



Published in final edited form as:

Mol Genet Genomics. 2009 February ; 281(2): . doi:10.1007/s00438-008-0400-z.

Adult-specific over-expression of the *Drosophila* genes *magu* and *hebe* increases life span and modulates late-age female fecundity

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Abstract

During *Drosophila* aging mortality rate increases exponentially and progeny production per animal declines dramatically, correlating with decreased number and division of somatic and germ-line stem cells in the gonads. To search for genes that might promote both longevity and fecundity, a P element transposon (*PdL*), containing an outwardly directed, doxycycline-inducible promoter was used to generate conditional mutations. Mutant females were screened for increased fecundity at late ages in the presence of doxycycline. Two genes were identified, named *hebe* (CG1623) and *magu* (CG2264), that when over-expressed in adult flies could increase life span by ~5–30% in both sexes and increase female fecundity at late ages. Transcripts for *magu* are enriched in the *Drosophila* stem cell niche region, and *magu* encodes a protein related to the human SMOC2 regulator of angiogenesis. While moderate over-expression of *magu* in adult females increased fecundity at late ages, high-level over-expression of *magu* was maternal-effect lethal. The data demonstrate that adult-specific over-expression of *hebe* and *magu* can increase life span and modulate female fecundity, and provide further evidence against obligatory trade-offs between reproduction and longevity.

Keywords

Aging; P element; Oogenesis; Stem cell; Trade-off

Introduction

Among the defining characteristics of aging across species are an exponential increase in mortality rate and reduced fecundity (progeny production), and it is possible that these conserved features of aging might have a common underlying mechanism (Hekimi 2006). *Drosophila* is a well-developed model system for the study of both aging and reproduction (Partridge et al. 2005; Partridge and Tower 2008), thereby facilitating the search for regulators that might be shared between these processes. For example the insulin/IGF1-like signaling (IIS) pathway negatively regulates life span in *Drosophila* (Clancy et al. 2001; Tatar et al. 2001) and other species (Kenyon 2005; Selman et al. 2008), and positively

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regulates *Drosophila* oogenesis in response to dietary protein (Drummond-Barbosa and Spradling 2001; Hsu et al. 2008). *Drosophila* gametogenesis involves the continued division of both germ line and somatic stem cell populations located at the anterior tip of the gonads (Fuller and Spradling 2007). The germ-line stem cells give rise to the oocyte and sperm, while the somatic stem cells produce short-lived tissues that provide nourishment and support to the developing germ-line cells. In response to a protein-rich diet, the *Drosophila* nervous system produces insulin-like peptides (DILPs) that activate the IIS pathway in ovarian stem cells to increase their rate of division (LaFever and Drummond-Barbosa 2005). Gametogenesis and reproduction in *Drosophila* responds to additional hormonal and circadian inputs, and is stimulated by mating (Howlader and Sharma 2006; Toivonen and Partridge 2008).

Decreased gamete production during *Drosophila* aging corresponds with decreased number and division of stem cells in both the ovary (Waskar et al. 2005; Pan et al. 2007; Zhao et al. 2008) and testes (Wallenfang et al. 2006; Boyle et al. 2007). Both intrinsic changes (within the stem cells) and extrinsic changes (systemic signals) are implicated in the decline in stem cell function during aging, including alterations in the stem cell niche, which is the specialized microenvironment that confers upon stem cells the ability to self-renew (Morrison and Spradling 2008).

The relationship between reproduction and life span is not entirely clear. In *C. elegans* signals from both germ-line and somatic tissues in the gonad can act through the IIS pathway to affect the life span of the animal (Yamawaki et al. 2008). In *Drosophila* it has been reported that ablation of germ-line stem cells does not affect life span (Barnes et al. 2006), however, more recently it is reported that ablation of germ-line stem cells increases life span and alters IIS (Flatt et al. 2008). While trade-offs between reproduction and life span have often been reported in *Drosophila* and other organisms, it has also been reported that under certain circumstances these traits can be uncoupled (Partridge et al. 2005; Barnes et al. 2006; Flatt and Kawecki 2007).

Conditional transgene over-expression systems, where transgene expression is induced by a heat pulse, or by feeding flies a drug such as DOX (Bieschke et al. 1998) or RU486/Mifepristone (Ford et al. 2007), provide a powerful method to test genes for effects on life span and reproduction. With the conditional systems, the control and experimental (gene over-expressing) groups have identical genetic backgrounds, and therefore the effects of the transgene can be identified without confounding effects of genetic background (Sun and Tower 1999; Tower 2000; Curtis et al. 2007; Giannakou et al. 2008; Min et al. 2008). Placing a conditional (DOX-dependent) promoter facing outward from a P element transposon (the “*PdL*” P element) allows for the efficient screening of *Drosophila* genes for over-expression phenotypes, including effects on life span (Landis et al. 2001; Landis et al. 2003) and oogenesis (Khokhar et al. 2008). In particular, the dominant and conditional nature of the *PdL* gene-over-expression mutations allows screening for effects on reproduction at the level of the individual (F1) mutant female fly. Here we report the use of *PdL* mutagenesis to search for genes that are positive regulators of both female fecundity and life span.

Materials and methods

Drosophila strains and culture

Drosophila strains and culture conditions are as previously described (Ford et al. 2007). Experiments were performed at 25°C using a standard cornmeal/agar media (Ren et al. 2007). To cause conditional gene expression using the Tet-on system, flies were cultured on media containing doxycycline and ampicillin each at a final concentration of 64 µg/ml

("+DOX"). For the control groups ("-DOX"), flies were cultured on media containing ampicillin only.

Generation of wild type female fecundity curve

A cohort of 40 Oregon-R virgin female flies was collected over a period of 48 h and designated as 1 day old. They were separated into 10 vials with 4 virgins in each vial together with 4 young Oregon-R males and kept in 25°C. Flies in each vial were transferred to fresh food vials every other day and the number of dead flies was recorded, and the old vials were kept at 25°C for subsequent progeny counts. Every month 4 young Oregon-R males were added to each vial to replenish the old males, and the experiment was performed until all the females were dead. The pupae number in each vial was counted 10 days after each transfer. For each vial, the number of pupae was divided by the number of females in that specific period to yield progeny per female. The average pupae number per female \pm SEM was calculated across the 10 replicate vials. A preliminary version of this data was presented in a review article (Waskar et al. 2005), and the data is included here with further statistical analysis for comparison purposes.

Genetic screen for new *PdL* mutations

Virgin flies from strain *PdL45C1* were crossed to males from strain delta2-3. The male progeny containing both the *PdL45C1* insertion and delta2-3 were selected by their phenotypic markers and crossed to virgins of strain rtTA(3)E2. The virgin progeny flies containing a new *PdL* insertion and rtTA(3)E2 have the ability to over-express the gene immediately downstream of the *PdL* insertion, and ~8,000 of these mutants were generated and analyzed. Each individual mutant female was combined with three young Oregon-R male flies and cultured on -DOX food for the first 35 days of their life span. During this process the flies were transferred to fresh food vials every 2 days. At the end of the 35 day period, the flies were then cultured on -DOX food for 4 days ("time period 1"). The flies were then cultured on +DOX food for 8 days to allow any gene over-expression and phenotypes to become maximal. The flies were then cultured on +DOX food for an additional 4 days ("time period 2"). During these steps the flies were always transferred to fresh food vials every other day. Pupal number was counted in vials 10 days after transfer. Because control flies showed significant decrease in fecundity between time period 1 and time period 2, any mutants that did not show a significant decrease in fecundity in this interval were considered potential positives and were further characterized. The site of insertion of *PdL* was determined using inverse PCR, sequencing, and comparison between the *PdL* flanking sequence and fly genomic DNA databases using NCBI-BLAST, as previously described (Landis et al. 2001).

Creation of constructs and transgenic flies

The full-length cDNA clones for the *magu* and *hebe* genes were ordered from BACPAC (<http://bacpac.chori.org/>) (BACPAC Resources Center at Children's Hospital Oakland Research Institute in Oakland, California). The cDNA was amplified from its clone vector POT1. For *magu* the forward primer was 5'-TGACGAATTCGAACTGCT AAG-3'; for *hebe* the forward primer was 5'-TTCAAAGG CAGACAGACATGG-3'; the reverse primer anneals to the POT1 vector and was 5'-CGTTAGAACGCGGCTACAA TT-3'. The amplified cDNAs were ligated into the USC1.0 vector that contains the mini-*white*⁺ marker gene (Allikian et al. 2002) to create P{magu} and P{hebe} transformation constructs, respectively. Multiple independent transgenic strains were generated for each construct by embryo micro-injection using standard protocols (Rubin and Spradling 1982) and the *y-ac-w* recipient strain (Patton et al. 1992).

