

# A search for doxycycline-dependent mutations that increase *Drosophila melanogaster* life span identifies the *VhaSFD*, *Sugar baby*, *filamin*, *fwd* and *Cct1* genes

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## Abstract

**Background:** A P-type transposable element called *PdL* has been engineered with a doxycycline-inducible promoter directed out through the 3' end of the element. Insertion of *PdL* near the 5' end of a gene often yields doxycycline-dependent overexpression of that gene and a mutant phenotype. This functional genomics strategy allows for efficient screening of large numbers of genes for overexpression phenotypes.

**Results:** *PdL* was mobilized to around 10,000 new locations in the *Drosophila melanogaster* genome and used to search for genes that would extend life span when overexpressed. Six lines were identified in which there was a 5-17% increase in life span in the presence of doxycycline. The mutations were molecularly characterized and in each case a gene was found to be overexpressed using northern blots. Two genes did not have previously known phenotypes and are implicated in membrane transport: *VhaSFD* encodes a regulatory subunit of the vacuolar ATPase proton pump (H<sup>+</sup>-ATPase), whereas *Sugar baby* (*Sug*) is related to a maltose permease from *Bacillus*. Three *PdL* mutations identified previously characterized genes: *filamin* encodes the homolog of an actin-polymerizing protein that interacts with presenilins. *four wheel drive* (*fwd*) encodes a phosphatidylinositol-4-kinase (PI 4-kinase) and *CTP:phosphocholine cytidyltransferase-1* (*Cct1*) encodes the rate-limiting enzyme in phosphatidylcholine synthesis. Finally, an apparently novel gene (*Red herring*, *Rdh*) was found in the first intron of the *encore* gene.

**Conclusions:** Screening for conditional mutations that increase *Drosophila* life span has identified genes implicated in membrane transport, phospholipid metabolism and signaling, and actin cytoskeleton organization.

## Background

*Drosophila melanogaster* has been a leading model for the study of aging for over 80 years [1-5]. The intensive use of *Drosophila* as a model for developmental biology has

produced a wealth of genetic and molecular biological tools that are readily adapted to the study of aging. Aging is associated with characteristic changes at the physiological and molecular level, however organismal life span is still the best

measure of aging rate. The most successful studies of aging generally involve manipulations that increase life span. Experimental alterations of environment or genetic makeup that cause decreased life span might create novel diseases or pathologies that do not usually limit life span in a normal individual. In contrast, a manipulation that increases life span is thought to be more likely to identify mechanisms that normally limit the life span of the organism.

Laboratory selection of *Drosophila* populations for late-life reproduction results in extended life span [6-8]. The genetic selection experiments demonstrate that life span has a large genetic component and is highly plastic. Increased life span was generally correlated with increased stress resistance including increased oxidative stress resistance [9,10]. An important question in the study of aging in invertebrates is whether life span can be increased without a trade off with metabolic activity. Certain manipulations that decrease metabolic activity cause increased life span, such as lower culture temperature [11]. In the genetic selection experiments, life span is increased without a reduction in metabolic activity [12,13].

Single gene mutations have been identified that increase *Drosophila* life span. These mutations identify negative regulators of life span, as mutations expected to decrease activity of the gene lead to increased life span. *mth* encodes a protein related to G-protein coupled receptors, and appears to negatively regulate life span, stress resistance and body size [14]. *Indy* is related to a mammalian cotransporter involved in membrane transport of Krebs cycle intermediates, leading to the suggestion that *Indy* mutations might increase life span by decreasing the availability of nutrients [15].

Certain mutant alleles of either the *Inr* or the *chico* genes can increase *Drosophila* life span [16,17]. *Inr* is homologous to the mammalian insulin receptor and insulin-like growth factor receptor, while *chico* is homologous to the mammalian insulin receptor substrate. The data indicate that an insulin-like signaling pathway negatively regulates life span, as had previously been found for the nematode *C. elegans* [18]. This pathway for life span regulation may be even more highly conserved during evolution, as there are many similarities with life span regulation in mammals and in yeast. The increased life span regulated by the insulin-like signaling pathway in *C. elegans* is associated with increased resistance to stress, including oxidative stress [18-21]. This has led to suggestions that increased oxidative stress resistance and decreased amounts of oxidative damage may be the mechanism by which mutation of the insulin-like signaling pathway causes increased life span. Consistent with this idea, life-span extension in *C. elegans* is associated with increased expression of the gene encoding the mitochondrial antioxidant enzyme manganese-containing superoxide dismutase (MnSOD) [22]. In *Drosophila*, mutations of *chico* that extend life span

increase total SOD enzyme activity; however, resistance to oxidative stress was not detectably increased, at least not as assayed by paraquat resistance [16]. Therefore it remains to be determined if mutation of the insulin-like pathway in *Drosophila* increases life span by increasing SOD activity or by some other mechanism(s).

Increased life span caused by overexpression of a gene by definition identifies a positive regulator of life span. Several genes involved in oxidative stress defense have been found to increase life span when overexpressed in transgenic flies. These include genes for both forms of SOD, the Cu/ZnSOD found in the cytoplasm and outer mitochondrial space, and MnSOD found in the inner mitochondrial space [23-25]. These enzymes convert the most common oxygen radical produced by the mitochondria, superoxide, into hydrogen peroxide and water. Abundant catalase enzyme then converts the hydrogen peroxide into molecular oxygen and water. Overexpression of Cu/ZnSOD or MnSOD increases life span up to about 40%, and the amount of life span increase is found to be proportional to the amount of enzyme increase in each case. The life span increase caused by SOD overexpression is not associated with a detectable tradeoff in metabolic activity, as oxygen consumption was normal throughout the extended life span of the flies [24]. Overexpression of the antioxidant enzyme peptide methionine sulfoxide reductase is also associated with increased life span [26]. Finally, several genes implicated in repair and stress responses have been shown to be able to extend *Drosophila* life span when the flies are cultured at elevated temperatures [27-30].

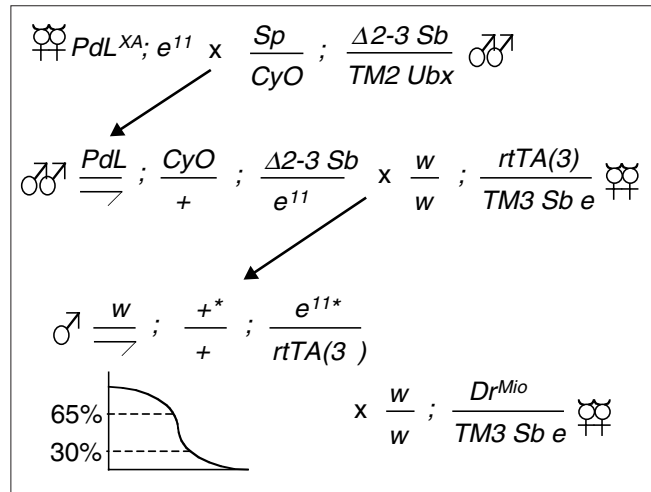
Testing specific genes by overexpression in transgenic flies is a candidate gene approach, meaning that the investigator must make an educated guess as to which genes are worth assaying by this relatively labor-intensive method. Recently genetic methods have been developed that allow for the efficient overexpression of random genes in *Drosophila*. P type transposable elements have been engineered to have transcriptional promoters directed out through the end of the element [31,32]. These elements will often insert upstream of gene coding regions, causing overexpression of the gene and mutant phenotypes. The *PdL* P element contains an outwardly directed tet-on promoter [33,34]. The transcription of this promoter is activated upon feeding the fly doxycycline, leading to conditional gene overexpression and conditional mutations. Conditional gene overexpression mutations have several potential advantages for study of aging and life span. By waiting until flies are young adults to activate gene overexpression, effects can be identified that are specific to adult aging. The system also provides powerful controls for genetic background effects, because control (no DOX) and over-expressing (+DOX) flies have identical genetic backgrounds and therefore any differences in life span must be due to DOX administration and gene overexpression. The *PdL* system allows the investigator to search for genes that can increase life span without having to guess ahead of time

which genes these might be. Therefore the approach has the potential to identify genes and pathways not previously known or suspected to be involved in life span regulation.

