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Physiological effects of manipulating the level of insulin-degrading enzyme in insulin-producing cells of Drosophila

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Abbreviations: IDE, insulin-degrading enzyme; IPC, insulin-producing cell; DILP, Drosophila insulin-like peptide

Insulin-degrading enzyme (IDE) degrades insulin and other peptides, including the Aβ peptide of Alzheimer's disease. However, the mechanism by which IDE acts on its substrates in vivo is unclear, and its role in pathogenesis of type 2 diabetes and Alzheimer's disease is controversial. Here, we show that in Drosophila knocking down IDE in insulin-producing cells (IPCs) of the brain results in increased body weight and fecundity, decreased circulating sugar levels and reduced lifespan. Moreover, knocking down and overexpressing IDE in IPCs have opposite physiological effects. As misregulated insulin signaling in peripheral tissues is known to cause similar phenotypes, our data suggest a role for Drosophila IDE in determining the level of insulin-like peptides made by IPCs that systemically activate insulin signaling.

Introduction

Insulin plays a critical role in a wide range of physiological processes, including growth control, metabolic regulation, fertility and aging.[1,2] Its physiological action is regulated by multiple mechanisms, including cellular removal and degradation, which can be disrupted in type 2 diabetes and other disorders.[3] The major activity in mammalian cells that degrades insulin is insulin-degrading enzyme (IDE), a metalloprotease of 110 kDa found in most tissues, including insulin responsive tissues such as liver and muscle.[4] IDE also degrades a variety of small peptides besides insulin in vitro, including the Aβ peptide that forms the hallmark amyloid plaques of Alzheimer's disease. Since type 2 diabetes could be a risk factor for Alzheimer's disease, IDE is a possible link between the two disorders.[5]

While IDE's biochemical properties have been extensively studied, its physiological role remains poorly understood. An IDE knock out mouse was described to have elevated plasma insulin and brain Aβ peptide, consistent with a physiological role for IDE in degrading insulin and Aβ.[6] However, an independent study reported increased Aβ peptide, but not insulin, in the same IDE knock out mouse.[7] Whether IDE is important in the pathogenesis of type 2 diabetes and Alzheimer's disease is also controversial, as multiple human genetic studies have reached opposite conclusions on this issue.[8,9] Moreover, the cellular location where IDE degrades insulin is unclear.[10] IDE is most often found in the cytosol, but is also found in mitochondria and peroxisomes, on the cell surface and outside the cell. Since IDE can degrade a variety of peptides in vitro, perhaps it is localized in different subcellular compartments where it degrades different physiological substrates depending on the tissue.

To better understand IDE's physiological role, we decided to study IDE function in Drosophila, a powerful model system for studying the insulin signaling pathway. Drosophila and human IDE have 48% amino acid sequence identity and many enzymatic properties in common, including the ability to degrade insulin in vitro.[11,12] Moreover, like mouse IDE, Drosophila IDE appears to be non-essential for organism viability, according to a recent study using a putative *Ide* gene knockout.[13] This study also provided evidence that Drosophila IDE antagonizes the insulin signaling pathway. Potential endogenous substrates for Drosophila IDE are the insulin-like peptides (DILPs), which are structurally homologous to human insulin and agonists of the insulin receptor.[14-16] Of the seven known DILPs in Drosophila, DILP1, 2, 3 and 5 are expressed in a cluster of brain neuroendocrine cells called insulin-producing cells (IPCs).[14,17] Deletion of DILPs or ablation of IPCs results in defects in glucose metabolism, organism growth and lifespan.[17-20] These and other studies suggest that IPCs, as a major source of DILPs that act systemically on peripheral target tissues, are functionally analogous to mammalian pancreatic islet β-cells.

Here, we report that altering IDE expression level in IPCs perturbed organism size, circulating sugar, fecundity and lifespan in Drosophila. As earlier studies have shown that misregulation of insulin signaling in peripheral tissues results in similar phenotypes, our results suggest that IDE expression in IPCs is