Northern blot

Northern blot assay was performed essentially as previously described (Landis et al. 2003). Briefly, strains bearing a *PdL* insertion or a transgene insertion were crossed with *rtTA(3)E2* flies. The male and female progeny were collected separately and cultured on +DOX and -DOX food for 10 days. Total RNA was extracted from 30 flies of each group using Trizol kit (Invitrogen), resolved on 2% agarose gels, and then transferred to Genescreen membrane (Dupont/NEN). Restriction fragments from the cDNA clones of the *magu* and *hebe* genes were radiolabelled and used as gene-specific probes. Ribosomal protein 49 (*Rp49*) probe was used as loading control. Transcript size was determined by comparison with 1 Kb RNA ladder (Gibco-BRL) according to the manufacturer's instructions.

Female fecundity assays

Female fecundity was assayed in a longitudinal protocol analogous to the screening procedure described above, except that multiple flies (four vials each containing four females) were used to provide replication and statistical inference (Fig. 1c). Female fecundity was also assayed in a parallel protocol, which involved four replicate vials, each containing four females and four wild-type males, for each of +DOX and -DOX conditions. Flies were transferred to fresh vials every other day. For the experiments with DOX administered beginning on day 1 (Fig. 5), the progeny numbers (pupae) were recorded in the old vials in the time intervals between day 10 and 20 ("time period 1") and between day 30 and 40 ("time period 2"). For the experiments with DOX administered beginning on day 40 (Fig. 6), the progeny numbers (pupae) were recorded in the old vials in the time intervals between day 35 and 39 ("time period 1") and between day 45 and 49 ("time period 2"). The average progeny per female for each time interval was calculated, and +DOX values were compared to -DOX values using unpaired, two-sided *t* tests. For certain experiments, the final concentration of DOX in the food was diluted 1:5 and 1:25 as indicated.

Egg hatch assay

The fraction of eggs hatching was assayed for the two strains in which *magu* cDNA over-expression caused decreased fecundity, P{*magu*}102 and P{*magu*}103. These strains were crossed to driver *rtTA(3)E2* to generate progeny containing both constructs, while controls were generated by crossing *rtTA(3)E2* to *w[1118]* to generate progeny containing only *rtTA(3)E2*, which are referred to in the text as "Control *w[1118]*". Progeny females were collected and at 4 days of age they were mated to Oregon R wild type males, and flies were cultured on food with and without DOX for 10 days, with transfer to fresh food every other day. Forty females plus ten males were then placed in an inverted bottle with a charcoal plate at the bottom overnight (12 h total). The dark charcoal plates facilitate visualization of eggs and hatched eggs. The bottles contained a cotton-stoppered hole for air exchange. Three replicate bottles were used for each condition. The charcoal plates were removed and scored for total number of eggs laid, and after an additional 12 h the plates were scored for the number of hatched eggs. Average and SD were calculated using the triplicate plates, and for each genotype the -DOX value was compared to the +DOX value using unpaired, two-sided *t* tests. We note that the flies lay significantly fewer eggs in the over-night charcoal plate collection assay than during day-long collections of eggs in culture vials.

Life span assays

Each transgenic strain was crossed to driver *rtTA(3)E2* to generate progeny containing both constructs, while controls were generated by crossing *rtTA(3)E2* to *w[1118]* to generate progeny containing only *rtTA(3)E2*, which are referred to in the text as "Control *w[1118]*", and by crossing *rtTA(3)E2* to Oregon R to generate progeny containing only *rtTA(3)E2*, which are referred to in the text as "Control Or-R". Life span assays were performed at 25°C

with passage to fresh food vials every other day as previously described (Ford et al. 2007). Each cohort consisted of ~125 flies at ~25 flies per vial, and median life spans of +DOX samples were compared to -DOX controls using log-rank tests.

Protein motif analyses

The protein motif search was performed using four different databases: InterPro (<http://www.ebi.ac.uk/interpro/>), GenomeNet (<http://www.genome.jp/>), ScanProsite (<http://expasy.org/cgi-bin/scanprosite>), and Sanger Institute Pfam (<http://pfam.sanger.ac.uk/>).

Results

Screening for gene over-expression mutations that increase late-age female fecundity using *PdL*

In *Drosophila*, female fecundity peaks in the first 2 weeks, and then declines dramatically with advancing age (Fig. 1) (Waskar et al. 2005; Zhao et al. 2008). To identify genes that might positively regulate both fecundity and life span, *PdL* mutagenesis was employed to create gene over-expression mutations. The *PdL* P element contains an outwardly directed, DOX-regulated promoter that will cause over-expression of genes located downstream of the 3' end of the element. The rtTA(3)E2 transgenic strain contains a construct in which the powerful, tissue-general promoter from the cytoplasmic actin gene (*actin5C*) drives expression of the artificial transcription factor rtTA. The rtTA transcription factor is activated upon interaction with DOX, and then binds to specific sites (Tet-operator sites) in the *PdL* promoter, thereby mediating DOX-inducible transcription. In this way feeding DOX to a fly containing both *PdL* and rtTA(3)E2 can cause robust over-expression of a gene located 3' to *PdL* in all of the somatic tissues of the fly.

Strain *PdL*[45C1] contains an insert of *PdL* on the second chromosome, which is also marked with the dominant mutation *Sternopleural* (*Sp*). Virgins of this strain were crossed to males from strain delta2-3, which expresses P element transposase ("Cross 1"; Fig. 1b). The male progeny containing both the *PdL*[45C1] insertion and delta2-3 were selected by their phenotypic markers. In these males the transposase will cause transposition of *PdL* to new sites in the genome in the germ line cells, and therefore these males are called "dysgenic". The dysgenic males were crossed to virgins of strain rtTA(3)E2 ("Cross 2"; Fig. 1b) to produce mutant female progeny bearing a new insertion of *PdL* on either the first, second or third chromosomes (indicated by asterisk), as well as the rtTA(3)E2 driver. The new insertions were identified by the presence of the mini-*white*⁺ marker gene in *PdL*, and the absence of the *Sp* mutation marking the chromosome bearing the starting *PdL* insertion. Approximately 8,000 of these mutant females were generated and analyzed. The mutant females were combined with Oregon-R male flies and cultured on -DOX food for the first 35 days of their life span. During this process the flies were transferred to fresh food vials every 2 days. At the end of the 35 day period, the female flies were then individually cultured in the presence of 3 Oregon-R wild type males on -DOX food for 4 days ("time period 1"), to allow measure of progeny production. The flies were then cultured on +DOX food for 8 days to allow any gene over-expression and phenotypes to become maximal. The flies were then cultured on +DOX food for an additional 4 days ("time period 2") to again allow measure of progeny production. During these steps the flies were always transferred to fresh food vials every other day. The number of progeny produced by the mutant females (pupae) was counted in each vial 10 days after transfer. Because control flies showed significant decrease in fecundity between time period 1 and time period 2 (Fig. 1c), any mutant females that did not show a significant decrease in fecundity in this interval were considered potential positives and were further characterized. Eight mutant females were identified where there was little or no decrease in fecundity between time period 1 and time

period 2. These eight promising new *PdL* insertions were balanced, and re-tested for fecundity effects using replicate females (Fig. 1c), and also tested for effects on life span (described below). Two mutants consistently gave positive results and were further pursued, hereafter referred to as P{PdL}magu and P{PdL}hebe, named for immortal deities from Chinese and Greek mythology, respectively.