**Results**

In the *rtTA* transgenic construct the powerful and tissue-general *actin5C* promoter drives expression of the *rtTA* transcriptional transactivator [33]. When flies are fed doxycycline, *rtTA* binds to target sequences in the synthetic tet-on promoter in the *PdL* P element. This outwardly-directed promoter often causes overexpression of genes near the *PdL* insertion site and yields various mutant phenotypes [34]. *PdL* is 8.4 kilobases (kb) long and contains the P element inverted repeats, the mini-*white+* transformation marker gene, and the 500 base-pair (bp) tet-on promoter directed out through the 3' end of the element. The tet-on promoter contains no open reading frame (ORF) or translation initiation sequences. Therefore *PdL* must insert upstream of an endogenous translation initiation site (ATG) to cause overexpression of a protein. The transcripts initiated within *PdL* will therefore contain 280 bp of 5' untranslated sequence derived from *PdL* itself.

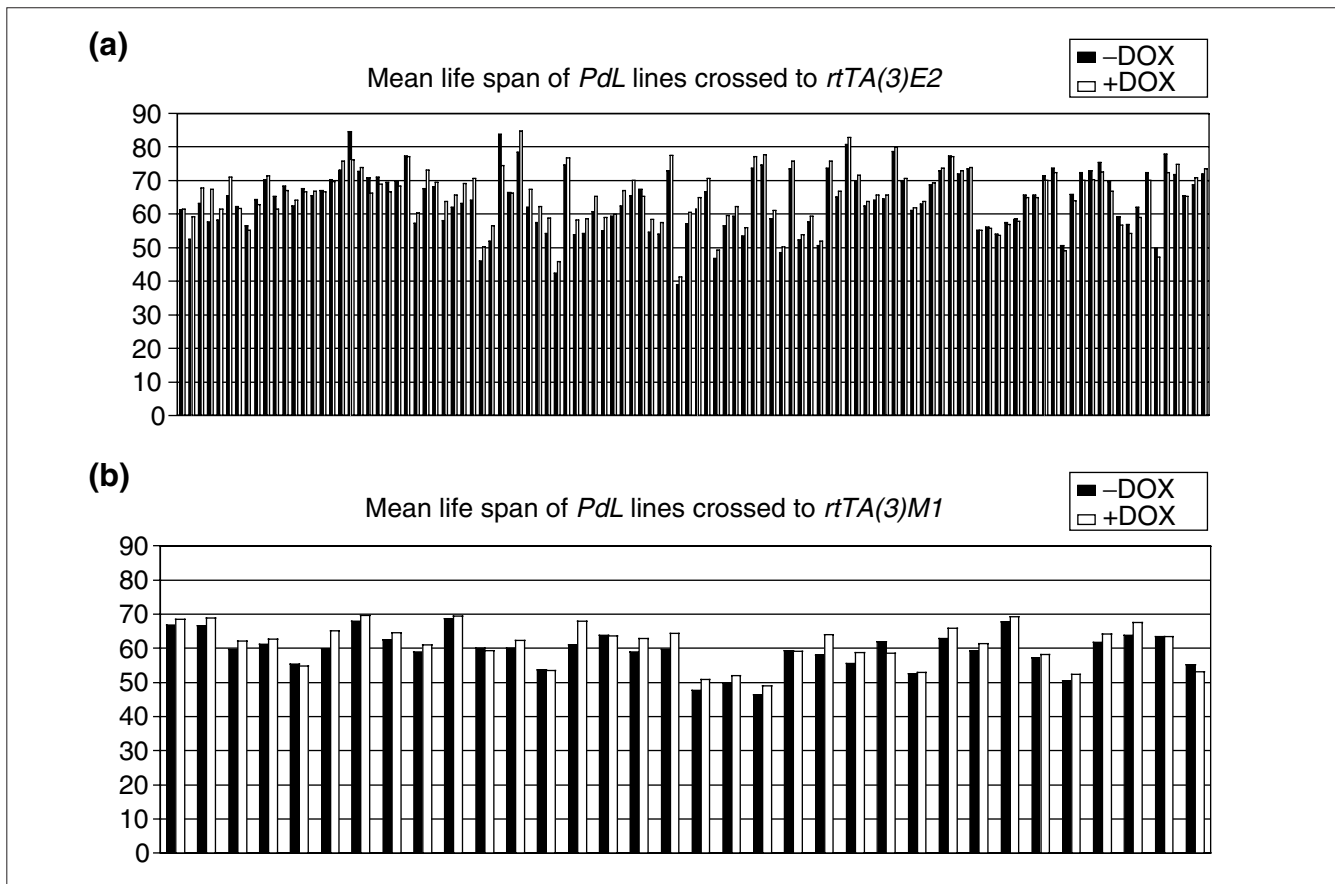
To search for genes that might extend life span, new insertions of *PdL* were created on the second and third chromosomes by appropriate crosses to a strain expressing P element transposase (Figure 1). Approximately 10,000 males bearing a new *PdL* insert and an *rtTA* transcriptional transactivator insertion were generated. The males were generated in eight staggered groups, each of around 1,200 individuals. In an attempt to increase the variety of mutations identified, two different *rtTA* insertion strains were used. The first six cohorts of males contained an insertion of the *rtTA* construct called *rtTA(3)E2* that is associated with moderate levels of target-gene expression and low levels of leaky expression in the absence of DOX [33]. The last two cohorts of males contained an *rtTA* insertion called *rtTA(3)M1* which is associated with a high level of target-gene expression and greater leaky expression. To obtain adult flies of defined age, the second cross was cultured at 25°C in urine specimen bottles (Figure 1). Prior to eclosion of the majority of pupae, bottles were cleared of adults and flies were allowed to emerge over the next 48 hours. The majority of the males will have mated during this time. The males only were then removed and were designated 1 day old, and were maintained at 25°C at 40 per vial in culture vials with food supplemented with 250 µg/ml DOX, and passaged to new vials every 48 hours. The expectation was that some of these flies might live longer due to the overexpression of a beneficial gene. For the first four cohorts, at approximately 30% survival of the cohort, each of the males was individually mated with four third-chromosome balancer stock virgin females. This was so that a stable line could be recovered later if that fly was found to be one of the longest lived. Only about 21% of these males were found to



**Figure 1**  
Genetic crossing strategy. The starting *PdL* insertion on the X chromosome *PdL(X)A* was mobilized using the  $\Delta 2-3$  transposase source to generate approximately 10,000 new *PdL* insertions on the second and third chromosomes. Chromosomes potentially bearing new *PdL* insertions are indicated by asterisks. Chromosomes potentially bearing new *PdL* insertions were recovered in males that also contained an *rtTA* transactivator construct. The males were allowed to age in the presence of DOX in staggered cohorts of approximately 1,200 each and were individually mated to balancer virgins. Stable *PdL* insertion lines were recovered for the longest-lived (approximately 1.4% of the males in each cohort). A total of 110 lines were identified using *rtTA* insertion *rtTA(3)E2*, and 34 lines with insertion *rtTA(3)M1*.

be fertile, meaning that there was selection for males that maintained fertility during aging. After 4 days, the fertile males were removed from the crosses, placed in individual numbered vials, and passaged every two days until they were all dead. For the rest of the cohorts the males were mated at approximately 65% survival of the cohort, which increased the frequency of fertile males to about 32%. Stable lines were recovered for the approximately 1.4% longest-lived flies from each cohort. These 144 lines should therefore be enriched for ones in which *PdL* is causing overexpression of a gene with a benefit for life span. In addition, lines in which *PdL* causes overexpression of a gene with large negative effects on life span should have been eliminated by this step.

