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# Misexpression screen delineates novel genes controlling *Drosophila* lifespan

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ABSTRACT

In an initial preliminary screen we identified factors associated with controlling *Drosophila* aging by examining longevity in adults where EP elements induced over-expression or antisense-RNA at genes adjacent to each insertion. Here, we study 45 EP lines that initially showed at least 10% longer mean lifespan than controls. These 45 lines and a *daughterless* (*da*)-Gal4 stock were isogenized into a *CS10* wild-type background. Sixteen EP lines corresponding to 15 genes significantly extended lifespan when their target genes were driven by *da-Gal4*. In each case, the target genes were seen to be over-expressed. Independently derived UAS-gene transgenic stocks were available or made for two candidates: *ImpL2* which is ecdysone-inducible gene L2, and *CG33138*, 1,4-alpha-glucan branching enzyme. With both, adult lifespan was increased upon over-expression via the GeneSwitch inducible Gal4 driver system. Several genes in this set of 15 correspond to previously discovered longevity assurance systems such as insulin/IGF-1 signaling, gene silencing, and autophagy; others suggest new potential mechanisms for the control of aging including mRNA synthesis and maturation, intracellular vesicle trafficking, and neuroendocrine regulation.



## 1. Introduction

Aging involves progressive functional deterioration accompanying reduced reproduction, increased mortality and sensitivity to diseases with the advance of age (Kirkwood and Austad, 2000). Multiple genetic and environmental factors are thought to influence the progress of these phenotypes (Finch and Tanzi, 1997), and in recent years work with model organisms has described numerous genes that increase lifespan when mutated or misexpressed. Genes affecting lifespan have been isolated from studies with yeast, *Caenorhabditis elegans*, *Drosophila* and mice (Guarente and Kenyon, 2000). Many of these longevity genes comprise cellular signaling pathways including: insulin/IGF-1 signaling (Kenyon et al., 1993; Tatar et al., 2001), target of rapamycin signaling (Harrison et al., 2009; Lee et al., 2010a; Luong et al., 2006), and the c-Jun N-terminal kinase pathway (Wang et al., 2003). Others involve genome regulation, stress responses and the integration of systems, including: gene silencing/deacetylation (Rosenberg and Parkhurst, 2002; Tanny et al., 1999), control of telomerase (Blasco, 2005), oxidation responses and chaperones (Orr and Sohal, 1994; Tatar et al., 1997), DNA or protein repair (Matheu et al., 2007), reproduction (Flatt et al., 2008; Hsin and Kenyon, 1999), and neuron function (Cvejic et al., 2004; De Luca et al., 2003; Lin et al., 1998).

A key approach of such analyses with *Drosophila* involves the P-element modular-misexpression system (Rørth et al., 1998). This allows a conditional over-expression or knock-down of genes tagged by transpositional insertion of an engineered P-element that carries the *e*nhancer and the basal *p*romoter, thereby designated as EP. The EP contains 14 copies of Upstream Activator Sequence (UAS), to which Gal4 binds and drives transcription of flanking genomic DNA downstream to the basal promoter. When a fly has an EP element inserted in the 5′ untranslated region (UTR)

*Abbreviations:* AD, Alzheimer's disease; *CalpA*, *Calpain* A; *da*, *daughterless*; DILPs, *Drosophila* insulin-like peptides; *Dlc90F*, *Dynein light chain 90F*; GBE1, 1,4-alpha-glucan branching enzyme 1; GS, GeneSwitch; *hnRNP L*, *heterogeneous nuclear ribonucleoprotein L*; *hsp70*, *heat shock 70*; *IMD*, *immune deficiency*; *kis*, *kismet*; MaxLS, maximum lifespan; *men*, *NADP-dependent malate dehydrogenase*; MLS, mean lifespan; PD, Parkinson's diseases; *PGRP-LF*, *peptidoglycan recognition protein*; PI, *pars intercerebralis*; SERAC1, serine active site containing 1; *SIFR*, *SIFamide receptor*; *sm*, *Smooth*; UAS, Upstream Activator Sequence.

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or promoter region of a gene in the orientation of normal transcription (+), the gene will be over-expressed in the progenies of EP flies mated with a fly expressing Gal4. When a fly has an EP element inserted within a coding region of a gene in the orientation opposite to normal transcription, antisense RNA is produced in the presence of Gal4 causing reduced expression of the corresponding gene (Rørth et al., 1998).

