**Drosophila** Cytokine Unpaired 2 Regulates Physiological Homeostasis by Remotely Controlling Insulin Secretion

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http://dx.doi.org/10.1016/j.cell.2012.08.019

**SUMMARY**

In *Drosophila*, the fat body (FB), a functional analog of the vertebrate adipose tissue, is the nutrient sensor that conveys the nutrient status to the insulin-producing cells (IPCs) in the fly brain to release *Drosophila* insulin-like peptides (Dilps). Dilp secretion in turn regulates energy balance and promotes systemic growth. We identify Unpaired 2 (Upd2), a protein with similarities to type I cytokines, as a secreted factor produced by the FB in the fed state. When *upd2* function is perturbed specifically in the FB, it results in a systemic reduction in growth and alters energy metabolism. Upd2 activates JAK/STAT signaling in a population of GABAergic neurons that project onto the IPCs. This activation relieves the inhibitory tone of the GABAergic neurons on the IPCs, resulting in the secretion of Dilps. Strikingly, we find that human Leptin can rescue the *upd2* mutant phenotypes, suggesting that Upd2 is the functional homolog of Leptin.

**INTRODUCTION**

Integration of information regarding nutrient status with other physiological processes, such as systemic growth, energy expenditure, feeding, and reproduction, is a complex function performed by multicellular organisms. Disruption of this fundamental homeostatic process can lead to a number of disorders, in particular obesity, anorexia, and diabetes (Morton et al., 2006). In addition, it can have profound effects on cancer and aging (Hurting et al., 2003).

Insulin peptides are key hormones involved in the regulation of carbohydrate and lipid metabolism, tissue growth, and longevity (Taguchi and White, 2008). Circulating insulin absorbs nutrients such as glucose and lipids, and stores them for later use in the form of glycogen and triacylglycerol (TAG). When insulin production from pancreatic beta cells is disrupted in mammals, as in the case of Type I diabetes, the body is unable to utilize the nutrients consumed and instead mounts a starvation response whereby stored lipids and glycogen are broken down to generate energy (Kahn et al., 2005). The production of insulin by pancreatic beta cells is tightly regulated to ensure that appropriate amounts are released into the blood depending on nutrient status and food intake.

The insulin pathway is highly conserved from mammals to *Drosophila* and serves fundamentally the same physiological functions (Taguchi and White, 2008; Wu and Brown, 2006). A main difference, however, is that the fly insulin-producing cells (IPCs), which are homologous to pancreatic beta cells, are found in the brain (Rulifson et al., 2002). These IPCs lie in the brain median neurosecretory cluster (mNSC) and produce at least three of the eight known *Drosophila* insulin-like peptides (Dilp2, Dilp3, and Dilp5) (Brogiolo et al., 2001; Ikeya et al., 2002). A deficiency that uncovers multiple Dilps (*Dilp1–Dilp5*) results in flies that, in addition to being smaller, have decreased TAG and increased circulating sugars (Kulkarni et al., 1997). Dilps secreted from the IPCs bind to the insulin receptor in peripheral tissues to promote growth and nutrient utilization.

The fat body (FB) functions as a key sensor of the nutritional status of the fly and couples systemic growth, metabolism, and stem cell maintenance with nutritional availability. Studies using ex vivo organ cocultures of FBs and larval brains proposed that the FB secretes growth-promoting factors (Britton and Edgar, 1998; Davis and Shearn, 1977). Suppression of amino acid (AA) transport in the FB by knocking down the AA transporter *slimfast (slif)* resulted in flies with systemic growth defects, suggesting that the FB acts as a nutrient sensor that nonautonomously modulates insulin signaling based on nutrient status (Colombani et al., 2003; Gémard et al., 2009). When flies were cultured on rich food, the amount of Dilp accumulation was considerably lower in the IPCs compared with the level of Dilps in IPCs of nutrient-deprived flies. Consistent with this, the level of Dilps in the hemolymph of fed flies was higher than in starved flies (Gémard et al., 2009). Altogether, these results suggest that the regulation of organismal growth in response to nutrient availability involves the control of insulin secretion from brain IPCs by factors originating from the FB.

Here, we identify Unpaired 2 (Upd2), a *Drosophila* cytokine, as a secreted factor produced by the FB in response to dietary fat and sugars. Upd2 activates Janus kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) signaling in...
Figure 1. Upd2 Controls Systemic Growth and Metabolism

(A) Adult flies with FB-specific knockdown of upd1, upd2, and upd3 using the ppl-GAL4 driver (ppl-GAL4 > upd-RNAi, subpanel a). RNAi of upd2 ( upd2-RNAi, indicated as upd2-i in the figure) using two independent lines (lines 1 and 2) results in size reduction of both adult males (subpanel a) and females (subpanel b). Note that in the subsequent figures, because upd2(1)-i and upd2(2)-i gave the same results, we used the upd2(1)-i line and simply refer to it as upd2-i. cg-GAL4 > upd2-RNAi third instar larvae are smaller and slimmer than control (subpanel c). Control-i is Luciferase-RNAi.

(B) Hemizygous adult males for the upd2 deletion allele ( upd2Δ) appear smaller and slimmer than the yw controls.

(C) Quantification of the wing area (subpanel a), cell size (subpanel b), and cell density (subpanel c) of upd2Δ males compared with y w control.
GABAergic neurons, relieving their inhibitory effect on the IPCs and in turn resulting in the secretion of Dilps into the hemolymph to promote systemic growth and fat storage. The similarities between Upd2 and vertebrate Leptin are striking because Leptin is secreted from the adipose tissue in mammals under conditions of nutritional surplus, and high fat levels in particular (Zhang et al., 1994). We show that human Leptin can functionally substitute for Drosophila Upd2 and can function as a ligand to the JAK/STAT receptor Dome Drosophila. Altogether, our studies illustrate how a cytokine-mediated pathway regulates the secretion of insulin to modulate systemic growth according to nutrient availability, in particular dietary fats, and provides evidence for the evolutionary conservation of this signaling module.

