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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.

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Keywords

Aging; Misexpression screen; Longevity genes; *ImpL2*

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, *C. 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) 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 backcrossed 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 to 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 hours after eclosion and maintained in a transparent polystyrene chamber with mesh ventilation (ø 40 mm, 72×72×100 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 day 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 $EP^{CS10}/da\text{-}Gal4^{CS10}$ to $EP^{CS10}/+^{CS10}$. Differences in mortality rate between genotypes were evaluated by Log-Rank tests (Rosner, 1995) for $EP^{CS10}/+^{CS10}$ versus $da\text{-}Gal4^{CS10}/EP^{CS10}$, $da\text{-}Gal4^{CS10}/+^{CS10}$ versus $da\text{-}Gal4^{CS10}/EP^{CS10}$, and $+^{CS10}/+^{CS10}$ versus $da\text{-}Gal4^{CS10}/EP^{CS10}$.

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 $da\text{-}Gal4^{CS10}/+^{CS10}$). 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 $da\text{-}Gal4^{CS10}/+^{CS10}$ 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 $da\text{-}Gal4^{CS10}/+^{CS10}$ cohorts (Table 1, Fig. 1). Heterosis is an unlikely explanation because these lines had been backcrossed, and the parental $da\text{-}Gal4^{CS10}/+^{CS10}$ 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 A. Supplementary table 1). Accordingly, here we conservatively quantify longevity assurance by comparing progeny that always carry the EP construct: $da\text{-}Gal4^{CS10}/EP^{CS10}$ versus $EP^{CS10}/+^{CS10}$, and we infer that an EP line increases MLS only when $da\text{-}Gal4^{CS10}/EP^{CS10}$ has greater MLS (Log-Rank test with $p < 0.001$) than $EP^{CS10}/+^{CS10}$, $da\text{-}Gal4^{CS10}/+^{CS10}$, 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. 1.1) and consistently reduce mortality rate

across adult ages (Fig. 1.2). These 16 EP lines represent 15 genes since GX2970 and GX6561 are inserted at *kismet* (*kis*). Only GX2970 was used for further validation. From Table 1 we do not include EP(2)2559 in the selected set because while these flies under *da*-Gal4 driver live significantly longer than $+^{CS10}/+^{CS10}$ and $EP^{CS10}/+^{CS10}$, they do not live longer than their *da*-$Gal4^{CS10}/+^{CS10}$ control.

To determine which gene was affected by the EP insertion of these 15 candidates, we compared mRNA levels of *da*-$Gal4^{CS10}/EP^{CS10}$ to $EP^{CS10}/+^{CS10}$ control (Fig. 3). mRNA from genes flanking the insertion on both strands were quantified by real time RT-PCR. When driven by *da*-Gal4 the EP insertions significantly increased the mRNA of the gene corresponding to the 5' to 3' direction of the insertion's orientation upstream of transcription start site of the target gene in all cases (Fig. 3). Thus, lifespan extension in the 15 EP lines is associated with over-expression of downstream target genes of the EP elements.

Stocks with UAS-transgene were available or generated for two of these longevity genes, UAS-*ImpL2* and UAS-*CG33138*. To further confirm the extension of lifespan by these two candidates we analyzed adult survival when the transgenes were driven in adults with the conditional GS driver system. This method produces control and experimental cohorts with identical genetic backgrounds and permits analysis of the transgene specifically in the adult. *CG33138* is a putative transcript for 1,4-alpha-glucan branching enzyme. Male offspring of the *da*-GS-Gal4>UAS-*CG33138* genotype showed 4% longer MLS when the transgene was induced (Fig. 2A). Longevity was likewise increased in the females, by 20% (Fig. 2B). When these flies were fed with less RU486, thus inducing less expression of *CG33138* gene, still MLS increased significantly in females (also refer to Supplementary Table 1). Mutations in 1,4-alpha-glucan branching enzyme 1 (GBE1), the human homologue of *CG33138*, cause glycogen storage disease type IV, which is characterized by tissue accumulation of abnormal glycogen accompanying liver disease, myopathy, or cardiomyopathy (Bruno et al., 2004; Tay et al., 2004). On the other hand, expression of GBE1 is dramatically enhanced by hypoxic stresses in mammalian cells and tissues (Zhao et al., 2004), which might be simply the consequence of increased anaerobic glycolysis and glycogen remodeling. Recently, it has been reported that hypoxia extends worm lifespan and this is mediated through increased expression of HIF-1α (Lee et al., 2010b). Thus, GBE1 might be a downstream target of HIF-1α through which it controls longevity.

The EP line GX4499 contains an EP adjacent to transcription start site of *ImpL2-RB* and causes significant induction of *ImpL2* mRNA when driven by *da*-Gal4 (Fig. 3D). *ImpL2* has 3 transcriptional isoforms, which are likely to produce similar proteins after post-translational modification (Flybase; http://flybase.org/). Lifespan was reduced when *ImpL2* was strongly over-expressed throughout the adult by the conditional GS drivers, *act*-GS-Gal4 or *da*-GS-Gal4 (data not shown). However, restricted over-expression of the *ImpL2* in fat cells by using *S106*-GS-Gal4 significantly extended lifespan in both sexes (Fig. 2D, 2E), and in repeated trials (Supplementary Table 2). *S106*-GS-Gal4>UAS-*ImpL2* in fat cells increased *ImpL2* mRNA about 6-fold (Fig. 2F). *ImpL2* was originally described as a gene induced by ecdysone (Natzle et al., 1986) and has more recently been recognized as an inhibitory insulin-like peptide binding protein (Honegger et al., 2008; Leopold and Perrimon, 2007; Sloth Andersen et al., 2000). A role for *ImpL2* in aging was previously suggested in the context of extended longevity when germline stems cells were genetically reduced (Flatt et al., 2008). Message of *Drosophila* insulin-like peptides (DILPs) was increased in those sterile, long-lived flies but insulin signaling at peripheral tissues appeared to be repressed. At the same time, mRNA for *ImpL2* was strongly elevated, suggesting that this binding protein might counteract the overproduction of DILPs and thus extend lifespan. Recently, over-expression of *ImpL2* was reported to extend *Drosophila* lifespan, including through some broadly expressed drivers that differed from our negative result with tubulin-