Conditional over-expression of *hebe* and *magu* cDNAs

Inverse PCR was used to clone the genomic DNA immediately adjacent to the 3' end of the *PdL* insertions in lines P{PdL}hebe and P{PdL}magu. Comparison of the flanking sequences to the GenBank database revealed that both inserts are located immediately upstream of the transcriptional start site for the respective genes (Fig. 2a): P{PdL}hebe is inserted 6,721 bp upstream of the translational start site (ATG) for gene CG1623 (*hebe*), while P{PdL}magu was located in the first intron of gene CG2264 (*magu*), 4,646 bp upstream of the translation start site. In both cases the 3' end of *PdL* was oriented towards the open reading frame, suggesting that DOX would cause over-expression of functional, full-length proteins. Full-length cDNA clones for both *hebe* and *magu* were obtained from a commercial source (BACPAC Resources Center at Children's Hospital Oakland Research Institute). Northern blot analysis confirmed the DOX-dependent over-expression of both the *hebe* and *magu* genes in the P{PdL}hebe and P{PdL}magu mutant strains, in both male and female adult flies (Fig. 3a, b).

To confirm the phenotypic effects of *hebe* and *magu* over-expression, the corresponding cDNAs were cloned into the USC1.0 vector, to allow for DOX-dependent over-expression in an otherwise wild-type genetic background (Fig. 2c). Multiple independent lines were generated for each over-expression construct (Table 1), and Northern blot analysis confirmed DOX-dependent over-expression of the transgenes in both male and female adult flies (Fig. 3c, d). The *magu* gene was also found to be expressed throughout the body of wild type flies (Fig. 3e).

Ubiquitous over-expression of *hebe* and *magu* in adults increases male and female life span

The rtTA(3)E2 ubiquitous driver was used to cause DOX-dependent over-expression of *hebe* and *magu* genes in adult flies. The original P{PdL}hebe and P{PdL}magu insertions were assayed, along with multiple independent cDNA over-expression construct insertions for each gene, in replicate experiments. Representative survival curves are presented (Fig. 4), and the statistical analysis is presented for each strain and replicate experiment (Table 2). Control genotypes were generated by crossing the Oregon-R wild-type and w[1118] injection strains to the rtTA(3)E2 ubiquitous driver strain, to generate progeny expressing rtTA but containing no target transgene. In these controls DOX treatment caused no significant change in life span of either male or female flies (Fig. 4a, b; Table 2). The P{PdL}hebe mutation yielded a 31% increase in median life in males and a 24% increase in mean life span in males. In females the P{PdL}hebe mutation yielded a 2.2% increase in median life span and a 11% increase in mean life span. The three independent *hebe* cDNA over-expression strains yielded increases in median life span in males ranging from 7 to 26%, while increases in females ranged from 6 to 23%. Similar but somewhat more variable results were obtained upon ubiquitous over-expression of *magu*. The P{PdL}magu mutation yielded 9.3% increase in median life in males and a 5.3% increase in mean life span in males. In females the P{PdL}magu mutation yielded a 2.2% increase in median life span and a 5.3% increase in mean life span. Five independent *magu* cDNA over-expression construct insertions were assayed in the first experiment, and three of these (P{magu}102, P{magu}67-1, and P{magu}39) yielded increases in median life span in both males and females ranging from 2 to 18%. In contrast line P{magu}103 yielded 14% increase in

median life span in males but no increase in females, while line P{magu}67-2 gave no increase in either sex. In the second experiment with the ubiquitous rtTA(3)E2 driver, the two *magu* cDNA lines tested (P{magu}102 and P{magu}67-1) each gave increases in median life span in both males and females, ranging from 2.6 to 21%. The specific chromosomal site of insertion of P element constructs can affect expression levels in various tissues, and we hypothesize that this may be why results varied for the five independent *magu* cDNA strains. However, two of the *magu* cDNA strains, P{magu}102 and P{magu}67-1, yielded significant increases for both males and females in the replicate experiments. Therefore, we conclude that while life span effects varied considerably across strains and experiments, ubiquitous over-expression of both *hebe* and *magu* can increase life span in both male and female adult flies.

Over-expression of *hebe* and *magu* preferentially in adult motor-neurons increases female life span

We have recently described a system consisting of three transgenic constructs that allows for tissue-specific, DOX-dependent transgene expression that can be modulated over three orders of magnitude (Ford et al. 2007). The D42 driver is a well-characterized GAL4/UAS system driver that yields expression of the yeast GAL4 transcription factor throughout the developing nervous system and preferentially in motorneuron tissue in adult flies (Parkes et al. 1998). The UAS-rtTA^{m2alt901} insertion (Stebbins et al. 2001) is a “bridge construct” that is regulated by GAL4 to yield expression of rtTA only in those same tissues. Finally, as discussed above, the Tet-on promoter in *PdL* (or USC1.0 for the cDNA constructs) will be activated by rtTA only in the presence of DOX. Therefore, the combination of these three constructs yields DOX-inducible expression of transgenes in adult flies, preferentially in motorneuron tissue (Ford et al. 2007).

The control genotype for the motorneuron expression experiments contained the motorneuron driver and the bridge construct, but no target construct, and in these flies there was no significant change in life span in males or females in the replicate experiments (Fig. 4i; Table 3). Two independent *hebe* cDNA lines were tested, P{hebe}DX2 and P{hebe}AX, and in the first experiment these lines yielded increases in median life span in females of 22 and 38%, respectively, while no significant effect was obtained in males. Two independent *magu* cDNA over-expression strains were also tested, P{magu}102 and P{magu}67-1, and these lines yielded increases in median life span in females of 28 and 28%, respectively, and no significant alteration in life span in males. Therefore, in the first experiment utilizing the D42 motorneuron driver, both *hebe* and *magu* over-expression increased life span in female flies but not male flies. In contrast, in the replicate experiment, both the *hebe* and *magu* cDNA over-expression constructs yielded increases in median life span in both males and females, ranging from 9 to 37%. In summary, while the life span increases varied considerably across strains and experiments, the conditional over-expression of *hebe* and *magu* in adult motorneuron tissue consistently increased life span in females, with less consistent increases observed in males.

Ubiquitous over-expression of *hebe* and *magu* in adult females modulates fecundity

To confirm effects of *hebe* and *magu* gene over-expression on adult female fecundity, the original *PdL* mutations, P{PdL}hebe and P{PdL}magu, as well as two independent cDNA expression lines for each gene were ubiquitously over-expressed in adult females, using varying amounts of DOX to vary the degree of over-expression. Adult females were cultured on –DOX media and media supplemented with varying concentrations of DOX, beginning at day 1 of age and continuing throughout the adult life span. Female fecundity was assayed at two time periods: day 10–day 20 (time period 1), and from day 30 to day 40 (time period 2) (Fig. 5). The control genotype contained the rtTA(3)E2 driver and no target

construct, and in these control flies DOX had no significant effect on female fecundity at either time point (Fig. 5a). In contrast the P{PdL}magu insertion increased female fecundity at late ages (Fig. 5b), consistent with the screening procedure by which it was identified above. The *magu* cDNA over-expression line P{magu}67-1 also increased female fecundity at late ages, however, in this case there was an apparent trade-off with reduced female fecundity at the earlier time point (Fig. 5c). Interestingly the *magu* cDNA line P{magu}102 caused dose-dependent decreases in female fecundity at both time points (Fig. 5d), and similar reductions were obtained with line P{magu}103 (data not shown). The reduced fecundity caused by over-expression of P{magu}102 and P{magu}103 was due primarily to maternal-effect lethality, in that relatively normal quantities of morphologically normal-appearing eggs were laid (Fig. 5h), but the majority of these eggs failed to hatch (Fig. 5i). The maternal-effect lethal phenotype observed with lines P{magu}102 and P{magu}103 may be because these lines yielded higher and perhaps toxic levels of cDNA over-expression in adult females; in contrast the P{PdL}magu and P{magu}67-1 lines that produced increased female fecundity were associated with more modest increases in *magu* expression (Fig. 3c). For the original P{PdL}hebe mutation, the change in late-age female fecundity did not reach statistical significance in this experiment (Fig. 5e). However, the *hebe* cDNA line P{hebe}DX2 did yield increased fecundity at late ages (Fig. 5f), and each *hebe* over-expression line was associated with decreased fecundity at the earlier time point, demonstrating that *hebe* over-expression can modulate female fecundity.

