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Aging: Reading, Reasoning, and Resolving Using *Drosophila* as a Model System

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Introduction

Aging in contemporary biology could be described as a collection of gradual senescence processes which operates at both physiological and cellular levels. In broadest sense, aging imitates all the changes that happen over the entire course of life. Interestingly, the evolutionary biologists define aging as an age-dependent decline in essential physiological and cellular functions, leading to decline in reproductive capability and an increase in age-specific mortality rate [67]. Therefore, the aging could be best defined as a persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration [226].

Aging is a complex process, involving both the genetic and environmental factors [250]. The phenomenon of aging is represented by some most prominent characteristics such as a progressive decrease in physiological capacity, reduced ability to respond adaptively to environmental stimuli, increased susceptibility to infection and complex diseases, and increased mortality [4, 97, 261]. Some irretrievable series of biological changes which occur during the advanced stages of aging inevitably result in the death of the organism. Although the exact cause of these changes is still unsolved and almost unrelated in different cases entailing no common mechanism, yet they often indicate some shared elements of descent. Several genes and many biological processes have been found to be associated with the phenomenon of aging; however, numerous unsolved questions remain to be deliberated. This is also largely due to absence of a large number of molecular markers which could be used to measure the aging process in a tissue-specific manner. Some of the critical questions include (i) How does the aging progress? (ii) Which biological processes lead to the age-dependent cellular and physiological dysfunction? (iii) Is it possible to target the molecular pathways to combat or restrict the aging phenomenon and

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associated impairments? (iv) Can we identify some genetic modifiers with the ability to recapitulate the after-aging effects? The researchers are still trying to disentangle the various aspects of aging phenomenon in different model organisms. Therefore, even after almost an era of attentiveness to the human race since the establishment of documented history, aging remained as a most enigmatic field of biomedical research.

The last decades have shown remarkable improvement in the genetic analysis of aging, with a greater prominence near interpretation of molecular mechanisms, pathways, and physiological processes associated with longevity. Since limitations associated with human genetics do not permit comprehensive analysis on the functional and mechanistic aspects of the candidate gene(s) in greater details, and with the fact that the basic biological processes remain largely conserved in various organisms, therefore utilization of model organisms to decipher the different aspects of aging phenomenon and modifier screening has emerged as a prime approach to study the in-depths of aging process. Extensive research has been performed utilizing several model systems such as *C. elegans*, *Drosophila*, and mice to elucidate the essential genetic/cellular pathways of aging. Subsequently, classical model systems such as *Caenorhabditis elegans* and *Drosophila melanogaster* have emerged as one of the prime organisms to elucidate the essential genetic/cellular pathways of human aging. *Drosophila*, particularly, holds tremendous promise for identifying genes and also to deduce other possible mechanisms which stimulate age-associated functional declines. Some of the most important features of *Drosophila* for aging studies have been discussed below:

Drosophila melanogaster as a Model Organism for Aging Research

Drosophila is one of the oldest and the most versatile model organisms to study a diverse range of biological processes including genetics, development, learning, behavior, and aging. For the first time, Thomas H. Morgan used the tiny invertebrate *Drosophila melanogaster* for his studies on the "chromosomal theory of inheritance" and this marks the beginning of an era of revolutionary research utilizing this humble organism in his "fly room" at the University of Columbia, USA. Subsequently, the researcher has traveled a long way, and *Drosophila* emerged as an excellent model system for aging studies due to a number of advantages, that is, short lifespan (50–70) days, high fecundity (female lay up to 100 eggs per day), availability of powerful genetic tools, accessibility of stocks with many different alterations, knowledge of the complete genomic sequence, and large homogeneous populations. In addition, ease of culturing, low maintenance costs, and affordability of maintaining large population within the confines of laboratory further make flies a remarkable model organism (Fig. 14.1). Further, the absence of meiotic recombination in male flies and availability of balancer chromosomes allow populations of flies carrying heterozygous mutations to be maintained without undergoing any constant screening for the mutations. Moreover, completely sequenced and annotated

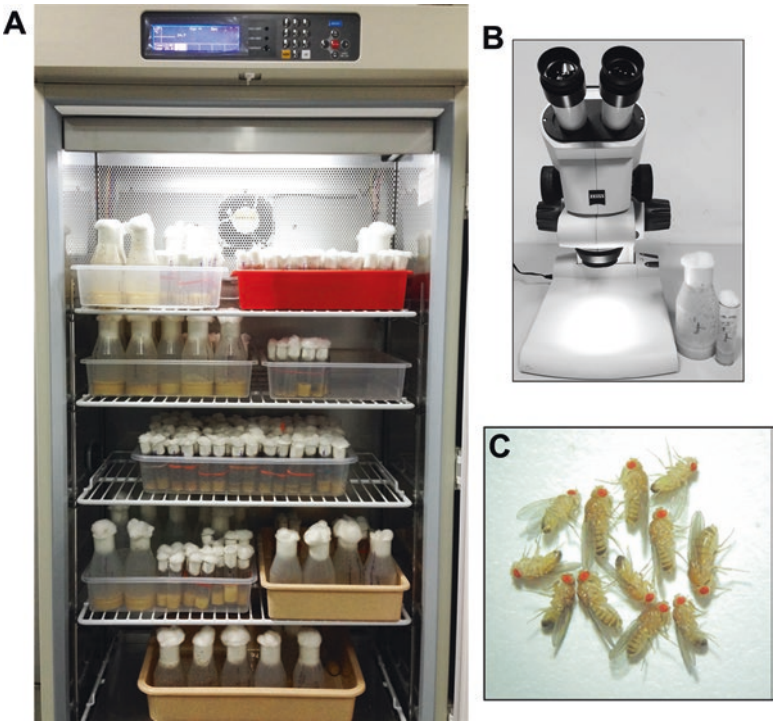

Fig. 14.1 Maintenance and handling of *Drosophila* in laboratory: (**a**) BOD incubator showing rearing of *Drosophila* at 25 °C in culture bottles and vials. (**b**) Stereo zoom binocular microscope used for routine fly work. (**c**) Stereo zoom binocular microscopic view of mixed population (male and female) of wild-type ($Oregon^{R+}$) *Drosophila* (Images not on scale)

genome distributed on four chromosomes make *Drosophila* a well-acceptable system to perform large-scale genetic screens for identification of potential modifiers of aging and disease-related phenotype(s). Due to existence of morphologically distinct developmental stages in *Drosophila* which include embryonic, larval, pupal, and adult phase (Fig. 14.2), it is easy to distinguish the sexually matured "aging" adults in the developing population. In several model organisms, it is not so convenient to visually distinguish the mature aging adults from immature or juvenile stage. *Drosophila* life cycle varies with temperature, and in the laboratories, it is generally maintained at 22 ± 2 °C. Since morphological features and developmental processes of *Drosophila* have been well documented, environmental and genetic manipulations which modulate the aging dynamics and lifespan could be easily performed and scored. Besides, availability of the large number of mutants and transgenic lines at several *Drosophila* stock centers further makes it a popular model organism [53, 231].

Interestingly, it has been estimated that more than 50% of the *Drosophila* genes have homologs in humans [2, 187], and nearly 75% of known human disease genes have functional homologs in the fly [221]. This striking similarity makes *Drosophila*

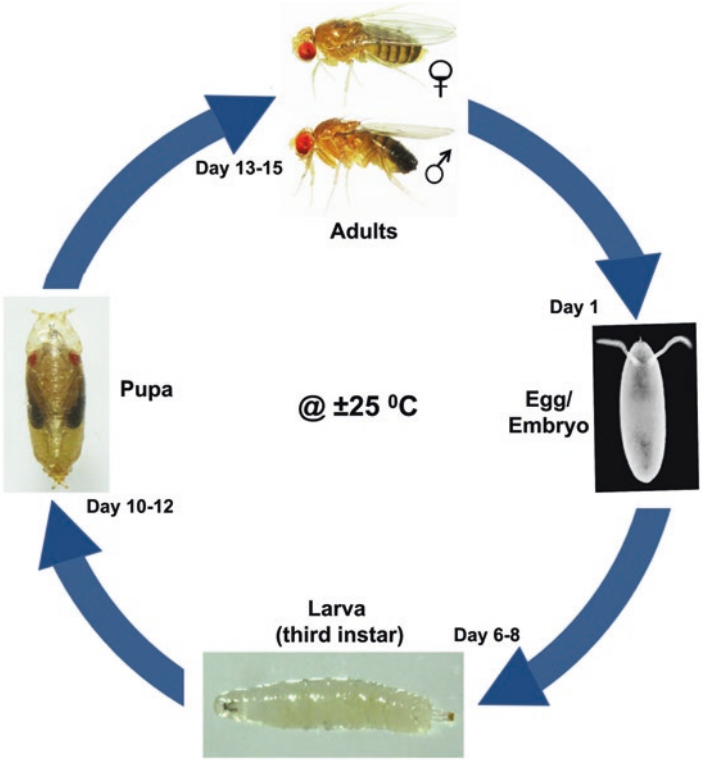

Fig. 14.2 Life cycle of *Drosophila melanogaster* at 25 °C

as the model organism of choice for several human-related studies such as aging and longevity. The adult fly harbors a well-coordinated sophisticated brain and nervous system, which makes it capable of displaying complex behaviors such as learning and memory, much like the human brain [123]. Disruption of this synchronized motor behaviors results in neuronal death and dysfunction. The aging-related characteristic phenotypes, such as locomotor and sensory impairments, learning disabilities, and sleep-like behavior, are well manifested in *Drosophila* [81]. *Drosophila* lacks a functional blood–brain barrier which could otherwise prevent access of drugs to the neuronal cells of central nervous system; and therefore, *Drosophila* has emerged extremely useful for pharmacological screening for identification of novel therapeutic drug targets [123]. In this context, it is interesting to note that response toward many drugs that has shown effects within the *Drosophila* CNS is reasonably comparable to the mammalian systems [201, 277].

Subsequent to above, *Drosophila* provides great genetic tools which facilitate manipulation of gene expression in a tissue-specific manner during various stages of life cycle. The *UAS-Gal4* system is a frequently used genetic tool to achieve ectopic expression of a gene of interest or to suppress it by *UAS-RNAi* transgene [27]. Furthermore, FLP-FRT (Flippase - Flippase Recognition Target) system, a

site-directed recombination technique, has been progressively used to manipulate the fly genome *in vivo* in somatic and germ cells [257]. Using this genetic tool, loss-of-function of a lethal gene could be easily studied in target organ or tissues [248]. The effect of altered gene expression can also be studied over time, by using an inducible promoter to trigger the recombination activity late in development. This prevents the genetic alteration from affecting overall development of the organ, and also allows single-cell comparison of the one lacking the gene to normal neighboring cells in the same environment [282].

