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Lack of Robustness of Life Extension Associated With Several Single-Gene *P* Element Mutations in *Drosophila melanogaster*

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The hypothesis tested in this study was that single-gene mutations found previously to extend the life span of *Drosophila melanogaster* could do so consistently in both long-lived *y w* and standard w^{1118} genetic backgrounds. GAL4 drivers were used to express upstream activation sequence (UAS)-responder transgenes globally or in the nervous system. Transgenes associated with oxidative damage prevention (*UAS-hSOD1* and *UAS-GCLc*) or removal (*EP-UAS-Atg8a* and *UAS-dTOR*^{FRB}) failed to increase mean life spans in any expression pattern in either genetic background. Flies containing a *UAS-EGFP-bMSRA*^{C} transgene associated with protein repair were found not to exhibit life extension or detectable enhanced green fluorescent protein (EGFP) activity. The presence of UAS-responder transgenes was confirmed by PCR amplification and sequencing at the 5′ and 3′ end of each insertion. These results cast doubt on the robustness of life extension in flies carrying single-gene mutations and suggest that the effects of all such mutations should be tested independently in multiple genetic backgrounds and laboratory environments.

Key Words: Drosophila—Transgenes—Longevity—Oxidation.

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OVER the past 20 years, a very large number of single-gene mutations have been reported to extend the life span of the fruit fly, *Drosophila melanogaster* (1,2). In many cases, life extension was observed after one or more P element insertions were used to increase or decrease the expression of an endogenous gene or to express a heterologous gene. Aging is therefore described by numerous investigators as a regulated process, controlled by genetic pathways (3–6). The strength of this inference depends on the reliability of the supporting evidence. Aside from trade-offs between beneficial effects on longevity and detrimental effects on fertility or other indicators of fitness, which are discussed extensively elsewhere (6,7), at least two potential problems must be considered.

First, it has become increasingly apparent that the effect of a genetic modification on longevity is often contingent on the strain and sex of the flies (1), the developmental stage and spatial pattern in which gene expression is altered (8), the magnitude of the change in gene expression (9), food concentration (10), temperature (11,12), the presence or absence of the endosymbiotic bacterium, *Wolbachia* (13), and especially on the use of appropriate control strains (14). Altering any one of these variables can diminish or eliminate the beneficial effect of increased or decreased gene expression or even shorten the life span. Where it occurs, this lack of robustness of life extension across genetic backgrounds or environmental conditions constrains the general conclusion that a gene governs the rate of aging or longevity in flies and may consequently be predicted to do so in organisms from other phyla.

A second consideration is the reference value to which extended life spans are compared. The life span of *D. melanogaster* is strongly dependent on the ambient temperature (15), among many other variables, and most studies of longevity are conducted at 25°C. Even at this constant temperature, the average life spans of unmodified, control populations vary widely, from less than 30 days to more than 70 days (8,16,17). Prior to the earliest reports of life extension associated with altered expression of one or two genes, several groups of investigators had reported average life spans ≥60 days for untreated flies (18–20). In many cases, the extended life spans of mutant flies have been observed in relation to much shorter-lived control populations, and their prolonged life spans have not surpassed the 60–70 day range (4,8,9,21–27). Given that aging limits the life spans of the longest-lived animals of a species, it is reasonable to question whether extension of relatively short life spans represents a delay of the aging process or an increase in resistance to some form of life-shortening challenge.

A number of single-gene mutations reported to extend the life span of *D. melanogaster* would be predicted to prevent molecular oxidative damage or to repair or remove

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the byproducts. The *GAL4/UAS* expression system (28) has been used frequently to achieve tissue-specific expression of the relevant transgenes. For instance, *UAS-hSOD1*, which encodes the human antioxidative enzyme, cytosolic superoxide dismutase, was expressed in flies using the neuronal *D42-GAL4* driver (24). The mean life span was initially found to be increased by up to 41% (from 45.1 to 63.7 days), although subsequent studies of the same transgenes in longer-lived backgrounds demonstrated smaller beneficial effects or in some cases no effect (29). *GCLc*, which encodes the catalytic subunit of glutamate-cysteine ligase, the rate-limiting enzyme in the biosynthesis of glutathione, was overexpressed using three different neuronal *GAL4* drivers (*Appl-GAL4*, *D42-GAL4*, and *elav-GAL4*) and a *UAS-GCLc* responder transgene (30). The mean life span was increased by up to 49% (from 55.9 to 83.5 days). Expression of *UAS-EGFP-bMSRA*^{C}, which encodes the bovine form of the protein repair enzyme, methionine sulfoxide reductase A, was driven by *elav-GAL4* and found to increase the median life span by more than 70%, from 42–48 days up to 77 days in males and from 55–61 days to a remarkable 95 days in females (31). *da-GAL4* was used to drive ubiquitous expression of *UAS-dTOR*^{FRB}, which encodes a dominant negative form of the target of rapamycin and is therefore believed to promote autophagy (32). The mean life span was increased by up to 31% (from 55–58 to 72 days). Finally, *Appl-GAL4*-driven expression of *EP-UAS-Atg8a*, which encodes a rate-limiting enzyme in the formation of autophagosomes, extended the mean life span of female flies up to 56% (from 44.6 to 69.5 days [25]).

The first objective of this study was to verify that the aforementioned transgenes could extend the life span in a background (w^{1118}) that has a life span typical of many standard laboratory strains and has served as the control background for several studies of longevity in mutant flies (5,25,27,32). A second objective was to test the same transgenes in a longer-lived background (*y w*), to assess whether their life-prolonging effect would show an inverse relationship to the control life span or whether they could extend the life span of flies beyond the range previously observed in *D. melanogaster*.

Materials and Methods

Fly Strains

The *y w* and w^{1118} genotypes have been used and described in previous studies in this laboratory and elsewhere (33). *Appl-GAL4*, *D42-GAL4*, *elav-GAL4*, *arm-GAL4*, and *da-GAL4* drivers and *UAS-GCLc* responder lines 3 and 6 were obtained from the laboratory of Dr. Bill Orr (Southern Methodist University, Dallas, TX). *UAS-hSOD1* flies were also provided by Dr. Bill Orr, who received them from Dr. Gabrielle Boulianne (University of Toronto, ON). *UAS-EGFP-bMSRA*^{C} flies were a gift from the laboratory of Dr. Toshinori Hoshi (University of Pennsylvania, Philadelphia, PA), *UAS-dTOR*^{FRB} lines 2 (II) and 3 (III) were generously provided by Dr. Thomas Neufeld (University of Minnesota, Minneapolis, MN), and the w^{1118}; *EP-UAS-Atg8a* stock (*EP(1)362*) was obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). Stocks w^{*}; *UAS-GFP.S65T* and w^{1118}; *UAS-lacZ* were also obtained from the Bloomington Stock Center. All stocks were passaged through media containing 0.25 mg/mL tetracycline to minimize the likelihood of perturbations of life span by the bacterial symbiont, *Wolbachia* (although *Wolbachia* was not known to infect any of these stocks). All of the driver and responder lines were then backcrossed simultaneously for 10 generations to both the *y w* and w^{1118} backgrounds, which have been shown not to contain *Wolbachia* (33).

DNA Sequence Analysis

Genomic DNA was purified from 50 flies per genotype in each background (*y w* and w^{1118}). The protocol included an obligatory extraction with phenol:chloroform:isoamyl alcohol (25:24:1) to obtain DNA of sufficient purity. Primers for PCR amplification and DNA sequencing (Supplementary Table 1) were designed using Oligo 6.8 software and purchased from Sigma-Aldrich. DNA sequencing was performed by Eurofins MWG Operon (Huntsville, AL). Sequences were obtained from the National Center for Biotechnology Information or FlyBase (34) and analyzed using web-based programs provided by the European Bioinformatics Institute (EMBL-EBI: ClustalW2), Baylor College of Medicine, and the Sequence Manipulation Suite (written by P. Stothard).