Each of the 144 new *PdL* insertion lines was crossed to the appropriate *rtTA* chromosome strain, and males containing both constructs were assayed in large cohorts (around 400 flies) for doxycycline-dependent effects on life span (Figure 2). The mean life spans of the different strains in the absence of DOX varied greatly, from 40 to 84 days. Both increases and decreases were observed in the presence of DOX. For the 110 lines tested with *rtTA(3)E2* there was an average 1.8% increase in the presence of DOX (Figure 2a), and for the 34 lines tested with *rtTA(3)M1* an average 3.0% increase in the presence of DOX (Figure 2b), consistent with previous results that DOX itself has little to no effect on life span. However, several lines were identified where there was

**Figure 2**

Life-span assay of new *PdL* insertion lines. The new *PdL* insertion lines were crossed to the appropriate *rtTA* insertion line, and male progeny were assayed for life span in the presence (+DOX) and absence of DOX (-DOX). Each line is represented by a pair of bars, black bars for -DOX and white bars for +DOX. (a) Lines crossed to *rtTA(3)E2*. The average life span -DOX was 64.7 days and the average +DOX was 65.9 days, for an average increase of 1.8%. (b) Lines crossed to *rtTA(3)M1*. The average life span -DOX was 59.4 days and the average +DOX was 61.2 days for an average increase of 3.0%.

a significantly greater than average increase in life span in the presence of DOX. To confirm these results, these lines were assayed one or more additional times (Table 1). As a control, Oregon-R wild-type flies were repeatedly crossed to the *rtTA* strains and assayed for life span in parallel. The Oregon-R controls exhibited changes in life span from -5.14% to +4.71%, with an average change of +0.45%. In contrast, six *PdL* lines were found to yield significant and reproducible increases in life span in the presence of DOX, ranging from 5 to 17%.

Line *PdL(3)3E36* was associated with the largest increase in life span (average 12%) (Table 1). The *PdL* insert in this line was located in the large first intron of the *encore* gene [35], with the promoter oriented in the sense direction (Figure 3a). *PdL* caused the expression of a pair of approximately 1.4 kb transcripts that contain a 44 amino acid ORF identified by the *Drosophila* genome project as *CG14975*, as determined by northern blot with a *CG14975* probe. These transcripts were not detected in the absence of DOX in the

adult males and this putative gene was named *Red herring* (*Rdh*). Northern analysis using a probe from *encore* exon 3 indicated that levels of the normal sized *encore* transcript were not detectably altered by DOX, as indicated by the arrowhead (Figure 3a). However, DOX appeared to induce expression of both larger and smaller RNAs containing *encore* coding-region sequences, resulting in a smear of hybridization in the upper part of the lane, as indicated by the asterisk. Representative survival curves are presented (Figure 3a).

Line *PdL(2)4G14* contains an insert at the 5' end of the *VhaSFD* gene, encoding the vacuolar proton pump ATPase ( $H^+$ -ATPase) SFD subunit [36]. DOX caused an approximately fourfold overexpression of the *VhaSFD* transcript and an average 8% increase in life span (Figure 3b). Representative survival curves for the *PdL(2)4G14* line are presented (Figure 3b). Line *PdL(3)2C33* contains an insert at the 5' end of a gene with homology to a maltose permease from *Bacillus stearothermophilus* [37], which was named

**Table 1**

Line name	Gene (enzyme)	Life-span phenotype*			Date of assay
		-DOX	+DOX	% Change (p)	
<b>PdL lines</b>					
PdL lines crossed to <i>rtTA(3)E2</i>					
<i>PdL(3)3E36</i>	<i>Red herring/core</i>	60.42±0.920	64.34±0.864	+6.50 (0.0021)	7 October 1999
		55.20±0.729	64.55±0.757	+16.95 (<0.0001)	6 July 1999
		66.91±0.980	75.59±0.910	+13.00 (<0.0001)	14 February 2001
		56.56±0.709	62.44±0.854	+10.38 (<0.0001)	7 August 2001
<i>PdL(2)4G14</i>	<i>VhaSFD</i>	76.05±1.039	82.71±1.088	+8.75 (<0.0001)	14 November 2000
		69.28±1.077	76.24±1.538	+10.6 (0.0002)	18 July 2000
		73.79±1.410	77.67±1.251	+5.27 (0.0404)	6 March 2001
		55.40±0.810	59.34±0.887	+7.11 (0.0011)	21 August 2001
<i>PdL(3)2C33</i>	<i>Sugar baby CG7334</i>	73.91±1.501	80.32±1.392	+8.67 (0.0019)	15 November 2000
		71.75±1.105	77.74±1.169	+8.35 (0.0002)	3 July 2000
		68.99±1.100	72.22±1.034	+4.68 (0.0329)	6 March 2001
		56.77±0.950	57.68±0.617	+1.59 (0.4241)	7 August 2001
Oregon-R		62.70±0.963	63.81±1.299	+1.76 (0.4954)	12 August 2001
Oregon-R		68.20±0.978	71.41±1.255	+4.71 (0.0438)	28 September 2000
Oregon-R		71.27±1.407	72.46±1.247	+1.66 (0.5278)	11 July 2000
Oregon-R		76.14±1.498	74.45±1.492	-2.23 (0.4236)	16 May 2000
Oregon-R		68.84±0.903	65.30±1.175	-5.14 (0.0176)	7 October 1999
PdL lines crossed to <i>rtTA(3)M1</i>					
<i>PdL(3)8S64</i>	<i>filamin</i>	58.87±1.295	64.11±1.066	+8.76 (0.0020)	13 December 2000
		56.00±1.202	60.68±1.103	+8.37 (0.0044)	3 May 2001
<i>PdL(3)8S25</i>	<i>fwd</i> (1-phosphatidylinositol 4-kinase)	58.90±0.868	62.99±0.912	+6.93 (0.0013)	22 December 2000
		56.25±0.940	61.31±1.028	+9.00 (0.0003)	3 May 2001
<i>PdL(3)8R128</i>	<i>Cct1</i> (cholinephosphate cytidyltransferase)	59.68±1.192	64.34±1.052	+7.80 (0.0035)	27 February 2001
		56.54±0.789	59.85±0.852	+5.93 (0.0041)	10 July 2001
Oregon-R		60.44±1.296	61.61±1.350	-1.96 (0.5337)	13 July 2001
Oregon-R		65.79±1.009	68.68±0.819	+4.39 (0.0266)	2 May 2001
Oregon-R		65.45±1.304	66.93±1.091	+2.20 (0.3824)	30 November 2000

\*Mean life span ±SEM, *p*-value for unpaired, two-sided *t*-test in parentheses.

*Sugar baby* (Figure 3c). DOX caused an approximately 8.5-fold overexpression of the *Sugar baby* transcript, and was associated with an average 6% increase in life span.

Three genes with previously characterized mutant phenotypes were also identified by *PdL* insertions. Line *PdL(3)8S64* contained an insert at the 5' end of the *filamin* gene 'A' transcript (Figure 3d). Filamin is an actin-binding protein involved in cytokinesis and ring canal formation [38,39]. *filamin A* was overexpressed approximately fivefold in the presence of DOX and associated with an average 8.5% increase in life span. Expression of the *filamin* 'B' transcript was not detectably altered. Line *PdL(3)8S25* contained an insert at the 5' end of the 'B' transcript of the *four wheel drive* (*fwd*) gene (Figure 3e). *fwd* encodes a 1-phosphatidylinositol

4-kinase (PI 4-kinase) that regulates actin organization and ring canal formation during germline cytokinesis [40]. *fwd B* was overexpressed approximately 10-fold and associated with an average 8% increase in life span. Finally, line *PdL(3)8R128* had an insert at the 5' end of the *CTP:phosphocholine cytidyltransferase 1* (*Cct1*) gene (Figure 3f). *Cct1* encodes a homolog of the rate-limiting enzyme in phosphatidylcholine biosynthesis [41]. *Cct1* was overexpressed approximately 1.7-fold and was associated with an average 7% increase in life span.