To determine if late age fecundity could be increased without a trade-off with earlier age fecundity, the fecundity assay experiment was repeated with DOX administration and gene over-expression initiated only at age 40 days (Fig. 6). Female fecundity was assayed at two time periods: day 35–day 39 (time period 1), and from day 45 to day 49 (time period 2). The control genotypes contained the rtTA(3)E2 driver and no target construct, and in these control flies DOX had no significant effect on female fecundity at either time point (Fig. 6a, b). In contrast, the original P{PdL}magu insertion and three independent *magu* cDNA lines, as well as the original P{PdL}hebe mutation and two independent *hebe* cDNA lines, all increased female fecundity at late ages at one or more DOX concentrations (Fig. 6c–i). As expected fecundity was not affected at time point 1 for any of the transgenic lines, since time point 1 was prior to drug administration. The late-age administration of drug had no negative effect on female survival (Fig. 6j–l), and in fact for P{PdL}magu survival was increased. These data demonstrate that over-expression of *hebe* and *magu* at late ages could increase female fecundity with no detectable trade-off with early age fecundity or life span.

Discussion

In this study 8,000 gene-over-expression mutations were generated in *Drosophila*, and screened for effects on late-age female fecundity. Two genes were identified; *hebe* and *magu*, that when over-expressed in adult flies could increase late-age female fecundity and extend life span. These reagents should be useful for the further study of aging mechanisms, and the results provide additional evidence against the existence of an obligatory trade-off between reproduction and longevity.

Because *PdL* gene over-expression mutations are both conditional (DOX-dependent) and dominant (only one copy of the gene needs be mutated), it means that new mutations do not need to be made homozygous, and phenotypes can be assayed in each mutant (F1) female (Khokhar et al. 2008). In the mutagenesis strategy employed here each mutant F1 female bearing a new *PdL* insertion was assayed longitudinally for fecundity phenotype before and after induction of gene over-expression by DOX. In this way the mutations of interest could be recovered from the progeny produced by the female prior to DOX treatment and induction of any phenotypes. However, even with this efficiency only 8,000 females have

been screened so far, meaning that only a fraction of the ~14,000 *Drosophila* genes have been tested, and therefore it is likely that additional genes remain to be identified that can positively affect both fecundity and longevity.

A negative correlation between reproduction and life span is often observed when comparing different species and different individuals within a species, suggesting the existence of a “cost of reproduction” (Williams 1966; Partridge et al. 2005). One idea is that the cost of reproduction results from a trade-off, in which limiting metabolic resources are partitioned between reproduction and somatic maintenance pathways required for optimal longevity (Kirkwood 2005). One way to test for such a trade-off is to experimentally alter life span and ask what is the effect on reproduction. For example, inhibiting the IIS pathway in *C. elegans* (Johnson et al. 1993; Kenyon et al. 1993; Dillin et al. 2002) or over-expressing dFOXO in adult *Drosophila* (Hwangbo et al. 2004) can increase life span without reducing reproduction, suggesting there is no obligatory cost of longevity. The other way to test for the trade-off is to experimentally manipulate reproduction and ask what is the effect on life span. Previous studies have involved experimentally decreasing reproduction (Sgro and Partridge 1999; Mair et al. 2004). However, experimental designs involving reduced reproduction provide limited inference, because reducing reproduction might not necessarily result in resources being redirected towards somatic maintenance in a usable way; reproduction might be decreased for a novel or pathological reason not related to processes that normally limit life span (Partridge et al. 2005). In contrast, the strategy employed in the present study works in the opposite direction: here P{PdL}magu over-expression throughout adulthood, as well as magu and hebe over-expression starting at late ages, could increase female reproduction with no detectable cost to life span, in fact life span was also increased. These results provide strong additional arguments against the existence of an obligatory trade-off between reproduction and life span (Partridge et al. 2005), and support the idea that aging-related mortality and fecundity decline may have a common underlying mechanism.

The magu gene sequence is most related to a human gene that stimulates angiogenesis, called SMOC2 (secreted modular calcium-binding protein 2) (Vannahme et al. 2003; Rocnik et al. 2006), including conserved EF-hand calcium-binding domains and a thyroglobulin type-1 repeat signature (Fig. 2). Angiogenesis is impaired during aging in humans and other organisms, and this decline may be related to senescence of the vascular endothelial cells, which must continually divide to maintain existing vessels and support new vessel growth (Reed and Edelberg 2004). SMOC2 is located in the extracellular matrix and enhances the stimulatory effects of growth factors on mammalian cell proliferation. One hypothesis is that in flies magu could act through a similar mechanism, and favor late-age fecundity by stimulating stem cell division in the gonads. In a recent study the *Drosophila* genome was screened for genes that are preferentially expressed in the stem-cell region of the testes, and the most enriched gene identified was indeed magu (CG2264) (Terry et al. 2006). If Magu protein is in fact extracellular, as suggested by its sequence and similarity to mammalian SMOC2, this would place Magu in an appropriate location to function as part of the stem cell niche. For hebe, sequence homology searches revealed that related genes of unknown function exist in *Drosophila pseudoobscura* (GA14053) and *Drosophila simulans* (CG1623), however, no additional hebe-related genes could be found in other *Drosophila* species or in any other genus. Moreover, no conserved protein motifs or possible functions could be identified for hebe. The one report of a mutant phenotype for hebe is for a P element insertion in the gene associated with decreased male aggression (Edwards et al. 2006).

It will be of interest in the future to determine how magu over-expression might be acting to increase life span. The preferential expression of magu in the gonad stem cell regions is consistent with its effects on fecundity; however, the gonad stem cells do not appear to be

the major determinant of animal longevity (Barnes et al. 2006; Flatt et al. 2008). Both the gut (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006; Choi et al. 2008) and the malpighian tubule (analogous to mammalian kidney) (Singh et al. 2007) are maintained by stem cell division in the adult fly, and the possible role of these stem cell populations in life span is unknown. Northern analysis revealed that *magu* is expressed in other tissues of the fly (Fig. 3e), and a previous genome-wide micro-array analysis of RNA levels in adult *Drosophila* tissues indicated that, in addition to ovary and nervous tissue, there was an enrichment for *magu* (*CG2264*) expression in the gut and malpighian tubule (Chintapalli et al. 2007), consistent with a correlation with stem cell locations.

It is notable that over-expression of *magu* and *hebe* preferentially in adult motor-neuron tissue was sufficient to produce increased life span. A small number of interventions have been shown to increase life span in *Drosophila* and other model organisms. These interventions include reduced insulin/IGF1-like signaling (IIS) (Clancy et al. 2001; Tatar et al. 2001; Kenyon 2005; Selman et al. 2008), activation of the IIS target transcription factor FOXO (Giannakou et al. 2004; Hwangbo et al. 2004), dietary restriction (DR) (Piper and Partridge 2007), reduced expression of mitochondrial genes during development (Dillin et al. 2002; Wolff and Dillin 2006), and altered redox homeostasis such as by over-expression of enzymes including SOD (Parkes et al. 1998; Sun and Tower 1999; Sun et al. 2002, 2004; Curtis et al. 2007; Hu et al. 2007). Interestingly, similar to *magu* and *hebe*, over-expression of Cu/ZnSOD preferentially in adult motor-neuron tissue is sufficient to extend life span (Parkes et al. 1998), with a preferential effect in females (Spencer et al. 2003). Future experiments will include further assay of tissue-specificity of effects of *magu* and *hebe* over-expression, for example by targeting over-expression to specific stem cell populations using the tissue-specific, DOX-regulated system (Ford et al. 2007). In addition it will be important to test whether the mechanism for life span increase might be related to one of the known pathways affecting life span.

Acknowledgments

This work was supported by a Sr. Scholar Award to JT from the Ellison Medical Foundation, and a grant from the Department of Health and Human Services to JT (AG11833).