β-Galactosidase

Adult flies, 4 days posteclosion (two per sex per genotype in each background), were flash frozen in liquid nitrogen, embedded in TissueTek O.C.T. (Fisher Scientific), and sectioned using a Leica CM1850 cryostat. Sections were fixed in 2% glutaraldehyde for 20 minutes, washed repeatedly with phosphate-buffered saline (PBS, pH 7.4), air-dried, and then exposed to 0.3% X-gal for 1.5–14 hours prior to image acquisition using a Nikon Eclipse 80i microscope.

Green Fluorescent Protein

Brains were dissected from adult flies 12–15 days posteclosion and mounted in 70% glycerol in PBS. Images were obtained using the FITC filter set on a Nikon Eclipse 80i fluorescence microscope (λ_{ex} = 465–495 nm, λ_{em} = 515–555 nm). A QImaging QICAM digital camera was used to acquire monochrome images, which were then pseudocolored.

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Life Span

Flies were reared and maintained on a medium containing 6.93% (w/v) cornmeal, 1.95% torula yeast, 1.63% sugar, and 0.655% agar, with 1.875 g/L methylparaben, 0.3% propionic acid, and 0.03% phosphoric acid (85%) to inhibit the proliferation of mould or bacteria. Adult flies were collected under brief carbon dioxide anesthesia. Males and virgin females of each genotype were collected separately and placed in four groups of 25 flies per vial. The flies were maintained at 25°C on a 12h:12h light:dark cycle at 40%–70% humidity. Fresh vials were provided and mortality was recorded every 2 days at younger ages (up to ~55 ± 10 days) and daily at older ages. The temperature was monitored daily during the light cycle and periodically during the dark cycle, using three thermometers of different makes in close proximity to the fly vials, to ensure that the life span was not prolonged in comparison with other studies due to low temperature.

Statistical Analysis

SYSTAT 12 software was used to compare distributions of survivorship and differences in mean life span among groups. For comparisons of survivorship distributions, Kaplan-Meier probability tables were constructed for each *GAL4/UAS* genotype and the corresponding *GAL4*/+ and +/*UAS* control groups. Log-rank tests were performed by the method of Mantel. *GAL4/UAS* survivorship curves were also compared with each control group separately. For comparisons of mean life spans, the mean age at death was calculated for flies in each vial. The four estimates of mean life span for each *GAL4/UAS* genotype were compared with those of the corresponding *GAL4/+* and *+/UAS* control groups of the same sex and genetic background, based on one-way analysis of variance (ANOVA). Kolmogorov-Smirnov tests were used to confirm the modeling assumption of normally distributed data, and Levene's test was used to confirm the assumption of equal variances. A significant difference was reported whenever ANOVA and post hoc pairwise comparisons (Tukey's Honestly Significant Difference test) showed an increase or a decrease in life span in comparison with both control groups. The significance threshold for all tests was $p < 0.05$.

Results

Sequence Analysis

Following backcrossing of each transgene into both *y w* and w^{1118} backgrounds for 10 generations, the insertions were partially or wholly sequenced, to verify the presence and molecular identity of each insertion. Results for every transgene were identical in both backgrounds, showing that any divergence from published results occurred prior to backcrossing.

Insertion of *Homo sapiens* soluble *superoxide dismutase 1* (*SOD1*) cDNA in *UAS-hSOD1* flies was verified by sequencing. The insertion consisted of 52 bp 5′ untranslated sequence, 465 bp coding sequence, and 307 bp 3′ untranslated sequence, cloned between the *Bgl* II and *Kpn* I sites of p*P*{*UAST*}. A K75R missense mutation identified previously in the ancestral stock (35) was confirmed in DNA isolated from transgenic flies in both the *y w* and w^{1118} backgrounds. This mutation is not among the 169 point mutations currently known to be associated with human familial amyotrophic lateral sclerosis (http://alsod.iop.kcl.ac.uk/Als/mutations/mutationsFoundGeneOnly.aspx?gene_id=SOD1, March 1, 2013).

Genomic DNA isolated from flies containing the *UAS-GCLc* insertion (lines 3 and 6) was shown to contain p*P*{*UAST*} sequences flanking both the 5′ and 3′ ends of *GCLc* transcription variant B cDNA, consistent with its construction from clone AT05811, as previously described (30). The insertion in clone AT05811 is distinguished from the reference sequence by a silent mutation in G106, which was verified in all four fly lineages. Otherwise, the inserted DNA and reference sequence were 100% identical at the 5′ and 3′ ends of the coding sequence. The 3′ untranslated region differed from the reference sequence at four loci: three single nucleotide substitutions and one insertion of four nucleotides 337 nt downstream from the stop codon.

Sequence analysis showed that the *bMSRA* component of the *UAS-EGFP-bMSRA*^{C} insertion in fly genomic DNA was identical to the *Bos taurus* MSRA mRNA (GenBank accession number: BC102980.1) and genome sequence (accession number: NW_003104074.1), except for an A4V substitution that is also found in the *Bos taurus* peptide MSRA mRNA (accession number: U37150.1). The latter sequence (accession number: U37150.1) contains four silent mutations that are not present in *UAS-EGFP-bMSRA*^{C}, BC102980.1, or the genome sequence. Therefore, the sequence in *UAS-EGFP-bMSRA*^{C} is a hybrid of the published sequences for *bMSRA*. This sequence was fused in-frame with the 3′ end of *EGFP*, with only the stop codon of *EGFP* and initiator Met codon of *bMSRA* deleted. The 5′ end of *EGFP* was also intact. The *EGFP-bMSRA* fusion, flanked by 12 bp of bMSRA 3′ untranslated sequence, and polylinker sequences (18 bp 5′ and 88 bp 3′) from another vector, was located at the *Xba* I site of p*P*{*UAST*}. All of the sequence data are fully consistent with the description given previously (31), showing that the insertion was not mutated or lost in the intervening years.

UAS-dTOR$^{FRB}_{II}$ (line "TOR2") and *UAS-dTOR*$^{FRB}_{III}$ (line "TOR3") flies contained 100% identical sequences that were almost as described earlier (36). In each case, the fly DNA contained p*P*{*UAST*} sequence with a 279 bp insertion between *Kpn* I and *Xba* I sites. The insertion encoded dTOR amino acids 1937-2026, flanked by start and stop codons and 3 bp 5′ untranslated sequence. This sequence corresponds closely with amino acids originally identified as dTOR^{FRB} but with primers listed for dTOR^{TED} (36).

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Flies carrying the *EP-UAS-Atg8a* transgene were confirmed to have a *P*{*EP*} insertion 146 bp downstream from the *Atg8a* transcription start site, as described (25).

Gene Expression

The expression of each *GAL4* driver was confirmed in both *y w* and w^{1118} backgrounds by crosses to a stock containing a *UAS-lacZ* responder transgene (Figure 1). *Appl-GAL4*, *D42-GAL4*, and *elav-GAL4* were expressed in the central nervous system, *arm-GAL4* was expressed in various tissues, mainly in the head and thorax (Figure 1D and results not shown), and expression of *da-GAL4* was widespread. β-Galactosidase activity was not detected in flies carrying the *UAS-lacZ* responder transgene in the absence of a driver or flies carrying any driver in the absence of the responder.