## Discussion

The genetic screen described here had several unusual aspects, and the potential to identify a novel set of genes



involved in life-span regulation. First, the screen was designed to identify positive regulators of life span, that is, genes that will increase life span when overexpressed. Most genetic screens for life-span regulators in *Drosophila* and other organisms, such as *C. elegans*, have involved loss-of-function mutations and therefore have identified negative regulators of life span [14,15,42,43]. Second, mutants were identified on the basis of the phenotype of increased life span under normal conditions, as opposed to some surrogate phenotype expected to correlate with life span, such as increased stress resistance. Because the *PdL* mutations generated here were screened directly for extended life span under normal conditions, the screen had the potential to identify genes and pathways not previously suspected to have a role in aging and life-span regulation.

Results were reported recently for a screen for positive regulators of life span using a different P-element-based gene overexpression strategy [29]. In that study a heat-stress inducible system was used, and lines were screened for life span at the moderate heat stress condition of 30°C. Several genes were identified that increased survival when overexpressed, including genes involved in heat stress and oxidative stress responses. It is interesting to note that there is no overlap with the genes identified here with *PdL* at 25°C. This may be due to the small fraction of the genome that has been tested so far, or it may indicate that different mechanisms limit life span under normal and moderate heat-stress conditions.

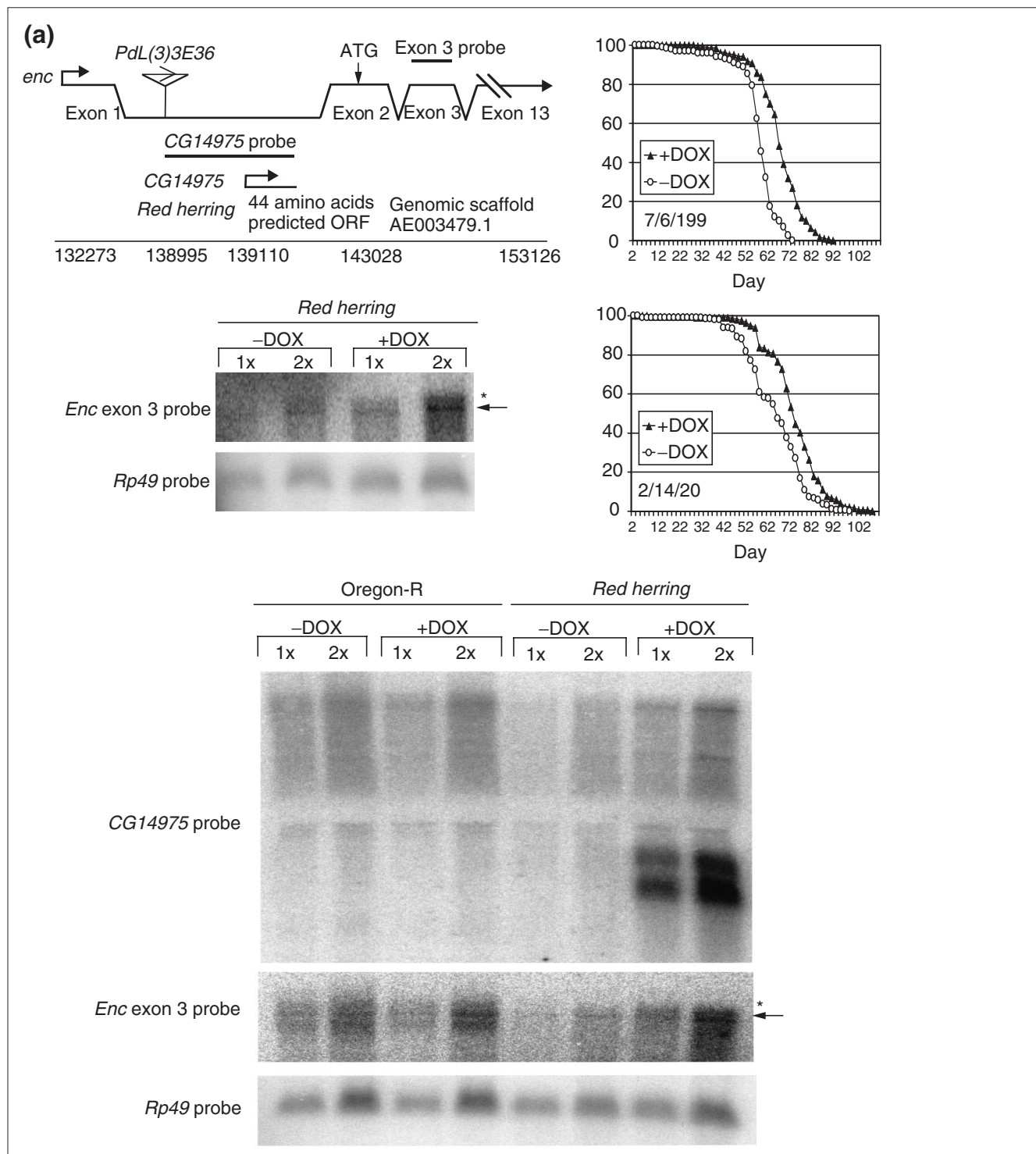
It is difficult to estimate the number of genes that were tested in the *PdL* screen, but it is certain to have been only a tiny fraction of the genome. In previous experiments approximately 7% of *PdL* insertions were found to cause lethal and visible phenotypes when the *PdL* promoter was activated during larval and pupal development [34]. Seven percent is certainly a large underestimate of the frequency at which genes are overexpressed, as embryogenesis was not tested, and not all genes will be lethal or have an obvious visible phenotype when overexpressed. The original 10,000 *PdL* insertions generated here went through an initial enrichment step of unknown efficiency, to yield the 144 lines that were tested in large cohorts. Lines in which a gene was overexpressed that had a positive effect on life span should have been enriched in the final 144 lines, whereas ones with a significant negative effect should have been eliminated. The enrichment step appears to have been at least partially successful, as the largest negative effect on life span observed among the 144 lines tested was -7.5%, and *PdL* is known to be able to create mutations with negative effects of up to -30% [34]. The molecular characterization of the mutations suggests a low frequency of false positives. If the six *PdL* insertion strains were a random set, then *PdL* would be expected to sometimes be in the wrong orientation and/or downstream of the ATG translation start. However, all six lines were found to have *PdL* insertions at the 5' end

of a gene and oriented in the sense direction, and in all six lines a transcript was overexpressed that was expected to contain a complete ORF.

