Drosophila insulin-like peptide-6 (dilp6) expression from fat body extends lifespan and represses secretion of Drosophila insulin-like peptide-2 from the brain

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Summary

Reduced insulin/IGF signaling extends lifespan in diverse species, including Drosophila melanogaster where the genome encodes seven insulin-like peptides (dilp1-7). Of these, reduced dilp2 expressed in the brain has been associated with longevity assurance when over-expression of dfoxo in fat bodies extends lifespan. Here, we show that the insulin-regulated transcription factor dFOXO positively modulates dilp6 mRNA in adult fat body. Over-expression of dilp6 in adult fat body extends lifespan and increases longevity-associated metabolic phenotypes. Adult fat body dilp6 expression represses dilp2 and dilp5 mRNA in the brain, and the secretion of Dilp2 into the hemolymph. The longevity benefit of expressing dfoxo in fat body, and the nonautonomous effect of fat body dfoxo upon brain dilp expression, is blocked by simultaneously repressing dilp6 by RNAi in fat body. dilp6 thus appears to bridge dFOXO, adipose tissue and brain endocrine function to regulate Drosophila longevity.

Key words: dilp6; dilp2; insulin/IGF; fat body; fruit fly; longevity.

Introduction

Insulin-like peptides are evolutionary conserved proteins that regulate growth, metabolism, reproduction, and longevity. Invertebrate genomes are notable for their many insulin-like peptide paralogs. The nematode Caenorhabditis elegans has some 40 insulin-like peptides (Pierce et al., 2001). Seven insulin-like peptides are encoded in Drosophila melanogaster (Drosophila insulin-like peptides, dilp1-7), and these are conserved across the genomes of 11 related Drosophilids (Brogiole et al., 2001; Gronke et al., 2010). Genes encoding insulin-like peptides have likewise been identified from the silkworm Bombyx mori (Bombyxim) and subsequently in orders spanning Orthoptera, Diptera, Lepidoptera, Coleoptera and Hymenoptera (reviewed in Wu & Brown, 2006)).

The seven Drosophila insulin-like peptides show diverse patterns of stage- and tissue-specific expression (Brogiole et al., 2001; Cao & Brown, 2001; Ikeya et al., 2002; Rulifson et al., 2002). dilp2, dilp-4, and dilp-7 are expressed in the mesoderm and midgut of embryos. dilp7 mRNA is also present in cells of the larval and adult ventral nerve cord (Brogiole et al., 2001; Yang et al., 2008). In larvae, dilp2, dilp-3, and dilp-5 are predominantly expressed in two clusters of brain neurosecretory cells (IPC, insulin producing cells); these cells are thought to have functional and developmental similarities to mammalian pancreatic β cells (Brogiole et al., 2001; Rulifson et al., 2002). Ablating the larval IPC delays metamorphosis, reduces body size and elevates hemolymph carbohydrates (Rulifson et al., 2002). In the adult stage, beside its expression in IPC, dilp5 transcripts were also detected in follicle cells of stage 10 oocytes (Ikeya et al., 2002). dilp3 mRNA is detected in visceral muscle cells of the midgut. dilp3 expression acts directly on midgut stem cells to regulate intestinal growth (Veenstra et al., 2008). dilp6 is strongly expressed in larval and adult fat body, a tissue with mammalian adipose and liver-like functions. In larvae, the expression of dilp6 is regulated by dFOXO and is required for pre-metamorphic growth (Okamoto et al., 2009; Slaidina et al., 2009). Recent studies with larvae revealed that dilp6 is also expressed in a subset of glia surrounding neuroblasts where this expression leads to neuroblast reactivation upon nutrient restriction (Chell & Brand, 2010; Sousa-Nunes et al., 2011).