Expression of EGFP was assessed by fluorescence microscopy in flies carrying the *elav-GAL4* driver and *UAS-EGFP-bMSRA*^{C} responder transgenes (Figure 2). *elav-GAL4* drove strong expression of a *UAS-GFP* positive control responder in isolated fly brains, which was consistent with the pattern of expression of the *UAS-lacZ* responder. A lower level of GFP activity was detected in a specific subset of cells in flies carrying the control *UAS-GFP* transgene in the absence of a driver, but the contrast between *elav-GAL4*-driven and "leaky" transgene expression was clear-cut in most of the brain. Brains from flies carrying the *elav-GAL4* driver and *UAS-EGFP-bMSRA*^{C} responder transgenes were indistinguishable from controls carrying either transgene alone or no transgene. In each of these cases, only faint autofluorescence was detected at long exposure times.

Expression of the *UAS-hSOD1*, *UAS-GCLc*, and *EP-UAS-Atg8a* transgenes has been demonstrated previously in other laboratories, using qRT-PCR to confirm transcription, Western blots to confirm translation, and/or functional assays of enzyme activity. In each case, the flies in this study were shown to carry the same transgene sequences that generated functional products in the earlier studies. However, expression of *UAS-dTOR*^{FRB} has not been demonstrated directly in *D. melanogaster*.

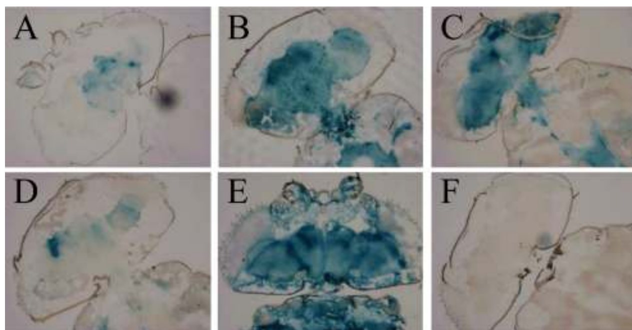

Figure 1. *GAL4* driver expression in the head and anterior thorax. Sections were obtained from progeny of flies containing a *UAS-lacZ* reporter gene crossed with each *GAL4* driver in the w^{1118} background. **(A)** *Appl-GAL4*, **(B)** *D42-GAL4*, **(C)** *elav-GAL4*, **(D)** *arm-GAL4*, **(E)** *da-GAL4* drivers, and **(F)** w^{1118} X *UAS-lacZ* (negative control). β-galactosidase staining was observed in the nervous system **(A–C)**, at trace levels **(D)**, or in a widespread distribution of tissues, particularly skeletal muscle **(E)**. No staining was detected in negative control flies obtained from crosses between w^{1118} and either *GAL4* driver (not shown) or *UAS-lacZ* reporter parents **(F)**. Similar images were obtained from sections of flies from crosses in the *y w* background.

Life Span—Experiment 1

An initial experiment was conducted to compare the life spans of a subset of flies derived from crosses between driver-*GAL4* females and *UAS*-responder male parents. Log-rank tests demonstrated differences in the distribution of survivorship among *GAL4/UAS* and control groups for 9/12 genotypes of male flies and 3/6 genotypes of females. Paired comparisons between *GAL4/UAS* and individual control groups showed significant differences in 17/36 cases. *GAL4/UAS* survivorship curves differed significantly from those of both control groups ($p < .05$) for 4/12 genotypes of male flies and 1/6 genotypes of females (Table 1; Figure 3). Among the five genotypes exhibiting significant differences from both control groups, two had mean or median life spans that were shorter than those of one control group and longer than the other control group.

ANOVA was performed to test the hypothesis that *GAL4/UAS* genotypes had significantly longer or shorter mean life spans than the control groups. In both backgrounds, *Appl-GAL4/UAS-GCLc* (line 6) male progeny were 10%–14% longer-lived than either *Appl-GAL4/+* or *+/UAS-GCLc(6)* control flies (ANOVA, $p < .01$; pairwise comparisons, $p < .03$; Table 1; Figure 3). Based on ANOVA, male flies expressing *UAS-GCLc(6)* driven by *D42-GAL4* or *elav-GAL4* or *UAS-EGFP-bMSRA*^{C} driven by *Appl-GAL4*, *D42-GAL4,* or *elav-GAL4* exhibited no consistent, significant difference in mean life span relative to both control groups in either background. Female flies expressing *EP-UAS-Atg8a* driven by *Appl-GAL4*, *D42-GAL4*, or *elav-GAL4* also exhibited no significant difference in mean life span relative to controls in either background.

The validity of conclusions based on ANOVA depends on the assumptions of a normal distribution within each set of data and equal variances among the groups that are compared. For 19/44 raw data sets, Kolmogorov-Smirnov tests demonstrated no departure of the life spans of individual flies from a normal distribution (Supplementary Table 2), consistent with the minimal early mortality and highly rectangular survivorship curves observed throughout this study (Figure 3). As would be predicted based on the central limit theorem (37), the mean life spans of vials of 25 flies were normally distributed in almost all cases (15/16 comparisons of groups containing at least three vials of flies). Likewise, in 15/16 cases, Levene tests confirmed the assumption of equality of variances among the groups that were compared. In the remaining case, for both Kolmogorov-Smirnov and

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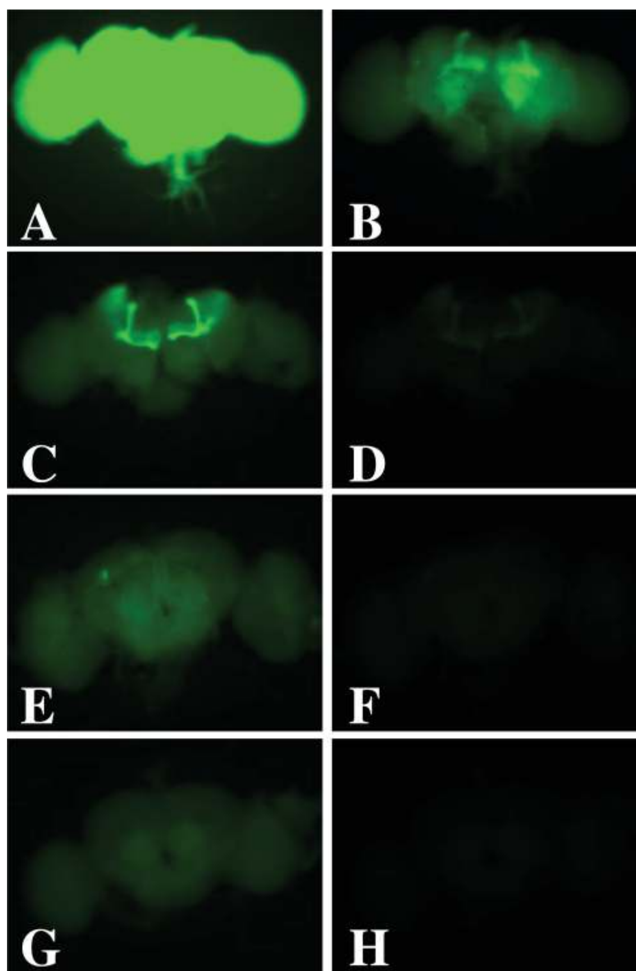

Figure 2. Green fluorescent protein. Brains were isolated from 13-day-old female flies in the w^{1118} background. **(A,B)** w^{1118} *elav-GAL4*; *UAS-GFP.S65T* (positive control). **(C,D)** w^{1118}; *UAS-GFP.S65T*. **(E,F)** w^{1118} *elav-GAL4*; *UAS-EGFP-bMSRA*^{C}. **(G,H)** w^{1118}; *UAS-EGFP-bMSRA*^{C} (negative control). Exposure times were 2 s (**A, C, E, G**) or 200 ms (**B, D, F, H**). Images were obtained using Nikon NIS Elements software. Similar images were obtained from flies of both sexes in both backgrounds.

