

# A Nutrient Sensor Mechanism Controls *Drosophila* Growth

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## Summary

Organisms modulate their growth according to nutrient availability. Although individual cells in a multicellular animal may respond directly to nutrient levels, growth of the entire organism needs to be coordinated. Here, we provide evidence that in *Drosophila*, coordination of organismal growth originates from the fat body, an insect organ that retains endocrine and storage functions of the vertebrate liver. In a genetic screen for growth modifiers, we identified *slimfast*, a gene that encodes an amino acid transporter. Remarkably, downregulation of *slimfast* specifically within the fat body causes a global growth defect similar to that seen in *Drosophila* raised under poor nutritional conditions. This involves TSC/TOR signaling in the fat body, and a remote inhibition of organismal growth via local repression of PI3-kinase signaling in peripheral tissues. Our results demonstrate that the fat body functions as a nutrient sensor that restricts global growth through a humoral mechanism.

## Introduction

In multicellular organisms, the control of growth depends on the integration of various genetic and environmental cues (Conlon and Raff, 1999; Stern, 2001). Nutrient availability is one of the major environmental signals influencing growth and, as such, has dictated adaptive responses during evolution toward multicellularity. In particular, complex humoral responses ensure that growth and development are properly coordinated with nutritional conditions.

In isolated cells, amino acid withdrawal leads to an immediate suppression of protein synthesis, suggesting that cells are protected by active sensing mechanisms that block translation prior to depletion of internal amino acid stores (Kimball and Jefferson, 2000). In many mammalian cell types, changes in amino acid diet affect the binding of the translation repressor 4EBP1 to initiation factor eIF4E and the activity of ribosomal protein S6 kinase (S6K) (Kimball and Jefferson, 2000). These two signaling events require the activity of TOR (target of rap-

mycin; for review, Gingras et al., 2001; Schmelzle and Hall, 2000), a conserved kinase recently shown to participate in a nutrient-sensitive complex both in mammalian cells and in yeast (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). Mutations in the *Drosophila* TOR homolog (*dTOR*) results in cellular and physiological responses characteristic of amino acid deprivation and establish that TOR is cell autonomously required for growth in a multicellular organism. (Oldham et al., 2000; Zhang et al., 2000). Furthermore, the TSC (tuberous sclerosis complex) tumor suppressor, consisting of a TSC1 and TSC2 heterodimer (TSC1/2), as well as the small GTPase Rheb, were recently shown to participate to the regulation of TOR function both in vitro and in vivo (Gao et al., 2002; Inoki et al., 2002, 2003; Garami et al., 2003; Saucedo et al., 2003; Stocker et al., 2003; Zhang et al., 2003). Overall, these data suggest that TSC, Rheb, TOR, and S6K participate in a conserved pathway that coordinates growth with nutrition in a cell-intrinsic manner.

In multicellular organisms, humoral controls are believed to buffer variations in nutrient levels. However, little is known about how growth of individual cells is coordinated. In vertebrates, growth-promoting action of the growth hormone (GH) is mostly relayed to peripheral tissues through the production of IGF-I (Butler and Le Roith, 2001). Binding of IGF-I to its cognate receptor tyrosine kinase (IGF-IR) induces phosphorylation of insulin receptor substrates (IRS), which in turn activate a cascade of downstream effectors. These include phosphoinositide 3-kinase (PI3K), which generates the second messenger phosphatidylinositol-3,4,5-P<sub>3</sub> (PIP<sub>3</sub>), and thereby activates the AKT/PKB kinase. Genetic manipulation of IGF-I, IGF-IR, PI3K, and AKT in mice modulates tissue growth in vivo thus demonstrating a requirement of the IGF pathway for growth (Efstratiadis, 1998; Shioi et al., 2000, 2002). In *Drosophila*, both loss- and gain-of-function studies have also exemplified the role of a conserved insulin/IGF signaling pathway in the control of growth (for reviews, Garofalo, 2002; Saucedo et al., 2003; Stocker and Hafen, 2000). Ligands for the unique insulin receptor (Inr) constitute a family of seven peptides related to insulin, the *Drosophila* insulin-like peptides (Dilps). Remarkably, three *dilp* genes (*dilp2*, *dilp3*, and *dilp5*) are expressed in a cluster of seven median neurosecretory cells (m-NSCs) in the larval brain, suggesting that they have an endocrine function (Brogiolo et al., 2001). Indeed, ablation of the seven *dilp*-expressing mNSCs in larvae induces a systemic growth defect (Ikeya et al., 2002; Rulifson et al., 2002).

Both in flies and mice, mutations in *IRS* provoke growth retardation as well as female sterility similar to what is observed in starved animals (Araki et al., 1994; Bohni et al., 1999; Tamemoto et al., 1994). Moreover, PI3K activity in *Drosophila* larvae depends on the availability of proteins in the food (Britton et al., 2002). Overall, this supports the notion that the insulin/IGF pathway might coordinate tissue growth with nutritional conditions. However, upon amino acid withdrawal, neither PI3K nor AKT/PKB activities are downregulated in mammalian or insect cells in culture, suggesting that this

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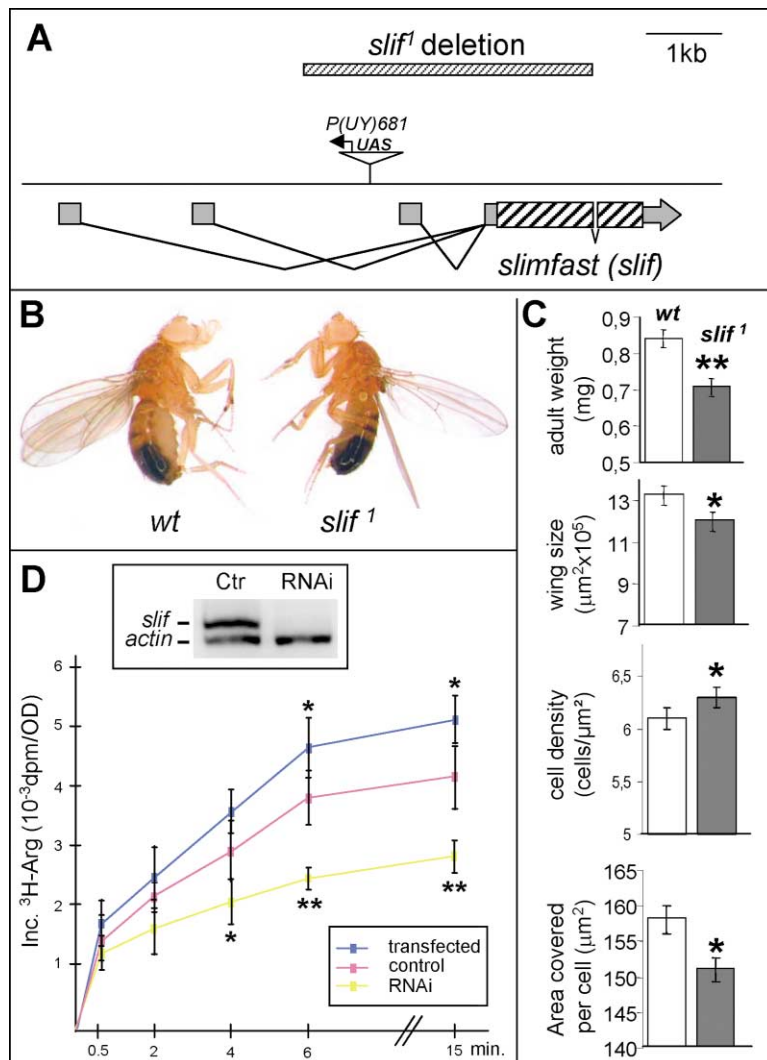


Figure 1. Characterization of the *slimfast* (*slif*) Gene

(A) The *slif* gene corresponds to CG11128 (hatched boxes: coding region). *GAL4*-dependent transcription of *P(UY)681* runs in reverse orientation of the gene (black arrow).

