Review article

Genetic approaches to study aging in *Drosophila melanogaster*

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Abstract

The process of aging can be described as a progressive decline in an organism's function that invariably results in death. This decline results from the activities of intrinsic genetic factors within an organism. The relative contributions of the biological and environmental components to senescence are hard to measure, however different strategies have been devised in *Drosophila melanogaster* to isolate and identify genetic influences on aging. These strategies include selective breeding, quantitative trait loci (QTL) mapping and single gene mutant analysis. Selective breeding effectively demonstrated a genetic, heritable component to aging while QTL mapping located regions within the *Drosophila* genome carrying loci that influence the aging process. Within the past decade, single gene mutant analysis has facilitated the identification of specific genes whose activities play a determinative role in *Drosophila* aging. This review will focus on the application of selective breeding, QTL mapping and single gene mutant analysis used in *Drosophila* to study aging as well as the results obtained through these strategies to date.

Abbreviations: CuZn – copper/zinc; DTT – DJ651-driven tetanus toxin; FLP – flippase; FRT – FLP recognition target; GPCR – (G-protein)-coupled receptor; Hsp – heat shock protein; Indy – I'm not dead yet; JNK – c-Jun N-terminal kinase; LD – linkage disequilibrium; PdL – Ponce de Leon; QTL – quantitative trait Loci; RI – recombinant inbred; rtTA – reverse tetracycline trans-activator; Sir2 – silent information regulator 2; SOD – superoxide dismutase; TetO – tetracycline operator; TOR – target of rapamycin; UAS – upstream activating sequence

Introduction

The time-dependent decline in overall biological function as an organism progresses through life, is the result of both extrinsic (i.e., disease, injury, wear and tear) and intrinsic (i.e., genes) factors (Arking 1998). Aging, if seen simply as the process of growing older, should not necessarily entail an accompanying deteriorative process. However, empirical observation leaves no doubt that living organisms break down naturally as they age, and that these biological degradations inevitably culminate in death. All organisms, even those raised in

laboratory conditions where harmful extrinsic factors are by and large absent, display a maximum species-specific life span. Aging therefore, is associated with the onset, typically at reproductive maturity, of an intrinsic biological phenomenon resulting in the reduction of organismal fitness and ultimately death. Unfortunately, disentangling the biological components from the environmental components of aging has proven to be a rather difficult task.

The common fruit fly, *Drosophila melanogaster*, has been a model organism of choice in the study of aging. Its easy upkeep and short life span, important features when one is scoring for length of life, initially

made *Drosophila* a very amenable organism to work with in aging research. The advent of modern molecular biology techniques, the in-depth knowledge of *Drosophila*'s genetics as well as the sequencing of its entire genome further extends its usefulness as an experimental system. This article reviews the different strategies employed to elucidate the genetic basis of aging in *Drosophila*: selective breeding, quantitative trait loci (QTL) mapping and single mutation approaches.

Selective breeding

Early investigations into a possible genetic basis of aging in *Drosophila melanogaster* relied on the use of selective breeding. *Drosophila* longevity is a quantitative trait that results in a high degree of variation in individual fly life spans. In any randomly mating population individual flies with relatively short, long and intermediate longevity phenotypes, as compared to the average *Drosophila* life span, will be found. The rationale behind selective breeding is that if these variations in longevity are partially genetically determined, then by applying artificial selective pressure either for or against long life, one should be able to experimentally manipulate fly longevity.

Initial selection studies in the mid-1960s by Wattiaux suggested that increased life span could be artificially selected for in *Drosophila* (Wattiaux 1968a, b). Using three different *Drosophila pseudoobscura* lines and two different *Drosophila subobscura* lines, he was able to demonstrate an increase in life span of progeny derived from older, longer lived parents when compared to progeny produced by younger parents. Subsequent selective breeding experiments however, produced perplexing and contradictory results. In the mid-to-late 1970s, the Lints lab was unable using both direct and indirect selective breeding strategies to significantly extend the average life span of experimental 'long-lived' flies compared to control flies (Lints and Hoste 1974, 1977; Lints et al. 1979). Soon thereafter several selective breeding studies were able to significantly increase the average life span by direct selection or indirectly by selecting late-reproducing flies (Rose and Charlesworth 1981; Rose 1984; Luckinbill et al. 1984; Arking 1987; Zwaan et al. 1995). A major difference in methodologies used was that the larval density was not identical between the different

reports. In an attempt to clarify these contradictory findings, selective breeding studies were carried out using high, low or uncontrolled larval density (Luckinbill and Clare 1985; Clare and Luckinbill 1985). Consistent with previous results, only the high and uncontrolled larval density environments could produce long-lived strains. In an attempt to rationalize these results, it is important to consider that lack of competition leads to canalization (i.e., uniformity of phenotype). Since a low larval density environment would be a low-competitive environment, it is predicted that this environment will result in a lack of phenotypic variety, a necessary precondition for any selection experiment. In other words, the inability to positively select for extended life span under low larval density is an experimental limitation produced by a lack of sufficient phenotypic variety to select from.

Selective breeding strategies have not only illustrated a malleable genetic aspect of longevity, but also demonstrated that laboratory derived long-lived flies actually live significantly longer than their wild counterparts (Khazaeli and Curtsinger 2000). This is important since concerns had been raised that laboratory selected long-lived flies had not acquired an above average life span, but rather were only recovering from reduced fitness resulting from inbreeding depression.

QTL mapping

Based on the underlying genetics, a phenotype of interest can be classified as either a Mendelian or a quantitative trait. Mendelian traits, the type studied by Mendel to elucidate the existence of heritable genetic factors, give rise to discrete, discontinuous phenotypes (i.e., pea seed shape) (Snustad and Simmons 2000). Quantitative traits in comparison give rise to a gradient, or continuous range of phenotypes. The variation in phenotypes observed for quantitative traits results from the influence of various genes, each of which make a small contribution to the phenotype in question. As well, many quantitative traits are also influenced by environmental and developmental factors. For example *Drosophila* life span, which is a quantitative trait, is the product of genetic, environmental and developmental factors. As a result of the interplay between genes, environment and development, different genotypes can give rise to identical phenotypes and thereby precludes any simple measurement of the genetic

contribution to a quantitative trait. One method used to isolate genomic regions affecting a quantitative trait of interest is quantitative trait loci (QTL) mapping (Mackay 2001).

Conceptually, QTL mapping is straightforward and is based on the assumption that linkage disequilibrium (LD) exists between marker loci and quantitative trait alleles. All that is needed for QTL mapping is a homogenous genetic background in which alleles affecting a phenotype of interest are linked to various polymorphic markers. To achieve this, two inbred lines of flies that have different QTL alleles fixed within their genomes are generated and then crossed to produce an F1 generation. Subsequently these F1 lines, in which recombinant allelic genotypes are generated through meiotic crossing-over within the female germ line, are backcrossed with the original inbred parental lines. The resultant F2 generation is allowed to inbreed for several generations in order to produce recombinant inbred (RI) lines; lines which genetically are virtually homozygous. These RI lines, in conjunction with polymorphic marker linkage maps, allow for the mapping of genomic regions influencing the quantitative trait of interest.

The idea is to associate, using statistical methods, specific QTL loci to marker alleles that can then be followed generation by generation, thereby enabling mapping of the QTLs influencing the phenotype of interest. Proper controls, such as outbred stocks must be used to account for any adverse fitness effects that could arise from intensive inbreeding. Practically, QTL mapping is done by classifying individual subjects according to the set of marker alleles they possess. The resulting marker genotype classes are then analyzed using correlative statistics to see if there is any significant difference between the classes with regards to the phenotype of interest. If a difference is observed between the marker genotype classes, then QTLs affecting the phenotype of interest are linked to the markers. With a large variety of visible mutant stocks along with numerous molecular marker genes covering most of its genome, *Drosophila* is an ideal model organism for QTL mapping.

It should be noted that QTL mapping does not identify specific genes, but rather, associates a specific chromosomal region with the quantitative trait in question. The size of the genomic region in which a QTL can be mapped to depends on the density and number of markers used in the linkage

study; the greater the number of markers and the denser these markers are, the smaller the chromosomal region a QTL can be resolved to. However, identified QTLs typically contain thousands of genes, each of which is potentially influencing the phenotype of interest. It is necessary to whittle down the number of QTL-associated genes to numbers that can be realistically investigated.