Insulin-like peptides of the adult help control many traits, including reproduction, metabolism, and lifespan (Hsu & Drummond-Barbosa, 2009; Gronke et al., 2010). Reducing insulin/IGF signaling (IIS) increases adult survival (Tatar et al., 2003; Giannakou & Partridge, 2007). Lifespan is increased in mutants and dominant-negatives of the Drosophila insulin receptor (Inr; Tatar et al., 2001; Slack et al., 2011), and by misexpression of insulin-receptor substrate (chico) and PTEN (Clancy et al., 2001; Tu et al., 2002, Hwangbo et al., 2004). Survival is increased when the insulin-producing neurons are ablated (Wessells et al., 2004, Broughton et al., 2005), which reduces multiple dilps as well as any other IPC-related neuropeptides. It has proved more difficult to analyze the impact of individual dilps on lifespan because the seven related genes exhibit compensatory expression (Broughton et al., 2008; Min et al., 2008). Nonetheless, analysis of homologous recombination knockouts of individual dilp genes revealed that loss of dilp2 was sufficient to increase survival (Gronke et al., 2010).

A role for dilp2 in the control of aging was also suggested by studies that extended lifespan through the IIS-related factors dFOXO (Hwangbo et al., 2004), Jun-N-terminal kinase (JNK; Wang et al., 2005), and short neuroreptide-F (SNPF, homolog of mammalian NPY; Lee et al., 2008). Work with dFOXO is notable because lifespan was extended when this IIS-regulated transcription factor was over-expressed in adult fat body in a diet-dependent manner (Min et al., 2008). Lifespan was extended and dilp2 mRNA was reduced in adults fed a low-yeat diet when dfoxo was over-expressed from abdominal fat body. In contrast, lifespan was extended and dilp2 was reduced in flies fed a high-yeast diet when dfoxo was over-expressed from head fat body. In both conditions, systemic IIS signaling in peripheral tissues was reduced, while dilp2 mRNA of the IPC was less abundant.

From this view, FOXO plays both autonomous and non-autonomous roles in aging. Work with the fly heart illustrates the autonomous role where increased dFOXO specifically within cardiac tissue is sufficient to slow heart functional aging (Wessells et al., 2004). dFOXO is proposed to
mediate feedback signaling between the IPC and fat bodies because dilp6 from the IPC could repress dFOXO within fat body while fat body dFOXO regulates dilp expression in the IPC. Besides non-autonomously modulating the expression of dilp mRNA in the adult brain, dFOXO has been reported to regulate the transcription of dilp6 within fat body, at least in the case of larvae (Okamoto et al., 2009; Slaidina et al., 2009). In larvae, dFOXO is required for starvation-activated dilp6 expression where dFOXO protein binds to the promoter region of the dilp6 locus. This observation stimulated the questions for the current work: Is dilp6 regulated by dFOXO in adult fat body, does dilp6 expressed from the adult fat body modulate aging, and if so, might it do so by regulating the expression and secretion of dilps produced by the adult IPC, especially dilp2?

To address these issues, we over-expressed or silenced dilp6 in specific adult tissues including fat body. Previous work that systemically and ubiquitously knocked out genomic dilp6 reported this to decrease larval growth but not to effect adult survival (Gronke et al., 2010). Here, we show that dilp6 over-expressed in adult fat body extends lifespan, elevates carbohydrate and fat storage, and improves oxidative stress resistance. 4ebp mRNA, a transcriptional target of dFOXO, is elevated in tissues aside from the site of dilp6 over-expression, suggesting that over-expression of fat body dilp6 systemically reduced insulin/IGF signaling. In these conditions, dilp2 and dilp5 mRNA of the brain were repressed, and hemolymph Dilp2 was significantly reduced as measured by an enzyme immunoassay. dilp6 RNAi in fat body blocks the longevity benefit typically observed when dfoxo is over-expressed in fat body, and this dilp6 RNAi likewise blocks the negative effect of fat body expressed dfoxo upon dilp2 mRNA. Fat body dilp6 forms part of the non-autonomous aging-control circuit between dFOXO, fat body, and brain.

