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A Fat Body-Derived IGF-like Peptide Regulates Postfeeding Growth in *Drosophila*

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SUMMARY

Members of the insulin family of peptides have conserved roles in the regulation of growth and metabolism in a wide variety of metazoans. Here we show that *Drosophila* insulin-like peptide 6 (DILP6), which is structurally similar to vertebrate insulin-like growth factor (IGF), is predominantly expressed in the fat body, a functional equivalent of the vertebrate liver and adipocytes. This expression occurs during the postfeeding stage under the direct regulation of ecdysteroid. We further reveal that *dilp6* mutants show growth defects during the postfeeding stage, which results in reduced adult body size through a decrease in cell number. This phenotype is rescued by fat body-specific expression of *dilp6*. These data indicate that DILP6 is a functional, as well as a structural, counterpart of vertebrate IGFs. Our data provide in vivo evidence for a role of ILPs in determining adult body size through the regulation of postfeeding growth.

INTRODUCTION

In vertebrates, insulin and insulin-like growth factors (IGFs) both play important roles in the regulation of metabolism, growth, and development, but function in different developmental or physiological contexts (Froesch and Zapf, 1985; Nakae et al., 2001). Insulin-like peptides (ILPs) have also been found in a variety of invertebrates, including insects (Nässel, 2002; Wu and Brown, 2006). Recent studies in the fruit fly (*Drosophila melanogaster*) have demonstrated that highly conserved insulin/IGF signaling (IIS) acts primarily to regulate growth, metabolism, fertility, and longevity (Tatar et al., 2003; Edgar, 2006; Géminard et al., 2006; Toivonen and Partridge, 2008). The *Drosophila* genome encodes seven *Drosophila* ILP (DILP) genes, *dilp1–7* (Brogiolo et al., 2001), and the most prominent *dilp* (*dilp1*, *2*, *3*, and *5*) expression is observed in the brain neurosecretory cells, called the insulin-producing cells (IPCs) (Brogiolo et al., 2001; Rulifson et al., 2002). IPC-derived DILP gene expression and peptide secretion is mainly regulated by nutritional cues associated with feeding (Ikeya et al., 2002; Géminard et al., 2009). Although some ILP genes are expressed outside the brain IPCs (Brogiolo et al., 2001; Riehle et al., 2006), previous studies on the regulation of insect growth through ILPs have focused almost exclusively on the IPC-derived ILPs.

In our recent study in the silkmoth (*Bombyx mori*), we identified a structural and functional counterpart of IGFs, *Bommo*-IGF-like peptide (*Bommo*-IGFLP or BIGFLP), which is predominantly produced in the pupal fat body in response to ecdysteroid and promotes the growth of adult-specific tissues in vitro (Okamoto et al., 2009). However, whether fat body-derived ILPs are widely present in other insects, and whether they indeed regulate growth during postfeeding development in vivo, remains unknown.

Here we report that DILP6 is the *Drosophila* IGFLP, which is expressed in the fat body during the postfeeding stage in response to ecdysteroid. We found that *dilp6* mutants exhibit growth defects during the postfeeding stage, which is rescued by fat body-specific expression of *dilp6* only during postfeeding development. These observations indicate that DILP6 serves as a growth factor to regulate postfeeding growth in *Drosophila*, and suggest that the IGFLPs have a conserved role in various insect orders.

RESULTS

dilp6 Is Predominantly Expressed in the Fat Body during Postfeeding Development

From our previous research (Okamoto et al., 2009), the characteristic feature of IGFLP is defined as its high expression in the fat body during pupa-adult development. Therefore, we investigated the expression patterns of all *dilps* to identify a *Drosophila* IGFLP. Real-time quantitative RT-PCR (qRT-PCR) analysis revealed that, among all *dilps*, only *dilp6* was expressed predominantly during late third instar (L3) and pupa-adult development at remarkably high levels (Figure 1A), suggesting its expression in a large tissue during this period. When the tissue-specific *dilp6* expression pattern was examined, high expression was detected in the fat body at 0 hr after puparium formation (APF) (Figure 1B), which was also confirmed by in situ hybridization (Figures 1C–1F). There was no detectable *dilp6* expression in

