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Drosophila foxo acts in males to cause sexual-dimorphism in tissue-specific *p53* life span effects

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Abstract

Sex-specific selective pressures are hypothesized to lead to sexually antagonistic gene functions that contribute to phenotypes such as aging and cancer. However, relatively little is known about the identity of such genes and possible mechanisms. Here we report that nervous system-specific over-expression of wild-type *p53* in *Drosophila* caused decreased life span in males and increased life span in females. In contrast, tissue-general over-expression produced the opposite pattern: increased life span in males and decreased life span in females. In a *foxo* null background, *p53* life span effects in males were reversed, becoming similar to the effects in females. In contrast, a *Sir2* null background tended to reduce the magnitude of *p53* effects. The data demonstrate that wild-type *p53* over-expression can regulate life span independent of *foxo*, and suggest that *foxo* acts in males to produce sexually antagonistic life span effects of *p53*.

Keywords

antagonistic pleiotropy; Sir2; aging

1. Introduction

The mammalian p53 transcription factor is a multi-functional tumor suppressor that regulates apoptosis, cell senescence, oxidative stress responses, and mitochondrial metabolism (Green and Kroemer, 2009), and it is preferentially required in females for neural tube closure, embryonic viability and adult fecundity (Chen et al., 2008; Hu et al., 2008; Kang et al., 2009). Several lines of evidence implicate *p53* in aging. Truncated forms of p53 protein in mice can produce a premature aging-like phenotype, apparently by causing a state of p53 hyperactivation (Maier et al., 2004; Moore et al., 2007), whereas increasing the dose of wild-type p53 along with the p53 activator p19ARF could delay aging (Matheu et al., 2007). These data suggest that increased activity of wild-type p53 can promote longevity, whereas mis-regulated and activated p53 forms can promote aging. Taken together, the results from mammals suggest that *p53* exhibits antagonistic pleiotropy, in that it favors development, fecundity and cancer resistance in young animals, but may promote aging in old animals (Hu et al., 2008; Kang et al., 2009; Rodier et al., 2007; Ungewitter and Scrable, 2009).

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Inhibition of the insulin/IGF1-like signaling (IIS) pathway increases life span in *C. elegans*, *Drosophila* and mice, and in *C. elegans* this has been shown to be dependent upon the forkhead-family transcription factor Daf16 (Kenyon, 2005; Murphy et al., 2007; Samuelson et al., 2007). The most closely related transcription factor in *Drosophila* is Foxo, which can increase life span when over-expressed in fly fat-body tissue (Giannakou et al., 2004; Hwangbo et al., 2004). It has been suggested that one way p53 might affect life span in mammals and other species is by interacting with the IIS pathway (Scrable et al., 2009). For example, in *C. elegans*, inactivation of the *p53* homolog *cep-1* causes increased life span, and this was dependent upon function of Daf16 (Arum and Johnson, 2007). The phenotype of a mutation, which will affect the animal throughout development and adulthood, can sometimes differ from the effects of transgenic and RNAi manipulations, which may be targeted to specific tissues and life-cycle stages; indeed, both IIS (Broughton and Partridge, 2009) and *p53* (this study; (Waskar et al., 2009)) manipulations can have contrasting effects on life span depending upon tissue, developmental stage and gender. Mutations in the p53 DNA binding domain can result in dominant mutant forms of the protein, that sometimes antagonize normal p53 function (Brodsky et al., 2000). Expression of a dominant mutant form of p53 in adult *Drosophila* has been shown to increase life span in females (Bauer et al., 2005; Shen et al., 2009), and this was found to correlate with decreased IIS (Bauer et al., 2007); however, it has not been determined if *Drosophila* life span regulation by *p53* requires Foxo.

One potential problem in interpreting the effects of dominant mutant *p53* transgenes is that the phenotypes might be neomorphic, i.e., not necessarily representative of the function(s) of wild-type *p53*. For example, dominant mutant forms of p53 containing a disruption of the DNA binding domain are expected to antagonize the transcriptional activity of p53, but may also promote one or more of the transactivation-independent effects of p53, in a tissue-specific manner (Green and Kroemer, 2009). We have recently shown that, in *Drosophila*, wild-type *p53* can cause increased life span when over-expressed during development, and that in adults, wild-type *p53* has sex-specific effects on life span: Over-expression of wild-type *p53* in a tissue-general pattern in adult flies caused decreased life span in females, and increased life span in males (Waskar et al., 2009); and consistent with these observations, null mutation of *Drosophila p53* caused increased life span preferentially in females.

Developmental stage-specific and sex-specific selective pressures are hypothesized to produce antagonistically-pleiotropic gene functions, that in turn affect phenotypes such as reproductive fitness, behavior and life span (Hughes and Reynolds, 2005; Leips et al., 2006; Long and Rice, 2007; Tower, 2006). Here we determine that the sexually antagonistic life span effects of *p53* are tissue-specific, and that these effects are modulated in a sex-specific way by *foxo* and *Sir2*.

2. Materials and Methods

2.1 Drosophila culture and strains

Drosophila melanogaster culture, life span assays, Geneswitch driver strains, and the multi-copy UAS-eGFP strain ("UAS-ultraGFP") are as previously described (Shen et al., 2009). Flies were cultured at 25°C until eclosion, and then adults were maintained at 29°C, or at 25°C as indicated for specific experiments; additional details are provided in Supplementary materials. UAS-*p53* transgenic lines and chromosomal deficiency lines that uncover the *foxo* and *Sir2* loci were obtained from Bloomington *Drosophila* Stock Center. "p53WT1" is *P{GUS-p53} 2.1*, "p53WT2" is *P{UAS-p53.Ex}2*, and "p53WT3" is *P{UAS-p53.Ex}3*. The *foxo[21rec7A]* and *foxo[w24]* null mutation lines were provided by M. Tatar (Min et al., 2008), and the *Sir2 [4.5]* and *Sir2[5.26]* null mutation lines were provided by S.L. Helfand (Rogina and Helfand, 2004). Certain genotypes were generated by chromosomal recombination and/or crosses to

double-balancer strains. Details of strain construction and characterization are provided in Supplementary materials; all strains and genotypes are listed in Supplementary Table S1.

2.2 Quantitative real-time RT-PCR

Total RNA was isolated from 15 male or female flies, using TRIzol reagent (Invitrogen). Flies were 12 days of age, and had been cultured for 10 days at 29oC on RU486 food, or on control (ethanol-only) food. Quantitative real-time RT-PCR was performed using the Bio-RAD MyiQ™ Real-time PCR detection system and SYBR green dye, according to the manufacturer's instructions, and *Rp49* gene expression was used as control. Values are plotted as mean ± SD of triplicate biological replicates. Additional details of methods and primer sequences are presented in Supplemental materials.

