Gbb/BMP signaling is required to maintain energy homeostasis in *Drosophila*

Shannon L. Ballard 1, Jana Jarolimova, Kristi A. Wharton *

Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02912, USA

**A R T I C L E  I N F O**

Article history:
Received for publication 10 June 2009
Revised 19 October 2009
Accepted 6 November 2009
Available online 13 November 2009

Keywords:

**A B S T R A C T**

The coordination of animal growth and development requires adequate nutrients. During times of insufficient food, developmental progression is slowed and stored energy is utilized to ensure that cell and tissue survival are maintained. Here, we report our finding that the Gbb/BMP signaling pathway, known to play an important role in many developmental processes in both vertebrates and invertebrates, is critical in the *Drosophila* larval fat body for regulating energy homeostasis. Animals with mutations in the *Drosophila* BMP-5,7 orthologue, *glass bottom boat* (gbb), or in its signaling components, display phenotypes similar to nutrient-deprived and *tor* mutant larvae. These phenotypes include a developmental delay with reduced overall growth, a transparent appearance, and altered total lipid, glucose and trehalose levels. We find that Gbb/BMP signaling is required in the larval fat body for maintaining proper metabolism, yet interestingly, following nutrient deprivation larvae in turn show a loss of BMP signaling in fat body cells indicating that Gbb/BMP signaling is a central player in homeostasis. Finally, despite strong phenotypic similarities between nutrient-compromised animals and gbb mutants, distinct differences are observed in the expression of a group of starvation responsive genes. Overall, our results implicate Gbb/BMP signaling as a new pathway critical for positive regulation of nutrient storage and energy homeostasis during development.

**Introduction**

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily of signaling molecules. These superfamily members generate secreted bioactive ligands that regulate critical cellular processes during the development of both vertebrates and invertebrates ([Chen et al., 2004; Herpin et al., 2004; Hogan, 1996; Nakayama et al., 2000]). In *Drosophila*, the vertebrate BMP2,4, and BMP5,6,7,8 orthologues are encoded by *decapentaplegic* (dpp) and *glass bottom boat* (gbb) genes, respectively. These two BMP ligands have pleiotropic functions during *Drosophila* development with overlapping roles in regulating growth and patterning of the wing imaginal disc, as well as non-overlapping roles in the specification of embryonic dorsoventral cell fates and retrograde signaling at the larval neuromuscular junction ([Chen et al., 2004; Herpin et al., 2004; Marques, 2005; Nakayama et al., 2000; O’Connor et al., 2006; Ray and Wharton, 2001]).

Gbb originally received its name due to the profound transparency of third instar larvae null for gbb function ([Khalsa et al., 1998]). Larval transparency is a phenotype long known to be associated with extended periods of nutrient deprivation ([Britton and Edgar, 1998; Hadorn, 1951]). Like starved larvae, we find that the transparency of gbb mutant larvae is largely due to a decrease in opacity of the larval fat body, a functional equivalent of the white adipose tissue and liver of vertebrates ([Arrese et al., 2001; Britton and Edgar, 1998; Canavoso et al., 2001]). Our observation of this notable phenotypic resemblance raised the possibility that a defect in Gbb signaling could impact the nutritional status of developing larvae, and we set out to investigate this possibility.

The ability of organisms to properly obtain, store, and metabolize nutrients is essential for their growth and development. When food is plentiful, sugars are stored as glycogen in the muscles and liver of vertebrates, with fats, or lipids, stored in the form of triacylglycerides (TAGs), in the liver and adipose tissue. Both fats and sugars are similarly stored in the insect liver and adipose organ equivalent, the *fat body* ([Arrese et al., 2001; Finn and Dice, 2006]). Under severe nutrient insufficiency, the fat body ([Arrese et al., 2001; Canavoso et al., 2001]) with sugar (trehalose) levels regulated by insulin and insect glucagon (adipokinetic hormone, AKH) secreting neurosecretory cells ([Leopold and Perrimon, 2007]). In the absence of adequate nutrient sources, animals will mobilize their internal nutrient stores in order to utilize their energy and ensure survival ([Finn and Dice, 2006; Wigglesworth, 1972]). TAGs are mobilized first to provide energy for high demand tissues, followed by the mobilization of glycogen and protein stores ([Arrese et al., 2001; Finn and Dice, 2006]). Under severe nutrient deprivation, not only are energy stores depleted but the time to complete development and reach maturity is significantly lengthened and the overall size of the developing animal can be dramatically reduced ([Edgar, 2006; Mirth and Riddiford, 2007]). The developmental pathways and molecular mechanisms involved in sensing available nutrients, as well as those involved in the regulation of lipid metabolism and energy homeostasis during the development are...
coming to light as is their conservation between invertebrates and vertebrates.

