mitochondrial activity in aged individuals. In that case there is no need for 'infection' of active mitochondria by severely affected mitochondrial species but rather for the occurrence of many mildly affected mitochondria that cannot efficiently perform mutual functional complementation.

Hypothesis: The Functional Role of Recurrent MtDNA Mutations (Homoplasy) in Aging and Age-Related Diseases

The above discussed findings led us to the hypothesis that mtDNA mutations with only subtle functional potential may partially impair mito-nuclear interactions and hence mitochondrial function; such partial impairment will not cause adverse acute phenotypes to both the cell and the whole organism, but would rather promote chronic health conditions, including ageing. With this in mind one could envision an integrative model of mitochondrial genetic involvement in ageing (Fig. 4.1). The model assumes that despite the negative selection acting against the accumulation of mtDNA mutations, some mildly deleterious mutations will be overlooked. The evidence discussed in this chapter supported the functional importance of a subset of ancient mtDNA common variants that were retained in the population for a long time; such mutations could be either beneficial (in certain environmental conditions) or mildly deleterious. The same logic that applies to the mtDNA mutational repertoire in the human population, applies also to the intracellular repertoire of heteroplasmic mutations. Accordingly, cellular mitochondria may harbor mutations with functional potential that could be beneficial in certain cellular conditions but would be mildly deleterious in others. Such heteroplasmic mutations could be

overlooked by the intracellular selective mechanisms. This view is supported by the accelerated ageing phenotype expressed by mice with many mtDNA mutations, of which many mutations have apparent minor effect [132]. Similarly, recurrent mutations that preferentially occur in aged individuals (such as the mtDNA mutations T152C and T414G), have unknown functional consequences, though localized near the promoter region [110, 133, 134]. Taken together, this opens a plausible consideration that certain mtDNA mutations with functional potential, including recurrent mutations, will be overlooked by the mechanisms of selection and will eventually play a role in chronic phenotypes including ageing.

Although mtDNA mutational accumulation apparently plays a role in the ageing process, mtDNA genetic information constitutes only a minor part of the factors required for mitochondrial function. It is thus possible that the accumulation of mtDNA mutations during the life of the individual, and during evolution, will not only exert functional impact on the factor in which they occurred but may also affect physical or epistatic mito-nuclear interactions. What would be a suitable approach to detect the effects on such interactions? Disease association studies of complex disorders would be the first step to start looking for such a possibility, mainly because these diseases are likely caused by combinations of mutations with subtle functionality rather than by a single causal genetic alteration [135, 136]. As of now most association studies identified the involvement of single genetic variants with marginal phenotypic impact, thus leaving a high percentage of the patients un-explained, and hence reflecting the 'missing heritability' problem [137]. In different from genome-wide association studies which are commonly performed in a hypothesis-free manner, there is an advantage in taking a candidate pathway approach, where one focuses on pathways with known biochemical/structural interactions (such as mito-nuclear interactions), thus increasing the power of the analysis. Additionally, although there was no clear association of age-related heteroplasmic mutations with certain mtDNA genetic backgrounds [137] there is still room to explore possible combinations between these mutations and certain nuclear-DNA encoded genetic landscapes. A partial support for this view comes from the study of Yao et al [138]. These researchers showed that the accumulation of age-related somatic mutations depended on the nuclear genetic background in mice and was not associated with increase in ROS or senescence. Thus, it is possible that some of the somatic mutations having only subtle effect will express their phenotypic impacts differentially depending on the associated nuclear genetic background. Although attractive, this speculation requires further experimental support.

Conclusions

All vertebrates age, thus making the mechanism underlying ageing difficult to investigate. In this chapter we emphasized the involvement of combinatorial genotypes involving mito-nuclear interactions in complex age-related disorders and ageing. We especially hypothesized, that subtle mutations that were either inherited

variants in the population or intra-individual heteroplasmic mutations may have been overlooked by selection and are candidates to affect the interaction (both physical and epistatic interactions) of mtDNA factors with certain nuclear genetic backgrounds, but not with others. These variants will either (A) escape selection due to their subtle effect, (B) will be functionally complemented via the mitochondrial network (fission-fusion cycles) or will be beneficial in certain environmental conditions. In either case, with the changing metabolic demands during the lifetime of the individual the subtle functional impact of these variants (either inherited or somatic) have the potential to affect chronic disease conditions, including ageing.

Acknowledgements The authors would like to thank Dr. Orian Shirihai (Boston University), Dr. Antonio Enriquez (CNIP, Madrid) and members of the Mishmar lab for critical discussions that led to better articulation of the arguments brought about in this chapter. The study was funded by an Israeli Science Foundation grant (610/12) awarded to DM.

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Chapter 5
GWAS and Meta-Analysis in Aging/Longevity

Linda Broer and Cornelia M. van Duijn

The Role of Genes in Longevity

In the past century, most Western countries have experienced substantial increases in life expectancy. This has been mostly due to a marked reduction in early life mortality during the first half of the twentieth century, followed by an almost twofold reduction in mortality at ages above 70 years in the past 50 years [1, 2]. Longevity is often defined as reaching extreme age. There is no single accepted age threshold and given the ever increasing life expectancy and the differences in life expectancy across countries, the definition is time and place dependent. At present, the 'oldest-old' in Western societies are often defined as individuals of 85 years and older and this cut-off has been used in genetic studies in the past [3].

However, the percentage of individuals reaching 90 years of age, or even 100 years of age, is growing enormously [4]. The proportions of individuals in a given birth cohort projected to reach 90 or 100 years of age are shown in Fig. 5.1 [4]. The figure illustrates that the proportion of individuals who survive to age 90 has increased dramatically over the past century. When we consider the elderly of today (born between 1919–1929), less than 5% of the women and men reached age 90 years. Also for more recent cohorts (now middle age), reaching 90 years of age is still relatively rare, and reaching 100 years of age even more so. For example, 10% of women from the 1959 birth cohort are projected to reach 90 years of age, and only 0.3% are projected to reach 100 years of age [4]. How difficult it is to reach age 100 can be seen by comparing the likelihood of making it from birth to age 90 with the likelihood of making it from age 90 to age 100. These are similar implying surviving from 90 to 100 years is as difficult as living from 0 to 90 years [4].

C. M. van Duijn (✉) · L. Broer
Department of Epidemiology, Erasmus University Medical Center, Dr. Molewaterplein 50, PO-Box 2040, 3000 CA Rotterdam, Netherlands
e-mail: c.vanduijn@erasmusmc.nl

L. Broer
e-mail: l.broer@erasmusmc.nl

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2_5

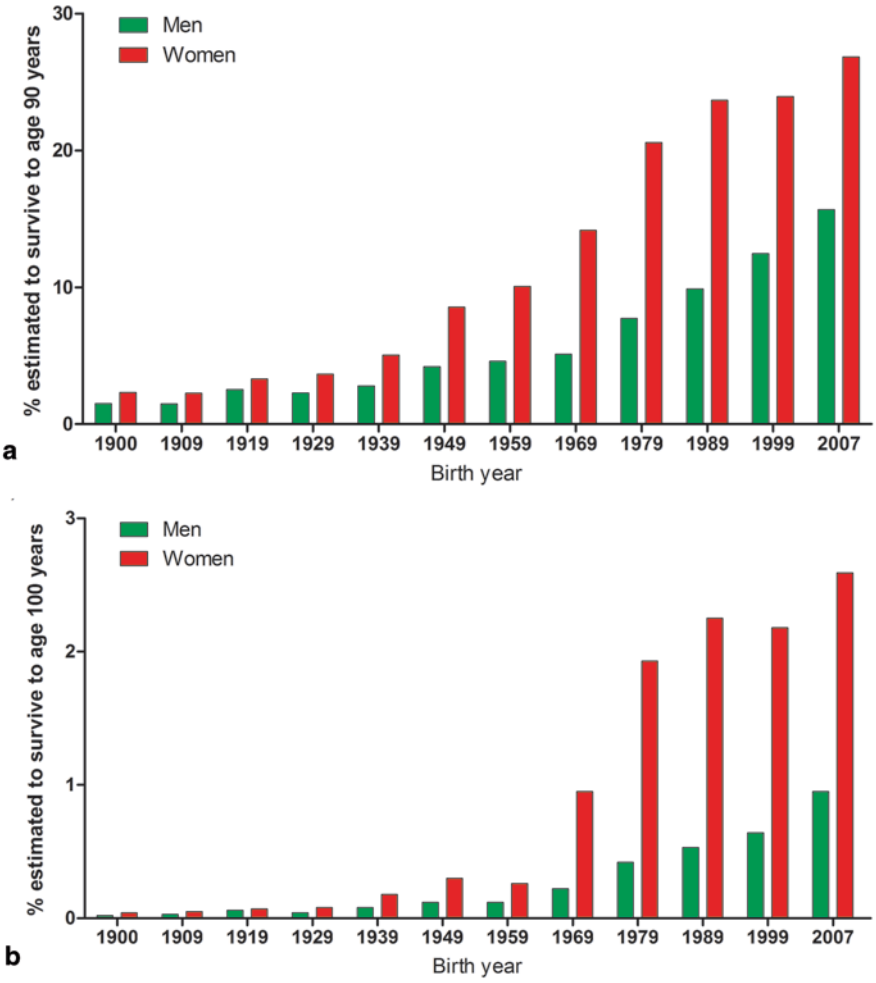

Fig. 5.1 Survivorship to ages 90 years (**a**) and 100 years (**b**) for the 1900–2007 birth cohorts by sex, United States. (Data were obtained from Arias [3])

Without a doubt, control of environmental risk factors (from hygiene to diet) and improved treatment of major diseases (cardiovascular and cancer) underlie the increase in life expectancy. Yet genes play a key role in reaching extreme age as shown by the fact that the heritability of age at death is higher at more exceptional thresholds for longevity [5]. Heritability estimates of age at death, range from 20 to 30% in twin registries [6, 7] and 15–25% in population-based samples [8, 9]. However, studying the birth cohort up that reached old age now, the heritability of surviving past 85 years was found to be 40% [10]. This is very similar to the heritability of other complex genetic traits such as blood pressure, lipids and diabetes [11, 12].

Despite the high heritability, in the previous century there has been little progress in unraveling the genetics of longevity. As has been the case for other diseases, candidate genes studies have limited few genes that have been replicated including apolipoprotein E and FOXO3a nor have family based studies yielded genes with major impact in the population [13–18]. Interestingly known age-related disease-causing genes have not been found to be associated with longevity [19, 20], suggesting there are specific domains in the genome that determine longevity beyond those that determine morbidity in the population. Genome-wide association studies (GWAS) have been able to identify hundreds of genetic loci for traits with similar or even lower heritability. The basic rationale of GWAS is that thousands to millions of genetic variants (single nucleotide polymorphisms (SNPs)) are measured across the genome and then associated to the phenotype of interest. Here we review the GWAS for longevity, distinguishing those using 85+ or 90+ as a cut-off and those studying more extreme phenotypes (100+ or centenarians). Further, we discuss an alternative approach to genetic studies of longevity using time to death as an outcome.

GWAS on Longevity (85+)

The Table 5.1 lists all currently performed GWAS on longevity, with their phenotype definition and sample sizes. The first GWAS investigating the longevity phenotype was by Newman et al [21]. This study consisted of 1836 individuals who achieved longevity, defined as 90 years and over. The comparison group consisted of 1955 individuals who died between 55 and 80 years of age [21]. The youngest age in the comparison group was set to match the minimum age at enrollment in one of the included cohorts. The maximal age at death in the comparison group was set arbitrarily at 80 years of age to include the majority of deaths, while excluding those individuals who survived far beyond average life expectancy for their respective birth cohort and nearly reached longevity [21]. None of the SNP-longevity associations achieved genome-wide significance (p-value $< 5 * 10^{-8}$). 24 independent regions with suggestive association levels (p-value $< 1 * 10^{-4}$) were identified (Table 5.2). 16 SNPs were successfully genotyped in a second stage including two independent cohorts. Only one of the SNPs had a smaller p-value after including the replication cohorts in the meta-analysis. This SNP, rs9664222, is located approximately 25 kb from the *MINPP1* gene and had an OR(odds Ratio) of 0.82 for the minor allele in the final meta-analysis (p-value $= 6.77 * 10^{-7}$) [21]. *MINPP1* encodes multiple inositol polyphosphate phosphatase 1, which is an enzyme that removes 3-phosphate from inositol phosphate substrates. MINPP1-deficient mice have no obvious defects, though targeted deletion *in vitro* is associated with slowed cellular proliferation [22].

Deelen et al published a longevity GWAS consisting of 4149 individuals over 85 years of age and a comparison group of 7582 younger controls [23]. In a first round including only one study (403 longevity cases and 1670 controls) no genome-wide significant SNPs were identified. For 58 out of 62 SNPs reaching a p-value $< 1 * 10^{-4}$

Table 5.1 GWAS studies on longevity phenotype

Author	Year	Phenotype definition	Sample size	Reference
Newman, AB	2010	Longevity: 90+ Comparison: died between 55–80	1836 cases 1955 controls	[21]
Deelen, J	2011	Longevity: 85+ Comparison: middle age	4149 cases 7582 controls	[23]
Nebel, A	2011	Longevity: 90 Comparison: middle age	763 cases 1085 controls	[27]
Malovini, A	2011	Longevity 90 Comparison: 18–45	582 cases 784 controls	[28]
Walter, S	2011	Survival: all-cause mortality Follow-up: 10.6 (5.4) years	25,007 total (8444 deaths)	[36]
Sebastiani, P	2012	Longevity 100+ Comparison: middle age	801 cases 914 controls	[30]

successful genotyping was obtained in the other cohorts (Table 5.3). One SNP on chromosome 19, rs2075650, was associated to longevity at genome-wide significance level (p-value = $3.39 * 10^{-17}$) with an OR of 0.71 [23]. This SNP is located in the *TOMM40* gene, next to the *APOE* gene. *APOE* had previously been identified as a longevity gene in candidate gene studies [24, 25], prompting the authors to test for independence of the signal. Conditional analysis confirmed the observed association was caused by the *APOE* locus. As *APOE* is known to be associated with Alzheimer's disease (AD), the authors investigated all other AD associated SNPs as summarized in AlzGene [26], but found no further significant associations.

In a GWAS including 763 longevity cases (90+) and 1085 control subjects of middle age Nebel et al tackled the longevity phenotype using both allele- and genotype-based case-control comparisons [27]. Their validation sample included 754 cases and 860 controls. 16 SNPs were selected for follow-up with *p*-values ranging from $3.7 * 10^{-10}$ to $9.1 * 10^{-6}$ (Table 5.4). Only rs4420638 was significant in the replication stage at a Bonferroni corrected level of significance (OR = 0.55; *p*-value = $1.9 * 10^{-8}$) [27]. This SNP is located 14 kb downstream of the *APOE* locus. Genome-wide haplotype analysis resulted in 13 significant haplotype pairs, but none were replicated.

Malovini *et al* used 582 longevity cases (90+) and 784 younger controls in their GWAS [28]. Three genetic models, allelic, dominant and recessive, were evaluated. In order to overcome the small sample size, resulting in a low power, a simulation study was performed which suggested that at a *p*-value cut-off for significance of 10^{-4} for at least one of the evaluated genetic models would guarantee a false-positive rate of approximately 2 in 10,000 independent tests. 67 SNPs with *p*-value < $1 * 10^{-4}$ were identified (Table 5.5). The authors claim that many of these SNPs mapped to genes potentially relevant to the aging process. One of the SNPs, rs10491334; *CAMKIV* had previously been associated with high diastolic blood pressure [29]. Replication of this SNP in 116 cases and 160 controls confirmed the finding (joint OR = 0.55; *p*-value = $1.68 * 10^{-6}$; dominant model) [28]. No replication for the other

Table 5.2 Top results of GWAS performed by Newman et al [21]

					Discovery			Replication	
SNP	Chr	Gene	EA	EAF	OR	*p*-value		OR	*p*-value
rs4443878	1	RGS7	T	0.04	0.41	$1.30*10^{-6}$		0.83	0.068
rs9825185	3	C3orf21	A	0.87	0.69	$2.50*10^{-6}$		0.91	0.045
rs954551	6	GRIK2	A	0.75	1.30	$5.30*10^{-6}$		NA	NA
rs7624691	3	IL20RB	T	0.57	1.25	$8.80*10^{-6}$		1.05	0.092
rs10888267	1	OR2W3	T	0.55	0.80	$9.70*10^{-6}$		NA	NA
rs9972933	17	ACCN1	T	0.23	0.77	$1.10*10^{-5}$		0.89	0.003
rs2739532	4		C	0.27	1.48	$1.10*10^{-5}$		NA	NA
rs8029244	15	LASS3	A	0.49	0.79	$1.20*10^{-5}$		0.90	0.002
rs16850255	1	PAPPA2	T	0.79	1.33	$1.20*10^{-5}$		1.09	0.041
rs1543505	14	REM2	A	0.72	0.79	$1.30*10^{-5}$		0.89	0.001
rs7321904	13	SPRY2	T	0.07	0.64	$1.30*10^{-5}$		0.92	0.179
rs17401847	1	OTUD3	A	0.85	0.72	$1.40*10^{-5}$		0.89	0.015
rs3124736	10	CASP7	A	0.03	2.30	$1.40*10^{-5}$		NA	NA
rs690232	9	DIRAS2	A	0.30	1.27	$1.60*10^{-5}$		NA	NA
rs9664222	10	MINPP1	A	0.21	0.77	$1.60*10^{-5}$		0.82	$6.8*10^{-7}$
rs11157721	14	LOC196913	T	0.39	0.79	$1.70*10^{-5}$		0.90	0.002
rs4690810	4	SC4MOL	T	0.65	1.27	$1.90*10^{-5}$		1.08	0.044
rs11605096	11	TMPRSS5	A	0.12	0.71	$1.90*10^{-5}$		NA	NA
rs16972414	18	PIK3C3	A	0.70	1.27	$2.00*10^{-5}$		NA	NA
rs12935091	16	ZNF19	A	0.93	1.61	$2.00*10^{-5}$		1.25	0.002
rs210332	14	BMP4	T	0.81	0.75	$2.30*10^{-5}$		NA	NA
rs17369174	8	CRISPLD1	T	0.90	1.45	$2.30*10^{-5}$		1.16	0.014
rs6721003	2	SCN7A	A	0.45	1.23	$2.40*10^{-5}$		1.09	0.006
rs4734457	8	ANKRD46	A	0.10	1.75	$2.50*10^{-5}$		1.10	0.098

EA effective allele, *EAF* effective allele frequency, *OR* odds ratio

suggested associations was attempted. Functional analysis showed that individuals homozygote for the polymorphism had significantly lower protein levels of CAMKIV compared to individuals carrying the wild-type gene. Additionally, *CAMKIV* incudes phosphorylation of a known longevity gene identified in candidate gene studies, *FOXO3* [28].