The transition from chromosomal region to candidate gene first involves refining the QTL to as small a genetic region as possible. This fine-scale mapping involves classical complementation analysis (Mackay 2001). Deficiency complementation analysis allows for the fine-scale mapping of recessive mutations through the use of well-defined chromosomal deletions. By using a collection of strains (deficiency stocks), each one missing a known chromosomal region, it is possible to test the whole *Drosophila* genome. By comparing complementation results between deficiency stocks carrying partially overlapping regions, a recessive mutant allele can be precisely mapped. If a limited workable number of candidate genes can be obtained, complementation tests can be carried out by crossing the RI strains with strains carrying a mutation in a single candidate gene.

However the number of genes within a QTL is often in the hundreds, and when obvious candidate genes are absent, choosing which genes to further evaluate is often dependent on biological intuition. A strategy to further reduce the number of candidate genes affecting a quantitative trait is to combine fine-scale QTL mapping with micro-array analysis (Wayne and McIntyre 2002). This strategy led to over 90% reduction of the number of potential candidate genes affecting ovariole numbers.

QTL strategies have met with some success in identifying genetic factors that affect life span. From 98 RI lines, QTLs having moderate effects on longevity, some of them in a sex, age or environment-dependent manner, were mapped to several genomic regions (Leips and Mackay 2000, 2002; Vieira et al. 2000; Nuzhdin et al. 1997). Deficiency complementation analysis of these QTLs revealed several candidate genes already known to affect *Drosophila* longevity, including genes encoding alcohol dehydrogenase, small heat shock proteins, superoxide dismutase, and catalase (Pasyukova et al. 2000): a great example of molecular and population genetics arriving at similar conclusions using dissimilar methodologies. In another analysis, a set of 114

RI lines was used to identify several QTLs on chromosomes II and III associated with longevity (Curtis and Khazaeli 2002). These QTLs also displayed pleiotropic effects, not only affecting life span, but female fertility during mid-life as well as the resistance to paraquat, a potent-free radical generator.

Unfortunately, only a few genes affecting *Drosophila* life span have so far been isolated using QTL mapping/complementation analysis. The only three genes identified so far through this strategy are *dopa-decarboxylase* (*Ddc*), *Shuttle craft* (*stc*) and *ms(2)35ci* (Pasyukova et al. 2004; De Luca et al. 2003). *Ddc* is an enzyme required for the production of dopamine and serotonin in the central nervous system and hypoderm. *stc*, a homologue of the human transcription factor NF-X1, encodes an RNA polymerase II transcription factor. *stc*, expressed in the embryonic, larval and adult stages of life, is found predominantly in the central nervous system as well as in the ovaries. Although *ms(2)35Ci* has an unknown molecular function, all characterized recessive alleles cause male sterility.

Single mutation approaches

Independently created long-lived *Drosophila* lines differ in a number of aspects of their physiology (Arking 1998; Mockett et al. 2001; Service et al. 1988; Force et al. 1995), emphasizing that there are a multitude of processes affecting aging and that life span may be altered significantly by activating or suppressing a variety of different mechanisms. Despite their success in demonstrating that genes influence longevity, both selective breeding and QTL mapping rely on strains carrying several allelic variations, preventing straightforward identification of genes affecting aging. Strategies based upon mutant strains resulting from a single mutation greatly facilitate and accelerate the discovery of such genes.

Phenotypic screens

Phenotypic screens aim to isolate mutant strains exhibiting modified features (phenotypes) that reflect a significant alteration of the rate of aging. The phenotypes scored in such screens can either be longevity or the pace of functional deterioration associated with aging. In both cases, the phenotypes examined during the screening process are strictly

associated with the rate of aging. Alternatively, in order to speed up the rate of discovery, stress response phenotypes have also been used because of the frequent association between extended longevity and increased resistance to environmental stresses.

Longevity screens

Since the longevity of an organism is linked to its rate of aging, the most obvious approach to identify genes affecting aging is to select genetic variants, generated through random mutation, that live longer than wild-type controls. Because of the limited nature of the molecular lesions that can be induced by any given mutagen, no genetic screen will be able to identify every age-influencing gene. In addition, the mechanism of action of a given gene will determine whether longevity can be affected either as the result of gene inactivation (loss of function) or inappropriate gene expression (gain of function). This issue can be largely solved by taking advantage of the wide panel of mutagens and screening designs available in *Drosophila* (St Johnston 2002). So far longevity screens have been used to isolate loss-of-function and gain-of-function single gene mutations.

Loss of function. Loss-of-function screens generally yield hypomorphic (partial loss of function) or null (complete loss of function) alleles of genes. Since mutagenesis by transposition simplifies the identification of the mutated gene, all published screens to date have relied on the use of transposons as a mutagen. Transposons are mobile DNA elements that can be used to introduce foreign DNA into the genome of model organisms, such as *Drosophila*. Random insertion of transposons into genes or their regulatory elements can be disruptive, resulting in reduced or abolished gene activity (Cooley et al. 1988). In 1998 Lin et al. conducted a genetic screen using a P-element transposon to isolate a third chromosome mutation, *methuselah* (*mth*), associated with a 40% extension of the average lifespan (Lin et al. 1998). In addition to its prolonged life, *mth* flies survive longer than controls under adverse conditions, including high temperature, low humidity, starvation and feeding on paraquat. This observation indicated that the alteration of the activity of a single gene can reproduce both the extension in longevity and the increase in resistance to external stresses obtained in selective breeding experiments reported previously. Both the longevity and stress resistance

were restored to normal when the P-element was precisely removed, indicating that the *mth* mutation is responsible for the phenotypes. The mutation is located inside a gene encoding a predicted protein of 514 amino residues with a putative leader peptide and seven hydrophobic regions; features that are indicative of membrane-spanning proteins. Comparison of the protein sequence with databases suggests that the *mth* gene encodes a GTP-binding regulatory protein(G-protein)-Coupled Receptor (GPCR). G-protein-coupled receptors are involved in a remarkably diverse array of biological activities, including neurotransmission, hormone physiology, ion transport across membranes, drug response, morphological differentiation, embryonic development, and transduction of stimuli, e.g., light and odorants (Milligan 1998; Watson and Arkinstall 1994). The molecular mechanisms are correspondingly complex. In principle, they receive information about the extracellular environment through the binding of a ligand and then undergo changes in conformation. The change in structure is transmitted to the cell interior by activating an associated heterotrimeric G-protein, which then transduces the signal via secondary messenger molecules. Since the receptors function at an upstream level of the signal transduction cascade, they have essential roles in the pathway. Overall, this implies that the MTH protein is a regulatory receptor that modulates stress response and biological aging. In agreement with this model, loss of function alleles of the MTH ligand are also associated with extended longevity and increased resistance to oxidative stress (Cvejic et al. 2004). Although the molecular mechanisms remains to be elucidated, the impaired neurotransmission observed in mutant *mth* larvae suggest that the MTH protein might be an environmental sensor that influence aging and stress response through neuronal or neuroendocrine signals (Song et al. 2002). This hypothesis is consistent with the longevity extension observed in sensory perception or neurosecretory *C. elegans* mutants (Ailion et al. 1999; Apfeld and Kenyon 1999).

Gain-of-function. Loss-of-function and gain-of-function studies provide complementary data that can be integrated into a detailed understanding of a genes function. In addition, gain of function screens can reveal genes involved in a particular process that could not have been identified by loss-of-function mutational

analysis. In *Drosophila*, gain of function screens have relied on the use of two different inducible gene expression systems that can be used in the overexpression or misexpression of any gene: The Tet-On system and the gene search system (Figure 1).

The Tet-On system combines two different transposons (Bieschke et al. 1998). One carries a transgene that constitutively and ubiquitously expresses the reverse tetracycline trans-activator (*rtTA*). The second contains a tetracycline-inducible promoter because of the presence of tetracycline operator sequences (TetO). Only in the presence of the inducer tetracycline (or its derivative doxycycline), will the TetO sequences be bound by *rtTA* leading to the activation of the promoter. By placing the TetO promoter at the end of the transposon and directing it outwards, a mobile inducible promoter known as *Ponce de Leon* (*PdL*) is generated. Once inserted in the fly genome, the *PdL*-transposon often causes overexpression of genes near the insertion site, resulting in various mutant phenotypes (Landis et al. 2001). This ingenious strategy is very suitable for the study of aging and longevity. By its ability to create conditional mutations, it is possible to avoid effects originating from the alteration of pre-adult development. Because control (inducer absent) and mutant (inducer present) have identical genetic backgrounds, an aging-related phenotype must be due to gene overexpression resulting from the administration of the inducer. Finally, the inducibility of the system allows the selection of mutations that may be developmentally lethal, therefore granting the possibility of analyzing the role in aging of genes functioning in multiple biological processes. By transposition, over 10,000 fly strains carrying a unique *PdL* insertion have been created (Landis et al. 2001, 2003). Once the *rtTA*-containing transposon is introduced in each strain through simple crosses, the ectopic expression of genes near the *PdL* integration site can be driven when flies are fed doxycycline. The candidate genes identified through the *PdL* transposon encode factors involved in protein glycosylation, membrane transport, actin cytoskeleton organization, phospholipids metabolism and signaling. Further study is needed to confirm and clarify how these factors may be influencing fly life span.