Mis-regulation of factors responsible for coordinating nutrient storage and metabolism in mammals increases the propensity of developing metabolic disorders, such as obesity and diabetes (Fingar and Blenis, 2004; Oldham and Hafen, 2003; Metzger et al., 2006). A recent study has shown that many of the pathways critical for metabolism are conserved between invertebrates and insects (Canavoso et al., 2001; Garofalo, 2002; Oldham and Hafen, 2003; Pan et al., 2004). This conservation and the tractable nature of Drosophila melanogaster genetics provide an ideal model system to identify key regulators of nutrient sensing, energy storage, and metabolism. Central to sustaining proper energy homeostasis during development is the larval fat body, where the energy released by \( \beta \) oxidation of fatty acids allows for continued growth and organismal survival (Arrése et al., 2001; Canavoso et al., 2001). This is most prominent in the larval fat body with lower levels of expression in the oenocytes (FB-Gal4) or in the brain and gut (ppl-Gal4). BO-Gal4 expression is high in embryonic and early first instar larval oenocytes but is undetectable in these cells during later larval stages. For UAS-dad FLP-out clones, hsFLP; UAS-dad flies were crossed to AyGal4 UASnGFP flies and allowed to lay on an apple juice plate for 6 h. First instar larvae were collected 0–2 h after hatching and placed in a 37 °C water bath for 10–15 min to induce UAS-dad, UAS-nGFP expressing clones. These larvae were then immediately placed on Bo-C12 containing food until dissection as crawling third instar larvae.

**Materials and methods**

**Drosophila melanogaster strains**

Flies were raised at 25 °C on standard cornmeal/sugar/agar food. Oregon R flies were used as wild-type control in all experiments. Alleles are described in Flybase (http://flybase.net) except for the hypomorphic Tor15 allele obtained from Sean Oldham. The following fly stocks were used for Gal4-UAS experiments: UASgbb9.9 (Khalsa et al., 1998), FB-Gal4 (Gronke et al., 2003), BO-Gal4 (Gutierrez et al., 2007), ppl-Gal4 (Colombani et al., 2003). The expression of FB-Gal4 and ppl-Gal4 is most prominent in the larval fat body with lower levels of expression in the oenocytes (FB-Gal4) or in the brain and gut (ppl-Gal4). BO-Gal4 expression is high in embryonic and early first instar larval oenocytes.

**Immunohistochemistry**

Fat bodies of crawling third instar larvae were dissected and fixed in 4% formaldehyde for 10 min, blocked in 1% Normal Goat Serum (NGS) and 0.1% Triton-X in PBS for 1 h. Primary antibody (anti-P51 1:1000) was used to analyze confocal Z-series/3D data sets.
instar larvae were used. Immunohistochemistry was carried out as for crawling) were dissected in PBS and stained with Oil Red O according to Gutierrez et al., 2007. Wing disc area was then determined using Image J (http://rsbweb.nih.gov/ij/). Wing disc area was traced three times, each time determining the area in pixels, and an average for each wing disc was calculated. 10–12 wing discs were examined for each genotype. For wild-type starved larval conditions, the larvae were placed on PBS soaked filter paper for 12 h prior to pupariation.

Oenocyte analysis

Crawling third instar larvae (either fed or starved up to 24 h before crawling) were dissected in PBS and stained with Oil Red O according to Gutierrez et al., 2007. Immunohistochemistry was carried out as for larval fat bodies (above), except the body wall of dissected third instar larvae were fixed for 20 min. Tissues were mounted in 80% glycerol, 0.5% n-propyl-galate, and images were taken using the same settings on a Zeiss Axiosvert 200M light microscope.

Construction of UASgbbRNAl

A ~400 bp region of gbb with little to no homology with other sequences in the genome was amplified by PCR using the following forward and reverse primers: 5′GAGAGATCTCGAACTGCGTCACG3′ and 5′CATGGGAATTCTGCGTCAAGG3′, and cloned into the BglII and EcoRI sites of SympUAST-w. Multiple transgenic lines were generated and tested for their ability to phenocopy gbb loss of function when crossed to various Gal4 lines.

