The Drosophila insulin-degrading enzyme restricts growth by modulating the PI3K pathway in a cell-autonomous manner

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ABSTRACT Mammalian insulin-degrading enzyme (IDE) cleaves insulin, among other peptide substrates, but its function in insulin signaling is elusive. We use the Drosophila system to define the function of IDE in the regulation of growth and metabolism. We find that either loss or gain of function of Drosophila IDE (dIDE) can restrict growth in a cell-autonomous manner by affecting both cell size and cell number. dIDE can modulate Drosophila insulin-like peptide 2 levels, thereby restricting activation of the phosphatidylinositol-3-phosphate kinase pathway and promoting activation of Drosophila forhead box, subgroup O transcription factor. Larvae reared in high sucrose exhibit delayed developmental timing due to insulin resistance. We find that dIDE loss of function exacerbates this phenotype and that mutants display increased levels of circulating sugar, along with augmented expression of a lipid biosynthesis marker. We propose that dIDE is a modulator of insulin signaling and that its loss of function favors insulin resistance, a hallmark of diabetes mellitus type II.

INTRODUCTION

In animals, proper balance of energy uptake, storage, and utilization relies on coordinated communication between different tissues and organs to adjust metabolism according to organism needs. This is achieved through paracrine and endocrine signals that act at the cellular level to activate signaling pathways that mediate cell and organism adaptation. Central among these pathways is the insulin signaling system (Pan, 2010). In mammals, glucose levels and metabolism are fine-tuned by insulin, which is secreted by pancreatic β-cells and delivered through the bloodstream to target tissues. Insulin binds its receptor on the plasma membrane of target tissues, activating the PI3K signaling pathway, which increases glucose uptake, storage, and utilization (Leto and Saltiel, 2012).

The insulin signaling pathway is highly conserved between mammals and Drosophila (Bohni et al., 1999; Edgar, 2006). In Drosophila, eight insulin-like peptides (dILPs 1–8), homologous to mammalian insulin, bind a single receptor, activating the canonical PI3K pathway (Brogiole et al., 2001; Gronke et al., 2010; Colombani et al., 2012; Garelli et al., 2012). In the fruit fly the insulin signaling pathway not only regulates metabolism through a unique insulin receptor, but also controls tissue growth in a similar way as insulin-like growth factors (IGFs) and IGF receptors do in mammals (Edgar, 2006). For this reason, the insulin cascade is known as the insulin/insulin-like signaling (IIS) pathway. dILP and IIS signaling is central for coupling growth and organismic adaptation. Insulin plays a major role in organism and developmental programs with the nutritional status of the organism (Edgar, 2006).

In recent years, significant information has been gained regarding the control of dILP transcription and release (Ikeya et al., 2001; Pan, 2010; Colombani et al., 2012; Garelli et al., 2012). This is achieved through paracrine and endocrine signals that act at the cellular level to activate signaling pathways that mediate cell and organism adaptation. Central among these pathways is the insulin signaling system (Pan, 2010). In mammals, glucose levels and metabolism are fine-tuned by insulin, which is secreted by pancreatic β-cells and delivered through the bloodstream to target tissues. Insulin binds its receptor on the plasma membrane of target tissues, activating the PI3K signaling pathway, which increases glucose uptake, storage, and utilization (Leto and Saltiel, 2012).

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In the present study we use the Drosophila system to explore the relationship between dIDE and the IIS pathway. Through a genetic approach, we analyze the ability of dIDE to regulate Drosophila insulin-like peptide 2 (dILP2) levels and thereby modulate metabolism and growth in a normal or a high-sugar diet. We propose that dIDE is a negative regulator of the PI3K pathway and that dIDE loss of function contributes to insulin resistance.