(B) Phenotype of *slif*<sup>1</sup> mutant: wild-type (*wt*) and homozygous *slif*<sup>1</sup> males are shown.

(C) Body weight, wing size, average wing cell density, and wing cell surface in *slif*<sup>1</sup> animals compared to *wt*.

(D) Measurement of arginine uptake in S2 cells. Schneider S2 cells were transfected with a control plasmid, a *slif* construct, or treated with double-stranded *slif* RNA (RNAi). In our conditions, only 3% of the cells are efficiently transfected by the *slif*-expressing plasmid. Average of triplicate experiments is presented (\**p* < 0.05; \*\**p* < 0.01). RT-PCR analysis shows *slif* expression in normal and RNAi-treated S2 cells; the actin gene is used as an internal control.

pathway does not directly respond to nutrient shortage (Campbell et al., 1999; Hara et al., 1998; Kimball et al., 1999; Patti et al., 1998; Radimerski et al., 2002). Hence, an intermediate sensor mechanism must link nutrient availability to insulin/IGF signaling.

An intriguing possibility is that specific organs could function as nutrient sensors and induce a nonautonomous modulation of insulin/IGF growth signaling in response to changes in nutrient levels. Here, we use a genetic approach in *Drosophila* to assess both the cellular and humoral responses to amino acid deprivation in the context of a developing organism. The insect fat body (FB) has important storage and humoral functions associated with nutrition, comparable to vertebrate liver and adipose tissue. During larval stages, the FB accumulates large stores of proteins, lipids, and carbohydrates, which are normally degraded by autophagy during metamorphosis in order to supply the developing tissues (Dean et al., 1985) but can also be remobilized during larval life to compensate transitory nutrient shortage. In addition to its storage function, the FB also has endocrine activity and supports growth of imaginal disc explants and DNA replication of larval brains in coculture

experiments (Davis and Shearn, 1977; Britton and Edgar, 1998). Here, we demonstrate that the FB operates as a sensor for variations in nutrient levels and coordinates growth of peripheral tissues accordingly via a humoral mechanism.

## Results

### A Tool to Study the Growth Response to Variation in Amino Acid Levels

In the course of a *P[UAS]*-based overexpression screen for growth modifiers (Raisin et al., 2003), a *P[UAS]*-insertion line (*UY681*) was found to cause growth retardation upon ectopic activation. Sequence analysis revealed that *P(UY)681* is inserted in a predicted gene (CG11128) that encodes a putative protein showing strong homology with amino acid permeases of the cationic amino acid transporter (CAT) family (Figure 1A and Supplemental Figure S1, online at <http://www.cell.com/cgi/content/full/114/6/739/DC1>). The *P[UAS]* element is inserted in the first intron of the CG11128 gene, potentially driving transcription of an antisense RNA in a *GAL4*-dependent manner (Figure 1A). To assess the function of this trans-

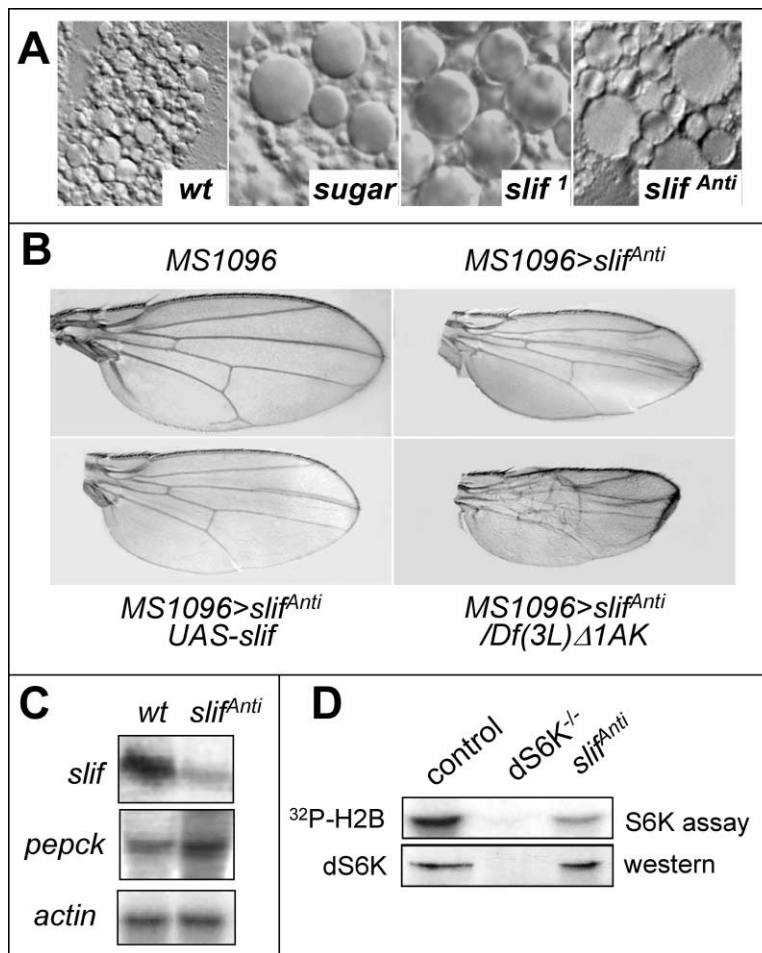


Figure 2. Loss of *slif* function mimics amino acid starvation

(A) Reduction of *slif* function in *slif*<sup>1</sup> or *da-GAL4>slif*<sup>Anti</sup> larvae induces a starvation response in the FB visible as storage vesicle aggregation, similar to that observed in animals raised on protein-free media (sugar). (B) Functional analysis of the *slif*<sup>Anti</sup> conditional allele: *GAL4*-activation of *P(UY)681* in the wing disc using the *MS1096-GAL4* driver reduces growth. This is partially rescued by coexpression of *slif* under *UAS* control or enhanced by reducing *slif* gene dosage with the deficiency *Df(3L)Δ1AK*. (C) Northern blot analysis indicates that in *da-GAL4>slif*<sup>Anti</sup> larvae (*slif*<sup>Anti</sup>), *slif* RNA levels are strongly reduced, whereas those of the starvation marker *PEPCK1* are increased. Actin is used as a loading control. (D) In *da-GAL4>slif*<sup>Anti</sup> larvae (*slif*<sup>Anti</sup>), S6 kinase activity is reduced by 80%; wt and *ds6k*<sup>-1</sup> mutant animals are used as controls. dS6K levels are compared using anti-dS6K antibodies.

porter, <sup>3</sup>H-arginine uptake was measured in S2 cells. Results indicate that amino acid uptake is either enhanced by transfection of a *CG11128* cDNA or suppressed by RNAi, indicating that the encoded protein presents CAT activity *ex vivo* (Figure 1D). In situ hybridization revealed basal levels of *CG11128* expression in most larval tissues but much higher levels in the FB and the gut, two tissues involved in amino acid processing (Supplemental Figures S2C and S2E).