Drosophila also offers some additional advantages for aging studies. The presence of almost fully differentiated post-mitotic cells throughout the adult stage represents synchronized aging [7]. The initiation of adulthood in *Drosophila* has been proposed to occur only after the pupal eclosion and the fly becomes sexually mature and competent to reproduce [237]. This provides a great advantage over other model systems where it is often difficult to exactly determine when the organism has attained the maturity [102, 103]. Further, the rarely dividing neurons of the *Drosophila* brain makes it an excellent model to study various aspects of human brain aging and neurodegenerative disorders [104]. Aging-mediated cellular and structural changes could be convincingly inferred by examining synchronously aging neuronal cells in adult *Drosophila* brain. Due to the lack of blood vessels in the fly brain, the pathological complexities due to blood vasculature can be excluded. Taken together, in view of above-noted several advantages, *Drosophila* has been extensively used to decipher various aspects of aging. A brief account of the *Drosophila* aging research has been provided below.

Drosophila in Aging Research: An Overview

Loeb and Northrop in 1916 reported the first use of *Drosophila* as a model organism for aging-related studies [148]. They demonstrated the effects of temperature and food on the lifespan and concluded that longevity of flies as poikilothermic organisms depends on the temperature of the environment [148]. In addition, they also studied the effect of starvation and sugar-rich diet on fly longevity [149]. Subsequently, it was demonstrated that longevity in flies is heritable [204, 205]. Consistent with the findings of Pearl and Parker, the role of genetic influence in regulation of lifespan of adult flies was validated further [45]. By utilizing *Drosophila* as a model system, several small compounds such as biotin, pyridoxine, and pantothenic acid were identified which extend the lifespan upon regulated feeding [71]. The relationship between reproduction and fly longevity was for the first time studied by J Maynard Smith in 1958 [241]. It was found that longevity could be considerably extended when female flies were selected late for fertility [153, 154, 227, 228].

The role of reproductive behavior on aging has been a topic of aging research since middle of the twentieth century when it was reported that longevity of *Drosophila* could be influenced by reproductive behavior [241]. This established *Drosophila* as an excellent model system to study the fitness trade-offs and lifespan [241].

Thereafter, studies on to establish the mechanistic correlation between reproduction and longevity has been a topic of immense interest in the aging research. Consistently, the plasticity behaviors between fly longevity and reproductive output was further confirmed by the selection experiments, which demonstrated that lifespan could be significantly extended when female flies were selected for late-life fertility [153, 154, 227, 228]. Michael Rose has reviewed the history of laboratory-based evolution experiments and the use of different genomic technologies to unravel the genetics of longevity in *Drosophila* [227, 228]. Interestingly, random mutagenesis approach during the end of twentieth century led to identification of two independent life-extending genes in *Drosophila*. Interestingly, *methuselah* (*mth*) was the first such gene in which P-element insertion-mediated downregulation was found to increase the lifespan [143]. In another study, it was found that five independent P-element insertional mutation in gene *I'm not dead yet* (*indy*) resulted in a near doubling of the average adult lifespan without making any negative impact on the fertility or physical activity of the flies [230]. *Drosophila* has further been used as a model to study the role of immune senescence and inflammatory responses in aging [87, 142]. Major milestone in aging research using *Drosophila* can be summarized as shown in Fig. 14.3.

In contemporary aging research, various approaches and strategies are being adopted to decipher the mechanistic insights of aging and longevity. Some of the widespread genetic approaches include random mutagenesis followed by forward genetic analysis, selective breeding, biochemical, cellular and molecular assays, and QTL analysis. These methods collectively have allowed identification of numerous genes those are involved in diverse cellular functions including aging and longevity in *Drosophila*. Table 14.1 provides a brief account of some genes and their

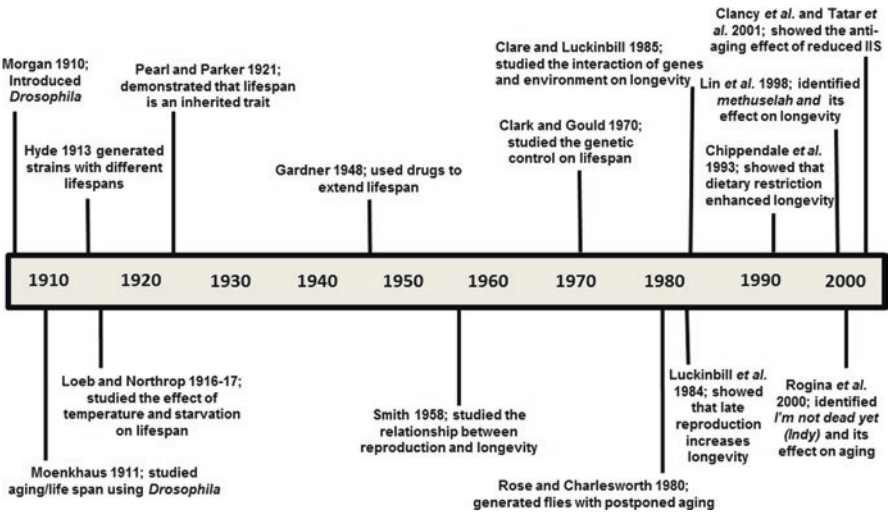

Fig. 14.3 Timeline representing historical overview of some major findings in *Drosophila* aging research

Table 14.1 A brief collection of some genes found to extend lifespan in *Drosophila melanogaster*

Gene	Endogenous function	Positive effect on longevity due to	References
Indy	Succinate and citrate transmembrane transporter	Knockdown	[225]
dsir2	Histone and nonhistone, NAD-dependent deacetylase	Overexpression	[14, 224]
gsh	Antioxidant enzyme involved in formation of reduced glutathione	Overexpression	[174]
mth	G-protein-coupled receptor	Knockdown	[143]
SOD	Antioxidant enzyme involved in partitioning of superoxide radicals to molecular oxygen	Overexpression	[202, 249]
Hep	JNK kinase	Overexpression	[270]
Puc	Inhibits JNK by specific JNK phosphatase activity in JNK signaling	Knockdown	[270, 287]
Hsp22	Molecular chaperons involved in stress response	Overexpression	[179]
Hsp23	Molecular chaperons involved in stress response	Overexpression	[177]
Hsp26	Molecular chaperons involved in stress response	Overexpression	[141]
Hsp27	Molecular chaperons involved in stress response	Overexpression	[141]
Hsp68	Molecular chaperons involved in stress response	Overexpression	[270]
Hsp70	Molecular chaperons involved in stress response	Overexpression	[253]
14-3-3ε	Antagonist to *dFoxo*	Knockdown	[192]
chico	Insulin receptor substrate in *Drosophila*	Knockdown	[44]
dFoxo	Forkhead transcription factor *in Drosophila*	Overexpression	[75]
dilps	Insulin-like peptides in *Drosophila*	Knockdown	[80]
InR	Insulin receptor *in Drosophila*	Knockdown	[254]
dPTEN	*Drosophila* phosphatase and tensin homolog control cell growth and proliferation by negatively regulating insulin signaling	Overexpression	[111]
dS6K	Important downstream kinase involved in TOR pathway	Knockdown	[124]
dTOR	It is a serine/threonine protein kinase which regulates cellular growth, proliferation, survival, transcription, etc.	Knockdown	[124]
dTsc1, dTsc2	Act synergistically to inhibit TOR	Overexpression	[124]

potential function(s) which have been found to modulate the aging and longevity in *Drosophila*. A brief overview of various techniques and approaches which have been adopted in *Drosophila* aging research is provided below.

Evaluating Aging in *Drosophila*

The rate of aging in *Drosophila* is affected by a combination of environmental and genetic factors. Thus, various approaches have been used to evaluate aging in *Drosophila*. Some of these approaches are briefly described below.

Environmental and Physiological Approaches

Environmental parameters which significantly influence lifespan in *Drosophila* include diet, oxidative stress, and conditions causing inflammation [99]. The following means can be exploited in order to analyze aging using physiological approaches.

Analyzing Demographics

This assay is based on the calculation of survivorship and mortality curves. A typical survivorship curve remains relatively flat for the early period of life and starts to decline at older ages, which corresponds to a period of low mortality followed by a period of an exponential increase in mortality (Fig. 14.4a). A stressful environment will usually manifest as an excess of early death in the population and an abnormal dip in the survivorship curve. Assuming that the shortening or lengthening of lifespan of an organism is the result of relative aging, comparative analysis among mean, median, and maximum lifespan of different populations under conditions could be treated as one of the factors to measure the aging process [145].

Dietary Restriction

Dietary restriction (DR) refers to a moderate reduction in food intake that leads to extension of lifespan beyond that of normal healthy individuals. DR in *Drosophila* usually involves reduction of the yeast and sugar components of the diet [203], and interestingly yeast appears to account for the majority of the DR effect on lifespan [42, 158]. It has been found that *Drosophila* fed on low yeast/low sugar diet has the highest lifespan [167]. DR impacts the physiology of flies in two major ways: extension of lifespan and reduction in reproductive ability [203]. It has been found that DR-mediated lifespan extension is controlled by metabolic pathways such as insulin/IGF-1 signaling and TOR pathway [203], as described in detail in the later sections. Two major methods have been generally used to measure the food intake in *Drosophila*. One method includes direct measurement of the amount of liquid food consumed by flies using a capillary feeder (CAFE) [116], and the second indirect method deals with the estimation of the food intake by measuring the uptake of a dye or radioactive tracer added in the food [278].