RESULTS

Upd2 Plays an FB-Specific Role to Control Systemic Growth

Cytokines are secreted molecules that communicate intercellular signals and thus are ideal candidates for remotely signaling nutritional status (Dinarello and Mier, 1986) and signal through the JAK/STAT pathway. In the Drosophila genome, three related Upd ligands—Upd1, Upd, and Upd3—have predicted secondary structures similar to that of type I cytokines (Boulay et al., 2003) and regulate the activity of the JAK/STAT pathway (Wright et al., 2011; Zeidler et al., 2000). They bind to the transmembrane receptor Domeless (Dome) (Brown et al., 2001), which in turn activates the JAK Hopscotch (Hop) (Binari and Perrimon, 1994). Activated Hop regulates STAT92E/Marelle (Hou et al., 1996). Because cytokines play central roles in mammalian nutrition sensing and metabolic homeostasis, we reasoned that their fly counterparts could be involved in similar processes.

To investigate the role of the Upd cytokines in the FB, and their effects on overall body size, we used RNA interference (RNAi) to knock down their expression. At least two independent RNAi lines per gene and two different FB GAL4 drivers (pol-GAL4; and cg-GAL4) were used to ensure that the knockdown was both gene and tissue specific. The efficiency of knockdown of all three genes in the larval FB was comparable (upd1, 80%; upd2, 60%; and upd3, 70%) as analyzed by quantitative PCR (qPCR). Of interest, only FB-specific knockdown of upd2, and not upd1 or upd3, resulted in smaller flies (Figures 1Aa and 1Ab) and larvae (Figure 1Ac), suggesting that this ligand alone plays an FB-specific role in regulating systemic growth. In further support of the model that the effect of Upd2 on systemic growth is specific to the FB, the knockdown of upd2 specifically in larval muscles did not affect body size (Figures S1A and S1B available online).

Previously, an upd2 homozygous deletion mutant (upd2∆) that removes the 5′UTR and the first 89 AAs was identified and reported to be viable and fertile (Hombría et al., 2005). An examination of the growth phenotypes of upd2∆ flies, compared with an age- and population-density-matched control, showed that the upd2∆ flies were significantly slimmer and smaller (Figure 1B), similar to the phenotype generated with FB-specific knockdown of upd2 (Figure 1Ab). An examination of the wings, a tissue that can be easily quantified for growth phenotypes, revealed that upd2 mutant wings have a 10% reduction in overall size (Figure 1Ca) and a significant reduction in both cell number (Figure 1Cb) and cell size (Figure 1Cc). Expression of an upd2 cDNA in the FB of upd2∆ flies was able to rescue the wing size phenotype (Figure 1D), providing further evidence that upd2 plays an FB-specific role in the regulation of systemic growth.

Upd2 Plays an FB-Specific Role to Control Metabolism

To test whether Upd2 plays a role in regulating energy metabolism, we measured the levels of triacylglycerol (TAG) in larvae with FB-specific knockdown of upd2. TAG levels were significantly reduced (Figure 1E). Similarly, upd2∆ larvae also showed a significant reduction in TAG levels (data not shown). Knockdown of upd2 specifically in other tissues, such as larval muscles, did not affect stored fat levels or body size (Figure S1), consistent with the model that the function of Upd2 is specific to the FB.

In Drosophila, organismal growth is restricted to larval stages, and genetic manipulations that affect nutrient sensing during adulthood lead to metabolic phenotypes. To assay whether Upd2 plays a specific role in nutrient sensing in adults, we used a GAL4 driver that is expressed only in the adult FB (yolk-GAL4; see Experimental Procedures) and measured TAG levels in flies 15 days after eclosion. FB-specific knockdown of upd2 (Figure 1G) was associated with an increase in the amount of circulating sugars in the hemolymph, suggesting that Upd2 plays a role in the FB to regulate overall energy metabolism. Consistently, upd2∆ adults displayed an increase in the levels of circulating sugars (Figure 1H), and a significant reduction in TAG levels (Figure 1I) that could be rescued by expressing an upd2-cDNA in the FB (Figure 1J). Of interest, knockdown of stat92E, the transcription factor that mediates JAK/STAT pathway activity, in the FB (efficiency of knockdown in the FB as assayed by qPCR > 90%) did not affect TAG storage (Figure 1F), indicating that Upd2 plays a nonautonomous role in regulating fat storage.

Altogether, these results indicate that Upd2 in the FB regulates systemic growth in larvae and energy metabolism in both larvae and adults. Further, the effect of Upd2 is likely FB
nonautonomous, as removal of stat92E from the FB is not associated with an effect on systemic growth.

**Upd2 Signals the Fed State and Senses Fat and Sugars**

To explore the relationship between Upd2 expression in the FB and the nutritional state, we examined upd2 transcript levels under starvation. Strikingly, we found that upd2 transcripts showed a 98% reduction (Figure 2A) in adult wild-type males that were starved on 1% sucrose agar for >72 hr. Because a reduction in size and energy metabolism could result from reduced nutritional intake, we measured fly feeding using both blue dye (Xu et al., 2008) and capillary feeder (CAFE) assays (Ja et al., 2007). Both tests, performed in three to five replicates on age- and population-density-matched adult male flies, showed that neither the FB-specific knockdown of upd2 nor the upd2 Δ flies had feeding-behavior defects (Figures 2B and 2C).