**RESULTS**

**dIDE is expressed ubiquitously**

To begin investigating the function of dIDE, we analyzed the temporal and spatial expression pattern of its transcript. We extracted total RNA at different stages of the fruit fly life cycle and analyzed dIDE transcript levels by real-time PCR. We observed that dIDE mRNA levels are maximal in adult males, although dIDE expression can be detected throughout the life cycle (Figure 1A). Next we compared the expression of the transcript in different larval tissues, finding that the highest expression occurs in the fat body, with significant expression in other tissues as well (Figure 1B). In publicly available databases on high-throughput transcriptomic analyses, those variations of dIDE expression in the life cycle and between organs have not been observed (Graveley et al., 2011; Marygold et al., 2013). To complete the analysis of dIDE transcriptional profile, we isolated a genomic region encompassing 2.9 kb upstream of the dIDE translation initiation site, including the dIDE putative promoter, and generated a dide–green fluorescent protein (GFP) reporter line. As depicted in Figure 1, C–J, the reporter was strongly expressed in salivary glands (Figure 1C), fat body (Figure 1D), gut (Figure 1E–G), imaginal disc (Figure 1H), tracheae (Figure 1I), and brain (Figure 1J). Taken together, these results suggest that dIDE is expressed in all Drosophila organs and cell types throughout the life cycle.

**dIDE can modulate dILP levels**

Because insulin is the most prominent substrate of mammalian IDE, we sought to analyze whether dIDE is capable of cleaving dILPs. Available anti-dILP antibodies are not sensitive enough to detect endogenous dILPs in Western blots, but one antibody can recognize overexpressed levels of this peptide (Figure 2A). We therefore performed an experiment in which dIDE and dILP2 were coexpressed ubiquitously and analyzed the levels of dILP2. dIDE was expressed throughout development in a UAS-dIDE transgenic line controlled by an actin-Gal4 driver in combination with the expression of dILP2 under direct control of a heat shock promoter. dILP2 was induced by exposing third-instar larvae to a 15-min heat shock, 4 h after which, whole-body homogenates were prepared for Western blot analysis. As shown in Figure 2, A and B, dILP2 levels were significantly reduced in larvae that overexpressed dIDE. These results suggest that the metalloprotease dIDE can cleave dILP2.

**FIGURE 1**: dIDE gene expression pattern. (A) Temporal variation of the expression of dide throughout the life cycle, as determined by qRT-PCR. Error bars represent SD (three independent experiments). (B) Expression of dide in third-instar larval organs. mRNA levels were determined by qRT-PCR. Error bars represent SD (three independent experiments). (C–J) Expression of dide in third-instar larval organs, as revealed by expression of a nuclear GFP reporter under the control of the dide promoter; representative organs. (C) Salivary glands, (D) fat body, (E) anterior gut and proventriculus, (F) midgut, (G) hindgut, (H) wing imaginal disc, (I) tracheae, and (J) brain. Bars, 50 μm.

Geminard et al., 2009; Gronke et al., 2010), but little is known about how dILP activity is terminated. This is an important aspect of IIS pathway regulation since, to attain a correct physiological outcome, not only is it important to have tight regulation of the initiation of the response, but it is also critical to shut down the cascade when it is no longer needed (Duckworth et al., 1998). Evidence for insulin degradation as a plausible mechanism for IIS down-regulation emerged with the description of the insulin-degrading enzyme (IDE), a zinc metalloprotease with the capacity to cleave a wide array of small peptides, with insulin being the substrate with highest affinity (Duckworth et al., 1998). Because insulin is the most prominent substrate of mammalian IDE, we sought to analyze whether dIDE is capable of cleaving dILPs. Available anti-dILP antibodies are not sensitive enough to detect endogenous dILPs in Western blots, but one antibody can recognize overexpressed levels of this peptide (Figure 2A).
dIDE provokes growth reduction in a cell-autonomous manner

Given that dIDE is capable of reducing dILP2 levels, we sought to determine whether expression of this metalloprotease affects growth. Initially, we expressed dIDE ubiquitously through an actin-Gal4 driver and observed 12.3% reduction of pupal volume in comparison to control individuals (Figure 3A). One possibility is that dIDE cleaves dILPs in the hemolymph, thereby limiting their access to target tissues. If this were the case, the effect of dIDE manipulation would be systemic rather than cell autonomous. We investigated this issue in wing discs. The adult wing is a bilayer of cells, so growth defects in the wing disc dorsal compartment give rise to a smaller dorsal wing layer that ultimately generates wings that are curved upward (Raisin et al., 2003). This was exactly what we observed after expressing dIDE in this disc territory: the wings were curved upward (Figure 3, B and C), indicating that dIDE can restrict growth in a cell-autonomous manner. To further analyze and quantify this effect, we expressed dIDE at the wing disc posterior compartment by using an engrailed-Gal4 driver (en-Gal4); we observed 19.1% reduction of region D of the wing at the posterior compartment (Birdsall et al., 2000), suggesting again that the effect of dIDE is cell autonomous (Figure 3, D–F). Because insulin signaling is known to regulate organ growth by controlling both cell number and cell size, we quantified the contribution to growth reduction of each of these two parameters. To this end, we determined cell density at the posterior compartment of the wing by counting the hairs in a fixed area. The quantification revealed that IDE overexpression increases cell density by 11.2%, and therefore the cells are on average 11.2% smaller than those of control wings (Figure 3G). Because cell size reduction accounts only partially for posterior compartment reduction (19.1%), we inferred that the latter originates from a combination of reduced cell size and reduced cell number. These conclusions were further confirmed by counting directly the cells after overexpression of dIDE under control of a patched-Gal4 driver in region C of the wing (Figure 3D). Also in this case, reduction of the area occurred specifically in the wing territory that overexpressed the enzyme, and this reduction resulted from a combination of decreased cell size and decreased cell number (Supplemental Figure S1).