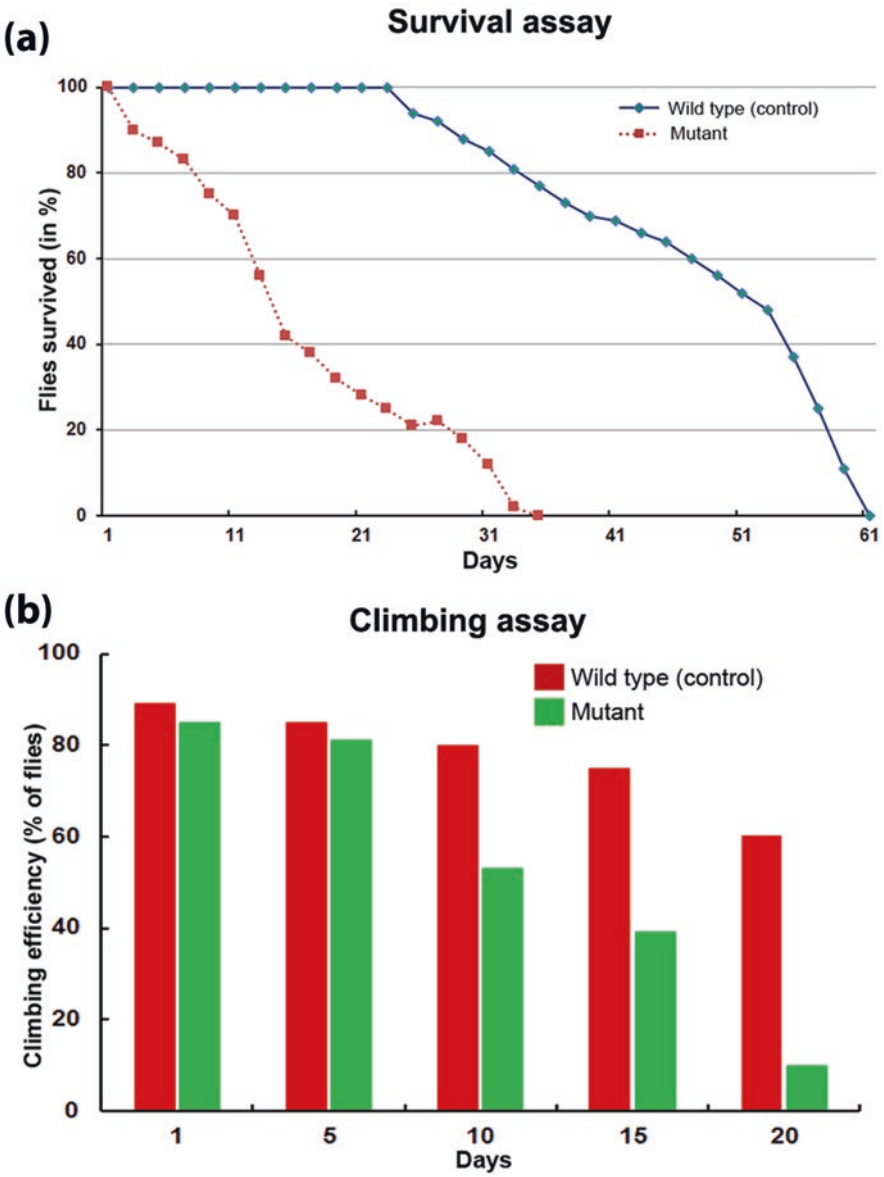

Fig. 14.4 Graphical representation of (**a**) survival assay and (**b**) climbing assay performed with wild type and a mutant line of *Drosophila.* In comparison to wild type, mutant population show increase in the mortality rate, and decline in the climbing efficiency with aging

Stress Resistance

Some amount of resistance could be observed when flies are exposed to the various physiological stresses such as oxidative stress, starvation, heat/cold shock, and desiccation. Level of such resistance has been found to be positively correlating with the longevity [88, 130]. Selection for increased resistance to starvation and desiccation in *Drosophila* has been shown to increase longevity [229]. The relative responses against desiccation and starvation and subsequent selection of lines with increased lifespan are partly independent of each other, indicating a multiplicity of physiological mechanisms involved in aging and longevity [229]. Oxidative stress resistance is usually measured in *Drosophila* by feeding paraquat (N,N′-dimethyl-4, 4′-bipyridinium dichloride), which produces ROS upon ingestion and consequently induces oxidative damage [8]. Starvation assay, on the other hand, is typically performed by measuring the survival of adult flies fed solely on water [109].

Reproductive Output

A negative correlation between reproductive output and lifespan was observed and termed as "cost of reproduction" [252]. Late reproduction in *Drosophila* has been found to increase lifespan [110], and decrease in early reproduction corresponds to long-lived flies [292]. Moreover, sterile flies tend to live longer than their fertile controls [13], and long-lived mutants flies have been shown to exhibit reduced fecundity [66]. The methods used to assess reproductive output in flies include measuring lifetime egg production in once-mated females or calculating progeny number from a pair mating of female and male. Other environmental factors such as temperature, humidity, and circadian light rhythm also affect longevity in flies, and thus can be used to study the process of aging like other environmental approaches.

Behavioral Approaches

Changes in behavioral pattern can also be used to study the cognitive functions during various stages of aging. Relationship between behavioral pattern and aging has been well established; for instance, *Drosophila* experiences a decline in sleep time with aging [132]. One such behavioral aspect is the locomotory activities. In general, there is a gradual fall in the locomotory activities with aging [114]. Two methods are commonly employed in *Drosophila* aging studies for assessment of locomotory behaviors.

Rapid Iterative Negative Geotaxis (RING) Assay

Based on the inherent negative geotaxis behavior of flies, this assay measures an innate escape response during which flies ascend the wall of a cylinder after being tapped to its bottom [72]. Climbing ability of *Drosophila* has been found to be deteriorating during the process of aging (Fig. 14.4b). In RING assays, digital photography could be used to document negative geotaxis in multiple groups of animals simultaneously [72].

Drosophila Activity Monitoring (DAM) System

In this method, flies are kept in sealed activity tubes individually and are placed in the DAM system [210]. Fly activity is measured by the frequency of an "activity event," which is recorded each time when a fly breaks an infrared light beam across the middle of the activity tube [210].

In addition to the above-noted two relatively simple systems, sophisticated video tracking systems have been developed to analyze various fly behaviors, such as movement pattern and courting, which can be potentially used to measure lifetime behavioral changes and locomotory activity-related health-span parameters [28, 82].

Genetic Approaches

Genetic approaches remain an invaluable method for identification of casual genetic factors which modulate the aging process. Single gene mutations and the resultant phenotypes help in determining the complex pathways of aging and longevity. Genetic approaches could also be adopted to confirm the already available hypotheses using candidate gene approaches, or to explore for novel ones, using random single-gene alteration approaches [102, 103]. Genetic approaches have also emerged as an important strategy for screening of the genetic interacting partners and modifiers. Several genetic tools are available in *Drosophila* to investigate the in-depths of aging and longevity. Some of the widely utilized genetic approaches being used in *Drosophila* aging research include selective breeding, quantitative trait loci (QTL) mapping [216], achieving ectopic expression of gene by *UAS-Gal4* system, inducible gene expression by gene-switch Gal4 (*GSG-UAS*) system, and gene knockdown by RNA interference (RNAi) strategy [250].

Cellular, Molecular, Biochemical, and Other Approaches

In addition to the above-mentioned strategies, various contemporary techniques are widely utilized to investigate the cellular, molecular, and biochemical aspects of aging-mediated changes in *Drosophila*. Several techniques such as reverse transcription PCR, real-time PCR, microarray, whole mount *in situ* hybridization, immunohistochemistry-based staining techniques, western blot analysis, co-immunoprecipitation, and microscopy techniques can be used appropriately to investigate different facets of aging in *Drosophila*. Examination of the systemic biomarkers of aging-like protein carbonylation, lipid peroxidation, protein aggregation, and accumulation of advanced glycation end products (AGEs) has also emerged as an important area to establish the age-related changes and cellular dysfunctions [99]. Further, status of gut homoeostasis and various organs such as heart, muscle, brain, etc. could be investigated in *Drosophila* to study the effect of aging-mediated changes on vital organs [99].

Cellular Pathways Affecting Aging in *Drosophila*

Aging is an inevitable phenomenon which affects all cell types in every hierarchy of organism. It is a natural process in which cells along with divisions accumulate certain defects leading to activation of some specific signaling pathways/stress responses that leads to senescence and finally cell death. There can be many stimuli or damages which can trigger the aging processes. Some of the key aging-related hallmarks are described below.

Genomic Instability

One of the common causes of aging is the progressive accumulation of genetic damages during the lifespan of an organism [182]. The veracity and stability of genomic DNA is being continuously affected by various exogenous stresses such as physical–chemical agents, and endogenous stimuli like DNA replication errors, spontaneous hydrolytic reactions, and ROS levels [105]. These stresses could cause damages encompassing point mutations, translocations, chromosomal gains, and losses. Here it is interesting to note that premature aging diseases such as Werner and Bloom syndromes are caused by accumulation of increased DNA damage [32]. However, the DNA repair systems of the organism collectively function with various cellular systems to minimize the nuclear damages [152]. Also, defects in nuclear architecture can be a cause of genomic instability which results in premature aging syndrome [280].

Defects in Nuclear Architecture

In addition to the genomic damage, defects in nucleus structure, that is, nuclear lamina, also contribute to genomic instability [52]. Nuclear lamins are the major components of nuclear lamina and they play an important role in maintaining the genomic stability by providing a scaffold for tethering chromatin and protein complexes, which are important for the maintenance of genomic stability [77, 146]. Lamins got attention in aging research recently after the discovery that mutation in lamins causes accelerated aging syndrome such as Hutchinson–Gilford (HGPS) and Néster-Guillermo progeria syndrome [33, 51, 58]. Accumulation of Progerin, an aberrant pre-lamin isoform, has been found during normal human aging [219, 233]. Interestingly, it has been reported that altered telomere function promotes production of Progerin in human fibroblast upon prolonged *in vitro* culture [34]. Aberration in nuclear lamina triggers various stress pathways such as activation of p53 [264], deregulation of somatotrophic axis [159], and attrition of adult stem cells [59, 233]. The role of nuclear lamins in aging is supported by the observation that decreased cellular level of pre-lamin A delays the onset of progeroid symptoms and extends lifespan in mouse models of HGPS [197]. An interesting approach using induced pluripotent stem cells (iPSCs) derived from HGPS patients has been developed to

correct the lamin A/C (*LMNA*) mutations by homologous recombination-based strategy, which could be utilized in future cell therapies [147].

