

# Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands

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The insulin/insulin-like growth factor-like signaling pathway, present in all multicellular organisms, regulates diverse functions including growth, development, fecundity, metabolic homeostasis, and lifespan. In flies, ligands of the insulin/insulin-like growth factor-like signaling pathway, the *Drosophila* insulin-like peptides, regulate growth and hemolymph carbohydrate homeostasis during development and are expressed in a stage- and tissue-specific manner. Here, we show that ablation of *Drosophila* insulin-like peptide-producing median neurosecretory cells in the brain leads to increased fasting glucose levels in the hemolymph of adults similar to that found in diabetic mammals. They also exhibit increased storage of lipid and carbohydrate, reduced fecundity, and reduced tolerance of heat and cold. However, the ablated flies show an extension of median and maximal lifespan and increased resistance to oxidative stress and starvation.

aging

The insulin/insulin-like growth factor (IGF)-like signaling (IIS) pathway is present throughout multicellular animals (1). Its functions include regulation of growth (2–5), development (6), and metabolic homeostasis (7). Also, the pathway has been shown to regulate adult lifespan, resistance to stress (8–10), and fecundity in *Caenorhabditis elegans*, *Drosophila*, and mice (11–14).

In mice, both deletion of the insulin receptor in white-adipose tissue and ubiquitous heterozygosity for a null mutation in the IGF-I receptor increase longevity, implying that both insulin and IGF-I signaling may regulate lifespan (10, 15). In *C. elegans* and *Drosophila*, mutation of the single IIS receptor (6, 16, 17), the single insulin receptor substrate (9, 18), or the PI3 kinase (19–22), the protein kinases Akt (23) and Sgk-1 (24), or overexpression of the forkhead transcription factor (25–27), all increase lifespan and delay or reduce fecundity. Thus, the role of reduced insulin signaling in lifespan-extension appears to be conserved. In light of the different roles of insulin signaling in diverse tissues within an organism, the array of phenotypes that result from altered systemic signaling may be separable.

In the genomes of the invertebrates *C. elegans* and *Drosophila*, single genes encode the intracellular components of the signaling pathway, with the exception of the triplication of the protein kinase B, Akt, SGK-1 in *C. elegans* (23). In contrast, there are multiple invertebrate-genes for the insulin-like ligands, with seven in the *Drosophila* genome (3) and 38 in *C. elegans* (28). The genes encoding the seven *Drosophila* insulin-like peptides (DILPs) were originally identified by a similarity search that used the four conserved cysteine residues within the insulin A chain as bait (3). DILP1–DILP7 are predicted to resemble proinsulin at the structural level, and therefore, they are considered orthologous to mammalian insulin, rather than IGF. The relative simplicity of the intracellular signaling-pathway, together with

the genetic diversification of DILPs, implies that the multiple functions of IIS may be mediated in part by functional diversification of the ligands. In support of this notion, the genes encoding the DILPs are independently transcriptionally regulated in response to nutrition, as well as in a tissue- and stage-specific manner during development (3, 5). However, the distinct biochemical roles that the different ligands play in these contexts remain to be identified.

The mechanisms by which IIS regulates lifespan and fecundity and the nature of any causal relationships between these phenotypes and the other traits regulated by this pathway are not understood. We have investigated the roles of the insulin-like ligands in adult *Drosophila*. We have determined that, in adults, three of the *dilp* genes are expressed in brain neurosecretory cells, two of them exclusively so. We have investigated the consequences of ablation of these cells for adult lifespan, fecundity, stress resistance, and metabolic phenotypes.

## Methods

**Fly Stocks and Maintenance.** The control *white*<sup>Dahomey</sup> stock was derived by backcrossing *w*<sup>1118</sup> into the outbred wild-type Dahomey background. Upstream activating sequence (UAS)-*reaper* (*rpr*) and *dilp2*-GAL (5) were backcrossed into *w*<sup>Dah</sup> five times. UAS-GFP (1521) and UAS-LacZ (1776) were obtained from Bloomington Stock Center. Stocks were maintained and experiments were conducted at 25°C on a 12:12-h light/dark cycle at constant humidity by using standard sugar/yeast medium (29).

**In Situ Hybridization.** RNA *in situ* hybridizations on whole-mount adult central nervous systems, ovaries, and other abdominal organs by using digoxigenin (DIG)- and FITC-labeled *dilp* probes were performed as described in ref. 5.

**Immunohistochemistry.** DILP2 expression was determined by using rabbit anti-DILP2 diluted 1:500 according to the procedure described in ref. 30 and a secondary Texas red-conjugated donkey anti-rabbit IgG (Jackson ImmunoLabs).