**Results**

**dilp6 is expressed in adult adipose tissue and is induced by fasting and dFOXO**

To date, dilp6 function has been best described in larval fat body (Okamoto et al., 2009; Slaidina et al., 2009). To study dilp6 in the adult, we measured its expression in several tissues (Fig. 1A). dilp6 mRNA is apparent in abdominal fat body, brain and head carcass (head fat body, compound eyes, antennae, and mouth parts) but is relative rare in midgut and ovary. This contrasts with dilp1, dilp2, dilp3, and dilp5, which are predominantly expressed in the brain at the central IPC (Fig. S1). dilp6 expression in abdominal fat body and head fat body is also observed from a dilp6-GAL4 insertion line (NP1079) driving UAS-GFP.nls, while in the brain, the expression pattern of dilp6 is distinct from primary insulin producing neurons (IPC; Fig. S2).

Slaidina et al. (2009) reports dilp6 mRNA in larval fat body was strongly induced by fasting. Here, we see dilp6 mRNA up-regulated in abdominal fat body of overnight fasted adults, while brain dilp5 mRNA was repressed and dilp2 mRNA was static (Fig. 1B). Unlike its expression in fat body, dilp6 mRNA in the brain does not change upon fasting (Fig. 1B). In late-stage larvae, fasting activates dFOXO and this induces transcription of dilp6 (Slaidina et al., 2009). To test whether such control operates in adults, we drove a constitutively active dFOXO (UAS–dFOXO-TM) in fat body, at least in the case of larvae (Okamoto et al., 2009; Slaidina et al., 2009). In larvae, dFOXO is required for starvation-activated dilp6 expression where dFOXO protein binds to the promoter region of the dilp6 locus. This observation stimulated the questions for the current work: Is dilp6 regulated by dFOXO in adult fat body, does dilp6 expressed from the adult fat body modulate aging, and if so, might it do so by regulating the expression and secretion of dilps produced by the adult IPC, especially dilp2?

To address these issues, we over-expressed or silenced dilp6 in specific adult tissues including fat body. Previous work that systemically and ubiquitously knocked out genomic dilp6 reported this to decrease larval growth but not to effect adult survival (Gronke et al., 2010). Here, we show that dilp6 over-expressed in adult fat body extends lifespan, elevates carbohydrate and fat storage, and improves oxidative stress resistance. 4ebp mRNA, a transcriptional target of dFOXO, is elevated in tissues aside from the site of dilp6 over-expression, suggesting that over-expression of fat body dilp6 systemically reduced insulin/IGF signaling. In these conditions, dilp2 and dilp5 mRNA of the brain were repressed, and hemolymph Dilp2 was significantly reduced as measured by an enzyme immunoassay. dilp6 RNAi in fat body blocks the longevity benefit typically observed when dfoxo is over-expressed in fat body, and this dilp6 RNAi likewise blocks the negative effect of fat body expressed dfoxo upon dilp2 mRNA. Fat body dilp6 forms part of the non-autonomous aging-control circuit between dFOXO, fat body, and brain.

**Over-expressing dilp6 in adipose tissue extends lifespan**

As a target of dFOXO in adult fat body, dilp6 may mediate longevity assurance conferred by over-expressing dfoxo. Consistent with this prediction, conditional expression of dilp6 in head and abdominal fat body extended female lifespan (Table 1). Notably, dilp6 from abdominal fat body extended lifespan (Fig. 2A) and consistently reduced age-specific mortality (Fig. S1A) in females maintained upon relatively low-yeast diet (2% yeast) but not on high-yeast diet (8% yeast; Fig. 2B). This pattern of diet dependence is similar to the nutrient conditions when dfoxo over-expression in abdominal fat body extends lifespan (Min et al., 2008). Likewise, dilp6 conditionally expressed in head fat body modestly increased lifespan in females upon high-yeast diet (Fig. 2D,F) and less so upon low-yeast diet (Fig. 2C). No detectable effect of dilp6 upon lifespan was seen for males on any diet or when expressed from either fat body (Table 1). In contrast to these results, lifespan was shortened by conditional expression of dilp6 with ubiquitous drivers, as well as when dilp6 was ubiquitously reduced by RNAi (Table 1; Figs S3 and S4). Lifespan was not affected when dilp6 was over-expressed by a conditional pan-neuronal driver or when silenced by RNAi in fat body (Table 1; Figs S3 and S4). As dfoxo over-expressed in fat body can extend lifespan (Hwangbo et al., 2004; Giannakou et al., 2005), we tested whether reducing dilp6 is sufficient to block the longevity benefit of the dfoxo transgene. As expected if dilp6 modulates the longevity benefit of dFOXO from the fat body, there was no survival or mortality differences between control and RU-induced cohorts of the genotype S106-Gal4 > UAS-dfoxo; UAS-dilp6 (RNAi; Figs 2E and S1C).
**Table 1** Median lifespan of adult flies with dilp6 over-expression and knockdown