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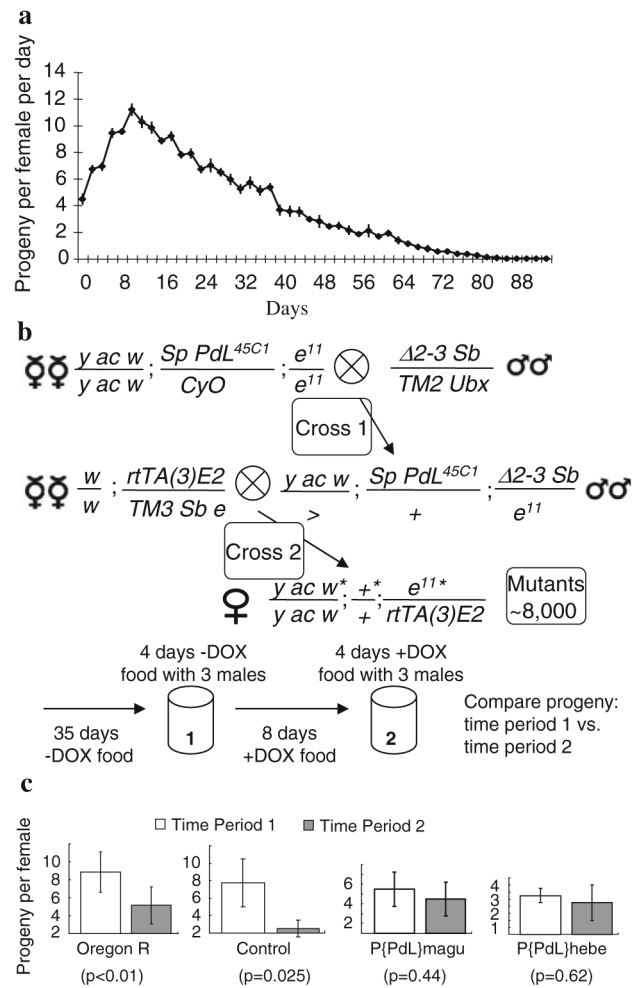


Fig. 1. Isolation of *hebe* and *magu* gene over-expression mutations. **a** Female fecundity of Oregon-R wild-type flies. Data are plotted as progeny per female per day versus age in days; *error bars* indicate SEM. **b** Genetic screen strategy. The starting *PdL* P element insertion on the second chromosome (*PdL*^{45C1}) was crossed to the *delta2-3* transposase source (cross 1). Male progeny containing both *PdL*^{45C1} and *delta2-3* were crossed to third-chromosome balancer strain virgins (cross 2). Female progeny bearing a new insertion of the *PdL* element on either the first, second or third chromosome (indicated by *asterisk*; ~8,000 total) were identified by presence of the *mini-white*⁺ marker gene in *PdL*. Each female was mated to three Oregon-R males and cultured for 35 days on -DOX food. Progeny production was then assayed for the next 4 days (“time period 1”) with addition of fresh, young Oregon-R males. The flies were then transferred to +DOX food for 8 days to induce any gene over-expression. Progeny production was then assayed for the next 4 days on +DOX food (“time period 2”). For females where the progeny count in time period 2 was equal to or greater than time period 1, the new *PdL* insertion was recovered from the progeny from time period 1. **c** Progeny per female per day during time period 1 and time period 2 for longitudinal assays of replicate flies of Oregon-R wild-type, Control (Oregon R x *rtTA(3)E2*), P{PdL}*magu* and P{PdL}*hebe* strains, as indicated

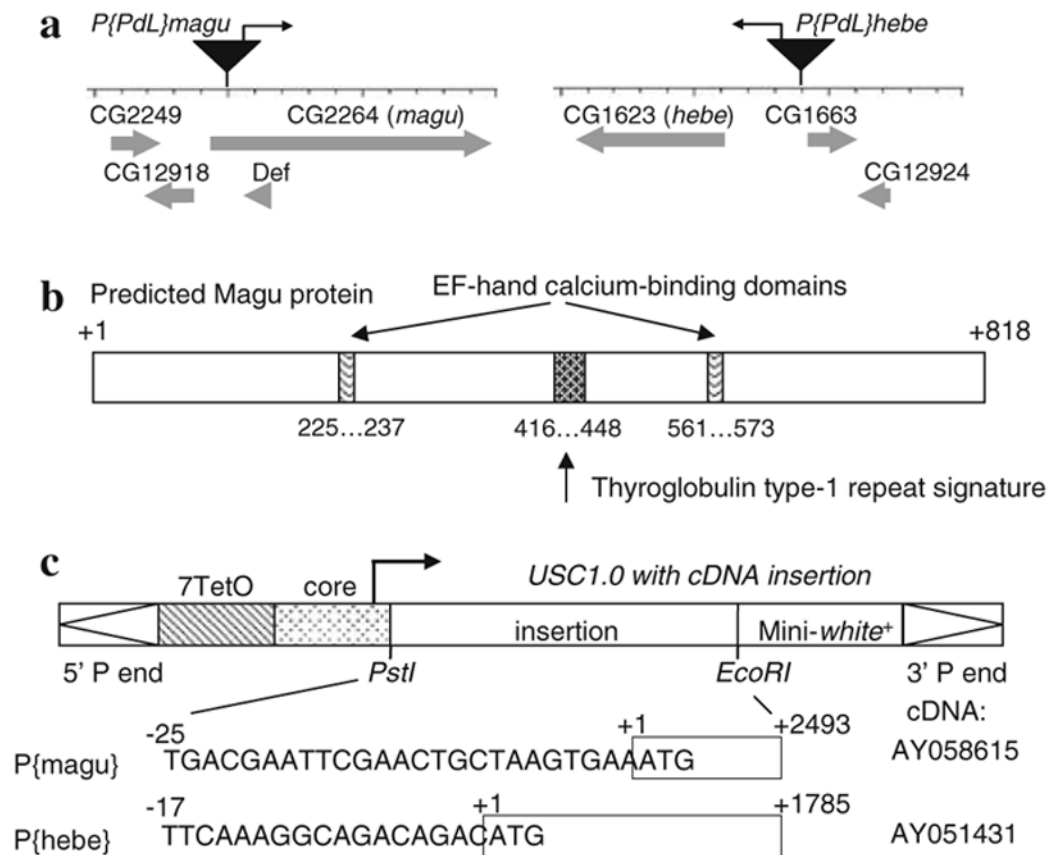


Fig. 2. Diagram of *magu* and *hebe* loci and transgenic constructs. **a** The location and orientation of the *P{PdL}magu* and *P{PdL}hebe* insertions relative to adjacent genes. **b** Protein motifs in the predicted Magu protein. **c** The over-expression constructs *P{magu}* and *P{hebe}*, generated by cloning of the indicated cDNA clones into the *USC1.0* vector

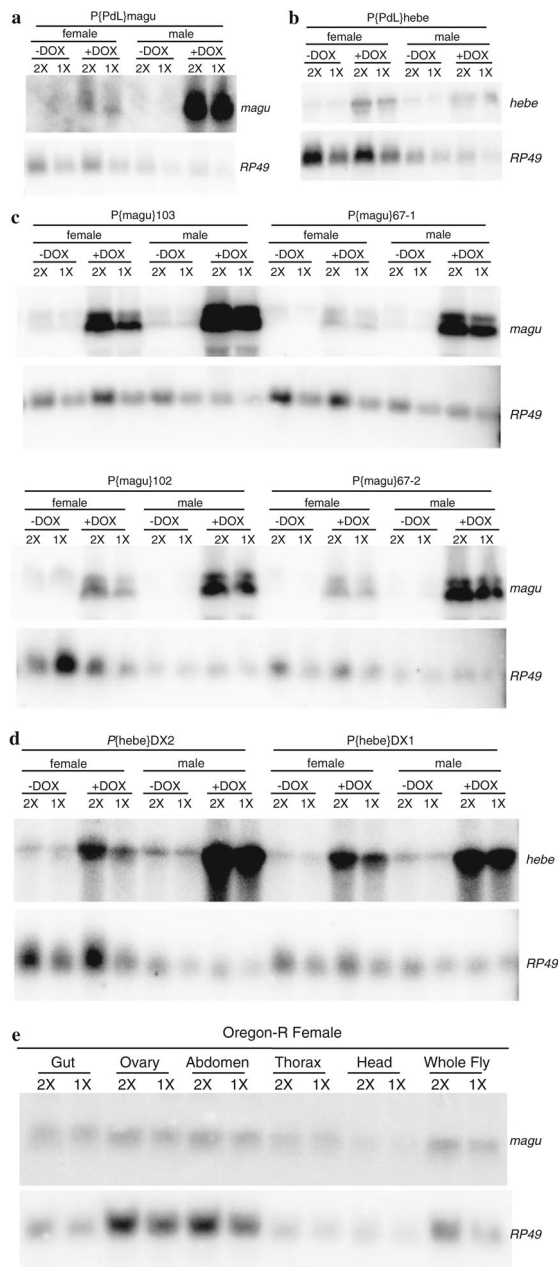


Fig. 3. Northern analyses. (a–d) DOX-dependent gene over-expression. The indicated strains were crossed to the rTA(3)E2 ubiquitous driver and male and female progeny containing both constructs were cultured as adults for 1 week, in the presence and absence of DOX, as indicated. Total RNA was isolated from 30 flies of each type, and 4 μ g (“1X”) and 8 μ g (“2X”) of each sample was fractionated and blotted, as indicated. Blots were hybridized sequentially with probes specific for genes *magu*, *hebe*, and *Rp49* loading control, as indicated. **a** The original P{PdL}magu insertion. **b** The original P{PdL}hebe insertion. **c** Two independent transgenic insertions of the *magu* cDNA over-expression construct, P{magu}103 and P{magu}67-1, as indicated. **d** Two independent transgenic insertions of the *hebe* cDNA over-expression construct, P{hebe}DX2 and P{hebe}DX1, as indicated. **e** Expression of *magu* in dissected tissues from Oregon R wild-type flies, as indicated