2.3 Western blot assay

Total protein was isolated by homogenizing 15 male or female flies in Laemmli sample buffer (Bio-Rad). Flies were 12 days of age, and had been cultured for 10 days at 29oC on RU486 food, or on control (ethanol-only) food. Antibodies were specific for the phosphorylated form of Akt (#4054, Cell Signaling Technology; 1:1,000 dilution), or for total Akt (#4691, Cell Signaling Technology; 1:1,000 dilution). Anti-beta-actin antibody (#4967, Cell Signaling Technology; 1:5,000 dilution) was used as a loading control. Additional details are provided in Supplemental materials.

2.4 Statistical analyses

For life span assays, mean, standard deviation, median, percent change in mean, percent change in median, and log rank (Breslow) *p* value were calculated using R 2.6.2 (RDevelopmentCoreTeam, 2006). Unpaired, two-sided t-tests were used to determine the significance of differences in mRNA levels between the RU486-treated and control groups; statistically significant differences ($p<0.05$) are indicated along with the fold change in mean above the bar graphs.

3. Results

3.1 Conditional over-expression of transgenes using the Geneswitch system

To over-express *p53* in adult flies, the Geneswitch system was utilized, where transgene expression is induced by feeding the flies the drug RU486 (Mifepristone) (Nicholson et al., 2008). Characterization of the system using UAS-LacZ and UAS-GFP reporter strains demonstrated that the Act-GS-255B driver yields target gene expression throughout all the tissues of male and female flies, and that the Elav-GS driver produces expression that is strictly limited to the central nervous system (Ford et al., 2007; Shen et al., 2009)(Supplemental Fig. S1). To control for any potential effects on life span of the drug, the driver lines were crossed to the *w[1118]* injection strain and to Oregon-R wild-type strain to produce progeny containing only the drivers and no target transgene (“Control” flies). In the first set of control experiments with the Elav-GS driver, the drug-treated flies exhibited a small increase in life span in males (+4%) and no significant change in females (Fig. 1 A, B). For both the Elav-GS driver and the Act-GS-255B driver the change in life span observed in the absence of a target transgene was on average approximately +3%, which we interpret as the background variability of the life span assay (results summarized in Fig. 4; details of statistical analyses for all life span data are presented in Table 1 and Supplemental Table S2).

3.2 Sexually antagonistic effects of p53 on adult life Span

When *p53* was over-expressed specifically in adult nervous tissue using the Elav-GS driver, and either one of two independent p53-WT transgenes, this produced decreased life span in

males (−9% and −13%, respectively), and increased life span in females (+11% and +12%, respectively)(Fig. 1C, D; Supplemental Fig. S2C, D; data summarized in Fig. 4 and Table 1). This result is in striking contrast to our previous observation that tissue-general over-expression of p53-WT3 using the Act-GS-255B driver produces the opposite effect: increased life span in males and decreased life span in females (Waskar et al., 2009). That result was confirmed here using three independent p53-WT transgenes, and life span was increased in males by +10 to +18%, and decreased in females by −4 to −6% (Fig. 1I, J; Supplemental Fig. S3C-F; data summarized in Fig. 4 and Table 1). Because tissue-general over-expression includes nervous-system expression, the opposite effects produced by nervous system-specific expression versus tissue-general expression suggests that the effect of *p53* in peripheral tissues dominates over the effect in nervous system, and/or that signaling between tissues is involved in producing the effects on adult life span. Quantitative real-time RT-PCR (qPCR) assays confirmed that *p53* RNA was being efficiently over-expressed in both males and females, using both the tissue-general driver (19-fold induction in males and 13-fold induction in females) and the nervous system-specific driver (~2-fold induction in both males and females; Supplemental Fig. S4).

3.3 Interaction of foxo *and* Sir2 *mutations with* p53 *life span effects*

To determine if the life span effects of wild-type *p53* expression observed here might be due to altered IIS, several experiments were conducted. First, it was asked whether the life span changes were dependent upon the IIS target transcription factor gene *foxo*. When *p53* was over-expressed in a *foxo* null background, the pattern and magnitude of effects in females remained essentially the same, i.e., increased life span with nervous system-specific expression (Fig. 1F; Supplemental Fig. S2F), and decreased life span with tissue-general over-expression (Fig. 1L; data summarized in Fig. 4 and Table 1). However, the *foxo* null background caused a reversal of the pattern in males, such that *p53* effects in males now had a pattern similar to females, i.e., increased life span with nervous system-specific over-expression (Fig. 1E; Supplemental Fig. S2E) and decreased life span with tissue-general over-expression (Fig. 1K; Supplemental Fig. S3G; data summarized in Fig. 4 and Table 1). These data demonstrate that wild-type *p53* over-expression can both positively and negatively regulate life span independent of *foxo*, while at the same time they suggest that *foxo* acts in males to modulate (reverse) the tissue-specific pattern of *p53* life span effects, thereby producing sexually antagonistic effects of *p53*.

Previously adult-specific over-expression of *foxo* in the fat-body (FB) tissue of the head using the S_1-32-GS driver (Hwangbo et al., 2004), or in the FB tissue of the thorax and abdomen using the S_1-106-GS driver (Giannakou et al., 2004) was found to cause increased life span. Here these FB drivers were used together to drive expression of the p53-WT1 transgene throughout the FB tissues of adult male and female flies. No life span increases were observed upon FB expression of *p53* in either males or females (Supplemental explanatory text; Supplemental Table S2). This suggests that FB is not likely to be the relevant tissue for the life span increase observed upon tissue-general *p53* over-expression.

The effects of adult-specific *p53* over-expression were also assayed in a *Sir2* null mutant background. *Sir2* encodes a conserved protein deacetylase known to regulate life span in several species (Finkel et al., 2009). For nervous system-specific over-expression of *p53* in the *Sir2* null background, the negative effect of *p53* on life span remained intact in males (Fig. 1G; Supplemental Fig. S2G), whereas the positive effect in females appeared to be reduced (Fig. 1H; Supplemental Fig. S2H; data summarized in Fig. 4 and Table 1). For tissue-general over-expression of *p53* in the *Sir2* null background, the effect of *p53* on life span was reduced in both males and females, such that no significant changes were observed (Supplemental Fig. S3I, J; data summarized in Fig. 4 and Table 1). Therefore, taken together, the data suggest that

the *Sir2* gene is required for the magnitude of some, but not all, of the observed effects of *p53* over-expression on life span.

