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Neuronal Susceptibility to GRIM in *Drosophila melanogaster* Measures the Rate of Genetic Changes that Scale to Lifespan

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Abstract

Gene expression in *Drosophila melanogaster* changes significantly throughout life and some of these changes can be delayed by lowering ambient temperature and also by dietary restriction. These two interventions are known to slow the rate of aging as well as the accumulation of damage. It is unknown, however, whether gene expression changes that occur during development and early adult life make an animal more vulnerable to death. Here we develop a method capable of measuring the rate of programmed genetic changes during young adult life in *Drosophila melanogaster* and show that these changes can be delayed or accelerated in a manner that is predictive of longevity. We show that temperature shifts and dietary restriction, which slow the rate of aging in *Drosophila melanogaster*, extend the window of neuronal susceptibility to GRIM over-expression in a way that scales to lifespan. We propose that this susceptibility can be used to test compounds and genetic manipulations that alter the onset of senescence by changing the programmed timing of gene expression that correlates and may be causal to aging.

Introduction

The idea of using the temporal pattern of specific gene expression changes to measure the rate of aging has been suggested and has in several cases correlated extremely well with percent lifespan and not chronological age (Helfand *et al.* 1995; Rogina *et al.* 1997; Rogina and Helfand 1995; Rogina and Helfand 1997). It is currently unknown whether these and other gene expression changes play some role in the causality of aging or if the rate of aging would be slowed if *Drosophila* could maintain their youthful levels of gene expression indefinitely.

It is intriguing to postulate that the negative aspects of aging begin only once *Drosophila* and other organisms undergo their full developmental program and that delaying particular genetic changes could prevent the onset of senescence and the accumulation of damage associated with it. In support of this theory, the accumulation of protein oxidation does not increase linearly with age, and shows an accumulation only later in life (Levine and Stadtman 2001). Lipid oxidation also shows a similar pattern, and the onset of this damage can be delayed by a reduction in ambient temperature and caloric intake, manipulations known to extend lifespan and delay particular genetic changes (Zheng *et al.* 2005b).

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The number of genes in *D. melanogaster* that change with age has been reported to be on the order of 10% with microarray studies (Kim *et al.* 2005; Landis *et al.* 2004; Pletcher *et al.* 2005; Pletcher *et al.* 2002). Over 80% of enhancer trap lines, however, show age-dependent changes in not only temporal expression but also in the tissues they're expressed (Seroude *et al.* 2002), suggesting that there are many tissue specific genetic differences that previous whole body and head and thorax microarray studies are incapable of identifying.

The use of a single gene as a biomarker of aging is problematic in that a genetic or environmental manipulation may basally alter the expression of a single gene and thus the identification of multiple genes that change expression in a way that scales to percent lifespan would be much more desirable. Another alternative would be to identify a physiological window that changes with age and also scales to lifespan.

D. Melanogaster reaches adulthood very quickly upon eclosion and can effectively mate and reproduce within 24hrs. The study of many enhancer trap lines indicates that there are many programmed genetic changes that occur during the first week of life (Seroude *et al.* 2002). One of these changes is the loss of endogenous caspase activity in brains (Zheng *et al.* 2005a). We explored the nature of the genetic changes involved in the loss of apoptosis in the brain and found that the ability to drive caspase activity in neurons is not initially lost. In this study we find that neurons retain the ability to undergo apoptosis into early adult life with an adequate pro-apoptotic stimulus but that this ability declines with age in a predictable manner with interventions known to extend lifespan. Expanding upon a previous study that found an age-dependent change in neuronal susceptibility to GRIM over-expression (Poon, 2007), we develop an assay that allows the measurement of the rate of particular genetic changes that appear to coincide and be predictive of the rate of aging. This assay will be useful for screening mutants and drugs that may extend lifespan in part by delaying the rate of gene expression changes.

Materials and Methods

Preparation of standard cornmeal food and other food types

Standard cornmeal food (1x food): For every 1L of food made, 8.9g of agar, 124.4g sucrose, 31.1g inactive yeast, and 53.6g of cornmeal was added, mixed and autoclaved. Food was cooled to 65°C and 2.7g tegosept, dissolved in 11.8mL of ethanol was added along with 5.3mL of propionic acid. For RU486 containing food 20ml of RU486 dissolved in etoh at either 200µM or 500µM concentration was added at temperature below 65C. In food without RU486, 20mL of etoh was added. 0.5x food was prepared similarly to 1x food except that only 50% of the yeast 15.6g and 50% of the sucrose 62.2g was added per Liter. 2x food was similar to 1x food except that twice as much yeast 62.2g and twice as much sugar 248.8g was added per Liter. 9% yeast food was prepared similarly to 1x food except that three times the yeast 93.3g was added per Liter. 18% yeast food was the same as 1x food except that 186.6g yeast was added per Liter.

Stocks

The ELAV-GeneSwitch line was from Haig Keshishian (Yale University, New Haven, CT). UAS-p35 and UAS-GFP were from the Bloomington Stock center lines 5072 and 1521, respectively. UAS-DIAP1 was from Bruce Hay (California Institute of Technology, Pasadena, CA). UAS-DRONC and UAS-GRIM were from John Abrams (UT Southwestern Medical Center, Dallas, TX). All stocks were grown up and used as received.

Fly Culture and Strains

All flies were kept in a temperature-controlled incubator at 60% humidity with 12 h on/off light cycle at 25°C in vials containing standard cornmeal medium with lightly sprinkled live yeast on top. For all data shown in the GRIM susceptibility experiments, UAS-GRIM virgins were used and crossed to GeneSwitch ELAV males.