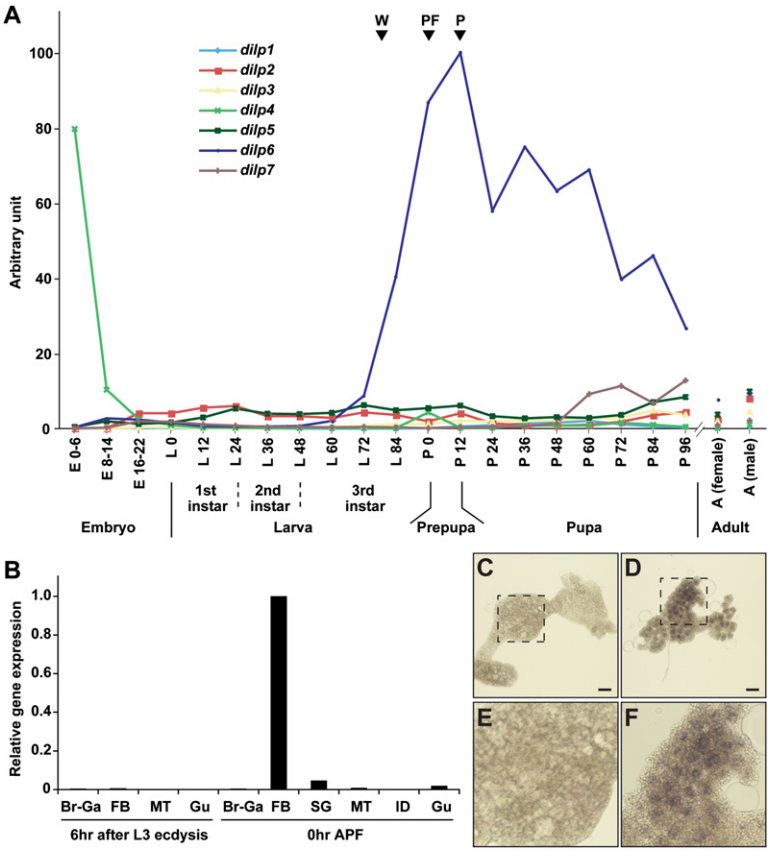

Figure 1. Expression Patterns of *dilp6*

(A) The whole-body transcript levels of seven *dilps* were examined by qRT-PCR. A, 24 hr after eclosion; En, n hr after egg laying; Ln, n hr after hatching; P, pupation; PF, puparium formation; Pn, n hr after puparium formation; W, beginning of wandering.

(B) Relative levels of *dilp6* transcript in various tissues at 6 hr after L3 ecdysis or 0 hr APF, as assessed by qRT-PCR. APF, after puparium formation; Br-Ga, brain-ventral ganglia complex; FB, fat body; Gu, gut; ID, imaginal disks; MT, malpighian tubule; SG, salivary gland.

(C–F) In situ hybridization of *dilp6*. Sense probe (C) or antisense probe (D) hybridization to 0 hr APF fat body. The boxed areas of C and D are magnified in E and F, respectively. Scale bars, 100 μm.

dilp6 Expression in the Fat Body Is Directly Induced by Ecdysteroid

The temporal expression pattern of *dilp6* during late L3 and pupa-adult development suggests that *dilp6* expression is regulated by ecdysteroid. Indeed, we found that the developmental profile of *dilp6* expression level tightly paralleled that of ecdysteroid titer (Figure 2A). Therefore, we tested the in vitro effect of 20-hydroxyecdysone (20E) on the expression of *dilp6* in the fat body of L3 larvae 30 hr after ecdysis, when *dilp6* expression was still at a low level. The expression of *dilp6* was induced by 20E in the fat body culture and continued to increase throughout the incubation period (Figure 2B). Although 20E regulates gene expression through binding to a nuclear receptor—the ecdysone receptor (EcR) that directly binds to specific DNA sequences—current knowledge about functional EcR-binding sites is incomplete, and it is still difficult to predict direct target genes of 20E from a genome sequence. Therefore, in order to

the brain IPCs (see Figure S1 available online). These results indicate that DILP6 is predominantly produced by the fat body during wandering and pupa-adult development, the postfeeding growth periods.