GWAS on Longevity (Centenarians)

To date, there is only the study of Sebastiani *et al* that included 801 unrelated centenarian cases and 914 population controls [30]. The controls were genetically matched to the cases that were either spouses of centenarian offspring ($n=241$) or

Table 5.3 Top results of GWAS performed by Deelen et al [23]

			Discovery		Replication	
SNP	Chr	Position	OR	p-value	OR	p-value
rs2075650	19	50087459	0.53	$2.65*10^{-6}$	0.71	$3.39*10^{-17}$
rs2003499	7	98852920	1.59	$8.07*10^{-5}$	1.19	$2.19*10^{-4}$
rs4736209	8	140208116	0.64	$9.50*10^{-6}$	0.90	$4.51*10^{-4}$
rs1516507	10	78707638	1.36	$9.67*10^{-5}$	1.09	0.002
rs4110518	10	96640560	1.52	$3.26*10^{-6}$	1.10	0.004
rs6577989	8	140182076	0.68	$3.24*10^{-5}$	0.92	0.006
rs7830605	8	140200576	0.69	$4.07*10^{-5}$	0.92	0.007
rs10401068	18	65414116	1.36	$9.62*10^{-5}$	1.08	0.008
rs1893132	18	2133155	2.07	$3.19*10^{-6}$	1.20	0.013
rs7661225	4	186275651	1.58	$6.59*10^{-5}$	1.12	0.013
rs886550	7	43302768	1.60	$5.75*10^{-5}$	1.12	0.019
rs11129533	3	32810964	1.39	$5.65*10^{-5}$	1.07	0.019
rs7005993	8	22845367	0.64	$5.38*10^{-5}$	0.92	0.021
rs2033563	8	103723247	1.48	$1.76*10^{-5}$	1.08	0.024
rs13248142	8	140182096	0.55	$1.43*10^{-5}$	0.92	0.024
rs625249	11	93149789	0.72	$9.29*10^{-5}$	0.94	0.027
rs1421746	5	127179875	1.38	$4.04*10^{-5}$	1.07	0.028
rs660100	1	4462210	1.48	$1.63*10^{-5}$	1.08	0.029
rs9827142	3	192599618	1.49	$4.88*10^{-6}$	1.07	0.029
rs9868286	3	192564180	1.48	$7.40*10^{-6}$	1.07	0.034
rs4681554	3	150992952	0.64	$9.82*10^{-5}$	0.94	0.050
rs642990	1	54461104	0.73	$9.81*10^{-5}$	0.95	0.052

Table 5.3 (continued)

			Discovery		Replication	
SNP	Chr	Position	OR	p-value	OR	p-value
rs12548929	8	140305428	0.55	$3.52*10^{-5}$	0.93	0.058
rs12548622	8	140190418	0.54	$8.78*10^{-6}$	0.93	0.059
rs6774262	3	32814515	1.40	$8.37*10^{-5}$	1.06	0.059
rs4133282	8	140287194	0.54	$2.02*10^{-5}$	0.93	0.066
rs2511703	8	103770272	0.73	$9.96*10^{-5}$	0.95	0.067
rs16861446	1	18221371	0.39	$8.47*10^{-5}$	0.91	0.071
rs970567	20	49281661	0.70	$3.50*10^{-5}$	0.95	0.075
rs10490478	2	207636308	1.63	$5.79*10^{-6}$	1.08	0.080
rs1859416	7	8866426	1.42	$5.04*10^{-5}$	1.06	0.080
rs11047358	12	24343978	1.47	$6.49*10^{-5}$	1.07	0.081
rs12101383	15	65055257	0.69	$9.53*10^{-5}$	0.95	0.112
rs268300	10	43900845	2.13	$4.05*10^{-5}$	1.14	0.113
rs12080088	1	230343981	1.45	$5.19*10^{-5}$	1.06	0.124
rs2290889	9	92679670	2.00	$9.14*10^{-6}$	1.11	0.125
rs2436932	8	103689078	1.59	$1.12*10^{-5}$	1.06	0.132
rs12892152	14	78049436	1.87	$5.78*10^{-5}$	1.11	0.162
rs11122430	1	230334906	1.50	$7.65*10^{-6}$	1.05	0.175
rs11776260	8	128451670	0.61	$4.30*10^{-5}$	0.96	0.250
rs2302951	19	53646233	1.51	$7.59*10^{-5}$	1.05	0.264
rs9662589	1	230344234	1.43	$9.68*10^{-5}$	1.04	0.268
rs7864625	9	92673769	2.00	$1.01*10^{-5}$	1.08	0.282
rs3959143	3	192600773	1.40	$2.61*10^{-5}$	1.03	0.290

Table 5.3 (continued)

			Discovery		Replication	
SNP	Chr	Position	OR	p-value	OR	p-value
rs10191593	2	207567835	1.49	$6.97*10^{-5}$	1.04	0.304
rs11782735	8	128435786	0.61	$4.92*10^{-5}$	0.97	0.369
rs6581191	12	57161965	1.38	$4.06*10^{-5}$	1.02	0.511
rs6852830	4	145726008	1.50	$7.17*10^{-5}$	1.02	0.641
rs7011660	8	30405716	1.42	$8.64*10^{-5}$	0.99	0.761
rs10502005	11	101985631	1.48	$2.42*10^{-5}$	1.01	0.784
rs6941242	6	48479887	1.97	$6.91*10^{-5}$	1.03	0.847
rs857788	1	157051761	1.41	$1.55*10^{-5}$	1.00	0.868
rs10931700	2	196176676	1.41	$6.06*10^{-5}$	1.00	0.869
rs857785	1	157050883	1.43	$2.68*10^{-5}$	0.99	0.877
rs12815289	12	102779349	0.69	$5.40*10^{-5}$	1.00	0.895
rs9473350	6	48475166	1.99	$5.68*10^{-5}$	1.02	0.943
rs10194564	2	142611043	1.55	$8.88*10^{-5}$	1.01	0.962
rs17154903	10	43839414	2.68	$7.31*10^{-5}$	1.03	0.976

OR odds ratio

Table 5.4 Top results of GWAS performed by Nebel et al [27]

				Discovery			Replication	
Chr.	Position	SNP	MAF	OR	*P*-value		OR	*P*-value
19	50114786	rs4420638	0.11	0.53	$3.70*10^{-10}$		0.55	$1.90*10^{-8}$
6	29778631	rs3129046	0.20	0.66	$1.20*10^{-7}$		0.95	0.523
6	29785931	rs1610742	0.20	0.66	$1.60*10^{-7}$		0.94	0.454
2	28515768	rs2338013	0.19	0.67	$7.40*10^{-7}$		1.06	0.496
6	29808162	rs1610601	0.20	0.68	$1.10*10^{-6}$		0.95	0.548
6	29753592	rs3129063	0.18	0.67	$2.20*10^{-6}$		0.92	0.353
6	29834025	rs1633063	0.20	0.68	$2.30*10^{-6}$		0.91	0.289
18	54052953	rs158869	0.48	1.37	$2.30*10^{-6}$		1.11	0.169
5	52022995	rs350450	0.22	0.69	$4.20*10^{-6}$		1.02	0.775
13	47001519	rs1575892	0.03	0.45	$4.50*10^{-6}$		1.13	0.442
6	29731718	rs29228	0.18	0.68	$4.70*10^{-6}$		0.93	0.446
16	73418057	rs16947526	0.06	0.56	$6.30*10^{-6}$		0.88	0.314
9	10715294	rs11790055	0.38	1.38	$7.10*10^{-6}$		1.01	0.942
1	175309154	rs12741354	0.45	0.74	$7.40*10^{-6}$		0.82	0.006
13	46905819	rs9595687	0.04	0.49	$8.00*10^{-6}$		1.13	0.470
9	10726580	rs10959258	0.37	1.36	$9.10*10^{-6}$		1.01	0.897

OR odds ratio

Table 5.5 Top hits of GWAS performed by Malovini et al [28]

SNP	CHR	Gene	Assoc Model	EA	EAF	p-value	OR
rs6504441	17	PRKCA	AM	T	0.30	$1.06 * 10^{-6}$	0.60
rs12413082	10	MSRB2	DM	T	0.32	$1.50 * 10^{-6}$	0.53
rs513154	3	IMPG2	DM	T	0.42	$2.14 * 10^{-6}$	0.52
rs4574762	7	–	RM	G	0.26	$2.29 * 10^{-6}$	0.19
rs1582594	16	–	DM	A	0.32	$3.00 * 10^{-6}$	1.87
rs10514626	1	–	AM	A	0.06	$4.65 * 10^{-6}$	0.38
rs10923806	1	–	RM	G	0.38	$7.24 * 10^{-6}$	2.30
rs2967137	16	–	DM	C	0.37	$8.00 * 10^{-6}$	1.83
rs12088486	1	–	DM	A	0.28	$8.83 * 10^{-6}$	0.56
rs11237644	11	–	AM	A	0.40	$9.24 * 10^{-6}$	1.52
rs2147556	13	–	AM	T	0.29	$9.59 * 10^{-6}$	0.63
rs6592810	11	–	AM	C	0.33	$1.10 * 10^{-5}$	1.54
rs7873259	9	ANKRD19	RM	G	0.27	$1.18 * 10^{-5}$	3.14
rs1563301	6	–	DM	G	0.13	$1.22 * 10^{-5}$	0.50
rs870959	11	–	AM	T	0.40	$1.27 * 10^{-5}$	1.51
rs571391	3	IMPG2	DM	G	0.43	$1.39 * 10^{-5}$	0.55
rs7915479	10	CDH23	DM	T	0.47	$1.47 * 10^{-5}$	1.92
rs10277343	7	–	AM	T	0.28	$1.79 * 10^{-5}$	0.64
rs888808	5	RHOBTB3	AM	G	0.41	$1.94 * 10^{-5}$	0.67
rs6769400	3	–	RM	A	0.48	$1.99 * 10^{-5}$	1.91
rs795602	4	MGST2	AM	T	0.47	$2.11 * 10^{-5}$	0.67
rs4938180	11	IGSF4	RM	T	0.49	$2.16 * 10^{-5}$	1.91
rs6701445	1	TAF5L	DM	T	0.15	$2.24 * 10^{-5}$	1.84

Table 5.5 (continued)

SNP	CHR	Gene	Assoc Model	EA	EAF	p-value	OR
rs10956502	8	FAM49B	DM	G	0.31	$2.25 * 10^{-5}$	0.57
rs4291539	1	–	DM	C	0.42	$2.37 * 10^{-5}$	1.81
rs1538287	1	KCNH1	DM	A	0.18	$2.64 * 10^{-5}$	0.55
rs10491334	5	CAMKIV	DM	T	0.18	$2.88 * 10^{-5}$	0.55
rs2670104	3	–	AM	C	0.36	$2.98 * 10^{-5}$	0.67
rs1556758	10	SORCS1	RM	C	0.45	$3.47 * 10^{-5}$	0.50
rs130329	22	–	RM	A	0.47	$3.52 * 10^{-5}$	0.50
rs1484583	8	–	AM	T	0.27	$3.74 * 10^{-5}$	1.53
rs285097	13	RNF113B	AM	G	0.08	$3.79 * 10^{-5}$	0.47
rs11738302	5	–	DM	T	0.27	$3.81 * 10^{-5}$	0.57
rs846427	7	–	RM	A	0.45	$3.82 * 10^{-5}$	1.94
rs2277472	14	MAMDC1	AM	T	0.09	$3.88 * 10^{-5}$	1.95
rs2495513	1	TMEM61	AM	G	0.20	$3.98 * 10^{-5}$	0.60
rs9315385	13	DCAMKL1	AM	G	0.14	$3.98 * 10^{-5}$	1.74
rs4740391	9	–	AM	G	0.11	$3.99 * 10^{-5}$	0.52
rs9366292	6	–	AM	G	0.25	$4.26 * 10^{-5}$	0.64
rs4727899	7	–	DM	G	0.48	$4.37 * 10^{-5}$	0.55
rs6540664	1	–	AM	A	0.50	$4.45 * 10^{-5}$	1.46
rs4777170	15	–	RM	C	0.43	$4.52 * 10^{-5}$	2.00
rs10513702	3	–	RM	T	0.48	$4.53 * 10^{-5}$	1.85
rs3134204	8	–	AM	G	0.28	$4.83 * 10^{-5}$	1.51
rs135416	22	–	DM	T	0.44	$4.86 * 10^{-5}$	0.57
rs4594173	14	MADMC1	AM	G	0.08	$4.88 * 10^{-5}$	1.94

Table 5.5 (continued)

SNP	CHR	Gene	Assoc Model	EA	EAF	p-value	OR
rs964403	3	SUMF1	RM	A	0.39	$4.91 * 10^{-5}$	2.07
rs1562688	3	–	RM	C	0.43	$4.93 * 10^{-5}$	0.50
rs712773	3	GRM7	AM	G	0.32	$4.98 * 10^{-5}$	0.66
rs697739	6	ATXN1	DM	A	0.34	$5.05 * 10^{-5}$	0.59
rs2111173	12	PTPRO	DM	C	0.39	$5.22 * 10^{-5}$	1.74
rs4282145	4	–	RM	T	0.33	$5.32 * 10^{-5}$	0.38
rs3864051	3	SUMF1	RM	T	0.39	$5.49 * 10^{-5}$	2.07
rs1428689	5	–	RM	C	0.49	$5.51 * 10^{-5}$	1.85
rs81647	16	–	DM	G	0.40	$5.53 * 10^{-5}$	0.58
rs10134056	14	–	RM	A	0.39	$5.61 * 10^{-5}$	0.46
rs2070325	20	LPLUNC4	RM	G	0.30	$5.98 * 10^{-5}$	2.42
rs2905476	9	–	RM	T	0.28	$6.35 * 10^{-5}$	0.29
rs7583529	2	CFLAR	RM	A	0.21	$6.45 * 10^{-5}$	3.15
rs7842001	8	–	RM	G	0.26	$7.12 * 10^{-5}$	0.32
rs969845	12	–	RM	A	0.18	$7.22 * 10^{-5}$	5.87
rs2354314	4	–	RM	T	0.43	$7.81 * 10^{-5}$	0.51
rs2073586	11	ABCC8	RM	T	0.40	$8.15 * 10^{-5}$	0.48
rs4505466	2	SH3BP4	RM	T	0.40	$8.31 * 10^{-5}$	2.06
rs1584547	14	–	RM	T	0.24	$8.52 * 10^{-5}$	2.96
rs3102484	8	–	RM	G	0.49	$9.20 * 10^{-5}$	1.82
rs731287	13	–	RM	T	0.28	$9.88 * 10^{-5}$	2.51

Assoc Model: *AM* allelic model [1 df]), *DM* dominant model [1, df]; *RM* recessive model [1 df]), *OR* odds ratio

came from the Illumina control database ($n = 673$). For replication two additional sets were used of 253 and 60 centenarians and 341 and 2863 population controls [30]. Four different genetic models (general/genotypic, allelic/additive, recessive and dominant) were investigated. A single SNP, rs2075650, in *APOE/TOMM40* reached genome-wide significance [30]. Table 5.6 contains the top 17 SNPs with a p-value $< 10^{-4}$ in the additive model. They further explored the hypothesis that different sets of SNPs that are associated with exceptional longevity, although with moderate effects, may jointly characterize the genetic predisposition to exceptional longevity [31, 32]. The authors included 281 predictive SNPs in the genetic risk profiles reaching 89% sensitivity and specificity for predicting centenarian status in the discovery sample [30]. In the replication samples the sensitivity was 60% and specificity was 58%. However, this set was slightly younger. In the older subjects sensitivity increased to 85%. The 281 predictive SNPs are located in 130 genes. Some of these genes are known for progeroid (premature aging-like) syndromes, like *LMNA* (Huthcinson-Gilford syndrome) and *WRN* (Werner's Syndrome) [33, 34]. 38 of the 130 genes were linked to AD in literature, 42 to dementia and 38 to tauopathies. The fact that so many genes play a role in dementia is consistent with the epidemiologic finding that dementia is absent or markedly delayed amongst centenarians [35]. Cluster analysis identified 26 groups of 8 to 94 centenarians (90% of the discovery set) with similar genetic risk profiles [30]. The genetic risk profiles associated with each cluster represent different genetic signatures of exceptional longevity. Some of the genetic signatures were significantly associated with different life spans, while others were associated with varying prevalences and ages of onset of various age-related diseases [30].

Alternative Approach

A different approach to study longevity was employed by Walter *et al* [36]. They employed a prospective follow-up design to investigate time to death as a continuous outcome (all-cause mortality) using a Cox proportional hazard model. The GWAS study included 25,007 participants including 8444 deaths. Mean follow-up time was 10.6 years. Mean age at death was 81.1 years of age. 14 SNPs were associated with time to death at a suggestive threshold of p-value $< 1 * 10^{-5}$ (Table 5.7). The strongest association was for rs4936894 (chromosome 11, near *VWA5A*) with a p-value of $3.4 * 10^{-7}$ [36]. Replication for the top 5 SNPs was sought in 4 independent samples ($n = 10{,}411$, deaths $= 1295$). None of the SNPs were consistently replicated. In the combined meta-analysis only rs1425609 near *OTOL1* showed a stronger association compared to discovery (p-value $= 1.61 * 10^{-6}$) [36]. Pathway analysis was applied to investigate the SNPs with p-values $< 1 * 10^{-3}$ in more detail. Relevant biological processes overrepresented in the results were developmental processes, neuronal activities, signal transduction, neurogenesis, ectoderm development and cell adhesion.