dIDE-dependent growth reduction is mediated by the PI3K pathway in a cell-autonomous manner

Given that dILPs activate the PI3K pathway upon binding the insulin receptor (InR), we sought to investigate whether regulation of this pathway mediates the effect of dIDE. *Drosophila* phosphatase and tensin homologue on chromosome 10 (PTEN) is a phosphatidylinositol (3,4,5)-triphosphate phosphatase that negatively regulates PI3K signaling downstream of the InR (Goberdhan et al., 1999; Huang et al., 1999), so it is expected that PTEN reduction of function will antagonize the effect of dIDE. Indeed, as depicted in Figure 4, A–C and E, loss of one dose of PTEN largely suppresses dIDE-dependent growth impairment, supporting the notion that dIDEs exerts its effect by modulating PI3K pathway activation.

The PI3K pathway signals in part through *Drosophila* forkhead box, subgroup O transcription factor (FOXO). Activation of the cascade leads to phosphorylation and nuclear exclusion of FOXO, resulting in reduced FOXO-dependent transcription (Junger et al., 2003; Puig et al., 2003). To investigate whether dILP-dependent reduction of growth is mediated in part by FOXO,
To confirm that dIDE can increase FOXO-dependent transcription, we examined the induction of the FOXO target gene thor (Teleman et al., 2005) by using a thor-LacZ enhancer trap. As depicted in Figure 4, G and H, β-galactosidase expression was detected only at the posterior wing disc compartment of en-Gal4/UAS-dIDE individuals, confirming that dIDE indeed induces FOXO-dependent transcription and that the effect of dIDE is cell autonomous. Consistent with the experiments described earlier, expression of thor-LacZ was largely suppressed in larvae that were heterozygous for FOXO (Figure 4, I and J). Taken together, these results suggest that dIDE-dependent growth reduction is mediated by a cell-autonomous mechanism that involves the negative regulation of the PI3K pathway and consequent activation of the transcription factor FOXO.

dIDE accumulates in the cytoplasm and plasma membrane of the cells in which it is expressed

The cell-autonomous effect of dIDE suggested that the enzyme is not secreted freely to the hemolymph but instead exerts its effect locally in the cell in which it is expressed. To analyze this, we generated a transgenic line expressing a dIDE:GFP fusion protein, which was initially induced at the wing imaginal disc under control of a ptc-Gal4 driver. dIDE:GFP did not diffuse away from the row of ptc-positive cells (Figure 5A). Instead, part of the fusion protein accumulated at the plasma membrane, and no protein could be detected at the lumen of the imaginal disc between the columnar epithelium and the peripodial membrane (Figure 5, B and C). The expression of dIDE:GFP in the patched expression pattern provokes reduction of the distance between veins III and IV, which is even more pronounced than the reduction elicited by the expression of the untagged dIDE (Figure 5, D–G). This indicates that dIDE:GFP is biologically active, suggesting that subcellular localization of the GFP fusion protein recapitulates the localization of endogenous dIDE. ptc-Gal4 drives transgene expression also in the salivary gland, so we studied subcellular localization of dIDE:GFP in this organ. In this case too, the fusion protein appeared to be retained in the cytosol and accumulated in the plasma membrane, whereas no protein appeared to be secreted to the extracellular milieu (Supplemental Figure S2, A–F). Remarkably, the size of the salivary gland expressing dIDE:GFP was autonomously reduced (Supplemental Figure S2, G and H), confirming again that the effect of dIDE is cell autonomous. Taken together, the foregoing experiments suggest that dIDE is retained in the cytoplasm and plasma membrane of the cell in which it is expressed.