Life Span Determinations

Newly eclosed adults were collected within 24 hrs, and 25 males and 25 females were placed into individual vials with the indicated food and temperature. Multiple independent vials were set up per experiment ranging from 4 to 10. Every 1–2 days flies were passed into new vials, and the number of dead flies was counted. The data from table 2 and figure 3a was collected from an earlier study and done separately from the other experiments. It was determined later that passing vials every day often extends the median lifespan of mated flies and in all other experiments vials were passed almost daily, while in the first study flies were passed every 2 days. We also began our experiments using 200µM RU486 but decided that 500µM RU486 was a more effective dose for inducing Grim expression and death.

QPCR

Total RNA was isolated by homogenizing 40–55 fly heads at the indicated date and temperature and purified using the RNeasy kit (Qiagen, Valencia, CA). 0.5–1µg of RNA was first DNase treated with DNA-free kit (Ambion, Austin, TX) and then cDNA was made using superscript SSII or SSIII (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. QPCR was performed on an ABI 7500 Real-Time PCR machine using the ABI SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Each PCR reaction was done in triplicate to control for pipetting errors using 20µM of pairs of the following primers:

DIAP1-F, AACAAATGTGCCGATCAATGC;

DIAP1-R, ATTAGCTGCGACATGGGTATGAC;

GRIM-F, GTTTGGATGCTGGGATCTTTTG;

GRIM-R, GTCGTCCTCATCGTTGTTCTGA;

Tubulin-F, ACATCCCGCCCCGTGGTC;

Tubulin-R, AGAAAGCCTTGCGCCTGAACATAG.

To obtain an estimate of relative expression levels we used the comparative cycle threshold (Ct) method with Tubulin as the endogenous reference gene.

Caspase assay

2–3 female heads or 4 male heads were homogenized on ice in 80–100µl of assay buffer. Assay buffer was composed of the following: 10mM tris-HCl (pH 8.4), 100 mM NaCl, 1 mM MgCl₂. Protease inhibitors pepstatin A, leupeptin, antipain, aprotinin and chymostatin were added immediately before use to a final concentration each of 2.5µg/mL. Samples were spun for 5 min at ~15,000g @ 4°C and 30µl of the supernatant was added to a costar white, opaque 96 well half area plate (Corning Incorporated, Corning, NY). 30µl of Glo reagent from the Caspase-Glo 3/7 Assay kit (Promega, Madison, WI) was added to each sample and mixed and incubated for 45 min to 1hr before luminescence was read with a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT). The remaining supernatant was used to quantify

protein/ μ l using a Bio-rad protein assay (Bio-Rad Laboratories, Hercules, CA) with Bovine Gamma Globulin (Pierce, Rockford, IL) as a standard measuring 595 absorbance with a Fisherbrand flat bottom clear 96 Well Plate.

GFP measurements

Single fly heads, bodies, or whole flies were homogenized in 80 μ l of assay buffer and spun for 5 min at 15000g @ 4C. 50 μ l of supernatant was used to add to a costar 96 well half area black opaque plate (Corning Incorporated, Corning, NY). Fluorescence was measured using a BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT) with (485 excitation/530 emission) filters. Protein/ μ l was quantified the same way as the caspase assay. For homozygous GFP; ES flies we detected ~30 fold increase in background GFP readings over buffer alone from eye pigment from the miniwhite gene. This background actually increases as the pigment changes color during the first week of life. To control for this we subtracted age matched controls not fed RU486. In contrast, bodies have ~0 background compared to buffer alone.

Statistics

Statistical analyses, including log rank tests, anova, and unpaired t-tests, were performed by using the Prism suite of biostatistical software (GraphPad, San Diego, CA). Error bars on graphs represent standard error mean while QPCR error bars were calculated as 95% confidence intervals using the software analysis package of the ABI 7500 Real-Time PCR machine.

Results

Induction of death by neuronal GRIM over-expression shows an age-dependent decline

Flies that allowed the inducible targeting of the pro-apoptotic gene, GRIM, selectively to the nervous system were created by mating female UAS-GRIM virgins to males containing the GeneSwitch ELAV driver (ES) (Osterwalder *et al.* 2001). This driver is active primarily in neurons where the modified yeast GAL4 transcription protein is expressed and can bind to the UAS promoter and activate transgene gene expression in the presence of the ligand RU486. When 500 μ M RU486 (to activate GRIM gene expression) was mixed into our standard cornmeal food and fed to flies during the first week after eclosion there was a significant increase in death compared to flies not fed RU486 (Figure 1a,b, Table 1). Within 48 hours of exposure to RU486, females did not lay any eggs and many displayed a bloated abdomen phenotype, which was a phenotype occasionally found in males. By delaying the initial feeding of RU486 to day 10, female flies no longer died significantly more than controls (figure 1a) although egg laying was still prevented and the bloating of the abdomen often remained. The change in susceptibility to death correlated with a loss of caspase induction in the heads of flies fed 500 μ M RU486 for 24 hours compared to age-matched controls (Figure 1c): d1 = 3.0 $\pm$ 0.2 p<.0001, d4 = 6.7 $\pm$ 1.5 p=.005, d7 = 1.1 $\pm$ 0.2, d10 = 0.8 $\pm$ 0.1, d13 = 0.7 $\pm$ 0.1, n = 5 each. The increase in caspase activity between day 1 and 4 may reflect an initial increase in GRIM susceptibility or possibly a diminished food intake on the first day of eclosion.