**Confocal Microscopy.** Confocal imaging of UAS-GFP expression and Fast Red fluorescence was carried out on an LSM 510 microscope (Zeiss). The fluorophore Fast Red was excited at

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Abbreviations: mNSC, median neurosecretory cells; IGF, insulin-like growth factor; DILP, *Drosophila* insulin-like peptide; IIS, insulin/IGF-like signaling; UAS, upstream activating sequence; DIG, digoxigenin; *rpr*, *reaper*.

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488 nm and fluorescence measured beyond 545 nm. Confocal image stacks were converted to projections by using LSM 510 software (Zeiss). Cell counts were performed on 3D reconstructions of expression in the mNSCs of *Drosophila* brains by using AXIOVERT software (Zeiss).

**Quantitative RT-PCR.** Total RNA was extracted by using TRIzol (GIBCO) according to the manufacturer's instructions. mRNA in total RNA was reverse transcribed by using oligo(dT) primer and the Superscript II system (Invitrogen). Quantitative PCR was performed by using the PRISM 7000 sequence-detection system (Applied Biosystems), SYBR Green (Molecular Probes), ROX ReferenceDye (Invitrogen), and Hot StarTaq (Qiagen, Valencia, CA) by following the manufacturers' instructions. For detailed information, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

**Lifespan.** Procedures for lifespan studies were as described in refs. 9 and 31. For detailed information, see *Supporting Materials and Methods*.

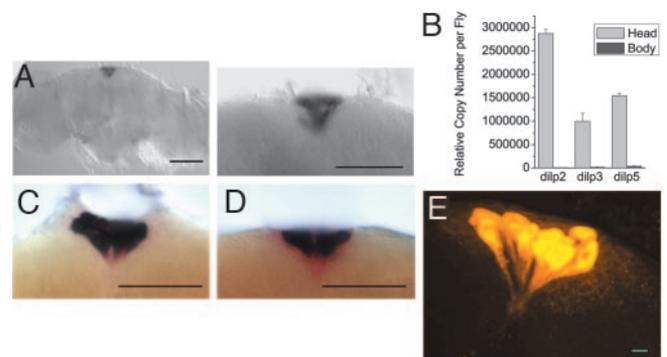
**Fecundity.** The number of eggs laid per vial of females was counted, and the number of females per vial and the number of days (3 or 4) over which they had laid were noted. Data are reported as the mean number of eggs laid per day per female  $\pm$  SEM over each 3- or 4-day period.

**Stress Tests.** UAS-*rpr*/+; *dilp2*-GAL/+ and UAS-*rpr*/+ mated females were generated as for lifespan. Survival was measured on (i) 20 mM paraquat in standard food (oxidative stress) or (ii) 1% agar (starvation). Time to knock-down was measured for flies exposed to 38.5°C (heat) and time to recovery from cold-shock was measured for flies exposed to 0°C for 4 h (32). Two experiments were performed for each treatment. For heat and paraquat, 6- and 10-day-old flies were tested in each experiment.

**Trehalose and Glucose Measurement.** Hemolymph was pooled from 8–10 females after a 5-h starvation on 1% agar. Flies were decapitated, and hemolymph was collected from the thorax by capillary action. Glucose amount was determined in  $\approx 0.3 \mu\text{l}$  of pooled liquid by using Infinity Glucose Reagent (ThermoElectron). Whole-fly trehalose was measured as described in ref. 33, by using the glucose assay outlined above. For detailed information, see *Supporting Materials and Methods*.

**Lipid and Glycogen Measurements.** The glycogen and lipid content of individual, adult female flies were measured 3 days after eclosion ( $n = 20$ ) according to the method described in ref. 34, which was modified to exclude protein separation. Data are expressed relative to the fresh body weight of each fly.

**Statistical Analyses.** All statistical analyses were performed by using JMP (version 4.0.5) software (SAS Institute, Cary, NC). Lifespan data were subjected to survival analysis (log-rank tests) and presented as survival curves. Mortality ( $\mu_x$ ) was estimated as  $\mu_x = -\ln(p_x)$ , where  $p_x$  is the probability of an individual alive at age  $x - 1$  surviving to age  $x$  days (35). Maximum-likelihood methods, executed in WINMODEST (36), were used to estimate mortality parameters of the Gompertz model:  $\mu_x = ae^{bx}$ . Fecundity, cold recovery, glucose, trehalose, lipid, and glycogen data were tested for normality by using the Shapiro–Wilk  $W$  test on studentized residuals (37). One-way ANOVA was used, and means comparisons were made by using Student's  $t$  test. Data are presented as means  $\pm$  SEM, and an asterisk indicates significant difference from control ( $P < 0.05$ ).