GS-Gal4 and, importantly, by *S106*-GS-Gal4, which concurs with our current report (Alic et al., 2011).

The remaining 13 longevity genes are distributed among functional categories (Table 1). The category 'transcription and translation' includes *kis, Sin3A*, and *Smooth* (*sm*). The Kismet protein is a DNA helicase containing an SNF2-like ATPase domain, and functions in trithorax mediated chromatin remodeling (Daubresse et al., 1999). Mutations of its human homologue, chromodomain helicase DNA binding protein 7 cause the CHARGE syndrome, of which clinical symptoms include developmental retardation, heart malformation, and coloboma (Lin et al., 1990). Reduced expression of *Drosophila kis* yields similar pathogenic phenotypes including defects in motor ability, neuronal development, and learning/memory (Melicharek et al., 2010). Kismet also functions in circadian photo-response and control of hedgehog expression (Dubruille et al., 2009; Terriente-Felix et al., 2011). Over-expression of Kismet may increase longevity by increasing chromatin silencing and homeostasis (Oberdoerffer and Sinclair, 2007). Likewise, Sin3 is a scaffolding protein that complexes with histone deacetylases (HDAC1/2) where it interacts with corepressors to control transcriptional silencing of genes (Grzenda et al., 2009). Notably, *rpd3*, which encode HDAC1 in *Drosophila*, is required for Sir2 expression to increase lifespan (Rogina et al., 2002). *Sm*, a homolog of the human heterogeneous nuclear ribonucleoprotein L (hnRNP L), is involved in mRNA synthesis and maturation. *Sm* is primarily expressed in chemosensory neurons and homozygous *sm* mutants show defects in axonal arborization of chemosensory neurons and in feeding behavior. These defects may be related to their early death after eclosion (Layalle et al., 2005). Interestingly, hnRNP L and hnRNP A2 are known to bind to the 3' UTR of glucose transporter-1 mRNA to repress its translation in the glioblastoma cells (Hamilton et al., 1999). Functions of *sm* in longevity control are unknown.

'Signal transduction' includes *ImpL2* as described above, *SIFamide receptor* (*SIFR*), *Calpain A* (*CalpA*) and *CG8155. SIFR is a* G protein-coupled neuropeptide receptor expressed in the intestines, brain and thoracicoabdominal ganglion of adult flies (Jorgensen et al., 2006; Veenstra et al., 2008). Its ligand, *SIFamide* is produced in *pars intercerebralis* (PI) of the brain. Reduced levels of the ligand causes hyperactive courtship behaviors in both sexes (Terhzaz et al., 2007). Increased *SIFR* may reduce reproductive behaviors and consequently extend lifespan. On the other hand, the PI secretes DILPs, and reducing secretion of these DILPs or ablation of PI increases lifespan (Broughton et al., 2005; Wessells et al., 2004). It remains to be investigated if SIFamide signaling influences the synthesis or secretion of DILPs in the PI. Calpains, calcium-dependent cysteine proteases, are implicated in protein turnover, intracellular cell signaling, cell cycle, apoptosis, and cell motility (Nixon, 2003). Calpain activity increases in normal brains with age (Benuck et al., 1996). Calpain is also elevated in the postmortem brain tissues of patients of Alzheimer's and Parkinson's diseases (AD and PD) (Crocker et al., 2003; Saito et al., 1993). Conversely, calpain activity is low in long-living bats (Baudry et al., 1986) and inhibition of calpain improves symptoms of AD (Trinchese et al., 2008) and PD (Crocker et al., 2003). Thus, while an increase of calpain activity should be adverse to longevity, our results suggest the opposite, and further investigation is needed to understand the effect of calpain function in aging. *CG8155* is a Rab GTPase activator and homologue of human TBC1 domain family member 25. It functions in intracellular trafficking of vesicles and signaling with phosphoinositides and growth factor receptors (Stenmark, 2009). High expression of Rab25, one of Rab GTPases, is found in ovarian and breast cancer (Cheng et al., 2004). Another subtype of Rab GTPase, Rab27B, is enhanced in senescent human fibroblast (Fujii et al., 2006). It is unclear whether increases of theses Rab GTPases are causes or consequences of aging. Our results suggest that certain types of Rab GTPases could be beneficial in longevity.

Dynein light chain 90F (*Dlc90F*) in 'cellular component movement' is a member of the dynein light-chain family (Davis and Smith, 2005). In aged monkey brain, dynein accumulates at the nerve endings and less dynein interacts with dynactin, causing accumulation of endogenous Tau and amyloid precursor proteins (Kimura et al., 2007). Mutations of Dynein are thought to reduce autophagic clearance of aggregate-prone proteins, leading to aggregation of GpC rich proteins such as Huntingtin (Ravikumar et al., 2005). Mutants of *Drosophila* dynein light chain 1 likewise reduce of autophagy in neurons and affect larval motility (Batlevi et al., 2010). We might predict, therefore, that over-expression of *Dlc90F* increases neuronal autophagic activity, and this might be sufficient to increase lifespan as has been observed for over-expression of *Atg8a* (Simonsen et al., 2008).