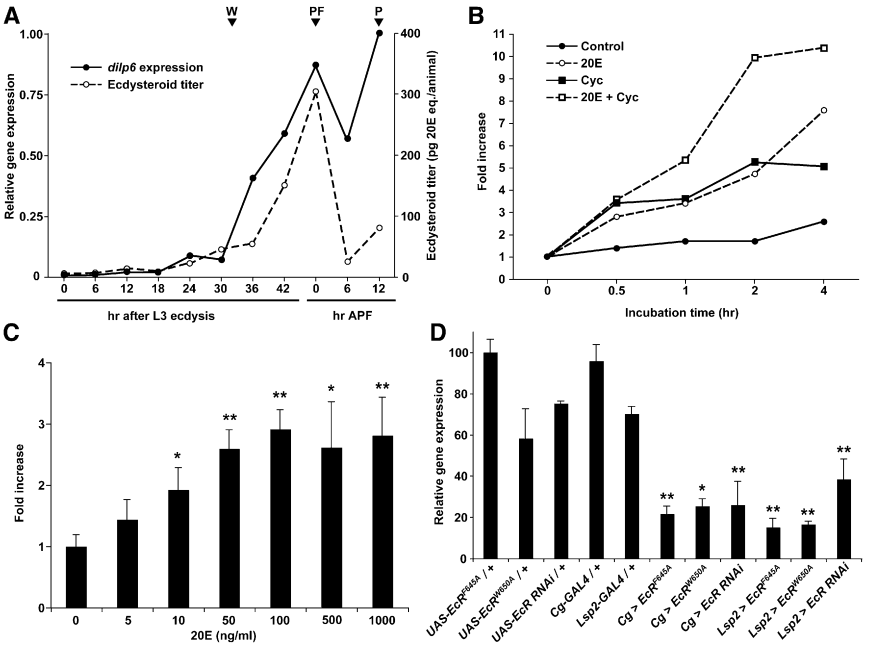

Figure 2. Direct Induction of *dilp6* Expression in the Fat Body by Ecdysteroid

(A) Developmental changes in *dilp6* expression level and ecdysteroid titer.

(B) In vitro induction of *dilp6* expression in the fat body by ecdysteroid. Fat bodies were cultured either with 20E alone (100 ng/ml), cycloheximide (Cyc) alone (25 μg/ml), or 20E plus cycloheximide (20E + Cyc) for 0–4 hr.

(C) Dose-dependent induction of *dilp6* expression by 20E. Fat bodies were cultured with various concentrations of 20E for 4 hr.

(D) Effects of dominant-negative EcR variants (EcR^{F645A} or EcR^{W650A}) or an EcR RNAi construct expression on the *dilp6* transcript level in the fat body at 0 hr APF. *Cg-* or *Lsp2-GAL4* was used as fat body-specific drivers. In all experiments, *dilp6* transcript levels were assessed by qRT-PCR. All values are means and SD (n = 3). Student's t test; *p < 0.05, **p < 0.01.