3.4 Effect of p53 *over-expression on phosphorylated Akt levels*

The activity of the Foxo transcription factor is regulated by IIS, whereby the phosphorylated and activated form of the protein kinase Akt acts to phosphorylate and inactivate Foxo. Phosphorylated Akt levels therefore provide one read-out of signaling through the IIS pathway. Phosphorylated Akt levels were assayed by western blot for control flies, and flies in which *p53* was over-expressed, in the wild-type background as well as in *foxo* null and *Sir2* null backgrounds. One striking result was that the *foxo* null mutation caused greatly reduced phosphorylated Akt levels in both males and females (Fig. 2A-D), whereas total Akt levels were unchanged (Fig. 2E; Supplemental Fig. S5A). This indicates that, in *Drosophila*, *foxo* positively regulates Akt phosphorylation level. This is consistent with a previous report that in cultured mammalian myocytes activation of either FoxO1 or FoxO3 caused increased Akt phosphorylation levels (Ni et al., 2007), as well as reports that in *Drosophila foxo* regulates expression of the upstream IIS gene *InR* (Puig et al., 2003), and that in *C. elegans* the *foxo* homolog *Daf16* regulates IIS (Murphy et al., 2007).

Interestingly, nervous system-specific over-expression of *p53* in adult flies in the wild-type background caused decreased Akt phosphorylation in males (Fig. 2A), and increased Akt phosphorylation in females (Fig. 2B), indicating that the sexually antagonistic effects of *p53* on life span can correlate with sexually opposite effects on physiology (data summarized in Fig. 4). However, the changes in Akt phosphorylation could be uncoupled from the regulation of life span by *p53* in several ways. First, the effects on life span produced by tissue-general *p53* expression did not correlate with any consistent alteration in Akt phosphorylation in males or females (Fig. 2C, D). Second, in the *foxo* null background, the increased life span produced by nervous system-specific *p53* expression and the decreased life span produced by tissue-general *p53* expression did not correlate with any consistent alteration in Akt phosphorylation in males or females (Fig. 2A, B); and this result was confirmed using two different *foxo*-null genetic backgrounds, as well as greater amounts of protein to increase the sensitivity of the assay (Supplemental Fig. S5B, C). Finally, the life span decrease produced in males by nervous system-specific *p53* over-expression in the *Sir2* null background also did not correlate with any consistent alterations in Akt phosphorylation (Fig. 2A, B). Note that the apparent alterations in Akt phosphorylation upon tissue-general *p53* over-expression in *Sir2* null background (Fig. 2C, D) were not observed in replicate experiments (Supplemental Fig. S5D, E). Therefore we conclude that the sexually opposite effects on Akt phosphorylation caused by nervous-system expression of *p53* are dependent upon *foxo* and *Sir2*, but are not required for *p53* to either positively or negatively regulate life span.

3.5 Effect of p53 on IIS pathway gene expression

Foxo is a transcription factor, and several positively-regulated Foxo target genes have been identified for *Drosophila*, including the small heat shock protein gene *l(2)efl* and the translation repressor gene *4E-BP* (Junger et al., 2003; Puig et al., 2003; Teleman et al., 2008; Wang et al., 2005). Over-expression of *l(2)efl* is reported to increase life span, and induction of *l(2)efl* has been suggested to mediate part of the life-span promoting effects of *foxo* (Wang et al., 2005). The *4E-BP* (or *Thor*) gene is induced by *foxo*, and is also a target of the TOR pathway that regulates translation, growth, and life span in response to diet in *Drosophila* (Kapahi et al., 2004; Teleman et al., 2008). qPCR was used to assay *l(2)efl* transcript levels upon *p53* over-expression in wild-type, *foxo* null and *Sir2* null mutant backgrounds (Fig. 3). Expression of *l(2)efl* was reduced in the *foxo* null background, particularly in males, consistent with the regulation of this gene by *foxo* (Fig. 3A-D). In addition, expression of *l(2)efl* was greater in males than females, suggesting the possibility of greater *foxo* activity in males; a similar set of

results were obtained for *4E-BP* (Supplemental Fig. S6). Conditional over-expression of *p53* in either the tissue-general or nervous system-specific manner in a wild-type background had no detectable effect on either *l(2)efl* or *4E-BP* expression levels, in males or females, consistent with the conclusion that *p53* regulates life span by a mechanism independent of *foxo* and the expression of *l(2)efl* and *4E-BP*.

The expression of two genes that act upstream in the IIS pathway, *Dilp2* and *InR*, were also assayed, in wild-type, *foxo* null and *Sir2* null mutant backgrounds. *Dilp2* encodes a *Drosophila* insulin-like peptide, and *Dilp2* expression is down-regulated in flies that are long-lived due to *foxo* over-expression in the head FB using driver S_1-32-GS (Hwangbo et al., 2004). *InR* encodes the *Drosophila* insulin-like receptor, and *InR* expression was reported to be positively regulated by *foxo* (Puig et al., 2003). *p53* over-expression had no consistent effects on the expression of either *Dilp2* (Fig. 3E-H) or *InR* (Supplemental Fig. S7), supporting the conclusion that *p53* over-expression affects life span through some mechanism other than altered IIS. Finally, expression of the *Sir2* gene was assayed upon *p53* over-expression in wild-type, *foxo* null and *Sir2* null mutant backgrounds, and no consistent changes were detected (Supplemental Fig. S8), suggesting that *p53* life span effects are not mediated by alterations in expression of *Sir2*.

4. Discussion

Here over-expression of wild-type *p53* in adult flies was found to regulate life span in a tissue-specific and sexually-dimorphic manner. Nervous system-specific over-expression of *p53* caused decreased life span in males and increased life span in females, whereas tissue-general over-expression produced the opposite pattern: decreased life span in females and increased life span in males (summarized in Fig. 4). In a *foxo* null background, *p53* life span effects in males were reversed, becoming similar to the effects in females. In contrast, a *Sir2* null background tended to reduce the magnitude of *p53* effects. Because life span alteration did not correlate with changes in expression of IIS pathway genes, did not require *foxo*, and could be uncoupled from changes in Akt phosphorylation levels, the data suggest that wild-type *p53* can both positively and negatively regulate life span independent of alterations in IIS. The fact that female life span was increased by adult nervous system-specific expression of both wild-type *p53* (this study) and dominant mutant *p53* transgenes (Bauer et al., 2007;Bauer et al., 2005) may indicate a transactivation-independent mechanism in that tissue.

Taken together, the present data demonstrate that wild-type *p53* over-expression can both positively and negatively regulate life span independent of *foxo*, and suggest that *foxo* acts in males to modulate (reverse) the tissue-specific pattern of *p53* life span effects, thereby producing sexually antagonistic effects of *p53*. This situation is somewhat reminiscent of the relationship of *foxo* to the DR response: *foxo* was not required for life span increase in response to DR, but was found to modulate the response (Giannakou et al., 2008; Min et al., 2008).