we took a genetic approach similar to that described for PTEN. We observed that FOXO reduction of function antagonized the effect of dIDE, as the wing curvature observed upon expression of dIDE in the dorsal compartment of the imaginal disc (A, B) is partially suppressed in flies lacking one dose of PTEN (C) or FOXO (D). (E, F) Reduction of the area of the posterior compartment provoked by the expression of dIDE under control of an en-Gal4 driver (assessed by determination of the wing region D) is partially reverted in individuals lacking one dose of PTEN (E) or FOXO (F). These results (A–F) indicate that the PI3K pathway mediates dIDE-dependent growth reduction through the regulation of FOXO. Error bars represent SEM (p < 0.05; one-way analysis of variance (ANOVA) with Tukey post hoc test; means with a letter in common are not significantly different; n ≥ 20 in three independent experiments). (G, H) The FOXO target gene thor is induced at the posterior compartment of the wing imaginal disc upon expression of dIDE in the same compartment, suggesting that dIDE-dependent inactivation of the PI3K pathway and activation of FOXO is cell autonomous. In foxo20 (I) or foxo21 (J) heterozygous mutant larvae, dIDE-dependent thor transcription is reduced, confirming that FOXO mediates thor-LacZ induction in this setting. Bar, 100 μm.
Down-regulation of dIDE provokes reduction of tissue growth and sensitizes developmental timing and metabolism to high-sucrose diets

Next we sought to study the effect of dIDE loss of function on growth and metabolism. We analyzed in detail a dIDE-null mutant line generated by gene targeting (Tsuda et al., 2010) and found that pupal volume of these mutants is significantly reduced in comparison to wild-type siblings (Figure 6A). dIDE RNA interference (RNAi) is efficient in mediating reduction of dIDE transcript levels (Supplemental Figure S3), so it was used to analyze whether the growth reduction observed in the dIDE mutants is cell autonomous. Indeed, this was the case, as expression of the RNAi in the wing disc dorsal compartment provoked a clear reduction of compartment size (Figure 6, B and C, and Supplemental Figure S4), which resulted in adult wings that were curved upward (Figure 6D and E). Similarly, expression of dIDE RNAi at the posterior compartment of the wing disc provoked a clear (13.9%) reduction of the area of the posterior compartment of the wing in comparison to control flies (Figure 6F). Taken together, the results indicate that dIDE loss of function provokes growth reduction in a cell-autonomous manner. As a next step, we investigated whether reduced activation of the PI3K pathway accounts for growth reduction also in this setting. Following an approach similar to the one utilized earlier, we expressed dIDE RNAi in the wing disc posterior compartment of larvae that were heterozygous for PTEN or FOXO mutant alleles. Area reduction of the posterior wing compartment was strongly suppressed in both PTEN (3.2%) and FOXO (3.9%) heterozygotes (Figure 6, F and G), suggesting that reduction of PI3K signaling accounts for growth impairment upon dIDE silencing. To get further evidence that FOXO mediates this growth reduction, we examined whether transcript levels of the FOXO target gene thor increase upon expression of dIDE RNAi. Silencing of dIDE in the fat body provoked strong up-regulation of thor transcription, and this up-regulation was completely suppressed in PTEN-heterozygous larvae (Supplemental Figure S5). Taken together, these results indicate that dIDE silencing provokes activation of the transcription factor FOXO through the inhibition of the PI3K pathway.

Given that dIDE can negatively regulate dILP availability and growth (Figure 2) and that this effect is mediated by the PI3K pathway (Figure 4), the reduction of growth observed upon dIDE knockdown seems paradoxical at first glance. However, it was recently shown that Drosophila larvae reared in a high-sucrose diet, albeit displaying augmented levels of circulating dILPs, exhibit reduced growth and delayed developmental timing (Musselman et al., 2011; Pasco and Leopold, 2012). Physiological characterization of these larvae revealed that growth impairment was due to a phenomenon known as insulin resistance (Musselman et al., 2011; Pasco and Leopold, 2012). We therefore investigated whether dIDE loss of function enhances insulin resistance in Drosophila larvae exposed to a high-sucrose diet.