Levene tests, exclusion of data for two flies (out of ~300 in the comparison) as outliers satisfied the modeling assumptions without affecting the decision to accept or reject the null hypothesis of no difference in mean life spans.

Life Span—Experiment 2

Subsequently, a single experiment was conducted in which the life spans of all driver-*GAL4* X *UAS*-responder combinations were measured concurrently, except those involving the *UAS-EGFP-bMSRA*^{C} transgene and *EP-UAS-Atg8a* males, which would be hemizygous (Figure 4; Table 2). Log-rank tests demonstrated differences between *GAL4*/*UAS* and both *GAL4*/+ and +/*UAS* controls for 6/30 female and 5/25 male groups in the *y w* background and 7/30 female and 6/25 male groups in the w^{1118} background (Table 2). When each *GAL4*/*UAS* and control group were compared separately, survivorship curves differed in 88 /220 comparisons ($p < .05$), including six for which median life spans differed by only 0–1 days; 49/88 of these differences were highly significant ($p < .0005$). Among the 24 groups exhibiting significant differences from both control groups, nine had mean or median life spans that were intermediate between the two controls.

For parametric testing, a total of 100/156 raw data sets for individual flies showed no significant departure from a normal distribution ($p \geq 0.05$ in the Kolmogorov-Smirnov test; Supplementary Table 2). Cumulatively, in Experiments 1 and 2, a total of 119/200 (60%) of the data sets for individual flies did not depart significantly from a normal distribution ($p \geq 0.05$); the proportion rose to 159/200 (80%) at a significance threshold of $p \leq .01$, 182/200 (91%) at $p \leq .001$, and 190/200 (95%) at $p \leq .0005$. Using the mean life spans of vials of 25 flies as input data, the modeling assumptions of normal distribution and equal variances were confirmed in 105/110 and 97/110 comparisons in Experiment 2, respectively. In all but one of the remaining comparisons, removing one to three flies (≤1% of the

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Table 1. Mean and Median Life Spans of Flies Containing *bMSRA*^{C}, *GCLc*(6), or *Atg8a* Transgenes*

Genotype	*y w* ♀	*y w* ♂	w^{1118} ♀	w^{1118} ♂
+/+	85.0 ± 1.1 (86)	76.9 ± 0.7 (79)	78.1 ± 2.8 (83)	66.0 ± 3.2 (68)
Appl-GAL4/+	86.6 ± 2.4 (88)	73.2 ± 2.4 (78)	80.8 ± 4.3 (83)	65.9 ± 1.8 (67)
D42-GAL4/+	84.7 ± 2.4 (88)	76.8 ± 3.2 (81)	76.9 ± 3.6 (77)	66.2 ± 4.5 (67)
elav-GAL4/+	80.6 ± 2.6 (84)	65.2 ± 0.8 (65)	82.3 ± 2.0 (85)	63.8 ± 7.5 (63)[†]
+/UAS-bMSRA^{C}		79.2 ± 2.3 (82)		70.5 ± 2.0 (73)
Appl-GAL4/UAS-bMSRA^{C}		75.0 ± 2.8 (76)		70.7 ± 3.1 (75)
D42-GAL4/UAS-bMSRA^{C}		80.0 ± 1.5 (83)		70.0 ± 2.9 (71)
elav-GAL4/UAS-bMSRA^{C}		69.6 ± 3.9 (71)		64.7 ± 1.8 (65)[§]
+/UAS-GCLc(6)		70.7 ± 2.1 (71)		64.2 ± 2.5 (68)
Appl-GAL4/UAS-GCLc(6)		80.7 ± 2.2 (81)		73.1 ± 4.5 (73)
D42-GAL4/UAS-GCLc(6)		73.9 ± 3.1 (75)		67.7 ± 1.7 (69)
elav-GAL4/UAS-GCLc(6)		63.7 ± 1.2 (68)[‡]		59.0 ± 5.8 (58)[§]
+/EP-UAS-Atg8a	84.6 ± 1.5 (86)		81.6 ± 4.2 (85)	
Appl-GAL4/EP-UAS-Atg8a	86.4 ± 2.5 (88)		79.8 ± 5.2 (81)	
D42-GAL4/EP-UAS-Atg8a	85.2 ± 3.0 (87)		76.9 ± 2.9 (81)	
elav-GAL4/EP-UAS-Atg8a	81.1 ± 1.6 (82)		81.2 ± 2.0 (84)	

Notes: Results are Mean ± *SD*, with medians in parentheses.

*Solid underlining designates genotypes that differed significantly from both controls in their survival distributions, based on log-rank tests, but not in their mean life spans based on analysis of variance (ANOVA). Dashed underlining designates genotypes that differed significantly from both controls in their survival distributions (log-rank) and in their mean life spans (ANOVA).

[†,‡,§] n = 2, 3, 5 vials, respectively. For all other groups, n = 4 vials of 25 flies.

raw data) as outliers resulted in data sets that conformed to the modeling assumptions. In the last case, removal of one vial (among 799 vials in the entire study) yielded normally distributed data with equal variances. In no case did the removal of outliers change the decision to accept or reject the null hypothesis that there was no difference between *GAL4*/*UAS* and both control groups.

ANOVA followed by pairwise comparisons demonstrated differences in mean life spans between *GAL4*/*UAS* and both *GAL4*/+ and +/*UAS* controls for 3/30 female and 1/25 male groups in the *y w* background and 1/30 female and 4/25 male groups in the w^{1118} background (Table 2). Among the nine groups that differed from both controls, only one had a mean life span that was intermediate between the two control groups.

The average life spans of ancestral control flies carrying no transgenes ranged from 64.3 days in w^{1118} male flies to 85.9 days in *y w* female flies. In no case was the mean life span of any transgenic group more than 10% longer than the ancestral control line of the same sex and background, except that *D42-GAL4*/*UAS-dTOR*^{FRB} and *D42-GAL4*/*UAS-hSOD1* males exhibited 11%–12% life extension in the w^{1118} background. The +/*UAS-dTOR*^{FRB} and +/*UAS-hSOD1* control males also lived 4%–10% longer than the ancestral controls in this background.

Significant increases or decreases in life span relative to both driver-*GAL4* and *UAS*-responder groups were not observed among *EP-UAS-Atg8a* females or *UAS-hSOD1* flies of either sex with any driver in either genetic background. *UAS-GCLc* (line 6) was associated with a significantly increased mean life span (9%) driven by *Appl-GAL4* in male but not female flies in both backgrounds. Flies containing the *UAS-GCLc* transgene at a different locus (line 3) showed no change in life span driven by *Appl-GAL4* in either sex or background. Flies containing the *UAS-GCLc* transgene (line 3 or 6) exhibited a slight (3%–10%) decrease in life span driven by *da-GAL4* in both sexes and backgrounds, but it was significant only in *y w* females. Line 3 (but not 6) also exhibited a significant 21% decrease in life span when driven by *elav-GAL4* in w^{1118} males but not in females or *y w* flies of either sex. *UAS-dTOR*^{FRB} expression was associated with significant life extension only in (i) line 2 (but not line 3) driven by *D42-GAL4* in w^{1118} males (12%) and *Appl-GAL4* in w^{1118} females (10%) and (ii) line 3 (but not line 2) driven by *elav-GAL4* in *y w* females (6%). No *GAL4* driver combined with *UAS-dTOR*^{FRB} led to life extension in both lines carrying the same insertion or in both sexes or in both backgrounds.