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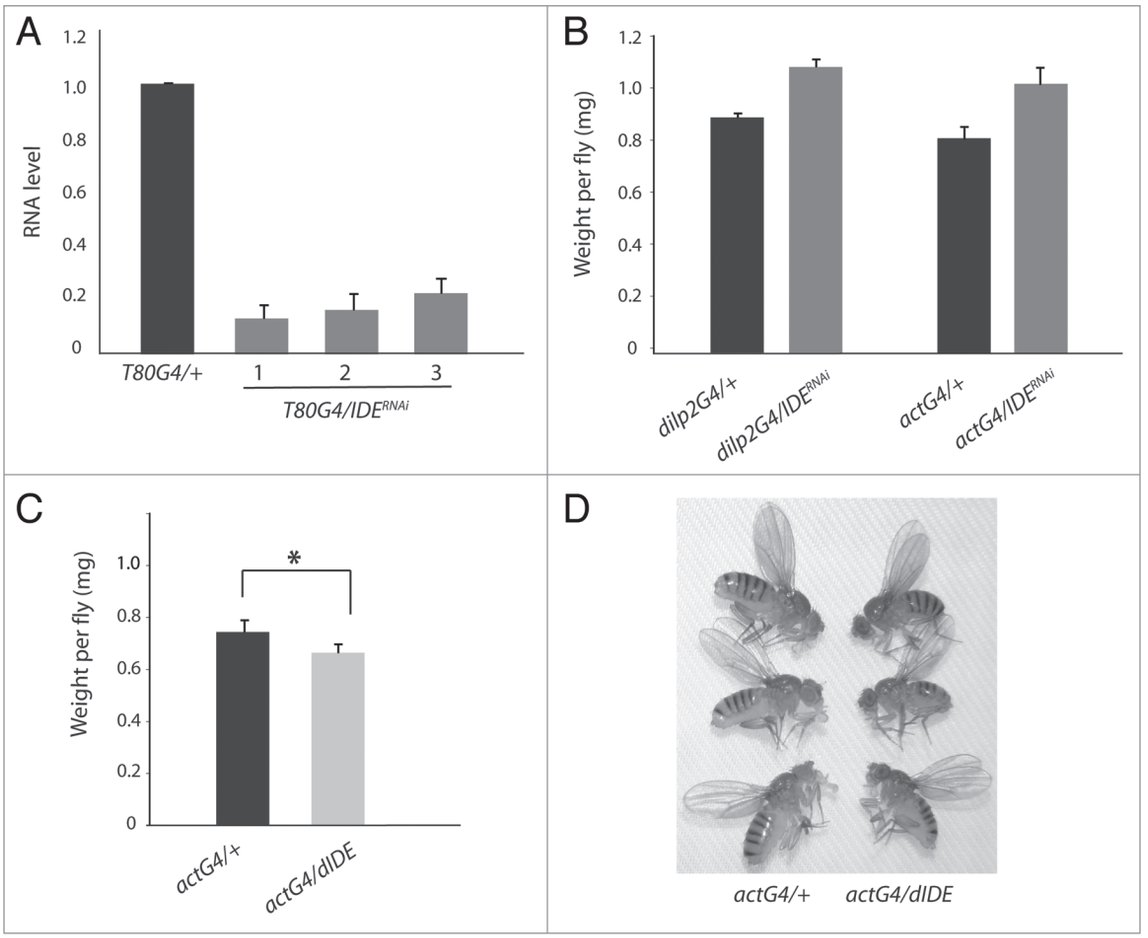

Figure 1. IDE is important for body weight. (A) The broadly expressed *T80-Gal4* driver was used to activate *Ide* RNAi in virtually all tissues during larval development. Quantitative RT-PCR analysis, using the same amount of total RNA isolated from 3rd instar larvae of the same age, indicated that *Ide* mRNA was reduced by 77–87% in three independent *Ide* RNAi lines (*T80G4/IDE*^{RNAi}) compared to the control where the driver was crossed into the *w*^{1118} background lacking the *Ide* RNAi construct (*T80G4/+*). (B) The broadly expressed *act-Gal4* driver or the IPC-specific *dilp2-Gal4* driver was used to activate *Ide* RNAi. The body weight increased by 25% in *actG4/IDE*^{RNAi} flies (n = 195) and by 21% in *dilp2G4/IDE*^{RNAi} flies (n = 134) when compared to the respective controls (*actG4/+*, n = 188; *dilp2G4/+*, n = 134). 7 day-old adult females were weighed. (C) Flies in which *Ide* expression is broadly activated (*actG4/dIDE*, n = 618) weighed 11% less than control flies (*actG4/+*, n = 878). Seven-day-old adult females were weighed. *p < 0.0002, two-tailed t-test. (D) IDE overexpressing flies (*actG4/dIDE*) were generally smaller than control flies (*actG4/+*). Flies of the two different genotypes were cultured separately but in parallel under identical conditions as possible to avoid over-crowding. The flies shown in this figure were randomly selected from culture vials and photographed about 1 day after eclosion.

important for controlling the level of DILPs that act systemically. We further speculate about the broader implication of our findings in the context of mammalian IDE and the control of insulin production by pancreatic islet β cells.