The category 'cellular metabolism' includes: aforementioned *CG33138, CG10383*, NADP-dependent malate dehydrogenase (*men*) and *CG30427. CG10383* is the homologue of human serine active site containing 1 (SERAC1) and is inferred to play a role in glycosylphosphatidylinositol metabolism, and has been associated with male sterility (Schimenti et al., 2005). No information is available about *CG10383. CG30427* is a homologue of human fatty acyl-CoA reductase, which converts long-chain aldehyde to long-chain acyl CoA, in the process producing NADPH (Riendeau et al., 1982). *men*, a key enzyme of the malate-pyruvate shuttle, converts malate to pyruvate to produce NADPH in the cytosol (Geer et al., 1979; MacDonald, 1995). The production of NADPH from over-expression of *men* and CG30427 may assist enzymes that scavenge cellular reactive oxygen species.

In the category 'immunity', peptidoglycan recognition protein LF (*PGRP-LF*) was identified from our screen. Unlike *PGRP-LC* and *-LE* that activate immune deficiency (IMD) signaling pathway, *PGRP-LF* inhibits immunity by sequestering circulating peptidoglycans (Aggarwal and Silverman, 2008; Maillet et al., 2008). While over-expression of *PGRP-LE* in *Drosophila* fat body enhanced pathogen resistance, this also shortened lifespan (Libert et al., 2006). Considered with our current results, activated immunity appears to represses lifespan, while suppressed immunity, all else being equal, slows *Drosophila* aging.

Two genes, *CG42663* and *CG10916*, are uncategorized. *CG10916*, a zinc ion binding protein, was identified as one of the genes that were significantly up-regulated under hyperoxia in *Drosophila* heads (Gruenewald et al., 2009). Taken together with our results, such increase may be protective from hyperoxic stress.

The wild-type *CS10* is established by backcrossing *Canton-S* to w^{1118} (Simon et al., 2003). The MLS of *CS10* (33 days) measured in the present study is similar to the other previous report (Yamazaki et al., 2007), but is much shorter than *Canton-S* and w^{1118} (Grandison et al., 2009; Lin et al., 1998). So, we compared fecundity, feeding behavior and locomotor activity of *CS10* with those of *Canton-S* and w^{1118} flies (Supplementary Fig. 1). But no difference in these criteria was found among these wild-type flies. The cause of shorter lifespan in *CS10* remains unclear.

One of the strongest assets of invertebrate genetic models of aging is their capacity for forward genetic screening. In this way, one can efficiently discover novel genes and pathways that assure longevity and thus lead to insights on the mechanism underlying senescence. This approach has been used several times with the nematode *C. elegans* where gene knockdown is rapidly induced by feeding *E. coli* engineered to produce specific dsRNA (Timmons and Fire, 1998). Screening with *Drosophila* is conducted by chemical mutagenesis or by the random insertion of engineered transposons, which have the capacity to produce both loss- or gain-of-function mutations (Ashburner et al., 2005; Rørth et al., 1998). Several previous studies have reported results from transposon screens. The first gene

described to affect *Drosophila* lifespan was *methusulah*, which was identified from a collection of P-element insertion mutants (Lin et al., 1998). Likewise, *indy* (*I* am *n*ot *d*ead *y*et) was found in a collection of P-element *lac-z* strains (Rogina et al., 2000). Seong *et al.* conducted a systematic gain-of-function screen for longevity benefits among 646 P-element insertions and reported 23 genes extending the lifespan (Seong et al., 2001). In an important experimental design, Landis *et al.* developed a system of doxycycline-inducible P-element insertion to make perfect genetic controls for each individual insertion genotype (Landis et al., 2003). They reported 6 longevity genes from a screen of approximately 10,000 mutants. Despite these collected efforts, it is clear that screening in *Drosophila* has not yet reached saturation for the aging phenotype because to date there is little to no overlap in the candidates so far described.