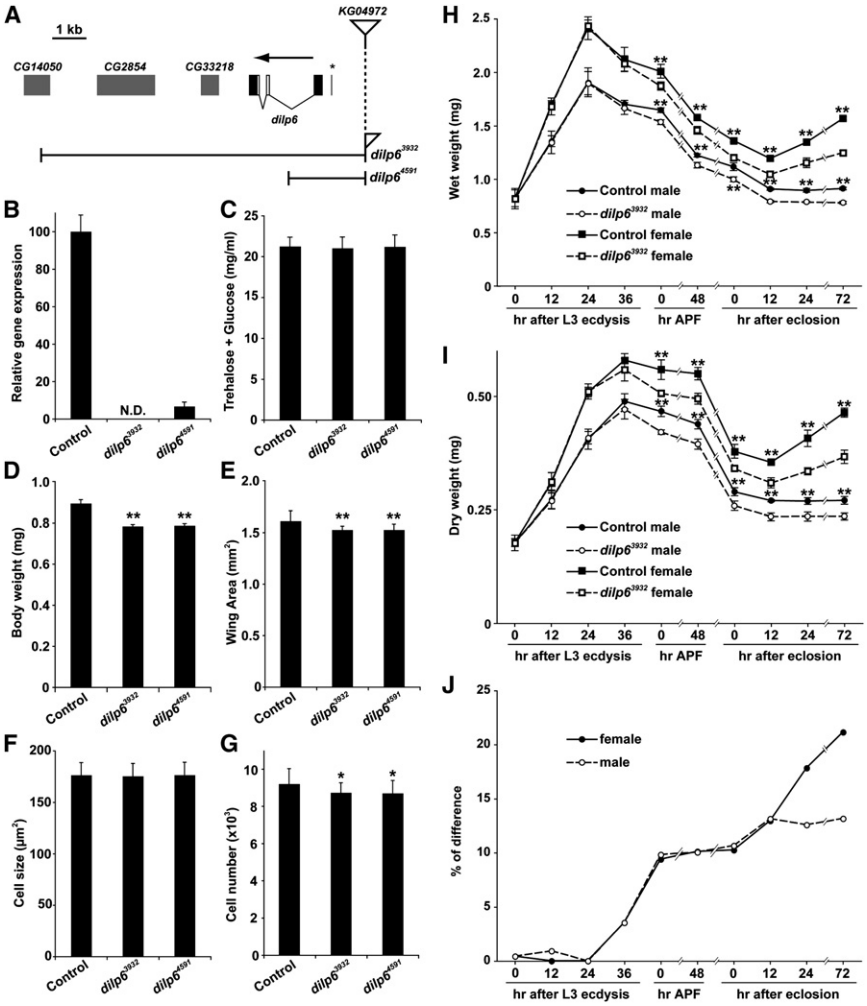

Figure 3. *dilp6* Mutant Phenotypes

(A) Schematic representation of the *dilp6* locus and molecular nature of the mutations. The gene structure of *dilp6* is shown, with protein coding regions represented by open boxes and untranslated regions by filled boxes. An arrow indicates the orientation of the gene. Three 3′ flanking genes are depicted in gray boxes, along with a short putative exon of a 5′ flanking gene, *phl*, marked with an asterisk. The site of *P* element insertion (KG04972) is marked with an inverted triangle. A part of the *P* element is still present in $dilp6^{3932}$ (open triangle).

(B) Relative *dilp6* expression levels in the mutants at 0 hr APF, as assessed by qRT-PCR. N.D., not detected.

(C) Hemolymph sugar (glucose + trehalose) concentrations of control, $dilp6^{3932}$, and $dilp6^{4591}$ male wandering larvae 36 hr after L3 ecdysis. Hemolymph was collected from batches of 15 larvae.

(D) Body weight of control, $dilp6^{3932}$, and $dilp6^{4591}$ male flies. Flies were weighed in batches of 10–30, and the average weight per fly was calculated.

(E–G) Wing area (E), cell size (F), and cell number (G) of control, $dilp6^{3932}$, and $dilp6^{4591}$ male flies.

(H and I) Developmental changes in the wet weight (H) and dry weight (I) of control and $dilp6^{3932}$ animals. Animals were weighed in batches of 10–50, and the average weight per animal was calculated.

(J) Developmental changes in the percentage of difference in dry weight between control and $dilp6^{3932}$ animals, calculated from (I).

All values are the means and SD (n = 3 batches [B], 4 batches [C, D, H, I], or 20 wings [E–G]; Student's t test; *p < 0.05, **p < 0.01).

investigate whether *dilp6* expression is directly induced by 20E, we performed the same assay in the presence of the protein synthesis inhibitor, cycloheximide, which should eliminate secondary effects by any transcription factors the expression of which is induced by 20E (Ashburner, 1974; Beckstead et al., 2005). The expression of *dilp6* was induced by 20E even in the presence of cycloheximide, indicating that the *dilp6* expression is directly induced by 20E. The levels of *dilp6* transcript in the presence of cycloheximide were higher than the levels of the control, probably due to the lack of putative repressors (Ashburner, 1974; Beckstead et al., 2005). The effect of 20E was dose dependent, with as low as 10 ng/ml still being effective (Figure 2C).

We further investigated the effects of fat body-specific loss of function of EcR on *dilp6* expression in vivo. Expressing dominant-negative forms of EcR (Cherbas et al., 2003) or *EcR RNAi* using two different fat body drivers significantly lowered the *dilp6* expression level (Figure 2D). Overall, these results suggest that the *dilp6* expression in the fat body is directly regulated by ecdysteroid.

dilp6 Mutants Show Reduced Adult Body Size through a Decrease in Cell Number

To investigate the loss-of-function phenotypes, we generated *dilp6* mutants by imprecise excision of a *P* element insertion. We obtained four deletion mutations, which, when homozygous, produce viable and fertile adult progeny. Two deletions ($dilp6^{3932}$ and $dilp6^{4591}$; Figure 3A) were selected for further characterization, and a precise excision line was used as a genotypically matched control. $dilp6^{3932}$ is a null allele with 9.5 kb deletion downstream of the insertion site that removes the entire *dilp6* locus along with adjacent genes. $dilp6^{4591}$ is a strong hypomorphic allele, with 2.3 kb deletion downstream of the insertion site that removes the entire *dilp6* 5′ untranslated region. qRT-PCR showed that, in $dilp6^{4591}$ homozygotes, *dilp6* mRNA level at 0 hr APF is decreased to approximately 7% of the control (Figure 3B).