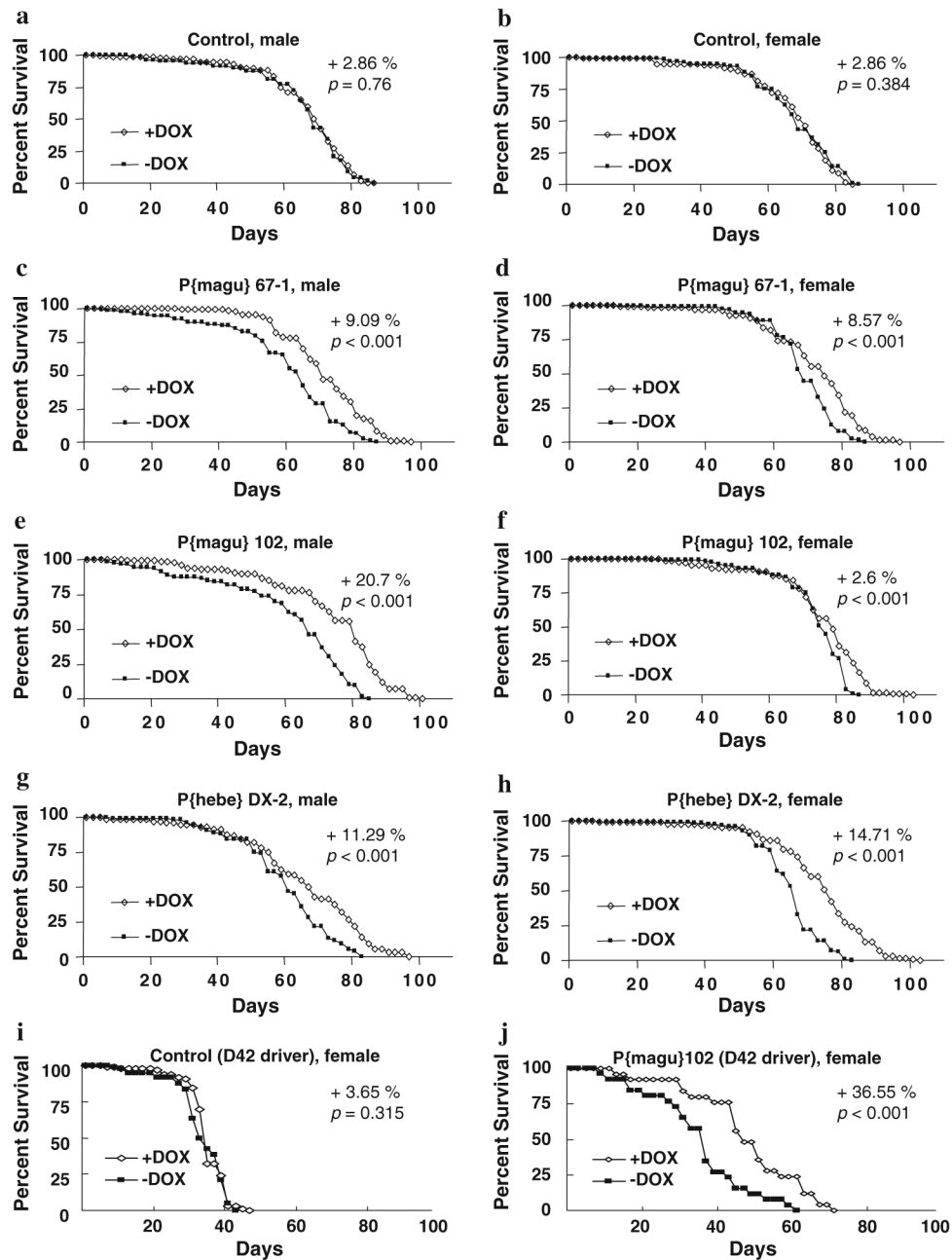


Fig. 4. Life span assays. The indicated strains were crossed to the rtTA(3)E2 ubiquitous driver (**a–h**) or D42 motor-neuron driver (**i, j**), and male (**a, c, e, g**) and female (**b, d, f, h, i, j**) progeny containing both constructs were cultured as adults, in the presence (open diamonds) and absence (*solid squares*) of DOX, as indicated. **a, b** Control flies; the progeny of Oregon-R wild-type crossed to rtTA(3)E2 driver. **c, d** P{magu}67-1 cDNA over-expression strain. **e, f** P{magu}102 cDNA over-expression strain. **g, h** P{hebe}DX2 cDNA over-expression strain. **i, j** The D42 motor-neuron driver crossed to w[1118] control (**i**) and P{magu}102 cDNA over-expression strain (**j**). The percent change in median life span +DOX is indicated, along with p value for log-rank test. Statistical data for these strains, additional independent strains, and replicate experiments is summarized in Tables 2 and 3