4. Conclusions

We studied the lifespan of 45 isogenic *Drosophila* EP lines to find novel genes that extend the lifespan, and confirmed 15 genes as longevity genes. Among these longevity genes, we also verified that gene-specific over-expression of *ImpL2* (ecdysone-inducible gene L2) and *CG33138* (1,4-alpha-glucan branching enzyme) by ubiquitous or tissue-specific GS-Gal4 drivers is sufficient to extend the lifespan. Extensive investigation of these longevity genes would fit some genes in current aging mechanisms, and offer opportunities to identify new systems that control aging processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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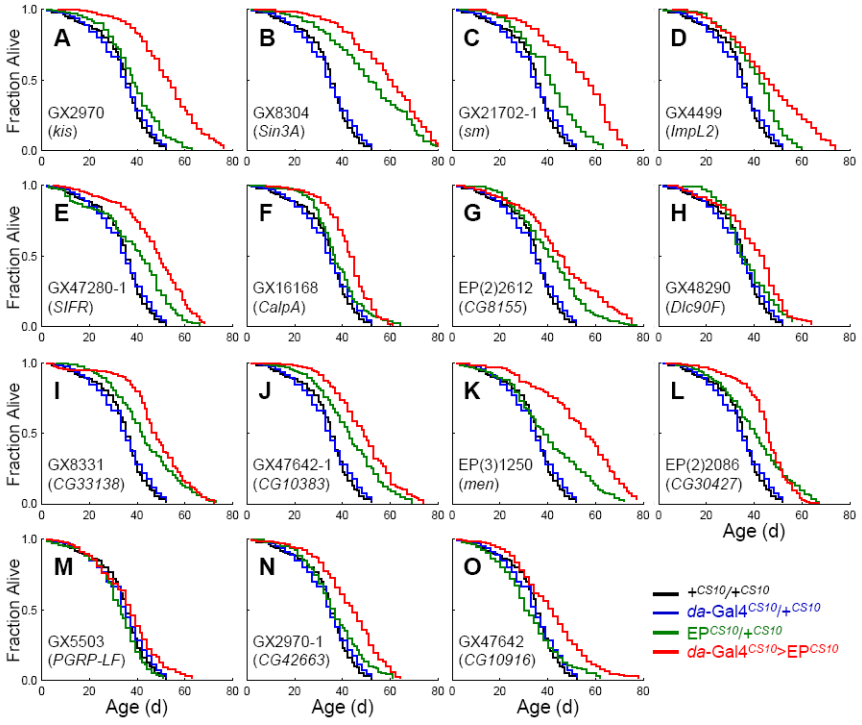
Abbreviations

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

1.1



1.2

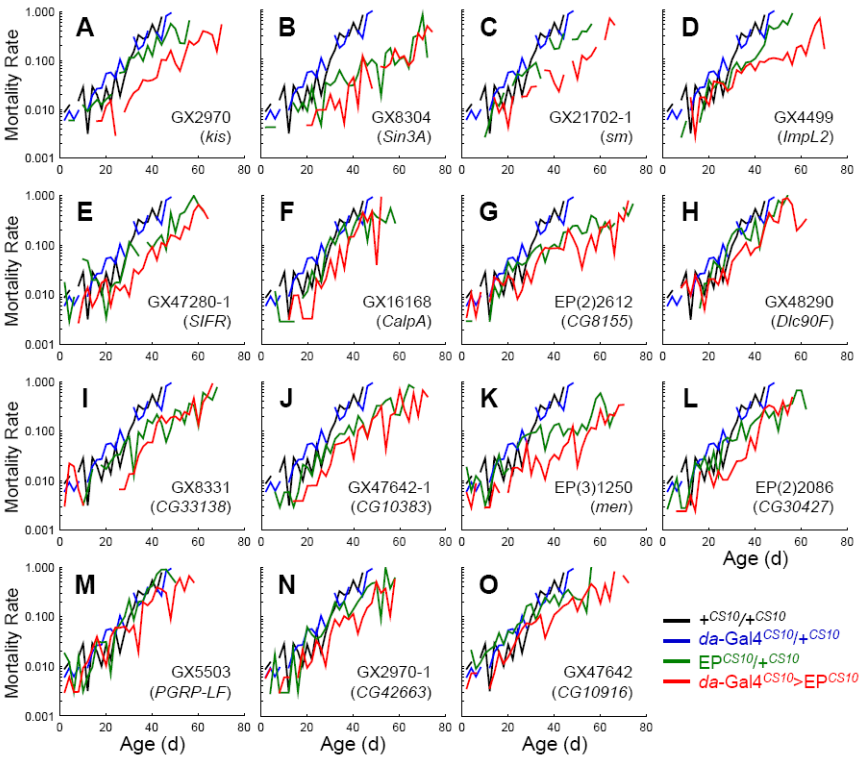
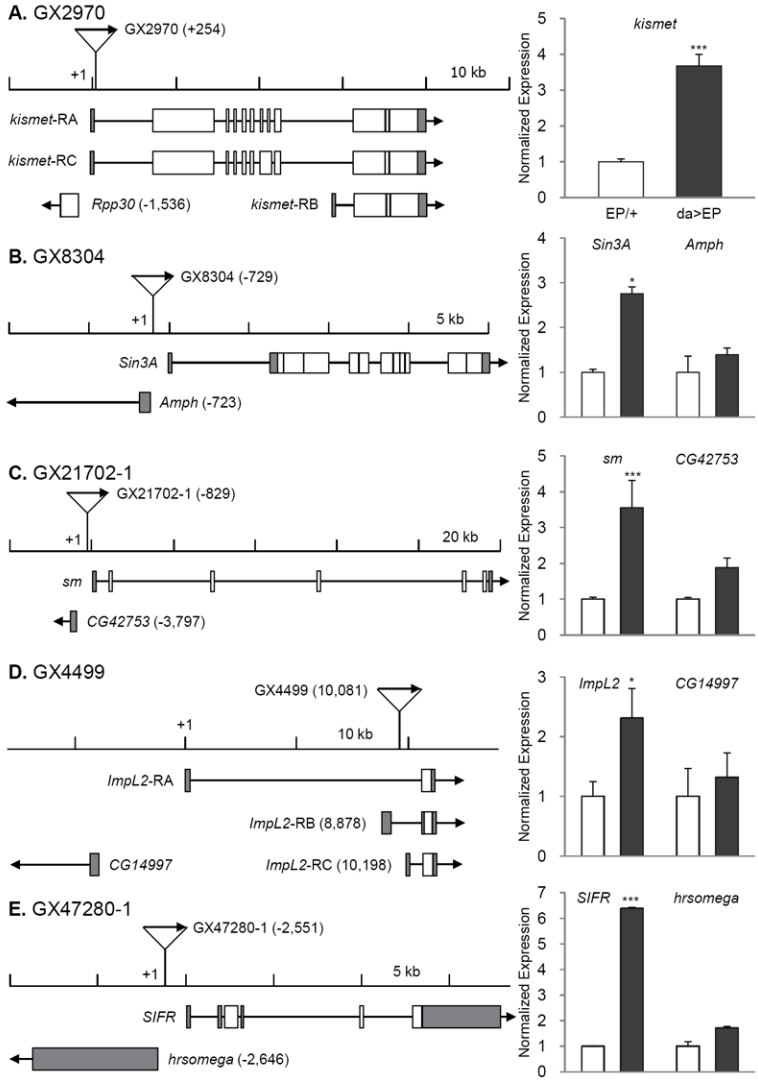
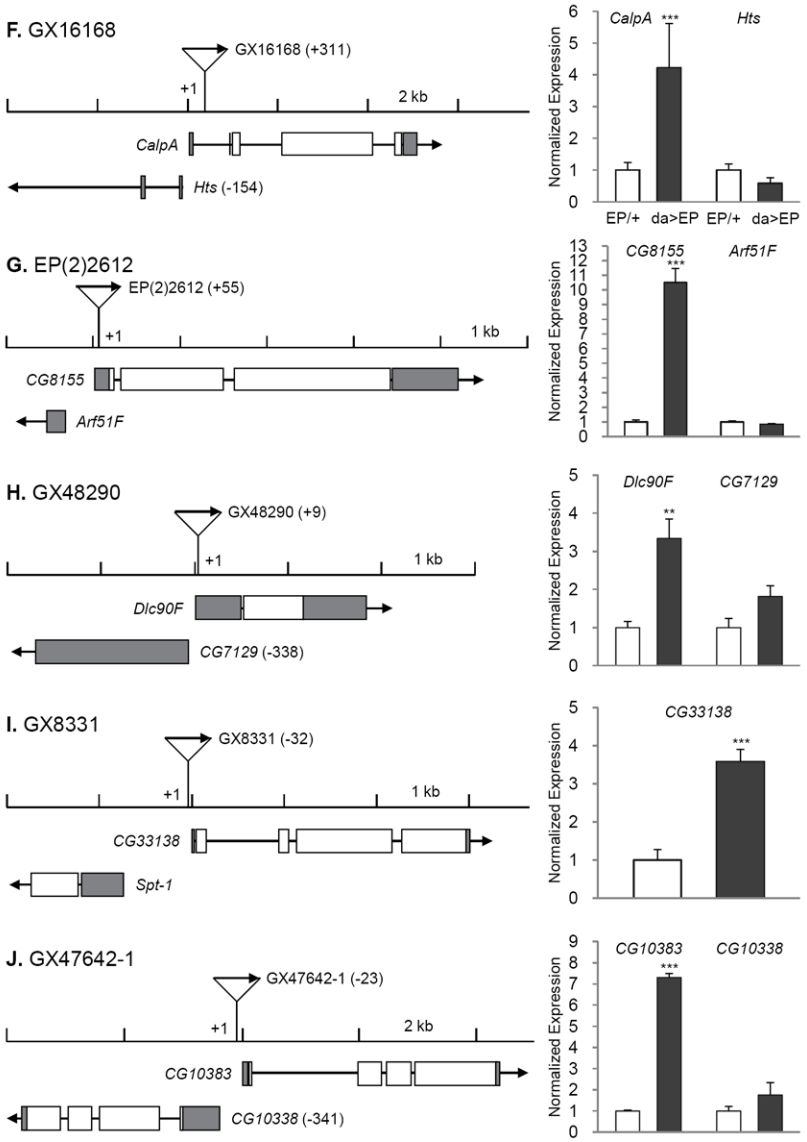