Male flies carrying the *elav-GAL4* driver in the *y w* background were consistently shorter-lived than the *UAS*-responder controls, and *elav-GAL4*/*UAS*-responder life spans closely matched those of the driver but not those of the responder control groups. This effect was sex- and background specific. During backcrossing to *y w*, the *elav-GAL4* driver, which was initially in a y^{+} background, was completely refractory to recombination between the linked *y* and *elav-GAL4* alleles, whereas recombination occurred readily between *y* and *Appl-GAL4* (which is adjacent to *elav-GAL4*) as *Appl-GAL4* was crossed from a *y w* into a w^{1118} background. The *y* marker was also eliminated in backcrosses of all of the other driver and responder alleles into the w^{1118} background. Therefore, backcrossing for 10 generations rendered the backgrounds largely but not completely isogenic. Inclusion of *elav-GAL4* along with

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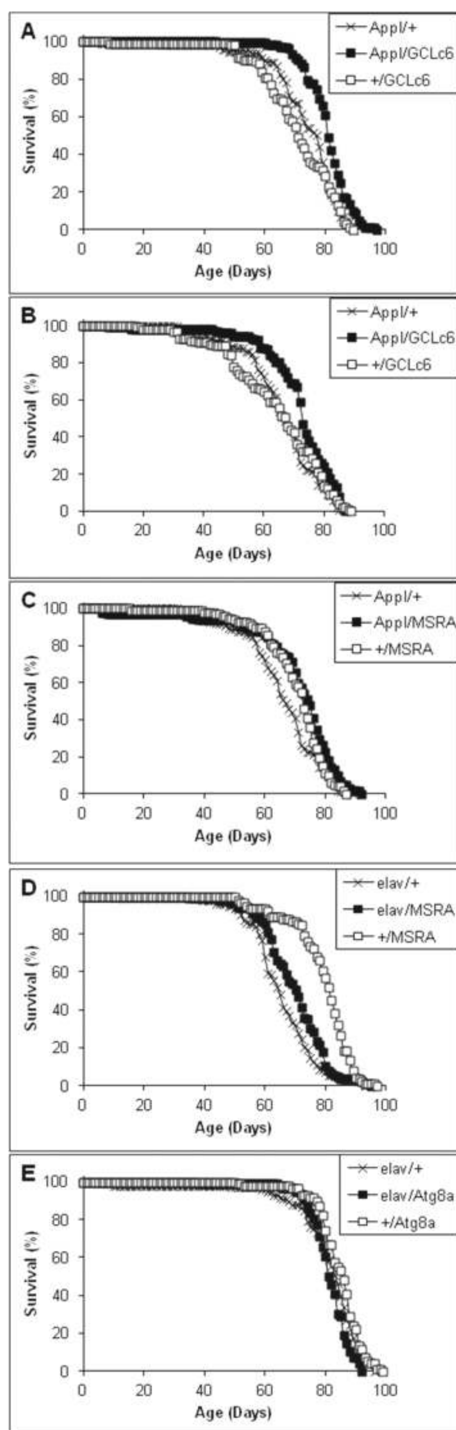

Figure 3. Survivorship curves. Results are shown for *GAL4/UAS* genotypes exhibiting significant differences in mortality distributions relative to both *GAL4/+* and *+/UAS* control groups, based on log-rank tests (Experiment 1). **(A)** *Appl-GAL4/UAS-GCLc(6)* (*y w* background, males), **(B)** *Appl-GAL4/UAS-GCLc(6)* (w^{1118} background, males), **(C)** *Appl-GAL4/UAS-bMSRA*^{C} (w^{1118} background, males), **(D)** *elav-GAL4/UAS-bMSRA*^{C} (*y w* background, males), and **(E)** *elav-GAL4/EP-UAS-Atg8a* (*y w* background, females). Based on analysis of variance (ANOVA), mean life spans were extended only for groups shown in panels **A** and **B**. Based on the log-rank test, the mortality distribution of *elav-GAL4/UAS-bMSRA*^{C} flies differed significantly from both controls (panel **D**), even though the *UAS-bMSRA*^{C} transgene was not expressed based on the EGFP assay.

UAS-responder controls demonstrated that the shorter life spans of *elav-GAL4/UAS*-responder flies in relation to ancestral and *+/UAS*-responder controls were due to genetic background variation, rather than an adverse effect of *elav-GAL4*-driven expression of these responder transgenes.

Discussion

It has become widely accepted that mutations affecting the expression of one or a few genes can extend the life span of the fruit fly, *D. melanogaster*. The principal result of this study was that tissue-specific expression of numerous transgenes using the *GAL4/UAS* activation system, which had been reported previously to extend the life span of *D. melanogaster*, generally failed to cause any significant change in longevity of flies of either sex, in two different genetic backgrounds. Some small differences in life span were observed, but none of them were reproducible in flies of the same sex and background containing the same *UAS*-responder insertion at a different locus or in flies of the opposite sex. A minor positive effect was observed in both backgrounds only for *Appl-GAL4/UAS-GCLc(6)* in two independent cohorts of male flies. Additionally, the hypothesis that life extension would be greater in a normal-lived than a long-lived genetic background under constant environmental conditions was not borne out. Instead, untransformed flies of both sexes in both backgrounds had mean life spans of more than 60 days, and the difference in longevity between these backgrounds was much smaller in female than in male flies.

Likely reasons for the general absence of life extension differ among the transgenes and may be considered for each transgene individually. A point in common for all of the transgenes is that the observation of life extension in some studies and not others might be explained either by differences in genetic background or environment (eg, the composition of the food provided to the flies). In either case, the lack of robustness of life extension under different conditions within a single species should constrain speculation that the same genes control the aging process in phylogenetically and physiologically remote species, including mammalian species.

Sod1 (encoding Cu-Zn SOD) is the archetypal example of a gene which, when overexpressed, is frequently associated with substantial life extension in short-lived flies but has either a much smaller effect or no effect in long-lived flies. Initially, two independent groups found that overexpression of the native *Drosophila dSod1* gene led to essentially no life extension in genetic backgrounds and environments where the mean life span of control flies was 61–63 days (19,20). Concurrently, a third group showed that expression of bovine *Sod1* in flies raised the mean life span of 4 out of 5 lineages from a lower baseline (44–46 days) up to 50–53 days (9). Later, to avoid adverse developmental effects of *Sod1* overexpression, FLP recombinase was