To assess whether upd2 senses the nutritional state of the organism, we analyzed lipid storage in hepatocyte-like cells/oenocytes that accumulate lipid droplets only under conditions of nutritional deprivation and not under a fed state (Gutierrez et al., 2007). Whereas the control larvae did not accumulate lipid droplets under fed conditions (Figure 2D), upd2 Δ larvae showed a striking accumulation of lipid droplets in oenocytes, as assayed by Oil Red O staining, even under conditions of nutritional surplus (Figure 2D). Because upd2 Δ flies feed normally, this lipid accumulation suggests that Upd2 is required to sense the appropriate nutritional state.

Given that knocking down the AA transporter silf mimics the starvation state (Colombani et al., 2003; Gutierrez et al., 2007), we examined the upd2 steady-state mRNA levels in FB-specific silf knockdown (upd1-GAL4 > UAS-silfΔam) by qPCR. Strikingly, the upd2 level increased by >2-fold, whereas another JAK/STAT ligand, upd1, and the downstream component STAT92E were not significantly altered (Figure 2E). Silf plays a role in transporting AAs, and its absence results in a protein-deprived state. Hence, the upregulation of upd2 under this state (Figure 2E) suggests that the upstream signal for upd2 expression is not nutrition-derived proteins.

We next assayed upd2 expression in adult male flies subjected to diets rich in protein, fat, or sugar, and compared upd2 mRNA levels under these conditions with respect to standard lab food. In many independent experiments, we consistently found that 5 days after exposure to high-fat and high-sugar diets, the normalized steady-state level of upd2 mRNAs went up by 4- to 6-fold, a statistically significant difference compared with levels obtained on standard lab food (Figure 2F). Taken together, these results strongly argue that upd2 senses the fed state downstream of fats and sugars.

To address whether Upd2 is sufficient to signal the fed state, we tested whether overexpression of upd2 in wild-type flies can suppress the starvation response associated with nutrient deprivation. We measured the breakdown of stored fat in starved flies overexpressing upd2 in the FB using thin-layer chromatography (TLC). TLC was performed on adult flies to measure the fat stores after a period of 24 hr on 1% agar. Whereas in control flies the stored fat levels were nearly depleted after 24 hr (Figure 2G; 98% reduction), the reduction was not significant in flies overexpressing upd2 in their FB (Figure 2G; 35% reduction). Thus, Upd2 can suppress stored fat breakdown under conditions of starvation, indicating that it signals a fed condition even when flies are deprived of nutrients.

Brain IPCs secrete Dilps in response to nutrition and accumulate Dilps under conditions of nutrient deprivation (Géminard et al., 2009). To test whether Upd2 overexpression in the FB can suppress accumulation of Dilps in the IPCs under conditions of starvation, age- and population-density-matched adult flies overexpressing Upd2 in the FB were starved for 24 hr on 1% agar. Then we dissected and stained the brains for Dlp2 and Dlp5, analyzed them by confocal microscopy, and calculated the mean Dilp fluorescence. Strikingly, Dlp2 and Dlp5 accumulation was significantly lower in brains of starved flies overexpressing Upd2 in their FB (Figure 2H, 78% less Dlp2; data not shown for Dlp5), revealing that Upd2 expression in the FB alters Dlp2 and Dlp5 accumulation in brain IPCs in response to the nutritional state. Note that in the experiments described below, the same results were observed for both Dlp2 and Dlp5. For simplicity, only the Dlp2 data are shown, and in the text Dlp2 and Dlp5 are referred to as Dilps.

The upd2 Δ mutants and FB knockdown of upd2 resemble the dlp1–dlp5 deletion flies with respect to their size and metabolic phenotypes. Hence, we tested whether the primary role of Upd2 is to signal the fed state to the IPCs. Two different FB-specific drivers, cg-GAL4 (Figure 3A) and ppi-GAL4 (data not shown), were used for these experiments. RNAi against upd2 in the FB resulted in a statistically significant increase in mean Dilp fluorescence in larval brains (Figure 3A). This increase in Dilp accumulation was most apparent when the larvae were actively feeding. We performed a qPCR analysis of Dilp in the brains of larvae with FB-specific knockdown of upd2 RNAi to ensure that the increase in mean Dilp fluorescence was not a result of increased transcription. No significant change in Dilp transcription was observed (Figure S2). In addition, FB-specific knockdown of either stat92E or upd1 did not result in increased accumulation of Dilp (Figure 3A), which is consistent with our observations regarding body size and metabolism (Figure 1). Further, the increase in Dilp accumulation in the IPCs was also observed in both larval (data not shown) and adult upd2 Δ homozygous mutant brains (Figure 3B). Finally, the accumulation of Dilp in the IPCs of upd2 Δ homozygous mutants could be rescued by expressing upd2 cDNA in the FB (Figure 3Bc), indicating that the inability to release Dilps underlies both the systemic growth and energy metabolism phenotypes of upd2 Δ mutants.

IPCs in the Drosophila brain use membrane voltage-dependent neurosecretory mechanisms to facilitate Dilp release in response to nutrition (Géminard et al., 2009). Thus, if Upd2 promotes Dilp secretion under fed conditions, activating Dilp secretion in upd2 Δ homozygous mutant brains should rescue the upd2 Δ growth phenotype. To achieve this, we depolarized the IPCs by expressing the bacterial sodium channel (NaChBac) (Luan et al., 2006; Ren et al., 2001) under the control of the Dlp3-GAL4 driver, which drives expression specifically in the brain IPCs during both larval and adult stages. Under these conditions, the level of Dilp accumulation in the IPCs of upd2 Δ was significantly reduced (Figure 3C). In addition, this artificial depolarization of the IPCs in an upd2 Δ homozygous mutant background rescued the small size of upd2 Δ mutants.
Figure 2. Upd2 Expression in the FB Signals the Fed State