One relevant issue to consider is how any differences in the level of *p53* expression achieved might affect the present results. The qPCR assay used to confirm *p53* over-expression was performed on whole-body RNA, and therefore the overall *p53* level observed is much greater with tissue-general over-expression (using driver Act-GS-255B) compared to the more limited nervous system-specific expression (using driver Elav-GS) (Supplemental Fig. S4). The expression level in the female brain is expected to be similar with these two drivers, as indicated by control experiments using GFP reporters (Shen et al., 2009). Therefore, at least for females, the opposite effect of *p53* produced using these two drivers is most likely due to the site of over-expression rather than any difference in the magnitude of over-expression. However, in males, the ActGS-255B driver produced a lower level of GFP reporter expression (Supplemental Fig. S1; and (Shen et al., 2009)). Therefore, conceivably, the opposite result

obtained upon tissue-general *p53* over-expression in males (positive effect) compared to females (negative effect) could result from a threshold effect of p53 expression, where moderate over-expression is positive and higher-level expression is negative. Indeed a recent study of *C. elegans* hermaphrodites is consistent with a threshold in *p53* effects: low levels of mitochondrial stress (suggesting modest p53 activation) caused increased life span, whereas high levels of mitochondrial stress (suggesting strong p53 activation) caused decreased life span (Ventura et al., 2009). However, such a threshold cannot explain the opposite effect of nervous-system expression observed here between male flies (negative effect) and females (positive effect), as that is in the wrong direction. The level of *p53* over-expression achieved in wild-type versus *foxo* null and *Sir2* null backgrounds was similar for male and female nervous-system expression, and for tissue-general expression in females (Supplemental Fig. S4), indicating that the different effects on life span observed in those instances are not simply due to variation in the level of *p53* over-expression. In contrast, for tissue-general over-expression of *p53* in males, the level of over-expression in wild-type background was moderate and produced increased life span, whereas over-expression in the *foxo* null background was greater and produced decreased life span, again conceivably consistent with a threshold effect. However, tissue-general over-expression of dominant-negative *p53* transgenes produced the opposite effect relative to wild-type *p53* transgenes, in both males and females (Shen et al., 2009; Waskar et al., 2009). This indicates that the opposite effects of *p53* transgenes on male and female life span are not likely to be due to some difference in the efficiency of transgene expression in males versus females, or to some differential toxicity of the encoded proteins in males versus females. Therefore, in summary, while differences in the magnitude of *p53* over-expression achieved may be contributing to the effects on life span, at least for males, they are not sufficient to explain the pattern of opposite effects on life span observed in males versus females, nor the opposite effects on life span observed with the two different drivers, nor the opposite effects observed with wild-type versus dominant-mutant *p53* transgenes; these results appear to require truly opposite effects of *p53* in the two sexes and in different tissues.

One way the present results might be interpreted is if *foxo* is activated in males to promote the differentiation of a male-specific metabolic state. This hypothesis is consistent with our observation that the Foxo target genes *l(2)efl* and *4E-BP* were expressed at higher levels in males than females in a *foxo* dependent manner. There is ample precedent for a role for IIS in *Drosophila* sexual differentiation. For example, disruption of IIS and mutations in the *InR* gene caused a feminization of male locomotor behaviors (Belgacem and Martin, 2006), and IIS is required for both male and female gametogenesis and fecundity (Hsu and Drummond-Barbosa, 2009; Toivonen and Partridge, 2008; Ueishi et al., 2009). In mammals there are four FOXO factors (FoxO1-4) that may be partially redundant in function, and among these, FoxO3 appears most related to *Drosophila* Foxo. FoxO3-null mice exhibit abnormal ovarian follicular development and age-dependent infertility (Hosaka et al., 2004), consistent with a role for FoxO3 in mammalian sexual differentiation. Finally, dramatically increased life span in the Ames dwarf mouse correlates with a loss of sexually dimorphic gene expression patterns in the liver (Amador-Noguez et al., 2005). Therefore, one way that IIS and *foxo* might regulate life span in *Drosophila* and other species is by promoting sexual differentiation, thereby revealing the sexually-antagonistic effects of genes such as *p53*.

An interesting question is what are the mechanism(s) by which *p53* is able to positively and negatively regulate life span independent of *foxo*. There are a number of possibilities, because *p53* is known to regulate several processes that might in turn affect life span, including autophagy, mitochondrial metabolism, apoptosis, and cell senescence (Green and Kroemer, 2009). Indeed, in *C. elegans*, the *p53* homolog *cep-1* mediates the life span promoting effects of mild mitochondrial stress (Ventura et al., 2009), and the life span increase due to *cep-1* mutation requires autophagy gene function (Tavernarakis et al., 2008). One possibility is if *Drosophila p53* over-expression caused alterations in feeding behavior or nutrient utilization,

this might regulate life span through the DR pathway. The Sirtuin gene family is implicated in mediating DR responses in several species (Finkel et al., 2009), and over-expression of *Sir2* is reported to increase life span in *Drosophila* females (Rogina and Helfand, 2004). Moreover, the life span increases caused by nervous system-specific expression of a dominant mutation *p53* transgene and a *Sir2* transgene in female flies were not additive, leading to the suggestion that these effects might occur through a common pathway, perhaps one related to DR (Bauer et al., 2009). Our data indicate that some, but not all, of the tissue-specific effects on life span of wild-type *p53* required *Sir2*. The positive effects of *p53* over-expression on adult fly life span were eliminated or reduced by *Sir2* mutation, consistent with a possible link to the DR pathway, whereas nervous-system-specific expression of *p53* in males decreased life span independent of *Sir2* (summarized in Fig. 4). This suggests the possibility that wild-type *p53* may be acting through more than one pathway to affect adult fly life span, perhaps by acting through different pathways in different tissues and/or in the two sexes.