By P element remobilization, we obtained an imprecise excision that deletes the sequences encoding the N-terminal half of the protein (Figure 1A). 87% of homozygous mutant animals die during larval stages. The few viable adults emerged after a 2 day delay and were smaller and markedly slimmer than control animals (Figure 1B). We named the associated gene *slimfast* (*slif*) and the excision allele *slif*<sup>1</sup>. Weight measurement indicated that homozygous *slif*<sup>1</sup> adult males displayed a 16% mass reduction compared to control (Figure 1C). Accordingly, adult wing size was reduced by 8% due to a reduction of both cell size and cell number (Figure 1C). When the *slif*<sup>1</sup> allele was in *trans* to *Df(3L)Δ1AK*, a deficiency covering the locus, larval lethality was slightly enhanced, suggesting that *slif*<sup>1</sup> corresponds to a strong hypomorphic allele. The amino acid transporter function of *slif*, as well as the phenotypes observed upon reduction of *slif* function suggest that *slif* mutant animals might

suffer amino acid deprivation. A major consequence of amino acid deprivation in larvae is the remobilization of nutrient stores in the FB, which typically results in aggregation of storage vesicles. Consistently, fusion of storage vesicles was observed in the FB of *slif*<sup>1</sup> larvae and was indistinguishable from that observed in animals fed on protein-free media (compare *slif*<sup>1</sup> and sugar, Figure 2A).

*GAL4* induction of *P(UY)681* resulted in a growth-deficient phenotype similar to that of *slif*<sup>1</sup> loss of function (Figures 2A and 2B). The antisense orientation of *P(UY)681* (Figure 1A) suggested that the growth defect following *GAL4* induction was due to an RNAi effect. Indeed, Northern blot analysis revealed that ubiquitous *GAL4*-dependent activation of *P(UY)681* using the *daughterless-GAL4* (*da-GAL4*) driver strongly reduced *slif* mRNA levels (Figure 2C). Only two of the three alternative first exons are potentially affected by the antisense RNA, possibly explaining the residual accumulation of *slif* mRNAs in *da-GAL4>P(UY)681* animals (Figure 1A). Most of these animals died at larval stage, similar to what was observed for *slif*<sup>1</sup> mutants. Specific induction of *P(UY)681* in the wing disc using the *MS1096-GAL4* driver provoked a reduction of the adult wing size, which could be either rescued by coactivation of a *UAS-slif* transgene or enhanced by reducing *slif* gene dosage with the heterozygous *Df(3L)Δ1AK* deficiency (Figure

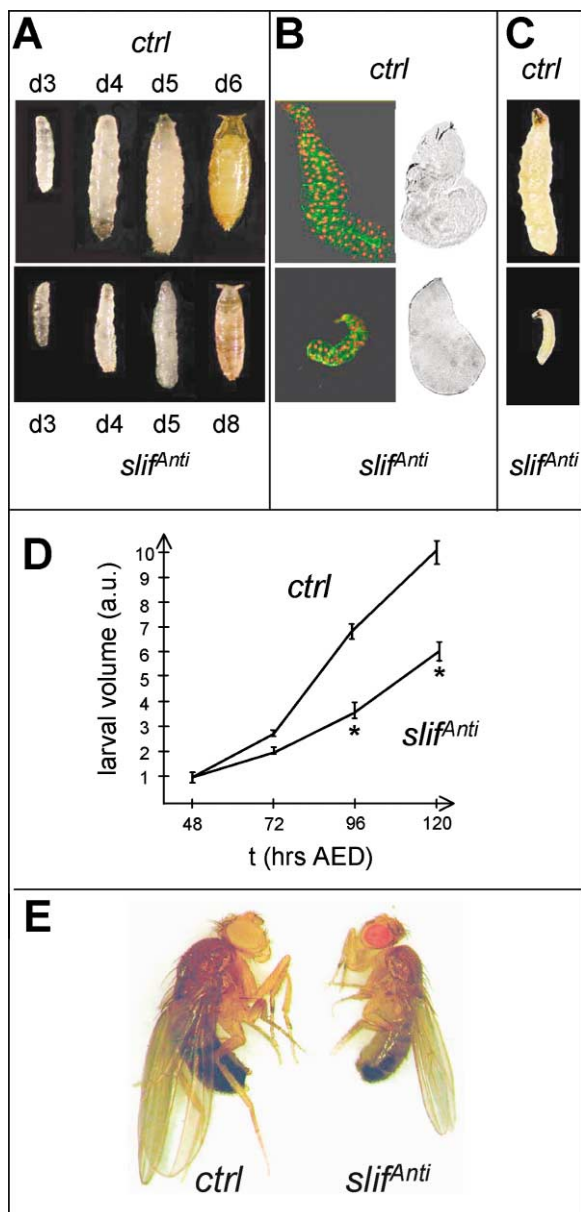
2B). Thus, *GAL4*-dependent activation of *P(UY)681* reduces *slif* function and defines a conditional loss-of-function allele hereafter termed *slif<sup>Anti</sup>*.

As expected, loss of *slif* function using the *slif<sup>Anti</sup>* allele also mimicked amino acid deprivation. Accordingly, ubiquitous *slif<sup>Anti</sup>* induction in growing larvae resulted in storage vesicle aggregation and strong reduction of global S6 kinase activity, similar to what was reported in animals raised on protein-free diet (Figures 2A and 2D) (Oldham et al., 2000; Zhang et al., 2000). Additionally, an increase in *PEPCK1* gene transcription was observed, similar to the effect of amino acid withdrawal (Figure 2C) (Zinke et al., 2002).

In summary, we have identified two loss-of-function alleles of the *slif* gene whose defects mimic physiological aspects of amino acid deprivation. Importantly, the conditional *slif<sup>Anti</sup>* allele provides a unique tool to mimic an amino acid deprivation in a tissue-specific manner.