**Fig. 1.** Characterization of expression of *dilp2*, *dilp3*, and *dilp5* in adult female *Drosophila*. Gene expression of *dilp2*, *dilp3*, and *dilp5* was determined in 5-day-old adult female brains by RNA *in situ* hybridization and quantitative RT-PCR of the control genotype (UAS-*rpr*/+). (A) Exemplar *in situ* hybridization using a *dilp2* DIG-labeled RNA probe and alkaline phosphatase-conjugated anti-DIG Ab. Cells containing the target gene constitute the median neurosecretory cells (mNSCs) in the pars intercerebralis of the adult brain. (Scale bars, 80  $\mu\text{m}$ .) (B) Separate RNA extracts from heads and bodies of adult female flies were assayed for the presence of *dilp2*, *dilp3*, and *dilp5* transcripts by RT-PCR. Data are shown as mean copy number  $\pm$  SEM. Double *in situ* hybridizations were performed by using a FITC-labeled *dilp5* probe and DIG-labeled *dilp2* and *3* probes, followed by alkaline phosphatase-conjugated anti-DIG and anti-FITC Abs. (C) Double staining for *dilp2* (visualized with fast red) and *dilp5* probes (visualized with BCIP/NBT) revealed their colocalization.  $n = 7$ . (D) Likewise, double staining using DIG-labeled *dilp3* (fast red) and FITC-labeled *dilp5* (BCIP/NBT) probes revealed their colocalization ( $n = 8$ ). (E) Fluorescent *in situ* hybridization was used to quantify *dilp*-expressing mNSCs. This example shows a fluorescent *dilp3* probe localized to six pairs of mNSCs. (Scale bar, 10  $\mu\text{m}$ .) Seven brains were examined by using the *dilp2* probe, 12 brains were examined by using the *dilp3* probe, and 11 brains were examined by using the *dilp5* probe.

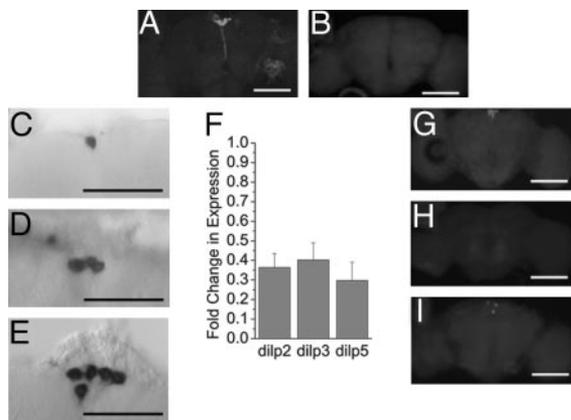
## Results

### Characterization of *dilp2*, *dilp3*, and *dilp5* Expression in Adult Flies.

Previous work has characterized the expression and distribution of the DILPs in *Drosophila* larvae (3, 5). However, little is known about these molecules in adult flies. To detect the presence and location of RNA transcripts of *dilp2*, *dilp3*, and *dilp5* in adults, RNA *in situ* hybridizations were performed, which revealed expression of all three genes in a cluster of cells in the adult brain that correspond to the median neurosecretory-cells (mNSC) of the pars intercerebralis (38) (Fig. 1A). Expression of the other four *dilps* was not detectable in these cells by *in situ* hybridization using probes complementary to *dilp1*, *dilp4*, *dilp6*, and *dilp7* (data not shown). *dilp5* expression was also detected in the ovary as noted in ref. 5, whereas *dilp2* and *dilp3* could not be detected anywhere else in the adult body (data not shown). These observations were supported by quantitative RT-PCR that revealed relatively strong expression of *dilp2*, *dilp3*, and *dilp5* in head extracts but not *dilp2* transcript, barely detectable levels of *dilp3*, and low levels of *dilp5* in extracts from bodies (Fig. 1B).

To determine whether the three *dilp* genes were all expressed in the same cells, double *in situ* hybridizations were performed. Combining the probes for *dilp5* with *dilp2* and *dilp5* with *dilp3* showed that all three genes were coexpressed in the same set of cells (Fig. 1C and D). The number of these *dilp*-expressing mNSCs per individual was determined by fluorescent *in situ* hybridization using probes for each of the three genes separately (Fig. 1E). Within each brain, the number of cells expressing these *dilps* was bilaterally symmetrical, and it varied from five to seven pairs.

**Targeted Disruption of *dilp* Expression by Cell Ablation.** All studies on the role of IIS in adult flies have manipulated the pathway components in target tissues. We sought to investigate the effects