Over a number of years we have performed a preliminary large-scale screen to find new longevity genes by analyzing lifespans of EP lines under control of a *heat shock 70* (*hsp70*)-Gal4 driver that moderately induces the EP UAS elements when flies are maintained at 29 °C. Other results of this initial study that dealt with more than 27,000 EP lines will be reported elsewhere. Here, 45 lines were non-systematically selected from a large set of potential candidate lines that show at least 10% longer mean lifespan (MLS) when driven by an *hsp70*-Gal4 driver (*hsp70*-Gal4 > EP) relative to controls (*hsp70*-Gal4/+). These 45 EP lines and flies possessing a ubiquitously expressing *daughterless* (*da*)-Gal4 driver were back-crossed to *CS10* wild-type flies. The survival of adults from these isogenic EP lines driven by *da*-Gal4 was analyzed at 25 °C. This analysis confirmed 15 genes from this set to extend *Drosophila* lifespan when misexpressed, including genes with functions in chromatin remodeling/silencing, cell matrix, metabolism, and insulin/IGF ligand binding. This longevity assurance was further confirmed for two genes, *ImpL2* and *CG33138*, with over-expression from independently generated UAS-transgenes.

## 2. Materials and methods

### 2.1. Fly stocks

EP of the GX series were generated at GenExel Inc. by mobilization of an EP element after crossing with P[*ry*$^{+}$,*Dr*;Δ2–3] (Rørth, 1996). Some GX series lines are currently available from KAIST Bio Medical Research Center (http://genexel.kaist.ac.kr/mapview3/) or the Bloomington *Drosophila* Stock Center. Lines labeled only with EP numbers were generated by Rørth and were provided by the Szeged Stock Center (Rørth et al., 1998). Lines with *hsp70*-Gal4 (Brand and Perrimon, 1993) and *S106*-GeneSwitch (GS)-Gal4 (Roman et al., 2001) were from the Bloomington *Drosophila* Stock Center. *CS10* wild-type, *da*-GS-Gal4, and UAS-*ImpL2* flies were obtained from Minoru Saitoe (Yamazaki et al., 2007), Véronique Monnier (Tricoire et al., 2009), and Hugo Stocker (Honegger et al., 2008), respectively. We generated UAS-*CG33138* (chromosome 3) for this study.

Males of all 45 homozygous EP lines and *da*-Gal4 flies were first crossed with virgin females of *CS10*. Their female progeny were mated with *CS10* males, and this backcross was repeated for 6–8 times. After the final cross, red-eyed males and virgin females were mated to make homozygous EP lines which were then approximately isogenic with *CS10*. These EP lines and *da*-Gal4 flies are designated as $EP^{CS10}/EP^{CS10}$ and *da*-$Gal4^{CS10}$/*da*-$Gal4^{CS10}$.

### 2.2. UAS-transgenic flies

To produce UAS-*CG33138*, the open reading frame of clone RE12027 (*Drosophila* Genomics Resource Center, Bloomington, USA) was inserted into *BamHI/XhoI* sites of *pUAST* vector. Transgenic flies were generated by standard germ line transformation in the $w^{1118}$ background. Positions of the inserted UAS sequence were mapped by inverse PCR.

### 2.3. Longevity

In the preliminary screen, males from 27,157 EP lines were crossed to *hsp70*-Gal4 females in a series of blocks. From each cross, between 20 and 255 F1 male progeny were maintained in vials of 20 flies, with deaths counted weekly when adults were transferred to new vials. In every block, lifespan was recorded for contemporary, similarly handled control adults (*hsp70*-Gal4/+ from the cross of *hsp70*-Gal4 females and $w^{1118}$ males). Overall, 8,736 EP lines had a MLS that was at least 10% greater than the across-block average MLS of the control cohorts. From these 8,736 EP lines, 45 lines were selected non-systematically for follow-up study in this report.

After backcrossing to *CS10*, virgins of *da*-Gal4 (*da*-$Gal4^{CS10}$/*da*-$Gal4^{CS10}$) were mated with males of each selected EP line ($EP^{CS10}/EP^{CS10}$). Male progeny of *da*-$Gal4^{CS10}/EP^{CS10}$ from each cross were collected within 48 h after eclosion and maintained in a transparent polystyrene chamber with mesh ventilation (∅40 mm, 72 mm × 72 mm × 100 mm; SPL, Republic of Korea). Near the bottom of the chamber, an adaptor connects to a vial of regular fly food. Each chamber contained about 100 (a range of 80–136) flies. Two to five chambers were allocated for each genotype. These chambers were maintained at 25 °C with 60% relative humidity and 12 h light: 12 h dark. Dead flies were counted every 2–3 days and removed from the chamber, when fresh food (3% cornmeal, 10% sucrose and 10% yeast) was supplied. Each EP line was also crossed to the coisogenic *CS10* ($+^{CS10}/+^{CS10}$) and to coisogenic *da*-Gal4 (*da*-$Gal4^{CS10}$/*da*-$Gal4^{CS10}$) to produce control $EP^{CS10}/+^{CS10}$ and *da*-$Gal4^{CS10}/+^{CS10}$ progeny. Lifespan data of all *da*-$Gal4^{CS10}/EP^{CS10}$ lines, their two controls and a cohort of the coisogenic *CS10* stock were collected simultaneously.