Table 5.6 Top hits of GWAS performed by Sebastiani et al [30]

SNP	Gene	EA	EAF	PVAL.GA	PVAL.AA	PVAL.DA	PVAL.RA
rs2075650	TOMM40/APOE	G	0.14	$2.89*10^{-9}$	$2.36*10^{-10}$	$2.50*10^{-4}$	$1.03*10^{-8}$
rs12629971	EIF4E3	G	0.82	$1.95*10^{-5}$	$1.90*10^{-6}$	$7.44*10^{-6}$	0.025
rs4977756	NA	G	0.37	$3.87*10^{-5}$	$7.97*10^{-6}$	$1.44*10^{-4}$	$5.88*10^{-4}$
rs6801173	EIF4E3	G	0.80	$6.81*10^{-5}$	$8.16*10^{-6}$	$2.20*10^{-5}$	0.041
rs1456669	NA	C	0.89	$1.94*10^{-5}$	$8.60*10^{-6}$	$4.05*10^{-6}$	0.374
rs4802234	CEACAM16	C	0.52	$3.06*10^{-5}$	$9.22*10^{-6}$	$3.25*10^{-5}$	0.003
rs1063192	CDKN2A	G	0.41	$9.70*10^{-5}$	$1.66*10^{-5}$	$6.33*10^{-4}$	$4.53*10^{-4}$
rs915179	LMNA	G	0.39	$1.03*10^{-4}$	$2.37*10^{-5}$	0.001	$3.18*10^{-4}$
rs2758331	SOD2	C	0.55	$1.31*10^{-4}$	$2.93*10^{-5}$	$4.39*10^{-4}$	0.001
rs4073968	NA	G	0.76	$5.38*10^{-5}$	$3.64*10^{-5}$	$1.24*10^{-5}$	0.129
rs1412832	NA	G	0.28	$2.18*10^{-4}$	$4.55*10^{-5}$	0.002	$6.89*10^{-4}$
rs9557276	CLYBL	C	0.50	$2.62*10^{-4}$	$4.65*10^{-5}$	$3.21*10^{-4}$	0.004
rs4722094	NA	G	0.18	$8.66*10^{-5}$	$5.00*10^{-5}$	0.239	$1.93*10^{-5}$
rs10483493	TTC6	C	0.25	$3.32*10^{-4}$	$5.96*10^{-5}$	0.004	$6.17*10^{-4}$
rs6997589	SH2D4A	G	0.22	$3.70*10^{-4}$	$6.25*10^{-5}$	0.017	$2.31*10^{-4}$
rs3763305	BTNL2	G	0.96	$1.62*10^{-4}$	$6.27*10^{-5}$	$4.51*10^{-5}$	0.810
rs277432	NA	G	0.62	$3.86*10^{-4}$	$9.12*10^{-5}$	0.001	0.002

PVAL.GA *p*-value for genotype association, *PVAL.AA* *p*-value for allelic association, *PVAL.DA* *p*-value for dominant association, *PVAL.RA* *p*-value for recessive association

Table 5.7 Top hits of GWAS performed by Walter et al [36]

SNP	Chr	Gene	EA	EAF	HR	p-value
rs4936894	11	VWA5A	A	0.23	1.11	$3.38*10^{-7}$
rs1425609	3	OTOL1	A	0.38	0.92	$1.46*10^{-6}$
rs766903	12	BIN2	A	0.83	0.90	$1.61*10^{-6}$
rs12042640	1	ATG4C	T	0.28	1.09	$1.71*10^{-6}$
rs17149227	7	HIP1	T	0.96	0.79	$3.56*10^{-6}$
rs3128591	9	COL5A1	A	0.75	0.92	$3.64*10^{-6}$
rs11582903	1	LMO4	A	0.15	1.12	$3.94*10^{-6}$
rs4850695	2	HECW2	A	0.77	1.09	$4.62*10^{-6}$
rs10259086	7	ORC5L	T	0.69	1.08	$5.16*10^{-6}$
rs2769255	1	KCNQ4	T	0.37	1.08	$5.17*10^{-6}$
rs17291546	6	LOC340156	A	0.96	0.82	$7.65*10^{-6}$
rs12606100	18	NETO1	T	0.20	1.11	$8.72*10^{-6}$
rs1274214	11	GRAMD1B	T	0.50	0.93	$8.87*10^{-6}$
rs10811679	9	SMARCA2	T	0.33	1.08	$9.53*10^{-6}$

HR hazard ratio

The Future of GWAS on Longevity

Published GWAS on longevity have so far failed to identify any new robust associations with longevity that have replicated over studies. The only loci robustly associated stem from candidate genes *APOE* and *FOXO3a* [13, 14, 16, 18, 24]. Though GWAS has proven to be a powerful approach to unravel the genetics of many complex traits, the longevity phenotype remains resistant to the efforts to uncover new genetic associations.

A reason for not finding any replicated associations for longevity could be the sheer complexity of the phenotype. Even centenarians fall into different groups in terms of age of onset of age-related diseases: survivors (onset of aging disease < 80 years), delayers (onset of aging disease between 80 and 100 years) and escapers (onset of aging disease > 100 years) [37]. Taking a younger age-cutoff for longevity cases (85+ or 90+), the number of cases will increase, which is very relevant for prospective cohort studies. However, along with an increase in power, the heterogeneity is expected to increase. The key to success in GWAS of other traits has been to increase samples size, ignoring issues of heterogeneity, which also occur in other complex outcomes such a blood pressure and cardiovascular disease. Without a doubt progress may be achieved by pooling the present studies in a joint analysis and adding as much as possible new studies available to increase the statistical power. Despite the robustness of GWAS to heterogeneity, there is a definite need to harmonize the longevity phenotype across studies. As seen in Table 5.1, almost every study investigating longevity uses different criteria for either longevity cases or the comparison group. This makes comparing the results between studies very difficult.

Why have we not identified new genes for longevity by GWAS? It has been argued that it may require a great number of 'protective' genes all with very small effects to have a genetic advantage to achieve longevity [30]. This model is also referred to as the infinitesimal model [38]. We have recently tested the infinitesimal model in the Rotterdam study and found that 81.3 % of the heritability in longevity defined as survival to age 90+ years is explained by common variants. Such a mechanism has been proposed for other complex traits including height. Though for highly heritable traits like height these genes are uncovered [39], in a trait like longevity this may require extremely large samples size to achieve sufficient statistical power, which have not been achieved yet. Using biomarkers of aging might be a more fruitful pursuit for finding associations with longevity. Unfortunately, no good biomarkers of aging currently exist, though many have been proposed [40]. Telomere length, a marker of cellular senescence, is one of the previously proposed biomarkers of aging [41] and has already proven successful in identifying genes for this trait [42]. As of yet, these genes have been associated with cardiovascular disease [42] but have not been found to associate with longevity [43]. These findings are not final as only a very small percentage of the telomere length variance can be explained by the currently uncovered genes [42].

An alternative approach to solving the heterogeneity issue in longevity is addressing healthy aging, as captured in a healthy aging index (HAI) [44]. The HAIs may include markers of 5 various organ systems that are known to predict mortality and disability. The systems included are vascular (carotid wall thickness), brain (white matter grade on MRI), kidney (cystatin-c), lung (forced vital capacity) and metabolic (fasting glucose levels) [44]. The HAI is able to distinguish a wide risk gradient, but is most remarkable for its advantage in identifying low risk individuals. As a single factor, the HAI prediction of mortality is similar in magnitude to age itself. When entered together, age remained partly independent, but the HAI explained 40 % of the effect of age [44].

Another potential reason for not finding any solid associations with the longevity phenotype stems back to the old debate of the role of common versus rare variants [45]. The common disease, common variant (CDCV) hypothesis states that common traits are caused by common variants with small effect sizes [46]. This theory is essentially targeted in GWAS. However, assuming a role of common variants may be an oversimplification of the true genetic architecture behind complex traits as longevity [47]. An alternate hypothesis is that rare phenotypes such as extreme longevity may be explained by rare variants with large effects that explain the high heritability and the clustering of nonagenarians and centenarians in families [48]. GWAS is not suited to identify rare variants as they are often not properly tagged by the variants present on genotyping arrays. Exome sequencing, or even genome sequencing, might help in uncovering such rare variants [49].

Although findings of GWAS to date have been disappointing, as discussed in this chapter there is ample opportunity to improve the statistical power of studies to find common variants with small effects that appear to explain over 80 % of the heritability in the Rotterdam study. Collaboration between various consortia is most likely the fastest way forward to success and may likely require some a priori titration on the definition of longevity cases and controls with the view to maximize the statistical power.

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Chapter 6
Exome and Whole Genome Sequencing in Aging and Longevity

Erik B. van den Akker, Joris Deelen, P. Eline Slagboom and Marian Beekman

Abbreviations

NGS	next generation sequencing
GWAS	genome-wide association studies
LOAD	late-onset Alzheimer's disease
FOA	familial generalised osteoarthritis
SKAT	sequence kernel association test
BBMRI-NL	Biobanking and Biomolecular Resources Research Infrastructure of the Netherlands

Introduction

A steadily growing life expectancy of the general Western population [1] urges research into age-associated mechanisms responsible for the gradual decline of health throughout the course of life. Calendar age is the major risk factor for the onset and

P. E. Slagboom (✉) · E. B. van den Akker · J. Deelen · M. Beekman
Department of Medical Statistics and Bioinformatics, Section of Molecular Epidemiology, Leiden University Medical Center, Postal zone S-05-P, PO Box 9600, 2300 RC Leiden, Netherlands
e-mail: p.slagboom@lumc.nl

IDEAL-consortium European Union FP7 framework, Leiden, Netherlands

E. B. van den Akker
Department of Intelligent Systems, The Delft Bioinformatics Lab, Delft University of Technology, Mekelweg 4, 2628 CD, Delft, Netherlands
e-mail: e.b.van_den_akker@lumc.nl

J. Deelen
e-mail: j.deelen@lumc.nl

M. Beekman
e-mail: m.beekman@lumc.nl

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2_6

progression of virtually all common diseases affecting the general population of the Western world today [2], suggesting that processes of aging are involved in the aetiology of many diseases. Indeed, aging is characterized by a progressive and systemic loss of function, which gradually leads to a state of senescence on the cellular, tissular and organismal level, thus affecting the general capacity for maintaining bodily homeostasis [3]. By studying factors affecting the rate of aging in humans, we aim to identify determinants that modulate the general capacity for maintaining the bodily homeostasis, and thus to gain insights in the common denominators underlying age-associated diseases.

Unlike most other traits, aging itself is not driven by any specific molecular mechanism per se, but instead seems to be the integrated result of all corrective and compensatory mechanisms failing to deal with the stochastic damage accumulated over life [4]. Despite this stochastic origin, the accumulation of damage does converge into consistently observed hallmark processes characterizing the aging phenotype, including cellular senescence, stem cell exhaustion, telomere attrition, and mitochondrial dysfunction [5]. Though the causality of some of these hallmarks has not yet been irrefutably proven, each of them is likely to occur during aging and is thought to at least aggravate the consequences of aging by further contributing to the loss of bodily homeostasis. Hence, the challenge in aging research is to identify those factors that causally affect the rate of aging amidst the myriad of processes that react in response to the widespread systemic changes that occur with aging.

Though seemingly inevitable, aging does not occur at an equal pace across species [6] or even within our own species. Whereas some experience an accelerated rate of aging, as exemplified by patients suffering from progeroid syndromes [7], others seem capable of delaying or evading at least some of the detrimental aspects of aging, as observed in members of long-lived families [8–11]. Unlike lifespan regulation, with its modest heritable component of about 25 % in the general population [12–14], age-related diseases and traits, such as Alzheimer's disease or coronary artery disease, have a considerable genetic component [15, 16]. Hence, most genetic studies into the aging rate and lifespan regulation have predominantly been based on three sorts of phenotype definitions reflecting functional decline: clinical or clinimetric endpoints of disease (e.g., diagnosis of dementia, diabetes, osteoporosis and cerebrovascular events), disease-related traits (e.g., blood pressure, cholesterol serum levels, bone mineral density) and mortality (i.e., age at death, disease free survival). In contrast, families composed of exceptionally long-lived individuals are also studied [11, 14, 17–20] (Table 6.1; Fig. 6.1), since the clustering of the longevity trait in these families suggests a somewhat larger heritable component for factors underlying the rate of aging and lifespan regulation. In summary, the genetics of the rate of human aging and life span regulation is studied either from the perspective of all that fails as age advances, or instead, by studying the exceptional cases that seem to evade or delay the onset of age-related decay.

Table 6.1 Overview of long-lived family studies

	Long-lived individuals		Off-spring	Controls		
Study	*n*	Type	*n*	*n*	Type	Reference
Ashkenazi Jews cohort	365	Centenarians	593	356	Spouses of off-spring + population controls	[18]
European Challenge for Healthy Ageing study	257	Centenarians	276	204	Cousins of offspring without centenarian parent	[19]
GEnetics of Healthy Ageing[a]	4,498	Nonagenarian siblings	~700	2,249	Population controls	[14]
Leiden Longevity Study	944	Nonagenarian siblings	1,671	744	Spouses of offspring	[11]
Long Life Family Study	1,373	Nonagenarian siblings	2,317	582	Spouses of offspring	[17]
New England Centenarian Study[b]	>1,800	Centenarians	>600	437	Population controls	[20]

[a] Offspring is recruited in the MARK-AGE project
[b] Recruitment still ongoing

Fig. 6.1 Centenarian twins participating in the Leiden Longevity Study

Common Variants Associated with Human Aging and Longevity

The hypothesis-free approach of genome-wide association studies (GWAS) focuses on the identification of common genetic variants involved in phenotypic variation. By comparing the frequencies of common variants (frequency > 1 %) between long-lived or disease-affected cases and population controls using GWAS, the location of numerous loci in the genome associated with age-related traits and diseases were identified [21, 22]. In contrast, GWAS-based studies aimed at identifying lifespan regulating loci, have only been mildly successful and have thus far yielded only three robust and independently confirmed loci influencing mortality: *FOXO3A* [23–26], *APOE* [27–31] and a novel intergenic locus on chromosome 5q33.3 [32]. GWAS for prospective survival did not provide additional robust loci [33].

Regarding the identification of loci involved in age-related diseases, GWAS discoveries in the field of dementia, for example, have been quite successful. Discovery of 19 susceptibility loci have shown to account for about 47 % of the population-attributable risk of late-onset Alzheimer's disease (LOAD) and about 61 % with the long-known *APOE* haplotype [34]. Usually, the effect of the variants marking the relevant genomic position is small and the position is often intergenic [35]. Hence, functional genomic studies are required, including resequencing carriers of disease susceptibility alleles, to answer which specific genetic variation influences the age-related trait.

Though many loci have been identified influencing age-related traits and diseases, only the *APOE* gene contributes to both age-related disease and population mortality by alleles associated with increased and decreased mortality risk. A set composed of GWAS-identified loci influencing the risk of cancer, diabetes, or cardiovascular disease did not reveal a detectable effect on mortality risk [36] in either longevity families nor in population-based individuals over 85 years of age. Since longevity in this study population was not compromised by the cumulative effect of this set of risk alleles for common disease, it was suggested that other genetic and environmental factors acting on individuals living to old age might counteract the detrimental effects of disease susceptibility alleles.

Family studies have also been performed to gain insight in the loci contributing to familial longevity. Genetic linkage studies in families with a history of extended lifespan [8, 11] have revealed a number of potential longevity loci [37–40]. However, none of these have been independently confirmed. In addition, fine-mapping of such linkage regions using GWAS data did not lead to significant findings, except for the *APOE* locus. Overall, genetic studies of human aging and longevity have not yet been very successful in identifying mechanisms underlying lifespan regulation.

The next step forward would be fine-mapping of GWAS and linkage regions using detailed DNA sequence information. This would result in putatively novel genetic variants, which may in the end be identified as the causal variants at disease associated and linked loci. Because GWAS for aging and longevity only revealed a few associated loci, the hypothesis is raised that rare genetic variants are likely to

be largely involved. Hence, the detection and analyses of rare genetic variation in aging and longevity research is currently ongoing.

Whole Exome and Genome Sequencing Technologies

Using next generation sequencing (NGS) techniques [41], the exome, whole genome, transcriptome, or methylome of multiple individuals can be (re)sequenced in fragments and mapped back to the reference genome. This technology provides more accurate and complete information about the genome in comparison to hybridization based chip-methods, like genetic and gene expression array data. However, NGS approaches have the drawback that the handling of its data is quite challenging, and thus requires bioinformatics expertise to handle and analyse the large amounts of data generated per individual sample. For instance, the identification of the causal mutations among the billions of base-pairs in whole genome sequencing data is not trivial. Fortunately, many algorithms exist for prioritising variants obtained from sequencing experiments, employing other data sources as prior information. Besides predicting the putative impact of coding variants using established gene models (e.g., SIFT [42] or PolyPhen [43]) or cross-species conservation [44]) more recent algorithms (e.g., CADD [45]) employ multiple sources of information and are also able to prioritise variants residing in non-coding regions.

Though the costs for exome and whole genome sequencing seem reasonable, it was at first not obvious why such data should be created for complete cohorts. Since it was hypothesized that the missing heritability in age-related disease, aging, or longevity would be explained by rare variants with a large effect [46], it was expected that study designs focussing on the most extreme cases only would be sufficient, and thus would require much smaller sample sizes than generally the case in GWAS for complex traits. Hence, the application of NGS technology for aging research as yet is modest. However a few larger initiatives are ongoing. Here we will discuss both published and ongoing NGS initiatives and their respective study designs aimed at revealing variants relevant for age-related disease and longevity.

Rare Germ Line Variation in Exceptional Longevity

It can be hypothesised that a contribution of rare loss of function variants to longevity might be explained in two ways. The human genome is reported to contain on average about 100 rare disruptive variants per individual, severely limiting or totally negating the functionality of the associated proteins [47]. So either a genome-wide depletion of such rare disruptive variants resulting in a more complete or better functioning proteome contributes to longevity or the targeted inhibition of a limited number of genes, as is the case in model organisms [48]. As of yet, little evidence exists on whether the genetic propensity for human longevity relates to a fitter proteome or the disruption of specific gene functions.

The first NGS efforts to study rare variants in longevity involve the study design of the extreme case. The genomes of super-centenarians and centenarians were sequenced in order to describe genetic features of exceptional longevity [49, 50]. Obviously, these analyses miss the statistical power to reveal broadly interpretable observations or evidence in favour of any of the two explanations mentioned above. In these studies of the whole genome sequence of the long-lived, the number of variants roughly seemed to resemble that of younger controls and genomes that carry GWAS-identified alleles to the same extent as younger controls. Using a more targeted approach, 988 candidate longevity genes were sequenced in 6 additional centenarians [51]. Novel high impact variants were subsequently validated in larger case control studies, suggesting that *PMS2* and *GABRR3* might be candidate longevity genes. These studies provide some initial insights into genetic backgrounds that are conductive to exceptional longevity.