| Sex     | GS-Gal4 | UAS | Diet % yeast | Median lifespan (E50 days) | 
|---------|---------|-----|--------------|---------------------------| 
| Male    | S32-GS  | UAS-dilp6 | 2% | 0 RU | 200 RU | E50 dif. (%) | P | Sample size (no. flies) |
| Male    | S32-GS  | UAS-dilp6 | 8% | 69 | 75 | −32.91 <0.0001 | 749 |
| Female  | S32-GS  | UAS-dilp6 | 2% | 79 | 79 | 15.58 <0.0001 | 753 |
| Female  | S106-GS | UAS-dilp6 | 4% | 97 | 87 | −11.76 <0.0001 | 749 |
| Female  | da-GS   | UAS-dilp6 | 4% | 67 | 73 | 11.76 <0.0001 | 749 |
| Female  | S32-GS  | UAS-dilp6 | 2% | 85 | 75 | 11.76 <0.0001 | 749 |
| Female  | S32-GS  | UAS-dilp6 | 4% | 71 | 71 | −2.60 <0.0001 | 650 |
| Female  | S106-GS | UAS-dilp6 | 8% | 77 | 53 | −10.31 <0.0001 | 741 |
| Female  | S106-GS | UAS-dilp6 | 2% | 77 | 89 | 15.58 <0.0001 | 753 |

**Summary:**

- **dilp6 regulates adult metabolism, stress resistance, and fecundity.** Manipulations that systemically reduce insulin/IGF signaling and extend Drosophila lifespan often induce metabolic and stress-resistant phenotypes. These stereotypic traits were seen when *dilp6* was expressed from adult fat body. This increased whole body triacylglycerides (TAG) and glycogen, and hemolymph trehalose (Fig. 3A–C). Nutrient storage is often associated with survival during fasting but only a modest fasting survival benefit was observed when *dilp6* was conditionally expressed from fat body (Fig. 3E). These females, however, exhibited elevated survival when challenged with H2O2 oxidative stress (Fig. 3F). Fecundity was slightly reduced when *dilp6* was expressed from adult fat body (Fig. 4D). These patterns of stress resistance, metabolite storage, and extended lifespan suggest that increased expression of *dilp6* may coordinate phenotypes by reducing insulin production from the brain.

- **Fat body *dilp6* modulates neuronal dilp2 mRNA and secreted dilp2 protein.** The transcription factor 4ebp is a direct transcriptional target of dFOXO that is induced when insulin signaling is repressed (Puig et al., 2003). Here, 4ebp mRNA was upregulated in abdominal fat body and thorax when *dilp6* was over-expressed in head fat body (Fig. 4A), while 4ebp message was increased in head tissues when *dilp6* was over-expressed in abdominal fat body (Fig. 4B). These effects at a distance from the site of *dilp6* over-expression suggest that peripheral insulin/IGF may be reduced in *dilp6* over-expression lines, and *dilp2* and *dilp5* mRNAs were indeed reduced in brains from females where *dilp6* was over-expressed in fat body (Figs. 4C,D). At the same time, *dilp2* peptides in IPC bodies were significantly reduced (Fig. 5A–C,G), while *dilp5* peptides were only modestly affected (Fig 5D–G). To determine whether *dilp6* of fat body represses the secretion of *dilp* protein, we measured hemolymph DILP titer by enzyme immunoassay (EIA) using antibodies against DILP2 or DILP5. When driven in abdominal fat body, *dilp6* over-expression strongly reduced the level of circulating DILP2 but this manipulation only modestly affected DILP5 (Fig. 5H). Furthermore, the ability of *dfoxo* expressed in the fat body to regulate the expression and secretion of DILP in the brain requires fat body *dilp6*. Simultaneously, driving UAS-FOXO and UAS-dilp6(RNAi) in fat body precludes the elevation of *dilp6* mRNA in this tissue (Fig. 6D) and prevents the expected reduction of brain *dilp2* mRNA when UAS-dfoxo alone is driven in fat body (Fig. 6E).