Telomere Abrasion

Telomeres are repetitive ends of chromosome whose length gets shortened by every cell division cycle. Progressive shortening of telomeric region has been observed during normal aging in human and other model systems [21]. Therefore, the length of the telomeres is quite heterogeneous from chromosome to chromosome and from cell to cell within a population. The telomeric ends of chromosomes are repetitive in nature and they are replicated by special DNA polymerase known as telomerase [79]. Since the normal mammalian somatic cells do not express telomerase, this leads to progressive shortening of the chromosomal ends, whereas in germline cells and many immortalized cell lines and cancers, telomere length is maintained by the enzyme telomerase [238]. Telomere abrasion explains the reason of limited proliferative capacity of *in vitro* cultured cells, this is called replicative senescence or Hayflick limit [98, 194]. Interestingly, ectopic expression of telomerase is sufficient to confer immortality to otherwise mortal cells [22].

In telomerase-positive cells, telomeres are maintained to a stable length resulting in the bypass of senescence and cellular immortalization. Both telomere protection and the regulation of telomere length are mediated by a stably associated complex, called shelterin. Mammalian shelterin masks the chromosome ends and makes them inaccessible for the telomerase and the DNA repair machinery [200]. Therefore, shelterins covered telomeres continuously accumulate exogenous DNA damage during cell divisions. Abnormalities in telomeres have been found to be associated with premature manifestation of many diseases in humans, such as pulmonary fibrosis, dyskeratosis congenita, and aplastic anemia [9]. Moreover, mutations in shelterins have been reported in some cases of aplastic anemia and dyskeratosis congenita [200]. In mice, shortened or lengthened telomeres exhibit decreased and increased lifespan, respectively [10, 230, 259]. Interestingly, human meta-analysis also suggests a strong correlation between short telomeres and mortality risk, particularly at younger age [24].

Nuclear–Mitochondrial (NM) Signaling in Aging

The mitochondria are double membrane-bound cytoplasmic organelles found in all eukaryotic organisms, which function as an energy production site of a cell. In addition, mitochondria are also involved in several other processes such as signaling, growth and differentiation, cell cycle, and apoptosis [6]. Mitochondria constantly maintain their morphology and function in response to changing microenvironment by multiple processes including fusion and fission, DNA repair, and mitophagy (clearance of damaged mitochondria). Impairment of any of these processes leads to mitochondrial dysfunction, which can be a causal for many mitochondrial

diseases, neurodegenerative disorders, cancer, diabetes, heart disease, immunodeficiency, and early aging [160, 220, 269, 285]. For instance, impairment of mitophagy has been reported in Parkinson's disease, Alzheimer's disease, and pathological aging [62, 166, 199]. Such dysfunctioning in mitochondria was suggested to be due to impairments in mitochondrial proteins and aberrant nuclear-to-mitochondrial signaling [63].

In view of the mitochondrial involvement in several cellular processes as noted above, proper functioning of a mitochondrion is important for the maintenance of the cellular homeostasis. Indeed, there are several evidences which show that damaged mitochondria accumulate with age from unicellular organisms to humans [47, 62, 183, 209]. Also, some recent reports suggest that compromised nuclear-to-mitochondrial signaling is a key component of mammalian aging, which initiates because of the nuclear damage accumulated over time due to progressive aging [63]. Number of factors which senses DNA damage includes poly(ADP ribose) polymerase 1 (PARP1), ataxia telangiectasia mutated (ATM), and transcription factor p53 [63]. Thereafter, NAD-dependent protein deacetylase sirtuin 1 (SIRT1) and AMP-activated protein kinase (AMPK) trigger chromatin remodeling through post-translation modification of histones and peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α); and subsequently, some other proteins assist in propagating the nuclear-to-mitochondrial signaling ahead [63]. Therefore, progressively accumulating DNA damages leads to downstream changes in cellular transcriptome, epigenome, metabolome, and in bioenergetics, which ultimately contribute to aging and its associated disorders.

Oxidative Stress

Almost about a century ago, it was observed that animals with higher metabolic activity generally have shorter lifespan, and this observation leads to the emergence of "the rate of living hypothesis" of aging [65]. Interestingly, in contrast to this theory, some species, that is, birds and primates, do not show such inverse correlation. Also, Denham Harman proposed a "Free radical theory," which postulates that ROS generated inside a cell results in oxidative damages to the cells, which in turn accelerates aging [92, 283]. Almost about a decade later, this theory was supported by the identification of superoxide dismutase (SOD) enzyme, which solely degenerate the superoxide anions [163]. Later, this theory was modified to oxidative stress theory which emerged as most convincing theory of aging [208]. Various studies attempted to substantiate this theory, however, the results were inconsistent and sometime challenging as well [136]. However, findings in several model organisms including *Drosophila* substantiate that a decrease in level of ROS is directly correlated with an increase in lifespan [23]. Hence, it appears that an intricate balance between ROS production and the ability of the cells to counteract it drives the progression of a cell toward aging.

D. melanogaster has been widely used to investigate mechanistic correlation between ROS levels and aging. In fly, the relationship between oxidative stress and

longevity was examined by modulating the expression levels of various antioxidant gene(s) by mutagenesis approaches. The driving hypothesis in finding this correlation postulates that factors which reduce the cellular levels of ROS should have beneficial effects against aging, and thus would increase the life expectancy. Supporting this claim, a positive correlation between decrease in the levels of ROS and increase in lifespan has been found in *Drosophila* [55]. Such *Drosophila* strains with extended lifespan either have low levels of ROS or have an increased level of antioxidant enzymes [55, 94]. For instance, P element insertion-mediated reduction in the level of *Methuselah* (a G-protein-coupled receptor) leads to ~35% increase in lifespan [143]. In addition to this, reduced level of *Methuselah* also increases the resistance of the fly toward various stresses such as high temperature, dietary paraquat (generates free radicals), and starvation [143]. Further, overexpression of an antioxidant gene glutathione reductase (GSH) leads to increased lifespan in hyperoxic conditions but no effect was evident under normoxic conditions [174]. Also, decrease in the expression of an antioxidant enzyme superoxide dismutase (SOD) and catalase (involved in H_2O_2 eradication) reduces the lifespan, which suggests a positive correlation between reduced level of cellular ROS and increased lifespan [129, 171, 211, 212]. In this context, it is important to note that since such mutation(s) are prevalent during the development of the fly, decrease in the lifespan could also be due to the cellular damages accumulated over time and not solely due to oxidative stress. However, studies conducted in the *Drosophila* lines in which antioxidant genes, that is, SOD and catalase, were overexpressed or SOD was overexpressed showed oxidative stress resistance and increase in the life expectancy [196, 202, 249]. Interestingly, the transgenic flies expressing human SOD gene in their motor neurons exhibited 40–50% increase in their lifespan [202]. These studies suggest specific role(s) of SOD and catalase in modulating the lifespan by regulating the cellular level of ROS. Here it is also important to note that some studies with several other antioxidant genes reported only slight or insignificant increase in the level of oxidative stress tolerance and life expectancy [195, 236]. Taken together, the above studies convincingly suggest a direct correlation between cellular level of ROS and progression of aging.

What Does Oxidative Stress Do?

Mitochondria produce ATP *via* the process of oxidative phosphorylation which involves consumption of oxygen, and therefore, surplus availability of oxygen in the mitochondria renders it to be site of ROS production. The major types of ROS found in living animals include superoxide anion ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH). In mitochondria, ROS is produced when (a) it is not generating ATP and thus has a high proton-motive force and a reduced level of coenzyme Q; and (b) there are high levels of NADH/NAD^+ in matrix [186]. Under normal physiological conditions, approximately 2% of the electrons leak from the electron transport chain (ETC) and account for ROS production [37]. When an electron encounters an oxygen atom, $O_2^{\bullet-}$ is formed due to a reduction reaction. $O_2^{\bullet-}$ is considered to be the most important oxygen-free radical and the source of other ROS molecules. $O_2^{\bullet-}$ is readily converted into H_2O_2 by superoxide dismutase (SOD)

enzyme which is in turn converted into •OH in the presence of ferrous (Fe^{2+}) or cuprous (Cu^{2+}) ions [54]. While •OH is highly reactive, H_2O_2 is more stable and membrane permeant. Although there are around eight known sites in the mitochondria which possess the ability to produce $O_2^{\bullet-}$, however, the two major sites in ETC include complex I (NADH dehydrogenase) and complex III (Ubiquinone–cytochrome c reductase) [26, 262]. In normal conditions, complex III is the major site of ROS production [37]. Non-ETC sources of mitochondrial ROS production include monoamine oxidase which locates in the outer mitochondrial membrane and produces H_2O_2 as a byproduct of oxidative deamination. Further, under the elevated level of NADPH/NADP+ ratio and calcium, glycerol-3-phosphate dehydrogenase (GPDH) and α-ketoglutarate dehydrogenase (α-KGDH) in the mitochondrial matrix produces $O_2^{\bullet-}$, and the both $O_2^{\bullet-}$and H_2O_2 respectively. In view of above, it is increasingly clear now that cellular level of ROS primarily depends upon the metabolic status of an individual. Intriguingly, ROS exhibit beneficial as well as deleterious effects. Since ROS are highly reactive in nature, excessive production or accumulation of ROS most often proves to be detrimental for the cell. The enhanced level of oxidative stress may show its effect on cells by damaging cellular components and/or by modulating some signaling cascades. A brief overview of the impact of ROS on cellular functioning is discussed below.

Effect of Oxidative Stress on Cellular Components

An increased level of ROS leads to oxidative damage to all the macromolecules such as nucleic acids, proteins, and lipids present in a cell, which in turn causes imbalance in the cellular homeostasis and instigates the aging process [138]. Interestingly, mitochondrion, in spite of being the major source of ROS, also becomes the key target of oxidants. Also, due to the close vicinity of mitochondrial elements to ROS production site, they are more susceptible to the damage by ROS. Lack of histone protection and repair mechanism in mitochondrial DNA further aggravates the susceptibility to ROS-mediated damages. Collectively, these factors add to the risk of mitochondrial dysfunction which has been greatly linked to manifestation of aging process [243, 268]. Several studies have been carried out in *Drosophila* which correlate age-associated changes with the structure and functions of mitochondria, which is indicative of the notion that gradual mitochondrial dysfunctioning is concomitant with aging [268]. For instance, one such study examining the effect of aging on *Drosophila* flight muscles demonstrated a specific "swirl"-like rearrangement of mitochondrial cristae under oxidative stress, with aging [266]. Interestingly, rapid and widespread accrual of similar pathological condition was perceived even in young flies under severe oxidative stress condition. Correlating with the functional aspects of this pathological condition, cristae with swirling pattern were found to have reduced enzymatic activity of cytochrome c (COX) or complex IV, which is an important enzyme complex in ETC involved in ATP production. Moreover, existence of swirls is accompanied by modifications in the structural conformation of cytochrome c, and extensive apoptosis of the flight muscles in *Drosophila* [43, 268].