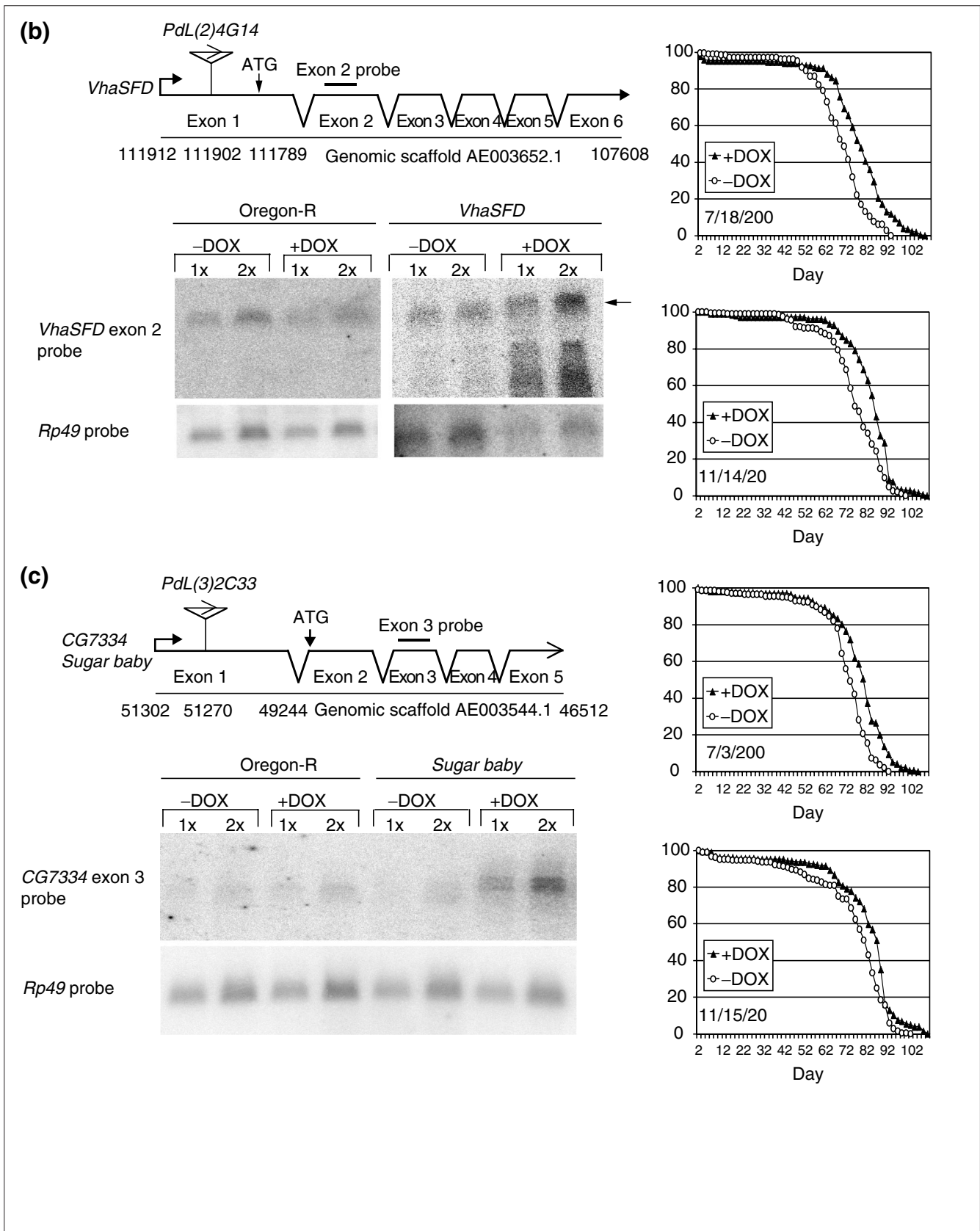
The magnitude of life-span increases caused by the *PdL* mutations was generally small, ranging from 5 to 17%. This is in contrast to overexpression of SOD genes or certain single gene mutations such as *mth*, where life-span increases range from 20 to 50% [14,23-25]. There are at least two possible reasons for the relatively small life span increases observed here. First, as only a fraction of the genome has been tested, it may be that genes with larger effects exist but have not yet been identified. Second, the tet-on system is sometimes leaky, meaning that at certain genomic locations there can be significant expression of the tet-on promoter in the absence of DOX [33]. It is possible that in some of the lines the increase in life span between -DOX and +DOX is reduced because there is already some gene overexpression and life-span extension in the -DOX flies. Such a line might be expected to have an unusually high -DOX life span relative to the other lines, and it is notable that this appears to be the case for the lines bearing mutations in the *VhaSFD* and *Sugar baby* genes. When this difference is included in the calculations, the life span increases for *VhaSFD* and *Sugar baby* are approximately 20% and 17%, respectively.

The largest average increase in life span caused by DOX (12%) was obtained for the *PdL(2)3E36* insertion in the large first intron of the *encore* gene. A pair of approximately 1.4 kb transcripts was expressed containing a 44 amino acid ORF with no detectable homologies. While there are a number of *Drosophila* genes encoding functional ORFs of this size, it is also possible that these transcripts do not encode a functional protein but rather function at the RNA level to affect life span. There are no cDNAs in the databases containing these intronic sequences, and we were unable to detect these transcripts in wild-type adult males using northern blots. The data suggest that these transcripts are not expressed under normal conditions, or are expressed at very low levels. Further experiments will be required to determine the mechanism of life-span extension in this line, and the elusive nature of the intronic gene suggested the name *Red herring*. The northern analyses also indicated induction of a small amount of a large transcript containing the *encore* coding region. Therefore it is also possible that the life span extension in this line is caused by an increase in the expression of *encore*. *encore* is a member of a novel family of proteins with multiple functions during *Drosophila* oogenesis [44].

Two of the genes identified appear to be involved in membrane transport. The *VhaSFD* gene encodes the *Drosophila* homolog of the vacuolar H<sup>+</sup>-ATPase SFD subunit. The vacuolar ATPase is an ATP-driven proton pump found in the endomembranes of eukaryotic cells [36,45-47]. It is composed of a cytoplasmic ATPase domain called V<sub>1</sub> that contains *VhaSFD* and other subunits, and a multisubunit