**Fig. 2.** The mNSC-specific expression of the *dilp2*-GAL driver in the adult brain and the effect of mNSC ablation on *dilp* expression. (A and B) Confocal image (z-stack projection) of *dilp2*-GAL-driven expression of UAS-GFP in control (UAS-GFP/+; *dilp2*GAL/+) (A) and ablated (UAS-*rpr*/+; UAS-GFP/+; *dilp2*GAL/+) (B) adult female brains. (C–E) RNA *in situ* hybridizations were performed by using DIG-labeled probes and an alkaline phosphatase-conjugated anti-DIG Ab to UAS-*rpr*/+; *dilp2*GAL/+ adult female brains. (C) *dilp2*. (D) *dilp3*. (E) *dilp5*. (F) The effect of mNSC ablation on *dilp* expression measured by RT-PCR. Data are mean fold differences in expression between the ablated genotype (UAS-*rpr*/+; *dilp2*GAL/+) and the control (UAS-*rpr*/+)  $\pm$  SEM. (G–I) Adult mNSCs immunostained with an Ab against DILP2 in a control brain (UAS-*rpr*/+;  $n = 5$ ) (G), and ablated brains (UAS-*rpr*/+; *dilp2*-GAL/+) (H and I). For sample sizes and residual mNSC numbers, see Table 2, which is published as supporting information on the PNAS web site.

of lowered IIS activity as a result of disruption of the insulin ligands. To achieve this aim, transcription of the proapoptotic gene *rpr* was driven by a GAL4 transgene whose expression was determined by a fragment of the *dilp 2* promoter (*dilp2*GAL4). This construct has been shown to express exclusively in the mNSCs of larval brains, starting during late third-instar (5). Fig. 2 shows expression in adult brains of UAS-GFP driven by this *dilp2*GAL4 construct in the absence (A) and presence (B) of UAS-*rpr*. *dilp2*-driven *rpr* expression clearly resulted in a complete ablation of the cells in the adult brain in which it was expressed, as shown by the absence of any fluorescence in the ablated flies.

However, *in situ* hybridizations using brains from ablated flies revealed some expression of each of the *dilp* genes in a variable number of residual mNSCs (Fig. 2 C–E). This expression was confirmed by RT-PCR data that showed that expression of each of the *dilps* was reduced to 30–40% of the levels in wild-type brains (Fig. 2F). Because only a fragment of the *dilp2* promoter was used to drive *rpr* expression, the expression pattern of the transgene may not always have fully replicated that found for the full-length promoter. This supposition was supported by immunostaining brains from control (Fig. 2G) and ablated (Fig. 2 H and I) animals by using a DILP2 Ab. Although in some brains there was no detectable protein (Fig. 2H), DILP-producing cells were still detectable in others (Fig. 2I). Attempts to quantify DILP2 levels in whole-body protein extracts by Western blot analysis failed because of the low abundance of the ligand (data not shown).

IIS activity is required for larval growth, and reduced activity in components of the signaling pathway have been shown to result in flies that are dramatically smaller and have delayed development (3, 39, 40). In contrast, the mNSC-ablated flies reported here were only slightly smaller than controls, and they showed no developmental delay, presumably because of the late developmental onset of expression of the GAL4 driver transgene used in these experiments (5).

### mNSC-Ablated Flies Have Longer Lifespan and Reduced Fecundity.

Reduced IIS activity has a conserved role in extending adult lifespan. To investigate the effects of mNSC ablation and the resulting reduced levels of *dilp2*, *dilp3*, and *dilp5* on lifespan, we assayed the longevity of female (mated and virgin) and male flies. The ablated flies exhibited a significant increase in median and maximum lifespan over that of control flies (Fig. 3 A–C). Median lifespan was increased by 10.5% in males and 18.5% and 33.5% in virgin and mated females, respectively. These increases in lifespan were accompanied by decreases in age-specific egg laying for virgin and mated females (Fig. 3 D and E).

Age-specific mortality analysis of lifespan data from mated female flies was performed (Fig. 3F). Over the linear portion of the mortality increase, fitting of a Gompertz model revealed a significant difference in the initial rate of mortality but no change in the slope of the mortality trajectory (Table 1). Thus, detectable aging-related mortality started later in the ablated flies but then proceeded at the same rate as in controls.

### Physiological Changes in mNSC-Ablated Flies: Altered Carbohydrate and Lipid Metabolism.

Insulin signaling is known to be involved in the regulation of energy homeostasis in mammals. One manifestation of this is that mammals with reduced systemic insulin signaling (such as that in diabetes) exhibit fasting hyperglycemia because of inappropriate regulation of glucose uptake from circulation and release from energy stores in the liver. To test whether reduction of insulin signaling has similar effects in *Drosophila*, we measured the levels of several molecules that are resources for energy generation.

Insects contain two types of sugar in circulation: glucose and trehalose. Glucose is obtained from the diet, and trehalose is used as a homeostatic molecule that originates from the fat body and is used to distribute sugar to peripheral tissues (41). Hemolymph was extracted from female flies that had been starved for 5 h, and the levels of these two sugars was measured. Glucose was elevated 2-fold above control values in mNSC-ablated flies (Fig. 4A), whereas trehalose was slightly (15%) but significantly lowered in ablated flies versus controls (Fig. 4B).