Although there was no detectable difference in hemolymph sugar levels (Figure 3C), the homozygous mutant adults showed a reduction in body size (Figures 3D–3G; Figure S2). Compared with control flies, $dilp6^{3932}$ and $dilp6^{4591}$ homozygous mutant males showed approximately 12%–13% reduction in body weight and 5%–6% reduction in wing area. We also analyzed the wing hair density. This analysis demonstrated that there is no reduction in cell size, but, instead, a decrease in cell number (Figures 3F and 3G), which likely accounts for the reduction in body weight. Similar results were obtained with female flies (Figures S2 and S3).

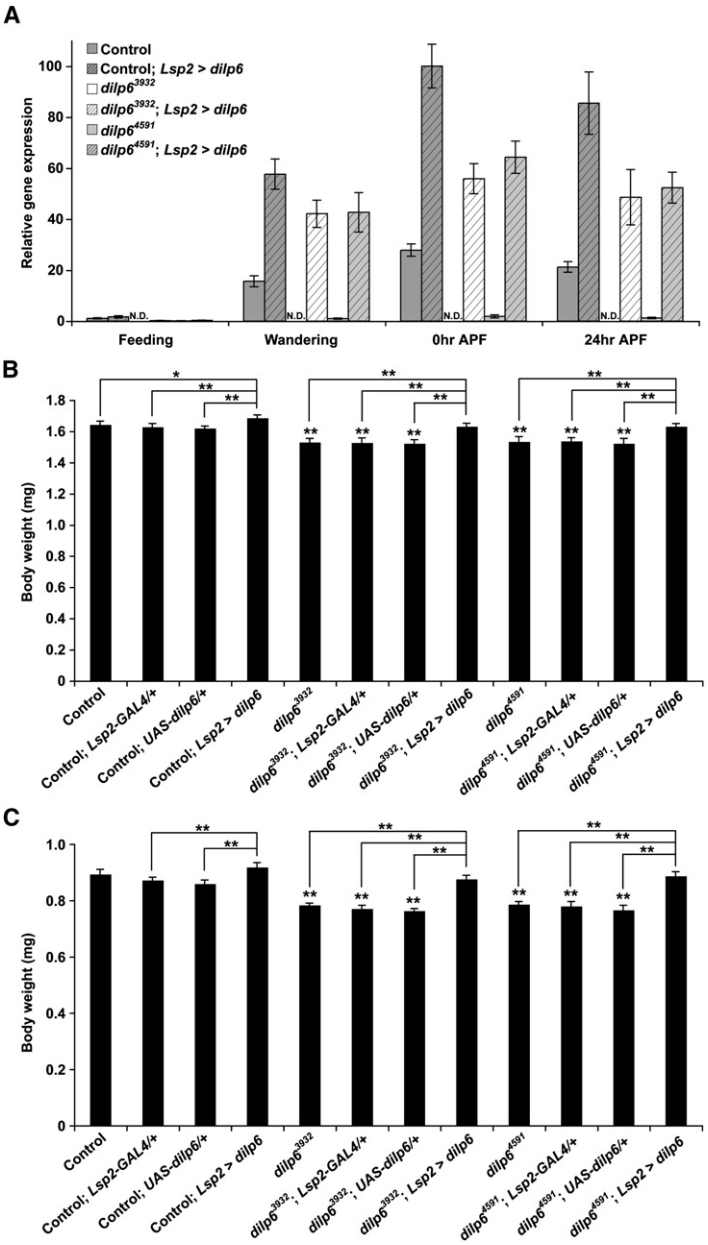

Figure 4. Rescue of the *dilp6* Mutant Phenotype by Fat Body-Specific Expression of *dilp6* during the Postfeeding Period

(A) Restored *dilp6* expression in the rescue crosses, as assessed by qRT-PCR. Feeding, 6 hr after L3 ecdysis; wandering, 36 hr after L3 ecdysis (all values are means ± SD; n = 3 batches). N.D., not detected. (B and C) The effects of fat body-specific expression of *dilp6* on body weight at 0 hr APF (B) and 24 hr after eclosion (C) in control, $dilp6^{3932}$, and $dilp6^{4591}$ male flies. Animals were weighed in batches of 10–30, and the average weight per animal was calculated. Student's t test; *p < 0.05, **p < 0.01. *Lsp2-GAL4* was used to drive *dilp6* expression in the fat body during the postfeeding period (all values are means ± SD; n = 4 batches).