The change in susceptibility to GRIM was gradual and showed individual variability in that the earlier a population of flies was fed RU486 for the first time, the more flies showed a high level of susceptibility. For example, there is a clear difference in the number of flies that die in the first 15–20 days post GRIM induction between day 4 and day 7 (Figure 1a). More flies are resistant at day 7, which suggests that susceptibility is determined by temporal genetic variation.

In contrast, males were still susceptible to GRIM induced death at day 10 (Figure 1b) and this correlated with the continued ability of GRIM induction to induce caspase activity in heads (Figure 1d). Males did, however, show a decrease in susceptibility with age as measured by the reduction in fold increase of head caspase activity (Figure 1d): d4 = 4.7 ± 0.8 $p < .003$ compared to uninduced control, d13 = 2.3 ± 0.3 $p = .005$ compared to uninduced control, $n = 5$ each and $p < .03$ that d13 is less induced than d4. Males also had a delayed onset of death at later ages. For example, it took only 6 days for males to start dying faster than uninduced controls when fed 500 μ M RU486 for the first time at day 6, compared to 12 days when induced for the first time on day 12 (data not shown). That the males are more susceptible than females may be due to a different expression pattern than females as GRIM-induced death rates are highly dependent on dose. It should be considered that RU486 and/or this particular over-expression system could enhance sensitivity to Grim and a different system is needed to confirm this that this is not the case.

It was previously reported that there is a dramatic decrease in endogenous caspase activity in the brain upon eclosion (Zheng *et al.* 2005a). We also found that this decrease was extremely rapid and correlated with the still developing morphology of the adult fly. The endogenous caspase activity (in arbitrary units) of w¹¹¹⁸ flies dropped significantly in only 10 hours; males 0–2 hrs old = 201.7 ± 48.2 , 10–22 hrs old = 29.3 ± 9.1 $p < .004$, and females 0–2 hrs old = 153.3 ± 33.1 , 10–22 hrs old = 18.3 ± 2.2 $p < .002$ $n=5$ each (Figure 1e).

The measured caspase activity in the brain of UAS-GRIM; ES female flies (not fed RU486) remained constant from 24 hours after emergence to at least day 13 (data not shown). We did however, detect at least a 3-fold increase in caspase activity compared to buffer alone with only two female heads and at least 10 -fold increase with 4 male heads, suggesting that there is a low but basal level of caspase activity due to the leakiness of the genetic system as endogenous caspase activity is not present in the adult fly brain (Zheng *et al.* 2005a).

The change in GRIM susceptibility is not related to a decrease in GAL4 driver expression

To determine if the change in GRIM susceptibility was intrinsic to the flies or if instead a decrease in the expression of the GAL4 driver was causing an apparent change in susceptibility we used GFP as a reporter to measure ELAV-GAL4 driver expression over time. To do this homozygous UAS-GFP; ES flies were fed 500 μ M RU486 continuously from the first day of eclosion and sampled throughout the first 38 days of life. Arbitrary units of GFP/ μ g protein was measured using a plate reader in both the head (Figure 2a, top): d6 = 63.3 ± 14.5 , d12 = 87.3 ± 5.8 , d18 = 155.2 ± 14.0 , d24 = 139.7 ± 6.4 , d31 = 168.6 ± 14.8 , d38 = 141.0 ± 37.5 ($n = 4$ each) d6 is significantly less $p < 0.04$ compared to all days except d12, and the body (Figure 2a, bottom): d6 = 4.0 ± 0.3 , d12 = 7.4 ± 0.4 , d18 = 9.8 ± 0.4 , d24 = 9.4 ± 0.5 , d31 = 10.8 ± 1.1 , d38 = 10.6 ± 1.9 . ($n = 4$ each) d6 is significantly less $p < 0.005$ compared to all days except for d12. Age-matched flies not fed RU486 were used to subtract out GFP auto-fluorescence from tissues and especially the eye pigment from the miniwhite gene. Plate reader background readings (in the absence of RU486) gave a 30-fold increase in GFP/ μ g protein in heads compared to bodies. This background actually increased during the first 10 days of life as the pigments darkened. In contrast, bodies did not have a significant increase in signal compared to buffer at any age.

GFP fluorescence increased greater than 2-fold from 6 days of feeding to 18 days in both the head and body and then reached a plateau. This suggested that driver expression was increasing during the first 1–2 weeks of life or that RU486 and/or GFP was accumulating. To determine this, mated female flies were fed RU486 for the first time at day 5 or day 10 for 1 week (Figure 2b). GFP expression was ~2x higher when initiated at day 10, suggesting that GAL4 expression was increasing in the brain with age: GFP/ μ g protein at day 5 = 72.7 ± 9.1 ($n = 6$), day 10 = 152.9 ± 40.8 ($n = 5$) $p < 0.07$.

To show that GFP did not remain indefinitely in the flies once expressed, female flies were removed from RU486 after 12 days of continuous induction and GFP expression was measured over time (Figure 2c). Fluorescence was significantly diminished after 6 days and was virtually gone by 12 days: d12 RU486 = 7.4 ± 0.4 , 6d etoh = 4.8 ± 0.3 , 12d etoh = 0.4 ± 0.3 , 26d etoh = 0 ± 0.1 . (n = 4 each) Each bar is different $p \leq 0.0002$ from every other bar except 12d etoh and 26d etoh. This result is consistent with western blot studies measuring the rate of decline of epitope tagged proteins (data not shown).