Rare High Impact Variants in Mendelian Age-Related Diseases

For the identification of rare high impact variants in rare age-related disease, the investigation of the genomes of affected distant relatives has shown to be a successful study design. In such designs it is hypothesised that affected members of the same family carry the same mutation causal to the disease. An example of this design is provided by the whole exome sequencing study of familial generalised osteoarthritis (FOA) patients, performed using two affected distant family members [52]. This study demonstrated that a heterozygous, probably damaging, read-through mutation (c.1205A=>T; p.Stop402Leu) in the *TNFRSF11B* gene encoding osteoprotegerin is likely to cause FOA in this family. In addition, the integration of exome sequencing data with other omics data has been shown to be very supportive in the filtering of potential causing mutations.

Sequencing efforts in the field of dementia and early onset Alzheimer's disease have been quite successful as well. In addition, exome sequencing of patients with atypical presentation of rare diseases, such as familial forms of dementia, occasionally indicate shared mechanisms between distinct age-related diseases, which may increase the insights into the basis of co-morbidity. Exome sequencing of Nasu-Hakola patients, for example, provided insights on the link between the bone and neurological manifestations of Alzheimer's disease [53].

Rare High Impact Variants in Common Age-Related Disease and Longevity

In terms of statistics, a fundamental problem lies in the analysis of extremely rare genetic variants and their potential for affecting the rate of aging and lifespan regulation. In many cases, the number of copies of the allele is too small to support robust

statistical inferences using only a single variant. Several solutions exist for dealing with rare variants and include for instance methods for the analysis of aggregates of variants, like the sequence kernel association test (SKAT) [54], or rely on very extensive family pedigrees for increasing the number of observed copies of alleles observed in the founder [55]. However, whole genome or exome analysis frameworks using only NGS data typically require comparable numbers of samples as single variant GWAS. Several other options exist for increasing the power of the analysis of rare variants and basically confine the initial number of variants by incorporating prior knowledge. For instance, NGS data is used for fine-mapping of regions with significant linkage, like in the case of familial type 2 diabetes, where significant linkage was observed at 4 loci that were fine-mapped using exome sequencing [56]. A N1072K variant of the early endosome antigen 1 (*EEA1*) gene was found to be more prevalent in diabetic patients than in controls using this approach.

The low frequency of rare variants dictates extremely large sample sizes to investigate the whole exome or genome for variation relevant to a common trait. Such studies therefore often focus on candidate genes or regions. For example, the coding variants in the *APP* gene associating to LOAD were investigated from a set of whole genome sequencing data in 1,795 Icelanders [57]. By combination with other phenotypic data, it was shown that mutations in *APP* contribute to the pathophysiology of Alzheimer's disease. Genome resequencing also revealed rare coding variants within the *TREM2* gene conferring increased risk for LOAD [53, 58]. In novel strategies whole exome sequencing is combined with deep phenotyping data such as neuroimaging data, for example. This applied in an extreme trait design resulted in identification of functional variants associated with the age-related rate of hippocampal volume loss in mild cognitive impairment [59].

Since current findings suggest that the rare variants often do not have the large effect that was expected, large genome sequencing initiatives have started. Whole genome sequencing data has been generated in the Genome of the Netherlands project in 250 nuclear families [60, 61] in the SardiNIA project of 1,000 individuals (http://genome.sph.umich.edu/wiki/SardiNIA), and in the Wellderly study of 2,000 individuals over 85 years of age (http://www.scripps.org/news_items/4757-scripps-wellderly-genome-resource-now-available-to-researchers). In addition, the genomes of 218 nonagenarians from the Leiden Longevity Study [10, 11] are being compared to 98 younger population controls of the Biobanking and Biomolecular Resources Research Infrastructure of the Netherlands (BBMRI-NL) consortium [60, 61] to identify different characteristics of their genomes. These studies will eventually contribute to our knowledge about genetic variation and its contribution to aging and longevity.

The Contribution of Somatic Mutations to Aging and Longevity

Whole exome and genome sequencing data is also used to investigate the occurrence of somatic mutations during lifetime and their impact on aging and longevity. In the 1960s, it was suggested that aging is the outcome of accelerated accumu-

lation of somatic mutations at the DNA level [62], which introduce errors in the primary structure of proteins [63]. These theories predict that somatic mutations appear stochastically from the beginning of life onwards and the accumulation of erroneous proteins and somatic mutations eventually affect key functions of somatic maintenance (synthesis, degradation, repair), leading to a cascade of detrimental consequences (error catastrophe). The technical limitations of detecting somatic changes and the lack of insight in the minimum level of erroneous proteins to affect organismal functions in aging hampered any firm conclusions on these theories [4].

To examine the level of detectable somatic variants that may occur in a lifetime, the genome sequences of DNA from whole blood of two randomly chosen monozygotic twin pairs of 40 and 100 years were generated by two independent next generation sequencing (NGS) platforms (Illumina and Complete Genomics) [50]. Potentially discordant single-base substitutions supported by both platforms were validated extensively by Sanger, Roche 454 and IonTorrent sequencing. The genomes of the two twin pairs were demonstrated to be germ line identical between co-twins, and the genomes of the 100-year-old MZ twins were discerned by eight confirmed somatic single-base substitutions, five of which within introns. Putative somatic variation between the 40-year-old twins was not confirmed in the validation phase. Thus, by using two independent NGS platforms somatic single nucleotide substitutions can be detected and a century of life did not result in a large number of detectable somatic mutations in blood. The low number of somatic variants observed by using two NGS platforms may provide a framework for detecting disease related somatic variants in phenotypically discordant monozygotic twins [64].

The accumulation of somatic mutations is thought to differ per tissue, since environmental exposure and self-renewal rate is different per tissue. Recently, whole genome sequences of DNA from several tissues of a 115-year-old Dutch woman were compared to the sequence of her brain [65]. The authors estimated that the blood tissue during 115-year lifespan accumulated 450 somatic mutations, but these did not seem to have shortened the life of this exceptionally old centenarian woman.

Summary

With the availability of affordable NGS data an unprecedented opportunity is now created for probing the genome for causal variants underlying the rate of human aging and life span regulation. However, the power to analyse such extremely rare genetic variants is generally very low, challenging the current methodologies for data analysis. Hence, with regard to the detection of germ line mutations involved in aging and longevity, it is important to generate data in much larger sample sizes than currently has been performed. Particularly, integration with additional sources of information, such as multi-level omics data on the same individuals will be useful for the interpretation whether a genetic variant may be the causal functional variant.

Thus far, most of the attention in NGS experiments has gone to variants in the coding domain, due to their ease of inference and interpretation. However, as most

of the established associations with common SNVs in GWASs generally seem to coincide with regulatory domains, like enhancers [35], rather than with coding domains, it seems reasonable to assume that the same holds for rare variants coming from NGS experiments into human aging and life span regulation. However, the extension of the analysis scope to variation beyond that in the coding sequence would even increase the demand for power and therefore would heavily rely on the availability of appropriate algorithms for prioritising genetic variants in non-coding sequence for their biological impact.

With regard to the detection of somatic mutations involved in aging and longevity, the evidence is too weak to draw firm conclusions on their contribution to aging processes. One of the potential hallmarks of human aging is stem cell exhaustion, for which some indications were found in the blood of the 115-year-old Dutch woman on the basis of detected somatic mutations [65]. The real impact of somatic mutations on human aging and longevity requires much larger sample sizes and study designs that allow investigations into the role of somatic mutation load or stem cell exhaustion in prospective survival studies of centenarians instead of cross sectional designs or the study of post mortem material.

Overall, the era of annotation of sequencing variants has just begun. Larger studies, meta-analyses and novel methodology for the analyses of sequencing and multilevel omics data will contribute to the elucidation of the mechanisms underlying human aging and longevity.

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Chapter 7
Models to Explore Genetics of Human Aging

David Karasik and Anne Newman

Introduction

There are multiple theories to explain the aging process. Although the processes and rates of aging are known to be at least partly genetically determined, the exact underlying molecular mechanisms of aging remain a subject of debate [12, 28]. The variability of aging among individuals reflects the precarious balance between the stochastic destruction, adverse environmental influences, and correcting effect of alleles responsible for repair [38]. Undoubtedly, there are genes that act to prevent an organism from destruction and disorganization; these genes may inhibit entropy, regulate inflammation, code for heat-shock proteins, maintain DNA repair (such as telomere maintenance factors), or provide anti-oxidant functions (e.g., antagonists of reactive oxygen species). Long and healthy life span and longevity may be related to absence of specific disease-causing alleles and/or the presence of protective, favorable alleles [25]. To reach advanced old age, it is essential that several of the major causes of death be avoided, requiring delayed damage in multiple organ systems. Furthermore, functional pathways regulating longevity are usually multigenic in living organisms [33, 74, 85]: transcriptomic and chromatin profiling studies imply that hundreds of genes show altered expression under longevity-inducing conditions [18]. We therefore can assume that longevity-related biological processes are highly integrated and have redundancy built into the homeostatic mechanisms [38].

Thus it might be further postulated that there are alleles in genes that confer a pleiotropic effect on the maintenance of structure and function of multiple organ systems during aging. These alleles should regulate the ability of an organism as a whole to withstand challenging endogenous and exogenous influences [38, 47]

D. Karasik (✉)
HSL Institute for Aging Research, 1200 Centre Street, Boston, MA 02131, USA
e-mail: karasik@hsl.harvard.edu

A. Newman
Graduate School of Public Health, University of Pittsburgh, A527 Crabtree Hall, 130 DeSoto Street Pittsburgh, PA 15261, USA

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2_7

in order to maintain homeostasis. Genetic studies in animal models have provided insight into the biological mechanisms underlying inter-individual differences in susceptibility to (or resistance to) aging. The identified genes may or may not have a direct relevance to humans. Reciprocal relationships between organ systems, which are more complex in humans than in flies or worms, are probably responsible for non-replication of genes found in model organisms which govern the human unique complexity. Advances in molecular and genetic epidemiology over the last decade provide tools to perform a study of the genetic sources of the variability in biological aging in humans as well. However, in order to be successful, the genetic study of a complex condition such as aging requires better definition of heritable phenotypes that characterize the aging process itself. Human genetic studies of aging and longevity are impeded by the overly simplified phenotypes, such as age at death, that do not take into account the interwoven nature of multiple structural and functional parts, whose relationships are characterized by redundancy and reciprocity as well as interactions with the environmental factors that are well known to affect age at death [56].

In this chapter, we will focus on approaches that can improve our chances of identifying genetic factors relevant to aging and longevity in humans. By this we can come closer to the understanding of the variation in the aging of the human organism and its molecular mechanisms [38].

Current Progress and Problems of Genetic Studies of Aging and Longevity

It has been demonstrated that human longevity has moderate heritability ($h^2 = 0.20$–0.30) [20, 30, 50] and that longevity is more heritable at greater extremes of survival [32]. To date, genome-wide association studies have converged on only a few common variations related to longevity while genome wide linkage analyses identified several chromosomal loci in which supposedly rare variants seem to be linked to the aging and longevity phenotypes. Thus, in the most recent linkage analysis which included > 2100 Caucasian nonagenarian sibling pairs from 11 European countries (part of the Genetics of Healthy Aging (GEHA) project), four regions showed linkage to longevity [2]. Notably, however, LOD scores (a measure of linkage strength) were always lower than 4.0, which is sobering. Similar "strongly suggestive" linkage of "excess longevity" *phenotype* to chromosome 3p24.1 *was* recently reported [42]. What makes this study [42] notable is that this is a replication of a locus obtained by another, independent study [5]. Similarly, linkage to "successful aging" was found in Amish kindreds for a region on 6q25, with LOD score = 3.2 (this linkage was, however, confirmed by a SNP which had regionally significant evidence of association) [15]. Linkage analyses of complex phenotypes are rarely sufficiently powered to find strong-effect variants or to map them to narrow chromosomal regions, but do provide potential leads.

To date, the GWAS approach has been proven productive in uncovering multiple genes responsible for complex diseases [31]. We focus on features of this technique below. GWAS can be applied to the studies of aging and longevity, however, the choice of a phenotype is critical for the study of a complex genetic process, such as aging [38, 52]. For example, there are several challenges in using longevity as a phenotype for a quantitative-genetic study, in spite of a simplicity of phenotype definition [6, 39]. The arbitrary nature of the "extreme longevity" phenotype can be seen from a sex-specific heritability of living to at least 100, which has been estimated at 0.48 in men but only 0.33 in women [70]; this is expected, given that reaching any given extreme age is more exceptional for men than for women [6].

In a recent-most GWAS meta-analysis of long-lived individuals of European descent ($n=5406$ who survived to ages ≥ 90 years, and 16,121 younger controls (<65 years old)), a novel locus was identified an intragenic variant on chromosome 5q33.3 ($P=1.74\times10-8$). The study also confirmed association of a TOMM40/APOE/APOC1 locus on chromosome 19q13.32 ($P=3.40\times10-36$) with longevity. The minor allele of rs2149954 on chromosome 5q33.3 associated with increased survival ($HR=0.95$, $P=0.003$). Notably, this allele has previously been associated with low blood pressure in middle age and with decreased cardiovascular mortality risk, even independent of blood pressure [14].

This study again testifies to the challenges in using longevity as a phenotype for a genetic study. Additionally, only a small subset of the general population have expressed this phenotype, thus it is very difficult to obtain a sufficiently large sample of parents and offspring who both possess extreme longevity [25]. Population control samples, such as average-life-span individuals are often derived from later birth cohorts than centenarians making birth cohort matching problematic. Within long-term cohort studies, birth cohort matching is more feasible, but the proportion of individuals in a cohort surviving to age 100 years is too small to permit studying longevity *per se.* An additional question remains as to how results obtained from the extreme samples reflect the aging process more generally [38, 56]: it is not clear whether results replicate beyond the centenarian cohorts. Continued follow-up of older individuals will someday provide adequate sample sizes for study in a cohort design [56], as well as for larger meta- and even mega-analyses.

"Healthy Aging", "Successful Aging" and "Exceptional Survival" Phenotypes

An alternative strategy would be to investigate individuals destined to be long-lived by characterizing those who are exceptionally healthy for their age. A major goal of aging research is aimed at compressing morbidity and preventing functional decline along with prolonging a healthy life span, rather than just increasing life expectancy [21, 38]. Indeed, people who live to an older age are likely to be disabled, and thus in need of caregiver's assistance, many months or years prior to death [76]. Thus "healthy aging" is of great interest regardless of its relationship to longevity. It can

be said that the study of longevity focuses on lifespan, whereas "healthy aging" is focused on health-span [6]. Numerous prospective population-based cohorts have been scrutinized to characterize persons with long-term survival. These studies also have defined the rates and risk factors for survival with intact health or function, such as survival free of chronic disease or survival with high levels of physical and cognitive function (reviewed in [56]). Various combinations of factors have been related to survival with intact health and function. These outcomes have been termed "healthy aging", "successful aging", or "exceptional survival". These phenotypes vary in construction and they are not necessarily interchangeable [66]. Moreover, there is no consensus for these classifications, especially as phenotypes for genetic study.

Another strategy would be to begin following even younger-old adults for their changes in functions, developing aging-related diseases, and ultimately, survival. Many researchers [11, 59, 82] have argued that studies of aging genetics should be initiated earlier in life. From the genetic-epidemiological point of view, such a study would benefit for larger samples to follow, since there are life expectations permissive of longitudinal studies, as well as information on life-long environmental exposures that are up-to-date and not based on a recall [38]. However, at the present, there is no clarity about how to measure aging in midlife, despite a plethora of publications on the individual biomarkers and risk factors of aging [57].

Endophenotypes and "Biomarkers" of Aging

A discernable decline occurs in function and morphology of most bodily systems with advanced age, including metabolic, cognitive, reproductive, and endocrine systems. For years, there has been an idea that an aging phenotype may be captured by an individual or a combination of several biological parameters of an organism, serving as "biomarkers of aging". Some biomarkers comprise functional parameters that are recognized as risk factors for age-related degeneration and diseases [4], predictably change with age [13, 37], manifest ability to be tested non-invasively, repeatedly and accurately [66], and are closely related to the maintenance of life. The ability to predict life span is also a traditional but imperfect criterion used to validate these biomarkers of aging; arguably it may be more important for a good biomarker to be able to discriminate between adverse aging-related events, such as frailty [54], immobility [75], and propensity to fall [46], as well as predict mortality.

There are additional considerations when choosing biomarkers to characterize aging. First, biomarkers measured at a given age are merely snapshots of important regulatory systems [72] and there is no information on system dynamics if each biomarker is measured only once. Having longitudinal measures should be important to approximate trajectories of change in each organ system. There had been attempts to develop a statistical approach in order to investigate both a cross-sectional measure at a given point of ontogenesis as well as dynamic trajectories of

its age-related change; for example, the longitudinal trajectories of blood glucose differed among cross-sectional age patterns in a population [88].

Secondly, how specific a biomarker should be (e.g. organ specific or systemic) and which bodily system or systems best represent general aging, is questionable [38]. Despite 2 decades of research and debates, the search for new biomarkers of aging is still continuing, as new works keep appearing: recently, skin/facial appearance [22] and waist circumference [90] were among newly proposed or validated. Telomeres remain an interesting, although challenging, measure: if telomere length is a biomarker of human aging, it is a weak biomarker with poor predictive accuracy [66].

In the Study of Osteoporotic Fractures, successful skeletal aging, defined as maintenance of bone mineral density for up to 15 years, served as a marker for longevity [8]. Similarly, in a longitudinal cohort study of more than 2700 participants with a mean age of 74 years at baseline and 80 years at follow-up, participants who maintained cognitive function over this period had a lower mortality risk and less decline in physical function [87]. These studies seem to be able to discern a lack of decline in an organ system as an important endophenotype of longevity. Endophenotypes are defined as intermediate components of the phenotype of interest. Development of endophenotypes of preservation of function across multiple systems likely will be needed to define health with intact physical and cognitive function into old age [49, 56]. These endophenotypes can be shared among organ systems and therefore, be potentially pleiotropically regulated. For example, reproductive aging phenotypes could serve as important endophenotypes for genetic studies of longevity. Evolutionary theories of aging support a tradeoff between fertility and survival, with associations between reproduction and life span being observed in both animal models [41, 83] and humans [56]. Centenarian women, for example, are more likely to delay childbirth until late in life than women who died at an earlier age [61, 79]. These data support the hypothesis that late fertility and slower somatic aging might share underlying genetic determinants [56], although the potential for bias due to social factors for having a late pregnancy has not been fully studied.

To add to the uncertainty of what biomarker or combination of biomarkers can serve as phenotypes, there is a conceptual and analytical challenge. Most biomarkers are complex traits that are polygenically regulated. The genetic component of each heritable biomarker is complex; it encompasses contributions from multiple genetic sources, not necessarily related to aging, but reflecting genes involved in maintenance of the organ system's homeostasis or even in early-life functions. Furthermore, change with age in biomarkers is a challenging phenotype for genetic research in general: the heritability of rate of change is usually lower than the cross-sectional "snapshot" [38, 53].