ETC in mitochondria is associated with energy production in the cell, which is the most vital process required for the maintenance of cellular homeostasis. Studies in *Drosophila* reported an overall decrease in various aspects of ETC with aging [64]. Interestingly, compared to the several other mitochondrial ETC enzymes that were examined, age-associated reduction was predominantly found in the activity of COX [64]. Correspondingly, drug-mediated impairment of COX in young flies results in enhanced ROS production in mitochondria [50]. These observations suggest that ROS-induced mitochondrial impairment results in further enhanced production of ROS which exaggerates the mitochondrial damages, forming a "vicious cycle" and thereby acting as driving force in aging and age-associated impairments [161].

Signaling Cascades Activated by ROS

Several stress pathways like the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) signaling cascades, the phosphoinositide 3-kinase [PI(3)K]/Akt pathway, the nuclear factor (NF)-kappaB signaling system, p53 activation, heat shock response, etc. get activated as a mechanism to combat oxidative stress. Here it is important to note that in addition to stress response, these pathways also play essential role(s) during normal growth and metabolism [65]. Among them, the JNK pathway has been identified as an evolutionarily conserved cascade which can potentially increase the lifespan in flies by activating a set of protective genes to mitigate the toxic effects of oxidative stress [270, 271].

Compared to JNK pathway in vertebrates which is relatively complicated due to involvement of huge gene families, JNK signaling in *Drosophila* is significantly less complicated, which makes the genetic analysis much simpler than other model organisms [113, 122]. The JNK signaling pathway in *Drosophila* constitutes various JNK kinase kinases (JNKKK) such as TGF-β-activated kinase1 (TAK1), mixed lineage protein kinase 2/slipper (MLK), MEK kinase 1 (MEKK1), apoptotic signal regulating kinase 1 (ASK1), two JNK kinase (JNKK; Hemipterous and dMKK4), and one JNK [Basket (Bsk)] [19, 25, 40, 74]. Oxidative stress results in activation of transcription factors AP-1 and dFoxo (*Drosophila* forkhead transcription factor) by Bsk phosphorylation, which in turn triggers stress-specific cellular responses by activating several response genes. JNK pathway is negatively regulated by a key target of AP-1 known as puckered (*puc*), which reduces JNK signaling because of JNK-specific phosphatase activity [19, 271]. Genetic manipulations of genes dosage in *Drosophila*, that is, downregulation and overexpression of the *puc* and JNKK/Hep, respectively, enhances the basal JNK signaling levels and leads to enhanced JNK signaling, which in turn result in improved oxidative stress tolerance and increased lifespan [270, 287]. On the contrary, mutant flies for JNKK/Hep gene displayed higher sensitivity toward oxidative stress and were observed incapable of eliciting JNK signaling-dependent transcriptional factor-induced stress response (Fig. 14.5) [275].

It has been found that adequate availability of dFoxo is essential to accomplish JNK signaling-mediated increased longevity in *Drosophila*. This suggests an

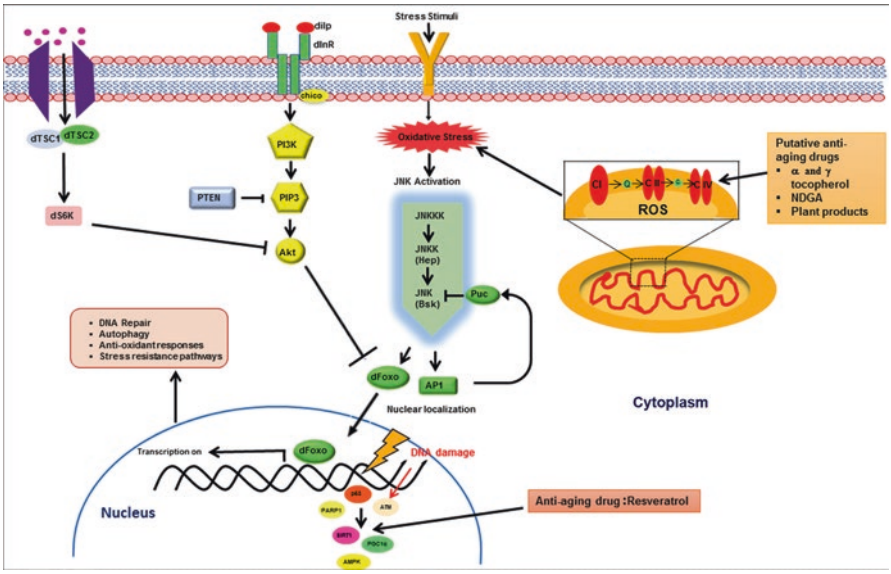

Fig. 14.5 Schematic diagram of various stimuli, participating signaling cascades, and putative drug targets which could modulate progression of aging and longevity in *Drosophila*

antagonistic relationship between JNK and insulin/insulin-like growth factor (IGF)-like signaling (IIS) pathways [271]. In *Drosophila*, JNK inhibits IIS pathway autonomously and/or systemically (endocrine mechanism) to regulate the life expectancy. While functioning cell autonomously, JNK inhibits insulin signaling by promoting nuclear localization of dFoxo, which subsequently activates transcription of genes involved in stress response, damage repair, and growth control [19, 106, 271]. In addition, JNK also inhibits insulin signaling systemically by repressing the expression of its ligand, *Drosophila* insulin-like peptide2 (dilp2) in insulin-producing neuroendocrine cells present in the fly brain [125, 271]. Therefore, antagonistic relationship between JNK and insulin pathway has emerged as an important aspect to combat oxidative stress during progressing of aging.

Proteostasis Loss During Aging

Proteostasis or protein homeostasis deals with the quality control process which regulates the complex signaling pathways to control biogenesis, folding, signaling, and degradation of proteins inside and outside the cell. The process of proteostasis involves various mechanisms such as stabilization of correctly folded protein, refolding of denatured protein, and/or degradation of misfolded proteins by proteasome or lysosomal pathways to remove them from the cell [95, 131, 173]. Moreover, there are regulators such as MOAG-4 which deal with age-related proteotoxicity and act *via* a different pathway from that of molecular chaperones and proteases

[151]. Aging and some age-related diseases impair the cellular protein homeostasis, and defect in these pathways result in progressive accumulation of abnormally folded or misfolded proteins in cellular compartments and triggers pathogenesis of several neurodegenerative disorders [218]. Molecular chaperones represent a major group of proteins which regulate the cellular proteostasis. A brief overview of the molecular chaperones and their involvement in regulation of cellular homeostasis and aging has been discussed below.

Molecular Chaperones Facilitated Protein Folding

It is increasingly clear now that aging is driven by both the genetic and nongenetic factors. In addition to the *in vivo* factors, several environmental stimuli such as cytokines, UV radiation, chemotherapeutic agents, hyperthermia, and some growth factors can also lead to enhanced production of ROS, which may potentially disturb the balance between normal redox levels and oxidative stress [65]. Molecular chaperones are ubiquitous and highly conserved protein families which utilize cycles of ATP-driven conformational changes to either stabilize the nascent and/or stresses-mediated unfolded proteins, or unfold them to translocate across membranes, or mark them for degradation [232]. Molecular chaperones are also regarded as Heat shock proteins (Hsps) or stress proteins because of their induced expression during stress condition(s). With the discovery of the molecular chaperones for the first time in *Drosophila* [222, 223], functions of these proteins and their correlation with aging and longevity have emerged as a prime area of research. Based on their amino acid sequence homologies, molecular weight, and functional aspects, Hsps have been divided into 5 major families: Hsp100 (100–104 kDa), Hsp90 (82–90 kDa), Hsp70 (68–75 kDa), Hsp 60 (58–65 kDa), and small Hsps (15–30 kDa) [232]. As in other model organisms, fly also has homologs of Hsp families, for example, Hsp83 (Hsp90 family), Hsp/Hsc 70 complex (Hsp 70 family), Hsp60, Hsp40, and small Hsps [177]. Expression of the Hsps is facilitated by binding of Heat Shock Factor (HSF) to the heat-shock response elements localized at promoter region of genes, and induction of their high-level transcription [265]. Since the basic property of Hsps involves refolding of protein denatured due to stressors, enhanced expression of Hsps could be observed due to increased level of cellular ROS [175, 185]. It has been reported that subsets of Hsps are also induced by oxidative stress through dFoxo transcription factor and the JNK pathway [271].

Interestingly, constitutive expression of Hsps exhibits well-regulated and stage-specific expression pattern during development; however, enhanced expression of several Hsps could be seen upon exposure to environmental stresses like heat [177]. Some Hsps such as Hsp70 do not express during normal physiological conditions and demonstrate stress-induced expression pattern. The definite involvement of Hsps in aging and increased sensitivity of the aged flies to environmental stimuli has originated from the comparative analysis of the stress response between young and old flies [68]. Subsequently, studies in different animal models support the idea of causative impact of chaperones decline in longevity. For example, transgenic *C.*

elegans and *Drosophila* with increased level of molecular chaperones show relatively longer lifespan [179, 267]. Also, mutant mice with reduced level of co-chaperones show enhanced rate of aging and manifestation of age-associated phenotypes [168].

Comparative analysis of old and young flies showed a greater abundance of damaged proteins in the old flies. Induction of the identical set of proteins in young *Drosophila* fed with canavanine (an arginine analog used to mimic accumulation of damaged proteins which were otherwise present only in old flies) suggests an increased sensitivity due to accumulation of aging-mediated damaged proteins [68, 191]. Moreover, tissue-specific enhanced expression patterns of several Hsps have been reported during normal fly aging [177]. For instance, enhanced expression of *hsp22* and *hsp70* at both RNA and protein level and upregulation of *hsp23* at RNA level could be observed during *Drosophila* aging [177]. Further, with the aim to elucidate transcriptional dynamics due to aging, genome-wide gene expression profiling in *Drosophila* has also revealed age-associated upregulation of several Hsps [49, 215, 290]. Interestingly, in addition to the notable upregulation of subsets of Hsps including Hsp70 and sHsps, enhanced expression of the genes for innate immune response has also been reported in old flies [49, 215, 290]. On the contrary, downregulation of the genes involved in energy synthesis and mitochondrial ETC was found in the same set of flies [49, 215, 290]. In this context, it also important to note that an extensive overlap between the gene expression profile of aged flies and the young flies exposed to oxidative stress further establishes the potential relationship between aging and oxidative stress [290].