(A) The steady-state mRNA expression of STAT92E and upd2 was analyzed by qPCR.
(B) Blue dye feeding assay in adult males with FB-specific knockdown of upd2.
(C) CAFE assay in adult males with FB-specific knockdown of upd2 or upd2D.
(D) Oil Red O staining of oenocytes in third instar male larvae shows that upd2D animals accumulate more Oil Red O droplets under fed conditions compared with yw controls.
(E) Steady-state mRNA levels in adult males with FB-specific knockdown of slif compared with control (UAS-slifanti).
(F) qPCR performed on RNA extracted 5 days after adult males were exposed to different diets. The normalized expression level of upd2 is shown.
(G) TLC was used to assay the amount of stored TAG in adult male flies under fed and starved conditions. TAG was extracted from ppl-GAL4 > 10D4 upd2-cDNA or GFP (control) adult males. S indicates the mobility of coconut oil used as a marker in the TLC plate.
(H) Dilp2 immunostaining in the IPCs of starved adult male flies expressing GFP (control) or 10D4 upd2-cDNA using ppl-GAL4. Black error bars represent the SD. Blue error bars represent the standard error of the mean; p values were calculated using Welch’s t test (**p < 0.01, ***p << 0.0001).
Figure 3. Upd2 Remotely Controls Dilp Accumulation in IPCs

(A) Maximum intensity XY projection (MIP) of cg-GAL4 male larval brains stained with Dilp2 driving the expression of either white-RNAi (control) or upd2-RNAi, and quantification of the mean Dilp2 immunofluorescence in larval brains of cg-GAL4 animals driving RNAi of JAK/STAT pathway components.

(B) MIP of adult male brains stained with Dilp2. The brains were dissected from age-matched upd2 and yw control (subpanel a). A significant increase in Dilp2 fluorescence is observed in upd2 male brains compared with yw (subpanel b). In subpanel c, rescue of Dilp2 accumulation in upd2 flies by FB-specific expression of the 10D4 upd2 cDNA using cg-GAL4. Brains from cg-Gal4 flies were used as positive controls (gray bar).
(Figure 3D). Of interest, hyperpolarizing the neurons by expressing the potassium channel rectifier EKO (White et al., 2001) did not exacerbate the upd2Δ size phenotype (Figure 3D), indicating that in the absence of upd2, the IPC neurons are already depolarized. In summary, the reduction in TAG levels, increase in circulating sugars, and systemic reduction in body size in the absence of upd2 resemble a reduction in insulin signaling, suggesting that the primary role of Upd2 is to remotely control the secretion of Dilps from the IPCs in response to nutrient intake.

### A STAT Reporter Is Expressed near the IPCs and Is Altered in Response to Starvation

To elucidate how Upd2 regulates Dilp secretion in response to nutrition, we examined the activity of a STAT reporter (10XSTAT::GFP; see Experimental Procedures) that was previously shown to recapitulate JAK/STAT pathway activation (Bach et al., 2007) in the adult brain. We found that STAT::GFP is widely expressed throughout the adult brain in both neurons and glia (Figure S3), and more specifically, it is expressed in the olfactory lobe (Figure 4A, asterisk), the optic lobe (Figure 4A, hash), and the mNSCs (a region that includes the IPCs plus other neurons; see arrow in Figure 4A) of the adult brain. Of importance, an analysis of single optical sections revealed that the STAT reporter is expressed in neurons juxtaposed with the IPCs (arrow, Figure 4B) in the mNSC region of the adult brain and in the larval brain (Figure S4).

Next, we addressed whether the expression of the STAT reporter is altered in response to nutritional deprivation. Under fed conditions, the STAT reporter is expressed in the cell body of neurons immediately adjacent to the Dilp-expressing IPCs (Figure 4C, red arrow). In addition, the STAT reporter is expressed along the neurites that run parallel to the median arborizations of the IPCs (Figure 4C, yellow arrow). Along the neurites, the reporter is enriched in vesicular structures that colocalize with the presynaptic marker Synapsin (Klagges et al., 1996; Figure S5), suggesting that they may correspond to the sites of synaptic contact with the IPCs. Following nutrient deprivation (adult males kept on 1% sucrose agar at 25°C for >72 hr), STAT reporter expression in neurons juxtaposed with the IPCs is altered, and both the expression in the cell body (Figure 4D, red arrow) and the vesicular enrichment along the median branches of the IPCs is reduced or lost (Figure 4D, yellow arrow). Altogether, the alterations in reporter activity in these neurons in response to the nutrient status suggest that they influence Dilp secretion in the fed condition.

If Upd2 controls Dilp levels in the IPCs by activating STAT signaling in the mNSC neurons, then compromising upd2 function should phenocopy the effect of starvation on STAT reporter expression. We analyzed STAT reporter expression in the adult brains of flies that express upd2-RNAi in the FB and compared it with a luciferase-RNAi control. Indeed, although the expression of the STAT reporter in the controls was very similar to that observed in the fed condition (Figure 4E), both the expression of the reporter in the cell body (red arrow, Figure 4F) and the vesicular enrichment along the IPCs (Figure 4F, yellow arrow) were much reduced or lost when upd2 was knocked down in the FB, as observed under starvation.