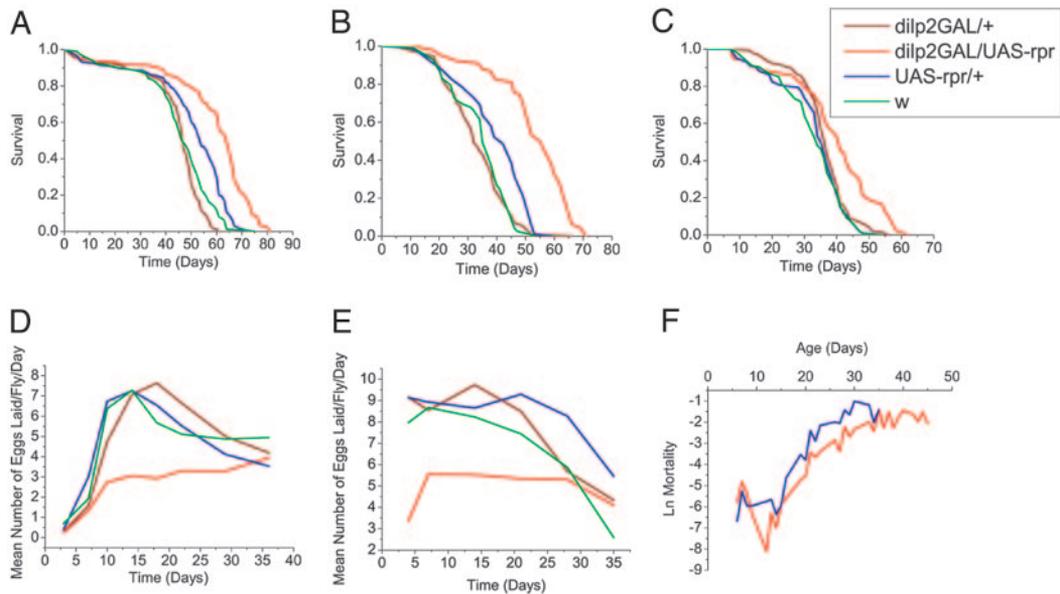
Examination of stored energy pools by using whole-body extracts revealed that mNSC-ablated flies contained significantly higher levels of trehalose (64% higher), glycogen (44% higher), and lipids (10% higher) relative to body mass when compared with controls (Fig. 4 C–E). Under normal conditions, these three storage molecules represent the largest readily accessible energy resource available to the fly. Thus, mNSC-ablated flies exhibit generally higher levels of stored energy and an altered profile of circulating carbohydrates compared with controls.

**Altered Resistance to Stress.** Enhanced resistance to various stresses is often associated with interventions that extend lifespan. We found that mNSC-ablated flies survived longer than controls when fed paraquat in their food. Assuming equal rates of paraquat ingestion, this indicates enhanced resistance to oxidative stress (Fig. 5A). Furthermore, the ablated flies were moderately starvation resistant when compared with controls (Fig. 5B). In contrast, the ablated flies showed greater sensitivity to heat shock (Fig. 5C) and took more time to recover from cold shock (Fig. 5D).

### Discussion

Several genetic interventions that have been shown to extend lifespan in worms (6, 16, 21), fruit flies (9, 17, 26, 27), and mice (10, 15) reduce the activity of the insulin-like signaling pathway. Here, we report extension of *Drosophila* lifespan by ablating cells in the adult brain that produce three of the seven DILPs.

During the preadult period, these peptides activate the IIS pathway in tissues expressing the insulin receptor to promote



**Fig. 3.** mNSC-ablated flies are long-lived and females show reduced fecundity. (A) Survival of UAS-*rpr*/+; *dilp2*-GAL/+ virgin females (red), and *dilp2*-GAL/+ (brown), UAS-*rpr*/+ (blue), and *w* (green) controls. Median lifespans are as follows: UAS-*rpr*/+; *dilp2*-GAL/+, 64 days (18.5% increase over UAS-*rpr*/+ control,  $P < 0.0001$ ;  $n = 173$ ); *dilp2*-GAL/+, 47 days ( $n = 187$ ); UAS-*rpr*/+, 54 days ( $n = 232$ ); and *w*, 47 days ( $n = 202$ ). (B) Survival of UAS-*rpr*/+; *dilp2*-GAL/+ mated females (red), and *dilp2*-GAL/+ (brown), UAS-*rpr*/+ (blue), and *w* (green) controls. Median lifespans are as follows: UAS-*rpr*/+; *dilp2*-GAL/+, 56 days (33.5% increase over UAS-*rpr* control,  $P < 0.0001$ ;  $n = 93$ ); *dilp2*-GAL/+, 32 days ( $n = 115$ ); UAS-*rpr*/+, 42 days ( $n = 88$ ); and *w*, 35 days ( $n = 112$ ). (C) Survival of UAS-*rpr*/+; *dilp2*-GAL/+ males (red), and *dilp2*-GAL/+ (brown), UAS-*rpr*/+ (blue), and *w* (green) controls. Median lifespans are as follows: UAS-*rpr*/+; *dilp2*-GAL/+, 41 days (10.5% increase over *dilp2*-GAL/+ control,  $P < 0.0001$ ;  $n = 140$ ); *dilp2*-GAL/+, 37 days ( $n = 150$ ); UAS-*rpr*/+, 35 days ( $n = 128$ ); and *w*, 34 days ( $n = 195$ ). (D and E) Fecundity of females from experiment shown in A (D) and B (E). Data are given as mean number of eggs laid per female per day  $\pm$  SEM. \*,  $P < 0.05$ , compared with controls. (F) Age-specific mortality analysis of UAS-*rpr*/+ ( $n = 1,186$ ) and *dilp2*-GAL4/UAS-*rpr* ( $n = 1,143$ ) mated females. Natural log of the mortality rate ( $\mu$ ) is plotted.