### A Nutrient Status Sensor in the FB

Several experiments suggest that in multicellular organisms, nutrient signals are not sensed at the cell level but, rather, relayed to peripheral tissues by a central sensing mechanism (Britton and Edgar, 1998). In *Drosophila* larvae, the FB plays important roles in the nutritional response and could therefore sense and relay nutrient availability to coordinate growth of the whole organism. To test this hypothesis, we induced *slif* down-regulation specifically in this organ using the *pupless (ppl)*-*GAL4* driver (see Experimental Procedures). As expected, *slif* expression was strongly reduced in the FB, but not in other tissues of *ppl>slif<sup>Anti</sup>* larvae (compare Supplemental Figures S2E and S2F with gut internal control S2C and S2D). Under these conditions and despite normal feeding behavior and processing of food along the digestive tract (Supplemental Figure S2A), larval development was delayed and accompanied by significant growth defects (Figure 3A). Measurement of larval volumes indicated that the growth rate was strongly reduced throughout larval life, as compared to control animals (Figure 3D). Under mild *slif<sup>Anti</sup>* induction (18°C), emerging adults were remarkably reduced in size and body weight (54% of wt, Figure 3E and Table 1). Stronger induction (25°C) provoked a longer developmental delay associated with pupal lethality (not shown). Interestingly, a 90% reduction of arginine concentration in the food did not affect the growth of control animals but led to a dramatic aggravation of the growth defect of *ppl>slif<sup>Anti</sup>* animals, with most animals dying as small L2 larvae (Figure 3C). This aggravation was not observed after starvation for the neutral amino acid valine (not shown), consistent with the fact that in the FB, *Slif* functions as a transporter of cationic amino acids. Comparison of larval endoreduplicating tissues (ERTs), as represented in the case of salivary glands, and imaginal discs revealed that in *ppl>slif<sup>Anti</sup>* animals, growth of discs was less affected than growth of the ERTs (Figure 2B). A similar phenotype was previously described in larvae starved for protein and in *dTOR* loss-of-function mutants (Britton and Edgar, 1998; Zhang et al., 2000). Overall, these results suggest that amino acid deprivation within the FB triggers a systemic response, which restricts growth of larval tissues.



**Figure 3. Amino Acid Deprivation in the FB Induces a Developmental Delay and a General Growth Defect**

(A) Growth of *ppl-GAL4>slif<sup>Anti</sup>* animals was monitored and compared to control raised in parallel (*ppl-GAL4*) at 25°C (days AED are indicated). Pupal weight measurement shows 35% reduction compared to control.

(B) Comparison of salivary glands and wing imaginal disc size before pupariation. *ppl-GAL4>slif<sup>Anti</sup>* wing discs (day 7) are 20% reduced as compared to *ppl-GAL4* control (day 5). Salivary glands at the same developmental times are more affected. Control gland is from *pGawB-GAL4>slif<sup>Anti</sup>*, which present a leaky *GAL4* expression in salivary glands similar to *ppl-GAL4>slif<sup>Anti</sup>*.

(C) A 10-fold decrease of arginine concentration in the food has no significant effect on the development of *ppl-GAL4* control animals, but lethality of *ppl-GAL4>slif<sup>Anti</sup>* animals occurs earlier (early L2 stage instead of late pupa). Experiments were conducted at 25°C; larvae are shown at the time of pupariation for control.

(D) Measurement of larval volumes ( $n = 10$  per time point;  $p < 0.05$ ) indicates a significant reduction in growth rate of *ppl-GAL4>slif<sup>Anti</sup>* larvae (25°C).

(E) *ppl-GAL4>slif<sup>Anti</sup>* adult males are 48% reduced in mass with normal proportions (18°C).

Table 1. Systemic Growth Control by the FB

	Mean Adult Emergence Time (Days)	Adult Weight (%)	FB Cell Size (%)	Nuclear Volume (%)	n
Wt	11	100	100	100	150
Sugar (PBS 20% sucrose)	—	—	69	21	—
ppl>slif <sup>Anti</sup>	12.5 (18°C)	54 (18°C)	90	25	120
ppl>dPTEN	11	99	70	10	120
ppl>dTOR <sup>TEd</sup>	12	72	84	26	120
ppl>dTSC1 + dTSC2	12.5	82	78	19	150
ppl>slif <sup>Anti</sup> + S6K-D4	12.5 (18°C)	68 (18°C)	—	—	60

Different constructs were specifically expressed in the larval FB using the *ppl*-*GAL4* driver: *dTOR*<sup>TEd</sup> (Hennig and Neufeld, 2002), *dTSC1* and *dTSC2* (Tapon et al., 2001), *dPTEN* (Huang et al., 1999), and mammalian activated *S6K-D4* (Zhang et al., 2000). Adult emergence time (days at 25°C) and adult weight (% of wt) were determined. n, number of animals for each experiment. FB cell size and nuclear volume were measured 5 days AED from images of FB tissue stained with phalloidin-rhodamin and propidium iodide. All experiments were carried at 25°C, except when indicated. In this case, emergence time and weight were compared to wt flies maintained at the same temperature, and emergence time was converted taking into account the increased development time of wt flies at 18°C.

Induction of *slif*<sup>Anti</sup> in the FB autonomously provoked a remobilization of lipid vesicles in FB cells, as well as a reduction in cell size and endoreduplication levels comparable to the effect of a diet lacking protein (Figures 4B–4C' and Table 1). Since the FB has been proposed to promote growth of larval tissue through endocrine signals (Davis and Shearn, 1977; Britton and Edgar, 1998; Martin et al., 2000), reduction of FB growth might directly affect hormone secretion and generate systemic growth defects. To investigate this issue, larval development was analyzed following restriction of FB growth in response to various growth antagonists. Using the *ppl*-*GAL4* driver, overexpression of either *dPTEN* or a dominant-negative form of the *Drosophila* PI3K (*dPI3K*<sup>DN</sup>, not shown) led to a pronounced reduction of FB cell size and DNA endoreduplication (Figure 4D, Table 1). Although this defect was stronger than that induced by *slif*<sup>Anti</sup>, aggregation of storage vesicles was not observed (Figure 4D'). Furthermore, in these conditions, the flies were neither delayed at eclosion, nor reduced in size (Table 1). This indicates that the systemic growth defects induced by *slif*<sup>Anti</sup> expression in the FB are not due to a moderate limitation of FB growth.

In conclusion, our data suggest that a sensor mechanism, specifically triggered by amino acid availability, originates from the larval FB to orchestrate developmental growth.

#### Downregulation of TOR Signaling Activates the FB Amino Acid Sensor

The growth defects induced by FB-directed *slif* downregulation at 18°C were partially rescued by coexpressing *Drosophila* S6 kinase (not shown) or an activated form of mammalian S6 kinase (*S6K1-D4*, Table 1). Similarly, the strong pupal lethality induced by *slif* downregulation at 25°C was totally rescued by coexpression of *Drosophila* S6 kinase (not shown). This suggests that dS6K is an important effector of the amino acid sensor mechanism. In flies, dS6K is a major downstream target of the TOR signaling pathway and overexpression of an activated form of S6 kinase was shown to partially rescue a hypomorphic *dTOR* heteroallelic combination (Oldham et al., 2000; Zhang et al., 2000). Growth defects and aggregation of FB storage vesicles have also been reported in *dTOR* mutants (Zhang et al., 2000), compara-

ble to the phenotypes observed in *slif* loss-of-function larvae. Taken together, these results prompted us to test whether the TOR pathway might participate in the FB amino acid sensor mechanism. For this purpose, we used a truncated version of dTOR (corresponding to a 754 amino acid central region described as “toxic effector domain”), which dominantly suppresses *dTOR* function in vivo (*dTOR*<sup>TEd</sup>) (Hennig and Neufeld, 2002). FB-specific overexpression of *dTOR*<sup>TEd</sup> triggered vesicle aggregation and restricted endoreduplication in FB cells (Figures 4E and 4E', Table 1). Interestingly, this was accompanied by a developmental delay and a growth defect comparable to those induced by a mild *slif* downregulation (Table 1). A similar phenotype was observed by cooverexpressing *dTSC1* and *dTSC2*, which inhibit the TOR pathway (Figures 4F and 4F'; Table 1). These phenotypes strikingly resemble those induced by *slif* downregulation, demonstrating that inhibiting dTOR activity specifically in the FB is sufficient to trigger the amino acid sensor.