Taken together, the data are consistent with the conclusion that *p53* exhibits antagonistic pleiotropy, and in particular, that *Drosophila p53* and *foxo* exhibit sexual antagonistic pleiotropy with regard to life span. These results suggest the potential usefulness of gender-specific manipulation of *p53* and IIS in future interventions in aging and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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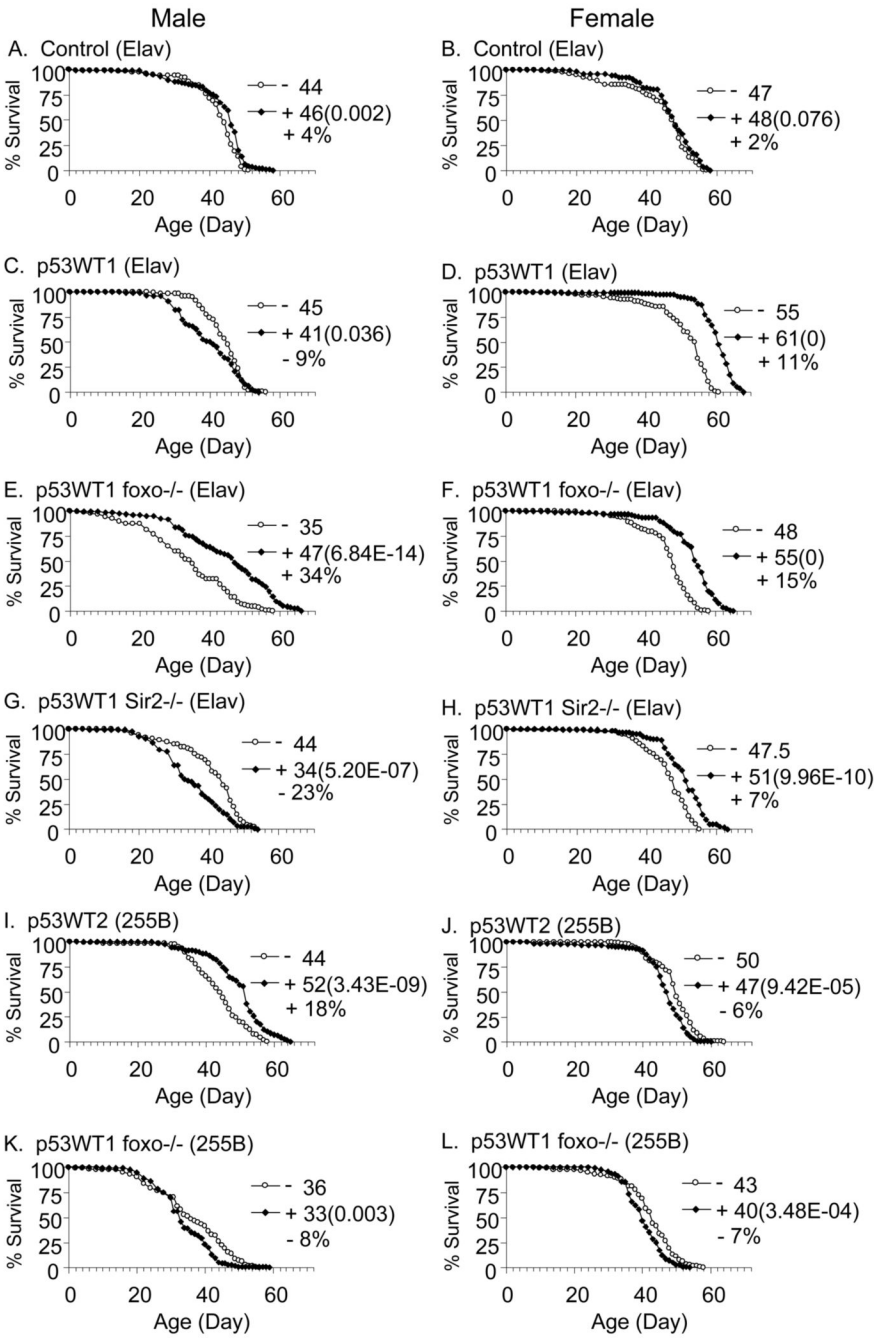

Fig. 1.

Effect of adult-specific *p53* over-expression on life span. The indicated wild-type *p53* transgenes (p53WT1 or p53WT2) were over-expressed using the nervous system-specific driver Elav-GS ("Elav") and the tissue-general driver Act-GS-255B ("255B"), as indicated. *p53* over-expression was carried out in wild-type background ("+/+"), *foxo* null background ("*foxo−/−*") and *Sir2* null background ("*Sir2−/−*") as indicated. The life span assays were performed at 29°C. Solid diamonds represent adults treated with drug ("+", ~125 flies), open circles represent the no-drug controls ("–", ~125 flies). Survival curves are plotted as percent survival versus adult age in days. Mean life span for each cohort is presented along with the *p* value for log rank test (in parentheses). Details of statistical analyses are presented in

Supplementary Table 2. (A, C, E, G, I, K) male flies. (B, D, F, H, J, L) female flies. (A, B) Control flies containing the Elav-GS driver and no target transgene, genotype *w[1118]/yw ; +*; *Elav*/+. (C, D) Elav-GS driver plus p53WT1, genotype *w[1118]/yw ; p53WT1*/+; *Elav*/+. (E, F) Elav-GS driver plus p53WT1, in the *foxo* null background, genotype *w[1118]; p53WT1*/+; *Elav, Df(3R)Exel8159/foxo[21]*. (G, H) Elav-GS driver plus p53WT1 in the *Sir2* null background, genotype *w[1118]; p53WT1, Df(2L)BSC344/ Sir2[4.5]; Elav*/+. Survival curves for Elav-GS plus p53WT2, in wild-type, *foxo* null and *Sir2* null background are presented in Supplementary Figure S2. (I, J) Act-GS-255B driver plus p53WT2, genotype *w [1118]; 255B/p53WT2; +*. (K, L) Act-GS-255B driver plus p53WT1 in the *foxo* null background, genotype *w[1118];255B/p53WT1; Df(3R)Exel8159/foxo[21]*. Survival curves for Act-GS-255B driver plus p53WT1 and p53WT3 in wild-type background, and p53WT2 in *foxo* null bankground, and p53WT3 in *Sir2* null background are presented in Supplementary Figure S3.