dilp6 Mutants Show a Growth Defect during the Postfeeding Period

Based on the temporal expression pattern and the mutant phenotype of *dilp6*, we hypothesized that *dilp6* serves as a growth factor that regulates postfeeding growth. To determine when this size difference becomes apparent, we measured the changes in body weight from L3 ecdysis to 72 hr after eclosion. We found that both control and $dilp6^{3932}$ animals gain body weight at approximately the same rate during the feeding period (Figures 3H and 3I). Although no significant difference between the control and $dilp6^{3932}$ in the timing of puparium formation was observed, body weight at 0 hr APF of the homozygous mutant was reduced by approximately 6%–7% (wet) and 9%–10% (dry) in both sexes (Figures 3H–3J). This result indicates a role for *dilp6* during the wandering stage, when larvae never feed, in regulating utilization of stored nutrients accumulated during the feeding period. Moreover, the weight difference between the control and the *dilp6* mutant further increased to approximately 12%–13% in wet weight and 13%–14% in dry weight 12 hr after eclosion. Once again, this is likely caused by a defect in the ability of *dilp6* mutants to utilize stored nutrition efficiently during pupa-adult development, and this inefficiency becomes evident after eclosion when the meconium is excreted. In female flies, the weight difference further increased 24 hr after eclosion, probably due to the reduced ovary volume in the *dilp6* mutant. It should also be noted that the percentage of water in the animals showed no significant difference between the control and $dilp6^{3932}$ throughout development (Figure S4). Overall, these results suggest that *dilp6* is required for postfeeding growth regulation.

The *dilp6* Mutant Phenotype Is Rescued by Fat Body-Specific Expression of *dilp6* during the Postfeeding Period

To further confirm that the lack of *dilp6* is indeed responsible for the mutant phenotype, we next examined whether fat body-specific expression of *dilp6* during the postfeeding period could rescue the phenotype of the *dilp6* mutants. For this purpose, we used the *GAL4/UAS* system with the fat body-specific *Lsp2-GAL4* as a driver. *Larval serum protein2 (Lsp2)* is an ecdysteroid-inducible gene predominantly expressed in the fat body during late L3 and pupa-adult development like *dilp6* (Lepesant et al., 1986), and *Lsp2-GAL4* reproduces this expression pattern (Figure S5).

Expression of *dilp6* under the control of *Lsp2-GAL4* driver completely restored the *dilp6* transcript level in the *dilp6* mutant backgrounds during the postfeeding period (Figure 4A). In accordance with the recovery of *dilp6* transcript level, body weights of the mutant males at 0 hr APF and 24 hr after eclosion were substantially rescued (Figures 4B and 4C; Table S1). Similar results were obtained with female flies (Figure S6 and Table S1). Furthermore, when *dilp6* was overexpressed using the same driver, body weight was increased compared with control animals, showing that the effect of *dilp6* on body weight is proportional to its transcript level (Figures 4A–4C; Figure S6 and Table S1). Taken together, the above results demonstrate that *dilp6* serves as a growth factor to regulate postfeeding growth in *Drosophila*.

DISCUSSION

In the present study, we demonstrated that DILP6, one of seven ILPs in *Drosophila*, is produced primarily in the fat body to regulate postfeeding growth without affecting the timing of metamorphosis. This observation is interesting to consider in light of previous findings that suggest that IIS affects both the timing of metamorphosis and the rate of growth (Shingleton et al., 2005; Edgar, 2006; Mirth and Riddiford, 2007). Our results thus clearly demonstrate that different ILPs have distinct temporal roles during development. Similar results are also presented by Slaidina et al. (2009).

Insects utilize larval accumulated nutrients for the development of adult-specific tissues during the postfeeding period. How DILP6 mediates this tissue-specific growth remains unknown, but previous reports indicate interplays between 20E and IIS involved in this process. In the fat body, 20E antagonizes IIS (Rusten et al., 2004; Colombani et al., 2005), which probably blocks an autocrine effect of DILP6. On the other hand, 20E synergistically enhances IIS in the imaginal disks to promote growth (Nijhout et al., 2007). It is also interesting to note that the downregulation of IIS by 20E in the fat body activates autophagy, which promotes the release of stored nutrients (Rusten et al., 2004). We suggest that these tissue-specific effects of 20E on IIS facilitate the directional transfer of nutrients from storage organs (fat body) to developing disks to promote adult-specific tissue growth. DILP6 appears to play a pivotal role in this process, and its loss leads to enhanced excretion of unused materials during wandering and after eclosion.