The gene search system is based on a similar design with the same pros and cons, but differs by the nature of the molecular components involved. This system also relies on the presence of two

transposons in the experimental flies. One transposon contains a transgene in which the cDNA encoding the GAL4 transcription factor is under the control of a heat-inducible *hsp70* promoter. The second transposon carries at each end a GAL4-dependent UAS promoter directed outward, into the adjacent genomic regions. Upon heat shock, GAL4 will be produced which will then bind to the UAS promoter and activate it, thereby driving the overexpression or misexpression of gene(s) present in the flanking genomic regions (Toba et al. 1999). Compared to the Tet-On system, the presence of two UAS promoters in the mutagenic transposon has the advantage of increasing the frequency of mutant phenotypes, but as a consequence, complicates the identification of the gene(s) affected. It should also be noted that the Tet-On system inducer does not alter longevity while the temperature used to induce the gene search system can profoundly affect lifespan (David 1988). This can potentially introduce a bias toward heat-resistant mutants. Using the gene search system, 646 strains harboring a single insertion of the UAS promoters-containing transposon as well as the GAL4 transposon were screened for increased life span. Twenty-three strains exhibited an extension of life span of which five as well showed a significant higher resistance to oxidative stress (Seong et al. 2001a). The molecular characterization of the UAS promoters insertions identified 23 candidate longevity-regulation genes, including 13 previously known genes related to stress response, cytoskeleton organization and the JNK signal transduction pathway. One candidate gene encoding the *Drosophila Plenty Of SH3s (DPOSH)* protein has been verified to influence longevity potentially by modulating the activity of the JNK signaling pathway (Seong et al. 2001b). The JNK pathway has been independently involved in aging by a candidate gene approach discussed in the last section (Wang et al. 2003).

Biomarker screens

Longevity studies are time consuming, lasting as long as the flies stay alive. In addition the use of mortality as an indicator of aging is blind to the many events that contributed to that eventuality. Age-related changes that can be used as indicators of the 'aging state' of an organism at any time during its life cycle (i.e., biomarkers of aging) allow the identification of organisms whose rate of aging is altered as well as the testing of mutations suspected

to be linked to the aging process. Because biomarkers can predict how long the organism will live or how well it will age, they expedite the identification of aging mutants. Two general classes of biomarkers can be distinguished: behavioral biomarkers and molecular biomarkers.

Behavior biomarkers. The rate of aging is reflected by how long the organism lives (the lifespan) as well as the age of onset of behavioral and physiological manifestations of decline (for which has been recently coined the terms healthspan or functional senescence). Functional senescence can be estimated by measuring behavior and/or physiological performances across the lifespan. As expected, the healthspan, quantified by measuring the capacity to perform stereotypic behaviors, declines with age in a predictive manner (Cook-Wiens and Grotewiel 2002). The selection of mutant flies exhibiting a delayed functional senescence is an innovative and promising alternative strategy for dissecting the genetic components of aging. Long-lived strains isolated by selective breeding differ from control strains only in the chronological age at which the performance in behavior and physiological tests has degraded to a point where it is not measurable anymore (Arking and Wells 1990). Interestingly, single-gene mutations have shown that it is possible to genetically dissociate lifespan and healthspan determination. Long-lived *mth* flies show no significant difference in the rate of functional decline in odor avoidance, negative geotaxis or exploratory activity relative to controls with normal life span (Cook-Wiens and Grotewiel 2002). Reciprocally, flies with mutations in the cell surface receptor molecule β -integrin retain significantly better locomotor activity at older ages while their maximum lifespan and stress-resistance are unaffected (Goddeeris et al. 2003). Although the rate of functional decline is a possible phenotype to use in the isolation of mutant flies that are genetically predisposed for long-life, its main benefit is the potential to identify aging genes that would not be selected in longevity studies Table 1.

Molecular biomarkers. Traditionally, analysis of genetic screens for mutations that extend life span are done by identifying long-lived individuals; a time consuming process even in organisms with relatively short life spans like *Drosophila*. It would be very advantageous to use an unambiguous biomarker of

aging that can be scored early in life, well before the death of the animal. In 2004, Bauer et al. designed an assay system to accelerate the identification of life span extending mutations in *Drosophila* (Bauer et al. 2004). The strategy uses the DJ651 GAL4 biomarker: a transgene associated with increased GAL4 expression during the first few weeks of life (Seroude 2002; Seroude et al. 2002) (see also enhancer-trap section). A second transgene containing the cDNA encoding the tetanus toxin light chain under the control of a GAL4-dependent UAS promoter (UAS-TNT-E) is combined with the DJ651 transgene to create the DTT system (DJ651-driven Tetanus Toxin). This toxin is capable of causing death in *Drosophila* at very low concentrations. As the animal ages, GAL4 increases and consequently leads to the production of the toxin that will kill all flies reaching a lethal toxin-level threshold. A long-lived mutant will delay the time at which GAL4 will reach sufficient levels to drive the expression of the toxin above the lethal threshold. Consequently, in presence of the DDT transgenes, long-lived mutants outlive normal animals within two weeks rather than months. The DDT system also detects several kinds of lifespan-extending environmental interventions including low temperature, caloric restriction and reproduction prevention. It is also capable of detecting the life span extending effects of single-gene mutants. Finally it has been used successfully to screen lifespan-extending drugs. This system will expedite the isolation of genetic, environmental and pharmaceutical interventions that influence life span (Figure 1).

Indirect screens

Similar to selective breeding studies where late-reproductive fecundity was used to indirectly select for extended life span, selection based on traits associated with extended longevity has been attempted to identify strains of flies carrying mutations that impact the rate of aging.

Stress resistance. Selected long-lived strains often associate extended life span with increased resistance to environmental stresses, including dietary paraquat, starvation, heat and desiccation (Mockett et al. 2001; Force et al. 1995; Service et al. 1985; Nghiem et al. 2000; Arking et al. 1991). To forego laborious longevity screens, this observation prompted the use of stress resistance as a paradigm instead

of the life span (Arking et al. 1991). Long-lived strains have been reported from the outcome of selective breeding for starvation, desiccation or paraquat resistance (Vettraino et al. 2001; Rose et al. 1992). However, other reports failed to generate long-lived strains using stress resistance (Harshman et al. 1999). This discrepancy can be easily explained if the correlation between stress and longevity in animals obtained through selective breeding simply results from the selection of multiple mutations. The analysis of single-gene mutants poorly supports the correlation between increased stress resistance and longevity. Mutations selected for increased resistance to a single kind of stress rarely increased life span as well (Wang et al. 2004) (unpublished data). Reciprocally, only five out of 23 mutations selected for extended longevity displayed an affiliated increase in paraquat resistance (Seong et al. 2001a). Furthermore stress resistance can also be genetically dissociated from healthspan (Goddeeris et al. 2003). These observations also imply that many aging-influencing genes do not have pleiotropic effects, making single stress resistance a poor strategy for isolating longevity-influencing genes.

Since some genes such as *mtl* can have pleiotropic effects, it might still be possible to find them by modifying the selection paradigm. In order to increase the correlation with longevity, the solution would be to select mutants resistant to multiple kinds of stress. Since this could become as laborious as selecting for longevity, cDNA subtraction was first applied to identify genes whose expression is upregulated by paraquat, heat and starvation treatments (Wang et al. 2004). Thirteen genes were uncovered and not surprisingly they are involved in free radical scavenging, chaperoning, detoxification, transcription and translation. Gain of function mutations were subsequently generated and tested for multiple stress resistance and longevity. This approach demonstrated that the *hsp26* and *hsp27* genes influence life span and resistance to heat-shock, paraquat and starvation.

Oxygen resistance. The oxidative damage theory of aging postulates that the use of molecular oxygen by aerobic organisms generates reactive oxygen species (ROS) that negatively influence life span by damaging many macromolecules including nucleic acids, proteins and lipids (Beckman and Ames 1998; Harman 1956). The exposure to a pure oxygen atmosphere (hyperoxia) could be a better single-stress to use as a paradigm, since unlike dietary free radical generators

Table 1. Aging-related genes identified using various genetic approaches.