Fig. 1. Survival and mortality of long-lived 14 EP lines induced by *da*-Gal4

Survivorship (fraction alive, Fig 1.1) and mortality rate (Fig 1.2) of $+^{CS10}/+^{CS10}$ (black line), *da*-Gal4$^{CS10}/+^{CS10}$ (blue line), EP$^{CS10}/+^{CS10}$ (green line), and *da*-Gal4^{CS10}>EP^{CS10} (red line). Survival plots are estimated by the Kaplan-Meier method. Mortality rate (μ_x) is estimated as $ln\,(-\,ln\,p_x)$, where p_x is the age-specific probability of survival.





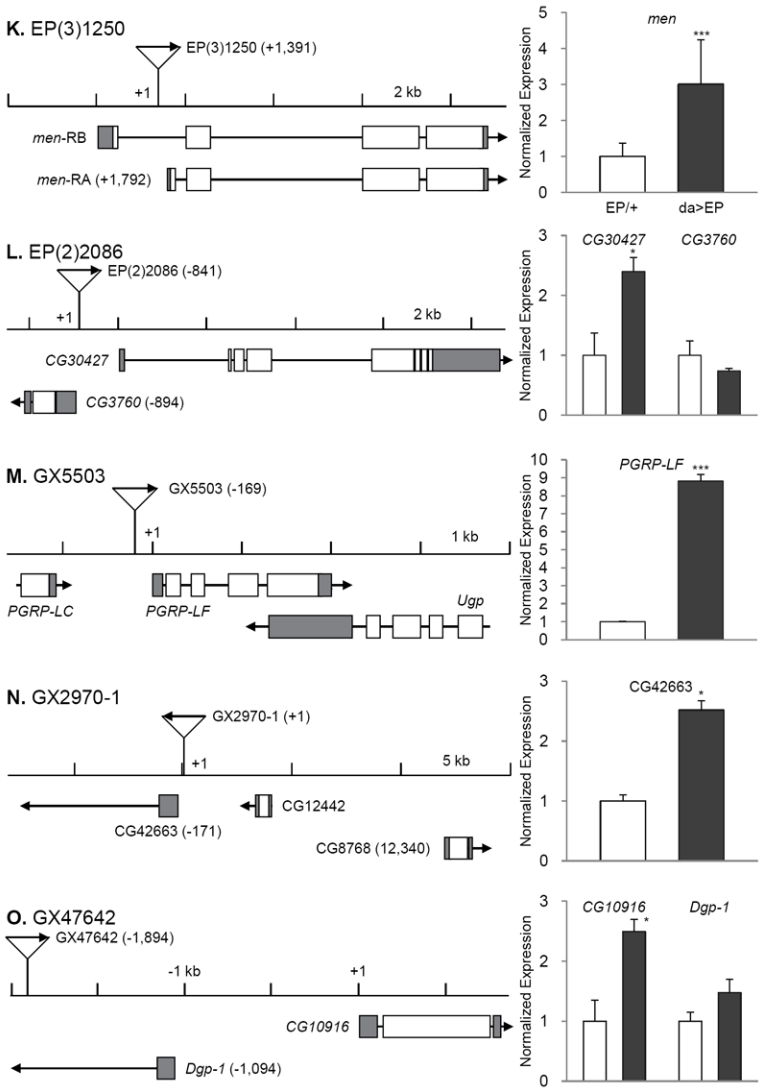


Fig. 2. Insertion position of each EP line and gene expression induction by *da*-Gal4

The transcription start sites are set as +1 and the relative positions of P-element are indicated in parentheses. Direction of transcription from EP and that of adjacent gene are marked with arrows. Induction levels of the target genes were measured by real time RT-PCR in $EP^{CS10}/+^{CS10}$ and *da*-$Gal4^{CS10}$ >EP^{CS10} flies and normalized to those of each $EP^{CS10}/+^{CS10}$. Statistical significance in induction levels between $EP^{CS10}/+^{CS10}$ and *da*-$Gal4^{CS10}$ >EP^{CS10} was obtained by chi-square test. In cases an EP is inserted in the region where it could affect two genes transcribed to the opposite directions, we measured expression levels of both genes (B, C, D, E, F, G, H, J, L, and O). Data are presented with mean and standard error bars. Asterisks indicate significant changes by $p < 0.05$-0.001.

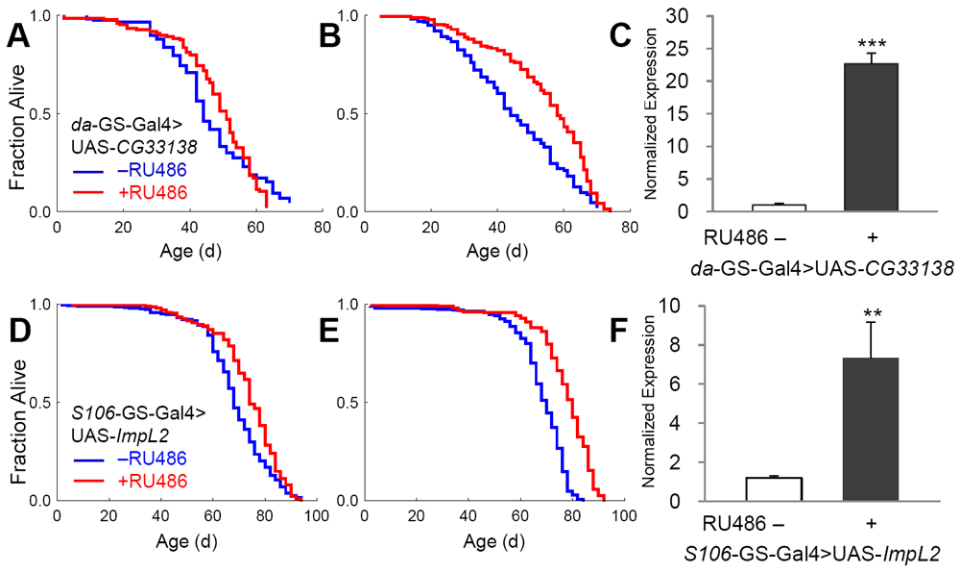


Fig. 3. Over-expression of *CG33138* and *ImpL2* during adulthood extends *Drosophila* lifespan

Using the inducible and ubiquitous *da*-GS-Gal4 with *CG33138* and the adult fat cell specific *S106*-GS-Gal4 with *ImpL2*, over-expression was induced by feeding flies with 200 μM RU486 after eclosion in males (A and D) and females (B and E). Gene expression of *CG33138* was enhanced more than 20 fold (C). Gene induction levels reached more than 7 fold by over-expression of *ImpL2* (F). Survival curves show control cohorts treated with vehicle only (blue) and with RU486 (red). Asterisks indicate statistical significance by chi-square test (**, $p < 0.01$; ***, $p < 0.001$).