Remarkably, overexpression of *dPTEN* in FB cells did not induce vesicle aggregation. Moreover, although it induced a more pronounced growth inhibition of FB cells than *dTOR*<sup>TEd</sup>, *dTSC1*, *dTSC2*, or *slif*<sup>Anti</sup> expression, it did not affect general growth of the flies (Figures 4D and 4D', Table 1). This indicates that inhibition of the PI3K signaling pathway within the FB fails to activate the amino acid sensor and suggests that PI3K signaling does not participate in the amino acid sensing mechanism per se.

#### *Inr*/PI3K Signaling Is Downregulated in Peripheral Tissues in Response to FB Amino Acid Sensor Activation

Using a GFP-PH domain fusion protein (tGPH) as a reporter, it has recently been shown that PI3K activity is downregulated in larvae deprived of dietary protein/amino acids (Britton et al., 2002). We therefore investigated whether activation of the FB amino acid sensor could remotely control *Inr*/PI3K signaling in other larval tissues. To test this hypothesis, tGPH was used as a cytological marker to monitor PI3K activity in larval cells. FB induction of *slif*<sup>Anti</sup> resulted in a faint decrease of tGPH-associated fluorescence at the membrane of FB cells, reflecting only a mild downregulation of PI3K activ-



ity in response to sensor activation (Figure 5B). In contrast, a complete loss of membrane-associated tGPH fluorescence was observed in epidermal and salivary gland cells (Figures 5E and 5M), indicating that PI3K activity is strongly suppressed in distant endoreplicative tissues in response to activation of the FB amino acid sensor. Only a slight decrease in tGPH-associated fluorescence was observed in imaginal wing disc cells (Figure 5H), supporting the notion that even under amino acid restriction, PI3K activity is partially maintained in mitotic tissues.

Specific induction of *dPTEN* in the FB provoked the disappearance of the membrane-associated tGPH fluorescence in FB cells (Figure 5C), indicating that in these conditions PI3K signaling is severely blocked. Nonetheless, tGPH localization in epidermal cells or in wing imaginal disc cells was not affected (Figures 5F and 5I). This correlates with the absence of nonautonomous growth defects in these animals.

In conclusion, our results indicate that activation of the FB amino acid sensor does not rely on a modulation of PI3K activity within FB cells but induces a remote inhibition of the *Inr*/PI3K pathway in targeted ERTs. Persistent growth of imaginal disc cells after sensor activation correlated with the maintenance of a minimal activation threshold of *Inr*/PI3K signaling in these tissues.

#### The FB Amino Acid Sensor Does Not Act upon Neuronal Dilp Expression

One hypothesis to explain the nonautonomous suppression of *Inr*/PI3K signaling in ERTs is that *Drosophila insulin-like peptide* (*dilp*) genes could be downregulated in response to activation of the FB sensor. Indeed, it has recently been shown that expression of *dilp3* and *dilp5* in seven median neurosecretory cells (m-NSCs) of the brain is strongly reduced when larvae are grown on a water-only diet (Ikeya et al., 2002). However, in *ppl-GAL4>slif<sup>Anti</sup>* larvae, expression of *dilp3* and *dilp5* was only marginally affected as visualized by in situ hybridization (Figure 6C). Such a small decrease in neuronal *dilp* expression is not sufficient to produce a general growth defect (see below), suggesting that the FB remote control is not mediated by a modulation of *dilp* expression in m-NSCs.

It has also been proposed that the *dilp*-producing m-NSCs could directly sense nutrition and control *dilp* gene expression accordingly. To test this hypothesis, both a *dilp3-GAL4* driver (see Experimental Procedures) and a *dilp2-GAL4* driver (Rulifson et al., 2002) were used to selectively induce *slif<sup>Anti</sup>* in the seven *dilp*-expressing m-NSCs. Under these conditions, expression of *dilp3* and *dilp5* was slightly reduced (Figure 6D, comparable to that observed in *ppl-GAL4>slif<sup>Anti</sup>* larvae), and no larval growth defect was observed. However, the mNSCs themselves were reduced in size, indicating that they were indeed amino acid deprived. This indicates that amino acid levels are not the main regulator of *dilp* expression in m-NSCs. Consistently, raising wild-type larvae on a protein-free diet only resulted in a mild reduction of *dilp3* and *dilp5* expression levels, comparable to the effect of *slif<sup>Anti</sup>* induction in FB cells or in m-NSCs (Figure 6E). In contrast, *dilp3* and *dilp5* expression in m-NSCs was almost entirely abolished when larvae were

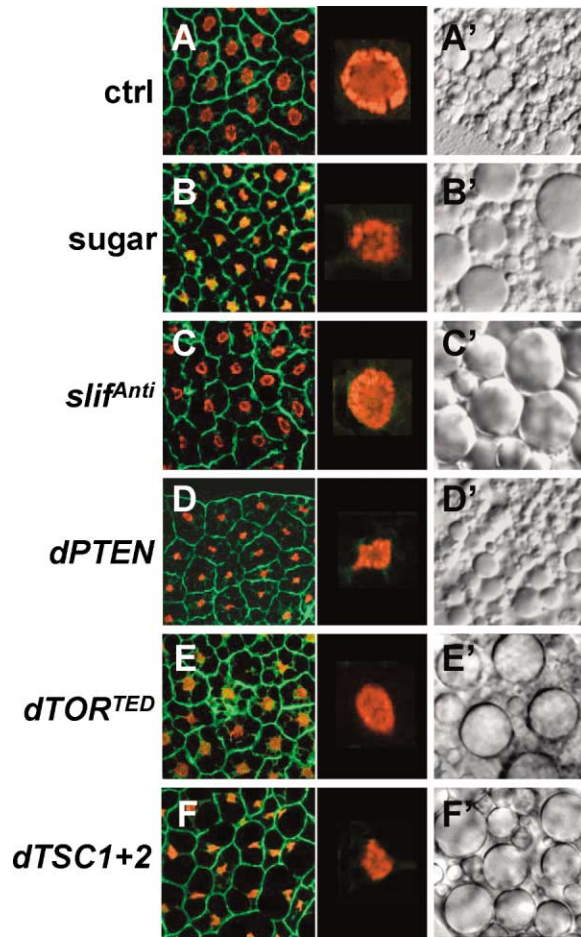


Figure 4. Loss of *slif* Function or Inhibition of the *TOR*/*TSC* Pathway in FB Cells Induces a Cell-Intrinsic Response Similar to Starvation (A–F) FB cell size and endoreduplication analyses. Membranes are stained in green with phalloidin-FITC and nuclei in red with propidium iodide. Closeups of representative nuclei are shown at constant magnification on the right. (A'–F') FB cell morphology under Nomarski optics. All *UAS* constructs are driven by *ppl-GAL4*. “Control” and “sugar” indicate larvae raised on normal diet or transferred on protein-free diet at L1 stage, respectively. Larval FB are dissected and fixed 4 days after egg deposition (AED).

fed a protein- and sugar-free diet (Figure 6F), suggesting that *dilp* expression in mNSCs might be predominantly modulated by carbohydrate rather than by amino acids.