RT-PCR analysis

mRNA was isolated using RNAeasy kit (Qiagen 74104) from wild type or mutant early third instar or second instar larvae that had been fed or starved for 12 h after the third instar or 4 h after the second instar molt, respectively. cDNA was produced by oligo dT and MLV-reverse transcriptase (Sigma). The cycling profile was optimized for each gene primer set and for amplification within the logarithmic phase (see also Zinke et al., 2002). All primers (Supplemental Table 2) span an intron(s) except gbb, which does not have an intron. PCR products from at least 2 separate RNA samples were run on 0.8% agarose gel, which was stained with Vistra Green (Amersham RPN5786 1:10,000) for 1 h. Gels were scanned on a Typhoon 9410, and ImageQuant software was used to determine the intensity of each band. The ratio of intensities of bands for the gene of interest to actin was then determined in each sample.

TAG analysis

Lipids were isolated from 10 wandering third instar larvae using an assay modified from Bligh and Dyer (1959). Lipids were spotted on a Silica chromatography plate (Baker Si250, Mallinkrodt Chemicals) and resolved in a chamber lined with Whatman paper and in heptane: isopropyl ether:acetic acid (60:40:4) using lipid standards (1787-1AMP Sigma). TLC plate was allowed to dry and placed in a chamber with iodide crystals for at least 1 h and immediately scanned. ImageQuant software was used to analyze intensity and area of bands for TAGs. TAG amount was normalized for weight of the larvae, and each genotype was compared to the TAG levels of normally fed wild-type larvae.

GC-MS

Lipids were isolated according to Kunte et al. 2006 using a known amount of C15 as an internal control. To yield fatty acid methyl esters, the lipid extract was dried under helium and incubated at 50 °C with 50 μl of benzene, 50 μl of methanol, and 5 drops of trimethylsilyldiazomethane (Sigma 362832). GC-MS analysis was performed using a JEOL JMS-600 mass spectrometer operated in the electron impact ionization mode. The GC was equipped with a 30-m HP-5MS column. Amount of each fatty acid class was compared to C15, and total lipid was compared to C15 relative to the wild-type fed larvae sample.

Statistics

Data presented are mean ± SEM. The Student’s t-test was used for comparisons between 2 groups. Significance was assumed for p values <0.05 unless otherwise noted.

Results

gbb mutant larvae exhibit growth defects

Gbb mutants progress through development at a slower rate than wild-type larvae and gbb null animals die prior to pupation (Wharton et al., 1999). During the third larval instar, gbb mutants appear morphologically distinct from their wild-type counterparts. At equivalent developmental times during the third instar, gbb mutant larvae are slightly smaller than wild-type larvae but exhibit a markedly transparent appearance (Figs. 1A, D). This transparency is primarily due to a change in opacity of the fat body. All regions of the fat body are present in gbb mutant larvae, however, the size of individual cells is reduced (Fig. 1B) such that the entire organ is somewhat smaller in overall size. In addition to the smaller size and the dramatic transparency of gbb mutant fat bodies, the intracellular lipid droplets in mutant fat body cells are more variable in size than that seen in wild type fat bodies, with mutant cells often having only 2–4 very large lipid droplets per cell (Figs. 1B, C). A change in lipid droplet size is often associated with a change in lipid metabolism (Brasamele, 2007) and could be an indication that in gbb mutants, the metabolism of lipids, or metabolism in general, is in some way altered.

We had previously observed a general reduction in the size of gbb mutant larval organs, such as the brain and imaginal discs (Khalsa et al., 1998). In some tissues such as the larval salivary glands where it is possible to count all cells, we have noted that gbb1 mutants have a 14 ± 3 % reduction in cell number (n = 28). Here, we document that late third instar gbb mutant wing imaginal discs are in fact 50% smaller in overall area compared to the area of wild-type wing discs of the same developmental stage (Fig. 1E). These findings are consistent with a requirement for gbb in ensuring normal growth of cells and tissues. Like gbb mutants, larval mutant for genes encoding other BMP signaling components, sax (a type I receptor) and Mad (encoding the R-Smad) also show larval transparency and a reduction in organ size (Fig. S1).

Loss of Gbb signaling leads to a reduction in metabolic stores

Larval transparency, reduced cell and tissue growth, and a change in lipid droplet size are all phenotypes strikingly similar to those observed in wild-type larvae that have been deprived of nutrients (Britton and Edgar, 1998) (Figs. 1D, E; Figure S1; data not shown). These same phenotypes have also been reported in animals that lack the activity of the TOR (target of rapamycin) kinase, known to play an important role in the regulation and sensing of nutrient stores within
the Drosophila fat body (Britton and Edgar, 1998; Butterworth et al., 1965; Colombani et al., 2003; Zhang et al., 2000) (Fig. 2B; data not shown). The similarity of gbb mutant larval phenotypes with those of nutrient deprived wild type larvae and Tor mutant larvae led us to investigate the status of nutrient stores in gbb mutant larvae.