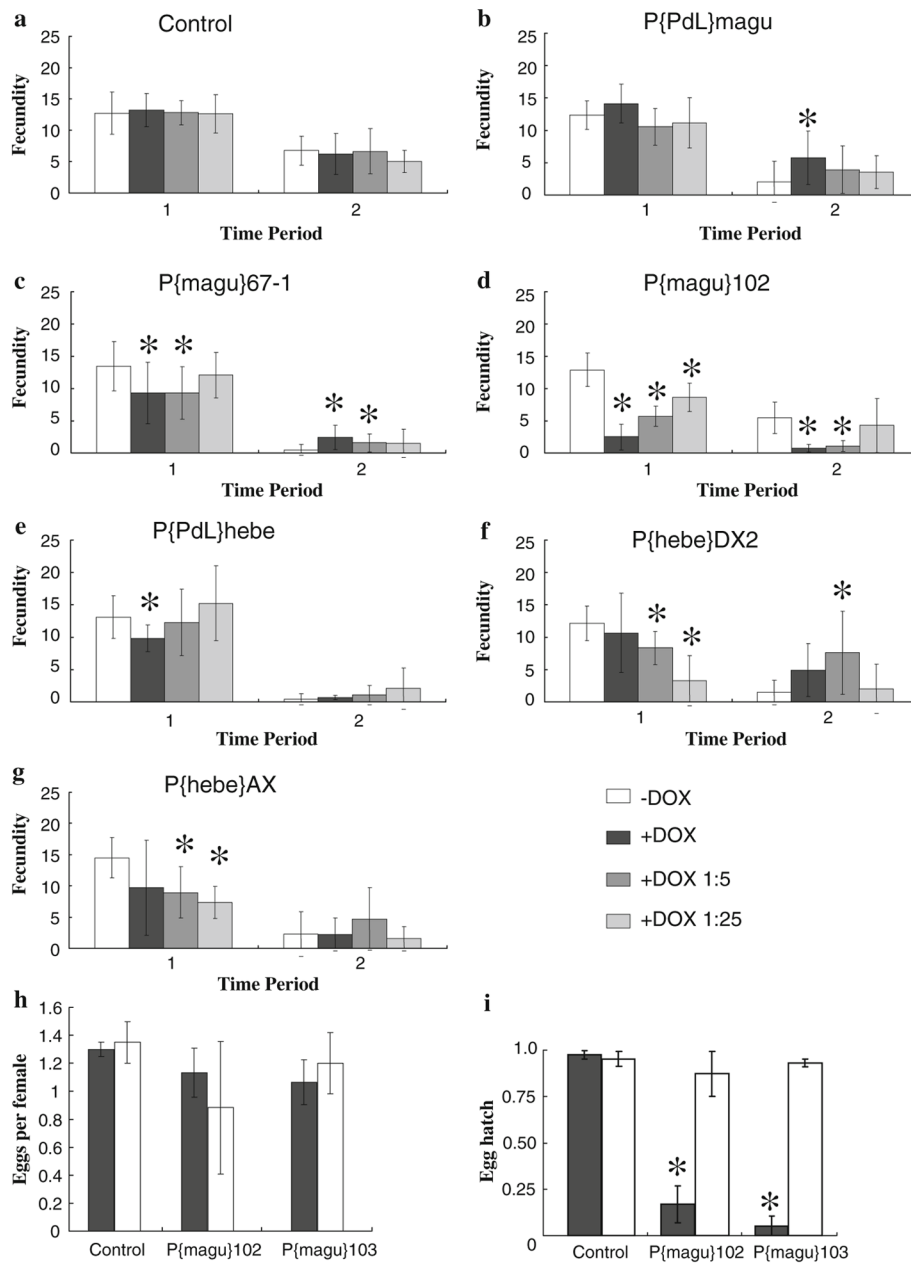


Fig. 5. Fecundity assay with gene over-expression throughout adulthood. **a–g** Production of viable progeny. The indicated strains were crossed to rt-TA(3)E2 ubiquitous driver and female progeny containing both constructs **b–g** and controls containing only the driver (**a**) were cultured as adults, in the absence of DOX (*white bars*), presence of DOX (*black bars*) and presence of DOX diluted 1:5 (*dark gray bars*) and DOX diluted 1:25 (*light gray bars*), as indicated. Drug treatment began on day one and continued throughout the adult life span. Fecundity (average pupal-stage progeny per female per day) is plotted for time period 1 (days 10–20) and time period 2 (days 30–40), as indicated. *Error bars* indicate standard deviation. The results for DOX-treated flies were compared to –DOX control using unpaired, two-sided *t* tests, and statistically significant changes ($p < 0.05$) are indicated with asterisks. **a** Control w[1118]. **b** P{PdL}magu. **c** P{magu}67-1. **d** P{magu}102. **e**

f P{PdL}hebe. **g** P{hebe}DX2. **h** Egg production for Control w1118, P{magu}102 and P{magu}103, in presence and absence of DOX, as indicated. **i** Fraction of eggs hatching for Control w[1118], P{magu}102, and P{magu}103, respectively, in presence and absence of DOX, as indicated. The data for **h** and **i** are from the same experiment

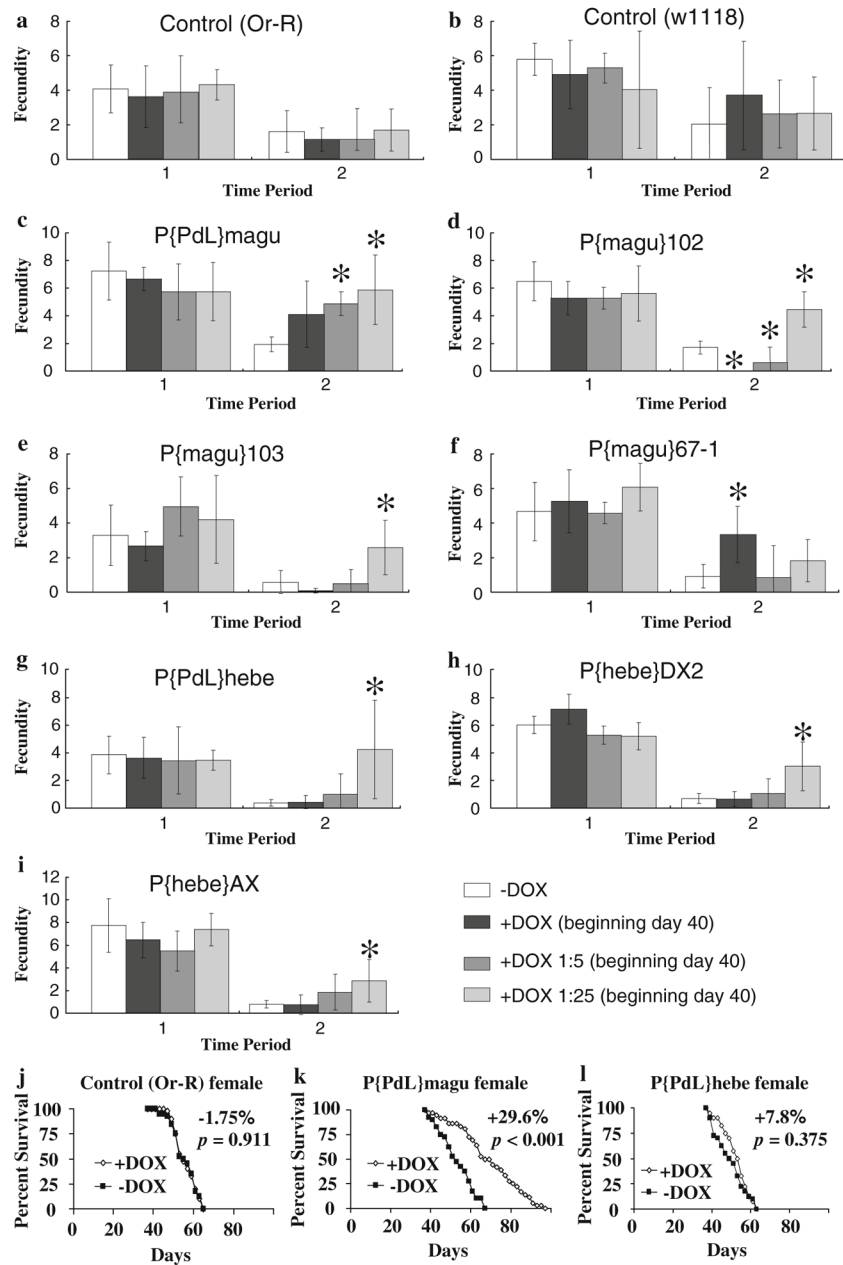


Fig. 6. Fecundity and life span with gene over-expression initiated at age 40 days. The indicated strains were crossed to rtTA(3)E2 ubiquitous driver and female progeny containing both constructs (**c–i, k, l**) and controls containing only the driver (**a, b, j**) were cultured as adults in the absence of DOX for 39 days. From day 40 onwards the flies were cultured in the absence of DOX (*white bars*), presence of DOX (*black bars*), presence of DOX diluted 1:5 (*dark gray bars*), and presence of DOX diluted 1:25 (*light gray bars*), as indicated. Fecundity (average pupal-stage progeny per female per day) is plotted for time period 1 (days 35–39) and time period 2 (days 45–49), as indicated. *Error bars* indicate standard deviation. The results for DOX-treated flies were compared to –DOX control using unpaired, two-sided *t* tests, and statistically significant changes ($p < 0.05$) are indicated with *asterisks*. **a** Control Or-R. **b** Control W[1118]. **c** P{PdL}magu. **d** P{magu}102. **e**

P{magu}103. **f** P{magu}67-1. **g** P{PdL}hebe. **h** P{hebe}DX2. **i** P{hebe}AX. (**j–l**) Survival data for flies cultured in the presence (*white diamonds*) or absence (*black squares*) of DOX from day 40 onwards. The percent change in median life span is presented, along with *p* value for log-rank tests. **j** Control (Or-R) females. **k** P{PdL}magu females. **l** P{PdL}hebe females

Table 1

Transgenic strains

Construct	Line name	Chromosome
PdL	magu	2nd
	hebe	2nd
P{magu}	39	2nd
	102	3rd
	103	2nd
	67-1	3rd
	67-2	3rd
	P{hebe}	DX1
DX2		2nd
AX		3rd