Death by GRIM over-expression is dose dependent

Our studies were now aimed at determining whether reduced temperature and a change in caloric intake could alter the window of neuronal GRIM susceptibility in a predictable manner that correlated with predicted lifespan. To do this, it was necessary to determine whether these two interventions themselves altered GRIM expression. It was previously shown that flies had a greater death rate with 500 μ M RU486 dissolved in standard cornmeal food compared to 200 μ M (Poon, 2007), which is consistent with greater GRIM gene expression as estimated using UAS-GFP flies as a surrogate reporter: whole body GFP/ μ g fluorescence (in arbitrary units) of homozygous mated female GFP; ES flies maintained on 200 μ M RU486 1x cornmeal food for 10 days post eclosion = 12.0 ± 1.0 (n = 8) vs. 500 μ M = 23.6 ± 2.2 (n = 8) $p = .0003$.

We next tested the death rates of flies fed the same concentration of RU486 on different caloric foods. The rate of death was much greater on the lower calorie food compared to the higher (Figure 3a, Table 2) $p < .0001$ that median lifespan of flies maintained on 0.5x with RU486 is less than 1x and 2x with RU486. To determine if this was related to a difference in intrinsic susceptibility due to increased protection from greater caloric intake or was instead related to GRIM dose (either from a difference in RU486 intake or a change in GAL4 driver expression), 200 μ M RU486 was fed continuously for 10 days to UAS-GFP; ES female flies on different caloric food media and GFP was measured. Flies raised on the food with the lowest caloric content had the greatest GFP expression: 0.5x food = 17.3 ± 1.1 , 1x food = 12.0 ± 1.0 , 2x food = 7.6 ± 0.5 n = 8 each, $p < .01$ that all means are different from each other (Figure 3b), which is consistent with compensatory feeding behavior on a low yeast diet (Carvalho *et al.* 2005; Min *et al.* 2007; Min and Tatar 2006).

Temperature also strongly influenced the inducible GAL4/UAS gene expression system. UAS-GRIM; ES flies fed 500 μ M RU486 at 18°C from day 1 of life did not die faster than controls not fed RU486. This is consistent with the inefficiency of the genetic expression system at low temperature (Figure 3c). 10-day GFP/ μ g expression of homozygous UAS-GFP; ES of female flies at 18°C is less than 50% that of age-matched flies maintained on the same food conditions at 25°C: 18°C = 10.4 ± 0.8 , at 25°C = 23.6 ± 2.2 n = 8 each, $p < .0001$.

To address the differences in gene expression caused by changes in ambient temperature and caloric intake, flies were initially maintained using different conditions (food and temperature) for a number of days and then switched to a uniform food for GRIM induction (Figure 4a). This food was our standard cornmeal food and 500 μ M RU486.

When flies were maintained on several different initial conditions for 10 days and then switched for 10 days to the standard RU486 food, GFP expression of UAS-GFP; ES flies was similar (Figure 4b) n = 5 each. To test whether GRIM expression was similar to that of GFP, and also was not initially different immediately after switching conditions, UAS-GRIM; ES flies were maintained for either 3 or 12 days at 18 or 25°C on standard cornmeal food without RU486 and then both were switched to 25°C on standard cornmeal food with 500 μ M RU486 for 24 hrs. RNA from the heads of female flies was extracted and analyzed for fold GRIM induction and found to be increased at day 12 (consistent with the results from Figure 2b) but the prior

temperature maintenance had no bearing on current GRIM induction with RU486 (Figure 4c). These results indicate that this experimental protocol was adequate for similar GRIM induction despite an initial difference in environmental conditions.

Lowering ambient temperature extends the window of GRIM susceptibility

The window of susceptibility for females on standard cornmeal food at 25°C ended by day 10, with very minimal susceptibility to death at day 9 (Table 1). We hypothesized that this change in susceptibility is due to intrinsic genetic expression changes that decrease susceptibility over time and that lowering ambient temperature would slow down the rate of temporal gene expression, thus extending the chronological time the flies were susceptible (Figure 4d).

Lifespan of UAS-GRIM; ES flies in the absence of RU486 was increased dramatically at 18°C compared to 25°C and also reduced significantly at 29°C (Table 3). To test if there were age dependent changes in susceptibility of flies maintained at different temperatures, flies were switched to 500µM RU486 at 25°C at day 4 (Figure 5a), day 7 (Figure 5b), or day 10 (Figure 5c). At day 4, flies maintained at 29°C had less susceptibility to GRIM than flies maintained at 18 or 25°C, which were similar. By day 7, flies previously maintained at 29°C were no longer susceptible compared to uninduced controls while flies maintained at 25°C were now clearly less susceptible as a population compared to flies maintained at 18°C. By day 10, only flies maintained at 18°C for that time remained susceptible. By day 20, flies maintained at 18°C also showed no susceptibility and actually outlived the 25°C control flies (Figure 5d, Table 3) as expected if aging was slowed during the first 20 days of life. Flies maintained for 16 days at 18°C did not live significantly shorter than the 25°C control curve but did live less than the 20-day switch flies, suggesting that the susceptibility window at 18°C ends completely between day 17 and 19 on standard cornmeal food.