Table 1

Longevity of 45 EP lines induced by *da*-Gal4

Forty-five isogenic EP lines, in which an EP is inserted in the same orientation of normal transcription, were crossed with either isogenic *CS10* or isogenic *da*-Gal4. Lifespans of their male progeny were measured at 25°C. The target genes of these EP lines are categorized into 8 groups, based on their functions. Fifteen EP lines that as *da*-Gal4^{CS10}>EP^{CS10} presented significantly longer mean lifespan than EP^{CS10}/+^{CS10}, *da*-Gal4^{CS10}/+^{CS10} (33.6 d) and +^{CS10}/+^{CS10} (33.4 d) were selected as longevity candidates (bold type; $p < 0.001$ by Log-Lank test). Five EP lines (GX193, GX7554, GX5503-1, GX8277, and GX6548) were lethal when induced by *da*-Gal4. Human homologs of these genes are indicated in the parentheses. Mean lifespan (MLS), maximum lifespan (MaxLS, day when the final survivor died), and increased lifespan (ILS, ratio of *da*-Gal4^{CS10}>EP^{CS10} to EP^{CS10}/+^{CS10}) are presented with the number (n) of flies tested.

EP line	Gene	Description (Human Homolog)	/+^{CS10}			/*da*- Gal4^{CS10}			ILS	*Log-Rank Probability*		
										+^{CS10} /+^{CS10}	Gal4^{CS10} /+^{CS10}	EP^{CS10} /+^{CS10}
			MLS	MaxLS	(n)	MLS	MaxLS	(n)		*vs. da* -Gal4^{CS10} >EP^{CS10}		
Transcription & translation												
GX193	*CG10209*	DNA binding	39.4	63.0	(361)		*lethal*		–	–	–	–
GX2970	***kis***	**DNA helicase (CDH7)**	**37.8**	**62.0**	**(357)**	**51.4**	**76.0**	**(382)**	**1.36**	**< 0.001**	**< 0.001**	**< 0.001**
GX6561	***kis***	**DNA helicase (CDH7)**	**36.6**	**59.0**	**(348)**	**45.3**	**76.0**	**(345)**	**1.24**	**< 0.001**	**< 0.001**	**< 0.001**
GX7554	*dan*	Transcription factor	42.7	71.0	(360)		*lethal*		–	–	–	–
GX8261	*gug*	Histone deacetylase	38.7	62.0	(385)	37.7	80.0	(358)	0.97	< 0.001	< 0.001	0.293
GX8304	***Sin3A***	**Transcription repressor (SIN3A)**	**50.5**	**78.0**	**(251)**	**57.2**	**80.0**	**(237)**	**1.13**	**< 0.001**	**< 0.001**	**< 0.001**
GX8403	*RpL13A*	Ribosomal protein L13 (RPL13A)	44.0	66.0	(172)	44.2	66.0	(220)	1.00	< 0.001	< 0.001	0.687
GX21702	*Rat1*	5’-3’ exoribonuclease (XRN2)	39.4	62.0	(285)	34.8	56.0	(323)	0.88	0.051	0.320	< 0.001
GX21702-1	***sm***	**Heterogeneous nuclear ribonucleoprotein L (HNRNPL)**	**40.4**	**62.0**	**(383)**	**52.1**	**72.0**	**(257)**	**1.29**	**< 0.001**	**< 0.001**	**< 0.001**
GX25754	*CG4901*	ATP-dependent RNA helicase (DHX33)	37.5	70.0	(613)	34.6	63.0	(594)	0.92	0.008	0.034	< 0.001
GX31631	*Thor*	Eukaryotic initiation factor 4E binding	46.2	75.0	(593)	46.7	77.0	(567)	1.01	< 0.001	< 0.001	0.567
GX46252	*mam*	Transcription coactivator	43.3	70.0	(611)	41.8	72.0	(586)	0.97	< 0.001	< 0.001	0.107
EP(2)2559	*CG3927*	RNA binding	31.4	60.0	(559)	34.9	65.0	(602)	1.11	0.013	0.080	< 0.001
Cell cycle												
GX822	*CycG*	Cyclin-dependent protein kinase regulator (CCNG2)	40.2	69.0	(577)	38.1	73.0	(549)	0.95	< 0.001	< 0.001	0.420
GX11242	*CycB3*	Cyclin B3	43.6	68.0	(362)	39.4	64.0	(327)	0.90	< 0.001	< 0.001	< 0.001
Signal transduction												
GX4499	***ImpL2***	**Ecdysone-inducible gene L2**	**39.8**	**60.0**	**(391)**	**45.7**	**74.0**	**(385)**	**1.15**	**< 0.001**	**< 0.001**	**< 0.001**