We raised wild-type individuals and larvae homozygous for the dIDEKO null allele either in regular food (0.2 M sucrose) or in a high-sucrose culture medium (1 M sucrose). As depicted in Figure 6H, under regular feeding conditions, wild-type and IDEKO larvae developed almost at the same rate. As previously reported (Musselman et al., 2011; Pasco and Leopold, 2012), in high-sucrose medium wild-type larvae exhibited a conspicuous developmental delay, pupariating 3 d later than in normal culture medium. Of interest, the delay provoked by the high-sugars diet was enhanced in dIDEKO larvae, which pupariated >1 d later than wild-type individuals (Figure 6H). Similar results were obtained when reduction of dIDE levels were attained by ubiquitous expression of dIDE RNAi (Supplemental Figure S6).
In mammals, insulin resistance provokes hyperglycemia in fasting conditions, derived in part from reduced capacity of the tissues to incorporate glucose, resulting in higher-than-normal circulating sugar levels. To test whether dIDE individuals display this metabolic phenotype, we subjected wild-type and dIDE adult flies to 24-h starvation, after which we measured circulating levels of the disaccharide trehalose, the main circulating sugar in insects. As depicted in Figure 6I, in wild-type flies trehalose levels in hemolymph dropped significantly after 24-h starvation; in dIDE individuals subjected to starvation, trehalose levels remained similar to those of well-fed animals. Error bars represent SEM (ns, non significant; *p < 0.05; two-way ANOVA with Student's t test; n = 60 in six independent experiments). (J) dIDE flies display augmented levels of the acetyl CoA carboxylase (ACC) transcript, used as an indicator of TAG metabolism. In high-sugar medium, ACC expression increased significantly in wild-type larvae, but ACC transcript levels in dIDE individuals were even higher. Error bars represent SEM (**p < 0.001; two-way ANOVA with Tukey post hoc test; n = 3).

**DISCUSSION**

In this work we showed that dIDE has the capacity to modulate dILP2 levels, regulate the PI3K pathway, and affect growth. The growth phenotypes observed upon dIDE manipulation depend at least in part on the transcription factor FOXO, one of the main effectors of the PI3K pathway. Because dIDE-null mutants are homozygous viable to adulthood, dIDE is probably not a core regulator of the PI3K pathway but instead a modulator of this cascade that contributes to fine-tuning fly growth and metabolism. We showed that both dIDE gain and loss of function bring about diminished growth, results that, at first glance, seem paradoxical. Nevertheless, diminished signaling in conditions of increased insulin has been reported.
and is known as insulin resistance, a hallmark of human diabetes mellitus type II (DMII; Zimmet et al., 2001). In a DMII Drosophila model, larvae fed with a high-sugar diet display higher-than-normal levels of circulating dILP2, along with reduced PI3K signaling in target tissues (Musselman et al., 2011; Pasco and Leopold, 2012).

The mechanisms of the genesis and progression of DMII are unclear. Although obesity, high-sugar diets, and fat-rich diets are well-established predisposition factors for the disease (Zimmet et al., 2001; Venables and Jeukendrup, 2009), genetic risk factors have also been proposed (Stumvoll et al., 2008; Riddrasterle and Groop, 2009). It is noteworthy that association between IDE loss-of-function and DMII has been reported, as human populations with mutations in the IDE locus display increased incidence of this disease (Karamohamed et al., 2003; Rudovich et al., 2009). In keeping with this observation, the Gk rat, an animal model that recapitulates most of the features of human DMII (Goto et al., 1976), was found to carry loss-of-function mutations in the IDE locus (Fakhrai-Rad et al., 2000; Farris et al., 2004). Experimental evidence for a role of IDE in the etiology of DMII came shortly afterward with the generation of IDE-knockout mice, which display hyperinsulinemia and glucose intolerance (Farris et al., 2003; Abdul-Hay et al., 2011). Even though the mechanisms linking IDE to the genesis of DMII are elusive, a simple explanation would be that reduced degradation of insulin leads to increased insulin sensitivity due to increased receptor internalization. In this simplistic model, IDE is predicted to operate in the bloodstream, mediating insulin clearance from circulation. If this model is correct, IDE should be secreted from the cell and mediate insulin degradation extracellularly (i.e., in the bloodstream), behaving in a nonautonomous manner.