The independent role of DILP6 compared to IPC-derived DILPs is reminiscent of the roles of IGFs compared to insulin in mammals. There are three major aspects of their similarities. First, we showed that *dilp6* is predominantly expressed in the fat body, a functional equivalent of the mammalian liver and adipose tissue, and the liver is the principal source of circulating IGFs in mammals (LeRoith, 1997). Second, our data revealed that the expression of *dilp6* is directly regulated by the steroid hormone, 20E, when growth is independent of extrinsic nutritional input. Although the expression of IGFs can be regulated by nutrition (Thissen et al., 1994), high concentrations of IGF-I and -II are observed during pubertal and fetal development, respectively, reflecting their importance in these key developmental transitions in mammals (Daughaday and Rotwein, 1989). Moreover, *igf-I* expression in several organs is induced by sex steroids (LeRoith, 1997), further supporting the analogy between DILP6 and IGFs. Third, the predicted peptide structure of DILP6 is distinct from other DILPs in that it has a short C peptide, which is more similar to vertebrate IGFs than to insulin (Brogiolo et al., 2001; Riehle et al., 2006). Moreover, the short C peptide is likely to remain in the mature form like IGFs, because of the lack of a cleavage site (Brogiolo et al., 2001). Thus, the structural aspect also favors the analogy between DILP6 and IGFs. From all these similarities between DILP6 and IGFs, we propose that DILP6 is a functional as well as a structural counterpart of vertebrate IGFs, and therefore we define DILP6 as a *Drosophila* IGFLP. It should be noted here that, in parallel with the analogy between DILP6 and IGFs, there are several analogies between IPC-derived DILPs and insulin in terms of the source tissues and the nutritional regulation of the expression and peptide secretion (Wang et al., 2007; Ikeya et al., 2002; Géminard et al., 2009).

Together with our previous characterization of IGFLP in *Bombyx* (Okamoto et al., 2009), it is highly likely that IGFLP is widely present in divergent insect orders. Surprisingly, however, phylogenetic analysis supports no orthology between BIGFLP and DILP6 (Figure S7), suggesting that BIGFLP and DILP6 have evolved independently. We hypothesize that, in ancestral insect species, there was a single ILP that was expressed both in the brain IPCs and in the fat body. This ancestral ILP was probably under distinct regulatory mechanisms (nutritional and developmental) in these tissues, which facilitated functional diversification of IPC-derived ILPs and fat body-derived ILPs after a gene duplication event(s) that happened independently in each insect order. In our previous study in *Bombyx*, we demonstrated that BIGFLP is released as a single-chain polypeptide, despite having two potential cleavage sites within the C domain (Okamoto et al., 2009). This suggests the lack of processing enzymes to generate mature insulin in the fat body, which probably explains why fat body-derived ILPs in different species have attained similar structural features as IGFLPs (shortened C-peptide and/or the loss of cleavage sites) despite their independent lineages. Studies in orthopteran species (which are considered closer to earlier insect species), where there is only one identified ILP the expression of which is differentially regulated in the brain IPCs and in the fat body (Kromer-Metzger and Lagueux, 1994; Badisco et al., 2008), support our hypothesis.

Since most insect genomes contain a single insulin/IGF-like receptor gene, IGFLPs and the other ILPs presumably activate the same receptor, although its binding affinities for different ligands likely vary according to the distinct structural features of the ligands. In contrast, mammalian genomes contain multiple receptors, each of which responds to one primary ligand. Therefore, there also appears to exist a clear difference between mammalian IGFs and insect IGFLPs. Considering the pivotal role of IGFs/IGFLPs during development in both of these animal groups, further investigations of the similarities as well as the differences in these signaling pathways should enrich our understanding of the underlying mechanisms that control development throughout the animal kingdom.