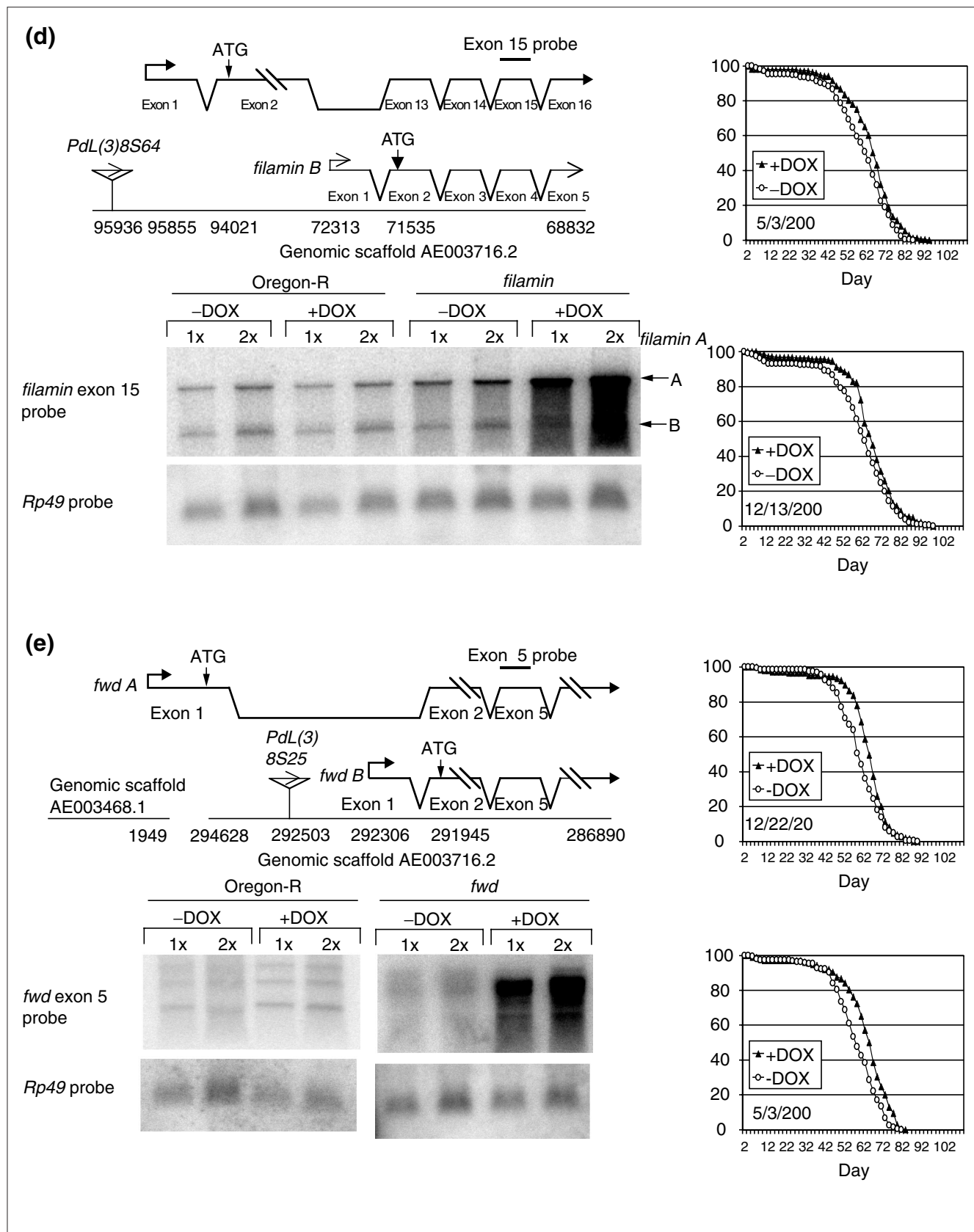


**Figure 3** (See parts b-f on the following pages) Characterization of new PdL mutations. **(a)** PdL(3)3E36 (*Red herring* and *encore (enc)*); **(b)** PdL(2)4G14 (*VhaSFD*); **(c)** PdL(3)2C33 (*Sugar baby*); **(d)** PdL(3)8S64 (*filamin*); **(e)** PdL(3)8S25 (*four wheel drive (fwd)*); **(f)** PdL(3)8R128 (*CTP:phosphocholine cytidyltransferase-I (Cct1)*). The molecular organization of each mutated gene is diagrammed. The PdL insertion is indicated by a triangle, with an arrow indicating the direction of transcription of the PdL tet-on promoter. Transcript start sites and ATG translation start codons are marked by arrows. The location of PdL insertions, transcription start sites, ATG translation start sites, and transcript termination sites are indicated by genomic scaffold numbers. mRNA was isolated from whole adult male flies cultured ±DOX and analyzed by northern blot. Hybridization with ribosomal protein gene *Rp49* probe was used as a loading control. The locations of gene-specific DNA probes used for northern analyses are indicated. Transcript sizes were estimated using RNA markers run in adjacent lanes (data not shown). Two representative survival curves are presented for each line.



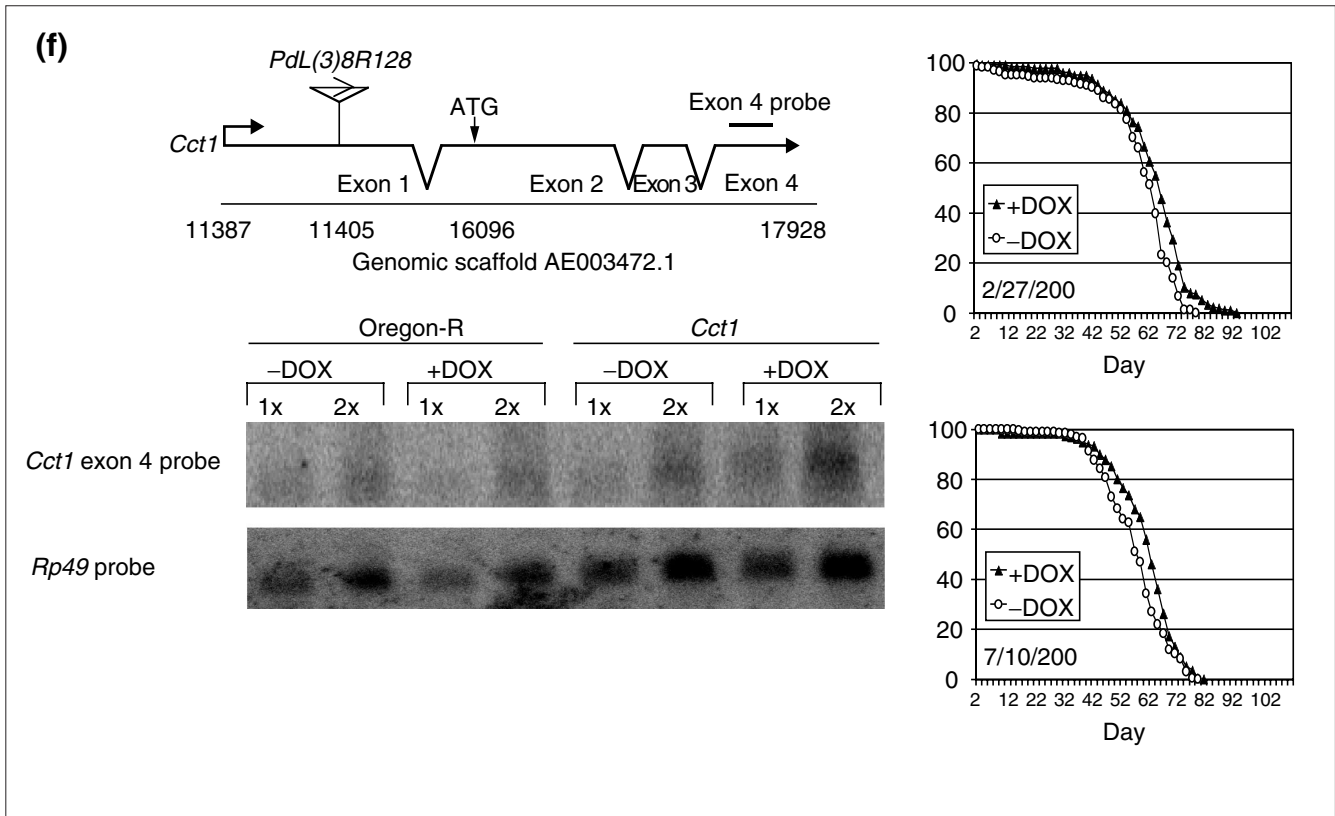
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membrane-channel component called  $V_o$ . The vacuolar ATPase is involved in the acidification and energization of organelles such as Golgi-derived vesicles, clathrin-coated vesicles, synaptic vesicles and lysosomes. The proton pumping is required for processes such as protein trafficking, receptor-mediated endocytosis, neurotransmitter release and intracellular pH regulation and waste management. The free membrane  $V_o$  domain has also been implicated in membrane fusion events. In higher organisms the vacuolar  $H^+$ -ATPase is also found in the plasma membrane of specialized epithelial cells where it functions in bulk transport. In *Drosophila* this is the Malpighian (or renal) tubule. The *VhaSFD* subunit is required for the ATPase activity and assembly of the vacuolar  $H^+$ -ATPase and may function as a dissociable regulatory subunit. Therefore, overexpression of *VhaSFD* may affect life span by increasing activity of the vacuolar  $H^+$ -ATPase.

The *Sugar baby* gene is related to a maltose permease from *Bacillus stearothermophilus* [37], suggesting that it may function in transport of maltose or other sugars across a membrane. Recently, loss-of-function mutations in another putative membrane transporter, *Indy*, have been found to increase life span [15]. It is possible that overexpression of the putative transporter encoded by *Sugar baby* could increase life span by increasing uptake of a particular sugar and altering energy metabolism pathways.

Filamin is an actin-binding protein with a homolog in humans called actin-binding protein 280 (ABP280) [38,39]. Filamin binds to several cell-membrane proteins and intercellular ligands involved in signal transduction, and appears to act as a downstream effector in remodeling of the actin cytoskeleton. During *Drosophila* oogenesis, filamin localizes to the ring canals, which are membrane-associated actin cytoskeletal structures that create intercellular bridges between germline cells. Loss-of-function mutations of *filamin* disrupt the normal function of these actin cytoskeletal structures. Filamin has several additional interactions with potential relevance to aging studies. Presenilins are membrane proteins found in both humans and *Drosophila*, and mutations in human presenilins are associated with early onset familial Alzheimer's disease (FAD) [48]. *Drosophila* filamin binds to both *Drosophila* and human presenilins, and *filamin* interacts genetically with *presenilin* in *Drosophila* [49]. Dominant adult phenotypes produced in *Drosophila* by overexpression of *presenilin* can be suppressed by overexpression of *filamin*. Filamin also interacts specifically with *Drosophila* Toll and Tube proteins [50]. Toll and Tube are members of a signal transduction pathway that activates the Rel transcription factor Dorsal and is involved in developmental patterning and in the adult immune response. Several of the immune-response genes regulated by Rel transcription factors are dramatically

induced during *Drosophila* aging (G.L., J. Carrick and J.T., unpublished data).