In insects, triacylglycerides (TAGs) stored in fat body lipid droplets are the main form of stored energy (Arrese et al., 2001; Canavoso et al., 2001). Protein and glycogen deposits are also found in the fat body and the major form of circulating carbohydrates are glucose and trehalose. During periods of inadequate food, the lipids and sugars are the first stored reserves to be mobilized to ensure that there is continued growth and progression through development (Finn and Dice, 2006; Wigglesworth, 1972). We did not find a significant difference in overall protein levels between wild-type and gbb mutant third instar larvae when normalized for body mass (Fig. 2D). However, we found that both lipids and sugars were reduced in gbb mutants (Figs. 2A–C). We also assayed for the level of nutrient stores in wild-type larvae that had been deprived of nutrients for 12 h as well as in normally fed Tor mutant larvae (Figs. 2F, G). Tor mutant larvae have been shown previously to have reduced glucose and lipid levels (Luong et al., 2006). And the reduction we observe in short-chain FAs in Tor mutants, starved larvae as well as gbb mutants, supports the possibility that gbb mutant, Tor mutant, and fasting larvae could be metabolizing short-chain FAs as a means of obtaining energy (Gutierrez et al., 2007; Kompore and Rizzo, 2008; Reddy and Hashimoto, 2001). Consistent with these changes in nutrient stores observed in gbb mutants is the alteration in fat body morphology, specifically the change in lipid droplet size, an alteration that has been suggested to reflect changes in both lipid droplet protein content and modifications in lipid metabolism (Brasaemle, 2007).

gbb mutant larvae mount a starvation response

In principle, depleted nutrient stores in gbb mutants may result from a failure of mutant larvae to ingest food. We made use of a fluorescently labeled fatty acid, BODIPY-tagged dodecanoic acid (Bo-C12), mixed into standard Drosophila food to investigate this possibility. If gbb mutants are defective in their ability to ingest food, we predicted that they would have less Bo-C12 labeled food within their guts. We observed no difference in Bo-C12 fluorescence within the lumen of wild-type and gbb mutant larval midguts after equivalent feeding regimes (Figs. 3A, B), indicating that gbb mutants have no obvious defect in their ability to ingest food. However, in these experiments we observed a difference in Bo-C12 fluorescence within the midgut epithelia and fat body lipid...
droplets between wild type and gbb mutant larvae. When larvae were fed food with 5 μM Bo-C12, gbb mutants exhibited significantly higher levels of Bo-C12 fluorescence than wild type larvae (Fig. 3, Fig. S2A). This difference in Bo-C12 fluorescence did not reflect an inability of Bo-C12 to be taken up by cells of the wild type midgut epithelium since dissected midguts simply incubated with Bo-C12 readily take it up into their cells (Figure S2C). Furthermore, wild-type larvae raised on food containing higher concentrations of Bo-C12 (10 μM) showed high fluorescence within both the midgut epithelium and fat body lipid droplets (data not shown).

Bo-C12 is regularly used to monitor fatty acid transport in a number of systems (Tong et al., 2006; Spanier et al., 2009) and give the difference in Bo-C12 fluorescence observed between wild type and gbb mutant larvae following ingestion of Bo-C12-containing food, we

![Image](https://example.com/image1)

**Fig. 2.** gbb mutant larvae have reduced metabolic stores. (A) Total lipids are reduced in early third instar fed gbb null and starved wild-type larvae. (B) After normalization for larval weight, fed gbb null larvae and starved wild-type larvae display reduced TAG levels relative to fed wild-type larvae. (C) Glucose and trehalose levels are decreased in wild-type larvae starved for 12 h and gbb mutant larvae. (D) There is no significant change in the protein:mass ratio in gbb mutant larvae compared to wild-type larvae. Tor mutant larvae exhibit a reduced protein:mass ratio. (E) GC-MS analysis indicates that early third instar fed gbb null and wild-type larvae starved for 12 h have lower levels of short-chain fatty acids compared to wild-type fed larvae. Percent of each fatty acid type in wild-type fed larvae is given in parentheses. (F, G) Short-chain fatty acids are also reduced in Tor mutant larvae.