It is safe to assume that specific phenotypes that focus on "successful" or "healthy" aging and are also clinically relevant to the length of life measured in years, will provide fruits of discovery. To obtain a more comprehensive picture, it was proposed to combine individual biomarkers of aging into a composite score to identify individuals who are healthy in multiple organ systems. Thus, in the Cardiovascular Health Study (CHS), [57] developed a physiological index of aging by

combining information across 5 major organ systems, whose aging well predicts death and disability. The index was constructed with the use of noninvasive testing from the vascular, pulmonary, brain, kidney, and metabolic systems and resulted in a wide range of values, from 0 (all systems normal) to 10 (clinical disease). Using continuous measures allowed us to distinguish individuals with exceptionally low mortality risk. There was no clustering of healthy aging traits in the CHS: the distribution was following a normal pattern, with very few individuals who were healthy in all 5 systems. In order to assess the heritability of such an index, a derived index was created using less precise but standard measures of these same systems, harmonized across cohorts. This modified "Healthy Aging Index" was moderately heritable in a family study of longevity, the Long Life Family Study [67]. The measures in this modified index are available in many cohorts, allowing application to other studies. Having a continuous phenotype has its benefits: first, it may be more powerful than dichotomous/ordinal (or non-normally distributed) ones and secondly, it is more straightforward to pool data from several studies for GWAS meta- or mega-analysis across many cohort studies. Importantly, the modified index was as predictive of mortality as the original one. The latter requirement is important for developing indices of biological aging. Considerations include the use of clinical biomarkers that are commonly measured in clinical practice settings and that predict an individual's health outcomes [1]. Additionally, a question might come up about the input data for any physiologic index of aging - whether there should be an age-adjustment for what is "normal" in older adults. In brief, one way to measure "health" is relative to one's age group and one is in absolute, homeostatic terms. If absolute definition is used, then "health" is rare in older adults. Alternative methods used age-adjusted values to assess specific aspects of health. These methods are useful but do not allow a comparison of the phenotypes to chronological age in predicting outcomes.

Biomarkers that comprehensively reflect organ systems involved in aging at multiple levels of biologic organization, including the molecular [80] and cellular levels, should be included (e.g. senescent secretory phenotype [7]). Further development of composite phenotypes is needed. Any new aging score needs to be validated by determining whether it predicts all-cause mortality as well as incidence of major age-related chronic disease and disability late in life.

Genome-Wide Association Study (GWAS) Approach to Discover Aging Genes

A genome-wide search offers advantages over candidate gene association studies because it provides more extensive coverage of the genome and the opportunity for truly novel gene discoveries which are unconstrained by existing knowledge. Hundreds of articles from GWAS demonstrate the potential of this approach to identify novel gene associations for multiple complex diseases and quantitative phenotypes. However, GWAS has its limitations too: being focused on common single nucleo-

tide polymorphisms (SNPs), the associations discovered do not explain the total genetic variance of a trait. This phenomenon is known as "missing heritability". Similarly, gene-by-environment interactions are not typically included in the GWAS design. New discoveries by GWAS and replication of association findings, especially for complex quantitative phenotypes, require large sample sizes [38], while studies of gene-by-environment interactions and rare variants require even more powerful designs [48]. In order to uncover "missing heritability", attention should be shifted to the many other variants in the human genome, such as rare polymorphisms (not only SNPs), copy number variants, and combinations of thereof [9]. Another statistical challenge the GWAS has to deal with is a need to correct for the multiple comparisons generated by scanning millions of SNPs.

Nevertheless, to date, the GWAS approach has been productive in uncovering multiple genes responsible for complex diseases; moreover, GWAS has proven itself able to uncover pathways with therapeutic potential and to provide drug targets [63]. Interestingly, many biological candidate genes (sometimes described as the "usual suspects") have generally not been confirmed by subsequent GWAS of the same phenotypes (see [62] for musculoskeletal and [55] for psychiatric phenotypes). Similarly, FOXO3 *gene, which was associated with* human longevity [84]*, did not appear as* a genome-wide significant (GWS) *signal with any aging related traits, aside of the* borderline statistically significant association with IGF-I concentration ($p = 5.1 \times 10^{-7}$). Other examples include genes coding for sirtuins or cholesteryl ester transfer protein (CETP), which did not appear on the top list of aging GWAS hits (despite the latter which was associated with lipid profile traits in multiple GWAS).

However, GWASs have been less successful for diseases in which phenotypes have been more difficult to define and to standardize, such as cognitive traits and mental-health-related diseases, behavioral traits, and osteoarthritis [16]. Similarly, in the field of aging and longevity, the progress is painfully slow. In general, results of the associations for phenotypes such as centenarian status, age at death, and morbidity-free survival at age 65 were far from genome-wide significant (the latter being defined by consensus as p-values $< 5*10^{-8}$). For example, in spite of relatively large sample in a meta-analysis by the CHARGE consortium (longevity "cases", defined as survival to age 90 and older, $n = 1836$, and population comparison "controls", $n = 1955$), did not reach the genome-wide significance threshold [58]. Genome-wide association studies with extreme long-living individuals to date did provide only a handful of longevity-associated genes [14, 71].

We therefore searched the database of published GWAS, using the Catalog of Published Genome-Wide Association Studies (www.genome.gov/gwastudies [31]), focusing of aging- and mortality-related traits. Apart from a well-known *TOMM40/APOE* locus, which is associated with Alzheimer's disease, age-related macular degeneration, triglycerides, cholesterol (total, HDL and LDL), C-reactive protein, and other aging-related traits, there are several other potentially-pleiotropic loci such as *MECOM, CAMK4, TMTC1, CCDC60, HNF1A (TCF1), GPR133*, and *STK24*. These loci might pin-point the new biological pathways or processes involved in human aging. Thus for example, *STK24* codes for a serine/threonine-protein kinase

Table 7.1 Potentially-pleiotropic loci identified by GWAS in aging-related traits. From the catalog of published genome-wide association studies. Available at: www.genome.gov/gwastudies. Accessed on June. 2013

Disease/Phenotype	Chr	Position	Reported Gene(s)	Strongest SNP	p-Value	OR or beta	95% CI	Reference
Aging traits(age at natural menopause)	2	81668808	Intergenic	rs10496265	*1E-8*	NR	NR	
	2	81751124	Intergenic	rs10496262[a]	3E-7	NR	NR	47
Pulmonary function decline (unit decrease)	2	81856526	Intergenic	rs12615721	8E-6	0.30	[0.17–0.44]	91
Aging (time to event)	3	168686676	MECOM	rs16852912	3E-6	1.18	[NR]	92
Blood pressure (mmHg increase)	3	169100886	MECOM	rs419076	*8E-13*	0.34	[0.25–0.43]	93
Longevity	5	110772404	CAMK4	rs10491334	2E-6	1.82	[1.43–2.33]	94
Blood pressure (DBP)	5	110772404	CAMK4	rs10491334	4E-6	NR	NR	95
Longevity	5	158393594	LOC101927697	rs2149954	1E-8	1.10	[1.06–1.14]	96
Blood pressure	5	158377449	LOC101927697	rs9313772 [a]	1E-11	0.335	[0.24–0.44]	94
Heart failure	12	30104142	TMTC1	rs2046383	3E-6	1.39	[0.97–1.97]	97
Aging traits (biologic age)	12	30113883	Intergenic	rs1463605[a]	7E-8	NR	NR	47
Aging traits (walking speed)	12	119989646	CCDC60	rs7137869	6E-7	NR	NR	47
Longevity	12	121363724	HNF1A, TCF1	rs6489785[a]	1E-6	NR	NR	98
C-reactive protein (unit increase)	12	121402932	HNF1A	rs7305618[a]	*1E-8*	0.27	[0.18–0.36]	99 (similar finding in 100–103)
C-reactive protein	12	121403724	HNF1A, TCF1	rs7953249[a]	1E-6	NR	NR	47
C-reactive protein (unit decrease)	12	121420260	HNF1A	rs7979473[a]	*1E-10*	0.12	[0.082–0.156]	103 (similar finding in 104–107)
Coronary heart disease	12	121435587	HNF1A, OASL, C12orf43	rs2259816[a]	5E-7	1.08	[1.05–1.11]	108

Table 7.1 (continued)

Disease/Phenotype	Chr	Position	Reported Gene(s)	Strongest SNP	p-Value	OR or beta	95% CI	Reference
Cardiovascular disease risk factors (GGT, units/L decrease)	12	121471337	OASL	rs3213545	*4E-15*	0.12	[0.092–0.150]	109
Longevity	12	131525053	GPR133	rs3847687	1E-6	NR	NR	98
Mortality among heart failure patients	12	131862903	LOC338797	rs7965445	2E-6	1.30	[0.99–1.72]	110
Protein QTLs (Erythropoietin)	12	131939920	GPR133	rs10466868	1E-6	NR	NR	111
Longevity	13	99126303	STK24	rs9517320	1E-6	NR	NR	98
Alzheimer's disease	13	99131294	STK24	rs912330[a]	4E-6	1.85	[1.43–2.44]	112
Longevity	19	45395619	TOMM40, APOE[b]	rs2075650	*3E-17*	1.41	[1.30–1.54]	113
Longevity	19	44919689	APOE	rs4420638	3E-36	0.72	[0.68–0.76]	96
Longevity	19	45422946	APOC1	rs4420638	*2E-16*	NR	NR	114

NR not reported, *DBP* diastolic blood pressure, *GGT* glutamyltranspeptidase, *QTL* quantitative trait loci

[a] this SNP is in LD with the one above

[b] APOE locus is associated with Alzheimer's disease, age-related macular degeneration, Brain imaging/hippocampal atrophy, triglycerides, cholesterol (total, HDL and LDL), C-reactive protein

that mediates oxidative-stress-induced cell death by modulating phosphorylation of JNK1-JNK2 and p38 during oxidative stress.

Along with the studies listed in Table 7.1, a recent GWAS on leukocyte telomere length [10], which is thought to be a marker of biological aging, identified TERC gene, which codes a telomerase RNA component.

There is a hope that GWAS can point out robust novel associations with regards to common etiology and potential shared biologic pathways between phenotypes and conditions [29, 68, 51, 69]. Presently, the potentially-pleiotropic signals mostly appear by a "semi-serendipity" (Table 7.1). One recent example is from the above-mentioned GWAS meta-analysis of the longevity in Europeans, which found an intragenic variation which also shared association with blood pressure [14]. Exploring pleiotropic relationships among the organism's systems and processes is challenging both methodologically and conceptually; this would need more method development, such as the Bayesian approaches of bi-clustering [24] and test for co-localisation of GWAS signals [19].

Identifying Pleiotropic Loci for Aging Phenotypes

There are benefits in considering pleiotropy for the genetic study of complex phenotypes, including utility of genetic relationships between the phenotypes to better define and explore the "phenome of aging" [86] or to approximate "endophenotypes" of aging, which are not measurable in most studies. For example, phenotypic and genetic overlap between psychiatric disorders was recently studied, based on an assumption that the estimates of genome-wide SNP-based genetic correlation between disorders reflect the pleiotropy of causal variants tagged by common SNPs [43, 77]. Empirical evidence of shared genetic etiology for psychiatric disorders has a potential to inform nosology and encourages the investigation of common pathophysiologies for related disorders [77].

As the genetic effects for most complex traits are small, combining results across studies of different phenotypes can improve the power of detecting potentially-pleiotropic associations [78]. It should be pointed out that the number of possible combinations to be adjusted for exponentially increases with the number of traits studied so that power of detection decreases for even moderate phenotype counts [78]. Therefore, multiple methods were proposed to identify pleiotropic candidate loci by identifying overlapping genetic variants between multiple independently run analyses on the same dataset or by meta-analyses of multiple correlated phenotypes (reviewed in [73, 78]). Multivariate analyses jointly analyze more than one phenotype in a unified framework and test for the association of multiple phenotypes with a given SNP [78].

For example, Gupta et al. [24] used bi-clustering of markers associated with at least one trait from multiple correlated phenotypes to identify clusters of potentially-pleiotropic markers, in the Framingham Osteoporotic Study sample. 2038

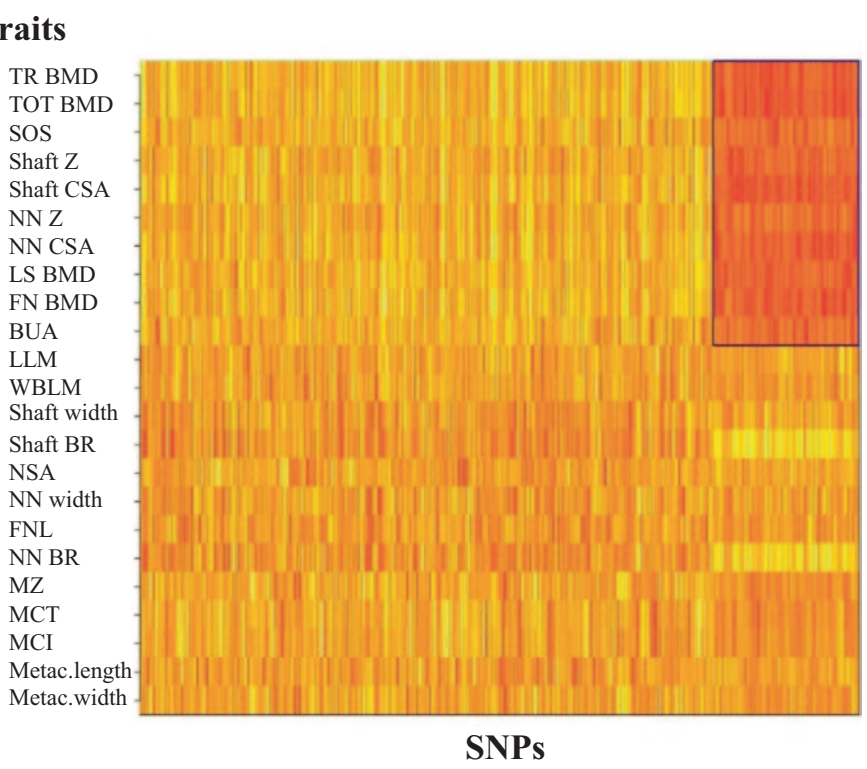

Fig. 7.1 Block cluster found in SNP data from the Framingham Osteoporosis Study. Cluster is outlined in blue; the horizontal and vertical axes denote the SNP IDs and measured variables, respectively. Heat map: color scale for a sign and strength of SNP-phenotype association (*yellow* color corresponds to the positive, *red* color corresponds to the positive, ber> Block cluster found in SNP data from the Framingham Osteoporosis

FHS women were measured for 21 bone traits (BMD of the hip and spine, heel ultrasound, hip and metacarpal geometry), and whole body and leg lean (muscle) mass by DXA. We first performed univariate GWAS of each 23 musculoskeletal phenotypes and extracted statistics for SNPs associated with at least one phenotype at a predefined threshold of significance (a α level $< 10-4$). A data matrix of standardized regression coefficients was partitioned along both axes - SNPs and phenotypes, with each partition representing a distinct cluster of SNPs that have similar effects over a particular set of phenotypes (the hypothesis here is that a perturbation in one phenotype—represented by an associated genetic variant - should impinge on other correlated traits). For efficient sampling, we adapted the originally one-dimensional evolutionary Monte Carlo procedure [44] for dealing with binary matrices in 2 dimensions. Application of this method to the Framingham Osteoporotic Study data showed several SNP-multiple-phenotype connections: we found

a strong cluster of association coefficients of high magnitude for a subset of traits (Fig. 7.1).

Application of this method to our data shows several interesting SNP-phenotype connections: the clustered traits were highly genetically correlated in members of the families [40]. Moreover, gene-set enrichment analyses indicated that genes that correlate with clustered traits are augmented in several pathways relevant to skeletal homeostasis. In spite of its application with musculoskeletal traits in the original paper, this Bayesian method might be also applied to other aging-related biomarkers to approximate the "phenome" of aging, based on the tight clustering of genetically-related biomarkers of aging. Furthermore, this knowledge may prove helpful in identifying novel genes and pathways that govern correlated phenotypes.

Approaches such as above are necessarily limited by the power and precision of the univariate analyses upon which they are based; therefore, focusing on top signals at GWS threshold would not be sufficient to discover pleiotropic signals. *De-novo* re-analysis of multiple phenotypes was also proposed [34, 35, 45, 89], however, these methods are usually applicable to bivariate scenarios (only 2 traits at a time) due to computational restrictions. Further development of the pleiotropy-based approaches will be useful for other studies of multiple related phenotypes, which employ the genome-wide associations to decipher genetics of human aging.

Future of Genetic Study in Human Aging

In absence of a consensus on an ideal, measurable single phenotype or set of phenotypes for human aging, genetic research is impeded [52]; longevity phenotype *seems* impractical for the study of biological processes. Moreover, it is difficult to determine whether preventative and therapeutic strategies (such as caloric restriction, vitamin and hormone replacement, or change of habits) have beneficial effects in humans because there are no unequivocal biomarkers that can serve as surrogate markers of aging. Any proposed treatments to delay or alleviate aging require that validated outcomes exist, which can be preferably measurable earlier rather than later in life.

Knowledge of genetic inter-relationships between the biomarkers of aging may lead to discovery of common pathways that summarize aging processes. The GWAS approach is considered to be productive in uncovering multiple genes responsible for diseases of aging and should be able to help discern the molecular mechanisms leading to variation in aging and longevity. Comprehensive databases on biomarkers of aging in multiple large cohort studies, along with information on various health outcomes, are needed to validate the proposed phenotypes of biological aging [38]. Genetic association data can be then mined for enrichment of biological candidate pathways. Similarly, prediction of "successful aging" can be attempted using a genetic risk score based on alleles associated with aging biomarkers [17]. Several population-based cohorts such as the Framingham Study, Rotterdam Study,

AGES-Reykjavik Study [27], and many others, are therefore uniquely suited to propel the field with abundant genome coverage and longitudinal phenotyping done by standardized methods [38].