growth (4, 5, 42). During adulthood, flies and worms with reduced IIS activity exhibit higher lipid levels, increased oxidative stress resistance, and longer lifespan (6, 9, 16, 17, 21, 26, 27, 43). Each of these phenotypes is shared by the mNSC-ablated flies reported here, supporting the idea that the cell ablation is acting by reducing one or more of DILP2, DILP3, and DILP5, leading to lowered systemic insulin signaling. These data are supported by lowered levels of the *dilp* transcripts in the ablated flies. Although it remains possible that other unknown effects of the cell ablation are responsible for the observed lifespan extension, data from *C. elegans* further support IIS involvement, because RNA interference to specifically reduce *ins-7* (1 of 38 genes encoding IIS ligands) has been shown to increase adult lifespan (44). These data support a model in which specific insulin-like ligands are involved in shortening adult lifespan, because their reduction is sufficient for lifespan extension despite the persistence of the remaining ligands.

Reduced insulin signaling in mammals is associated with the disease-state diabetes, characterized by high blood glucose, because of its decreased uptake from circulation into tissues such as muscle and liver, as well as increased glucose release into the blood from catabolism of glycogen and protein by the liver (45).

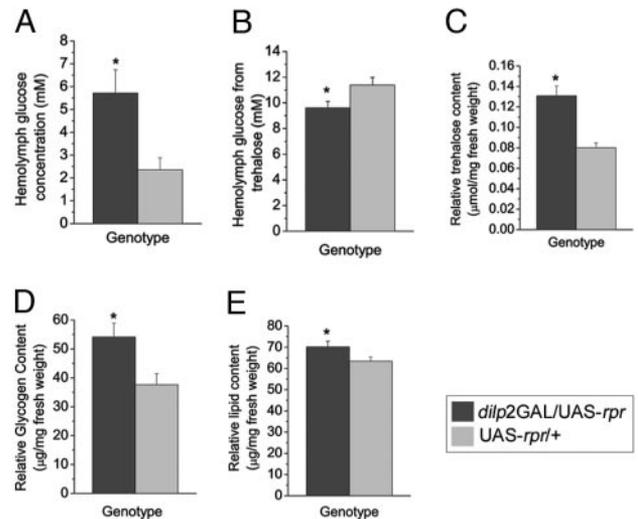
**Table 1. Estimates of the parameters of the Gompertz mortality model with 95% lower and upper confidence intervals**

Genotype	$a^*$	LCI	UCI	$b^†$	LCI	UCI
<i>dilp2</i> GAL/ <i>rpr</i>	0.00015	0.00010	0.00023	0.21128	0.19835	0.22505
UAS- <i>rpr</i> /+	0.00037	0.00028	0.00049	0.22061	0.21012	0.23162

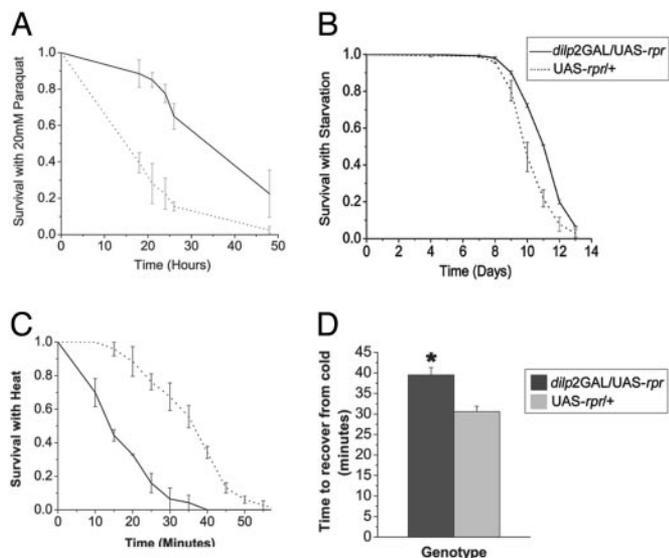
Mortality models and maximum-likelihood analysis were executed by using WINMODEST (36). LCI, lower confidence interval; UCI, upper confidence interval. \*Initial mortality rate.