Gene	Strategy used	Experimental systems	Molecular function	Biological function	Reference
β -integrin	Candidate gene	Behavior biomarkers/ Loss of function	Cell surface receptor	Behavior, development, immunity, tumour metastasis	(Goddeeris et al. 2003)
Catalase	Candidate gene	Gain of function: FLP-OUT Gain of function: multiple gene copies	H ₂ O ₂ catabolism	ROS scavenging	(Griswold et al. 1993; Mackay and Bewley 1989; Orr and Sohal 1994; Orr and Sohal 1992; Mockett et al. 2003; Durusoy et al. 1995; Sun and Tower, 1999)
Ctll	Longevity screen	Gain of function: PdL	CTP:phosphocholinecytidyltransferase-I	Phosphatidylcholine synthesis	(Landis et al. 2003)
Chico	Candidate gene	Loss of function	Insulin receptor substrate	Insulin/IGF signaling pathway	(Clancy et al. 2001)
dDmrt2	Candidate gene	Loss of function	DNA methyltransferase	DNA methylation	(Lin et al. 2005)
dFOXO	Candidate gene	Gain of function: UAS/GAL4	Forkhead transcription factor	Insulin/IGF signaling pathway	(Hwangbo et al. 2004)
Ddc	QTL mapping	Gain of function: GeneSwitch	DOPA decarboxylase	Catecholamines metabolism	(Giannakou et al. 2004)
DPOSH	Longevity screen	Complementation tests Gain of function: GeneSearch	Rac1 interacting protein	Stress response signaling pathways	(Seong et al. 2001a; Seong et al. 2001b)
dS6K	Candidate gene	Gain of function: UAS/GAL4	Ribosomal protein S6 kinase	Nutrient-sensing and growth control	(Kapahi et al. 2004)
dTOR	Candidate gene	Gain of function: UAS/GAL4	Serine/threonine kinase	Nutrient-sensing and growth control	(Kapahi et al. 2004)
dTsc1	Candidate gene	Gain of function: UAS/GAL4	Unknown	Nutrient-sensing and growth control	(Kapahi et al. 2004)
dTsc2	Candidate gene	Gain of function: UAS/GAL4	Putative GTPase-activating protein	Nutrient-sensing and growth control	(Kapahi et al. 2004)
Exuperantia	Longevity screen	Gain of function: GeneSearch	Microtubule-dependent mRNA localization	Microtubules organization	(Seong et al. 2001b)
Filamin	Longevity screen	Gain of function: PdL	Actin-binding protein	Cytoskeleton organization	(Landis et al. 2003)
Fwd	Longevity screen	Gain of function: PdL	Phosphatidylinositol 4-kinase	Male meiosis	(Landis et al. 2003)
GstS1	Longevity screen	Gain of function: GeneSearch	Glutathione S transferase	Stress response, redox balance	(Seong 2001b)
Hep	Candidate gene	Loss of function	JNK kinase	Stress response signaling pathways	(Wang et al. 2003)
hsp22	Selective breeding	Gain of function: UAS/GAL4 Northern blot between normal and long-lived strains	Protein folding and molecular chaperone	Stress response	(Kurapati et al. 2000; Morrow et al. 2004a; Morrow et al. 2004b)
	Candidate gene	Loss of function			
hsp23	Selective breeding	Gain of function: UAS/GAL4 Northern blot between normal and long-lived strains	Protein folding and molecular chaperone	Stress response	(Kurapati et al. 2000)
hsp26	Longevity screen	Gain of function: GeneSearch	Protein folding and molecular chaperone	Stress response	(Seong et al. 2001b; Wang et al. 2004)
hsp27	Candidate gene	Gain of function: UAS/GAL4	Protein folding and molecular chaperone	Stress response	(Wang et al. 2004)

hsp68	Candidate gene	Gain of function: UAS/GAL4	Protein folding and molecular chaperone	Stress response	(Wang et al. 2003)
hsp70	Candidate gene	Gain of function: multiple gene copies	Protein folding and molecular chaperone	Stress response	(Tatar et al. 1997)
Indy	Gene expression screen	Enhancer-trap	Metabolite transporter	Metabolites trafficking	(Rogina et al. 2000)
		Loss of function			
InR	Candidate gene	Loss of function	Insulin receptor	Insulin/IGF signaling pathway	(Tatar et al. 2001)
JNK	Candidate gene	Loss of function	Stress-activated protein kinase	Stress response signaling pathways	(Wang et al. 2003)
Jumpy	Gene expression screen	Gain of function: GeneSwitch			
		Gain of function: UAS/GAL4			
		Enhancer-trap			
		Loss of function	Inositol lipid phosphatase	Putative role in muscle contraction and relaxation.	(Seroude, 2002; Selaya, 2000)
Klönking	Longevity screen	Gain of function: GeneSearch	Immunoglobulin super family	Development, cell-cell interactions	(Seong et al. 2001b)
LK6	Gene expression screen	Enhancer-trap	Serine/threonine kinase	Microtubules and cytoskeleton organization	(Seroude 2002)
Methuselah	Longevity screen	Loss of function	G-protein-coupled receptor	Neurotransmission	(Lin et al. 1998; Song et al. 2002)
ms(2)35Ci	QTL mapping	Complementation tests	Unknown	Male fertility	(Pasyukova et al. 2004)
mtrA	Candidate gene	Gain of function: UAS/GAL4	Peptide Methionine Sulfoxide Reductase A	Repair of oxidized methionine	(Ruan et al. 2002)
MyO3IDF	Longevity screen	Gain of function: GeneSearch	Class I myosin	Microtubules organization	(Seong et al. 2001b)
Nla	Longevity screen	Gain of function: GeneSearch	Unknown	Stress response	(Seong et al. 2001b)
PGRP-LC	Gene expression screen	Enhancer-trap Microarray	Peptidoglycan recognition protein	Immunity	(Seroude, L. 2002)
Puckered	Candidate gene	Loss of function	JNK phosphatase	Stress response signaling pathways	(Wang et al. 2003)
Rpd3	Candidate gene	Loss of function	Histone deacetylase	Chromatin remodeling	(Rogina et al. 2002)
Sema-5C	Longevity screen	Gain of function: GeneSearch	Class V semaphorin	Development, cell-cell interactions	(Seong et al. 2001b)
Sir2	Candidate gene	Loss of function	Histone deacetylase	Chromatin remodeling	(Rogina and Helfand, 2004)
Shuttle Craft	QTL mapping	Gain of function: GeneSwitch			
SOD1	Candidate gene	Complementation tests	RNA polymerase II transcription factor	Transcription	(Pasyukova et al. 2004)
		Loss of function	Superoxide anion catabolism	ROS scavenging	(Parkes et al. 1998; Phillips et al. 1989;
		Gain of function: multiple gene copies			Orr and Sohal, 1994)
SOD2	Candidate gene	Gain of function: UAS/GAL4	Superoxide anion catabolism	ROS scavenging	(Sun and Tower, 1999; Kirby et al. 2002)
		RNA interference			
Stadmin	Longevity screen	Gain of function: FLP-OUT	Putative microtubule binding protein	Microtubules organization	(Seong et al. 2001b)
Sugar baby	Longevity screen	Gain of function: GeneSearch	Putative maltose permease	Metabolites trafficking	(Landis et al. 2003)
Trx-2	Longevity screen	Gain of function: PdL	Thioredoxin	Redox balance	(Seong et al. 2001b)
VhaSFD	Longevity screen	Gain of function: PdL	Vacuolar H ⁺ -ATPase subunit	Proton pumping	(Landis et al. 2003)

Approaches through which these genes were identified are listed, as well as their characterized enzymatic activity (in the context of the cited work) and their biological role. See text and references for further details.

the adverse effects are generated by the endogenous metabolic pathways (Walker and Benzer 2004). Under hyperoxia, life span is reduced to seven days and a progressive degeneration of mitochondria featuring cristae derived swirls is observed. These swirls, first appearing after four days exposure, progressively increase in number as the period of hyperoxia is lengthened. Under normal oxygen concentration (normoxia), mitochondria remain intact throughout the first week of life, but as the fly ages, mitochondria progressively accumulate swirls. Since the mitochondrial electron transport chain consumes the vast majority of molecular oxygen, the mitochondria are exposed to high levels of ROS and therefore would be expected to be a primary site of ROS-induced aging defects. This suggests that these swirls may be a life span limiting, age-acquired mitochondrial defect. Survival under hyperoxia is a promising paradigm to isolate life extension genes since short-lived and long-lived mutants under this stress turned out to be also short-lived and long-lived under normoxia (Walker and Benzer 2004).

Gene expression screens

Gene expressions screens nicely complement phenotypic screens by selecting genes according to their expression pattern instead of an associated mutant phenotype. Two methods have been used to identify genes with interesting age-related expression patterns: the enhancer-trap technique and microarray analysis. While microarray analysis has the ability to screen all the genes within the genome, mutations affecting identified candidate genes are not readily available. On the other hand, the enhancer-trap analysis is only capable of screening a limited number of genes but it does allow for the quick isolation of mutations in genes of interest.