Taken together, these results demonstrate that activation of the FB amino acid sensor does not induce a modulation of neuronal *dilp* expression. Furthermore, the FB appears to be a major amino acid sensing tissue in larvae, since *dilp*-expressing m-NSCs did not trigger a systemic growth inhibition in response to amino acid deprivation.

#### A Putative Dilp Cofactor Is Expressed in the FB

The *dilp* expression results suggest that the general growth defects observed in FB starved animals might be mediated through other diffusible factors linked to insulin/IGF signaling. In mammals, most circulating IGF-I is stoichiometrically associated in a ternary complex with IGF-BPs and a third partner called acid labile sub-

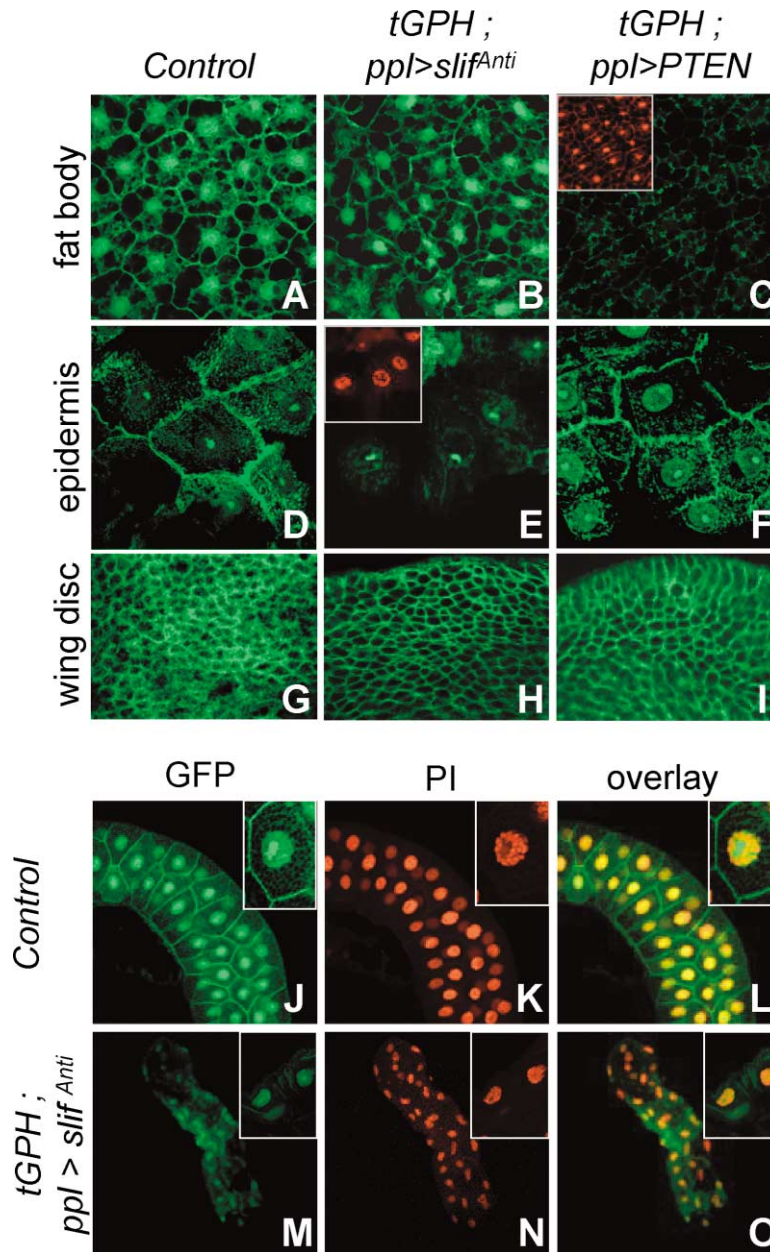


Figure 5. Activation of the FB Amino Acid Sensor Triggers a Shutdown of PI3K Activity in Larval ERTs

(A–I) tGPH fluorescence (green) in the FB (A–C), epidermis (D–F), and wing disc (G–I) of L3 larvae raised at 25°C. Genotypes are: *ppl-GAL4* (A, D, and G), *ppl-GAL4>slif<sup>Anti</sup>* (B, E, and H), and *ppl-GAL4>PTEN* (C, F, and I). In (C) and (E), membranes (rhodamin-phalloidin, [C] inset) and/or DNA (propidium iodide, [C] and [E], insets) were counterstained.

(J–O) tGPH fluorescence (GFP, green) and propidium iodide (PI, red) in salivary glands of control (*pGawB-GAL4>slif<sup>Anti</sup>*) and *ppl-GAL4>slif<sup>Anti</sup>* L3 larvae.

unit (ALS) (Boisclair et al., 2001; Duan, 2002). This ternary complex is known to regulate most of IGF-I biological functions and plays an important role in the stabilization of circulating IGF-I. ALS is a liver-secreted glycoprotein whose concentration in the serum varies with nutritional conditions. We identified a *Drosophila* ALS ortholog gene (*dALS*), which encodes a protein presenting 46% homology with human ALS in a 444 amino acid central region (Supplemental Figure S3). Strikingly, the gene is expressed in the same seven *dilp*-expressing m-NSCs in each larval brain lobe, reinforcing the notion of a functional link with Dilps (Figures 7E and 7F). In contrast to the *dilp* genes, *dALS* is also strongly expressed in the larval FB (Figure 7A), but not in any other larval tissue (not shown). In response to different starvation conditions, *dALS* is severely downregulated in the FB

and in the mNSCs (Figures 7C, 7D, and 7G). Interestingly, *dALS* expression in the FB is strongly suppressed when amino acid restriction is induced in this tissue using *slif<sup>Anti</sup>* (Figure 7B), suggesting that it is a direct target of the FB sensor mechanism.

## Discussion

Developing animals must be able to tolerate variations in nutrient availability. In *Drosophila* larvae, amino acid restriction arrests growth and endoreplication of larval tissues and slows down growth and proliferation of the central nervous system and imaginal discs (Britton and Edgar, 1998). If starvation is temporary, development is delayed but metamorphosis eventually proceeds to give rise to adult flies. Emerging adults are severely reduced

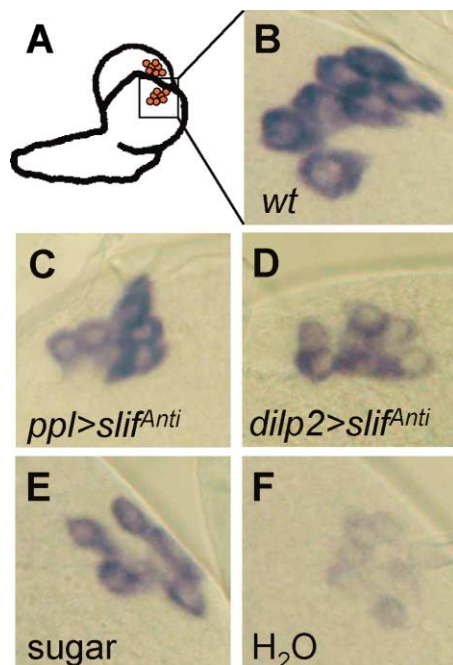


Figure 6. *dilp5* Expression Is Differentially Modulated by Distinct Nutritional Conditions

(A) Schematic localization of m-NSCs expressing *dilp3* and *dilp5* in larval brain.