Although several studies revealed that IDE can be secreted to the extracellular milieu (Goldfine et al., 1984; Seta and Roth, 1997; Vekrellis et al., 2000; Bulloj et al., 2010), other works reported the occurrence of the enzyme at other locations, such as the plasma membrane, endosomes, peroxisomes, mitochondria, and cytosol (Hamel et al., 1991; Authier et al., 1994; Morita et al., 2000; Leissring et al., 2004). In rat liver parenchymal cells the early endosome is a major site of degradation of insulin, although IDE was found to be cytosolic and thus is apparently not involved in endosomal degradation in this tissue (Pease et al., 1985; Backer et al., 1990; Doherty et al., 1990; Authier et al., 1994).

In the present study, we found that IDE is in part associated with the plasma membrane, without any evidence of its being secreted. We provided clear evidence that its effect on growth regulation is cell autonomous, and so it is its capacity to modulate activation of the PI3K pathway. Thus, based on our genetic evidence, an attractive possibility is that dIDE is associated to plasma membrane domains that are in close proximity to insulin receptors, and so after binding of the ligand, dIDE is endocytosed together with the receptor–ligand complex. Once in early endosomes, dIDE cleaves the ligand, contributing to recycling the receptor to the plasma membrane.

Of interest, it was suggested for other receptor tyrosine kinases that cleavage of the ligand at the early endosome is important for recycling the receptor to the plasma membrane (Sorkin and Von Zastrow, 2002). So, if Drosophila IDE does indeed exert its function at early endosomes, knockdown of dIDE is expected to cause reduction of dILP proteolysis in this compartment, leading to impairment of recycling of insulin receptors to the membrane, ultimately provoking insulin resistance. In this scenario, overexpression of dIDE may increase dILP proteolysis at the plasma membrane before ligand binding to the receptor, thereby reducing PI3K signaling. Detailed biochemical and cellular studies are required to define the precise subcellular location of dIDE and particularly the functional relevance of its possible role in early endosomes.

**MATERIALS AND METHODS**

**Fly stocks**

For the generation of UAS-dIDE lines we amplified the dide open reading frame by PCR from the EST RE1745B (DGRC). For generation of the dide nGFP expression reporter we cloned a 2-kb fragment upstream of the translation initiation site with the primers 5′-GGG-TACCCCTTGGTTTATAAAGGCTGACATC-3′ and 5′-GAAAGAT-CTTCAATACATGGCAATCGTGTTCCCAC-3′, which include sites for KpnI and BgII, in a pStinger (Barolo et al., 2000) nGFP vector. For the UAS-dIDE:GFP transgenic line we cloned the dide gene by removing the stop codon by PCR using the primers 5′-GACGAATTCATG-TACTCTACGTGCAGAAAATCG-3′ and 5′-CCGGGTACCGTAAAGCT- TGCTCGTGTGCTCCCTCG-3′ (including sites for EcoRI and KpnI restriction enzymes, respectively) into a pEGFP-N1 plasmid and then subcloned the fusion protein construct into a pUAST-attB plasmid (Bischof et al., 2007). Transgenic lines were generated by embryonic germ-line transformation (Spradling and Rubin, 1982).

We used the following stocks from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN): En-Gal4 (#6356), MS1096 (#8698), Ap-Gal4 (#3041), Act-Gal4 (#3954, #4414), ptc-Gal4 (#2017), and thor′ (#9558). The following stocks were from the Vienna Drosophila RNAi Center: UAS-dIDERNAi (#15957 and #15958) and UAS-Dcr-2 (#60009). The following stocks were obtained from different colleagues: PTEN117 (Oldham et al., 2000), FOXO31 and FOXO5 (Junger et al., 2003), hs-dilp2 (Rullifson et al., 2002), and IDEKO (Tsuda et al., 2010). Controls for the experiments with IDEKO mutants were performed in the y,w background line.