- **Discussion**

The Drosophila genome contains one insulin-receptor gene with several isoforms, and seven *dilp* loci (Brogiole et al., 2001). Among these *dilps*, reduced *dilp2* has been consistently associated with increased lifespan. Homologous recombination to knockout *dilp2* increased longevity (Gronke et al., 2010), and mRNA levels of *dilp2* were reduced and longevity was increased in genotypes that misexpressed JNK (Wang et al., 2005). *sNPF* (Lee et al., 2008), and *dfoxo* (Hwangbo et al., 2004; Giannakou et al., 2005) where *dfoxo* misexpression from fat body alone was sufficient to slow aging. The targets of dFOXO responsible for its non-autonomous control of aging are unknown, and there are potentially many candidates. dFOXO can bind to at least 700 promoter regions (Alic et al., 2011), and this factor has been associated with the transcriptional control of more than 1000 genes (Zinke et al., 2002; Gershman et al., 2007). At least in larvae, *dilp6* is a target of dFOXO and through its expression in fat body *dilp6* modulates growth during post-feeding development (Okamoto et al., 2009; Sladina et al., 2009).

**Note:**

Diets contained cornmeal, sugar, agar and either 2%, 4% or 8% yeast.

Probability is based on chi-square distribution from log-rank test between control (0 RU) and induced (200 RU) cohorts. GeneSwitch-Gal4 (GS) divers were used for *dilp6* over-expression: S32-GS (head fat body), S106-GS (abdominal fat body), Tub-GS (ubiquitous), da-GS (ubiquitous), Elav-GS (pan-neuronal).
signaling. Decreased systemic insulin signaling is not likely to arise because dilp6 acts as an antagonist, unlike as has been suggested for ins-1 of C. elegans (Pierce et al., 2001). In the fly, dilp6 expressed within fat body increases insulin-receptor-mediated phospho-signaling and decreases 4ebp mRNA of the fat body, indicating dilp6 is a local insulin signaling agonist. Rather, decreased systemic insulin is associated with a 50% reduction in circulating DILP2 peptide. Reduced circulating DILP2 may account for the observed increase in lifespan when dilp6 is expressed in fat body, and for the correlated changes in storage lipids and carbohydrates, oxidative stress resistance, and fecundity.

dFOXO control of dilp6 and the subsequent effects of dilp6 upon DILP secreted from IPC may contribute to an insulin-regulatory feedback loop between fat body and insulin producing neurons of the brain (Fig. S10). dFOXO expressed in adult fat body represses dilp2 mRNA in the IPC (Hwangbo et al., 2004). When DILP2 secretion is reduced, the fat body will further induce dFOXO and thus reinforce the repression of dilp2 in the IPC. This circuit will reinforce a state of low circulating DILP and thus promote longevity assurance. Events that stimulate DILP synthesis in the IPC could shift the feedback to an alternative stable state with active DILP secretion. Elevated DILP secretion would thus suppress fat body dFOXO and thus release the dFOXO suppression of dilp expression in the IPC. dilp6 may play an intermediary role in this regulatory circuit because it is a target of fat body dFOXO, while it is also upstream of dilp expression and secretion in the IPC.