The susceptibility to death by GRIM induction at different temperatures correlated with the ability of the 24hr feeding of 500µM RU486 to induce caspase activity in female heads compared to age-matched uninduced controls (Figure 5e): d4, 29°C = 1.4 ± 0.1 $p < 0.01$, 25°C = 6.7 ± 1.5 $p < 0.003$, 18°C = 5.2 ± 0.8 $p = .0004$; d7, 29°C = 0.8 ± 0.2 , 25°C = 1.1 ± 0.2 , 18°C = 5.8 ± 2.8 $p < 0.07$; d10, 25°C = 0.8 ± 0.1 , 18°C = 2.0 ± 0.4 $p < 0.04$; d13, 25°C = 0.7 ± 0.1 , 18°C = 2.3 ± 0.7 $p < 0.06$, $n = 5$ each. We confirmed that susceptibility to GRIM and the induction of caspase activity in female heads was not due to an increase in total GRIM expression (Figure 5f). Normalizing GRIM mRNA expression levels to day 3 induction of flies previously maintained at 18°C did not demonstrate any correlation between total GRIM levels and the ability to induce caspase activity or death. The values of relative GRIM mRNA expression after 24 hrs of 500µM RU486 feeding were 18°C d3 = 1 (± 0.1), 25°C d3 = 1.4 ± 0.2 , 29°C d3 = 0.9 ± 0.1 , 18°C d12 = 2.4 ± 0.2 , 18°C d20 = 2.5 ± 0.2 , 25°C d12 = 2.3 ± 0.1 , 29°C d6 = 1.3 ± 0.1 .

Increasing the caloric intake of yeast shortens the window of GRIM susceptibility

We next tested whether increasing the percentage yeast in the food medium would shorten the window of GRIM susceptibility due to an increase in caloric intake. All flies were raised on the same sugar and cornmeal diet but were supplemented with different percentages of yeast (3%, 9%, and 18%). When flies were switched after 4 days to standard cornmeal food (3% yeast) and fed 500µM RU486, flies that were previously maintained on 18% yeast showed a reduced susceptibility to GRIM, with no difference between 3% or 9% (Figure 6a).

If the switch to RU486 food was made on day 7, only flies maintained on the lowest caloric food had a reduced median lifespan (Figure 6b). These data suggest that caloric restriction is able to delay some of the same genetic changes that a reduction in temperature does but to a lesser extent.

The single control curve of 3% cornmeal food appeared adequate and did not require the additional control curves of 9% yeast switched to 3% yeast and 18% yeast switched to 3% yeast without RU486 because the time window of only 4 and 7 days was short enough to minimally effect lifespan. Evidence for this is found by examining the data in Tables 1 and 4 and comparing the median lifespans of flies maintained on 3%, 9% and 18% food before being switched to 3% food with RU486 after a time when the flies are no longer susceptible to GRIM over-expression. Median lifespans are all similar to the median lifespan of the 3% ethanol control, ~45 days when switched within 10 days.

Discussion

We have shown that the ability to induce death by apoptosis in the adult nervous system with the pro-apoptotic gene GRIM diminishes with age and that this period of change correlates to percent lifespan such that the time window of inducing death can be dramatically extended by lowering ambient temperature and slightly extended by lowering the percentage yeast in the diet. An interesting finding is observed by looking at mortality curves of flies subjected to GRIM over-expression. Under a particular temperature or food condition, each successive day that GRIM induction is delayed results in fewer of the population being affected (figure 1a, 5d) suggesting that stochastic genetic changes are occurring and there is variability in the regulation of these changes that can be manipulated.

The nature of the genetic change in GRIM susceptibility

There are seven known caspases in *Drosophila*; these include the initiator caspases DRONC, Strica, and Dredd and the executioner caspases Dcp-1, Decay, Drice, and Damm. Dark is also a player in apoptosis and is necessary for DRONC activation (Hay and GUO 2006). It is the relative levels of DIAP1 and active caspases that regulate apoptosis in *Drosophila*. DIAP1 is essential for inhibiting cell death and inhibits many caspases either by driving their degradation (Chai *et al.* 2003; Olson *et al.* 2003) or rendering them inactive. GRIM, along with the proteins Hid and REAPER are upstream of these factors and bind to DIAP1 and hinder its ability to disrupt caspase activity (Hay and Guo 2006).

We did not detect an increase in DIAP1 mRNA or any decrease in the mRNA of 8 pro-apoptotic genes tested during the first 12 days of life (data not shown). This does not exclude the possibility that protein levels are unchanged during this time, as DIAP1 protein levels are highly regulated.

To determine if death could still be induced in the adult fly downstream of the GRIM-DIAP1 interaction we over-expressed DRONC in the nervous system and observed a significant although not huge increase in death compared to ethanol control at days 6 and 12: control median lifespan = 38 days (n = 48), d6 = 30 days (p < .002, n = 96), d12 = 29 days (p < .0001, n = 97). Previous studies indicate that 200μM RU486 is enough to induce death at day 6 but not day 12 (Poon, 2007). That death was not more rapid or severe is consistent with endogenous DIAP1 levels being high enough to partially inhibit excess DRONC.

In support of increasing neuronal DIAP1 levels mediating the switch in GRIM susceptibility with age, the simultaneous neuronal over-expression of DIAP1 and GRIM inhibited death and caspase activity (figure 7a,b) as well as the bloating and inhibition of egg laying phenotypes observed in females when GRIM alone is expressed. The baculovirus p35 protein, which inhibits apoptosis, (Lannan *et al.* 2007) was also able to prevent death and other GRIM-induced phenotypes when over-expressed simultaneously with GRIM (Figure 7a,b), proving that GRIM is killing flies due to the activation of caspases.