To measure lifespan of the independently derived UAS-*CG33138* and UAS-*ImpL2* lines, females from these stocks were crossed to males from the *da*-GS-Gal4 (ubiquitous) and *S106*-GS-Gal4 (abdominal fat body-specific) stocks. Male and female offspring were maintained separately in cages as above but with food that either contained 200 μM RU486 (mifepristone, Sigma, USA) to induce gene expression or vehicle only (ethanol) as control. RU486 at this concentration alone does not affect longevity (Tricoire et al., 2009).

### 2.4. Real time RT-PCR

Adults, 3–5 days after eclosion, were frozen in liquid nitrogen and stored at −80 °C until analysis. After treating with DNase I (Invitrogen, USA) to remove trace genomic DNA, total RNA from homogenized whole body lysates was prepared with RNAiso reagent (Takara, Japan). Total RNA (5 μg) was reverse-transcribed using the PrimeScript RT reagent Kit (Takara, Japan). Real-time RT-PCR was performed using SYBR Premix Ex-Taq II (Takara, Japan) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). Mean induction folds were calculated from values of 3–6 independent experiments and statistically evaluated by chi-square test.

### 2.5. Survival statistics

Data from the assays with the 45 selected lines and with the UAS-transgene lines were converted to life tables by the extinct cohort method, and the mean life span (MLS) was estimated from Kaplan–Meier life tables (Rosner, 1995). The proportion change in MLS (increased lifespan, ILS) was estimated from the ratio of the MLS of $EP^{CS10}$/*da*-$Gal4^{CS10}$ to $EP^{CS10}/+^{CS10}$. Differences in mortality rate between genotypes were evaluated by Log-Rank tests (Rosner, 1995) for $EP^{CS10}/+^{CS10}$ versus *da*-$Gal4^{CS10}/EP^{CS10}$, *da*-$Gal4^{CS10}/+^{CS10}$ versus *da*-$Gal4^{CS10}/EP^{CS10}$, and $+^{CS10}/+^{CS10}$ versus *da*-$Gal4^{CS10}/EP^{CS10}$.

## 3. Results and discussion

Forty-five EP lines (Table 1) were selected from the preliminary subset that lived at least 10% longer than controls. In the preliminary screen, the MLS of the control (*hsp*-Gal4/+) was 27.8 days, on average across blocks. The MLS of the 45 selected lines ranged from 30.5 to 43.1 days. The purpose of the current study was to determine for this subset whether and which of these preliminary longevity differences can be verified through independent, robust genetic experiments.

After backcrossing, the 45 EP lines were crossed to driver and wild-type control stocks to produce the $Gal4^{CS10} > EP^{CS10}$ genotype and two control genotypes ($EP^{CS10}/+^{CS10}$ and *da*-$Gal4^{CS10}/+^{CS10}$). Five EP lines were lethal when driven by *da*-Gal4 and were excluded from further study. Forty EP lines produced adults for survival analysis (Table 1). Wild-type *CS10* ($+^{CS10}/+^{CS10}$) and driver *da*-$Gal4^{CS10}/+^{CS10}$ cohorts had nearly identical MLS (33.4 and 33.6 days, respectively). Some $EP^{CS10}/+^{CS10}$ cohorts lived longer than the parental, coisogenic *CS10* or *da*-$Gal4^{CS10}/+^{CS10}$ cohorts (Table 1 and Fig. 1). Heterosis is an unlikely explanation because these lines had been backcrossed, and the parental *da*-$Gal4^{CS10}/+^{CS10}$ genotype was also a composite genotype with chromosomes from two lines. Rather, several $EP^{CS10}/+^{CS10}$ flies appear to induce some over-expression of the target gene in the absence of Gal4 (Appendix C. Supplementary Table C). Accordingly, here we conservatively quantify longevity assurance by comparing progeny that always carry the EP construct: *da*-$Gal4^{CS10}/EP^{CS10}$ versus $EP^{CS10}/+^{CS10}$, and we infer that an EP line increases MLS only when *da*-$Gal4^{CS10}/EP^{CS10}$ has greater MLS (Log-Rank test with $p < 0.001$) than $EP^{CS10}/+^{CS10}$, *da*-$Gal4^{CS10}/+^{CS10}$, and $+^{CS10}/+^{CS10}$.

We identified 16 EP lines that met these criteria (Table 1). These lines also extend maximum lifespan (MaxLS; Table 1 and Fig. 1 A1–O1) and consistently reduce mortality rate across adult ages (Fig. 1 A2-O2). These 16 EP lines represent 15 genes since GX2970