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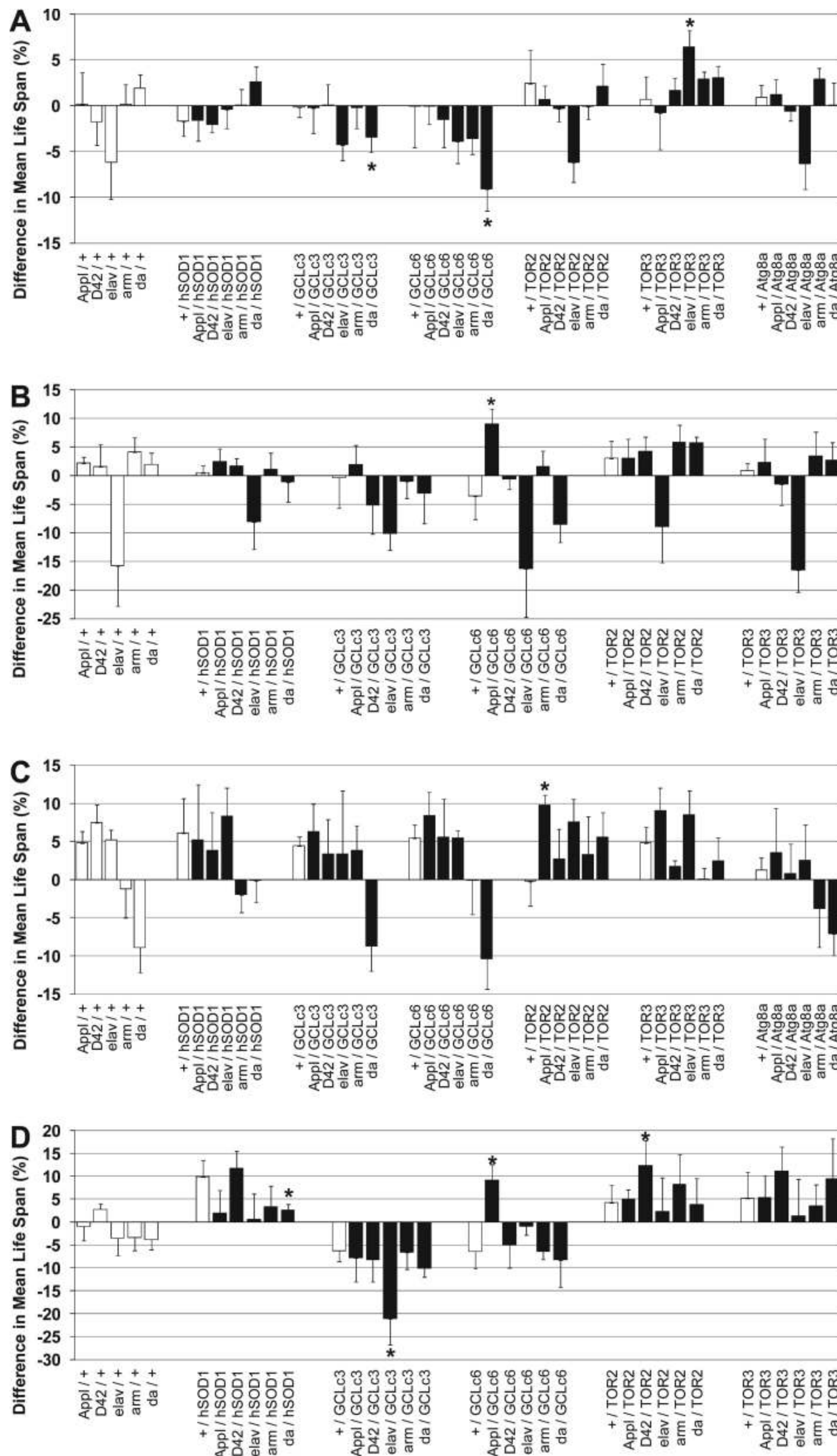

Figure 4 . Effects of *Atg8a*, *GCLc*, *dTOR*^{FRB}, and *hSOD1* transgene expression on mean life span. Results are expressed as percent life extension (Mean ± *SD*) in comparison with ancestral control flies (+/+) of the same sex and background. Open bars: control groups (driver = *GAL4*/+; responder = +/*UAS*). Filled bars: experimental groups (*GAL4/UAS*). **(A)** *y w* background, female flies. Control life span (+/+) = 85.9 ± 2.4 days. **(B)** *y w* background, male flies. Control life span (+/+) = 77.8 ± 5.2 days. **(C)** w^{1118} background, female flies. Control life span (+/+) = 80.8 ± 4.2 days. **(D)** w^{1118} background, male flies. Control life span (+/+) = 64.3 ± 3.1 days. $*p < .05$ in comparisons with both driver and responder control groups. Numeric mean and median life span data for all groups are provided in Table 2.

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Table 2. Mean and Median Life Spans of Flies Containing *hSOD1*, *GCLc*, *TOR*^{FRB}, or *Atg8a* transgenes*

Genotype	*y w* ♀	*y w* ♂	w^{1118} ♀	w^{1118} ♂
+/+	85.9 ± 2.4 (87)	77.8 ± 5.2 (81)	80.8 ± 4.2 (84)	64.3 ± 3.1 (66)
Appl-GAL4/+	86.0 ± 3.0 (87)	79.5 ± 0.8 (81)	84.7 ± 1.2 (88)	63.7 ± 2.0 (65)
D42-GAL4/+	84.4 ± 2.2 (85)	79.0 ± 3.1 (82)	86.8 ± 1.9 (89)	66.1 ± 0.8 (68)
elav-GAL4/+	80.6 ± 3.4 (81)	65.5 ± 5.4 (65)	85.0 ± 1.1 (86)	62.0 ± 2.4 (61)
arm-GAL4/+	86.0 ± 1.8 (88)	81.1 ± 2.0 (82)	79.8 ± 3.1 (82)	62.1 ± 1.9 (64)
da-GAL4/+	87.6 ± 1.3 (88)	79.4 ± 1.6 (82)	73.6 ± 2.6 (76)	61.9 ± 1.5 (62)
+/UAS-hSOD1	84.5 ± 1.4 (84)	78.2 ± 1.0 (81)	85.7 ± 3.7 (89)	70.6 ± 2.3 (71)
Appl-GAL4/UAS-hSOD1	84.5 ± 2.0 (85)	79.7 ± 1.7 (81)	85.0 ± 5.9 (87)	65.6 ± 3.1 (64)
D42-GAL4/UAS-hSOD1	84.1 ± 0.8 (84)	79.1 ± 1.0 (81)	83.9 ± 4.0 (86)	71.8 ± 2.4 (71)
elav-GAL4/UAS-hSOD1	85.6 ± 1.8 (85)	71.5 ± 3.7 (71)	87.5 ± 3.0 (89)	64.7 ± 3.6 (65)
arm-GAL4/UAS-hSOD1	86.0 ± 1.5 (85)	78.7 ± 2.2 (82)	79.2 ± 1.9 (83)	66.4 ± 2.9 (65)
da-GAL4/UAS-hSOD1	88.1 ± 1.4 (89)	77.0 ± 2.7 (81)	80.7 ± 2.3 (82)	66.0 ± 0.8 (65)
+/UAS-GCLc(3)	85.7 ± 0.9 (87)	77.6 ± 4.2 (80)	84.4 ± 0.9 (86)	60.3 ± 1.5 (61)
Appl-GAL4/UAS-GCLc(3)	85.7 ± 2.4 (86)	79.3 ± 2.6 (81)	85.9 ± 2.9 (88)	59.3 ± 3.3 (60)
D42-GAL4/UAS-GCLc(3)	86.0 ± 1.9 (86)	73.8 ± 4.0 (75)	83.5 ± 3.7 (85)	59.0 ± 3.1 (61)
elav-GAL4/UAS-GCLc(3)	82.2 ± 1.5 (83)	70.0 ± 2.3 (70)	83.5 ± 6.7 (87)	50.7 ± 3.7 (50)
arm-GAL4/UAS-GCLc(3)	85.7 ± 2.0 (87)	77.1 ± 2.3 (79)	83.9 ± 2.6 (85)	60.0 ± 2.4 (58)
da-GAL4/UAS-GCLc(3)	83.0 ± 1.4 (82)	75.5 ± 4.2 (78)	73.7 ± 2.6 (78)	57.8 ± 1.2 (59)
+/UAS-GCLc(6)	85.9 ± 3.9 (86)	75.1 ± 3.2 (77)	85.2 ± 1.4 (87)	60.2 ± 2.4 (60)
Appl-GAL4/UAS-GCLc(6)	85.9 ± 1.7 (87)	84.9 ± 2.1 (87)	87.5 ± 2.5 (90)	70.1 ± 2.2 (71)
D42-GAL4/UAS-GCLc(6)	84.6 ± 2.6 (86)	77.4 ± 1.4 (78)	85.3 ± 4.0 (87)	61.1 ± 3.2 (63)
elav-GAL4/UAS-GCLc(6)	82.5 ± 2.1 (82)†	65.1 ± 6.6 (65)	85.2 ± 0.8 (86)	63.7 ± 1.3 (64)
arm-GAL4/UAS-GCLc(6)	82.8 ± 1.5 (83)	79.1 ± 2.1 (81)	80.7 ± 3.6 (85)	60.2 ± 1.1 (60)
da-GAL4/UAS-GCLc(6)	78.1 ± 2.0 (79)	71.2 ± 2.5 (73)	72.4 ± 3.2 (77)	58.9 ± 3.8 (60)
+/UAS-TOR^{FRB}(2)	88.0 ± 3.1 (88)	80.2 ± 2.4 (83)	80.6 ± 2.6 (84)	67.0 ± 2.4 (68)
Appl-GAL4/UAS-TOR^{FRB}(2)	86.5 ± 1.3 (86)	80.2 ± 2.6 (81)	88.6 ± 1.1 (92)	67.5 ± 1.3 (70)
D42-GAL4/UAS-TOR^{FRB}(2)	85.6 ± 1.2 (85)	81.2 ± 2.0 (84)	83.0 ± 3.1 (84)	72.2 ± 3.5 (72)
elav-GAL4/UAS-TOR^{FRB}(2)	80.6 ± 1.8 (82)	70.9 ± 4.9 (72)	86.9 ± 2.4 (90)	65.7 ± 4.8 (65)
arm-GAL4/UAS-TOR^{FRB}(2)	85.8 ± 1.2 (85)	82.4 ± 2.3 (82)	83.4 ± 4.1 (86)	69.6 ± 4.2 (70)
da-GAL4/UAS-TOR^{FRB}(2)	87.7 ± 2.1 (88)	82.3 ± 0.8 (84)	85.3 ± 2.6 (86)	66.7 ± 3.7 (68)
+/UAS-TOR^{FRB}(3)	86.5 ± 2.1 (88)	78.5 ± 1.0 (82)	84.7 ± 1.7 (85)	67.6 ± 3.7 (68)
Appl-GAL4/UAS-TOR^{FRB}(3)	85.2 ± 3.4 (87)	79.6 ± 3.2 (81)	88.1 ± 2.4 (89)	67.7 ± 3.1 (68)
D42-GAL4/UAS-TOR^{FRB}(3)	87.3 ± 1.2 (87)	76.7 ± 2.9 (82)	82.2 ± 0.6 (83)	71.4 ± 3.3 (72)
elav-GAL4/UAS-TOR^{FRB}(3)	91.4 ± 1.5 (90)	65.0 ± 3.0 (62)	87.6 ± 2.5 (90)	65.1 ± 5.1 (62)
arm-GAL4/UAS-TOR^{FRB}(3)	88.4 ± 0.7 (88)	80.5 ± 3.2 (83)	80.9 ± 1.1 (86)	66.5 ± 3.1 (68)
da-GAL4/UAS-TOR^{FRB}(3)	88.5 ± 1.1 (88)	79.9 ± 2.4 (84)	82.8 ± 2.4 (84)	70.4 ± 5.7 (72)
+/EP-UAS-Atg8a	86.7 ± 1.1 (88)		81.8 ± 1.3 (83)	
Appl-GAL4/EP-UAS-Atg8a	87.0 ± 1.4 (87)		83.7 ± 4.7 (86)	
D42-GAL4/EP-UAS-Atg8a	85.4 ± 0.9 (86)		81.4 ± 3.2 (83)	
elav-GAL4/EP-UAS-Atg8a	80.5 ± 2.4 (80)		82.8 ± 3.7 (83)	
arm-GAL4/EP-UAS-Atg8a	88.4 ± 1.1 (88)		77.7 ± 4.1 (79)	
da-GAL4/EP-UAS-Atg8a	85.9 ± 2.1 (88)		75.1 ± 2.3 (75)	