Enhancer-trap

The enhancer-trap technique is a powerful genetic method to identify gene expression patterns *in vivo* through the detection of the activity of DNA transcriptional regulatory sequences known as enhancers (O'Kane and Gehring 1987). This method has been employed in *Drosophila* to characterize gene activity as a function of age (Seroude 2002; Seroude et al. 2002; Helfand et al. 1995). Briefly, a transgene containing a gene encoding a measurable product under the control of a minimal promoter is randomly inserted in the *Drosophila* genome. The transcription of the transgene is dependent on proximity to an enhancer normally controlling an endogenous gene. Therefore, the temporal and spatial activities of regulatory sequences can be measured at the single-cell level. The identification of enhancers with stereotypic age-dependent activity is an entry point to the isolation of the corresponding genes whose role in aging can be quickly assessed by mutational analysis.

Stephen Helfand pioneered the use of this technique to screen for age-regulated genes in the fly antenna (Helfand et al. 1995). The antenna, a tissue consisting entirely of postmitotic cells, eliminates the problem that mitotic cell replacement poses in aging-related gene expression research. Of 49 transgene insertions examined, 80% showed dynamic reporter gene expression across the life span. Measurement of the transgene product in flies with different life spans, demonstrated that changes in reporter gene expression scaled with life span (Helfand et al. 1995; Rogina and Helfand 1995; Rogina et al. 1997). In other words, the alteration of longevity does not affect the pattern of changes but the rate at which they occur. This is a critical observation since it demonstrates that gene expression is linked to aging and reflects the physiological age rather than the chronological age. This implies that gene expression

Figure 1. Single-gene transgenic strategies. Losses of function mutational analyses are complemented by strategies based on the manipulation of the expression of a single gene through the use of transposon-based transgenes. Inverted sequences indicated by the 5' P and 3' P black boxes define the boundaries of the transposon. Right-angled arrows designate promoters and the direction of transcription. Promoters are indicated as active with a wavy line illustrating the production of mRNA. Oval and rectangle boxes denote protein products. Refer to text for the details of the experimental system for each strategy. The left column shows the genetic crosses done to obtain the progeny (right column) with the appropriate combination of transgenes. In the UAS/GAL4 and DTT systems, the progeny is born in an active state (indicated by the white background) that will express the protein product according to the activity of an endogenous enhancer, independently of any further intervention by the experimentalist. In the other systems, the progeny is born in an inactive state (indicated by the light grey background) until an appropriate intervention (stated in the boxes) is applied. See text for the applications and findings associated with each system.

changes can be used as aging biomarkers to monitor the progression of the aging process well before the death of the individual, and that the characterization of the genes under age-dependent regulation provides an efficient way to identify the molecular changes associated with senescence.

A thorough investigation into the genetic basis of aging requires extending this type of gene expression analysis to the whole fly. This was accomplished by analyzing over 180 GAL4 enhancer-trap strains (Brand and Perrimon 1993; Duffy 2002) in which the transgene product is the GAL4 transcription factor (Seroude et al. 2002). The GAL4 transcription factor binds to a DNA element known as an Upstream Activating Sequence (UAS). Genes under the control of a promoter containing an UAS element will only be expressed upon GAL4 binding. In this case, the expression of the enhancer-trap transgene product is indirectly measured by introducing a UAS promoter linked to a reporter gene. Like in the antenna, over 80% of the strains showed whole-body age-dependent fluctuations in reporter gene expression. In most cases, the reporter gene expression is regulated by the physiological age of the fly. Whole-body examination revealed that the spatial pattern of reporter gene expression changed over time as well. Cloning of the genomic regions surrounding the transgene insertion site identified candidate aging-influencing genes involved in immunity, cytoskeleton organization and phospholipids-related enzymes.

Since transgene insertion is potentially mutagenic, the enhancer-trap technique allows for the quick transition from candidate gene identification to mutational analysis. Enhancer-trap strains associated with transcriptional up-regulation has led to the identification of genes regulating longevity. Enhancer-trap insertions in the *Indy* (*I'm Not Dead Yet*) gene are responsible for a dominant long-lived phenotype (Rogina et al. 2000). The *Indy* gene encodes a polypeptide with some homology to the human sodium dicarboxylate cotransporters. Sodium dicarboxylate cotransporters are membrane proteins involved in the uptake of Krebs cycle intermediates. The *jumpy* enhancer-trap insertion in the *EDTP* (Egg-Derived Tyrosine Phosphatase) gene is responsible for a recessive short-lived phenotype (Selaya 2000). Although originally suspected to encode a tyrosine phosphatase, the EDTP protein is biochemically characterized as a phospholipid phosphatase (Unpublished) (Alonso et al. 2004).

Microarray analysis

The *Drosophila* genome contains over 13,000 genes, precluding any possibility of investigating each one for a role in aging by single gene mutant analysis. Approaches need to be devised in order to facilitate the timely and efficient isolation of genes potentially involved in aging. By using microarray analysis, the transcript profile of every gene in the genome can be examined for expression patterns that exhibit age-related fluctuations. Because of the exhaustive nature of this approach, too many potential candidate genes will be obtained to effectively investigate. To reduce the number of candidates, strategies have been devised that integrate profiles associated with aging and profiles associated with interventions related to aging. Two such interventions are caloric restriction and oxidative stress.

Both free radicals and aging are capable of regulating gene expression. Although the nature of the relationship is under debate, they are related. Gene expression profiles of approximately 30% to 40% of the fly genome were carried out at different ages as well as after paraquat feeding (Zou et al. 2000). Out of more than 4,500 tested, 42 genes exhibited both age-dependent and oxidative-stress-dependent fluctuations in expression. The majority of these candidate genes encode proteins with unknown biological functions. The known candidates encode serine proteases, reproductive factors and mitochondrial enzymes involved in energy production.

In 2002, Pletcher et al. compared genome-wide gene expression profiles between different ages and caloric restriction (Pletcher et al. 2002). Caloric restriction, which involves maintaining flies on a nutrient media with a 35% less yeast and sugar than standard laboratory media, has been shown to extend *Drosophila* life span (Chapman and Partridge 1996). Following the same logic of the enhancer-trap studies, caloric restriction offers a robust and reproducible life-extending intervention to identify those changes in gene expression associated with physiological rather than chronological age. Nearly 23% of the genome exhibited age-related changes in transcript abundance. Many genes displayed rate of changes in expression that were reduced by caloric restriction proportionally to the life span expansion resulting from this treatment. Although this study yielded too many genes whose expression is dependent on physiological age to identify the critical ones on a gene-by-gene basis, they could be classified into

groups by considering their biological/molecular functions and their expression patterns. By doing this, it was possible to link several biological functions with aging including reproduction, enzyme inhibition and immunity.

In 2004, Landis et al. compared the transcription of over 13,000 genes in young and old animals as well as animals maintained under a pure oxygen atmosphere for seven days (Landis et al. 2004). The level of expression of several genes responded similarly to old age and oxygen treatment. The upregulated genes encoded factors involved in the purine biosynthetic pathway and the heat-shock, oxidative stress and immune responses. Old age and caloric restriction also shared the downregulation of genes encoding proteasome subunits, proteases, alkaline phosphatases and triacylglycerol lipases.

While genome-wide gene expression profiling is a very fashionable method to survey the molecular changes associated with aging, it is important to keep in mind its limitations. To date, it has only been applied on whole body RNA isolations, masking the tissue specific fluctuations in gene expression observed with enhancer-trap strains. To be applicable to the analysis of spatial changes, dissection and isolation of RNA from every cell or tissue will be required. Though RNA amplification techniques are available, characterizing the gene expression profile of a single cell would require enormous resources. This is a critical drawback since it is known that some genes influence aging in a tissue-specific manner. For instance, the targeted over-expression of the human CuZn superoxide dismutase (SOD) gene in motoneurons significantly extends longevity whereas whole-body, central nervous system or muscles targeted expression does not (Parkes et al. 1998, 1999). In addition, gene expression patterns result from the combinatorial action of several regulatory components whose individual activities are not accessible by the microarray technology. Finally, this technology depends on statistical analyses that are trustable only when a gene exhibits drastic temporal changes (in general at least two folds). It would be silly to assume that only the genes with large fluctuations in expression will influence aging. Although aging may be characterized by the loss of some kinds of homeostasis, it is not associated with a pervasive decline of regulation of gene expression (Pletcher et al. 2002; Rogina et al. 1998). Gene expression appears to be well regulated even

at older ages when biological performance is generally diminished. Therefore, it is more likely that the outcome of aging originates from the additive effect of small changes in transcript levels of numerous genes.