EXPERIMENTAL PROCEDURES

Fly Stocks and Mutants

Unless otherwise indicated, wild-type strain Canton-S was used. A transposable *P* element insertion *KG04972* (Bloomington stock no. 13,536) was used to generate *dilp6* mutants by imprecise excision. The progeny were first screened for the loss of a body color marker (y^+), and the extent of deletion in each mutant was determined by PCR and subsequent DNA sequencing. *UAS-dilp6* (no. 617; a gift from Ernst Hafen) or *Lsp2-GAL4* (a gift from Thomas Neufeld) transgenes were crossed into the control or *dilp6* homozygous mutant ($dilp6^{3932}$ or $dilp6^{4591}$) backgrounds by standard methods. *Cg-Gal4* line was a kind gift from T. Neufeld; $UAS\text{-}EcR^{F645A}$ (no. 6869) and $UAS\text{-}EcR^{W650A}$ (no. 6872) were obtained from Bloomington stock center; *UAS-EcR RNAi* (no. 37,059) was obtained from Vienna *Drosophila* RNAi Center.

Developmental Staging

Before starting timed egg collections, adults were allowed to lay for 1 hr in order to remove held eggs. Egg laying was performed for 6 hr (for collecting embryos) or 2 hr (for collecting larvae). After egg laying, 30–40 eggs were

transferred to fresh standard *Drosophila* culture medium to avoid overcrowding and maintained at 25 ± 1°C under a 12 hr light/dark cycle. Stages were determined by observing spiracles and mouthhook morphology (Ashburner et al., 2005). Newly ecdysed L3 larvae were transferred to medium containing 0.05% bromophenol blue (Wako, Osaka, Japan) to facilitate staging of wandering larvae (Warren et al., 2006). Animals were resynchronized at 0 hr APF, and stages during pupa-adult development were determined by multiple markers (Ashburner et al., 2005). Eclosion was checked at 15 min intervals, and male and female animals were lightly anesthetized with carbon dioxide and separately transferred to the medium supplemented with yeast paste.

qRT-PCR

Total RNA was prepared by using RNeasy mini kit (QIAGEN, Germantown, MD) and RNase-Free DNase Set (QIAGEN), and reverse transcription was performed using PrimeScript RT reagent Kit (TaKaRa Bio, Shiga, Japan). qRT-PCR was performed on ABI PRISM 7500 Real-Time PCR Systems (Applied Biosystems, Foster City, CA) using SYBR Premix Ex TaqII (TaKaRa Bio). For absolute quantification of mRNAs, serial dilutions of plasmids carrying cDNAs were used for standards. After the molar amounts were calculated, transcript levels of the *dilps* were normalized with *rp49* levels in the same samples. The primers used for qRT-PCR are listed in Table S2.

In Situ Hybridization

In situ hybridization was performed as previously described (Yamanaka et al., 2006). Specific primers used for the production of a probe are listed in Table S2. Tissues were observed using a Nikon ECLIPSE E800 microscope (Nikon, Kawasaki, Japan).

Whole-Body Ecdysteroid Titer Determination

Frozen wild-type larvae or pupae (10 animals/tube) were individually homogenized and extracted as previously described (Warren et al., 2006). The extracts were evaporated, redissolved, and subjected to time-resolved fluoroimmunoassay (TR-FIA) for ecdysteroid determination. The TR-FIA was performed in a competitive assay format using anti-20E rabbit antiserum, ovalbumin-conjugated 20E, and 20E (Sigma, St. Louis, MO) as the detection antibody, immobilized antigen, and standard hormone, respectively. The rabbit antibody bound to the well was quantified by DELFIA system (Wallac Oy).

In Vitro Culture of Fat Body

Fat bodies of wild-type female larvae 30 hr after L3 ecdysis were dissected in Schneider's medium (Sigma), rinsed in fresh medium twice, and precultured for 1 hr in the same medium. Preculture medium was replaced with fresh medium with or without 20E (100 ng/ml, except for the dose-response experiment) and/or cycloheximide (25 μg/ml; Sigma). Cultures were maintained at 25 ± 0.5°C under 40% oxygen partial pressure.

Weight Determination

Larvae and pupae were washed with water and carefully blotted. Adult flies were lightly anesthetized with carbon dioxide. Pools of known numbers of animals were weighed (for wet weight), frozen at −80°C, lyophilized overnight, further dried at 110°C for 12 hr, and weighed again (for dry weight).

Wing Size and Cell Density Determination

Microscopic images of wings mounted in 70% glycerol were captured using a Nikon ECLIPSE E800 microscope, and the area of the wing was measured using ACTII software (Nikon). Cell density was analyzed by counting the number of wing hairs in 0.01 mm^2 area of the wing (Brogiolo et al., 2001).

Hemolymph Sugar Measurement

Hemolymph sugar (glucose + trehalose) concentrations were measured as previously described (Teleman et al., 2003). D-trehalose and D-glucose were used as standards.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and two tables and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00430-4.

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