The factors transmitting signals from dilp6 of fat body to the IPC of the brain are unknown. Fat body-derived DILP6 itself may be secreted and function as this hormone. Alternatively, dilp6 may regulate a downstream adipokine that circulates to affect the brain IPC, or it may affect the brain through changes in systemic metabolism to which the IPC are sensitive. In general, the roles and functions of the adult fat body of Drosophila are enigmatic. This tissue shares functions found in mammalian liver as well as adipose tissue. Drosophila fat body participates in immune function (antimicrobial peptide expression), yolk protein synthesis, and energy homeostasis and storage, although metabolic data are mostly known from studies of larvae (Colombani et al., 2003; Okamoto et al., 2009; Slaidina et al., 2009). In larvae, the amino acid transporter Slimfast and TOR function in the fat body as nutrient sensors to coordinate systemic growth (Colombani et al., 2003). On the basis of data from co-culture experiments, amino acid-restricted fat body secretes a hormonal factor (an adipokine) to remotely control DILP secretion from IPC (Geminard et al., 2009), and this process requires TOR signaling in the fat body. Whether TOR signaling from adult fat body has the capacity to regulate lifespan has not been satisfactorily resolved (erratum to Kapahi et al., 2004).

Fig. 2 dilp6 expressed in fat body extends lifespan. (A) dilp6 over-expressed in abdominal fat body (via S106-GS-Gal4) extends lifespan in flies maintained on low-yeast diet, but not (B) when maintained on high-yeast diet. (C) dilp6 over-expressed in head fat body (via S32-GS-Gal4) does not extend lifespan on low-yeast diet but (D) moderately extends lifespan on high-yeast diet. (E) Simultaneous induction of UAS-dfoxo and UAS-dilp6 RNAi inhibits the survival benefit expected from over-expressing dfoxo alone in fat body.

Fig. 3 dilp6 expressed in abdominal fat body modulates metabolism, fecundity and stress resistance. dilp6 over-expressed in abdominal fat body increases fat body (A) triglycerides, (B) glycogen, and (C) trehalose in hemolymph, while (D) repressing fecundity. Asterisk indicates significant difference between treatment and control, $P < 0.05$. dilp6 over-expressed in abdominal fat body modestly increases the resistance (E) to starvation (log-rank test, $P < 0.048, n = 150$) and (F) to H2O2 ($P < 0.0003, n = 130$).
The potential for adipokines to modulate longevity assurance may be conserved across taxa. In mammals, systemic insulin signaling is influenced by fat body-derived hormonal factors, such as tumor necrosis factor-alpha (TNF-α), leptin and adiponectin. Inflammatory cytokine TNF-α and TNF-α receptor knockout mice present increased insulin sensitivity (Schreyer et al., 1998). Leptin, first observed in mutant obese mice, regulates food intake by acting on hypothalamic neuropeptide Y signaling (Friedman & Halaas, 1998). In pancreatic islets and β cells culture, leptin inhibits glucose and glucagon-like peptide-1 stimulated insulin secretion (Zhao et al., 1998). Although the Drosophila genome reveals no obvious homolog of leptin, over-expression of Drosophila sNPF, an ortholog of mammalian neuropeptide Y, increases adult dilp1 and dilp2 mRNA, and conversely, knock-down of sNPF decreases these dilps and extends lifespan (Lee et al., 2008). Notably, dilp6 expressed from adult abdominal fat body reduces expression of sNPF from the brain (Fig. S7), and this reduction may contribute to the suppression of dilp2 in the IPC (Lee et al., 2008). In addition to leptin, mammalian adiponectin secreted from adipose tissue profoundly affects insulin resistance and type 2 diabetes (Kadowaki et al., 2006). Transgenic mice expressing human adiponectin in the liver have increased circulating adiponectin, reduced fasting glucose, insulin, and leptin and improved survival when fed high-fat/high-sucrose diet (Otabe et al., 2007). Intriguingly, elevated adiponectin and polymorphisms of the adiponectin gene (ADIPOQ) are enriched in human centenarians (Atzmon et al., 2008). Drosophila contains a predicted homolog of the adiponectin 1 receptor (CG5315) that has cell-autonomous and non-autonomous roles in regulating oogenesis in response to diet (LaFever, 2010), but an adiponectin-like ligand has yet to be identified.