EP line	Gene	Description (Human Homolog)	/+^{CS10}			/*da*- Gal4^{CS10}			ILS	*Log-Rank Probability*		
										+^{CS10} /+^{CS10}	Gal4^{CS10} /+^{CS10}	EP^{CS10} /+^{CS10}
			MLS	MaxLS	(n)	MLS	MaxLS	(n)		*vs. da* -Gal4^{CS10} >EP^{CS10}		
GX47280-1	***SIFR***	**Neuropeptide receptor (NPFFR2)**	**38.4**	**66.0**	**(364)**	**47.1**	**68.0**	**(381)**	**1.23**	**< 0.001**	**< 0.001**	**< 0.001**
GX5503-1	*Rapgap1*	Ras GTPase activator (RAP1GAP)	36.3	58.0	(350)		*lethal*		–	–	–	–
GX6589	*cv-2*	Cysteine-rich domain (BMPER)	34.6	60.0	(388)	34.8	58.0	(377)	1.01	0.028	0.082	0.548
GX8277	*gom*	Calcium ion binding	37.1	56.0	(317)		*lethal*		–	–	–	–
GX8630	*Lrch*	Leucine-rich-repeats and calponin homology domain protein (LRCH2)	39.4	69.0	(631)	40.0	65.0	(539)	1.02	< 0.001	< 0.001	0.673
GX8689	*vimar*	Ral GTPase binding (RAP1GDS1)	33.6	66.0	(330)	33.6	60.0	(371)	1.00	0.597	0.977	0.474
GX16168	***CalpA***	**Calcium-dependent cysteine-type endopeptidase (CAPN9)**	**37.5**	**64.0**	**(368)**	**42.0**	**60.0**	**(347)**	**1.12**	**< 0.001**	**< 0.001**	**< 0.001**
EP(2)2612	***CG8155***	**Rab GTPase activator (TBC1D25)**	**39.7**	**76.0**	**(369)**	**45.6**	**74.0**	**(294)**	**1.15**	**< 0.001**	**< 0.001**	**< 0.001**
Cellular component movement												
GX48290	***Dlc90F***	**Dynein intermediate chain binding (DYNLT1)**	**36.5**	**56.0**	**(197)**	**39.8**	**64.0**	**(224)**	**1.09**	**< 0.001**	**< 0.001**	**0.002**
Cellular metabolism												
GX1008	*nemy*	Carbon-monoxide oxygenase	33.0	56.0	(375)	35.3	59.0	(296)	1.07	0.017	0.072	0.035
GX1008-1	*CG42708*	Glutaminase	40.4	66.0	(317)	40.3	61.0	(342)	1.00	< 0.001	< 0.001	0.731
GX4385	*CG13890*	Dodecenoyl-CoA delta-isomerase	36.8	60.0	(626)	36.5	63.0	(546)	0.99	< 0.001	< 0.001	0.297
GX8295	*arf51F*	NAD(P)$^{+}$-protein-arginine	35.2	62.0	(280)	33.9	58.0	(336)	0.96	0.032	0.518	0.111
GX8331	***CG33138***	**1,4-alpha-glucan branching enzyme (GBE1)**	**41.9**	**72.0**	**(340)**	**46.4**	**72.0**	**(354)**	**1.11**	**< 0.001**	**< 0.001**	**< 0.001**
GX47642-1	***CG10383***	**Hydrolase (SERAC1)**	**42.0**	**68.0**	**(372)**	**47.2**	**74.0**	**(285)**	**1.12**	**< 0.001**	**< 0.001**	**< 0.001**
GX56643	*eco*	Acetyltransferase	47.3	72.0	(223)	36.8	66.0	(204)	0.78	< 0.001	< 0.001	< 0.001
GX62810	*fabp*	Fatty acid binding	41.9	71.0	(593)	42.1	72.0	(585)	1.00	< 0.001	< 0.001	0.219
EP(3)1250	***men***	**NADP-dependent malate dehydrogenase (ME3)**	**39.5**	**72.0**	**(350)**	**52.4**	**76.0**	**(374)**	**1.33**	**< 0.001**	**< 0.001**	**< 0.001**
EP(2)2086	***CG30427***	**Oxidoreductase**	**38.1**	**66.0**	**(380)**	**44.9**	**66.0**	**(464)**	**1.18**	**< 0.001**	**< 0.001**	**< 0.001**
Transport												
GX26268	*Atpα*	Na pump α subunit (ATP1A3)	52.2	78.0	(619)	49.9	77.0	(597)	0.96	< 0.001	< 0.001	0.012
EP(3)3232	*drip*	Water channel (AQP4)	48.0	76.0	(330)	42.3	74.0	(344)	0.88	< 0.001	< 0.001	< 0.001
Immunity												
GX5503	***PGRP-LF***	**Peptidoglycan recognition protein LF (PGLYRP3)**	**31.3**	**51.0**	**(356)**	**35.5**	**63.0**	**(351)**	**1.13**	**< 0.001**	**0.002**	**< 0.001**
GX6548	*PGRP-LC*	Peptidoglycan recognition protein LC	44.3	70.0	(372)		*lethal*		–	–	–	–

EP line	Gene	Description (Human Homolog)	/+^{CS10}			/*da*- Gal4^{CS10}			ILS	*Log-Rank Probability*		
										+^{CS10} /+^{CS10}	Gal4^{CS10} /+^{CS10}	EP^{CS10} /+^{CS10}
			MLS	MaxLS	(n)	MLS	MaxLS	(n)		*vs. da* -Gal4^{CS10} >EP^{CS10}		
Others												
GX1042	*CG42268*	Unknown	36.3	60.0	(345)	35.6	63.0	(362)	0.98	< 0.001	< 0.001	0.606
GX2970-1	***CG42663***	**Unknown**	**36.0**	**60.0**	**(368)**	**42.5**	**64.0**	**(356)**	**1.18**	**< 0.001**	**< 0.001**	**< 0.001**
GX3571	*Mbs*	Myosin phosphatase (PPP1R12A)	55.9	83.0	(387)	47.2	78.0	(350)	0.84	< 0.001	< 0.001	< 0.001
GX8400	*CG5861*	Transmembrane protein (TMEM147)	40.3	66.0	(347)	37.3	62.0	(324)	0.93	< 0.001	< 0.001	< 0.001
GX47642	***CG10916***	**Zinc ion binding**	**32.0**	**62.0**	**(361)**	**40.8**	**78.0**	**(293)**	**1.28**	**< 0.001**	**< 0.001**	**< 0.001**
GX62808	*CG5807*	Limb region 1 homolog-like (LMBR1L)	47.5	75.0	(596)	42.6	70.0	(572)	0.90	< 0.001	< 0.001	< 0.001