### STAT Signaling in mNSC Neurons Regulates Systemic Growth and Metabolism

To further test the model that JAK/STAT signaling plays a role in the mNSCs to regulate systemic growth and metabolism, we assayed whether knocking down STAT92E in mNSC neurons would affect systemic growth and fat storage. To knock down STAT92E, we used dome-GAL4 (Mandal et al., 2007), which we found drives expression in a number of neurons in the adult brain, including STAT-reporter-positive mNSCs (Figure 5A, white arrow). At 25°C, dome-GAL4 > stat92E-RNAi adults to 29°C for 7 days to allow for a more significant knockdown of STAT92E expression, and assayed for stored fat by performing a TLC assay. Strikingly, the level of stored fat was significantly lower in flies with compromised STAT92E function (Figure 5C). A similar experiment performed with upd2-RNAi flies failed to reveal a role for Upd2 in the dome-GAL4 neurons, as there was no effect on stored fat (Figure 5C) or body size (data not shown).

To examine whether compromising the function of the receiving end of the JAK/STAT pathway results in increased Dilp accumulation in the IPCs, we used dome-GAL4; tub-GAL80Ts to express stat92E-RNAi or dome-RNAi only during adulthood (see Experimental Procedures). We assayed the expression of Dilp2 in the IPCs of these flies 7 days after transfer at the restrictive temperature and compared it with a GFP-RNAi control. Dilp2 accumulation significantly increased when stat92E or dome function was compromised in adults (Figure 5D), suggesting that the JAK/STAT pathway plays a role in promoting Dilp2 secretion from the IPCs.

The dome-GAL4 driver is expressed not only in the brain but also in other tissues of the fly (e.g., gut and muscle). Hence, to address whether JAK/STAT signaling in the mNSC neurons activates/depolarizes or inhibits/hyperpolarizes neuronal activity, we expressed the bacterial sodium channel (NaChBac) using dome-GAL4 at the restrictive temperature (dome-GAL4; tub-GAL80Ts > NaChBac). This forced depolarization resulted in reduced fat storage (Figure 5E) and increased Dilp2 accumulation (Figure 5F), suggesting that activation of the JAK/STAT pathway in mNSC neurons prevents their neuronal firing.

### JAK/STAT Plays a Role in Neurons Expressing Vesicular GABA Transporter to Modulate Systemic Growth and Metabolism

IPCs express receptors for serotonin and GABA, and both of these neurotransmitters exert an inhibitory effect to prevent...
Figure 4. STAT Reporter Expression in the Adult Brain and its Response to Starvation

(A) Anterior (subpanel a) and posterior (subpanel b) MIPs of adult male brains expressing the 10XSTAT::GFP reporter. nc82 (magenta), a presynaptic active-zone protein marker, identifies the neuropils. STAT::GFP (green) is widely expressed, including in the olfactory lobe (asterisk), optic lobe (hash), and mNSCs (arrow).

(B) Single optical section along the XY axis of an adult male brain immunostained for Dilp2 (magenta) and STAT::GFP (green). Note that the cell bodies of the STAT reporter-expressing neurons are located right next to the Dilp2-expressing IPCs (arrow points to the juxtaposition of these neurons).

(C–F) Three-dimensional projections along the XY axis of adult male brains stained for Dilp2 (magenta) and STAT::GFP (green).

(C) Under fed conditions, the STAT::GFP reporter is expressed in neurons immediately adjacent to the Dilp2-expressing IPCs (red arrow). The STAT reporter is expressed along the neurites that run parallel to the median arborizations of the IPCs (yellow arrow).

(D) Under starved conditions, the GFP reporter expression is reduced in the cell body (red arrow) and along the neurites (yellow arrow). Dilp2 fluorescence in the IPCs is increased under starved conditions (D) compared with fed conditions (C).
Dilp release (Enell et al., 2010; Luo et al., 2012). Thus, we examined whether the cells in the mNSCs that express the STAT reporter could exert their effects through those neurotransmitter systems. However, although neurons that express the STAT reporter do not express the GABA receptor (Figure 6A), a subset of them expressed the Drosophila vesicular GABA transporter (dVGAT; Figure 6B, yellow arrows) that is required for GABA release (Fei et al., 2010). We found that reducing the activity of dome and STAT in dVGAT neurons affected systemic growth at 25°C (Figure 6C). Next, we assayed the level of stored fat in dVGAT-GAL4 > dome-RNAi adult flies at 29°C (because high levels of lethality were observed for dVGAT-GAL4 > STAT-RNAi at this temperature, we analyzed dome flies in the TAG and Dilp assays). The knockdown of dome in dVGAT neurons resulted in a significant decrease in stored fat (Figure 6D) and an increase in Dilp2 accumulation in the IPCs of adult females (Figure 6E), indicating that JAK/STAT signaling in GABAergic neurons relieves their inhibitory effect on IPCs. Consistent with our relay model, loss of JAK/STAT components in the Dilp neurons themselves did not affect systemic growth (data not shown) or metabolism (as assayed by stored fat; Figure S6).

**Human Leptin Can Functionally Substitute for Upd2 by Signaling via the JAK/STAT Receptor Dome**

Upd2 encodes a type I cytokine that presents structural features similar to those of Leptin (Boulay et al., 2003). Because our finding that Upd2 influences insulin secretion by impinging on STAT signaling in GABAergic neurons in order to disinhibit Dilp neurons is reminiscent of a recent finding that Leptin activates STAT signaling in GABAergic neurons and in turn results in the release of STAT reporter expression in the brain (F) compared with Luciferase-RNAi control (E). Note that the bouton-like enrichment of the STAT::GFP reporter along the neurites, juxtaposed with the IPCs, is lost in flies expressing FB-specific upd2-RNAi (compare yellow arrow in E with F). STAT::GFP expression in the cell body is reduced in flies expressing FB-specific upd2-RNAi (compare red arrow in E with F). Dilp2 fluorescence is increased in the IPCs of adult males expressing FB-specific upd2-RNAi compared with control (compare E with F).