Candidate gene approaches

Rather than searching for genes randomly through the genome, an alternative strategy is to focus on genes already suspected to influence aging. Such genes can be chosen from observations made in other model organisms, aging theories or simply by using common sense. *Drosophila* homologs of these genes make ideal candidates for investigation into the role they may play. In addition, once a factor involved in a particular pathway or biological function has been identified, genes within the same pathway or analogous pathways become obvious candidates for investigation as well. Alternatively, genes shown to be involved in biological processes linked with aging, such as the stress response, can also be tested.

Stress-related

Heat stress. The phenomenon of hormesis, which is the increase in the life span on return to normal conditions after mild heat shock, led to question a link between increased heat tolerance and extended longevity. In many organisms, including *Drosophila*, the level of misfolded and abnormal proteins increases with age. Chaperon proteins, such as the heat shock proteins, are involved in maintaining the proper folding of cellular proteins in normal and high-stress environments. In *Drosophila*, the heat shock proteins have age-specific expression patterns, with hsp22, hsp23, hsp26, hsp27 and hsp70 levels increasing with age (Seroude et al. 2002; King and Tower 1999; Wheeler et al. 1995). Selected long-lived strains have higher levels of hsp22 and hsp23 RNAs than short-lived controls (Kurapati et al. 2000). Small heat shock proteins were also positively associated with the natural variation in longevity observed in wild *Drosophila* strains (Geiger-Thornberry and Mackay 2004). Recently, a study of the *Drosophila* DNA methyltransferase dDnmt2 indicated that overexpression extended lifespan and increased resistance to starvation and oxidative stress while reducing expression had the opposite effects (Lin et al. 2005). Upregulation of dDnmt2 resulted

in a concomitant increase in the levels of the small heat shock proteins hsp22, hsp23 and hsp26 while downregulation resulted in a concomitant decrease in hsp22, hsp23 and hsp26 protein levels.

These observations suggest that the heat shock response pathway may be a mechanism through which life span is regulated. If true, one would predict that increased expression of heat shock proteins should extend longevity. Indeed, raising the number of hsp70 genes in the genome extends the life span extension observed in response to mild heat shock (Tatar et al. 1997). Similarly, GAL4-driven *hsp22* overexpression increased life span and heat resistance while loss of function mutations had the opposite effects (Morrow et al. 2004a, b). In contrast, whole-body *hsp22* overexpression using the Tet-On system decreases life span as well as the resistance to oxidative and heat stresses (Bhole et al. 2004). A possible explanation for these contradictory results is that the beneficial effects of increased hsp22 protein levels are only realized when hsp22 is found in specific stoichiometric ratios with other small heat shock proteins. Over accumulation of hsp22 disrupts this optimal ratio and/or active hsp22 complex, thereby resulting in decreased longevity. Alternatively, the different expression systems used may have different tissue specificities, strengths of induction and temporal patterns of *hsp22* expression.

Oxidative stress. The observation that oxidative damage increases with age in many organisms led to the assumption that oxidative stress was an influential factor in determining life span. If so, then all other things being equal, individual organisms better able to handle oxidative stress should live longer than individuals less able to cope with ROS. Consistent with this expectation, mammalian longevity can be correlated with cellular stress resistance (Kapahi et al. 1999). *Drosophila* strains unable to produce CuZn SOD1 and catalase, two key enzymes required for ROS scavenging, are short-lived (Phillips et al. 1989; Griswold et al. 1993; Mackay and Bewley 1989). Reciprocally, simultaneous elevated activities of CuZn SOD1 and catalase extend life span by over 30% and reduced the accumulation of oxidative damage (Orr and Sohal 1994). None of these effects were obtained when the activity of only one of these enzymes is elevated. However, using the UAS/GAL4 system (Brand and Perrimon 1993;

Duffy 2002), it is possible to observe a 40% life span extension by manipulating SOD1 expression in motoneurons (Parkes et al. 1998). Solely increasing catalase activity and/or expression reduces life span or has neutral effects (Orr and Sohal 1992; Mockett et al. 2003; Durusoy et al. 1995; Sun and Tower 1999).

The SOD1 results led to the investigation of a second major form of *Drosophila* SOD known as *SOD2*. Through RNA interference techniques using the ubiquitous *daughterless* promoter to express Sod2 interfering RNAs throughout the adult, *SOD2* expression was decreased, resulting in severe life span reductions (Kirby et al. 2002). Increased *SOD2* expression has been achieved with the FLP-OUT inducible system (Sun and Tower 1999) (Figure 1). Like the gene search and Tet-On systems previously introduced, this strategy relies on two transgenes. One transgene places the flippase (FLP) recombinase cDNA under the control of a heat shock promoter. The second transgene carries the *SOD2* cDNA under control of a constitutive promoter. To prevent any constitutive expression, the cDNA is interrupted by a piece of DNA bordered by FLP recognition targets (FRT). Upon heat shock, the FLP recombinase is expressed, binds to FRTs and excises the interrupting DNA resulting in *SOD2* expression. Although this strategy has the advantage of avoiding developmental effects and genetic background issues, like the gene search system, the inducer can influence longevity. As expected, life span is extended in proportion to the increase in enzyme activity achieved through this strategy. Overall, oxidative stress candidate approaches have clearly established that SOD enzymes have a critical role in determining *Drosophila* longevity.

Despite the presence of enzymes dedicated to eliminate ROS, oxidative damages can still occur. For instance, methionine is readily oxidized to met sulfoxide (met(O)). The presence of met(O) in a variety of proteins affects biological activity. Since the reduction of met(O) back to methionine is catalyzed by the enzyme peptide methionine sulfoxide reductase A (MSRA), one would expect that this enzyme could significantly impact the rate of aging. Using the GAL4-UAS system, the overexpression of the bovine MSRA can be obtained by combining a UAS-MSRA transgene with a GAL4 enhancer-trap. This manipulation did increased longevity and the resistance to dietary paraquat (Ruan et al. 2002).

Insulin/IGF signaling pathway

The association between insulin signaling and life span was first uncovered in experiments on dauer formation in the nematode *C. elegans* (Johnson 1990; Kenyon et al. 1993; Larsen et al. 1995; Morris et al. 1996). Dauer formation, in which *C. elegans* larvae enter an extended, developmentally arrested larval stage, is a response to conditions of high population density or low food. Although the dauer stage can last for many months, upon proper nutritional cues, the animal will resume development and yield to an adult with normal life span. Genetic screens based on dauer phenotypes isolated the *daf-2* gene encoding a homologue of the mammalian insulin/IGF (insulin growth factor) receptor. Some larvae carrying *daf2* mutations that cause constitutive dauer formation can be induced to develop into adulthood if raised at low temperatures, resulting in adults with significant extensions in life span. Subsequent work demonstrated that reduced levels of *daf-2* or other components of the insulin pathway results in extended life span while reduction in the levels of a negative regulator of insulin signaling, *daf-18*, a PTEN phosphatase homologue, decreased life span (Guarente and Kenyon 2000).

Studies in *Drosophila* demonstrated that the insulin/IGF signaling pathway also regulates the life span of the fly. Null mutations in *chico*, a *Drosophila* insulin receptor substrate significantly increases female (and to a lesser extent male) life span (Clancy et al. 2001). A similar mutational strategy confirmed that the gene *InR* encoding the insulin receptor is a negative regulator of longevity (Tatar et al. 2001). In this case, homozygous hypomorphic *InR* alleles are often lethal during development, but female flies heteroallelic for the two hypomorphic alleles *InR^{E19}* and *InR^{p5545}* exhibit an 85% increase in life span while males show a decrease in late age-specific mortality.

Life span extensions produced in the nematode by mutations in the insulin/IGF signaling pathway require the presence of a functional *daf-16* gene (Ogg et al. 1997; Lin et al. 1997). *daf-16* encodes a forkhead transcription factor whose activity and nuclear localization are regulated by the insulin/IGF signaling pathway. Overexpression of *daf-16* alone is sufficient to extend *C. elegans* life span (Henderson and Johnson 2001). To demonstrate that the insulin/IGF signaling pathway influences *Drosophila* life span through similar mechanisms, two recent studies

(Hwangbo et al. 2004; Giannakou et al. 2004) used the GeneSwitch system (Roman et al. 2001; Osterwalder et al. 2001) (Figure 1) to show that dFOXO, a *Drosophila* *daf-16* homolog, has the same life-extending properties. The GeneSwitch system is a derivative of the UAS/GAL4 system in which the GAL4 component is replaced by a chimeric GAL4 gene that encodes the GAL4 DNA binding domain, the human progesterone receptor ligand binding domain and the activation domain from the human protein p65. Only in the presence of the antiprogesterin, RU486, will the chimeric molecule bind to a UAS transgene and activate its transcription. This modification of the UAS/GAL4 system adds the ability to control the onset of the UAS transgene expression to prevent developmental effects. Like the Tet-On system, the GeneSwitch inducer has no effect on adult longevity.