Results and Discussion

We decided to use RNAi to perturb IDE activity in Drosophila and therefore generated transgenic lines that can express double-stranded RNA targeting endogenous *Ide* mRNA under *Gal4-UAS* system control.[21,22] Using a bioinformatics off-target search tool (available at http://flyRNAi.org), no potential off-target of this *Ide* RNAi construct was identified.[23] To test knock down efficiency, expression of the *Ide* RNAi construct was activated with the *T80-Gal4* driver, which is ubiquitously expressed in the embryo and larva.[24] Quantitative RT-PCR analysis of three different *Ide* RNAi lines revealed that the *Ide* mRNA level was significantly reduced by 77–87% in RNAi larva when compared to control larva carrying the *T80-Gal4* driver but not the *Ide* RNAi construct (**Fig. 1A**). *Ide* RNAi larvae developed into viable adults with no obvious morphological abnormality. In all subsequent experiments, flies carrying two copies of the *Ide* RNAi construct were used in order to maximize knock down efficiency.

Earlier studies showed that overexpression of DILPs results in a 10–50% increase in the body weight of flies depending on the DILP isoform that is overexpressed.[14,15] If IDE degrades DILPs in vivo, then flies with reduced IDE activity would be expected to have a greater than normal DILP level and body weight. Indeed, adult flies in which *Ide* RNAi was broadly activated with the *act-Gal4* driver weighed 25% more than control flies (**Fig. 1B**). IDE overexpression with the same driver, which reduced adult fly viability by up to 92%, had the opposite effect. Surviving IDE