Using the UAS-GRIM; GeneSwitch ELAV system for longevity screens

Using our standard cornmeal food, mated female GRIM; ES flies maintained at 25°C no longer show an increase in death rate between days 9 or 10 when fed 500µM RU486 compared to uninduced controls. This was extremely consistent and repeatable in 3 independent experiments. The reduction of ambient temperature to 18°C extends this window over 50%, while changing the caloric composition of the food altered the window by up to 25%. Using UAS-GRIM; ES flies in combination with a large-scale drug screen may identify compounds capable of extending or accelerating the time period of GRIM susceptibility and give key insights into processes that mediate the rate of aging at the genetic level. These experiments could be done by using standard cornmeal food with particular drugs mixed in for five to ten days before switching the food media to RU486 and comparing survivorship curves to a no drug control population.

GRIM susceptibility is only one specific genetic change or group of genetic changes that happens to scale to lifespan. It is unclear whether this specific change can be used as a marker for interventions that delay the timing of gene expression in general. That this assay works in a predictable manner for both dietary restriction and temperature shifts is strong evidence that the nature of the changes associated with neuronal GRIM susceptibility can predict of the rate of aging.

The UAS-GRIM; GeneSwitch ELAV system could also be used in transposon-based mutagenesis screens to attempt to identify novel genes involved in regulating the timing of gene expression. One method of performing the screen would be to create a fly line with a piggyBac transposable element with homozygous GeneSwitch ELAV and a piggyBac transposase homozygous for GeneSwitch ELAV. These flies could then be crossed together and the resulting offspring crossed to a homozygous UAS-GRIM line so all resulting offspring would express UAS-GRIM and GeneSwitch ELAV. Flies with the transposase would be discarded via the use of a specific selectable marker and inverse PCR would be done on single female flies that were still susceptible to GRIM induced death after day 10 on standard cornmeal food at 25°C.

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Literature Cited

- Carvalho GB, Kapahi P, Benzer S. Compensatory ingestion upon dietary restriction in *Drosophila melanogaster*. *Nat Methods* 2005;2:813–815. [PubMed: 16278649]
- Chai J, Yan N, Huh JR, Wu JW, Li W, et al. Molecular mechanism of REAPER-GRIM-Hid-mediated suppression of DIAP1-dependent DRONC ubiquitination. *Nat Struct Biol* 2003;10:892–898. [PubMed: 14517550]
- Hay BA, Guo M. Caspase-dependent cell death in *Drosophila*. *Annu Rev Cell Dev Biol* 2006;22:623–650. [PubMed: 16842034]
- Helfand SL, Blake KJ, Rogina B, Stracks MD, Centurion A, et al. Temporal patterns of gene expression in the antenna of the adult *Drosophila melanogaster*. *Genetics* 1995;140:549–555. [PubMed: 7498736]
- Kim SN, Rhee JH, Song YH, Park DY, Hwang M, et al. Age-dependent changes of gene expression in the *Drosophila* head. *Neurobiol Aging* 2005;26:1083–1091. [PubMed: 15748788]
- Landis GN, Abdueva D, Skvortsov D, Yang J, Rabin BE, et al. Similar gene expression patterns characterize aging and oxidative stress in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 2004;101:7663–7668. [PubMed: 15136717]

- Lannan E, Vandergaast R, Friesen PD. Baculovirus caspase inhibitors P49 and P35 block virus-induced apoptosis downstream of effector caspase DrICE activation in *Drosophila melanogaster* cells. *J Virol* 2007;81:9319–9330. [PubMed: 17582002]
- Levine RL, Stadtman ER. Oxidative modification of proteins during aging. *Exp Gerontol* 2001;36:1495–1502. [PubMed: 11525872]
- Min KJ, Flatt T, Kulaots I, Tatar M. Counting calories in *Drosophila* diet restriction. *Exp Gerontol* 2007;42:247–251. [PubMed: 17125951]
- Min KJ, Tatar M. *Drosophila* diet restriction in practice: do flies consume fewer nutrients? *Mech Ageing Dev* 2006;127:93–96. [PubMed: 16256171]
- Olson MR, Holley CL, Yoo SJ, Huh JR, Hay BA, et al. REAPER is regulated by IAP-mediated ubiquitination. *J Biol Chem* 2003;278:4028–4034. [PubMed: 12446669]
- Osterwalder T, Yoon KS, White BH, Keshishian H. A conditional tissue-specific transgene expression system using inducible GAL4. *Proc Natl Acad Sci U S A* 2001;98:12596–12601. [PubMed: 11675495]
- Pletcher SD, Libert S, Skorupa D. Flies and their golden apples: the effect of dietary restriction on *Drosophila* aging and age-dependent gene expression. *Ageing Res Rev* 2005;4:451–480. [PubMed: 16263339]
- Pletcher SD, Macdonald SJ, Marguerie R, Certa U, Stearns SC, et al. Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. *Curr Biol* 2002;12:712–723. [PubMed: 12007414]
- POON, PC. PhD dissertation. University of Connecticut; 2007. Neuronal Apoptosis in Adult *Drosophila melanogaster*.
- Rogina B, Benzer S, Helfand SL. *Drosophila* drop-dead mutations accelerate the time course of age-related markers. *Proc Natl Acad Sci U S A* 1997;94:6303–6306. [PubMed: 9177212]
- Rogina B, Helfand SL. Regulation of gene expression is linked to life span in adult *Drosophila*. *Genetics* 1995;141:1043–1048. [PubMed: 8582611]
- Rogina B, Helfand SL. Spatial and temporal pattern of expression of the wingless and engrailed genes in the adult antenna is regulated by age-dependent mechanisms. *Mech Dev* 1997;63:89–97. [PubMed: 9178259]
- Seroude L, Brummel T, Kapahi P, Benzer S. Spatio-temporal analysis of gene expression during aging in *Drosophila melanogaster*. *Aging Cell* 2002;1:47–56. [PubMed: 12882353]
- Zheng J, Edelman SW, Tharmarajah G, Walker DW, Pletcher SD, et al. Differential patterns of apoptosis in response to aging in *Drosophila*. *Proc Natl Acad Sci U S A* 2005a;102:12083–12088. [PubMed: 16099837]
- Zheng J, Mutcherson R 2nd, Helfand SL. Calorie restriction delays lipid oxidative damage in *Drosophila melanogaster*. *Aging Cell* 2005b;4:209–216. [PubMed: 16026335]