Table 2

Lifespan assay using ubiquitous driver rtTA(3)E2

Strain	DOX	Sex	Median	Mean	SD	Median (%)	Mean (%)	p value
Experiment 1								
w1118	+	M	92	87.2	14.5	0	0.12	0.448
w1118	-	M	92	87.0	14.9			
w1118	+	F	76	80.1	14.2	0.00	0.21	0.672
w1118	-	F	76	79.9	14.3			
Or-R	+	M	92	85.7	16.8	2.22	-0.69	0.678
Or-R	-	M	90	86.3	13.8			
Or-R	+	F	76	78.2	13.4	0.00	-2.04	0.977
Or-R	-	F	76	79.9	12.3			
P{PdL}magu	+	M	94	88.1	15.8	9.30	8.59	4.50E-06
P{PdL}magu	-	M	86	81.1	15.2			
P{PdL}magu	+	F	92	90.7	8.7	2.22	5.28	4.85E-05
P{PdL}magu	-	F	90	86.1	9.0			
P{PdL}hebe	+	M	92	88.8	15.6	31.43	24.21	9.10E-15
P{PdL}hebe	-	M	70	71.5	18.5			
P{PdL}hebe	+	F	92	90.7	10.9	2.22	11.25	1.02E-07
P{PdL}hebe	-	F	90	81.6	14.7			
P{magu}102	+	M	98	96.0	17.7	8.89	23.77	1.11E-16
P{magu}102	-	M	90	77.5	22.8			
P{magu}102	+	F	90	86.8	11.0	7.14	9.34	0.00176
P{magu}102	-	F	84	79.4	15.9			
P{magu}103	+	M	96	88.2	18.1	14.29	20.13	5.47E-05
P{magu}103	-	M	84	73.4	26.9			
P{magu}103	+	F	72	76.7	14.3	0.00	4.66	0.652
P{magu}103	-	F	72	73.36	19.9			
P{magu}67-1	+	M	96	93.6	17.8	2.13	11.64	6.79E-07
P{magu}67-1	-	M	94	83.8	20.7			
P{magu}67-1	+	F	90	86.9	13.1	18.42	13.85	8.91E-08
P{magu}67-1	-	F	76	76.3	15.4			

Strain	DOX	Sex	Median	Mean	SD	Median (%)	Mean (%)	p value
P{magu}67-2	+	M	86	84.6	15.8	-4.44	-3.91	0.439
P{magu}67-2	-	M	90	88.0	7.9			
P{magu}67-2	+	F	90	81.3	23.4	0.00	0.12	0.688
P{magu}67-2	-	F	90	81.2	18.8			
P{hebe}DX1	+	M	90	83.5	21.5	7.14	9.14	0.00028
P{hebe}DX1	-	M	84	76.5	20.9			
P{hebe}DX1	+	F	72	77.8	13.6	5.88	9.16	0.000506
P{hebe}DX1	-	F	68	71.2	14.8			
P{hebe}DX2	+	M	96	85.5	23.4	26.32	15.62	2.34E-10
P{hebe}DX2	-	M	76	74.0	21.3			
P{hebe}DX2	+	F	84	81.3	14.1	20.00	15.65	1.10E-09
P{hebe}DX2	-	F	70	70.3	13.5			
P{hebe}AX	+	M	92	88.6	15.3	21.05	12.60	3.24E-08
P{hebe}AX	-	M	76	78.6	15.1			
P{hebe}AX	+	F	86	83.1	14.6	22.86	11.80	6.16E-08
P{hebe}AX	-	F	70	74.3	14.7			
P{magu}39	+	M	86	85.0	20.1	7.50	-3.98	0.762
P{magu}39	-	M	80	88.5	18.1			
P{magu}39	+	F	94	88.7	16.7	4.44	0.67	0.0405
P{magu}39	-	F	90	88.1	11.9			
Experiment 2								
w1118	+	M	72	68.0	14.2	2.86	1.61	0.76
w1118	-	M	70	66.9	15.3			
w1118	+	F	72	68.2	14.1	2.86	-0.52	0.384
w1118	-	F	70	68.6	13.7			
P{magu}102	+	M	82	75.3	18.1	20.59	20.72	1.06E-12
P{magu}102	-	M	68	62.4	20.0			
P{magu}102	+	F	80	77.0	14.2	2.56	2.63	0.000502
P{magu}102	-	F	78	75.0	10.7			
P{magu}67-1	+	M	72	72.7	12.4	9.09	18.39	1.73E-07
P{magu}67-1	-	M	66	61.4	17.3			

Strain	DOX	Sex	Median	Mean	SD	Median (%)	Mean (%)	p value
P{magu}67-1	+	F	76	73.1	14.3	8.57	4.60	3.64E-05
P{magu}67-1	-	F	70	69.8	9.8			
P{hebe}DX-2	+	M	69	66.8	18.6	11.29	9.54	2.39E-05
P{hebe}DX-2	-	M	62	61.0	14.0			
P{hebe}DX-2	+	F	78	75.1	14.8	14.71	14.07	4.50E-13
P{hebe}DX-2	-	F	68	65.8	10.1			
P{hebe}AX	+	M	70	65.7	19.7	12.90	7.04	2.09E-05
P{hebe}AX	-	M	62	61.4	13.0			
P{hebe}AX	+	F	74	72.2	13.0	8.82	8.57	4.70E-07
P{hebe}AX	-	F	68	66.5	11.1			

Table 3

Life span assay using motor-neuron driver D42

Strain	DOX	Sex	Median	Mean	SD	Median (%)	Mean (%)	p value
Experiment 1								
w1118	+	M	38	35.8	11.1	11.76	0.00	0.809
w1118	-	M	34	35.8	11.1			
w1118	+	F	34	34.1	6.1	6.25	3.65	0.315
w1118	-	F	32	32.9	7.3			
P{magu}102	+	M	38	37.2	14.5	11.76	-0.53	0.884
P{magu}102	-	M	34	37.4	13			
P{magu}102	+	F	46	46.7	14.7	27.78	36.55	0.000893
P{magu}102	-	F	36	34.2	13.2			
P{magu}67-1	+	M	44	41.9	15.7	0.00	-3.68	0.547
P{magu}67-1	-	M	44	43.5	11.1			
P{magu}67-1	+	F	46	45.3	13.5	27.78	23.43	2.14E-06
P{magu}67-1	-	F	36	36.7	13.7			
P{hebe}DX2	+	M	38	39.4	12.9	11.76	10.36	0.127
P{hebe}DX2	-	M	34	35.7	13.9			
P{hebe}DX2	+	F	44	42.3	13.9	22.22	17.83	0.00121
P{hebe}DX2	-	F	36	35.9	10.9			
P{hebe}AX	+	M	28	32.7	14.1	-22.22	-8.91	0.187
P{hebe}AX	-	M	36	35.9	15			
P{hebe}AX	+	F	44	39.6	16	37.50	24.53	0.00887
P{hebe}AX	-	F	32	31.8	14.4			
Experiment 2								
w1118	+	M	50	48.4	10.5	0	-1	0.948
w1118	-	M	50	48.9	8.8			
w1118	+	F	50	51.7	7.6	-3.8	5.5	0.583
w1118	-	F	52	49.0	11.5			
P{magu}102	+	M	52	45.9	17.6	36.8	32.7	1.34E-07
P{magu}102	-	M	38	34.6	16.4			
P{magu}102	+	F	48	45.3	16.3	9.1	13.0	4.29E-07

Strain	DOX	Sex	Median	Mean	SD	Median (%)	Mean (%)	p value
P{magu}102	-	F	44	40.1	11.2			
P{magu}67-1	+	M	50	44.8	18.4	31.6	29.5	7.16E-08
P{magu}67-1	-	M	38	34.6	17.3			
P{magu}67-1	+	F	46	41.3	15.6	21.0	15.0	5.83E-07
P{magu}67-1	-	F	38	35.9	12.0			
P{hebe}DX2	+	M	50	48.4	12.4	19.0	17.8	2.46E-09
P{hebe}DX2	-	M	42	41.1	10.0			
P{hebe}DX2	+	F	54	48.4	16.6	17.4	15.2	1.75E-05
P{hebe}DX2	-	F	46	42	16.2			
P{hebe}AX	+	M	46	45.8	12.0	9.5	13.6	2.45E-06
P{hebe}AX	-	M	42	40.3	9.8			
P{hebe}AX	+	F	52	44.9	17.6	18.2	13.7	0.000207
P{hebe}AX	-	F	44	39.5	16.5			