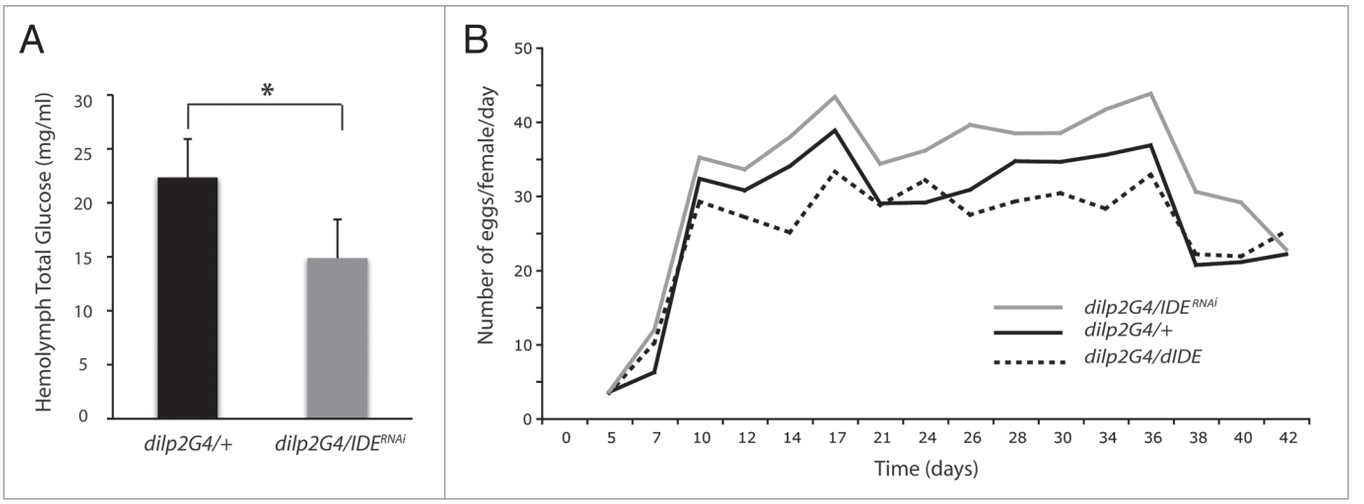

Figure 2. IDE is important for circulating sugar level and fecundity. (A) Hemolymph total glucose, derived mostly from trehalose and representing ≤1% free glucose, was about 30% lower for flies in which *Ide* RNAi was activated with the *dilp2-Gal4* driver (*dilp2G4/IDE*^{RNAi}) when compared to the control (*dilp2G4/+*). *p = 0.0127, one-tailed t-test. The circulating sugar level was not determined for IDE overexpressing flies. (B) Using the *dilp2-Gal4* driver to activate *Ide* RNAi (*dilp2G4/IDE*^{RNAi}) or IDE overexpression (*dilp2G4/dIDE*) in IPCs resulted in flies with increased and decreased fecundity, respectively, compared to control flies (*dilp2G4/+*). Eggs laid by individual virgin females were counted, with a plot showing the average of data from 50–60 females per genotype minus any flies that died during the experiment. The *dilp2G4/+*, *dilp2G4/IDE*^{RNAi} and *dilp2G4/dIDE* females, respectively, laid per female an average of about 1,090, 1,250 and 970 eggs over 39 days, or about 28, 32 and 25 eggs per day. Preliminary experiments with mated females showed similar results.

overexpressing flies weighed 11% less and were visibly smaller than control flies (**Fig. 1C and D**). As a comparison, flies lacking DILPs 1–5 were reported to have around 50% of the normal body weight.[20] The opposing effects on body weight from RNAi and overexpression approaches argue that altering IDE activity is responsible for the observed changes in body weight.

Insulin producing cells (IPCs) of the Drosophila brain are a major source of systemically acting DILPs.[17-20] By RNA in situ hybridization analysis, IDE appears to be broadly expressed in Drosophila tissues, including the brain (data not shown). To examine whether IDE expression is required in IPCs, we activated *Ide* RNAi using the *dilp2-Gal4* driver, which is specific for IPCs during late larval to adult stages.[15] Surprisingly, such IPC-specific *Ide* RNAi resulted in flies that weighed more than control flies by 21%, nearly comparable to the increase seen when *Ide* RNAi was more broadly activated (**Fig. 1B**). This result suggests that IDE expression is required in IPCs for normal body weight in Drosophila.

In Drosophila, reducing DILPs or ablating IPCs results in elevated circulating sugar, reduced fecundity and increased lifespan, all phenotypes associated with lowered insulin signaling.[15,17-20] We therefore investigated whether changing the IDE level in IPCs, by RNAi or overexpression with the *dilp2-Gal4* driver, affects these physiological parameters. To assay circulating sugar, we measured the hemolymph level of trehalose, a glucose disaccharide representing the predominant circulating sugar in Drosophila and other insects.[17,20] We found that IPC-specific *Ide* RNAi flies had about 30% lower hemolymph trehalose when compared to control flies (**Fig. 2A**), as would be predicted if these flies had higher than normal DILP levels. In addition, when compared to the control, IPC-specific *Ide* RNAi females laid more eggs while IPC-specific IDE overexpressing females produced fewer eggs (**Fig. 2B**). For example, in terms of the average number of eggs laid per female per day, IPC-specific *Ide* RNAi flies laid 14% more and IPC-specific IDE overexpressing flies laid 11% fewer than control flies; by comparison, flies lacking DILP1-5 were reported to lay 90% fewer eggs than normal.[20] While modest, the differences that we observed were reproducible and consistent with the changes that would be predicted from increasing or decreasing DILP levels. Finally, we found that IPC-specific *Ide* RNAi female flies had a median lifespan 18% shorter than control female flies (**Fig. 3A**), and a similar result was obtained with male flies (data not shown). Conversely, IPC-specific IDE overexpressing female flies had a median lifespan 14% greater than control females (**Fig. 3B**), while no consistently reproducible lifespan extension was observed for IPC-specific IDE overexpressing males. In comparison, ablation of IPCs was reported to increase the median lifespan of females and males by 33.5% and 10.5%, respectively, also revealing a substantial difference in effect between females and males.[18] Ectopic expression of human IDE in IPCs resulted in an 18% extension of median lifespan, again only in female flies, demonstrating that Drosophila and human IDE have similar in vivo activities (**Fig. 3C**).