**Anti-dILP2 Western blots**

First-instar larvae overexpressing or not dILP2 were sorted and placed in groups of 60 individuals/vial. First, larvae were exposed to a 15-min heat shock at 37°C and then placed at 25°C for 4 h. Larvae were then recovered, rinsed in phosphate-buffered saline (PBS), and homogenized in lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 1% NP-40, Sigma Protease Inhibitor Cocktail). After centrifugation, samples were loaded in 10% SDS–PAGE gels, run, and transferred to an Immobilon-P SQ membrane (Millipore, Billerica, MA). Membranes were incubated with 1:1000 anti-dILP2 antibody (gift from Hugo Stocker, ETHZ, Zürich, Switzerland), revealed with Amersham ECL Prime (GE Healthcare, Pittsburgh, PA), and scanned. Bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

**Immunostaining and X-Gal staining**

For immunostaining, larvae were dissected in PBS and fixed in PBS/Triton 0.1% (PBST) and formaldehyde 4% for 20 min at room temperature. Samples were washed with PBST and blocked with PBST bovine serum albumin 5%, followed by incubation with a primary antibody overnight at 4°C, washed, and incubated 4 h at room temperature with secondary antibodies conjugated to fluorophores. Phalloidin (1:200; Sigma, St. Louis, MO) was added during the last 30 min of secondary antibody incubation. Samples were then washed and mounted in PBST glycerol 80%. Antibodies used in this study were anti-GFP (1:1000, A-11122; Invitrogen, Carlsbad, CA) and anti-Wingless (1:1000, 4D4; Developmental Studies Hybridoma Bank, Iowa City, IA). Images were captured in a Carl Zeiss LSM510 Meta Confocal Microscope.

For X-Gal staining, larvae were dissected in PBS and fixed 10 min in a 16.67 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 6.8), 75 mM KCl, 25 mM NaCl,
3.33 mM MgCl₂, 5% glutaraldehyde buffer at room temperature. Samples were then washed with PBST and incubated overnight with the staining solution (5 μM K₃Fe(CN)₆, 5 μM K₄Fe(CN)₆, 0.2% X-Gal) at room temperature. Samples were then washed and mounted for observation and photographed using an Olympus BX60 microscope equipped with an Olympus DP71 digital camera.

Quantitative real-time PCR
The following primers were used for quantitative real-time PCR (qRT-PCR): for dIDE, 5′-CAACCGCCGACAAGACATCC-3′ and 5′-AGATTT-TACCAGATTGATTCC-3′; for dlLP2, 5′-ATCCTGATTCCACAACAAG-3′ and 5′-GCGGCTCTGACGAGT-3′; for ACC, 5′-GCTGGGTATGCCCTGTT-3′ and 5′-GATATGGGTATGCGACCAGAT-3′; and for Rpl29 to normalize the quantifications. qRT-PCR was performed using a Mx3005P QPCR System (Stratagene, La Jolla, CA).

Wing measurements
First-instar larvae of the different genotypes were sorted and placed in groups of 60 individuals/vial and allowed to develop to adulthood. The 2- to 4-d-old adults of each genotype were placed in vials in groups of 60 individuals/vial and allowed to develop to adulthood. The 2- to 4-d-old adults were collected and conserved in 70% ethanol until analysis. The right wings of female individuals were removed and mounted in a lactic acid:ethanol (1:1) mixture. Photographs of the wings were taken with an Olympus MVX10 stereomicroscope with an Olympus DP71 digital camera. Quantification of the areas was performed with ImageJ.

Developmental timing and pupal size
First-instar larvae of the different genotypes were sorted against fluorescent balance chromosomes and placed in groups of 60 individuals/vial and allowed to develop to adulthood. The 2- to 4-d-old adults from each genotype were placed in vials in groups of 60 individuals/vial and allowed to develop to adulthood. Standard and high-sugar culture media were prepared as previously described (Pasco and Leopold, 2012).

Trehalose determinations
First-instar larvae of the different genotypes were sorted against fluorescent balance chromosomes and placed in groups of 60 individuals/vial and allowed to develop to adulthood. The 2- to 4-d-old adults were placed in vials in groups of 60 individuals/vial and allowed to develop to adulthood. The 2- to 4-d-old adults were collected and conserved in 70% ethanol until analysis. The right wings of female individuals were removed and mounted in a lactic acid:ethanol (1:1) mixture. Photographs of the wings were taken with an Olympus MVX10 stereomicroscope with an Olympus DP71 digital camera. Quantification of the areas was performed with ImageJ.

Statistical analysis
Infostat Statistical Software and Excel (Microsoft, New York, NY) were used for statistical analysis where appropriate.

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