See also Figures S3, S4, and S6.
Figure 6. Role of the JAK/STAT Pathway in GABAergic Neurons Affects Systemic Growth and Metabolism by Influencing Dilp2 Accumulation in the IPCs

(A) XY slice of the mNSC region of 10XSTAT::GFP male adult brains expressing dsRed2 under the control of GABA-B-R2-GAL4. Immunostaining for Dilp2 (blue), GFP (green, STAT::GFP expression), and dsRed2 (red, GABA-B-R2 expression domain) shows that Dilp2 IPCs are positive for the GABA receptor, and that STAT reporter neurons do not overlap with the GABA-B-R2-GAL4 expression domain.

(B) XY slice of the mNSC region of 10XSTAT::GFP male adult brains expressing the membrane-associated dendritic marker DenMark under the control of the dVGAT-GAL4 driver. Immunostaining for Dilp2 (blue), GFP (green, STAT::GFP expression), and DenMark (red, dVGAT expression domain) shows that STAT reporter neurons are positive for the vesicular GABA transporter dVGAT (yellow arrow).
deregression of a neuronal group called proopiomelanocortin (POMC) neurons, allowing them to fire (Vong et al., 2011), we tested the possibility that Leptin can substitute for Upd2. We generated transgenic flies that expressed human Leptin under the control of the UAS promoter (see Experimental Procedures) and expressed this transgene in the FB of upd2Δ flies. Strikingly, in similarity to flies expressing upd2 cDNA in the FB, human Leptin cDNA was able to rescue the growth (Figure 7A), fat storage (Figure 7B), and Dilp2 accumulation phenotypes (Figure 7C) of upd2Δ flies. In addition, as documented in previous studies in mice (Caton et al., 2011; Ni et al., 2008; Rodgers and Shearn, 1977), injecting a physiologically relevant dose of recombinant human Leptin (see Experimental Procedures) into upd2Δ flies significantly reduced the accumulation of Dilp2 in upd2Δ brains (Figure 7D) and solved the primary defect underlying a loss of Upd2 function. These results suggest that exogenous Leptin signals through the same pathway as Upd2.

To test whether human Leptin is a bona fide ligand of the JAK/STAT pathway receptor Dome, we used a well-established JAK/STAT reporter assay (10XSTATLuc) in Drosophila Kc cells (Baeg et al., 2005; Hombria et al., 2005; Wright et al., 2011). Drosophila Kc cells were transfected with 10XSTATLuc and Actin-promoter driving Renilla luciferase (Act-Renilla) together with dsRNAs against dome or control (GFP) or without dsRNA treatment. The cells were then incubated with 4 nM recombinant human Leptin, a concentration at which Upd ligands effectively activate the Dome receptor, only Upd2 plays a significant role in communicating the nutritional status from the FB. This is somewhat surprising, because all three Upd ligands are secreted JAK/STAT pathway agonists and are able to activate the JAK/STAT pathway nonautonomously in vivo. However, the signal sequences of the different Ups appear to confer different biophysical properties upon them, as illustrated by tissue-culture assays showing that although Upd1 and Upd3 associate primarily with the extracellular matrix, Upd2 is easily detectable in the media (Wright et al., 2011). In addition, secretion assays showed that Upd2 is able to condition tissue-culture media more potently than either Upd1 or Upd3. Altogether, these results suggest that Upd2 activates JAK/STAT signaling at greater distances than Upd1 or Upd3.

As evidenced by the growth and metabolic phenotypes of FB-specific knockdown, Upd2 seems to be required only in the FB; however, the reason for this tissue specificity is unclear. In a previous study, Hombria et al. (2005) analyzed the Upd2 protein using a hidden Markov model, and suggested that Upd2 is probably not secreted via the classical Golgi-ER machinery because it lacks a signal peptide. In fact, other type I cytokines involved in interorgan crosstalk also lack the signal peptide and are secreted by unconventional secretory pathways (Haas et al., 2011). Thus, a possible explanation for the tissue specificity of Upd2 is that the FB is the only tissue that can secrete this protein in an active form. Future work, contingent on the development of techniques and reagents to detect Upd2 in the fly hemolymph, will clarify this issue.

**DISCUSSION**

Previous investigators have postulated the existence of secreted factors produced by the FB that stimulate systemic growth by stimulating cell proliferation, and proposed that the FB (the fly nutrient sensor) couples Dilp secretion from brain IPCs depending on the nutritional status (Britton and Edgar, 1998; Colombani et al., 2003; Davis and Shearn, 1977; Gémardin et al., 2009). Here, we show that the JAK/STAT ligand Upd2, a type 1 cytokine signal, is involved in the interorgan communication between the FB and the brain IPCs. We demonstrate that human Leptin can rescue the upd2 mutant phenotypes, which implies that an invertebrate model system can be used to address questions pertaining to Leptin biology.

**Upd2 Plays an FB-Specific Role in Communicating the Fed State to the Brain IPCs**

Upd proteins have secondary structures that are predicted to have α helices similar to that of type I cytokines belonging to the IL-6 family, and sequence alignments suggest that they show some similarity to vertebrate Leptins (Boulay et al., 2003; Harrison et al., 1998). Among the three Upd ligands that activate the Dome receptor, only Upd2 plays a significant role in communicating the nutritional status from the FB. This result is somewhat surprising, because all three Upd proteins are secreted JAK/STAT pathway agonists and are able to activate the JAK/STAT pathway nonautonomously in vivo. However, the signal sequences of the different Ups appear to confer different biophysical properties upon them, as illustrated by tissue-culture assays showing that although Upd1 and Upd3 associate primarily with the extracellular matrix, Upd2 is easily detectable in the media (Wright et al., 2011). In addition, secretion assays showed that Upd2 is able to condition tissue-culture media more potently than either Upd1 or Upd3. Altogether, these results suggest that Upd2 activates JAK/STAT signaling at greater distances than Upd1 or Upd3.