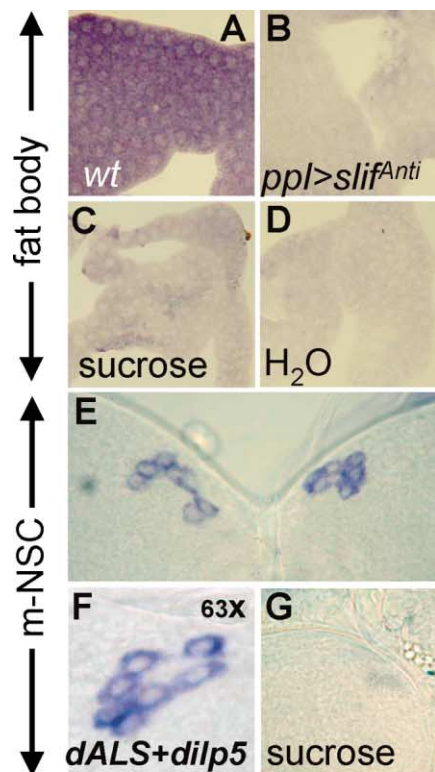
(B–F) In situ hybridization of *dilp5* mRNA on third instar larval brains. Similar results were obtained with a *dilp3* probe (not shown). “Sugar” and “H<sub>2</sub>O” indicate carbohydrate-only and water-only diets, respectively.

in size, but their relative body proportions are maintained. These regulations occur in most metazoans whose life spans over fasting and feeding phases. Young rats weaned onto a low-protein diet display proportionate growth defects accompanied by a cell size reduction (Crace et al., 1991). The preserved proportions of these animals indicate that under nutrient restriction, an adjusting mechanism coordinately restrains growth of the various body parts.

Here, we present a study of growth control by nutrition in *Drosophila*, which highlights the importance of systemic regulations.

#### The Role of the FB in the Nutritional Response

We have established that the FB is a sensor tissue for amino acid levels, as downregulation of the Slif amino acid transporter within the FB is sufficient to induce a general reduction in the rate of larval growth. In contrast, specific disruption of *slif* in imaginal discs, larval gut, or salivary glands did not induce a nonautonomous growth response (not shown), suggesting that these tissues do not participate in the systemic control of growth. The *dilp*-expressing m-NSCs also affect growth control, since selective ablation of these cells in the larval brain induces an overall reduction of animal size (Ikeya et al., 2002; Rulifson et al., 2002). In response to complete sugar and protein starvation, the m-NSCs stop expressing *dilp3* and *dilp5* genes, suggesting that these neurons also sense nutrient levels (Ikeya et al., 2002). We show



H

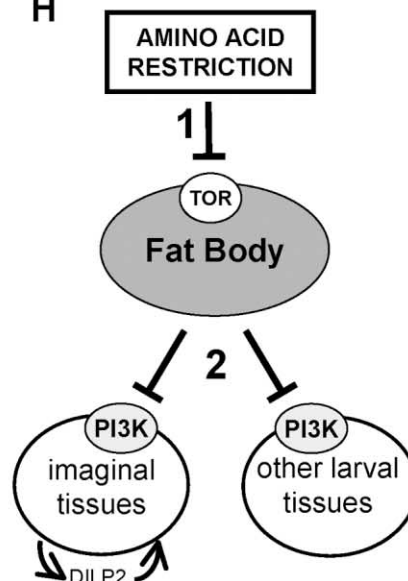


Figure 7. *dALS* Is Expressed in the FB and Repressed by Starvation

During larval development, *dALS* is expressed only in the FB (A) and the seven *dilp*-expressing mNSCs (E). Double labeling of *dALS* and *dilp5* shows colocalization in the same seven mNSCs in the brain lobes (F). *slif<sup>anti</sup>* expression (B), protein-free diet (C), or complete starvation (D) abolish *dALS* expression in the FB. Amino acid deprivation also strongly reduces *dALS* expression in the mNSCs (G). (H) Our results establish that the larval FB is a central sensor role for amino acids levels. Once triggered in FB cells through a modulation of TOR signaling (1), a starvation signal is relayed to peripheral tissues to suppress the Inr/PI3K pathway (2). Imaginal tissues would be partially protected from this regulation by an autocrine production of Dilp2.



here that the selective reduction of *slif* function in these cells has no obvious effect on tissue growth and animal development. This indicates that the seven *dilp*-expressing m-NSCs do not constitute a general amino acid sensor. In contrast, the role of m-NSCs in carbohydrate homeostasis (Rulifson et al., 2002) and the observation that they stop expressing certain *dilp* genes when larvae are deprived of sugar (Ikeya et al., 2002) rather suggests that these cells have a role in sensing carbohydrate levels.

Our analysis also provides a framework in which to understand the phenotype of *minidisc*, a mutation in an amino acid transporter gene that exhibits nonautonomous growth defects in imaginal discs (Martin et al., 2000).

#### Interplay between TOR and Inr/PI3K Pathways in the Starvation Response In Vivo

In a number of model systems, both PI3K and TOR have been implicated in linking growth to nutritional status and, until recently, were considered as intermediates of a common regulatory pathway. In yeast, the TOR kinase is part of a cell-autonomous nutrient sensor, which controls protein synthesis, ribosome biogenesis, nutrient import, and autophagy (Gingras et al., 2001). Genetic analysis in *Drosophila* indicates that *dTOR* is required for cell-intrinsic growth control. Our results obtained using the *slif<sup>Anti</sup>* allele in the wing disc indicate that individual tissues have indeed the potential to respond to amino acid deprivation in a cell-autonomous manner. Nonetheless, we also demonstrate that the TOR nutritional checkpoint participates in a systemic control of larval growth emanating from the FB. Within a developing organism, each cell may integrate these two distinct inputs regarding nutritional status, one originating from a systemically-acting FB sensor, and the other from TOR-dependent signaling in individual cells. One can further speculate that depending on the strength and duration of starvation, different in vivo nutritional checkpoints will be hierarchically recruited to protect the animal and that the systemic control might, in most physiological situations, override the cell-autonomous control. Indeed, as our data demonstrate, the FB sensor is sufficient to induce a general and coordinated response to starvation without calling individual cell-autonomous mechanisms into play.