The beneficiary effects of Hsps on longevity was also confirmed by the "Hormesis" in *Drosophila*, in which mild dosage of stressors are used to activate stress response without causing cellular damages [169]. Exposure to sublethal levels of stress induces hormetic effect which in turn modulates the heat shock response and this helps the organism to survive longer by reducing the negative effects generated due to aging [169]. Also, *Drosophila* strains with improved lifespan also show intrinsic increased level of cellular sHsps, which further correlates enhanced expression of Hsps with aging [135]. Among multiple sHsp in *Drosophila*, Hsp27, Hsp26, Hsp23, and Hsp22 have been established to increase the lifespan significantly upon tissue-specific overexpression [177, 260, 270]. Enhanced expression of Hsp22 in motor neurons has been found to increase the lifespan by 30%, and these flies also exhibited improved stress tolerance and locomotor activity [179]. In agreement to the above observation, mutation in *hsp70* or *hsp22* has been found to be associated with decreased lifespan and increased sensitivity to stress. The beneficial effect of Hsps in longevity was further demonstrated by the fact that histone deacetylases (HDAC) inhibitors-mediated enhanced expression of Hsp70 and sHsps increases the lifespan of adult flies [288]. Independent studies have revealed decreased survival rate of *Drosophila* when *hsp22* or all six copies of *hsp70* [76, 178] were mutated and exposed to heat and/or other stresses. In addition, it has been found that *hsp83* mutant *Drosophila* becomes more sensitive to the toxic effects of stresses such as sleep deprivation [237]. Unlike sHsps, major Hsps such as Hsp70 and Hsp60 have failed to make any notable effect on longevity, except causing reduced

mortality rates upon mild stress, improved heat tolerance, and an insignificant increase in overall lifespan [170, 253]. Therefore, due to ubiquitous nature of Hsps and their crucial involvement in a variety of cellular processes by interacting with various cellular proteins, it can be concluded that the prevalent outcome of aging could be the consequence of the associated chaperone failure, and therefore, molecular chaperones itself represent one of the vital inherent regulators of aging and longevity.

Impact of Epigenetic Changes on Aging

Age-dependent changes in the chromatin configuration and subsequent amendment in gene expression is primarily regulated by various epigenetic modifications. However, establishing a direct correlation between aging and epigenetic modification is complex. The aging-induced epigenetic changes are largely mediated via methylation of the regulatory regions of genes, modification of the core histone proteins, and by controlled expression of several regulatory noncoding RNAs [60]. Different epigenetic changes affect all types of cells in an organism throughout the life [251]. In agreement to this, epigenetic changes in the genome also affect the expression of the genes involved in aging and longevity [89, 133], which subsequently leads to various molecular and physiological changes during the aging process [258]. Some epigenetic modifications like increased H4K16 acetylation, H4K20 or H3K4 trimethylation, and decreased H3K9 methylation or H3K27 trimethylation constitute the hallmark of aging-mediated epigenetic changes [70, 89]. The enzymes involved in generation and maintenance of such epigenetic hallmarks include DNA methyltransferases (Dnmts), histone deacetylases (HDACs) and acetylases, histone methylases and demethylases, as well as some other proteins involved in chromatin modifications. A brief overview of various epigenetic changes and its impact on aging has been provided below.

DNA Methylation

The degree of DNA methylation in genome is inversely proportional to the number of activated genes. Although establishing one-to-one correlation between methylation status and aging is complex, however, some reports have revealed locus-specific hypermethylation of various tumor suppressor genes and polycomb target genes, with advancing age [156]. With aging, the number of methylated cells and the extent of methylation in the CpGs of various promoters increases, which in turn cause reduced gene expression [189]. A connection between chronological age and 5-methylcytosine DNA methylation has been observed in humans [18, 91, 172], and therefore, methylation status can serve as an "aging clock" for determining the chronological age of an individual.

It is increasingly accepted now that DNA methylation regulates the process of aging [17]. Several studies have reported that DR, which is a major risk factor for aging, causes changes in the DNA methylation pattern at specific loci of some cancer-causing genes such as increased methylation of proto-oncogene ras [96].

Also, a study demonstrates that normal cells subjected to 4 weeks of glucose restriction shows increased methylation of tumor repressor $p16^{INK4\alpha}$ [140]. Interestingly, it was found that gene expression of Dnmt 1 (prime methyltransferase under normal conditions) and Dnmt3a significantly declines in aged ells, which is paradoxical to the finding that widespread hypermethylation occurs during aging [35]. This finding was explained based on transcriptional upregulation of the third methyltransferase Dnmt3b which could be responsible for causing increased methylation with advancing age [35].

Histone Modification with Aging

The extent of expression of a gene is also regulated by histone modifications carried out by various acetyl transferases, deacetylases, methyltransferases, and demethylases [133, 181]. Additionally, histone demethylases can modulate lifespan by targeting key components of several pathways affecting longevity [121]. The ADP ribosyltranferases and sirtuin family of NAD-dependent deacetylases have been extensively studied as potential antiaging factors due to their role in chromatin remodeling. This was further supported by the fact that overexpression of dSir2 in *Drosophila* also extends the lifespan [224]. However, these findings came into question with a report showing that perplexed genetic background was the leading factor to cause the increase lifespan, and not only the dSir2 overexpression [30]. Several of the mammalian sirtuins have been reported to delay various factors of aging in mice [107, 235]. Among them, the mitochondrial-located SIRT3 has been shown to mediate some of the beneficial roles of DR in longevity [245]. Interestingly, SIRT3 has also been reported to converse the regenerative capacity of aged hematopoietic stem cells [30].

Levels of some chromosomal enzymes such as heterochromatin protein 1α (HP1α), and chromatin remodeling factors like Polycomb group of proteins, or the NuRD complex get diminished both in normal and pathologically aged cells [206, 217]. This was also supported by the finding that loss of function of HP1α in flies leads to early death, and on the contrary, its overexpression increases longevity and delays muscular deterioration (a characteristic of old age) [137].

Noncoding RNAs

Noncoding genes have emerged as important regulators of the aging-associated epigenetic changes. A variety of noncoding RNAs such as microRNAs (miRNAs), siRNAs, piwi interacting RNAs, QDE-2 interacting RNAs (qiRNAs), and long noncoding RNAs have been found to regulate the epigenetic aspects of aging. They affect various biological processes by regulating the gene expression and also assist in maintenance of the integrity of genome.

Reduced expression of argonaute-like gene-1 (alg-1) affects the lifespan in *C. elegans* [126]. Adequate expression of alg-1 is required for the processing and functioning of miRNAs in *C. elegans* [126]. In *Drosophila*, human homolog of miR-200, i.e., miR-8 affects the aging process by inhibiting PI3K kinase of the insulin-signaling pathway [112]. The miR-8 knockout flies were smaller in size which is also an indicative of defective insulin signaling pathway [111, 112]. The

above reports suggest the involvement of miR-8 and miR-200 in regulation of aging in *Drosophila* and human aging, respectively [112].

Long noncoding RNAs have emerged as one of the important regulators of the gene expression. Several noncoding RNAs have been found to control various aging-associated cellular activities such as proliferation, differentiation, quiescence, and stress response [78, 120]. Long noncoding RNAs such as Telomerase RNA component (TERC), Telomeric repeat-containing RNA (TERRA) control telomere length during aging; *Airn*, *PTENpg1-AS*, and *H19* regulate the epigenetic changes; *lncRNA-p21* deals with proteostasis, and *MALAT1*, *ANRIL*, *eRNAs*, and 7SL control cell division; Kcnq1ot1, NeST, and ANRASSF1 regulate histone modifications, and linc-RoR, ES1, ES2, and ES3 have been found to regulate the stem cell behavior [78, 128].

Dietary Restriction

Diet is one of the major factors affecting the quality and duration of life in various living organisms. DR refers to the reduction in energy intake without being malnourished. More explicitly, during DR, calorie intake is restricted by about 30–40% in comparison to controls fed *ad libitum* [115]. Several studies have demonstrated lifespan extension in response to DR [256]. The first report of this kind was published in 1935 in which it was demonstrated that rats subjected to DR displayed increased mean as well as average lifespan [162]. Several hypotheses have been put forward to explain this effect of DR on longevity. One of such hypothesis states that DR slows down the metabolism which in turn restricts the production of reactive oxygen species (ROS), thereby decelerating the aging process [84], whereas the other asserts that DR extends lifespan by delaying the onset of age-related disorders [247]. The latter has been elucidated in David Sinclair's unified theory of aging which perceives DR as a highly complex yet conserved stress response that increases an organism's likelihood of surviving adversity by modulating key cellular processes like cell protection, repair mechanisms, and metabolism [239]. DR has also been shown to prevent muscle damage [165] and inhibit aging cardiomyopathy [12, 284].

The phenomenon of lifespan extension by DR is conserved between species; however, the mechanisms underlying such prolongation may not be conserved which intensifies the problem of comparative DR studies [213]. In case of *Drosophila*, DR effect is brought about by using a new medium for feeding that has controlled concentration of nutrients, which allows determination of specific nutrients that are essential for the organism's response to DR [214]. Since flies visit the food several times each hour to eat, and since restricting the access to food by intermittent starvation leads to considerable deaths [193], diluting the food proved to be more practical and effective [203]. Flies subjected to DR exhibit increased lifespan as compared to their control counterparts [39]. In fact, when flies were shifted from DR food to control food, they adopted an increased mortality rate when compared with the flies subjected to continued DR diet [157]. This suggests that death-causing

damage accumulates in both DR and control flies at the same rate, but a high-nutrient diet increases the risk of death. Moreover, it has been suggested that the protein/carbohydrate ratio also plays a role in modulating longevity in *Drosophila* [244]. A higher ratio tends to shorten the lifespan and *vice versa*. Furthermore, it has been demonstrated that within the protein component of the diet also, specific amino acids have crucial roles to play. For example, restriction of methionine extends average and maximal lifespan in *Drosophila* [1]. Additionally, whole genome transcript profiling in *Drosophila* has shown that DR is capable of reverting aging-specific transcriptional changes and limits cell growth, metabolism, and reproduction [215].