Notes: Results are Mean ± *SD*, with medians in parentheses.

*Solid underlining designates genotypes that differed significantly from both controls in their survival distributions, based on log-rank tests, but not in their mean life spans, based on analysis of variance (ANOVA). Dashed underlining designates genotypes that differed significantly from both controls in their survival distributions (log-rank) and in their mean life spans (ANOVA). Dotted underlining designates genotypes that differed significantly from both controls in their mean life spans (ANOVA) but not in their survival distributions (log-rank).

†*n* = 3 vials. For all other groups, *n* = 4 vials of 25 flies.

used to overexpress *dSod1* only in adult flies (8). The life span of male flies was raised by 48%, from a baseline of only 25 days up to 37 days. Within the same study, overexpression of *dSod1* raised the life span of female flies by 10%–20% in a short-lived background (30–36 days), but it had small, inconsistent effects (−3% to +13%) in a long-lived background (54–59 days). Finally, spatially restricted overexpression of human *hSod1* using the *GAL4/UAS* system has been examined. Overexpression of the *UAS-hSod1* transgene in the male nervous system, driven by *D42-GAL4*, was reported to raise the mean life span by up to 41%, from 45 to 64 days (24). Overexpression of the same transgenes in long-lived, wild-caught flies (mean life span: ca. 65–85 days at 24°C) yielded statistically significant life extension in only 1/10 backgrounds in male flies and 6/10 backgrounds in females (29). Substantial life extension was again observed in a short-lived background, but in no case was the magnitude of life extension in the long-lived backgrounds greater than ~20%. More recently, the *UAS-hSod1* transgene was found to have no effect on the

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longevity of male flies in a single genetic background when driven by *D42-GAL4*, *Appl-GAL4*, *elav-GAL4*, or glial- or muscle-specific drivers, but global expression using *da-GAL4* increased the mean life span by 30% (the median was raised from ~55 to ~74 days), and *actin-GAL4* had a smaller beneficial effect (38). In this study, no life extension was observed in either male or female flies expressing the same *UAS-hSod1* transgene driven by *D42-GAL4*, *Appl-GAL4*, *elav-GAL4* (consistent with 38), or *da-GAL4*, in two different genetic backgrounds with control life spans ranging from 64 to 86 days. Some of the contrasting effects might result from differences in the timing, spatial distribution or magnitude of gene expression, or in genetic background, housing, or diet. Nevertheless, where positive effects of *Sod1* on longevity have been observed, they were almost always small in magnitude except in short-lived lineages, confined to a fraction of the lineages that were studied, and sometimes contradicted by the results of seemingly similar experiments using the same or similar strains. On the premise that aging limits the life spans of relatively long-lived animals within a species, the results are not sufficiently consistent to support a general conclusion that *Sod1* is an antiaging or "longevity assurance" gene.

The results of GCLc overexpression in this study, showing almost no effect on life span, were most surprising. Overexpression of the same transgenes in essentially the same background, using the same food medium and several of the same *GAL4* drivers, was found previously to cause substantial life extension (30). Both studies were performed on a large scale, with extensive replication. Premature, age-independent death (before ~40 days) was negligible in both studies. The control life span was fairly long in both cases, which would seem to rule out elevated stress, poor husbandry, and genetic defect as explanations of either set of results. Furthermore, the genetic construct was shown to be the same in both cases, and the enzymatic activity of the gene product was demonstrated in the original study. Perhaps the same set of genotypes should be examined in a third, independent laboratory to establish whether either set of results was an outlier.