Figure 1.

Susceptibility to death by GRIM over-expression in the nervous system shows age-dependent changes and correlates with the induction of changes in caspase activity in the head. A) Mated female UAS-GRIM; GeneSwitch ELAV (ES) flies or B) mated male GRIM; ES flies were maintained on standard food media (1x cornmeal) without RU486 until the day indicated; red (day 4), blue (day 7), orange (day 10), or black (no RU486) when they were switched to food with 500 μ M RU486 and maintained on that for the remainder of life. C) Fold increase in female or D) male head caspase activity/ug after a 24hr 500 μ M RU486 feeding starting at the indicated day compared to aged matched flies not fed RU486. E) Endogenous caspase activity/ug, in arbitrary units, of w1118 female heads collected 0–2 hrs post eclosion vs. 10–22 hrs post eclosion.

Figure 2.

GeneSwitch ELAV (ES) driver expression increases and then remains constant with age. A) Homozygous UAS-GFP; ES mated female flies were continually fed 500 μ M RU486 from the first day of eclosion and GFP expression/ug protein of the head (top) or bodies (bottom) of single flies was measured on the days indicated. B) 7 day feeding of 500 μ M RU486 begun on either day 5 or day 10 shows GFP expression increases in female brains during early life. C) GFP fluorescence from the bodies of mated female UAS-GFP; ES flies after 12-day induction with 500 μ M RU486 and after the subsequent removal of RU486 shows GFP is virtually absent by 12 days.

Figure 3.

Caloric content and temperature affect GRIM-induced mortality due to alterations in gene expression. A) Lifespan curves of mated female UAS-GRIM; GeneSwitch ELAV (ES) flies fed 200 μ M RU486 from the first day of eclosion dissolved in 0.5x (triangles), 2x (diamond) or 0.5x cornmeal food in the absence of RU486 (black). B) Whole body GFP/ μ g fluorescence of mated female homozygous GFP; ES flies fed 200 μ M RU486 dissolved in 2x, 1x, or 0.5x cornmeal food. C) Whole body GFP/ μ g fluorescence of mated female homozygous UAS-GFP; ES flies fed 500 μ M RU486 dissolved in 1x cornmeal food maintained at 18°C or 25°C for 10 days post eclosion.

Figure 4.

Initiating RU486 feeding at the same temperature and caloric condition normalizes gene expression despite differences in prior temperature and caloric conditions. A) An illustration of an example protocol where flies are maintained on several different conditions in the absence of RU486 for a number of days before being switched to the same condition on the day when RU486 feeding is initiated. B) GFP/μg fluorescence of mated female homozygous UAS-GFP; ES flies maintained with 0.5x cornmeal food at 25°C, 1x cornmeal food at 25°C, or 1x cornmeal food at 18°C for 10 days before being switched to 500μM 1x cornmeal food for 10 days. C) Fold increase in GRIM mRNA after 24hrs of 500μM RU486 feeding on 1x cornmeal food measured by quantitative PCR and compared to age matched controls in the absence of RU486 maintained at 25°C (gray) or 18°C (white) for either 3 or 12 days before switching to 25°C and fed RU486. D) An illustration of the experimental hypothesis. Mated female flies maintained on standard cornmeal food stop being susceptible to 500μM RU486 feeding by day 10 at 25°C. We hypothesize that there are intrinsic gene expression changes that mediate this change that are altered as the fly ages. At 18°C the genetic changes that mediate the change in GRIM susceptibility may be delayed and thus flies maintained for 10 days at 18°C would still be susceptible to GRIM and have increased mortality compared to controls.

Figure 5.

Temperature shifts alter susceptibility to neuronal GRIM over-expression in a way that scales to lifespan A) Lifespans of mated UAS-GRIM; GeneSwitch ELAV (ES) females after induction of GRIM by 500 μ M RU486 feeding at 25°C on the fourth day post eclosion after being maintained at the indicated temperatures before the switch: red (29°C), blue (25°C), green (18°C), black curve is uninduced. B) Same as in A) but switched on the seventh day post eclosion. C) Same as in A) but switched on the tenth day post eclosion. D) Lifespan curves of mated female UAS-GRIM; ES flies maintained at 18°C on standard cornmeal food for the indicated number of days: green (7 days), blue (12 days), red (20 days) before being fed 500 μ M RU486 at 25°C. The black curve is the control curve maintained at 25°C E) Fold

increase in caspase activity 24hrs post induction of 500 μ M RU486 compared to uninduced age-matched control at different temperatures and ages: orange (day 4), grey (day 7), red (day 10), green (day 13). F) Relative GRIM expression after 24hr 500 μ M RU486 feeding after being maintained on different conditions all normalized to 18°C d3 before induction. Red bars show conditions and relative GRIM expression levels at which flies die significantly faster than uninduced controls. Blue bars show conditions at which flies do not die significantly faster than uninduced controls.