†Rate of exponential increase in mortality with age early in life.

Like diabetic mammals, mNSC-ablated flies showed higher starving levels of circulating glucose, indicating that one or more of DILP2, DILP3, and DILP5 may be required to stimulate



**Fig. 4.** The effect of mNSC ablation on hemolymph glucose and trehalose levels, and whole-body trehalose, glycogen, and lipid content. (A) Hemolymph glucose concentration in UAS-*rpr*/+ and UAS-*rpr*/+; *dilp2*-GAL/+ flies. (B) Hemolymph trehalose concentration in UAS-*rpr*/+ and UAS-*rpr*/+; *dilp2*-GAL/+ flies ( $n = 8$ ). (C) Whole-fly trehalose content in UAS-*rpr*/+ and UAS-*rpr*/+; *dilp2*-GAL/+ flies per mg of fly (fresh weight) ( $n = 10$ ). (D) Glycogen content of UAS-*rpr*/+ and UAS-*rpr*/+; *dilp2*-GAL/+ flies per mg of fly (fresh weight) ( $n = 21$ ). (E) Lipid content of UAS-*rpr*/+ and UAS-*rpr*/+; *dilp2*-GAL/+ flies per mg of fly (fresh weight) ( $n = 17$ ). Data are shown as means  $\pm$  SEM. \*,  $P < 0.05$ , compared with control.



**Fig. 5.** Long-lived mNSC-ablated adults are resistant to oxidative stress and starvation but show reduced thermotolerance. (A) Survival of 10-day-old adult female flies on 20 mM paraquat added to standard food. Sample sizes were  $n = 100$  for each genotype in the first experiment and  $n = 85$  for each genotype in second. (B) Survival of females during starvation (1% agar).  $n = 144$  for *dilp2GAL/UAS-rpr*;  $n = 150$  for *UAS-rpr/+* in experiment 1;  $n = 182$  for *dilp2GAL/UAS-rpr*; and  $n = 154$  for *UAS-rpr/+* in experiment 2. For both experiments, the median lifespans were as follows: *dilp2GAL/UAS-rpr*, 12 days; and *UAS-rpr/+*, 10 days. (C) Survival of 6-day-old females with 38.5°C heat.  $n = 20$  for each genotype/experiment. (A–C) Data are shown as mean survival for each time point over two experiments  $\pm$  SEM.  $P < 0.05$ , compared with control. (D) Mean time to recover from cold treatment of 9-day-old females  $\pm$  SEM ( $n = 50$  for each genotype).

glucose uptake in adult flies in a manner similar to mammalian insulin. Such a homeostatic control of blood sugar has been demonstrated in *Drosophila*, in which the early larval ablation of the DILP-producing mNSCs results in increased hemolymph sugar levels during development (4).

In flies and mammals, certain components of the insulin signaling pathway are represented by several genes. In *Drosophila*, the insulin-like ligands are encoded by seven genes, whereas in mammals the intracellular signaling components are multiply encoded. For some of these genes, it is also known that paralogues have different spatiotemporal expression patterns, indicating that they have functionally distinct roles (46). In general, functional diversity among IIS pathway paralogues raises the intriguing possibility that the lifespan-extension phenotype associated with lowered IIS activity may be separable from the various accompanying metabolic defects.

In stark contrast to reduction of systemic insulin-signaling in mammals, which results in diabetes, whose associated pathologies shorten lifespan, targeted ablation of the insulin receptor in white adipose-tissue in the mouse results in lifespan extension (15). Interestingly, reduced IIS activity in fat body (analogous to the mammalian adipose-tissue and liver) alone is also sufficient to elicit lifespan extension in flies (26, 27) and worms (47). However, in contrast to mammals, reduction of insulin ligands as a result of the cell ablation reported here or by RNAi for *ins-7* in *C. elegans* (44) resulted in animals that were longer lived than controls. Although the exact relevance of the invertebrate models to the mammalian disease state is uncertain, a possible explanation is that the mortality caused by diabetes in mammals may be due to physiological damage that neither *Drosophila* nor *C. elegans* are at risk of contracting, such as the microvascular and macrovascular damage that results from hyperglycemia (45).