*fwd* encodes a phosphatidylinositol 4-kinase (PI 4-kinase) that converts phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PIP) [40]. This is the first step in the synthesis of the key regulatory membrane phospholipid PIP<sub>2</sub>, which is generated from PIP by a PI 4,5-kinase. PIP<sub>2</sub> interacts directly with a number of proteins, regulating both their subcellular localization and their activity. Data from yeast and mammalian systems implicates homologous PI 4-kinases and PIP<sub>2</sub> in regulation of secretion and membrane vesicle trafficking [51,52]. In *Drosophila*, *fwd* gene function is required for the formation of ring canals and cytoplasmic bridges during cytokinesis in male meiosis. PIP<sub>2</sub> is also the precursor to the important second messengers PIP<sub>3</sub> and diacylglycerol. PIP<sub>3</sub> is involved in the insulin-like signaling pathway that affects life span in *Drosophila* and *C. elegans* [53], and it will be of interest to determine if *fwd* overexpression is somehow affecting this pathway.

CTP:phosphocholine cytidyltransferase (Cct) catalyzes the conversion of phosphocholine to CDP-choline and is the rate-limiting enzyme in the phosphatidylcholine biosynthetic pathway [41]. Phosphatidylcholine is a major membrane phospholipid and is a precursor to second messengers involved in signal transduction at the membrane, including the PIP, PIP<sub>2</sub> and PIP<sub>3</sub> discussed above. There are two genes encoding Cct in *Drosophila* (*Cct1* and *Cct2*), and mutations in the *Cct1* gene disrupt patterning during oogenesis (T. Gupta and T. Schupbach, personal communication). Overexpression of *Cct1* might affect life span by increasing synthesis of phosphatidylcholine and altering membrane structure and/or phospholipid signaling pathways.

It is striking that most of the genes identified in this screen as positive regulators of *Drosophila* life span are implicated in functions at the membrane, including regulation of transport, phospholipid metabolism, signal transduction and actin cytoskeleton organization. It is tempting to speculate that some of these genes may be acting in the same pathway(s) for life-span regulation. For example, both *filamin* and *fwd* are involved in the function of ring canals, which are membrane-associated actin cytoskeletal structures that create intercellular bridges between germline cells during early mitotic divisions [38-40]. *encore* is also required for normal early mitotic division of the germline cells [44]. In *C. elegans*, germline cells have recently been shown to send signals that regulate life span of the adult organism [54-56], and it will be of interest to determine if any of the genes identified here increase life span through effects on the germline. Both *fwd* and *Cct1* are involved in phospholipid metabolism, and phospholipid signaling pathways are implicated in life-span regulation in *Drosophila* and other organisms [53]. The conserved insulin-like life-span regulatory pathway includes a PI 3-kinase that converts PIP<sub>2</sub>

**Table 2**

**ANOVA results for 110 lines crossed to *rtTA(3)E2***

Source	Degrees of freedom	Mean square	F	p
Line	109	30,407.071	134.854	< 0.001
Treatment	1	19,763.341	87.650	< 0.001
Line x treatment	109	720.007	3.193	< 0.001
Error	40,129	225.481		

into PIP<sub>3</sub>. The enzymes encoded by *fwd* and *Cct1* are both in the biosynthetic pathway for PIP<sub>2</sub> and therefore might affect the insulin-like pathway by altering the availability of this substrate. Further experiments will be required to confirm the role of these genes in life-span regulation, and to determine their interactions with each other and in known or novel life-span regulatory pathways.

## Materials and methods

### *Drosophila* strains

All *D. melanogaster* strains are as described [33,34,57,58].

### *Drosophila* culture and life-span assays

*Drosophila* were cultured on standard agar/molasses/corn meal/yeast media [59]. To obtain adult flies of defined age, the indicated *PdL* lines and Oregon-R control strain were crossed to an *rtTA* stock and cultured at 25°C in urine specimen bottles. Prior to eclosion of the majority of pupae, bottles were cleared of adults and newly eclosed flies were allowed to emerge over the next 48 h. The majority of the males will have mated during this time. The males only were then removed and were designated 1 day old, and were maintained at 25°C at 40 per vial in culture vials with food. At 4 days of age the males were split into control and experimental groups of 200 males each, with experimentals (+DOX) placed on culture media supplemented with 250 µg/ml DOX. Dead flies were counted at each passage, and the number of vials was progressively reduced to maintain approximately 40 flies per vial. To calculate mean life spans for the experimental (+DOX) and control (-DOX) cohorts, each fly's life span was tabulated and their life spans were averaged and the standard error of the mean (SEM) calculated. Statistical significance of differences in mean life span was calculated for each experiment using unpaired two-sided *t* tests.

### ANOVA

Two-way analysis of variance (ANOVA) was performed for each life-span dataset. The first dataset was the 110 lines crossed to *rtTA(3)E2* (Figure 2a). Sources of variance were line, treatment (DOX), and line x treatment. Results are presented in Table 2. A *P*-value ≤ 0.05 was considered statistically significant. The significant line x treatment interaction

for dataset 1 indicated that DOX affects life span only in certain lines. The significance and 95% confidence interval were calculated for each line with experiment-wise error rate for multiple comparisons reduced using the Bonferroni method (see supplementary table in Additional data files). DOX decreased life span in 6 lines and increased it in 34 lines. The second dataset was the 34 lines crossed to *rtTA(3)M1* (Figure 2b). Results were line  $F = 57.168$ ,  $p < 0.001$ ; treatment  $F = 48.131$ ,  $p < 0.001$ ; line x treatment  $F = 1.898$ ,  $p = 0.001$ . The significant line x treatment interaction again indicates that DOX affects life span only in certain lines. The significance and 95% confidence interval were calculated for each line, with experiment-wise error rate reduced using the Bonferroni method (see supplementary table in Additional data files). DOX decreased life span in one line, and increased it in seven lines.

Each line in which DOX increased life span was crossed to the appropriate *rtTA* strain and assayed one to three additional times. For six lines there was a reproducible increase in life span with DOX (Table 1). As an additional control, Oregon-R wild-type was repeatedly crossed to *rtTA(3)E3* and to *rtTA(3)M1* strains and assayed for life span in the presence or absence of DOX. These datasets were analyzed using two-way ANOVA with date of assay, treatment (DOX), and date x treatment as the sources of variance. Dataset 3 was the four independent assays of line *PdL(3)E36* (*Red herring/encore*) (Table 1). Results were date  $F = 100.690$ ,  $p < 0.001$ ; treatment  $F = 129.780$ ,  $p < 0.001$ ; and date x treatment  $F = 5.387$ ,  $p = 0.001$ . Dataset 4 was the four independent assays of line *PdL(2)4G14* (*VhaSFD*). Results were date  $F = 145.348$ ,  $p < 0.001$ , treatment  $F = 43.294$ ,  $p < 0.001$ , date x treatment  $F = 1.057$ ,  $p = 0.366$ . Dataset 5 was the four independent assays of line *PdL(3)2C33* (*Sugar baby*). Results were date  $F = 121.648$ ,  $p \leq 0.001$ ; treatment  $F = 24.728$ ,  $p \leq 0.001$ ; date x treatment  $F = 2.283$ ,  $p = 0.077$ . Dataset 6 was the five independent assays of Oregon-R crossed to *rtTA(3)E2* (Table 2). Results were date  $F = 28.171$ ,  $p < 0.001$ , treatment  $F = 0.005$ ,  $p = 0.944$ , date x treatment  $F = 2.313$ ,  $p = 0.056$ . Dataset 7 was the two independent assays of line *PdL(3)8S64* (*filamin*). Results were date  $F = 7.192$ ,  $p = 0.007$ , treatment  $F = 17.871$ ,  $p < 0.001$ , date x treatment  $F = 0.057$ ,  $p = 0.812$ . Dataset 8 was the two independent assays of line *PdL(3)8S25* (*fwd*). Results were date  $F = 5.319$ ,  $p = 0.021$ , treatment  $F = 23.784$ ,  $p < 0.001$ , date x treatment  $F = 0.271$ ,  $p = 0.603$ . Dataset 9 was the two independent assays of line *PdL(3)8R128* (*Cct1*). Results were date  $F = 15.405$ ,  $p < 0.001$ , treatment  $F = 17.177$ ,  $p < 0.001$ , date x treatment  $F = 0.455$ ,  $p = 0.500$ . Dataset 10 was the three independent assays of Oregon-R crossed to *rtTA(3)M1*. Results were date  $F = 16.611$ ,  $p < 0.001$ , treatment  $F = 3.777$ ,  $p = 0.052$ , date x treatment  $F = 0.315$ ,  $p = 0.730$ .