As evidenced by the growth and metabolic phenotypes of FB-specific knockdown, Upd2 seems to be required only in the FB; however, the reason for this tissue specificity is unclear. In a previous study, Hombria et al. (2005) analyzed the Upd2 protein using a hidden Markov model, and suggested that Upd2 is probably not secreted via the classical Golgi-ER machinery because it lacks a signal peptide. In fact, other type I cytokines involved in interorgan crosstalk also lack the signal peptide and are secreted by unconventional secretory pathways (Haas et al., 2011). Thus, a possible explanation for the tissue specificity of Upd2 is that the FB is the only tissue that can secrete this protein in an active form. Future work, contingent on the development of techniques and reagents to detect Upd2 in the fly hemolymph, will clarify this issue.

**Existence of Nutrient-Specific FB-Derived Signals**

The identification of Upd2 as a nutrient-regulated signal from the FB that does not depend on AAs but is produced in response to dietary fats and sugars reveals that different nutrient-specific secreted factors exist in the fly. Of interest, the upregulation of upd2 levels in FB knockdown of silf suggests the existence of a homeostatic feedback loop whereby Upd2, in the context of low protein, promotes the utilization of fat and carbohydrate energy sources. High-sugar diets in flies have been shown to
Figure 7. Human Leptin Can Rescue the Upd2Δ and Signals through Domeless
(A) Quantification of wing area in upd2Δ males with FB-specific expression of upd2 cDNA (10D4) and human Leptin cDNA.
(B) The amount of TAG measured by TLC is significantly increased in adult males expressing human Leptin cDNA in the FB.
(C) Quantification of Dilp2 accumulation in FB-specific expression of the 10D4 upd2 cDNA and human Leptin cDNA in upd2Δ background. Brains from cg-Gal4 flies were used as positive controls (gray bar).
(D) MIP of upd2Δ adult male brains stained with Dilp2 after injection of human Leptin or control (PBS). The difference in Dilp2 fluorescence is quantified.
(E) Response of the 10XSTATLuc reporter in Kc cells upon incubation with 4 nM human Leptin. The ratio of Renilla to firefly luciferase activity is measured at regular intervals from 24–72 hr and quantified as a percent fold change in relative units with respect to cells incubated with media alone in the presence of no dsRNA, control, or dome dsRNA.
Error bars represent the SD; p values were calculated using the t test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.00001). See also Figure S7.
trigger a lipogenesis program akin to high-fat diets in mammals (Musselman et al., 2011; Zhou et al., 2005), suggesting that Upd2 is most likely downstream of signals that are produced by increased fat stores. This is a highly significant finding because it brings into question the broadly prevailing view that one dominant secreted factor downstream of AAs governs nearly all aspects of systemic growth and metabolism in flies. Our findings support a model in which the fly FB secretes numerous factors that regulate systemic growth and metabolism downstream of various components of the fly diet.

**JAK/STAT Activation in GABAergic Neurons and Parallels the Adipose-Hypothalamic Circuit in Mammals**

Our results indicate that STAT activation in GABAergic neurons inhibits their firing. Previous work implied that the GABA-B-receptors in Dilp neurons inhibit Dilp release (Enell et al., 2010). Given that these GABAergic neurons are presynaptic to the IPCs, we propose that activation of STAT in GABAergic neurons relieves the IPCs from repression, thus resulting in Dilp release. This is reminiscent of the observation that first-order neurons that respond to adipose-derived Leptin are inhibitory GABAergic neurons expressing LepRs (Vong et al., 2011). When LepRs are activated by Leptin, they regulate Stat3 phosphorylation, which inhibits the firing of the GABAergic neurons via an unknown mechanism. This in turn relieves the repression on the POMC neurons, allowing them to fire (Vong et al., 2011). Thus, this circuit module is strikingly similar to what we observe in the fly.

Many questions remain to be resolved regarding the signaling mechanisms by which the JAK/STAT pathway regulates GABAergic neurons. The target(s) of the JAK/STAT pathway in regulating neuronal firing in mammalian GABAergic neurons remains to be identified (Vong et al., 2011). It was suggested that Leptin activation of STAT signaling may be required for the long-term effects of Leptin’s action on energy homeostasis, rather than for the acute effects of Leptin (Karsten et al., 2006), and that the acute effects of Leptin on the membrane potential of certain neuronal groups require activation of PI3-K signaling rather than STAT (Schober et al., 2005). However, the role of JAK/STAT versus PI3-K signaling in modulating the electrophysiology of the presynaptic GABAergic neurons has not yet been clarified (Vong et al., 2011), in large part because previous studies were done on non-GABAergic neuronal groups. Further investigations into the role of JAK/STAT signaling in modulating neurotransmission in GABAergic neurons will be necessary to clarify how JAK/STAT signaling regulates their activities. Of importance, based on the similarity of the circuits and the conservation of the signaling pathways, studies in the fly are likely to provide insights relevant to mammalian neurophysiology.

**Parallels between Upd2 and Leptin**

The physiology of Leptin signaling is undoubtedly more complex in vertebrates than in flies, and differs in several ways. For example, upd2Δ mutant flies are smaller and leaner, whereas mutations in Leptin in mammals are associated with obesity. However, there are some striking parallels. We find that under starvation, upd2 mRNA steady-state levels drop significantly (Figure 2A), and there is a significant increase of upd2 mRNA expression under high-fat diets (Figure 2F). This is similar to the alteration in Leptin levels observed during starvation and high-fat diets by Ahima et al. (1996). These authors examined the role of Leptin in the physiology of starvation by providing mice with exogenous Leptin during periods of nutrient restriction, and found that the primary physiological role of Leptin is to regulate the neuroendocrine system during starvation. Leptin reduced the animals’ reproductive capacity and increased stress hormone levels, which in turn increased the survival capacity of the organism under adverse nutrient conditions (Ahima et al., 1996). Consistent with this, flies with ablated IPCs, which are unable to produce insulin, were shown to perform much better under starvation conditions and increased stress conditions (Broughton et al., 2005). Given that the role of Upd2 is to promote insulin secretion, the reduction of Upd2 levels during starvation should decrease Dilp secretion and increase the chances of survival under starvation (upd2Δ mutants were more starvation resistant than the wild-type controls; Figure S7B). Hence, in this context, the primary physiological roles of Upd2 and Leptin converge.