A feature that has been shared by all long-lived IIS mutant flies, including the mNSC-ablated flies described here, is increased lipid content (17, 39). Although also true of long-lived IIS-mutant worms (6), this elevation of lipid content may not be essential for the longevity phenotype (48). In mammals, reduced activity of the insulin pathway can also be associated with dyslipidemia. Neuron-specific disruption of the insulin-receptor gene in mice (NIRKO mice) resulted in decreased fertility and diet-dependent increased adiposity (49). In *Drosophila*, fat storage is mainly in the fat body. Thus, the role of IIS in generation and storage of fat in this tissue may be evolutionarily conserved. In addition, mNSC-ablated flies possessed increased relative levels of storage carbohydrates that could also be expected of NIRKO mice. The increased fat, trehalose, and glycogen stores seen in the mNSC-ablated flies are also unlikely to be causal in the extension of lifespan, because long-lived *chico* (*Drosophila* IRS) flies show no increase in glycogen content (39). Interestingly, studies of long-lived insulin-receptor mutant mice indicate that decreased fat levels are associated with longevity (15).

IIS is required for normal growth in *Drosophila* (50), and lifespan extension by this pathway has been associated with reduced body size and delayed development time, reminiscent of the constellation of phenotypes seen in long-lived dwarf mice (51). However, the reduction in body size caused by reduction of IIS signaling in development is not necessary for extension of lifespan. *chico* heterozygotes are wild type in body size and yet show extended lifespan (9), as do flies with adult-specific overexpression of the IIS transcription factor dFOXO (26, 27).

Increased resistance to various forms of physiological stress is often correlated with life-extending genetic alterations and has been proposed to be causal (52). The increased thermotolerance and accumulation of heat-shock proteins observed in IIS-mutant worms is consistent with this hypothesis (53, 54). However, we have found that long-lived mNSC-ablated flies are more sensitive to hot and cold treatments than controls, indicating that enhanced resistance to thermal stresses is not necessary for lifespan extension. One possible cause for the ablated flies reduced thermotolerance is their lowered circulating trehalose concentrations. Trehalose has been proposed to play a protective role against anoxia in *Drosophila* by preventing protein aggregation (55) and is a requirement for the stress-resistance of stationary-phase yeast cells (56, 57). However, this attractive hypothesis remains questionable because the ablated flies only contain lowered trehalose in circulation, while possessing higher levels of whole-body trehalose. Thus, any role for this disaccharide in thermal tolerance would be limited to its levels in the hemolymph. Another aspect of stress resistance that apparently correlates with longevity in mNSC-ablated flies and other long-lived mutant IIS flies is enhanced resistance to oxidative stress (9, 26, 27). However, note that in each case the prooxidant paraquat was administered by feeding. Therefore, it is possible that unquantified feeding-rate differences between genotypes alter the dose received by each treatment group. Notwithstanding this possibility, increased oxidative stress resistance has been found for long-lived IIS-mutant worms (43) and mice (10), and in each case, it is associated with increased activity of the antioxidant enzyme superoxide dismutase (SOD). Also, increased activity of the JNK signaling pathway in *Drosophila* results in both increased resistance to paraquat and extended lifespan (58). Although some conflicting results exist in the literature regarding the sufficiency of overexpression of these systems to extend the lifespan of *Drosophila* (59–62), recent data support the hypothesis that SOD activity is indeed limiting for lifespan (63). Therefore, these studies agree that enhanced oxidative stress resistance correlates with and may be both necessary and sufficient for retarded aging.

Reduced female fecundity is also often correlated with lifespan extension in IIS-mutant animals (for review, see ref. 64).

The mNSC-ablated flies presented are not sterile but have reduced fertility that could possibly contribute to the long lifespan of these flies. In worms and flies, reduced fertility has been uncoupled from longevity caused by reduced IIS activity (9, 16). Furthermore, control of fertility can be effected by only a subset of insulin signaling functions in mammals because knock-outs of different IRS proteins in mice yield different effects on fertility (65, 66). In worms, reduced IIS activity limited to larval stages was shown to reduce fecundity without increasing lifespan, whereas lowering IIS during the adult period only extended lifespan without lowering fecundity (67). It is possible that a similar effect is also at work in *Drosophila* because our mNSC ablation occurred during the final larval stage (5), resulting in lowered IIS activity during the late larval and pupal periods as well as during adulthood. However, in *Drosophila*, IIS acts at least in part in the adult to determine fecundity, because lifespan extension by overexpression of dFOXO that was confined to the fat body of adult flies led to reduced fecundity (26). It would be interesting to characterize the relationship between this inter-

vention and mNSC-ablation, because their phenotypes are consistent with a role for the DILPs signaling to the fat body to elicit alterations in lifespan and fecundity.

Together, the data presented here demonstrate that lowered levels of a subset of the ligands that are responsible for systemic insulin signaling in flies results in lifespan extension. Therefore, only a subset of the roles of IIS needs to be lowered to effect longevity. This extension of lifespan occurred despite a doubling of blood glucose levels, an interesting phenomenon when viewed in light of the pathological effects of diabetes in mammals. Further characterization of the mechanisms by which this intervention works is well suited to the omic techniques because at least some of the observable physiological correlates of longevity apparently do not indicate causation.

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