DR-mediated effects on longevity have been shown to be brought about by a set of molecular effectors including FOXO transcription factor, AMP kinase (AMPK), sirtuins, Heat shock factor-1 (HSF1), and NRF-2 transcription factor [69]. Inhibition of Akt due to DR activates the transcription factor FOXO which is involved in the upregulation of several pathways such as DNA repair, autophagy, antioxidant responses, stress resistance, and cell proliferation, which in turn promote longevity [272–274]. Similarly, ectopic overexpression of a few sirtuins like SIRT1, SIRT3, and SIRT6 reduces NF-κB signaling, increases genomic stability, and improves metabolic homeostasis via histone deacetylation [83]. Combined activation of SIRT1 and AMPK activates PGC-1α, which is a major transcriptional regulator of mitochondrial function and antioxidant defense [281]. Further, DR-mediated upregulation of HSP70 and p62 activates the transcription factors HSF-1 and NRF-2, which are also involved in enhancing antioxidant responses, preventing age-dependent impairment of proteostasis and promoting maintenance of cell structure and metabolism [3]. Thus, multiple yet parallel processes contribute to DR-mediated lifespan extension.

Insulin Signaling/mTOR Network

The **I**nsulin/**I**nsulin-like Growth Factor (IGF) **S**ignaling (IIS) pathway is one of the major pathways involved in cellular metabolism and growth and differentiation of somatic cells, whereas the mTOR pathway is vital for nutrient/energy/redox sensing and control of protein synthesis in the cell. DR has been suggested to exert its modulation on lifespan mostly via the IIS/mTOR network [56]. DR reduces plasma insulin/IGF levels in humans [275], and evidences comply that compromised insulin signaling results in increased lifespan in various model organisms [73]. Partridge and coworkers reported the first IIS mutation that extends lifespan which was present in the *Drosophila* homolog of the insulin receptor substrate CHICO [44]. The *chico* null flies were found to exhibit up to 48% increased median lifespan in homozygous females, 31% in heterozygous females, and 13% in homozygous males. Subsequently, it was also found that a hypomorphic mutation in the *Drosophila* insulin receptor (dINR) also affects the longevity positively [254]. Interestingly, these mutants also display increased triglyceride content and super oxide dismutase (SOD) activity pointing toward enhanced stress response, and thus increased lifespan. Similarly, reduced expression of the *Drosophila* insulin-like peptides (dilps),

the ligands for dINR, also extends lifespan [80]. In fact, ablation of dilp-producing cells and median neurosecretory cells (MNCs) in the late-staged larval brain also produces an analogous effect [29], as does the deletion of dilp-encoding genes [80]. Furthermore, enhanced expression of dPTEN (*Drosophila* phosphatase and tensin homolog), a negative regulator of IIS signaling, has been shown to bring about lifespan extension by antagonizing the action of phosphatidylinositol-3-kinase (PI3K) and promoting nuclear localization of FOXO [111]. This, in turn, causes an upsurge in transcriptional activity of FOXO. Since inhibition of the activity of *Drosophila* FOXO homolog *daf-16* leads to decreased lifespan [127], it is affirmatory that the key molecular effector of IIS in context of aging is dFOXO. Inevitably, downregulation of 14-3-3Ɛ, the negative regulator of FOXO, also tends to extend lifespan [192].

IIS has been suggested to exert its effects, in part, by the mTOR pathway. It has been observed that systemic overexpression of dTSC1 and dTSC2, antagonists of TOR activity, increases lifespan [124]. Similarly, expression of a dominant negative form of TOR or mutating the major downstream effector of this pathway, S6 kinase (S6K), also extends lifespan [124]. It was also demonstrated that reduced TOR activity exhibits ~20% increase in median lifespan without any associated stress resistance, as compared to the controls [156]. Moreover, rapamycin-mediated inhibition of mTORC1, the chief signaling complex of TOR pathway, also increases lifespan [20]. Inhibition of mTORC1 enhances processes like proteostasis, autophagy, and stem cell functions [69]. Since autophagic processes are activated in response to damaged or malfunctioning of proteins and/or organelles, they play a vital role in eliminating the damaged macromolecules and/organelles that contribute to intensifying the aging process. In fact, it has been demonstrated that inhibition of autophagic processes makes positive impact on longevity [20].

Another important cellular pathway involved in stress response against DR and inducing longevity is the Jun-N-terminal kinase (JNK) pathway. Although it acts as an independent pathway in the cell, it ultimately converges at the same molecular effectors as the IIS and mTOR pathway. JNK primarily antagonizes IIS and causes FOXO to localize to the nucleus and activate its downstream gene targets [271]. Taken together, it is increasingly clear now that several pathways act synergistically in the cells to bring about lifespan extension without making any adverse effects or fitness cost.

Aging-Associated Diseases

The risk of developing several diseases such as diabetes type 2, heart diseases, obesity, cancer, arthritis, kidney, and neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disorder (AD) increases with aging. With a rapidly growing aging population, these disorders have become a prodigious economic burden on the society. Therefore, due to absence of effective therapies, it has become even essential to find effective strategies for the benefit of the aging population. As discussed earlier, manifestation of aging-associated impairments could be minimized to certain extent by genetic, dietary, and pharmacological interventions,

which generally target different molecular pathways involved in aging, because these diseases have shown interference with age-related molecular mechanisms. Advancing mechanistic understanding of aging-associated diseases might be a clue for development of new therapeutic strategies.

Interestingly, age comes out as a critical factor for the onset of several human neurodegenerative disorders. Neuronal loss, shrinkage of cell bodies and axons of neuronal cells, and loss of synapse collectively lead to reduced brain volume and weight in aging individuals, who are cognitively normal [221]. Neurofibrillary tangles and senile plaques that show sparse distribution are neuropathological hallmark of AD, which have been found to accumulate in cortical region and adversely affect the cognitive function of the individual [86]. Similarly, common pathology of polyglutamine [poly(Q)]-mediated neurotoxicity in a variety of poly(Q) disorders is presented by degeneration of neuronal cell bodies, axons, synapse, and specific parts of the nervous system [61]. Moreover, it is still enigmatic whether both aging and disease-associated proteins act synergistically to extend neuronal dysfunctions, or only aging-related changes are accountable for driving the neuronal pathology.

It appears relatively coherent to hypothesize that disease-related proteins enhance disease toxicity by accelerating the aging process. For instance, in *C. elegans*, mutation that increases longevity in poly(Q) disease divulges age-dependent reduction in protein aggregate formation and toxicity, subsequently affirming the effect of aging in poly(Q)-mediated cellular dysfunction [176]. Several reports including our own findings demonstrate gradually aggravating poly(Q)-mediated neurotoxicity in an age-dependent manner [240]. Targeted expression of SCA-78(Q) in *Drosophila* eye causes manifestation of poly(Q) disease in form of cellular degeneration, retinal depigmentation, and neurotoxicity [240]. Our studies on flies expressing SCA3-78(Q) transgene during aging suggest that the extent of retinal depigmentation and cellular toxicity gradually increases with age. Similarly, in *Drosophila* human neuronal tauopathy models, tissue-specific expression of human tau (h-tau) transgene causes severe degradation of neuronal tissue [38]. Figure 14.6 depicts extensive degeneration of mushroom body upon pan neuronal expression of h-tau transgene in 3-day-old *Drosophila* adult brain. Mushroom body of *Drosophila* is a specialized structure which functions as a center of associative learning and also regulates a wide range of behaviors including habituation, olfactory learning, temperature preference, and sleep [164]. Contribution of common signaling networks in longevity and alleviation of neurodegenerative disorders further suggests that slowing down the aging process may act as a neuroprotective measure. Therefore, in order to develop novel strategies to obstruct onset and progression of such deadly disorders, it will be interesting to walk around how aging dysfunction and neuropathology are intertwined, and how they act together during disease pathogenesis.

As stated earlier, all eukaryotic life forms have well-regulated protein quality control system which includes chaperone network, ubiquitin–proteasome system, and lysosome-mediated autophagy. Proper functioning of this system is essential to achieve post-translational modifications, protein folding, stress response, and clearance/translocation of damaged proteins [11, 246]. It has been found that process of aging deteriorates the functional capacity of the cellular protein folding machinery,

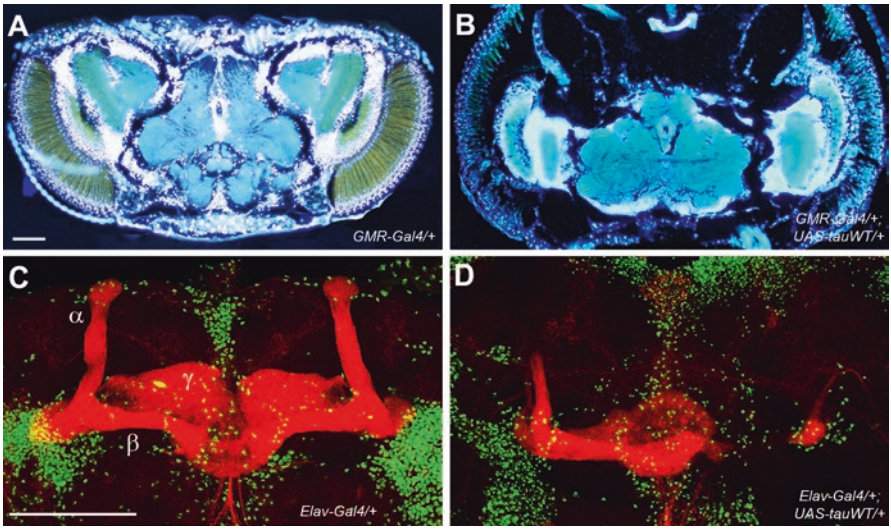

Fig. 14.6 Paraffin sections of a 3-day-old adult head across the midbrain stained with DAPI. In comparison to wild-type (**a**), eye-specific expression of human tau (h-tau) transgene results in severe tissue degeneration (arrows in **b**). Anti-Fasciclin II (FasII) staining shows that compared to the wild-type mushroom body with distinct presence of α, β, and γ lobes (**c**), pan neuronal expression of h-tau transgene results in notable degeneration of mushroom body (**d**) as distinctly seen in α, β, and γ lobes (arrowhead in **d**) (Scale **a**, **b** = 100 μm; **c**, **d** = 100 μm)

proteosome activity and the stress response; therefore, the post-mitotic neurons become susceptible to toxic protein aggregates and ultimately lead to cell death [184]. Several studies have been performed using *Drosophila* to illustrate the potential role(s) of molecular chaperones in suppression of neurodegenerative disorders. Not surprisingly, tissue-specific upregulation of molecular chaperones ameliorates the disease toxicity and also minimizes age-related cellular impairments. Targeted upregulation of Hsp70 along with Hsp40/DnaJ (HJD1) suppresses neurodegenerative phenotypes and also improves the lifespan in *Drosophila* Machado–Joseph disease (MJD) and Huntington disease (HD) model [184]. Further, role of Hsp70 and Hsp40 in regulation of poly(Q) aggregation and cellular toxicity has been further validated in *S. cerevisiae*, *C. elegans*, and mouse [48, 185].