Several lines of evidence indicate that the PI3K pathway is not part of the sensor mechanism in FB cells. First, tGPH fluorescence in the FB is only marginally affected by amino acid deprivation in that tissue, indicating that the cell-autonomous response to amino acid starvation does not directly influence PI3K signaling. This is reminiscent of previous observations in mammalian cultured cells, showing that PI3K activity does not respond to variations in amino acid levels (Kimball and Jefferson, 2000). Moreover, inhibition of PI3K signaling by *dPTEN* expression in the FB is not sufficient to trigger the sensing mechanism. Although, *dPTEN* overexpression causes a complete disappearance of tGPH accompanied by growth suppression of FB cells, the FB maintains a critical mass that allows for normal larval growth. In contrast, the regulatory subunit p60 whose overexpression potentially inhibits PI3-kinase in flies was pre-

viously described to induce a systemic effect on larval growth when overexpressed in the FB using an *Adh-Gal4* driver (Britton et al., 2002). We found that *ppl-GAL4*-directed expression of p60 also provokes a strong suppression of larval growth and a dramatic inhibition of FB development in young larvae (J.C. and P.L., unpublished data, and not shown). Thus, the systemic effect on growth observed upon p60 overexpression most likely results from a drastic reduction of FB mass, which then fails to support normal larval growth.

Our results further indicate that PI3K signaling is a remote target of the humoral message that originates from the FB in response to amino acid deprivation. This is in agreement with previous data showing that PI3K activity is downregulated by dietary amino acid deprivation and explains why global PI3-kinase inhibition mimics cellular and organismal effects of starvation (Britton et al., 2002). The existence of a humoral relay reconciles these in vivo studies with the absence of direct PI3K responsiveness to amino acid levels.

The relative resistance of imaginal disc growth to the systemic control exerted by the FB correlates with maintenance of PI3K activity in these tissues. This is in agreement with previous observations by Britton and Edgar (1998) that cells in the larval brain and in imaginal discs maintain a slow rate of proliferation under protein starvation, while ERTs arrest. This difference might be attributed to the basal levels of *dilp2* expression observed in imaginal discs (Brogiolo et al., 2001), allowing a moderate growth rate of these tissues through an autocrine/paracrine mechanism. It was recently shown that clonal induction of PI3K potentially induces cell-autonomous growth response even in fasting larvae (Britton et al., 2002), indicating that some nutrients are still accessible to support cell growth within a fasted larva. The main function of a general sensor could be to preserve these limited nutrients for use by high priority tissues. In this context, local PI3K activation through an autocrine loop in imaginal tissues could favor the growth of prospective adult structures in adverse food conditions. Thus, the FB would have an active role in controlling the allocation of resources depending on nutritional status. In this respect, it is noteworthy that FB cells are relatively resistant to the FB-derived humoral signal, since tGPH is not drastically affected in the FB of *ppl>slif<sup>Anti</sup>* animals (Figure 5A). Thereby, essential regulatory functions of the FB could be preserved even in severely restricted nutritional conditions.

#### How Does the FB Signal to Other Tissues?

Our work suggests that a humoral signal relays information from the FB amino acid sensor and systemically inhibits PI3K signaling. In addition, this downregulation is not due to a direct inhibition of *dilp* expression by neurosecretory cells in the brain. Nevertheless, we cannot rule out that the secretion of these molecules is subjected to regulation in the mNSCs. Both in vivo and in insect cell culture, several imaginal discs growth factors (IDGF) secreted by the FB have been proposed to function synergistically with Dilp signaling to promote growth (Kawamura et al., 1999). However, we did not find any modification of IDGF expression in the FB of larvae raised on water- or sugar-only diet, or upon FB induction

of *slif<sup>anti</sup>* (not shown). In vertebrates, the different functions of the circulating IGF-I are modulated through its association with IGF-BPs and ALS (Boisclair et al., 2001; Duan, 2002). In particular, the formation of a ternary complex with ALS leads to a considerable extension of IGF-I half-life (Boisclair et al., 2001). Our finding that a *Drosophila* ALS ortholog is expressed within the FB in an amino acid-dependent manner provides a new avenue to study the molecular mechanisms of nonautonomous growth control mediated by the FB.

### Concluding Remarks

Our present study highlights the contribution that genetics can provide to unravel the mechanisms of physiological control. Using a genetic tool to mimic amino acid deprivation, we demonstrated that nutrition systemically controls body size through an amino acid sensor operating in the FB. We propose that (1) in metazoans, a systemic nutritional sensor modulates the conserved TOR-signaling pathway, and (2) the response to sensor activation is relayed by a hormonal mechanism, which triggers an Inr/PI3K-dependent response in peripheral tissues (Figure 7H).

### Experimental Procedures

#### *Drosophila* Stocks

*w<sup>1118</sup>* (control in most experiments), *tGPH* (Britton et al., 2002), *UAS-dS6K* (Montagne et al., 1999), *UAS-p70<sup>S6K1-D4</sup>* (Zhang et al., 2000), *UAS-PI3K<sup>DN</sup>* (originally called *Dp110<sup>DB45A</sup>*) (Leevers et al., 1996), *UAS-dPTEN* (Huang et al., 1999), *UAS-TSC1*, *UAS-TSC2* (Tapon et al., 2001), *UAS-dTOR<sup>TEO</sup>* (Hennig and Neufeld, 2002), *dilp2-GAL4* (Rulifson et al., 2002), *ppl-GAL4* (gift from M. Pankratz), and *pGAWB-GAL4* (gift from J. Graff) are as described. *MS1096-GAL4*, *da-GAL4*, *en-GAL4*, *Df(3L)Δ1AK*, and others lines were obtained from the Bloomington stock center.

#### Genetic Experiments

*UY681* line was isolated in a gain-of-function screen (Raisin et al., 2003). Molecular characterization of the *slif<sup>f</sup>* intragenic deletion was performed by PCR analysis. The *dilp3-GAL4* line was generated as follows: a 1570 bp genomic fragment immediately 5' of the *dilp3* ORF was amplified by PCR and StuI-cloned in the modified pCasper plasmid *pC3-GAL4* (gift from M. Bourouis) to establish transgenic lines. The *pumpless-GAL4* (*ppl-GAL4*) line allows *GAL4* expression under control of the FB-specific *pumpless* promoter (Zinke et al., 1999). Expression is strongly induced in larval FB at all stages, with leaky expression in salivary glands due to some intrinsic enhancer activity in sequences 5' of the *GAL4* gene. All experiments using this driver were confirmed with *F30*, another FB-specific *GAL4* line (a generous gift from J. Graff), and repeated with a control *pGawB-GAL4* line expressing *GAL4* in the salivary glands with similar timing and intensity as *ppl-GAL4* and *F30*.

#### Molecular and Biochemical Analysis

The *slif* gene (CG11128) was identified by reverse PCR on genomic DNA isolated from the *UY681* line. For Northern analysis, affinity-purified poly(A<sup>+</sup>) RNAs were isolated from *da-GAL4>slif<sup>anti</sup>* F1 larvae grown at 25°C. S6 kinase assays were performed as reported (Radimerski et al., 2002), with addition of a protease inhibitor cocktail (Complete Mini, Roche) to the extraction buffer. Kinase activity and Western blotting of dS6K were performed as described (Oldham et al., 2000).

Additional Experimental procedures are available online as Supplemental Data.

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