In conclusion, we found that knocking down IDE in IPCs resulted in phenotypes previously associated with the upregulation of DILPs and insulin signaling, while overexpressing IDE in IPCs resulted in opposite phenotypes. Thus, our findings are consistent with the idea that IDE degrades DILPs made by IPCs that activate insulin signaling. A role for IDE in degrading DILPs is also supported by a recent study showing that, upon coexpression, IDE can antagonize the activity of DILP2 in promoting tissue growth.[13] However, the same study reported

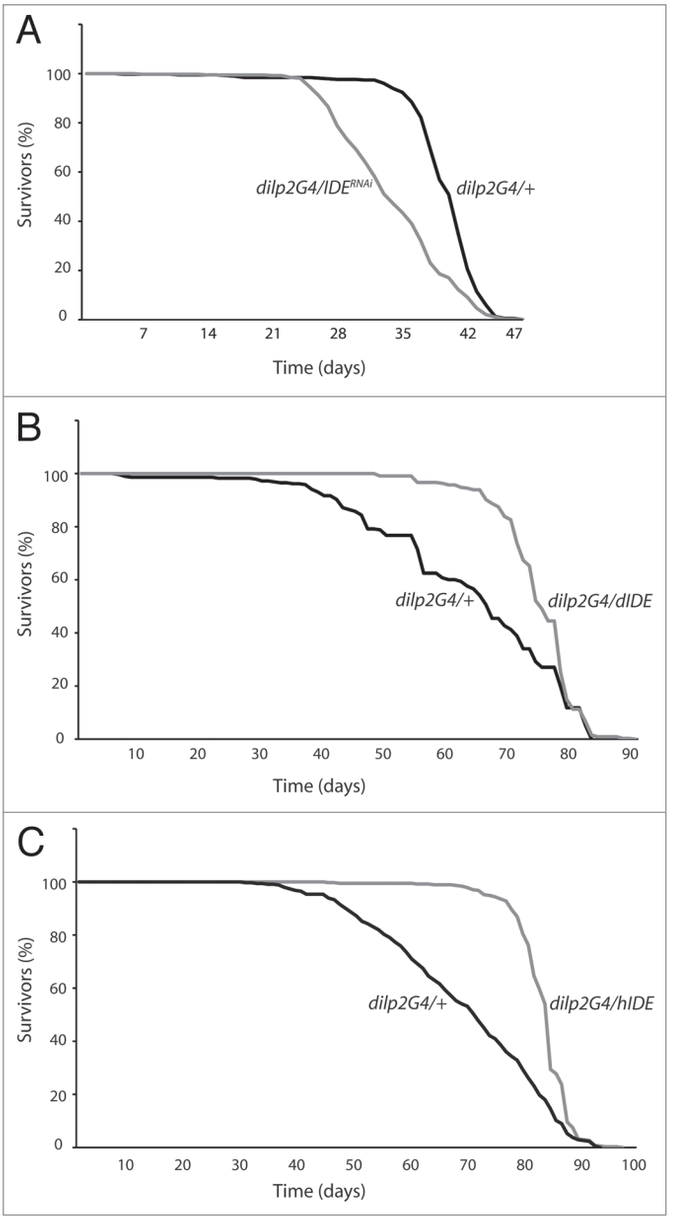

Figure 3. IDE is important for lifespan. (A) Flies in which IDE was knocked down in IPCs with the *dilp2-Gal4* driver (*dilp2G4/IDE*^{RNAi}, n = 381) had 18% lower median lifespan compared to control flies carrying the driver alone (*dilp2G4/+*, n = 383). Results are shown for female flies cultured at 29°C to enhance knockdown efficiency. Similar results were obtained with male flies. (B) Flies in which Drosophila IDE was overexpressed in IPCs with the *dilp2-Gal4* driver (*dilp2G4/dIDE*, n = 328) had 14% greater median lifespan compared to control flies carrying the driver alone (*dilp2G4/+*, n = 288). Results are shown for female flies cultured at 25°C. (C) Flies in which human IDE was overexpressed in IPCs with the *dilp2-Gal4* driver (*dilp2G4/hIDE*, n = 374) had 18% greater median lifespan compared to control flies carrying the driver alone (*dilp2G4/+*, n = 344). Results are shown for female flies cultured at 25°C.

that, while IDE overexpression lowered the body weight of flies as we observed, a putative *Ide* knockout mutation did not alter body weight, or other physiological parameters, in the manner that we observed with *Ide* RNAi. One possible explanation for the apparent discrepancy between the *Ide* knockout and RNAi results is that *Ide* knockdown causes an upregulation of DILPs, whose phenotypic effect is masked by upregulation of a distinct substrate or multiple substrates to a level only achieved upon complete elimination of *Ide* activity. Drosophila IDE likely degrades other substrates besides DILPs, just as mammalian IDE degrades multiple substrates.[3]