In Drosophila and mammals, there are reciprocal interactions between fat body and systemic insulin signaling. Fat body can produce adipokines to modulate systemic IIS sensitivity and stimulation, while insulin signaling within the fat body itself responds to these systemic insulin changes. Identifying the roles and functions of adipokines in Drosophila may provide an avenue to understand central regulatory mechanisms of aging and its interaction with metabolism.

**Experimental procedures**

**Fly stocks and husbandry**

RU486 (RU, mifepristone)-induced drivers (all referred to here as Gene-Switch, GS) were as follows: Tub-GS-Gal4; Elav-GS-Gal4 (Roman et al., 2001); da-GS-Gal4 (Tricoire et al., 2009); S32-GS-Gal4; S106-GS-Gal4 (Roman et al., 2001); dilp2-GS-Gal4 (kindly provided by Dr. Heinrich Jasper). dilp6-Gal4 (#103877, DGRG-Kyoto stock center) is a constitutive driver. Transgenes responding to Gal4 were as follows: UAS-GFP.nls (gift from Bruce Edgar, Heidelberg, Germany); UAS-dfoxo-TM and UAS-dfoxo...
overnight by transferring flies to glass vials with 1% agar media.

**Expression and knock-down (Hwangbo et al., 2014)**: The effects of UAS-dilp6 (RNAi) (#31379, Bloomington stock center) on gene expression were assessed using Western blotting. The expression of phospho-AKT, AKT, phospho-FOXO, and FOXO was increased when dilp6 is over-expressed in the brain, and decreased when dilp6 was over-expressed in the fat body. The expression of these genes was quantified in (B; n = 4). (C) 4ebp mRNA in abdominal fat body is reduced when dilp6 was over-expressed in this tissue. Asterisk indicates significant difference between treatment and control (P < 0.05). Simultaneous expression of dfoxo and dilp6(RNAi) in fat body (D) prevents blocks the normal over-expression of dilp6 induced by dfoxo and (E) prevents the repression of dilp2 mRNA at a distance in the brain.

**Demography and survival analysis**

Two- to three-day-old adult flies were collected with CO₂ anesthesia and pooled in 1 L demography cages at a density of 100–125 flies per cage, with three independent cages per genotype. Food vials with media containing vehicle only or RU486 were changed every 2 days, at which time dead flies were removed and recorded. Survival analysis was conducted with JMP statistical software with data from replicate cages combined. Survival distributions were compared by the log-rank test.

**TAG, glycogen, and trehalose**

Triglycerides (TAG) and glycogen were quantified from abdominal fat body dissected from 10 female adults and homogenized in lysis buffer (0.2% Tween-20 in PBS). The lysate was heated for 5 min at 70 °C and then centrifuged at 14 000 g for 5 min. TAG was measured from 10 μL of supernatant with Infinity Triglycerides Reagent (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Protein was quantified via BCA Protein Assay (Pierce, Thermo Fisher Scientific Inc.). To measure glycogen, 30 μL of the supernatant was incubated with amylglucosidase (Carolina Biological, Burlington, NC, USA) for 30 min at 37 °C to convert glycogen to glucose; glucose was measured with Infinity Glucose Reagent (Thermo Fisher Scientific Inc.).

To measure trehalose, hemolymph was collected from 20 centrifuged, decapitated flies (Broughton et al., 2008; Demontis & Perrimon, 2010). 0.3–0.5 μL of hemolymph was diluted in PBS, and the glucose concentration was measured with Infinity Glucose Reagent (Thermo Fisher Scientific Inc.) after incubation with porcine trehalase (Sigma) at 37 °C overnight.

**Female fecundity**

Three-day-old mated female flies were maintained on food with or without RU486 for 5 days at one female per vial and 10–15 vials per group. Flies were daily passed to new vials over 3 days (with or without RU486), and eggs were counted daily. RU486 alone had no effect on fecundity as shown in previous studies. RU486 and RU486 + RU486 alone had no side effects upon egg production, TAG, dilp2 or dilp5 expression or adult survival.