Chromatin remodeling

Previous work in *S. cerevisiae* and *C. elegans* has shown that factors involved in chromatin-dependent transcriptional silencing are involved in determining life span (Guarente and Kenyon 2000). Abolishing expression of the Rpd3 deacetylase (Kim et al. 1999) or increasing expression of the Sir2 deacetylase (Kaeberlein et al. 1999) extends life span in the yeast *S. cerevisiae*. Increased levels of Sir2 results in long-lived *C. elegans* as well (Tissenbaum and Guarente 2001). In *Drosophila*, both Rpd3 and Sir2 influence fly life span. Hypomorphic Rpd3 mutants are long-lived, although excessive reduction of Rpd3 levels is lethal (Rogina et al. 2002; Mannervik and Levine 1999). UAS/GAL4 or GeneSwitch-mediated *Sir2* gain of function increases the life span (Rogina and Helfand 2004). Since the life span extension associated with a *Rpd3* mutation is prevented by *Sir2* loss of function, Sir2 is likely acting downstream of Rpd3.

Tor pathway

The Tor (Target of Rapamycin) protein, a serine/threonine kinase, is a component of a nutrient-sensing signaling pathway involved in the regulation of *Drosophila* growth (Hafen 2004). By regulating the activity of downstream factors such as the ribosomal S6 kinase (S6-kinase) and the 4E-binding protein (4E-BP), TOR ultimately influences translation and therefore growth. TOR activity is repressed by a protein complex composed of TSC1 and TSC2 (tuberous sclerosis complex genes 1 and 2). The

insulin/IGF signaling pathway is also involved in regulating the growth and size of *Drosophila* and not surprisingly, it interacts with the Tor pathway. Based on the observation that mutations in many of the components of the insulin/IGF signaling pathway extend life span, the UAS/GAL4 system has been used to investigate whether TOR signaling had any influence in determining fly longevity (Kapahi et al. 2004). In agreement with this hypothesis, Tor-deficient mutant nematodes live more than twice as long as wild-type controls (Vellai et al. 2003). Like the insulin/IGF pathway, the Tor pathway is expected to negatively regulate longevity. Indeed life span extensions were associated with increased levels of negative regulators of the pathway: TSC1, TSC2 and dominant-negative variants of Tor and S6-kinase. Reciprocally, the overexpression of a constitutively active form of S6-kinase decreased the mean life span by 34%.

JNK pathway

The Jun N-terminal kinase (JNK) is a stress sensing kinase activated by various insults including UV radiation and oxidative stress. In addition, gain of function analysis suggests that DPOSH influences longevity by altering the JNK signaling pathway (Seong et al. 2001b). Mutational analysis verified that increased JNK signaling can raise resistance to oxidative stress and extend life span while decreased JNK signaling had the opposite effects (Wang et al. 2003).

Conclusion

Aging, the slow deteriorative process that culminates in death is a reality of life for all organisms. Nonetheless, work done in various organisms, ranging from yeast to mammals, has revealed that the aging process has a genetic component that may be amenable to intervention. Even if this intervention does not lead to large extensions in the length of human life, it may allow for significant improvement of the quality of life in the elderly.

Intensive investigations into the biological causes of aging have rewarded us with the isolation of pathways and factors that affect aging in numerous model organisms. The fruit fly, *Drosophila melanogaster*, has been an important experimental system in which to carry out aging research. The full

sequencing of its genome coupled with a wide panel of molecular genetics tools, makes *Drosophila* an ideal organism for aging research. Initially, selective breeding studies demonstrated that aging, a quantitative trait, was influenced by heritable, genetic factors. Subsequent mapping techniques allowed researchers to localize these quantitative trait loci to specific chromosomal regions within *Drosophila*'s genome. However, the application of single gene mutant analysis has been the most effective method to date for isolating specific genes whose activities influence the rate of aging. As these techniques are refined and applied on a greater number of genes, it seems likely that a comprehensive understanding of the genetic component of aging will be within our grasp by using the power of *Drosophila*.

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References

- Ailion M, Inoue T, Weaver CI, Holdcraft RW and Thomas JH (1999) *Proc Natl Acad Sci USA* 96: 7394–7397
- Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I and Osterman A et al. (2004) *Cell* 117: 699–711
- Apfeld J and Kenyon C (1999) *Nature* 402: 804–809
- Arking R (1987) *Exp Gerontol* 22: 199–220
- Arking R (1998) *Biology of Aging: Observations and Principles*. Sinauer Associates, Sunderland, Massachusetts
- Arking R and Wells RA (1990) *Dev Genet* 11: 141–148
- Arking R, Buck S, Berrios A, Dwyer S and Baker GTD (1991) *Dev Genet* 12: 362–370
- Bauer JH, Goupil S, Garber GB and Helfand SL (2004) *Proc Natl Acad Sci USA* 101: 12980–12985
- Beckman KB and Ames BN (1998) *Physiol Rev* 78: 547–581
- Bhole D, Allikian MJ and Tower J (2004) *Mech Ageing Dev* 125: 651–663
- Bieschke ET, Wheeler JC and Tower J (1998) *Mol Gen Genet* 258: 571–579
- Brand AH and Perrimon N (1993) *Development* 118: 401–415
- Chapman T and Partridge L (1996) *Proc R Soc Lond B Biol Sci* 263: 755–759
- Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H and Hafen E et al. (2001) *Science* 292: 104–106
- Clare MJ and Luckinbill LS (1985) *Heredity* 55(Pt 1): 19–26