#### Southern analysis of *PdL* copy number

DNA was isolated from *PdL* lines and restriction digested with *Xba*I, *Hind*III, *Pst*I and *Taq*I in separate reactions.

DNA was transferred to a Southern blot and hybridized with a radiolabeled 172 bp fragment from the 3' P end of *PdL*. This probe fragment was generated by PCR amplification with primers located within the 3' P end, IRREV (ATGATGAAATAACATAAGGTGGTCCCG) and P3MCSREV (ATGAGTTAATTCAAACCCACGGACAT).

#### Inverse PCR amplification of *PdL* flanking sequences

Protocols were as previously described [60,61]. Briefly, DNA equivalent to one fly was restriction digested with *Taq*I. The DNA was extracted with phenol/chloroform, precipitated with ethanol, resuspended and treated with T4 ligase overnight at 16°C. PCR amplification was performed using primers P<sub>ry1</sub> (CCTTAGCATGTC-CGTGGGGTTTGAAT) and IR (CGGGACCACCTTATGT-TATTTTCATCATG) located in the 3' P end. PCR protocol was as follows: step 1, 95°C for 5 min; step 2, 95°C for 30 sec; step 3, 51°C for 1 min; step 4, 72°C for 1 min; step 5, repeat steps 2-4 40 times; step 6, 72°C for 10 min. The PCR product was subcloned into the pCR2.1-TOPO cloning vector (Invitrogen). Dideoxy sequencing was carried out using the Sequenase version 2.0 DNA sequencing kit (US Biochemical) and the T7 and M13 reverse sequencing primers. Each inverse PCR product will contain 284 bp of 3' P-element sequences. The sizes of the products were: *PdL(3)E36* (*Red herring/encore*) 534 bp; *PdL(2)4G14* (*VhaSFD*) 465 bp; *PdL(3)2C33* (*Sugar baby*) 464 bp; *PdL(3)8S64* (*filamin*) 362 bp; *PdL(3)8S25* (*fwd*) 345 bp; *PdL(3)8R128* (*Cct1*) 298 bp.

#### DNA sequence analyses

*PdL*-flanking DNA sequences were used to query GenBank databases using BLASTN with default settings as provided at the National Center for Biotechnology Information (NCBI) website [62]. Genomic scaffold sequences and numbering and ORF designations are according to the NCBI databases and *Drosophila* genome sequence [63].

#### Northern blot analyses

RNA was isolated from adult *Drosophila* using the RNAqueous kit (Ambion), fractionated on 1.0% agarose gels and transferred to GeneScreen membranes (DuPont/NEN). 1x = 4 µg, and 2x = 8 µg, except for the analysis of *Sugar baby* where 1x = 8 µg. The DNA probe for exon 5 of the *fwd* gene was generated by PCR amplification from *Drosophila* genomic DNA using primers FWD FWD (TGCTTCCTCCATTTGGCGAAC) and FWDREV (ATCATCTGTGGCTCAGAGTCG). The probe for exon 2 of the *VhaSFD* gene was generated using primers VhaFWD (CCAGCTGATCCTTCAGGAACTGC) and VhaREV (ACCAGGACGATCAACTGGGCTTC). The probe for exon 15 of the *filamin A* gene was generated using primers FIL-AMINAFWD (GCCAATGTAGGCCCTTCTTCAG) and FIL-AMINAREV (ATCCATGCCATCCACGTCAAG). The probe for exon 1 of the *CG1049* gene was generated using primers CG1049EX1FWD (AGTTGTGTTTGTGTCCGACG)



and CG1049EX1REV (ATAAACAGAGCAGAGCAGAGC). The probe for exon 4 of the *CG1049* gene was generated using primers CG1049EX4FWD (TCTGTCCGATGAATTCATCGCC) and CG1049EX4REV (ATGATTCAGGTTCTCACGTCCG). The *CG14975* ORF probe was generated using primers CG14975FWD (TCAGCCGAGAGATTCTAGAGAG) and CG14975REV (CATCGACATTGTTCTCTCTCC). The 3E36 intergenic region probe was generated using primers 3E36-140120FWD (TTTCATTTCCCTTCCACTGCC) and 3E36-1406038REV (TTACAGCTGCTCACTCACTCAC). The probe for exon 3 of the *encore* gene was generated using primers ENCFWD (AATGAAGCGGAGTTCCCAAAGC) and ENCREV (ATAAAGCCCGAGGTGTTGTTGC). The probe for exon 3 of the *Hr39* gene was generated using primers Hr39FWD (ACATGTCCAGCATCAAAGCGG) and Hr39REV (TATCGTTGTAGTGCAGAC). The 8R96 intergenic region probe was generated using primers 8R96-225683FWD (CAAGTGGGCTCCATAATAGC) and 8R96-226069REV (TGGAGCTCTCGGTCTGTTAG). The probe for exon 4 of the *CG8677* gene was generated using primers CG8677FWD (ATCCGTACCAGTGGCTAAAAGG) and CG8677REV (TTCTTCAACAGCACCCTCGTC). The loading control was ribosomal protein gene *Rp49* [64]. DNA probes were <sup>32</sup>P-labeled using the Prime-It II DNA labeling kit (Stratagene). Hybridization was carried out in Church-Gilbert solution at 65°C overnight. Hybridization signals were visualized and quantitated using the phosphoimager and ImageQuant software (Molecular Dynamics), and exposure times were 4 days. Transcript size was determined by comparison with 1 kb RNA ladder (Gibco-BRL) according to the manufacturer's instructions.

Relative RNA levels and the fold induction of transcripts in the presence of DOX were estimated as follows. A box was drawn around the band for each gene transcript and intensity measured in arbitrary units using the phosphoimager and ImageQuant. An equal sized box was drawn around a region of the lane containing no bands and that value was subtracted as background. *Rp49* loading control was quantitated in the same way for each lane. Each *Rp49* intensity value was divided by the median *Rp49* intensity value to generate a loading correction factor for each lane. A normalized intensity value for each gene transcript was then calculated by multiplying by the *Rp49* correction factor for that lane. This quantitation was done twice for each phosphoimage. The 1x and 2x northern lanes for each RNA sample were quantitated and the numbers were averaged. The resulting relative expression levels are presented in arbitrary units  $\pm$  standard deviation (SD). *VhaSFD*: -DOX =  $4.8 \pm 1.2$ ; +DOX =  $20 \pm 5.5$ ; fold induction approximately 4. *Sugar baby*: -DOX =  $1.5 \pm 0.5$ ; +DOX =  $12.8 \pm 2.4$ ; fold induction approximately 8.5. *filamin*: -DOX =  $59 \pm 11$ ; +DOX =  $318 \pm 64$ ; fold induction approximately 5. *fwd*: -DOX =  $240 \pm 7$ ; +DOX =  $2,303 \pm 28$ ; fold induction approximately 10. *Cct*: -DOX =  $2.3 \pm 0.4$ ; +DOX =  $4.0 \pm 0.2$ ; fold induction approximately 1.7.

## Additional data files

A table showing the calculation of 95% confidence intervals for all the datasets is available with the online version of this paper.

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