Figure 6.

Increased yeast concentration in the food media shortens the period of susceptibility to GRIM over-expression in the nervous system. A) Lifespan curves of mated female flies maintained for 4 days on food conditions that differ only in the percentage of yeast in the food medium: 3% (green), 9% (blue), 18% (red) before being switched to standard cornmeal food (3% yeast) with 500μM RU486, uninduced etoh control on 3% yeast (black) B) Survivorship curves of mated female flies maintained for 7 days on the same food conditions as in A) before being switched to standard cornmeal food (3% yeast) with 500μM RU486.

Figure 7.

DIAP1 and p35 block the effects of neuronal GRIM over-expression. A) GRIM/CyO; GeneSwitch ELAV (ES)/ES males were crossed to w¹¹¹⁸, UAS-DIAP1, or UAS-p35 females to enable co-expression of GRIM and the anti-apoptotic factors. Flies were fed standard cornmeal food (etoh) or the same food containing 500μM RU486 within 24 hours of eclosion for 10 days and the number of dead flies were counted. Shown are mated females. GRIM; ES (etoh, n = 71; ru = 118), GRIM;DIAP1/ES (etoh, n = 45; ru = 77), GRIM/p35;ES (etoh, n = 84; ru, n = 49). B) Caspase activity/μg of female heads was measured by pooling several female heads together after 24hr induction of 500μM RU486 feeding within 48 hours of eclosion. Only flies without DIAP1 or p35 co-expression had a significant increase in caspase activity upon RU486 feeding ($p < .001$), n = 3 to 4 sets of grouped heads each.

Table 1

Median and mean lifespans of mated female (top) and male (bottom) UAS-GRIM; GeneSwitch ELAV *Drosophila* maintained on standard cornmeal food at 25°C. The etoh condition represents flies never fed RU486 while the day indicates the first day that 500µM RU486 was introduced into the food medium. P values are calculated using log rank analysis and the number of flies used in each study is shown.

Condition	median	mean	P	N
Etoh (F)	44	44.5		150
d4	13	18.7	<.0001	150
d7	20	30.4	<.0001	144
d9	39	39.2	.02	179
d10	44	39.5	ns	152
Etoh (M)	58	44.6		151
d4	12	12.4	<.0001	144
d7	15	17.8	<.0001	146
d9	21	22	<.0001	184
d10	23	23	<.0001	144

N = the number of flies used in the study, F = female, M = male.

Table 2

Median and mean lifespans of mated female and male UAS-GRIM; GeneSwitch ELAV flies maintained at 25°C on different caloric conditions with and without the feeding of 200µM RU486 within 24 hours of eclosion. P values are from log rank analysis and are calculated as different from etoh control.

Condition	median	mean	P	N
<i>0.5x cornmeal</i>				
Etoh (F)	38	38.8		93
200µM (F)	10	12	<.0001	100
Etoh (M)	34	33.5		96
200µM (M)	8	8.7	<.0001	96
<i>1x cornmeal</i>				
Etoh (F)	44	43.6		203
200µM(F)	22	29.4	<.0001	163
Etoh (M)	42	39.5		172
200µM (M)	20	23.8	<.0001	183
<i>2x cornmeal</i>				
Etoh (F)	40	41.5		95
200µM (F)	24	23.8	<.0001	101
Etoh (M)	25	27.5		91
200µM (M)	18	18.6	<.0001	105

N = the number of flies used in the study, F = female, M = male.

Table 3

Median and mean lifespans of mated UAS-GRIM; GeneSwitch ELAV female flies maintained at different ambient temperatures prior to the induction of 500 μ M RU486 at the indicated day, at which flies were changed to 25°C. Etoh indicates that these flies were not fed RU486 and were maintained at the indicated temperature for the duration of their lifetime. P-values are calculated using log rank analysis and are compared to 3% yeast controls at 25°C not fed RU486.

Condition	median	mean	p value	N
29°C etoh	32	30.8	<.0001	139
d4	34	33.9	<.0001	148
d6	46	44.2	ns	147
d7	49	46.7	ns	123
d8	46	45.2	ns	122
d10	51	48.5	ns	143
18°C etoh	87	89.4	<.0001	122
d4	14	16.2	<.0001	142
d7	16	20.6	<.0001	152
d10	21	26.1	<.0001	149
d12	25	33.5	<.0001	155
d16	38	42.1	ns	142
d20	55	54.5	<.0001	117

N = the number of flies used in the study.

Table 4

Median and mean lifespans of mated female UAS-GRIM; GeneSwitch ELAV flies on different food media prior to the induction of 500 μ M RU486 on standard cornmeal food (3% yeast) at the indicated day. All flies here were aged at 25°C. P-values are calculated using log rank analysis and are compared to 3% yeast controls not fed RU486.

Condition	median	mean	P	N
<i>9% yeast (RU486)</i>				
d4	14	19.3	<.0001	143
d6	29	31.8	<.0001	148
d7	43	41.3	<.007	141
d8	45	42.3	<.04	152
d10	44	46.4	ns	152
<i>18% yeast (RU486)</i>				
d4	27	27.7	<.0001	153
d6	36	35.9	<.0001	161
d7	45	42.1	.0009	145
d9	45	43.9	.008	135

N = the number of flies used in the study.