- Cook-Wiens E and Grotewiel MS (2002) *Exp Gerontol* 37: 1347–1357
- Cooley L, Kelley R and Spradling A (1988) *Science* 239: 1121–1128
- Curtsinger JW and Khazaeli AA (2002) *Mech Ageing Dev* 123: 81–93
- Cvejic S, Zhu Z, Felice SJ, Berman Y and Huang XY (2004) *Nat Cell Biol* 6: 540–546
- David JR (1988) in *Drosophila* as a Model Organsim for Ageing Studies. In: Lints FA and Soliman MA (eds) Blackie, Glasgow and London, pp 33–45
- De Luca M, Roshina NV, Geiger-Thornsberry GL, Lyman RF, Pasyukova EG and Mackay TF (2003) *Nat Genet* 34: 429–433
- Duffy JB (2002) *Genesis* 34: 1–15
- Durusoy M, Diril N and Bozcuk AN (1995) *Exp Gerontol* 30: 77–86
- Force AG, Staples T, Soliman S and Arking R (1995) *Dev Genet* 17: 340–351
- Geiger-Thornsberry GL and Mackay TF (2004) *Mech Ageing Dev* 125: 179–189
- Giannakou ME, Goss M, Junger MA, Hafen E, Leivers SJ and Partridge L (2004) *Science* 305: 361
- Goddeeris MM, Cook-Wiens E, Horton WJ, Wolf H, Stoltzfus JR and Borrusch M (2003) *Aging Cell* 2: 257–264
- Griswold CM, Matthews AL, Bewley KE and Mahaffey JW (1993) *Genetics* 134: 781–788
- Guarente L and Kenyon C (2000) *Nature* 408: 255–262
- Hafen E (2004) *Curr Top Microbiol Immunol* 279: 153–167
- Harman D (1956) *J Gerontol* 11: 298–300
- Harshman LG, Moore KM, Sty MA, Magwire MM (1999) *Neurobiol Aging* 20: 521–529
- Helfand SL, Blake KJ, Rogina B, Stracks MD, Centurion A and Naprta B (1995) *Genetics* 140: 549–555
- Henderson ST and Johnson TE (2001) *Curr Biol* 11: 1975–1980
- Hwangbo DS, Gersham B, Tu MP, Palmer M and Tatar M (2004) *Nature* 429: 562–566
- Johnson TE (1990) *Science* 249: 908–912
- Kaeberlein M, McVey M and Guarente L (1999) *Genes Dev* 13: 2570–2580
- Kapahi P, Boulton ME and Kirkwood TBL (1999) *Free Radic Biol Med* 26: 495–500
- Kapahi P, Zid BM, Harper T, Koslover D, Sapin V and Benzer S (2004) *Curr Biol* 14: 885–890
- Kenyon C, Chang J, Gensch E, Rudner A and Tabtiang R (1993) *Nature* 366: 461–464
- Khazaeli AA and Curtsinger JW (2000) *Genetica* 109: 245–253
- Kim S, Benguria A, Lai CY and Jazwinski SM (1999) *Mol Biol Cell* 10: 3125–3136
- King V and Tower J (1999) *Dev Biol* 207: 107–118
- Kirby K, Hu J, Hilliker AJ and Phillips JP (2002) *Proc Natl Acad Sci USA* 99: 16162–16167
- Kurapati R, Passananti HB, Rose MR and Tower J (2000) *J Gerontol Ser A Biol Sci Med Sci* 55: B552–B559
- Landis G, Bhole D, Lu L and Tower J (2001) *Genetics* 158: 1167–1176
- Landis GN, Bhole D and Tower J (2003) *Genome Biol* 4: R8
- Landis GN, Abdueva D, Skvortsov D, Yang J, Rabin BE and Carrick J et al. (2004) *Proc Natl Acad Sci USA* 101: 7663–7668
- Larsen PL, Albert PS and Riddle DL (1995) *Genetics* 139: 1567–1583
- Leips J and Mackay TF (2000) *Genetics* 155: 1773–1788
- Leips J and Mackay TF (2002) *Exp Aging Res* 28: 361–390
- Lin K, Dorman JB, Rodan A and Kenyon C (1997) *Science* 278: 1319–1322
- Lin YJ, Seroude L and Benzer S (1998) *Science* 282: 943–946
- Lin MJ, Tang LY, Reddy MN and Shen CK (2005) *J Biol Chem* 280: 861–864
- Lints F and Hoste C (1974) *Exp Gerontol* 9: 51–69
- Lints F and Hoste C (1977) *Evolution* 31: 387–404
- Lints F, Stoll J, Gruwez G and Lints C (1979) *Gerontology* 25: 192–2004
- Luckinbill LS and Clare MJ (1985) *Heredity* 55(Pt 1): 9–18
- Luckinbill LS, Arking R, Clare MJ, Cirocco WC and Buck SA (1984) *Evolution* 38: 996–1003
- Mackay TF (2001) *Nat Rev Genet* 2: 11–20
- Mackay WJ and Bewley GC (1989) *Genetics* 122: 643–652
- Mannervik M and Levine M (1999) *Proc Natl Acad Sci USA* 96: 6797–6801
- Milligan G (1998) *Trends Endocrinol Metab* 9: 13–19
- Mockett RJ, Orr WC, Rahmandar JJ, Sohal BH and Sohal RS (2001) *Exp Gerontol* 36: 441–463
- Mockett RJ, Bayne AC, Kwong LK, Orr WC and Sohal RS (2003) *Free Radic Biol Med* 34: 207–217
- Morris JZ, Tissenbaum HA and Ruvkun G (1996) *Nature* 382: 536–539
- Morrow G, Battistini S, Zhang P and Tanguay RM (2004a) *J Biol Chem* 279: 43382–43385
- Morrow G, Samson M, Michaud S and Tanguay RM (2004b) *FASEB J* 18: 598–599
- Nghiem D, Gibbs AG, Rose MR and Bradley TJ (2000) *Exp Gerontol* 35: 957–969
- Nuzhdin SV, Pasyukova EG, Dilda CL, Zeng ZB and Mackay TF (1997) *Proc Natl Acad Sci USA* 94: 9734–9739
- Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L and Tissenbaum HA et al. G (1997) *Nature* 389: 994–999
- O’Kane CJ and Gehring WJ (1987) *Proc Natl Acad Sci USA* 84: 9123–9127
- Orr WC and Sohal RS (1992) *Arch Biochem Biophys* 297: 35–41
- Orr WC and Sohal RS (1994) *Science* 263: 1128–1130
- Osterwalder T, Yoon KS, White BH and Keshishian H (2001) *Proc Natl Acad Sci USA* 98: 12596–12601
- Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP and Boulianne GL (1998) *Nat Genet* 19: 171–174
- Parkes TL, Hilliker AJ and Phillips JP (1999) *Neurobiol Aging* 20: 531–535
- Pasyukova EG, Vieira C and Mackay TF (2000) *Genetics* 156: 1129–1146
- Pasyukova EG, Roshina NV and Mackay TF (2004) *Aging Cell* 3: 297–307
- Phillips JP, Campbell SD, Michaud D, Charbonneau M and Hilliker AJ (1989) *Proc Natl Acad Sci USA* 86: 2761–2765
- Pletcher SD, Macdonald SJ, Marguerie R, Certa U, Stearns SC and Goldstein DB et al. (2002) *Curr Biol* 12: 712–723
- Rogina B and Helfand SL (1995) *Genetics* 141: 1043–1048
- Rogina B and Helfand SL (2004) *Proc Natl Acad Sci USA* 101: 15998–16003
- Rogina B, Benzer S and Helfand SL (1997) *Proc Natl Acad Sci USA* 94: 6303–6306
- Rogina B, Vaupel JW, Partridge L and Helfand SL (1998) *Curr Biol* 8: 475–478

- Rogina B, Reenan RA, Nilsen SP and Helfand SL (2000) *Science* 290: 2137–2140
- Rogina B, Helfand SL and Frankel S (2002) *Science*. Online 298: 1745
- Roman G, Endo K, Zong L and Davis RL (2001) *Proc Natl Acad Sci USA* 98: 12602–12607
- Rose MR (1984) *Evolution* 38: 1004–1010
- Rose MR and Charlesworth B (1981) *Genetics* 97: 187–196
- Rose MR, Vu LN, Park SU and Graves JL Jr (1992) *Exp Gerontol* 27: 241–250
- Ruan H, Tang XD, Chen, M-L, Joiner, M-LA, Sun G and Brot N et al. (2002) *Proc Natl Acad Sci USA* 99: 2748–2753
- Selaya A (2000) *Caltech Undergraduate Research Journal* 1: 42–47
- Seong KH, Matsuo T, Fuyama Y and Aigaki T (2001a) *Biogerontology* 2: 271–281
- Seong KH, Ogashiwa T, Matsuo T, Fuyama Y and Aigaki T (2001b) *Biogerontology* 2: 209–217
- Seroude L (2002) *Genesis* 34: 34–38
- Seroude L, Brummel T, Kapahi P and Benzer S (2002) *Aging Cell* 1: 47–56
- Service P, Hutchinson E, MacKinley M and Rose M (1985) *Physiol Zool* 58: 380–389
- Service PM, Hutchinson EW and Rose MR (1988) *Evolution* 42: 708–716
- Snustad DP and Simmons MJ (2000) *Principles of Genetics*. John Wiley and Sons Inc., New York
- Song W, Ranjan R, Dawson-Scully K, Bronk P, Marin L and Seroude L et al. (2002) *Neuron* 36: 105–119
- St Johnston D (2002) *Nat Rev Genet* 3: 176–188
- Sun J and Tower J (1999) *Mol Cell Biol* 19: 216–228
- Tatar M, Khazaeli AA and Curtsinger JW (1997) *Nature* 30
- Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM and Garofalo RS (2001) *Science* 292: 107–110
- Tissenbaum HA and Guarente L (2001) *Nature* 410: 227–230
- Toba G, Ohsako T, Miyata N, Ohtsuka T, Seong KH and Aigaki T (1999) *Genetics* 151: 725–737
- Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L and Muller F (2003) *Nature* 426: 620
- Vettraino J, Buck S and Arking R (2001) *J Gerontol A Biol Sci Med Sci* 56: B415–B425
- Vieira C, Pasyukova EG, Zeng ZB, Hackett JB, Lyman RF and Mackay TF (2000) *Genetics* 154: 213–127
- Walker DW and Benzer S (2004) *Proc Natl Acad Sci USA* 101: 10290–10295
- Wang MC, Bohmann D and Jasper H (2003) *Dev Cell* 5: 811–816
- Wang HD, Kazemi-Esfarjani P and Benzer S (2004) *Proc Natl Acad Sci USA* 101: 12610–12615
- Watson SP and Arkinstall S (1994) *The G-protein linked receptor factsbook*. Academic Press, London; San Diego
- Wattiaux J (1968a) *Evolution* 22: 406–421
- Wattiaux J (1968b) *Experimental Gerontology* 3: 55–61
- Wayne ML and McIntyre LM (2002) *Proc Natl Acad Sci USA* 99: 14903–14906
- Wheeler JC, Bieschke ET and Tower J (1995) *Proc Natl Acad Sci USA* 92: 10408–10412
- Zou S, Meadows S, Sharp L, Jan LY and Jan YN (2000) *Proc Natl Acad Sci USA* 97: 13726–13731
- Zwaan B, Bijlsma R and Hoekstra RE (1995) *Evolution* 49: 649–659