Further analysis is required to understand how IDE might degrade DILPs in vivo. Given that mammalian IDE rapidly degrades insulin but not proinsulin, Drosophila IDE would be expected to degrade mature and active DILPs rather than the pro form.[3] Thus, an antibody capable of detecting processed DILPs would be an important reagent in order to directly test IDE-dependent degradation of DILPs in vivo. However, the only western blot analysis of DILPs in Drosophila tissues reported so far detected the pro but not the processed form of DILP2, suggesting that processed DILPs are non-abundant.[19,25] Within IPCs, IDE could function in the secretory pathway during the secretion of DILPs. Alternatively, IDE could function in an endocytic pathway to degrade DILPs after they are secreted and internalized by the insulin receptor in IPCs, perhaps as part of an autocrine feedback loop as hypothesized to occur via insulin signaling in IPCs and in mammalian β cells.[19,26] By either mechanism, IDE could play an important role in IPCs to determine the level of DILPs that ultimately activate insulin signaling in peripheral tissues. Given the functional homologies between Drosophila and mammalian IDE, as well as between Drosophila IPCs and mammalian β-cells, we speculate that mammalian IDE could have a similarly important role in β cells in controlling insulin levels.

Materials and Methods

Fly strains. Transgenic lines carrying constructs encoding full-length Drosophila IDE (*UAS-dIDE*) on the second and third chromosomes, full-length human IDE (*UAS-hIDE*) on the third chromosome and double-stranded RNA specific for Drosophila IDE (*UAS-IDE*^{RNAi}) on the second and third chromosomes were made by standard P-element transformation of *w*^{1118} (Fbal0018186) flies.[27] The *dilp2-Gal4/CyO* line was from D. Bohmann (Univ. of Rochester). *T80-Gal4/CyO* (FBst0001878) and *act-Gal4/CyO* (FBst0004414) were from the Bloomington Stock Center (Bloomington, IN). In all RNAi and overexpression experiments using a given driver (e.g., *dilp2-Gal4*), parallel crosses between driver bearing flies and *w*^{1118} flies were used to generate control flies (e.g., *dilp2-Gal4/+*).

Molecular biology. The *UAS-dIDE* and *UAS-hIDE* constructs were made using Drosophila IDE cDNA (RE17458) and the pPWF vector from the Drosophila Genomics Resource Center (Bloomington, IN) and human IDE cDNA (BC096336) from Open Biosystems (Huntsville, AL). The *UAS-IDE*^{RNAi} construct contains sequences 1,343–1,856 from the Drosophila IDE cDNA in the pWIZ vector.[21] RT-PCR was performed using the Roche LightCycler® on total RNA isolated from third instar larvae with TRIzol (GIBCO-BRL), using ribosomal protein 49 as the internal normalization standard for both RNAi and control flies.

Hemolymph sugar assay. Hemolymph was isolated from adult females aged 2–4 days after eclosion essentially as described in reference 28. About 1.5 μl of hemolymph obtained from 40 flies was mixed with 20 μl PBS and incubated with 1 μl trehalase (3.7 units/ml, Sigma) overnight at 37°C in order to digest trehalose to glucose. After incubation, 5 μl of trehalase reaction in duplicate was incubated with 100 μl of Glucose Assay Reagent (Sigma) in a 96-well plate for 15 minutes at room temperature. The glucose concentration in these samples was determined by spectroscopic measurement against a glucose standard curve using a Bio-Rad microplate reader, and then used to determine the concentration of total glucose in hemolymph. Free glucose, the glucose in hemolymph prior to trehalase treatment, represented ≤1% of total glucose.

Fecundity measurements. Fecundity was determined by counting the number of eggs laid by females during a period of 3–42 days after eclosion. Females of a given genotype were placed in ten 100 ml beakers, each containing 5–6 females. Each beaker was inverted over an apple juice agar plate with yeast paste, which was exchanged every 2–3 days. The eggs laid on the plate were counted, with data presented as described in reference 19.

Lifespan measurements. Survival rate was determined as described in reference 19. Freshly eclosed adult flies were collected into vials, with each vial containing 25 flies. Flies were transferred to a fresh vial every other day and the dead flies remaining in the old vial were counted. This process was repeated until all flies of a given genotype had died. Flies were maintained at 25°C on standard medium.

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