**EXPERIMENTAL PROCEDURES**

**Drosophila Strains and Diets**

Details about the fly strains, transgenic fly construction, standard lab food composition, and temperatures for specific crosses can be found in Extended Experimental Procedures.

**Triglyceride Measurements**

Triglyceride assays were performed as previously described (Al-Anzi et al., 2009). Further details can be found in Extended Experimental Procedures.

**Hemolymph Glucose Measurements**

Glucose concentration in the hemolymph was quantified as described previously (Géminard et al., 2009).

**qPCR**

Total RNA was prepared from triplicates of 15 fed (standard lab food) or starved (>72 hr on 1% sucrose agar) age-matched adult males at 25°C. cDNA was prepared using 1 μg RNA, and qPCR was performed using iQ SYBR Green Supermix. SYBR Green Supermix. alpha-tubulin and rp49 were used to normalize the RNA levels. Relative quantification of mRNA levels was calculated using the comparative CT method. See Extended Experimental Procedures for information on the oligos.

**Feeding Assays**

The blue dye feeding assay was adapted from Xu et al. (2008). The CAFE assay was performed as described by Ja et al. (2007).

**Oil Red O Staining**

Oil Red O staining was performed as previously described (Gutierrez et al., 2007; Palanker et al., 2009).

**Immunostaining, Confocal Imaging, and Analysis**

Immunostaining of larval and adult brains was performed based on protocols described by Pfeffer et al. (2010). Images were captured with the use of Leica SP2 and Zeiss LSM 780 confocal systems, and analyzed using Zeiss ZenLite 2009, Leica LAS AF lite, and ImageJ. To calculate the intensity of Dilp staining, the mean gray value was calculated from maximum intensity projections (MIPs) of a similar number of confocal stacks using ImageJ. For details on the antibodies used, see Extended Experimental Procedures.
**Leptin Injections**

Human recombinant Leptin (#L4146-1MG; Sigma Aldrich) was dissolved per the package instructions in a solution of HCl and NaOH to a final concentration of 1 μg/μl to prepare the human Leptin stock solution, which was then frozen in aliquots at -20°C. Fresh aliquots were thawed on ice before each experiment. The working solution was made by diluting the human Leptin stock solution in PBS to a final concentration of 0.001 μg/μl and injected into 10-day-old adult males of y w and upd2 J. PBS was injected into mock controls. The injections were done by inserting the needle at the junction of the thorax and abdomen close to the haltere using a microinjector (Femtojet; Eppendorf) at a pressure of 305 kPa, using needles pulled in a pipette puller (vertical pipette puller, model 720; Kopf) at a heater setting of 14.1. At least 25–20 flies were injected per condition. The flies were allowed to recover for 24 hr and then the brains were dissected, stained, and analyzed, and Dilp2 levels were quantified as outlined in the previous section. A minimum of three to five brains were analyzed per experiment. The experiments were performed three independent times.

**Tissue-Culture and Luciferase Assays**

Drosophila Kc cells were maintained in Schneider’s medium (GIBCO), 10% heat-inactivated fetal bovine serum (FBS; Sigma), and 5% Pen-Strep (GIBCO) at 25°C. Experiments were run in 96-well plates (six replicates per condition), and 150 ng/μl of the appropriate dsRNA were seeded in the wells before the start of the experiment. Each well was transfected with 0.05 ng 10XSTATLuc, 14 ng Act-RENilla, and 106 ng pAC-PL (used as carrier DNA). Then, 96 hr after transfection, media were supplemented with 4 nM human recombinant Leptin or control media supplemented with no Leptin were added to the wells. Luciferase activity was measured using DualGlo reagents (Promega) per the kit instructions and measured using the Analyst GT plate reader. For details about the amplifiers used for dsRNA production, refer to Extended Experimental Procedures.

**Statistical Analysis**

Statistical analysis was performed using Welch’s t test with Excel.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.08.019.

**ACKNOWLEDGMENTS**

We thank M. Zeidler, E. Bach, B. Hassan, B. White, B. Al-Anzi, M. Panikratz, P. Shen, J. Wang, D. Krantz, J. Simpson, A. Gould, E. Hafen, H. Stocker, P. Leopold, G. Technau, and E. Olson for reagents. We thank the Transgenic RNAI Resource Project, NIG-Fly Stock Center (Japan), and the Bloomington Stock Center for flies, the Developmental Studies Hybridoma Bank for monoclonal antibodies, and the Drosophila RNAI Screening Center (Harvard Medical School) for dsRNA reagents and plate-reader equipment. We also thank C. Villalta for performing embryonic microinjections to create the human Leptin transgenic flies, F. Demonis and C. Pitsouli for critical comments on the manuscript, Y. Kwon for advice on the STAT luciferase assay, I. Droujinine for discussions and help with the diet experiments, and Y. Kwon for advice on the STAT luciferase assay. For details about the amplifiers used for dsRNA production, refer to Extended Experimental Procedures.

Received: March 13, 2012
Revised: June 1, 2012
Accepted: August 6, 2012
Published: September 27, 2012

**REFERENCES**