**Stress resistance**

Starvation resistance was measured in 5-day-old females previously maintained on food with and without RU486. Females were transferred into glass vials containing 1% agar with or without RU486. Dead flies were counted twice a day. To assess oxidative stress resistance, 5-day-old females previously maintained on food with and without RU486 were transferred into glass vials containing 1% agar, 5% sucrose, and 5% H₂O₂ (with or without RU486). Dead flies were counted twice a day. In
both assays, ~80 females distributed among 8 vials were tested in each group. Effects on survival were analyzed by the log-rank test.

**Antibodies and immunostaining**

Antibodies included anti-DILP2 (1:200), anti-DILPS (1:200; Geminard et al., 2009); anti-rat IgG-Alexa Fluor 594 (1:300); and anti-rabbit IgG-DyLight 488 (1:300; Jackson ImmunoResearch, West Grove, PA, USA). Samples were processed as described in Geminard et al. (2009) and imaged with a Leica SP2 laser scanning confocal microscope. To quantify fluorescence signaling, confocal Z-stack images were obtained with identical laser power and scan settings (Leica Microsystems Inc., Buffalo Grove, IL, USA). The integrated density for IPC was measured and subtracted from the density of background readings using ImageJ software.

**Enzyme immunoassay (EIA) for hemolymph dilp**

About 0.5 μL of hemolymph was diluted with PBS and incubated overnight in cells of a 96-well EIA/RIA plate (Corning Incorporated, Corning, NY, USA) at room temperature. Following incubation, cells were cleared of hemolymph, and bound material in the plate was blocked for 2 h with EIA buffer (10 mM NaH₂PO₄, 3 mM Na₂HPO₄, 150 mM NaCl, 1 mM NaEDTA -2H₂O, 0.2% Na azide) and 1% BSA. Blocked samples were washed three times with PBS-Tween. Except for the blank well, samples were treated with 100 μL of anti-DILP or anti-DILPS antibody at 1:2500 dilution, incubated 1 h at room temperature, washed three times with PBS-Tween, and treated with HRP-conjugated secondary antibodies, Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific Inc.) Band intensity was quantified with AlphaView software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

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**Author contributions**

H.B. and M.T. designed the experiments and wrote the manuscript; H.B. and P.K. performed the experiments.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site: 

**Fig. S1** *dilp1, dilp2, dilp3 and dilp5* mRNA measured from fat body, midgut, ovary, brain and head carcass.

**Fig. S2** *dilp6* reporter in adipose tissue and brain. 

**Fig. S3** Lifespan of *dilp6* over-expression using ubiquitous drivers: (A) *Tub-GeneSwitch-Gal4*, (B) *da-GeneSwitch-Gal4*, (C) neuronal *Elav-GeneSwitch-Gal4*.

**Fig. S4** Lifespan of *dilp6* silencing using ubiquitous drivers: (A) *Tub-GeneSwitch-Gal4*, and fat body (B) *S32-GeneSwitch-Gal4*, (C) *S106-GeneSwitch-Gal4*.

**Fig. S5** Specificity of *anl-DILP2* and *anl-DILPS* anl-bodies in EIA.

**Fig. S6** Upon 2% and 8% yeast diet, qRT-PCR verifies the induction of *dilp6* by UAS-*dilp6* (A,B), and *dilp6* knock-down by RNAi (C).

**Fig. S7** sNPF transcripts measured in flies with ubiquitous and tissue-specific *dilp6* overexpression.

**Fig. S8** *S106-gal4* does not induce transgene expression without RU.

**Fig. S9** RU486 alone does not induce aging or metabolic phenotypes.

**Fig. S10** Model for DILP6 to regulate lifespan by repressing DILP produced by antagonizing cellular and organism-wide responses to insulin signaling. *Cell* **121**, 115–125.

**Fig. S11** Mortality rate (estimated as –ln(ln(px)) for survival plots of text figure 2.