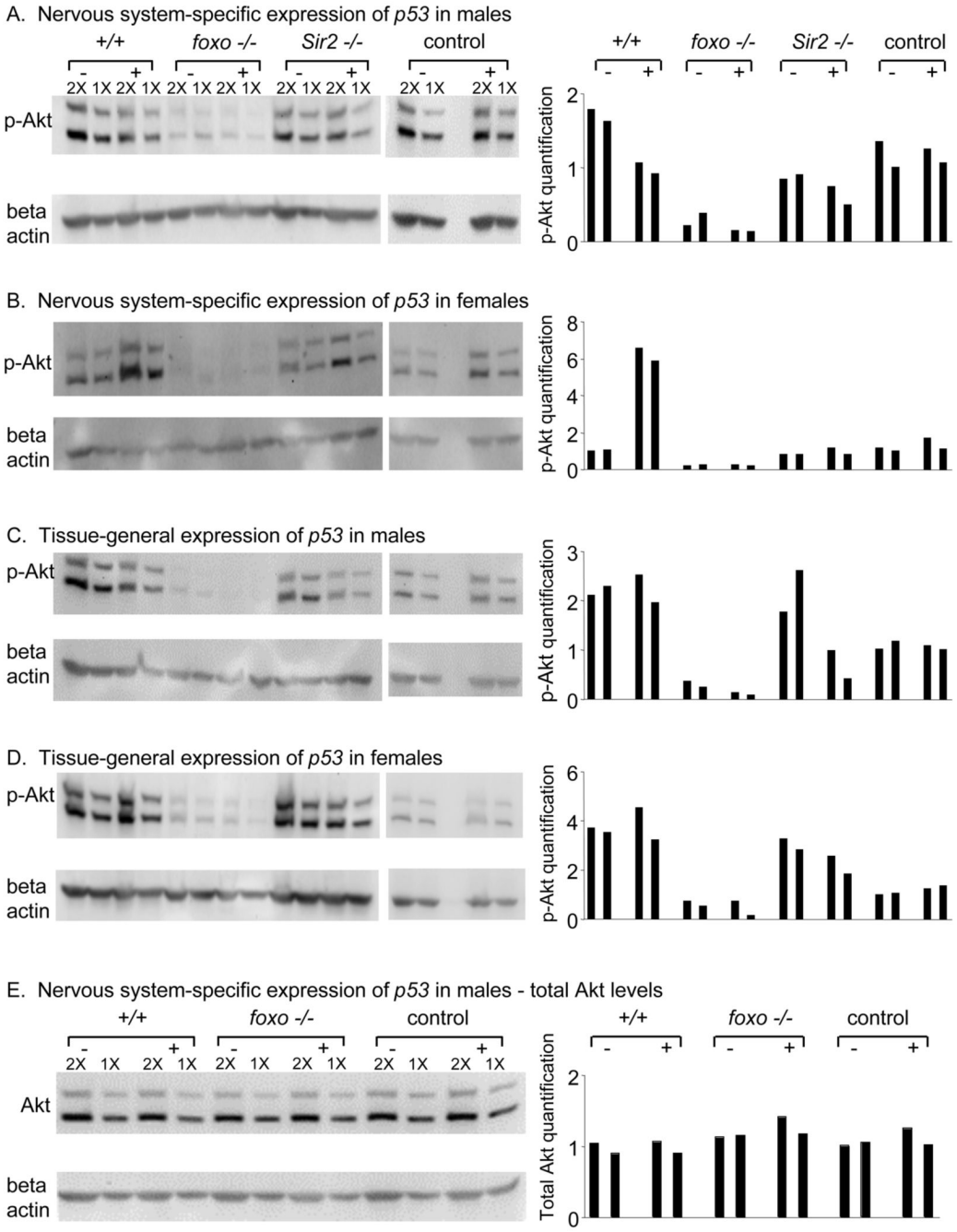

Fig. 2.

Effect of *p53* over-expression on levels of Akt and phosphorylated Akt. Total protein was isolated from 15 adult flies for each genotype and sex. "+" and "−" indicates 10 days treatment with drug, and no-drug controls, respectively. 2X and 1X volume from the same sample were loaded next to each other, as indicated, as controls for quantification. Samples were analyzed by western blot, using antibodies specific for phosphorylated Akt (panels A-D; two *Drosophila*-specific isoforms are recognized), and for total Akt (panel E), and for the beta-actin loading control, as indicated. Signals were quantified by phosphorimager, normalized to the loading control, and the results are plotted in relative units as bar graphs to the right of each western blot. (A, B) Nervous system-specific over-expression of p53WT2 using the Elav-GS

driver. Genotypes are indicated above the panels. "+/+" indicates *w[1118]/yw ; p53WT2*/+; *Elav*/+. *"foxo−/−"* indicates *w[1118] ; p53WT2*/+; *Elav, Df(3R)Exel8159/foxo[21]*. *"Sir2−/−"* indicates *w[1118] ; p53WT2, Df(2L)BSC344/ Sir2[4.5]; Elav*/+. "control" indicates *w[1118]/yw ;* +; *Elav*/+. (C, D) Tissue-general over-expression of p53WT using the Act-GS-255B driver. "+/+" indicates *w[1118]; 255B/p53WT2;* +. *"foxo−/−"* indicates *w[1118]; 255B/p53WT2; Df(3R)Exel8159/foxo[21]*. *"Sir2−/−"* indicates *w[1118]/y[1]w[1118];255B, Df(2L)BSC344/ Sir2[4.5]; p53WT3*/+. "control" indicates *w[1118]; 255B*/+; +. (E) Nervous system-specific over-expression of *p53* in males using the Elav-GS driver, assay of total Akt protein levels; genotypes as in (A, B).