We expect that integrated indices of biological age based clinical biomarkers, similar to the Healthy Aging Index [66], will be both genetically and clinically relevant [1]. Such an integrated phenotype, if measurable earlier in life, has a potential to identify rare variation, especially when applied to family studies. Sequencing in large samples, either in targeted regions or across the whole genome, can further identify rare variations that may contribute to the biological aging mechanisms. Notably, a good annotation for identified variants is still lagging behind the biological knowledge, making prediction of variant's role questionable. For example, by sequencing of 988 candidate genes in 6 Ashkenazi Jewish centenarians, Han et al. detected a p.P391L variant in *ZMIZ1* and p.L40F variant in *PLCD4* gene. Notably, the two annotation algorithms they used, SIFT and PolyPhen2, predicted those two amino acid substitutions to be "damaging" or "deleterious" [26]. This seemingly paradoxical finding fits into a 'buffering' paradigm proposed by Bergman et al. [3] (protective factors of some kind may prevent disease-associated risk variants from being manifested (penetrant)).

There are still too few genotyped longevity cases for mega-analyses, despite the field is moving in this direction. Meta-analysis of multiple GWAS has become common practice over the past few years [60]. The main advantage of this technique is the maximization of power to detect subtle genetic effects for common traits. An even more preferable option would be to combine individual data instead of the aggregated statistics. Thus, mega-analysis of individual phenotype and genotype data would allow applying more consistent quality control and analysis, disentangle the issue of pooling control subjects used by multiple studies, and to enable efficient secondary analyses, such as conditional analyses [64]. Mega-analysis and meta-analysis yield essentially identical results in theory and in practice; recent numerical studies and comprehensive reviews discuss some emerging logistical and practical issues related to the conduct of meta-analysis of GWAS [60, 64].

In parallel to the genome-wide exploration, the candidate gene analyses that search for absence of deleterious allele in longevity are performed (such as [26] above). However, these "hypothesis-based" approaches are largely not successful. Aging and longevity are thus similar to other common complex traits. Thus, in a recent study of European ancestry patients with knee and hip osteoarthritis, of the 199 studied candidate genes, SNPs in only 2 (COL11A1 and VEGF) were associated with osteoarthritis [65]. These results are reminiscent of the similar exercise by Richards et al. [62], where among 150 tested candidate genes for osteoporosis, only 9 were associated with BMD.

Further development of the pleiotropy-based approaches by capitalizing on shared genetic associations between phenotypes will be useful, as new candidate genes for global processes may emerge for further pursuit. There are accumulating examples that prove viability of the genome-wide approaches searching for pleiotropy, from multiple other fields, such as neurocognitive tests and learning abilities

[81], blood pressure and hematological traits [36], and bone size and body lean mass [23]. In its turn, the discovery of the "phenome of aging" may advance studies of the fundamental biological processes of aging and translate into innovative diagnostic and therapeutic interventions to improve the overall health of older men and women [38].

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Chapter 8
Systems Biology of Aging

Kendra Bolt and Aviv Bergman

Introduction

To consider the systems biology of aging, we must first abandon all preconceived notions of how aging presents itself. Is it wrinkled skin? Foggy memory? Stiff joints? Although these may be some of the phenotypes we associate with advanced age, the systems biology approach to aging is more concerned with why such biological changes occur across multiple systems in a coordinated, time-dependent manner. For example, why wrinkled skin, foggy memory and stiff joints may all begin to arise around the same time in a previously robust individual.

To the disciple of Natural Selection, aging presents itself as a paradox. How does a process that favors reproductive success and survival overlook a decline in fitness so great as to have death as its endpoint? In 1957, George C. Williams presented a theory that partially allayed his concerns: his theory of Antagonistic Pleiotropy(*1*). Though nuanced and complex, this theory introduced quantitative support for a much more basic premise that has reshaped the conceptualization of aging since: he proposed that death is simply the cost of living.

In the decades that followed, the scientific literature has been inundated with new theories of aging. They have varied widely in their purported mechanisms and major players, but share that they have strayed from Williams' charming passivity. Rather than accepting death as an outcome of life, more recent theories seek to identify specific purveyors of failure; some going so far as to suggest that aging pathways have evolved for the express purpose of terminating life.

Williams' quote is astute and his question retains validity, but only under the assumption that a complex metazoan harbors an explicit desire to stay alive. One could argue that, although the desire to *not die* seems inherent to most beings, the

A. Bergman (✉) · K. Bolt
Albert Einstein College of Medicine, Michael F. Price Center, 1301 Morris Park Avenue, Room 153C, Bronx, NY 10461, USA
e-mail: aviv.bergman@einstein.yu.edu

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2_8

desire to continue living is relatively new and uniquely human. It requires a level of foresight that the majority of species do not share with us. Furthermore, the question of "maintaining what is already formed" begs that we define the stage of formation that we would like maintained; as "what has already formed" is in constant flux. It seems that the *simpler task* is actually what we're already doing: proceeding, as we have been all our lives, through the developmental program that evolved to best serve our reproductive years. The task of "maintaining what is already formed" would require all biological processes involved in developmental progression (which is, in fact, *all* biological processes), to halt. So at what point in this ongoing developmental progression should we draw a line and call all subsequent processes "aging-related"; at what point might we refer to an individual as "aging"; or should we make a distinction like this at all? Perhaps the more appropriate conceptualization is that aging begins at conception and therefore death is simply the cost of life.

Williams understood that aging is not a disease so much as the colloquial term for a process of functional decline. In his theory of Antagonistic Pleiotropy, death and decline are incidental to an organism's pursuit of reproductive success. He suggests that a gene faces positive selection pressure to be maintained in the gene pool as long as its net effect on reproductive fitness is positive. Ergo, a gene exerting detrimental effects on fitness after the reproductive period would persevere in the gene pool so long as it also imbued a beneficial effect prior. The earlier a trait is expressed (and the longer its duration of influence), the more heavily it is weighted. He quantifies this as follows:

$$W = (1 + m_1 p_1)(1 + m_2 p_2)(1 + m_n p_n)$$

where m_i is the magnitude of gene i's effect on fitness, p_i is the proportion of an organism's reproductive potential while gene i is expressed, and W is the fitness. Although Williams' theory is not sufficient to untangle the aging paradox, it succinctly presents two premises that have guided, and should continue to guide, our research in the field of aging. First, Williams' theory emphasizes reproduction as the ultimate measure of success. Second, his theory makes the point that contributions to aging are made over the entire course of an organism's lifetime. Williams' theory resonates because both its rationale and its quantitative description of fitness (W) stress the likelihood that aging is something we are doing all our lives; that development is a continuous process and that the phenotypes associated with relatively older ages are the evolving outcomes of all prior processes and events. This is a significant point that seems to have been forgotten by many contemporary researchers who view aging as a determinant rather than an outcome.

An interdisciplinary effort to compile all aging and longevity related genes has recently been initiated by Human Ageing Genomic Resources (HAGR) and its GenAge database(*2*). The consortium is managed and advised by a reputable panel of individuals within the field of aging, who have scoured the scientific literature for genetic correlates of aging. The website's latest build (Version 18/12/13) contains 298 genes purportedly related to human aging. Rather than taking these genes association with aging for granted and looking into the dowstream effects they may have on a body over

time, we abandoned preconceived notions of aging and asked the reverse question: What are we looking for when we're looking for signs of age-related change, what genes did we identify by looking for those things, and why may they have been identified as aging related? What we found was similar to Daniel Promislow's earlier work with the "aging-related" gene products of *Saccharomyces cerevisiae*; genes related to developmental and aging processes showed increased levels of pleiotropy (are associated with a higher number of unique gene ontology processes) and connectivity (are involved in a higher number of unique protein-protein interactions)(*3*). By using genes defined by the GenAge database, we showed that the genes we have come to associate with aging also tend to have higher levels of connectivity, have higher degrees of pleiotropy and have a higher number of splice pattern variants (meaning that a higher number of proteins with unique amino acid sequences are produced by each gene). In network terms, this suggests that aging related genes are the highly connected "hubs", while less-connected genes are called "nodes". Since most biological networks follow a power-law distribution, they will contain a large number of genes with few connections, and a small number of genes with a very large number of connections. Due to being less common, the likelihood that a highly connected node is damaged or altered is small. However if alterations to a node were to occur, the consequences of that change would have far greater affects on a network than would alterations to an edge and that network would suffer more heavily.

It is likely that the pleiotropic and highly connected nature of these genes is both why they are involved with processes associated with aging, and why they were *identified* as being involved with processes associated with aging. Regardless, the findings support the premise of Williams' Antagonistic Pleiotropy theory by suggesting that if we want to understand aging, we have to look to the everyday processes of living, growth, and development. To this end, we suggest a novel aging theory, which modifies concepts from several leading theories to produce a single, cohesive one: the Burn Out Lifespan and Thermosensitivity, or BOLT theory.

The BOLT theory suggests that there is an additional cost associated with elevated metabolic rates, which, alongside high metabolic costs, establishes the upper limit of human body temperature and lifespan. It suggests that neither the production of heat nor the byproducts of heat production (such as reactive oxygen species), but *the presence of heat itself* which causes the damage that facilitates organismal decline. Therefore, lifespan is negatively correlated with the bodys production of heat (as through the processes of growth and metabolism) and positively correlated with its ability to utilize or disperse heat (body composition).

Simply stated, the BOLT theory suggests that minute quanta of thermal energy, produced as normal byproducts of metabolism, cause shifts in the energetic fields surrounding the atoms within a protein. These shifts are sufficient to alter the behavior of proteins, but too small to cause structural change that might be detected by repair mechanisms. Those altered behaviors lead to subtle changes in the way that proteins interact with one another, which lead to subtle changes in the topologies of protein-protein interaction networks. Emergent properties include functions, complex mechanisms and phenotypes, and are dependent on network topologies. Therefore topological changes will manifest as altered functions, mechanisms and phenotypes over time (Siegal, M. L., Promislow, D. E. L., & Bergman, A. (2007).

Functional and evolutionary inference in gene networks: Does topology matter? *Genetica, 129*(1), 83-103). Indeed, increased variability in a proteins interaction partners has been detected over time. Moreover, the time over which variability is measured outlasts the lifetime of a single protein, providing evidence for the heritability of such behavioral changes across protein and cellular generations (Southworth LK, Owen AB, Kim SK (2009) Aging Mice Show a Decreasing Correlation of Gene Expression within Genetic Modules. PLoS Genet 5(12): e1000776. doi: 10.1371/journal.pgen.1000776).

We will look at each facet of the BOLT theory in more detail, beginning with consideration of existing theories of human aging. Although most theories vary widely in their proposed mechanisms, they share some sort of failure as their eventual outcome and cause of death. Damage or mutation is said to accumulate within a cell until some critical level is reached and the cell dies. This continues with age until at some point, an entire organ system will fail and in turn, so too will the organism. However, these theories cannot elude certain criticisms: First, if damage occurs at random, why don't we see repair mechanisms overwhelmed in a similarly random and age-independent manner? Second, how can we account for differential rates of aging between but not within organs and tissue-types? Third, how can we account for the variation seen in human lifespans and the specific aging-related phenotypes each person expresses?

The answers to these questions are likely to come through our recent understanding of aging as a complex, epigenetic, and network-based phenomenon. Recent studies have shown that aging is an outcome of many small changes in a great number of cellular components, rather than any defined aging pathways [18]. Further, those cellular components and the extent to which they are affected will vary between tissue and organ types [19]. Promising new studies explore the network architectures of cellular interactomes and reveal that age-related changes involve a loss of coordination between constituent nodes [15]. It has been observed that the extent to which these age-related topological changes occur depends on cell type [16], but no causal mechanism has been implicated. The BOLT theory suggests that thermal energy is the catalyst behind age-related epigenetic changes, and that variations in its production and dissipation can explain different rates of aging.

It has been observed that lifespan scales with body mass, and that body mass scales with metabolic rate to the 3/4 or 2/3 power [1, 20]. Contentions exist as to whether this exponential term should be 3/4 or 2/3, with qualitative evolutionary explanations making up the bulk of support for the former. On the other hand, the Heat Dissipation Limit has been offered as support for the latter, with the relationship between body mass and surface area being credited for body mass-imposed limits on metabolic rate [5, 21, 22]; by proposing that an organism's ability to dissipate heat is what limits its growth and metabolism. This implies that an inability to effectively dissipate heat will have some consequences for an organism, some of which may lead to mechanistic and phenotypic variability that, simply due to its deviation from the norm, may be perceived as functionally detrimental. Because these alterations occur over time, they're referred to as signs of aging. Thus, we can say that lifespan and the age-related processes colloquially referred to as aging may be correlated

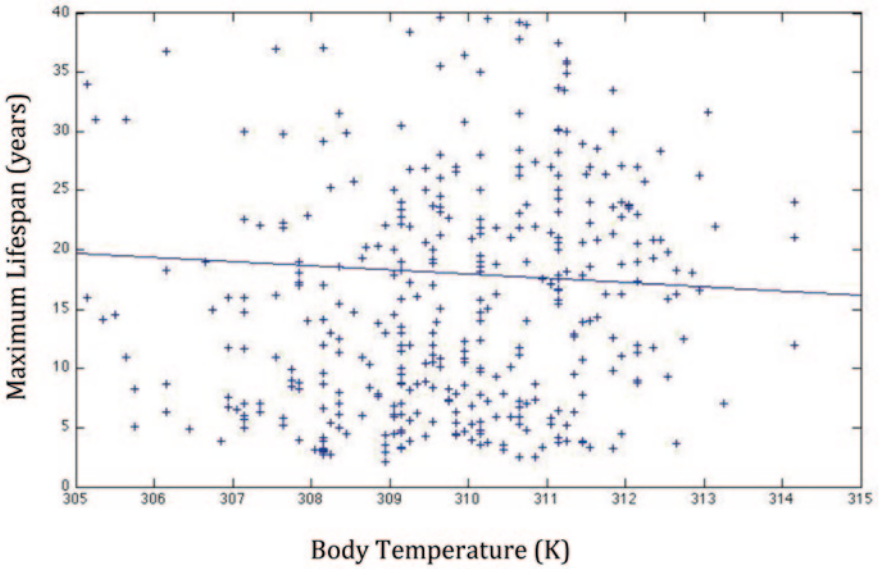

Fig. 8.1 Temperature versus lifespan. Life-expectancy scales with body temperature across species

with body temperature. A conglomerate analysis of raw body temperature and lifespan data from a wide range of taxa can be analyzed to suggest precisely this ([23] Fig. 8.1). It's likely that the weak albeit significant relationship shown in Fig 8.1 varies between one taxon and another, so a further analysis of interest would group data points by taxon and identify the outliers.

So far, we've suggested that heat is the unifying and overlooked contributor to most existing theories of aging. We've stated that the Burn-Out Longevity and Thermosensitivity theory may be able to bridge gaps in our current conceptualization of age-related decline. Now, we'll introduce and explore the BOLT theory in greater detail. To review, the BOLT theory suggests that minor fluctuations in thermal energy are the antagonizing agents of age-related changes that result in functional decline. In network terms, we can say that heat alters the properties of nodes (biomolecules) or hubs (highly connected biomolecules) such that the edges (the nature of the interactions) between them are modified and topological changes to network architectures allow new properties to emerge [13]. The extent to which molecules are susceptible to heat-induced damage (their thermosensitivity) will also contribute to the rate of a networks topological decline. In order to assess the validity of this theory, we question whether the energetics of aging related genes might have enhanced susceptibility to heat induced change, as predicted. We then ask whether such thermally-induced behavioral changes to gene products may be able to alter the topologies of biomolecular networks. Finally, we explore the feasibility of altered topologies giving rise to significantly altered phenotypes, such that lifespan or course of aging are affected.

The Mechanistic Theory Behind Thermosensitivity and Temperature Dependence : Do thermally induced changes occur?

Endothermic animals have evolved a system that enables them to maintain their bodies at a temperature that is optimal for their biological functioning. On the other hand, some ectotherms have developed the ability to conform to external temperatures so that their mechanisms function best at ambient temperatures. The bullhead catfish, for example, has developed the ability to adjust the fatty acid composition of its membranes and alter its transcription profile to generate enzymes with analogous functions but different temperature optima, and synthesize entirely new enzymes as needed for new temperature environment. By rendering the external environment irrelevant, the stubborn endotherm has bypassed the evolutionary opportunity to develop acclimating mechanisms. The downside is that endothermic mechanisms will only function appropriately within a narrow range of temperatures. And in the rare event that one departs from that narrow range, biological mechanism may not function correctly or at all. The upside, is that the endothermic body provides a consistent environment for its mechanisms almost all of the time. So while the ectotherm is able to adapt to its environment, the endotherm can, to an extent, control it. As endotherms, humans are very good at maintaining homeostasis, such that the human body is typically maintained within the range of 36 and 38 °C. Core temperatures are usually a bit higher, while the body's shell typical averages around 33 °C. The body is much more sensitive to increases in temperature than to decreases: an increase in core temperature as small as 2 °C can cause heat stroke, while 4 °C can cause death. A more extreme decrease of 8 °C can lead to semiconsciousness, while 12 °C below the normal range introduces the risk of death due to cardiac arrest. Our skewed tolerances are likely due to the rapid responsiveness of thermogenic mechanisms, while heat dispersal does not have finely regulated internal mechanisms and is dependent on external factors like ambient temperature and humidity. Luckily, core and peripheral thermosensors can be activated by fluctuations in temperature on the order of 1/10th–1/100th of a degree (27–29) , so that a healthy body is able to rapidly respond before body temperature steps outside the normal range. However, in order to activate a thermosensor, enough unbridled heat must first build up in the system. The fact that this heat comes into contact with thermosensors is incidental, and likely representative of the way that such quantities of heat come into contact with all other cellular components. The fact that these thermosensors alter their structure to perform a function (transmit a signal) when they come into contact with thermal energy is evidence of proteins having the ability to alter functionalities in response to minute temperature fluctuations without being recognized as damaged. We suggest that proteins that did not specifically evolve to respond to temperature changes the way that thermosensors have may endure subtle changes to their energetic fields that result in altered behaviors like binding affinities, motilities, reaction kinetics and so on. To test the hypothesis that proteins' behavioral integrities can be altered by heat, we look at the stability of several different groups of proteins in two different ways: energetic calculations of electrostatic interactions, and surrogate data on protein half-lives.