The results obtained with the *UAS-EGFP-bMSRA*^{C} transgene were also surprising. Expression of the bovine transgene was reported originally to extend the life span of flies by more than 60% (31). A more recent, small-scale study demonstrated slight, but statistically significant life extension when the *Drosophila MSRA* gene was overexpressed in adult neurons using the Gene Switch *elav-GS-GAL4* driver (39). In contrast, an extensive investigation of *Drosophila UAS-MSRB* or mouse mitochondrial *UAS-MSRB2* expression in flies driven by four different GAL4 drivers yielded no consistent, beneficial effect on life span (17). The present results for MSRA do not contradict the previous findings, because the EGFP activity data indicate that the transgene was not expressed in flies of either sex in either genetic background in this study. Nevertheless, the drastically divergent expression of the same combination of the *elav-GAL4* driver and *UAS-EGFP-bMSRA*^{C} responder transgenes in the separate studies is concerning. It is also noteworthy that with the exception of females in one experiment using *elav-GAL4*, the extended life spans of all *UAS-EGFP-bMSRA*^{C} flies in the original study were essentially the same as for both control and experimental groups here and in the MSRB report (17), whereas control life spans in the original background were much shorter. A hypothesis prompted by these observations is that the shorter life spans of some backgrounds result from greater oxidative stress. Increasing MSRA activity might be beneficial in such backgrounds, but not in others that experience either lower rates of oxidation or greater intrinsic resistance to oxidants.

The absence of any effect of *UAS-dTOR*^{FRB} or *EP-UAS-Atg8a* expression on life span in this study was perhaps less surprising than some of the other results. TOR^{FRB} is predicted to have a dominant negative effect on Target Of Rapamycin (TOR) activity (36). TOR activity is negatively regulated and autophagy is activated when nutrients are limited (25,40). Some investigators provide flies with media containing substantially higher concentrations of yeast and/or sugar than the media routinely provided in this laboratory and designate flies on lower concentrations of sugar/yeast as subject to "dietary restriction" ('DR'41). If the medium used in this study chronically diminishes TOR activity and enhances autophagy sufficiently to ensure a long life, then either *UAS-dTOR*^{FRB} or *EP-UAS-Atg8a* expression in the same flies could be redundant. A separate study of the relationship between food concentration and composition, TOR, and Atg8a activities in these genotypes would be of value.

The present findings raise, to at least 10, the number of genetic or dietary interventions reported in recent years to extend the life span of *D. melanogaster*, but which have failed to do so consistently in subsequent tests in the same species under conditions ranging from essentially the same as to substantially different than those of the original studies. Examples include supplementation with resveratrol (42,43) and altered expression of *dSir2* (14,26), *Indy* (13,21), *Hsp22* (44,45), *dCat* combined with *dSod1* (8,46,47), *hSOD1* (24,29, present study), *Atg8a*, *dTOR*^{FRB}, *GCLc*, and *bMSRA* (present study). Concerning the latter five transgenes, the apparent contrast in outcomes does not result simply from differences in methods of data analysis because the magnitude of the effect (or lack of effect) on longevity differed greatly between the past and present studies. Nevertheless, the output of the log-rank test and parametric tests in this study illustrates at least two precautions that should be taken to avoid assertions of life extension that might prove not to be reproducible in subsequent experiments. First, both *GAL4*/+ and +/*UAS* controls should be included in studies of *GAL4*/*UAS* transgenic flies (14). Survivorship curves of *GAL4*/*UAS*

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overexpressor groups were found to differ from both controls in a grand total of 29/128 cases based on log-rank tests and in 105/256 paired comparisons with individual control groups. Thus, in 47 cases, a significant difference would be reported if only one control group were used, and not if both controls were included. The results for *y w* male flies containing the *elav-GAL4* driver suggest that differences relative to only one of the two control groups may occur due to residual differences in genetic background after backcrossing, rather than the functioning of the *UAS*-responder transgene product.

Second, and perhaps more controversially, differences in distributions of survivorship based on the log-rank test should not be equated with effects on the average life span. The log-rank test is used very widely in studies of life span in *D. melanogaster* (4,6,13,14,17,23,26,30,32,38,39,43,48–50), and the output is routinely interpreted as showing that the life span was lengthened or shortened. In many cases, the magnitude of the increase in mean or median life span is large, but in other cases much smaller differences are reported to be highly significant. In this study, groups of flies that differed in mean and median life span by as little as 0.1 and 0 days, respectively, had significant differences in survivorship based on log-rank tests, which is possible because this test accounts only for the rank order in which the flies die, not their actual ages at death. Results shown in Figure 3C–E suggest that the log-rank test is highly sensitive to small differences in life span among batches of ≥100 flies. Such differences could easily result from minor, transient fluctuations in the health status or fertility of the parent flies used to establish control and experimental crosses or even from interactions among flies within individual vials.

ANOVA and *t* tests are sometimes used to compare survivorship between groups of flies (7,25,27,29,45,47,49), but this procedure is somewhat less common because raw survivorship data are often not normally distributed. A possible method to circumvent this problem, and to avoid conflating log-rank test output with differences in mean life span, was considered based on the central limit theorem, according to which means of large samples from a nonnormal distribution may themselves be normally distributed (37). In this particular study, a surprisingly large fraction of the raw data sets for individual flies were normally distributed. Parametric tests could be applied to the survivorship of individual flies in those data sets that were normally distributed, but a large minority of raw data sets showed at least some departure from the normal distribution. In contrast, after exclusion of a minuscule proportion of outliers, the mean life spans of groups of 25 flies conformed consistently to the modeling assumptions of normal distribution and equal variance. Therefore, comparisons of means of groups of flies were most appropriate for this study. The mean life spans based on ANOVA and pairwise tests differed in 11/128 cases after correction for multiple comparisons, and some of the differences that were statistically significant might be physiologically meaningful (Figure 3A and B). This method yielded fewer dubious positive results than the log-rank test, but it would still be appropriate to replicate the experiments in subsequent generations, if replication with independent insertions of the same transgenes had yielded positive results. It remains to be determined whether means of groups of survivorship data gathered under different laboratory conditions would be amenable to parametric testing. A separate study devoted specifically to this question would be of value.

Although negative results are not inherently more reliable than positive results, it seems judicious to refrain from describing any single gene or pathway as a controller or regulator of aging in flies, until life extension has been confirmed in multiple laboratories. The National Institutes of Health Interventions Testing Program for mice provides a model for studies in flies (51). In the case of flies, besides ensuring that comparisons are made in both sexes and more than one genetic background or strain, important variables to consider include the composition of the diet and consistency of results obtained at different times from independent cohorts of flies in the same or other laboratories. In the case of the *GAL4/UAS* expression system, both driver-*GAL4* and *UAS*-responder controls should be used, with flies in constant genetic backgrounds. Additionally, a major objective of this work is to identify methods to delay human aging and/or age-related disease in societies where average life spans are already exceptionally long by historical standards. Fly strains that are shorter-lived than others are not necessarily "sick," poorly maintained, or genetically defective, and life span data derived from them are not necessarily uninformative. Nevertheless, a complete investigation leading to inferences about the causes of aging should include an analysis of strains and environmental conditions conducive to relatively long life spans. It is noteworthy, notwithstanding the vast range of interventions reported to extend life span or slow the aging process in *D. melanogaster*, that no intervention has ever been found to extend the mean life span at 25°C beyond the 90–95 day range reported for wild-caught, inbred flies (33) or flies artificially selected for delayed reproduction (52).

At present, it seems premature to assert that the genes examined in this study belong to pathways that "control" or "regulate" the aging process. Such terminology implies that age-related degradative change at the organismal level is the consequence of an evolved program or sequence of events, in the same way as the degradation of a molecule in a metabolic pathway. Current evidence supports the more cautious conclusion that some single-gene mutations "influence" longevity in some fly lineages under some environmental conditions, which implies only that animals with different genotypes differ in their resistance to the underlying causes of aging and death under particular circumstances.

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Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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