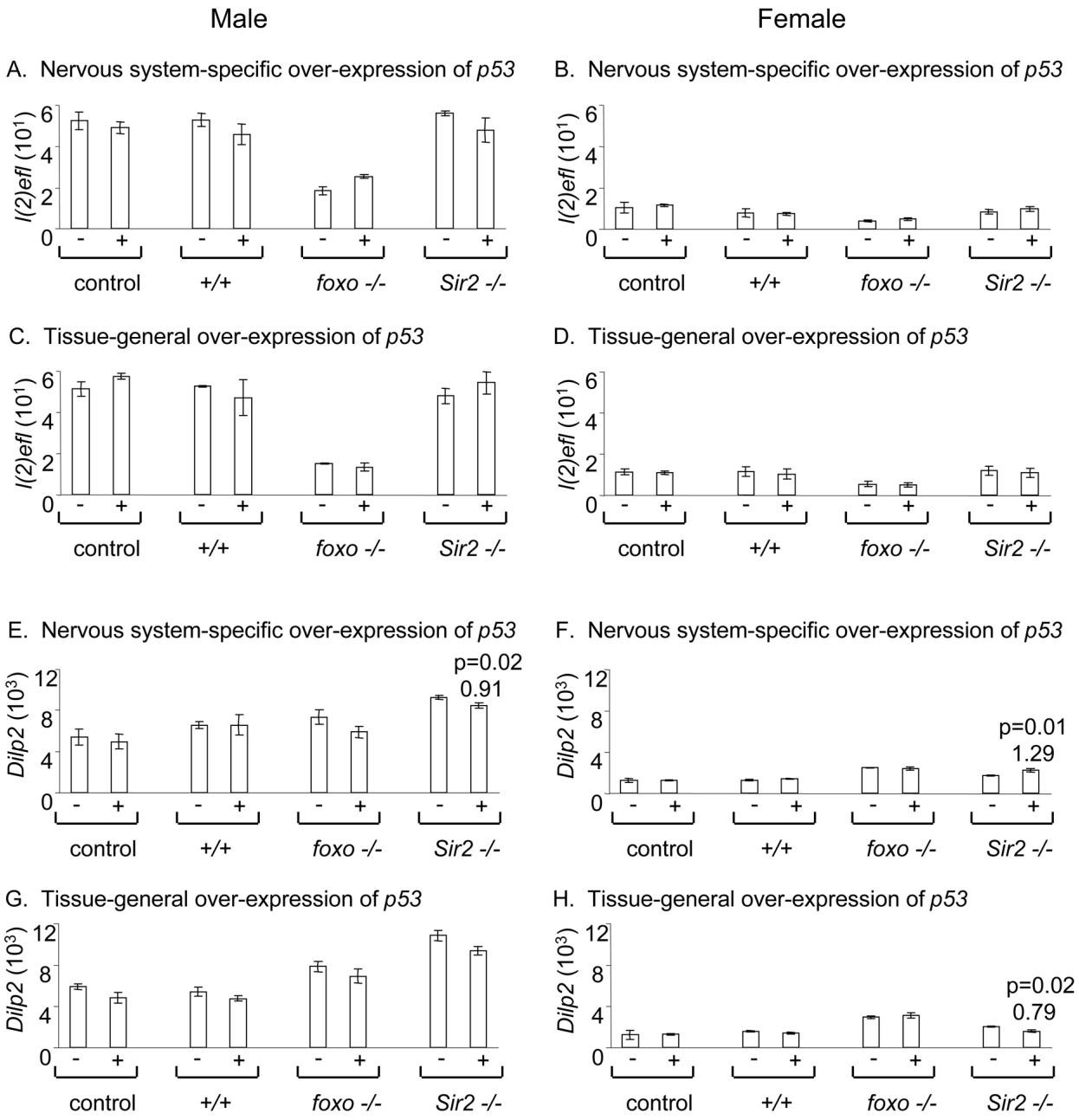

Fig. 3.

Effect of *p53* over-expression on endogenous *4E-BP* and *Dilp2* gene expression. Quantitative real-time RT-PCR assay was used to measure RNA levels for the *4E-BP* and *Dilp2* genes, as indicated. Mean ± SD of triplicate assays (biological replicates) is plotted as bar graphs. Statistically significant differences ($p<0.05$) were determined using unpaired, two-sided t-tests, and are indicated above the bars along with the fold change in mean. (A, B, E, F) Nervous system-specific over-expression of p53WT2 using the Elav-GS driver. (C, D, G, H) Tissue-general over-expression of p53WT2 using the Act-GS-255B driver. Specific genotypes are the same as in Figure 3. (A-D) Assay of *l(2)efl* RNA levels. (E-G) Assay of *Dilp2* RNA levels. (A, C, E, G) Males. (B, D, F, H) Females.

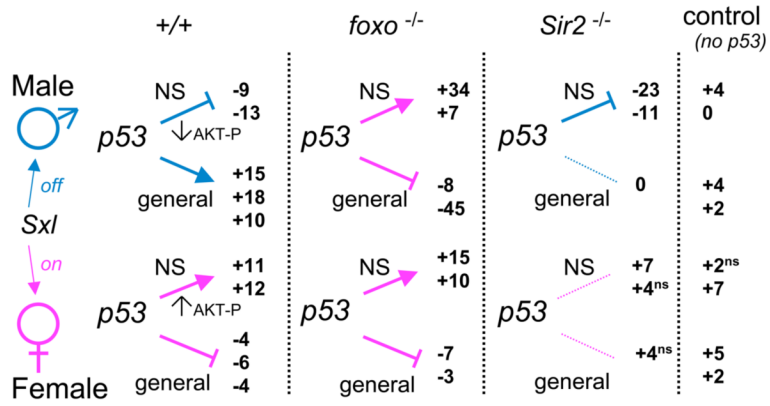

Fig. 4.

Summary of effects of wild-type *p53* over-expression. The life span data for nervous system-specific over-expression of *p53* using the Elav-GS driver ("NS"), and tissue-general over-expression of *p53* using the Act-GS-255B driver ("general"), are summarized for each sex, as indicated. Numbers in black type indicate the percent change in median life span obtained for replicate experiments using independent wild-type *p53* transgenes; ns = not significant. The summary includes all data for the wild-type background ("+/+"), the *foxo* null background ("*foxo−/−*") and the *Sir2* null background ("*Sir2−/−*"). *"Control (no p53)"* indicates the changes in life span observed in the absence of a *p53* target gene (background of the assay). Net positive effects are indicated with a blue or pink arrow, net negative effects are indicated with a blue or pink T-bar. Dotted lines indicate no effect, or reduced effect, relative to the wild-type background. The changes in life span caused by *p53* over-expression in the wild-type ("+/+") background are indicated in blue for males, and pink for females. In the *foxo* null background the effects in males are reversed in sign, becoming like the female pattern, and are therefore indicated in pink. Changes in phosphorylated Akt level ("AKT-P") are indicated by up and down black arrows, and changes were consistently observed only for nervous system-specific expression of *p53* in the wild-type background. Somatic sexual differentiation in *Drosophila* is controlled by the on/off status of the *Sex lethal* (*Sxl*) gene, as indicated.

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Table 1

Life span data with means, standard deviations, medians, percent change in mean and median, and log rank *p* value.

Cross MxF	RU486	*Genotype*	Sex	N	Mean[a]	Median	Δ Mean	Δ Median	Log Rank p Value
Exp1 Life span assay with GS255B driver at 29°C									
3-1	−	*w/Y; 255B/+; +*	M	126	52.52±10.68	56	--------	---------	---------
3-1	+	*w/Y; 255B/+; +*	M	132	56.02±7.79	57	6.68	1.79	1.30E-05
3-1	−	*w/+ ;255B/+; +*	F	126	57.96±10.65	61	---------	---------	---------
3-1	+	*w/+ ;255B/+; +*	F	124	60.33±9.89	62	4.09	1.64	5.92E-05
4-1	−	*w/Y; 255B/+; +*	M	130	48.11±8.56	48	---------	---------	---------
4-1	+	*w/Y; 255B/+; +*	M	131	50.52±9.15	50	5.01	4.17	0.008
4-1	−	*w ;255B/+; +*	F	131	52.82±5.31	53	---------	---------	---------
4-1	+	*w ;255B/+; +*	F	136	54.9±5.02	55.5	3.94	4.72	0.001
5-1	−	*w/Y; 255B/p53WT1; +*	M	128	47.01±4.97	46	---------	---------	---------
5-1	+	*w/Y; 255B/p53WT1; +*	M	130	51.85±7.44	53	10.29	15.22	1.02E-12
5-1	−	*w; 255B/p53WT1; +*	F	134	49.95±5.15	49	---------	---------	---------
5-1	+	*w; 255B/p53WT1; +*	F	134	46.61±5.53	47	−6.68	−4.08	1.22E-08
6-1	−	*w/Y; 255B/p53WT2; +*	M	125	43.59±8.45	44	---------	---------	---------
6-1	+	*w/Y; 255B/p53WT2; +*	M	137	49.58±8.39	52	13.73	18.18	3.43E-09
6-1	−	*w; 255B/p53WT2; +*	F	129	49.32±6	50	---------	---------	---------
6-1	+	*w; 255B/p53WT2; +*	F	129	46.29±7.86	47	−6.13	−6.00	9.42E-05
7-1	−	*w/Y; 255B/+; p53WT3/+*	M	134	46.84±9	48	---------	---------	---------
7-1	+	*w/Y; 255B/+; p53WT3/+*	M	125	51.61±9.04	53	10.19	10.42	6.986E-07
7-1	−	*w/yw; 255B/+; p53WT3/+*	F	130	51.72±9.32	54	---------	---------	---------
7-1	+	*w/yw; 255B/+; p53WT3/+*	F	136	50.43±6.06	52	−2.48	−3.70	5.64E-08
9-8	−	*w/Y;255B/p53WT1; foxo −/−*	M	118	35.7±11.26	36	---------	---------	---------
9-8	+	*w/Y;255B/p53WT1; foxo −/−*	M	115	33.65±8.07	33	−5.75	−8.33	0.003
9-8	−	*w;255B/p53WT1; foxo −/−*	F	129	42.02±8.3	43	---------	---------	---------
9-8	+	*w;255B/p53WT1; foxo −/−*	F	123	40.3±5.45	40	−4.10	−6.98	3.48E-04
10-8	−	*w/Y; 255B/p53WT2; foxo −/−*	M	104	30.05±12.47	33	---------	---------	---------
10-8	+	*w/Y; 255B/p53WT2; foxo −/−*	M	104	16.59±6.55	18	−44.80	−45.45	0
10-8	−	*w; 255B/p53WT2; foxo −/−*	F	128	36.98±7.6	37	---------	---------	---------