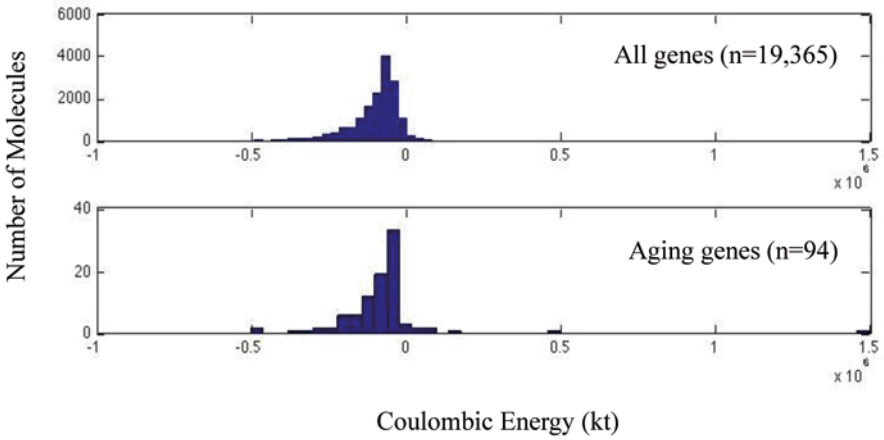

Fig. 8.2 Protein energetics. Proteins associated with aging have different energetic profiles to the rest of the protein set

Energetic calculations were done by looking at the strength of minute energetic fields surrounding the constituent atoms of a protein. This was done by measuring coulombic and Gibbs Free energy values for over 100,000 human protein structures obtained via high-resolution nuclear magnetic resonance (NMR) files from the Protein Data Bank. For coulombic energy values, PDB structures were hydrolyzed through public access software program Reduce (*http://kinemage.biochem.duke.edu/software/reduce.php*, [27]) and then assessed for coulombic energy values using the Poisson Boltzmann Solver and molecular electrostatics modeling package DelPhi (http://compbio.clemson.edu/delphi.php, [28]). A total of 19,365 coulombic energy values were produced, and the coulombic energy values for proteins associated with aging were compared to those of the rest of the human proteome (Fig. 8.2).

Three methods were used to calculate the Gibbs free energy values: the software packages Opus-C++, RF_CB_SRS_OD and RF_HA_SRS, which calculate energetic field strengths based on the orientation of alpha-carbons, beta-carbons, and all atoms, respectively [29, 30]. Although energetic comparisons between aging related and non-aging related genes suggested that a difference may exist, the these analyses were not able to address the specific hypothesis proposed by the BOLT theory. Again, the BOLT theory suggests that minute amounts of energy are able to facilitate a shift in protein behavior, while the visualization of stagnant protein structures is unable to reveal anything definitive about the functionality of each protein state or the ease with which it may shift between them [7]. Therefore, these stagnant images may not be a reliable surrogate for temperature dependent functional change.

A surrogate analysis was introduced to indirectly query the stability of different proteins, the hypothesis being that a protein with greater susceptibility to heat induced change will also naturally degrade more easily and therefore, have shorter half-lifes and higher production rates to compensate for high turnover. Raw values for half-lives, transcription rates, mRNA copy numbers and protein copy numbers were derived from previous studies [31]. Half-life and copy number values from

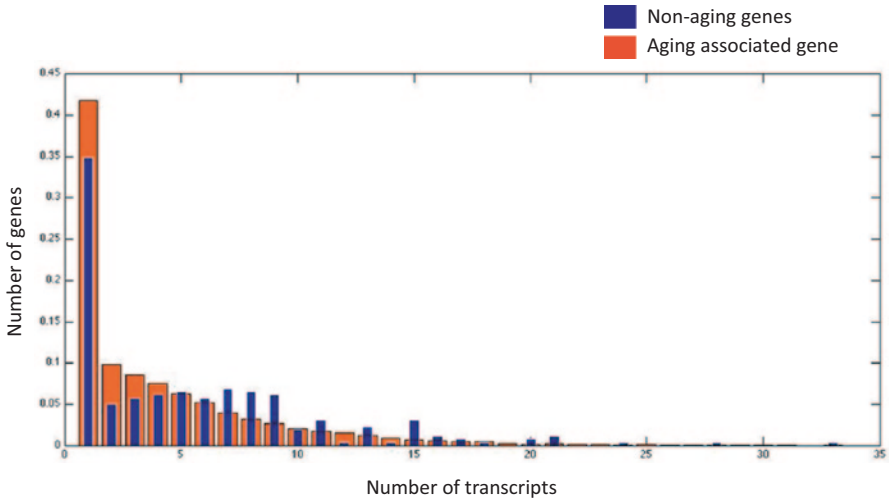

Fig. 8.3 Protein variants per gene, aging versus non-aging genes. For genes associated with aging, the number of protein variants produced by alternative splicing tends to be higher

these experiments were partitioned into functional groups based on queries performed in Ensembl and Ingenuity Pathway Analysis. By comparing the half-lives, transcription rates, mRNA copy numbers and protein copy numbers for several subsets of genes to the remainder of the human protein set, we found that although aging genes do not have shorter half lives, they do have relatively higher transcription rates ($p=2.3e-8$), mRNA copy numbers ($p=5.44e-7$), and translated protein numbers ($p=0.0076$) than other human gene products. Interestingly, the subset of human proteins that did show relatively shorter half lives was that containing proteins necessary for the spliceosome; those needed to complete the post-transcriptional processes of alternative splicing ($p=.0323$). This suggests that the altering effects that thermal energy has on aging-related genes may actually be indirect and mediated through spliceosomal complex formation, since we also show that aging related genes have a greater relative dependency on alternative splicing than the rest of the human genome (Fig. 8.3). Greater resolution of transcript expression assays will be necessary for the exploration of this hypothesis.

Topological Changes: Do thermally induced changes lead to topological network changes?

The BOLT theory is concerned with the modest affects of unbridled thermal energy that do not warrant intervention by repair mechanisms. This includes changes in binding affinities, kinetics, motilities, and functionalities, and can be dependent on minute energetic fields within molecules, localization, or concentration. Unlike changes to conformation or sequence, these changes persist unnoticed by heat shock proteins or other canalizing mechanisms [8, 9]. Indeed, the field of epigenetics has

revealed numerous mechanisms of phenotypic switching that do not rely upon detectable physical changes to molecules. Genetically identical monozygotic twins have shown increasing variability between their phenotypes with age, which can only be explained by epigenetic processes [32]. Moonlighting proteins provide a recently discovered example of how this may occur, as they are capable of switching between functions without modifying their structures in any way [17]. Proteins containing alpha-helices often contain stutter regions, which allow them to irreversibly change their conformation and stability, without eliciting a corrective response from chaperones or repair mechanisms [33]. Transcription factors have long evidenced the importance of concentration; the ratio of one transcription element to another is sufficient to flip a phenotypic switch [34]. Not to be mistaken with the physical modifications that occur over evolutionary time (a la Medawar's mutation accumulation theory), these behavioral modifications accumulate over the course of the individual's lifespan and must therefore be inherited by daughter cells. If, as in moonlighting proteins and transcription factors, molecular ratios dictate function and functional output, then alterations are passed down as ratios– even when cellular constituents are halved by division, their ratios will remain constant in daughter cells and alterations will be propagated.

Phenotypic Outcomes: Do topological changes induced by temperature fluctuation affect phenotype and function?

Studies have shown that transient exposure to heat can cause changes in developmental regimes that are propagated as growth proceeds [35], so that the extent to which a network deviates from its original topology and output will increase with time. Here, we provide support for the BOLT theory with a model that describes the corrosive role of heat in biological systems. The extent to which a cells emergent properties will change over time, D, will be proportional to dissipated energy, less repair and maintenance, and its metabolic rate, $B_0\, m^{\alpha}$, which scales with cell mass, to the power α. So that

$$D = \int dt\left[\left(ß - g\right)B_0 m^{a}\right] \tag{1}$$

B_0 is a taxon specific normalization constant that can be further detailed as

$$B_0 \text{ is proportional to } e\hat{}\left(E_i/kT_0\right) \tag{1.1}$$

where E_i is the taxon specific activation energy for biochemical reactions, k is the Boltzmann constant, and T is the temperature in Kelvin. With all else being equal, a change in temperature from B_0 to B_t would alter the taxon specific normalization constant according to:

$$B_t / B_0 = \ldots e\hat{}\left[\left(E_i / k\right)\left(T_T - T_0 / T_T T_0\right)\right] \tag{1.2}$$

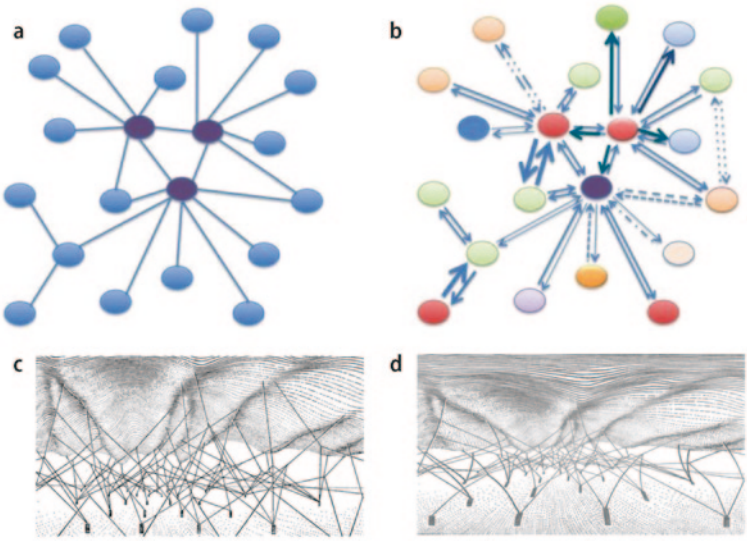

Fig. 8.4 Suggested network changes. Behavioral changes to network nodes may shift architectures and emergent properties, as visualized using Waddington's epigenetic landscape

so that wherever $T_T > T_0$, then $B_T > B_0$ and therefore $D_T > D_0$. This is to say that cellular function will deviate in proportion to temperature.

The mosaic pattern of aging can then be modeled based on divergent thermal properties between tissue types [36]. Metabolic rate, thermal conductance and heat capacity all vary with tissue type. The fact that different cell types have different components and morphologies means that they will be affected by these alterations to varying degrees. Since the metabolic properties of a given cell type are constant within cells of that type, there will not be a large discrepancy in the rate of age-related change in a single tissue type.

Waddington's theory of genetic canalization suggests that together, nodes and edges create something analogous to a scaffold supporting the epigenetic landscape [10]. We can think of a phenotype as the path taken by a ball rolling down that landscape. Just as in energetics, the ball will follow negative gradients; often moving through narrow canals formed by closely related but relatively unfavorable options. To alter the scaffold and landscape is to alter the route a ball will take and thus, an individual's phenotype. The BOLT theory suggests that thermal energy warps this scaffold to elicit different phenotypic outputs. Figure 8.4a presents a biological network as they are most often portrayed, while Fig. 8.4b hints at the point that network complexity is much greater than such cartoons can represent. We can imagine that in Fig. 8.4b, edge lengths represent physical proximity, node and hub colors represent electronegativities, arrow weights represent reaction kinetics, and so on. When interactions are strengthened or weakened due to any of the heat-induced "behavioral" changes suggested, we can imagine this taking the form of new edges appearing or existing edges dissolving. Figure 8.4c addresses this concept through

Waddington's original image of an epigenetic landscape [10], while Fig. 8.4d offers visualization of the change in topology once a few scaffolds (edges) have been added and removed.

Although a correlation between lifespan and body temperature has been made clear [37, 38], the mechanism behind it has remained elusive. Here, we provide a theoretical framework in which observed patterns of aging can be intuitively understood. The BOLT theory also opens a new avenue for understanding longevity mechanisms, by suggesting that an individual with reduced heat production or advanced heat dissipation may experience age-related changes more slowly. Many eurythermal organisms maintain function in changing thermal environments through the use of splice pattern variants. Post-transcriptional splicing modifications may produce isoforms which perform optimally in different temperature environments [39]. Humans may employ a similar mechanism; we've shown that genes associated with human aging and longevity ($n=261$) [40] have a significant tendency to be spliced into a higher number of splice pattern variants when compared to the rest of the human gene set ($n=22{,}187$; $p=5.96\times10^{-9}$). Future studies may be able to determine whether all variants of a given gene are produced in all individuals or if not, then whether the expression of a certain subset of gene products can be correlated with longevity data (Fig. 8.3).

Implications: Can these changes to phenotype and function account for the processes we call aging?

The Burn Out Lifespan and Thermosensitivity theory suggests that heat and thermosensitivity are the predominant determining factors in aging. Cellular components are almost literally burnt-out over the course of a lifetime. Minute temperature fluctuations alter the behavior of network components, which result in changes to the emergent properties of those networks. However, being a polygenic, multi-component network involving all gene products to some extent, it has been difficult to pinpoint a single conserved mechanism.

We know that heat can directly damage DNA and proteins, or it can denature a strand of DNA so as to leave it susceptible to damage by radiation, mutators, reactive oxygen species (ROS), etc. We have seen cases of cellular metabolism causing nucleotide damage, indeed, some studies have associated the production of ROSs with DNA damage [4] while others have argued that high numbers of ROSs have no discernible relationship with lifespan [5]. This contention may lie in the fact that while ROS's and DNA damage are both correlated with the heat dispersed by cellular metabolism, they are not directly associated with one another. Thus, a significant relationship is observed only part of the time. Meanwhile, the notion of accumulated change calls to mind Medawar's classic Mutation Accumulation theory of aging, while the BOLT theory differs in both mechanism and implication. Medawar suggested that mutations to DNA sequence grow too numerous for repair mechanisms to address, and that those *physical* changes are transcribed and translated into dysfunctional mRNA or protein products. Contrarily, the BOLT theory

suggests that alterations are initially *behavioral*; induced by small fluctuations in temperature. These transient *behavioral* changes to network components produce lasting changes in the emergent properties of those networks.

An exciting aspect of the BOLT theory is its ability to account for the large-scale, tissue-specific mosaicism that is empirically observed in aging. Cellular differentiation guides each cell and tissue type to develop a particular internal milieu reflecting its specialized tasks and transcriptional schema. The reactants, reactions and products present within a cell type, as well as the permeability of its membrane, water composition, et cetera will vary between cell types and therefore contribute to the discrepant effects that free thermal energy will exert, but not within, different cell types. Over the course of a lifetime, those discrepancies are sufficient to alter the kinetics, motility, binding affinities, and structures of essential cellular components(19), to the extent that within a body, different organs and tissue types will age with minor but noticeable differences in timing, while within an organ or tissue, aging progresses in an extremely concerted manner. Indeed, this is precisely what we see in the aging human body, and what no other theory of aging has yet been able to explain.

We and others have previously introduced the notion of buffering mechanisms imbuing longevity phenotypes, and here we present a mechanism with both heritable and environmental components. We suggest that some degree of deviation from a theoretical level of optimum health is unavoidable if progression through development is to occur.

Our work underscores the utility of a systems approach to cell and body, and reiterates the ease with which compounding factors are overlooked from the reductionist perspective. Direct experimental evidence is still needed to validate our proposed role of unbridled thermal energy, but we are optimistic that the results of such investigations will have significant implications for lifespan and healthspan management, as well as our understanding of a host of other medical conditions not necessarily correlated with age.

Data Resources

Aging genes

Lists were obtained from Human Ageing Genomic Resources, GenAge Build 16 (September 2012). [41] *http://genomics.senescence.info/help.html*

GenAge contains several lists, the following 4 were considered separately:

i. Genes that affect human lifespan by impacting overall health (111 Genes, 1734 PDB, 1280 Cou)
ii. Genes that are differentially expressed in humans at different ages (72 Genes, 1011 PDB, 848 Cou)
iii. Genes associated with aging in model organisms that may be relevant to human aging (288 genes, 3033 PDB, 2676 Cou)
iv. Genes that can appear to modulate higher mammalian aging (22 Genes, 23 PDB, 23 Cou)

PDB files n = 20115 Atomic coordinate files for all catalogued human proteins were obtained from the Worldwide Protein Data Bank. These consist of solitary and

complexed proteins, so that a single PDB ID for a protein complex will belong to the dataset of each of its constituent proteins. [42]
ftp://ftp.wwpdb.org

Reduce: Protonated PDB files n = 19365 output PDB files were protonated prior to DelPhi analysis by the software program reduce (per recommendation by DelPhi and Amber developers). [27]
http://kinemage.biochem.duke.edu/software/reduce.php

DelPhi: Coulombic energy values n = 19365 output Coulombic energy values were calculated using the Poisson Boltzmann Solver and molecular electrostatics modeling package, DelPhi. [28]
http://compbio.clemson.edu/delphi.php

RF HA SRS: Gibbs free energy values n = 2920 genes Gibbs free energy values were calculated based on PDB crystal structures with 50 % sequence and sequence length homology with human genes of interest, using the RF_HA_SRS program developed by Dmitry Rykunov and Andras Fiser [29].

Gene names, ID conversions, Transcript counts (20806 genes, 21355 PDB, 18393 Cou) Accessed through Ensembl US East release 71.37 (April 2013), *Homo sapiens* genome assembly GRCh37.p10 (February 2009). Copyright Wellcome Trust Sanger Institute/European Bioinformatics Institute. [43]
http://useast.ensembl.org/Homo_sapiens/Info/Index

Spliceosomal genes (143 genes, 305 PDB terms, 255 Cou values) Lists of genes associated with assembly and function of the human spliceosome were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems). This includes human genes categorized as having involvement in: splicing of primary transcript RNA, mRNA, hnRNA, tRNA, reporter mRNA; alternative splicing of mRNA; and spliceosome assembly.
www.ingenuity.com

Statistical Analyses All statistical analyses were performed in MATLAB (Version 7.10.0. Natick, Massachusetts: The MathWorks Inc., 2010). For comparing the distribution and correlations of GO terms, protein-protein interactions, splice pattern variants, and translated proteins, the Mann-Whitney U test or Wilcoxon rank-sum test was applied. This method of assessment is the gold standard for non-parametric statistical hypothesis testing. To determine correlation coefficients and their significance, the function corrcoef was applied to retrieve a matrix of p-values for testing the hypothesis of no correlation.

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Chapter 9
Epigenetics of Aging

Dan Ben-Avraham

Background

Aging has emerged as a major global public health issue [17]. Life expectancy has increased steadily over the last two centuries [90], however, a significant proportion of the extra life years is associated with many types of morbidity. Age-related diseases, including cancer, cardiovascular diseases and dementia, are now the dominant health problems among the elderlies in most western countries. Identifying the underlying molecular changes that occur as part of the aging process and how they contribute to the development of age-related diseases will be critical for improving the health outcome for elderly patients, and in potential preventative strategies. The molecular basis of human aging is currently being investigated in many experimental contexts, including telomere shortening, DNA damage, degeneration of cellular or organ structures, genomic studies, and changes in gene expression. Both genome-wide association studies and candidate gene approaches have provided valuable information on the role of DNA in aging and disease risk. These approaches, however, do not provide information on differential genetic expression due to developmental or epigenetic changes [84].