In order to explain the progressive decline of Hsps in neurodegenerative diseases, several mechanisms have been hypothesized, including transcriptional deficit of *hsps* expression via toxic misfolded protein, and sequestration of cellular soluble Hsps along with the toxic aggregates to form inclusion bodies [90]. Moreover, evidences like CBP-induced transcriptional impairment of Hsp70 in *Drosophila* via reduction of Hsf-1 activity further support the transcriptional deficit hypothesis [90]. It appears that misregulation of molecular pathways and several factors those are responsible for regulation of protein quality control mechanism at cellular level might be the risk factor for disease occurrence. Therefore, novel therapeutic strategies could be provided by rejuvenating the protein quality control machinery for restoration of cellular homeostasis and to delay the aging onset of diseases.

Focusing on the disease-associated stress condition, it was fascinating to find a correlation between insulin/IGF-1 signaling in protein aggregation and toxicity, as aging is the key factor for disease onset. For the first time, studies on the *C. elegans* provided a direct link between insulin/IGF-1 signaling and protein aggregation. It was demonstrated that reduced level of insulin/IGF-1 neutralizes the poly(Q) aggregation and protects the worms from motility impairment and neurotoxicity [255]. The above finding suggests that lowered level of insulin/IGF-1 signaling pathway restricts the neurodegenerative disease phenotypes by modulating the aging process. Subsequently, studies in other model systems also suggest neuroprotective properties of insulin/IGF-1 signaling, which is primarily achieved by modulating the aging processes. Therefore, insulin/IGF-1 signaling can be considered as a novel target to combat aging-mediated impairment.

Antiaging Drugs and Natural Products

As discussed earlier, aging is a complex process due to involvement of multiple factors which influence this phenomenon. Such factors include genetic components, environment, metabolism, as well as reproduction. These multiple factors generate logistical difficulties in the development and evaluation of antiaging compounds. Therefore, studies focused on relatively simpler model organisms such as *Drosophila* and *C. elegans* have emerged as excellent systems for screening of genetic modifiers and antiaging drug molecules. In these model organisms, longevity can be altered and scored by genetic manipulations, and potential drugs which can increase the lifespan. Such antiaging compounds could be identified and categorized based on their functioning and mode of action. In this context, it is also important to note that several physiological and biological pathways are conserved in humans and *Drosophila*. Several antiaging molecules such as anticonvulsants (ethosuximide), antidepressants (mianserin), antioxidants, and others such as inhibitors of histone deacetylase, and resveratrol, a sir2 activator, have been identified and characterized in these model organisms [134]. A brief collection of antiaging molecules which modulate aging in *Drosophila* and other model systems has been provided in Table 14.2.

It was reported for the first time in *Drosophila* that resveratrol extends lifespan by activating sirtuins, without making any negative impact on fecundity [279]. This finding was further supported by the fact that feeding of resveratrol and rapamycin to 1-year-old mice improves the lifespan and heath, respectively [15, 93]. In view of above findings, it was postulated that resveratrol-induced increase in lifespan was sirtuin-dependent, and functions through pathways related to caloric intake [279]. However, studies based on biochemical assays with native substrates suggest that resveratrol does not activate SIRT1 directly [198]. Therefore, it appears that pharmacologic–genetic interplay should be taken into account while investigating the antiaging compounds and their operating mechanism(s). Moreover, this could also facilitate screening of additional genes and pathways which influence the aging process.

Table 14.2 A brief collection of some drugs and natural products found to delay aging and extend lifespan

S.no.	Drug/antiaging compound	Molecular target(s)	Generalized function	References
1.	Metformin	Mitochondrial respiratory complex I, AKT/TOR signaling modulation	Treatment of type 2 diabetes, antitumor; extends life span and inhibit age-related centrosome amplification in *Drosophila*	[5, 188]
2.	NSAID (celecoxib)	3′-Phosphoisositide-dependent kinase-1 (PDK-1) component of insulin/IGF-1 signaling cascade	Increases life span in *C. elegance*	[41]
3.	NSAID (ibuprofen)	Inhibits the tryptophan permease Tat2p, a component of Pkh2-ypk1-lem3-tat2 signaling pathway	Increases life span in *S. cerevisiae*, *C. elegance*, and *Drosophila*	[101]
4.	Sc-560, trans resveratrol, and Valdecoxib Aspirin, and NS-398, APHS, valeryl salicylate	Inhibition of COX and reduction in the production of ROS	Increases life span in *Drosophila*	[50]
5.	Ethosuximide	Inhibitor of T-type calcium channel, anti-convulsant, inhibits the function of specific chemosensory neurons	Delay age-related changes and extend life span of *C. elegance*	[46]
6.	Lithium	Inhibits GSK3 and activates NRF-2	Extends life span of *Drosophila*	[36]
7.	Spermidine (natural polyamine)	Activates autophagic machinery	Extends lifespan of *S. cerevisiae*, *C. elegance*, and *Drosophila*	[57]
8.	Sodium butyrate	HDAC inhibitor	Promotes longevity in *Drosophila*	[263]
9.	Cranberry plant extract	Minimizes oxidative stress, activates ERK/MAPK signaling and AKT pathway	Promote longevity in *Drosophila* and *C. elegance*	[85, 272, 273]

(continued)

Table 14.2 (continued)

S.no.	Drug/antiaging compound	Molecular target(s)	Generalized function	References
10.	Blueberry plant extract	Upregulates superoxide dismutase (SOD), catalase (CAT), and Rpn11, and downregulates methuselah (*mts*)	Promotes longevity in *Drosophila*	[207]
11	Extract of *Rhodiola rosea*	Acts against oxidative stress and decreases the production of ROS	Extends lifespan in *Drosophila*, *C. elegance* and *S. cerevisiae*	[16, 118, 276]
12.	Extract of *Rosa amascena*	Acts against oxidative stress	Extends lifespan in *Drosophila*	[119]
13.	Curcumin	Activates TOR pathway	Extends lifespans in *Drosophila*	[139, 242]
14.	Extract of *Ludwigia octovalvis*	Activates AMP-activated protein kinase (AMPK) pathway	Extends lifespan in *Drosophila*	[144]

The genetic approach in *Drosophila* generally follows the effects of antiaging drugs or compounds on pathways those are potentially involved in the aging process; there are still several pharmacologic compounds that are supposed to exert an impact on aging; however, their mechanisms are yet to be determined. Such putative antiaging compounds may facilitate discovery of novel antiaging compounds and also help in unraveling additional insights into the antiaging pathways. Several studies were focused on to examine the antioxidant effects of some selected compounds such as tocopherol-p-chloro-phenoxy acetate, nordihydroguaiaretic acid (NDGA), and Mg-TCA, and α and γ-tocopherol in the *Drosophila* [286, 291]. Interestingly, Jafari and coworkers have identified several antiaging pharmaceutical and botanical agents using *Drosophila* as a model organism [117]. A few of such antiaging agents include extracts of the plants *Rhodiola rosea*, *Rosa damascene*, cinnamon, green tea, and antidiabetic drug pioglitazone [117, 150, 234]. Regulated dosage of above compounds has been suggested to decrease the mortality rate in male and female flies without making any significant negative impact [117, 150, 234]. Further, extracts from cranberry plant has been found to contain antiaging and anti-inflammatory bioactive compounds and found to extend the lifespan in *C. elegans* and *Drosophila* significantly [85, 100, 108, 190, 272, 273]. Age-related functional decline of pancreatic β-cells in rats has been found to be delayed by using cranberry extract [289]. Interestingly, extracts from cranberry plant have been suggested to induce some epigenetic changes in chromatin, which in turn alter the dynamics of the aging-related signaling pathways and minimize the cellular damages [250].

As discussed earlier, chronic inflammation is associated with development of several aging-related diseases, and therefore, pharmacological inhibition of inflammatory processes using certain drugs has emerged as an effective antiaging strategy. A large number of nonsteroidal anti-inflammatory drugs (NSAIDs) such as

CAY10404, aspirin, APHS, SC-560, NS-398, SC-58125, valeroyl salicylate, transresveratrol, valdecoxib, and licofelone have been screened using *Drosophila* [50]. It was deduced that regulated feeding of anti-inflammatory drugs to *Drosophila* results in extended lifespan, delayed age-dependent decline of locomotor activities, and increased stress resistance [50].

Although numerous pharmacological drugs and natural compounds have been studied using various model organisms to target the aging phenomenon individually, however, the major challenge remains that once an antiaging compound shows antiaging activity deprived of any impact on physiological processes, or any undesirable effects on health span, it may require further evaluation in other genetic backgrounds or in additional model organisms. Further, deciphering the mechanism of action of such compounds could be worthwhile for identifying added antiaging agents or assessing combination therapies. However, there are still quite a lot of challenges for studies in this field, which is a major prerequisite to overcome.

Concluding Remarks

Aging research has perceived a notable acceleration due to the inclusion of several model organisms and development of contemporary tools that permit rapid screening of genetic modifiers and novel drug molecules, and analysis of the genome, transcriptome, epigenome, proteome, and metabolome of aging cells and tissues. This information is now being utilized for development of possible therapies to minimize the deleterious aspects of aging. It is increasingly clear now that aging is not an irreversible process, and senescence is not the inevitable fate of all organisms and it could be significantly delayed without any significant fitness cost. However, in spite of a considerable advancement in aging research, several questions related to molecular and neurological aspects of aging remain to be answered. Besides, most of the life-extension molecules/mechanisms have been observed in simpler model organisms, and these have still to be verified as viable antiaging therapies in humans. Here, it is also worth considering that a number of genetic manipulations which extend lifespan in *Drosophila* and other species show ex-specific inclination. The histrionic progress made in recent years established the feasibility to disentangle the mysteries of aging and to reach to a logical and decisive conclusion.

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