Cross MxF	RU486	*Genotype*	Sex	N	Mean[a]	Median	Δ Mean	Δ Median	Log Rank p Value
10-8	+	*w; 255B/p53WT2; foxo −/−*	F	128	33.83±10.91	36	−8.51	−2.70	0.579
12-11	−	*w/Y;255B Sir2 −/−;p53WT3/+*	M	131	43.02±7.16	45	---------	---------	---------
12-11	+	*w/Y;255B Sir2 −/−;p53WT3/+*	M	122	43.37±10.12	45	0.80	0.00	0.003
12-11	−	*w/yw;255B Sir2 −/−;p53WT3/+*	F	127	47.25±12.11	50	---------	---------	---------
12-11	+	*w/yw;255B Sir2 −/−;p53WT3/+*	F	131	47.86±12.45	52	1.28	4.00	0.064
Exp2 Life span assay with Elav-GS driver at 29°C									
3-2	−	*yw/Y; +; Elav/+*	M	126	48.59±5.02	50	---------	---------	---------
3-2	+	*yw/Y; +; Elav/+*	M	127	49.61±7.56	50	2.10	0.00	0.004
3-2	−	*yw/+; +; Elav/+*	F	118	58.2±5.28	58	---------	---------	---------
3-2	+	*yw/+; +; Elav/+*	F	125	60.82±4.8	62	4.49	6.90	5.94E-05
4-2	−	*yw/Y; +; Elav/+*	M	124	42.07±7.61	44	---------	---------	---------
4-2	+	*yw/Y; +; Elav/+*	M	127	43.29±8.52	46	2.90	4.55	0.002
4-2	−	*yw/w; +; Elav/+*	F	127	43.99±10.55	47	---------	---------	---------
4-2	+	*yw/w; +; Elav/+*	F	129	46.58±8.29	48	5.89	2.13	0.076
5-2	−	*yw/Y; p53WT1/+; Elav/+*	M	122	43.94±5.46	45	---------	---------	---------
5-2	+	*yw/Y; p53WT1/+; Elav/+*	M	123	39.56±8.4	41	−9.97	−8.89	0.036
5-2	−	*yw/w; p53WT1/+; Elav/+*	F	125	50.78±9.47	55	---------	---------	---------
5-2	+	*yw/w; p53WT1/+; Elav/+*	F	120	60.21±6.33	61	18.56	10.91	0
6-2	−	*yw/Y; p53WT2/+; Elav/+*	M	122	43.27±5.86	43.5	---------	---------	---------
6-2	+	*yw/Y; p53WT2/+; Elav/+*	M	129	39.37±8.01	38	−9.01	−12.64	0.016
6-2	−	*yw/w; p53WT2/+; Elav/+*	F	125	44.34±13.5	49	---------	---------	---------
6-2	+	*yw/w; p53WT2/+; Elav/+*	F	122	53.89±10.37	55	21.54	12.24	1.49E-13
9-13	−	*w/Y;p53WT1/+; Elav foxo −/−*	M	127	33.46±12.42	35	---------	---------	---------
9-13	+	*w/Y;p53WT1/+; Elav foxo −/−*	M	124	44.91±12.96	47	34.21	34.29	6.84E-14
9-13	−	*w;p53WT1/+;Elav foxo −/−*	F	125	46.17±7.55	48	---------	---------	---------
9-13	+	*w;p53WT1/+;Elav foxo −/−*	F	127	52.94±8.82	55	14.68	14.58	0
10-13	−	*w/Y;p53WT2/+; Elav foxo −/−*	M	123	41.8±9.99	45	---------	---------	---------
10-13	+	*w/Y;p53WT2/+; Elav foxo −/−*	M	123	45.56±13.58	48	9.01	6.67	7.21E-08
10-13	−	*w; p53WT2/+; Elav foxo −/−*	F	120	40.49±8.19	42	---------	---------	---------
10-13	+	*w; p53WT2/+; Elav foxo −/−*	F	130	43.71±11.14	46	7.94	9.52	1.10E-05
16-14	−	*w/Y; p53WT1 Sir2 −/−;Elav/+*	M	132	40.83±9.21	44	---------	---------	---------

Cross MxF	RU486	*Genotype*	Sex	N	Mean[a]	Median	Δ Mean	Δ Median	Log Rank p Value
16-14	+	*w/Y; p53WT1 Sir2 −/−;Elav/+*	M	133	34.8±9	34	−14.75	−22.73	5.20E-07
16-14	−	*w; p53WT1 Sir2 −/−;Elav/+*	F	126	45.83±7.43	47.5	---------	---------	---------
16-14	+	*w; p53WT1 Sir2 −/−;Elav/+*	F	125	50.35±7.13	51	9.86	7.37	9.96E-10
16-15	−	*w/Y; p53WT2 Sir2 −/−; Elav/+*	M	121	35.26±7.3	36	---------	---------	---------
16-15	+	*w/Y; p53WT2 Sir2 −/−; Elav/+*	M	131	31.99±8.08	32	−9.28	−11.11	0.017
16-15	−	*w;p53WT2 Sir2 −/−; Elav/+*	F	126	46.19±7.97	47	---------	---------	---------
16-15	+	*w;p53WT2 Sir2 −/−; Elav/+*	F	132	47.61±8.86	49	3.06	4.26	0.084

[a]Mean life span, days +/− SD. All cross directions are presented with the male parent strain first, female parent strain second; details on strains and strain numbers are presented in Supplemental Table S1; additional life span data is presented in Supplemental Table S2.