Epigenetic changes refer to gene expression alteration that arise from chromosomal changes without DNA sequence modification. These changes, which collectively make up the epigenome, include; alternations in DNA methylation patterns, post translation modification of histones, chromatin remodeling, and non-coding RNAs (e.g., microRNAs) which in turn may affect gene expression and genomic instability during aging [64, 65]. Although the contribution of epigenetics to several human diseases such as cancer, metabolic diseases, and neurodegenerative disorders has been proved [6], the epigenetic variations in normal tissue due to aging is still poorly understood. Invertebrate model organisms, such as yeast, worms, or

D. Ben-Avraham (✉)
Departments of Genetics and Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
e-mail: danny.ben-avraham@einstein.yu.edu

G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2_9

flies, have been extensively studied in the context of longevity, reporting fundamental clues about the mechanisms through epigenetic factors that contribute to aging [7]. In contrast, the mechanisms by which epigenetics promote aging and age-related diseases in mammals are not well defined. In this regard, monozygotic twins are a good model for studying epigenetic changes linked to aging: they provide evidence that such changes can accumulate over time, as one individual may gradually undergo alterations that his or her twin (with identical DNA) does not [28, 77]. Inheritance of epigenetic modifications has been reported in a variety of taxa, ranging from plants [45], yeast [35], and flies [15, 49] as well as vertebrates such as mice [1, 37, 72] and humans [29]. In general, DNA hypermethylation leads to gene silencing, and DNA hypomethylation endorses gene activation. DNA hypermethylation causing silencing of genes involved in the cell cycle, apoptosis, detoxification and cholesterol metabolism [14] has been reported. Aberrant regulation of epigenetic mechanisms can result in genomic imprinting disorders, such as Angelman syndrome and Prader-Willi syndrome, and may contribute to the heritability of many forms of cancer, asthma, Alzheimer's disease, and autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis [39, 42, 44].

Role of Epigenomic Modification in Aging

Aging is a complex physiological process that results in compromise of biological functions, increased susceptibility to age-related diseases, and eventually death [7]. It is well recognized that human aging and longevity are influenced by both genetic and environmental factors. Inherited genetic mutations and polymorphisms resulting in alterations in gene function can explain some features of aging and age-related diseases. However, in addition to inherited genetic factors, aging is influenced by the gradual accumulation of molecular alterations since birth. Environmentally induced changes in the epigenetic processes involve alterations of gene expression without a change in DNA sequence, and can determine different aspects of aging, as well as pathogenesis of age-related diseases [33]. Epigenetic changes can specifically play a role in the modulation of aging processes and healthy life extension [87]. In particular, promoter methylation changes and associated gene silencing are the epigenetic changes seen in age-related diseases.

In addition to DNA methylation, several types of histone modifications have been demonstrated to occur both globally and at gene-specific loci during aging [33]. Other epigenetic processes including histone modifications of Polycomb group proteins, chromosomal position effects, and methylation of ncRNAs are modulated with aging.

Common human age-related diseases are accompanied by a loss of genomic DNA methylation. Progressive loss of genomic DNA methylation has been demonstrated throughout the human genome [12], in mice, and in cell lines [78, 93, 94], although this decrease may be tissue and/or gene specific [70, 71, 95]. Age-related

epigenetic changes have also been demonstrated in sperm cells; however, the direction of change over time appears to be gene specific [25]. Within pairs of twins, differences in DNA methylation are greater in older than in younger monozygotic twins [28]. The age-related decrease in long-term synaptic plasticity, especially long-term potentiation (LTP), manifesting as cognitive decline, is linked to histone acetylation and BDNF/trkB signaling. Indeed, treatment with histone deacetylase inhibitors or a neurotrophin receptor B agonist restores LTP in the hippocampus of old animals. These studies suggest that epigenetic changes may play a significant role in age-related diseases [61].

DNA Methylation and CpG Islands

DNA methylation occurs by the addition of a methyl group to the aromatic ring of a cytosine (5-mC), mostly located 5′ to a guanosine (CpG) [50]. In mammalian genomes, approximately 70–80 % of CpG dinucleotides are methylated. However, stretches of CpG-rich sequences with low levels of DNA methylation, known as CpG islands, exist [10, 20]. CpG islands make up only 1 % of the human genome but contain 5 % of the CpG dinucleotides [20]. CpG islands are often highly enriched at gene promoters, and approximately 60 % of all mammalian gene promoters are CpG-rich. The changes in DNA methylation patterns observed during aging are loss of DNA methylation at the genome-wide level in combination with gains in methylation levels in CpG islands in or near gene promoters [89]. DNA methylation also recruits methyl-CpG-binding proteins, which recruit additional proteins that add silencing modifications to neighboring histones. This coordination between DNA methylation and silencing histone marks leads to compaction of chromatin and gene repression [59].

The mechanisms that keep CpG islands free of methylation appear to involve binding of transcription factors and other transcriptional machinery, or the act of transcription itself. However, CpG islands can become hypermethylated [60, 63] to silence specific genes during cellular differentiation, genomic imprinting, and X chromosome inactivation.

The methylation status of a DNA sequence can be determined using a variety of techniques such as the use of restriction enzymes (REs), which recognize short DNA sequences and cleave double-stranded DNA at specific sites within or adjacent to these sequences [82]. Bisulfite sequencing refers to another technique that assesses DNA methylation through bisulfite conversion, which converts unmethylated cytosine residues to uracil residues. Methylated cytosine residues remain unmodified [5, 30].

An epigenome wide association scan (EWAS) identified age-related differentially methylated regions as well as differentially methylated regions associated with age-related phenotypes [4].

Because aging is associated with specific DNA methylation changes at specific CpG sites in the genome, some attempts were made to implement a model, or

epigenetic signature, to predict the biological age. Several CpG sites show almost linear DNA methylation changes during aging. In 2011, a study was published showing a model for saliva samples of 34 male identical twin pairs aged 21–55 years. 88 CpG sites changes correlated significantly with age [11]. Three of these sites were located in the promoters of EDARADD, TOM1L1, and NPTX2 genes. Other authors demonstrated that one single CpG site was associated with the age related gene ELOVL2 can explain 47 % of the variance of age with an accuracy of 3.7 years [26]. Another group built an epigenetic aging signature using five CpG sites located in the genes NPTX2, TRIM58, GRIA2, KCNQ1DN and BIRC4BP. The average absolute difference between predicted and real chronological age was about 11 years [51]. This model was improved, using only 3 CpG sites—located in the genes ITGA2B, ASPA, and PDE4C [92]. This epigenetic aging signature has an age prediction with a mean absolute deviation from chronological age of less than 5 years. Furthermore, patients with acquired aplastic anemia or dyskeratosis congenita—two diseases associated with progressive bone marrow failure and severe telomere attrition—are predicted to be prematurely aged [92]. Using human dermal fibroblasts, 75 CpG sites were differently methylated upon aging. From these sites, there was a large group within the INK4A/ARF/INK4b locus [52]. These epigenetic modifications at specific CpG sites support the notion that aging represents a coordinated developmental mechanism that seems to be regulated in a cell type specific manner.

Whole genome bisulfite sequencing of DNA from CD4+T cells of a centenarian and a newborn identified differentially methylated regions that were usually hypomethylated and less correlated with methylation of adjacent CpG dinucleotides in the centenarian [43]. These results support the idea that small cumulative DNA methylation changes accumulate over a lifetime. While it is challenging to quantify these changes of tissue aging within an organism, an epigenetic method for doing just that was recently published [46]. Using this method it was suggested for example, that heart tissue is actually characterized by a particularly slow aging rate. Age-related temporal changes in DNA methylation also show significant familial clustering, indicating that methylation maintenance is a familial trait [9]. A study of DNA methylation in centenarians and their offspring compared with the offspring of non-long-lived individuals and young individuals showed that the offspring of the centenarians demonstrate delay in age related methylation changes [31]. A paper by Hannum et al. [40] offers an explanation for this familial nature [40]; using methylome analysis to compare human aging rates in individuals of age 19–101, they identified specific methylation QTLs (including one at methyl CpG binding domain protein 4). Also, trans-generational epigenetic inheritance of extended lifespan has been demonstrated in *C. elegans* [34].

Aging of hematopoietic stem cells (HSCs) leads to functional impairment and is a possible cause for hematopoietic malignancies in the elderly. Examination of the HSC methylome from differently aged mice revealed an age-dependent hypermethylation of PRC2-regulated genes concomitant with a reduced transcription of genes Ezh2, Suz12 and Eed encoding PRC2 components [3]. The data suggest that PRC2-mediated gene repression may decrease upon aging, thus allowing the DNA methylation machinery to more readily methylate PRC2 target genes.

Early evidence demonstrated that there is a global decrease in DNA methylation in different human tissues during aging [9]. This loss was attributed to a progressive loss in DNA methylation in repetitive sequences—especially Alu elements—located throughout the genome [12]. Paradoxically, and similarly to what happens in cancer, certain genes are hypermethylated. Specific age-related hypermethylation has been described at various developmentally regulated genes in various human tissues, such as *MYOD1* in brain [16, 24, 41], *PCDH10,* and *P2RX7* in intestine [57] or *DDAH2* and *TET2* in skin [36]. The extent of this hypermethylation is not yet known, but *de novo* methylation in skin during aging was recently found to affect <1 % of genes [36]. Some authors have found significant enrichment of age-dependent CpG hypermethylation at DNA-binding factors and at transcription factors [41], suggesting that deregulation of these genes could affect a broad spectrum of biological pathways and, consequently, could explain the wide phenotypic alterations of aging. However, many of the genes that were demonstrated to be hypermethylated during aging belong to the senescence and apoptosis pathways [73]. Interestingly, some classic tumor-suppressor genes that are commonly hypermethylated in tumorigenesis also undergo *de novo* methylation during aging in normal tissues [73]. Thus, researchers have proposed a link between hypermethylation of specific tumor-suppressor genes (e.g., LOX, p16INK4α, RUNX3, and TIG1) and age in non-tumorigenic gastric epithelia [80]. The three well-known, epigenetically regulated tumor-suppressor genes RARβ2, RASSF1A, and GSTP1 also become hypermethylated in premalignant prostate tissues in an age-dependent manner [53]. Likewise, the putative tumor-suppressor gene TET2 is commonly hypermethylated in myeloproliferative tumors and in aged healthy skin [36].

Muscle atrophy, or sarcopenia is a degenerative loss of skeletal muscle mass, quality, and strength associated with normal human aging. In a genome wide study of DNA methylation in aged skeletal muscle, hypermethylation was found in comparison to samples of younger people [96].

DNA methylation dynamics can also influence brain function. 5-hydroxymethylcytosine (5-hmC) is a newly described epigenetic modification generated by the oxidation of 5-methylcytosine by the ten–eleven translocation family of enzymes [83, 88]. An increase of 5-hmC with age in the mouse brain as well as an age- and gene-expression level related enrichment of 5-hmC in genes implicated in neurodegeneration have been demonstrated. Many 5-hmC-regulated regions are dynamically modified during neurodevelopment and aging [83], suggesting that 5-hmC may play an important role in the etiology and course of age-related neurodegenerative disorders [88].

Histone Modification and Histone Variants

Epigenetic gene regulation is also controlled by changes in histones that make up the nucleosome. Chromatin, which is organized into repeating units called nucleosomes, is the complex of DNA, protein, and RNAs that comprises chromosomes

[8]. In mammalian cells, most of the chromatin exists in a condensed, transcriptionally silent form called heterochromatin. Euchromatin is less condensed, and contains most actively transcribed genes. Canonical nucleosomes consist of 147 bp of double-stranded DNA wrapped around an octamer of histone proteins, usually two copies of each of the core histones H2A, H2B, H3, and H4. However, there are several histone variants that can vary by a small number of amino acids or include large insertions [75]. Often these histone variants are found at specific locations within the chromatin or are used to demarcate boundaries between heterochromatin and euchromatin regions.

Identification of proteins that read, write, or erase these marks is critical to help unravel the complexities of epigenetic regulation. Chromatin immunoprecipitation (ChIP) is a powerful assay to identify proteins that bind to chromatin and map protein binding throughout the genome using techniques such as microarray analysis or high-throughput sequencing [5].

The majority of histone-mediated regulation stems from histone modification, most often modification of the exposed amino termini of histones protruding from the nucleosome core. The predominant histone modifications include acetylation [8], methylation [48], phosphorylation [68], ubiquitination [91], and sumoylation [66], with thousands of potential combinations of modifications within a single nucleosome. Of these, histone acetylation and methylation are the best understood, and some general trends have been observed. Tri-methylation of histone H3, specifically the lysine at position 4 (H3K4me3), is associated with transcriptionally active chromatin, whereas H3K27me3 leads to compact chromatin, which represses gene expression. The term "histone code" is used to describe how different combinations of histone modifications affect transcription levels.

Increased histone H4K16 acetylation, H4K20 tri-methylation, or H3K4 tri-methylation, as well as decreased H3K9 methylation or H3K27 tri-methylation, constitute age-associated epigenetic marks [27, 38]. Early studies showed that global levels of K20H4me3 were increased in several organs of rats older than 30 months [74]. An epigenetic change that may be linked to aging in mammals is decreased expression of sirtuin1 (SIRT1) resulting in the DNA damage-induced reorganization of chromatin (chromatin instability) [65, 81]. Most importantly, several studies on pharmacological activation of Sirtuins (e.g., using resveratrol) have revealed beneficial anti-apoptotic effects [54]. This evidence makes Sirtuins potential targets for anti-aging therapies.

Noncoding RNAs and Longevity

It is likely that the effects of epigenetic changes manifest in part by effects on gene expression. There is increasing evidence that expression of noncoding RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), play a role in epigenetic gene regulation [18]. miRNAs are small non-coding ncRNAs that were initially discovered in *Caenorhabditis elegans* and since, reported across the animal kingdom. In humans, thousands of miRNAs have been demonstrated in a variety

of tissues with major impact on transcription and translational repression or gene silencing. The role of miRNAs in aging was demonstrated recently in *C. elegans* and in mice [47, 69]. miRNAs affect gene expression during the aging process in mice and modulate senescence in human cell lines [79]. An miRNA expression array performed in the livers of mice aged 4–33 months old showed more upregulated than downregulated miRNAs during aging [58]. Four miRNAs (miR-93, miR-669c, miR-214, and miR-709) were especially upregulated, and proteomic profiling of the same samples demonstrated a significant correlation between the aforementioned miRNAs and expression of the corresponding gene targets associated with mitochondrial function, oxidative stress, and proliferation [58]. The list of miRNAs associated with mammalian aging is rapidly increasing. Some examples include: Link between upregulation of miR-143 and senescence-dependent growth arrest in human fibroblasts [13], increased expression of let-7 family members in skeletal muscle aging [22], and the role of miR-27 in the aging delayed model Ames mice [2]. Models of premature aging, such as the *Zmpste24*-deficient mice also showed miRNA deregulation (miR-29) [86]. Interestingly, miR-29 upregulation was described also in somatic tissues from old mice during physiological aging. Increased expression is strongly associated with DNA damage and the p53-pathway [86], which would reinforce the link between aging and tumorigenesis. Studies in *C. elegans* and mice have resulted with some important observations, such as; (a). miRNAs work in groups (packs) by coordinating and regulating gene expression/silencing resulting in age-dependent disease states or alternatively with longevity [55], (b). inherited epigenetic effects in miRNA loci lead to changes in gene expression that modulate longevity [56, 67], and (c). miRNAs that target members of the insulin/IGF-1 pathway (a known target for genetic disruption that leads to life extension) can predict up to 47% of lifespan differences [69]. The insulin/IGF pathway acts as a cascade of phosphorylation reactions that inactivates the transport of FOXO transcription factors (i.e., DAF-16) to the nucleus and the consequent inhibition of anti-aging genes such as oxidative stress or DNA damage genes. This observation on the role of IGF-1 was further supported by studies in long-lived mutant mice that showed that higher expression of three miRNAs altered IGF-1 signaling that in turn promoted long-lived phenomenon [56]. de Lencastre et al. demonstrated that miRNAs could affect lifespan through disruption of multiple loci that are not necessarily associated with the insulin/IGF-1 pathway [19]. Some loci illustrate positive effects on lifespan, promoting longevity, and some however demonstrate the opposite effect leading to a shorter lifespan [19]. Such observations are also reported by Ugalde et al.; altered expression of two miRNAs promoted progeroid phenotype in a mouse model for a progeria syndrome through the effect on key components of the DNA-damage response pathways [85]. A genome-wide miRNA screen for differential expression between long-lived individuals and controls revealed that 10% of the miRNA microarray (863 miRNAs) demonstrated significant alterations in expression, of which only 16 were upregulated in the exceptional long-lived individuals. Most of these differentially expressed miRNAs have been associated with genes linked to major age-associated diseases, suggesting that regulation of key genes by miRNAs could promote longevity in humans [23]. Longevity-selected lines of Drosophila show gene expression profiles that are similar to younger control flies [76].

In humans, a cross-sectional analysis of individuals aged 50–90 and centenarians was used to identify a miRNA, miR-363*, whose expression declined with age but was preserved at youthful levels in the centenarians [32].

By definition, lncRNAs have a length of >200 bp and no protein coding potential; moreover, they are more cell type-specific than protein coding mRNAs. lncRNAs participate in the regulation of protein localization, translation, posttranslational modifications, mRNA stability, and chromatin shaping, often by means of their secondary structure [62].

Using deep sequencing, it is possible to obtain information on Small noncoding RNAs that circulate in the blood. The serum levels of specific sncRNAs change markedly with age. The ability of circulating sncRNAs to transmit functions between cells and to regulate a broad spectrum of cellular functions, and the changes in their levels with age, implicate them in the manifestation of aging [21].

Conclusion

Aging epigenetics is an exciting new field that is rapidly evolving through significant progress in methods and analysis coupled with advancement in technology. Investigators have only just begun to explore the variation in the landscape of biological changes during aging. Probably the greatest barriers to progress in this area are the long period of time required for assessing longevity in humans and the enormous degree of variation among individual's aging owing to environmental factors. The integration of DNA modifications, histone marks, and alterations of non-coding RNAs will surely pave the way to define reference epigenomes of aging. This reference will help identify epigenetic alterations associated with the complex process of aging and will have important ramifications on age-related diseases and therefore, life span.

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G. Atzmon, PhD (ed.), *Longevity Genes,* Advances in Experimental Medicine and Biology 847, DOI 10.1007/978-1-4939-2404-2