![Image](https://example.com/image2)

**Fig. 3.** Loss of BMP signaling leads to alterations in lipid metabolism in the fat body. Midguts and fat bodies from wandering third instar larvae fed Bo-C12 (green) and stained with Hoechst (blue) and Phalloidin (red). (A, B) Third instar wild-type (A) and gbb1/gbb3 mutant (B) guts ingest Bo-C12 from the food. (A′, B′) Magnification to 300% of regions of the midgut lumen (L) and epithelium (E), indicated by white box, illustrates the increased uptake of Bo-C12 by gbb mutants in the epithelium. (C) Lipid droplets in fat body cells of wandering third instar wild-type larvae have little Bo-C12 fluorescence following continuous feeding of low levels of Bo-C12. (D–I) Wild-type larvae starved for 12 h (D), gbb1/gbb3 (E), Mad12/Mad2 (F), sax5/Df(2R)H23 (G), and Tor17004/TorK17004 (H, I) mutant fat bodies exhibit elevated levels of Bo-C12 fluorescence within lipid droplets indicative of increased uptake from ingested Bo-C12, likely in response to lower overall metabolic stores (see also text and Fig. S2). Scale bar = 47.62 μm. n>20 for each genotype. All images were taken with identical confocal settings.
considered a possible explanation for the apparent difference in fatty acid transport between wild type and gbb mutants. Given that gbb mutant larvae have reduced levels of total lipids (Fig. 2A), it is possible that the higher Bo-C12 fluorescence observed in gbb mutant midgut epithelia and fat bodies reflected an increase in nutrient uptake. An increase in Bo-C12 uptake would be consistent with an attempt by the mutant larvae to replenish any reduced lipid stores. Consistent with this interpretation, we found that when we fed Bo-C12 food to wild type larvae and then deprived them of any nutrients for 12 h they also displayed a higher level of Bo-C12 in their lipid droplets compared to the fat bodies of wild-type larvae continuously fed Bo-C12 containing food (Figs. 3C, D). These observations suggest that when food is withdrawn and/or available energy stores are reduced, larvae mount a starvation response by increasing the uptake of available nutrients remaining within the gut lumen, and thus, we detect an increase in Bo-C12 uptake and transport from the midgut lumen to the fat body. If the presence of Bo-C12 fluorescence in lipid droplets of fasting larvae (Fig. 3D) reflects an increase in the uptake of lipids from the gut, then a pulse of Bo-C12 food followed by a chase with standard food, whereby little to no Bo-C12 remains in the gut lumen, should result in no difference in lipid droplet fluorescence between fed and starved larvae. Indeed, no difference in the level of fat body Bo-C12 fluorescence is seen in these animals (Fig. S2A). Taken together, these results indicate that Bo-C12 is readily transported from the midgut to the fat body lipid droplets upon food withdrawal and that the presence of high levels of Bo-C12 fluorescence in fat body cells is indicative of animals with altered metabolic status. Consistent with mounting a starvation response, we also found that mutant larvae appear to continually mobilize their stores, as gbb mutant (as well as Mad mutant, see below) larvae show a decrease in Bo-C12 fluorescence despite being supplied with adequate food following feeding on Bo-C12-containing food (Fig. S2A).

Larva mutant for Gbb/BMP signaling components, sax (sax$^3$/Df (2R)H23) and Mad (Mad$^{12}$/Mad$^{a}$), also display high levels of Bo-C12 fluorescence in their fat body lipid droplets compared to wild-type larvae (Figs. 3F, G). The increased transport of Bo-C12 into fat body lipid droplets that we observed in gbb mutant larvae is also apparent in Mad mutants. Furthermore, timed feeding experiments with Mad mutant larvae show a significant increase in Bo-C12 fluorescence by 9 h compared to that seen in wild-type fat bodies (Fig. S2B).

Given that Mad is a downstream transducer for multiple ligands in the Drosophila BMP pathway, we considered the possibility that another BMP ligand, such as the BMP 2/4 orthologue Dpp, may cooperate with Gbb as it does in a number of other developmental processes (Kawase et al., 2004; Khalsa et al., 1998; O’Connor et al., 2006; Shimmi et al., 2005). However, in this case we found that larval-lethal alleles of dpp do not exhibit larval transparency or high levels of Bo-C12 in fat body droplets (Fig. S3; data not shown), suggesting that Dpp does not influence organosomal metabolic status or not to the same extent as Gbb.

Tor mutants are deficient in proper nutrient sensing and show a reduction in lipid stores (Colombani et al., 2003; Luong et al., 2006). Based on our studies with Bo-C12 as a probe for fatty acid transport, we tested Tor mutants for the level of Bo-C12 fluorescence in their fat bodies. Following feeding on Bo-C12-containing food, Tor mutants display high levels of fluorescence in their fat body lipid droplets (Figs. 3H, I). This high level of Bo-C12 fluorescence is indicative of increased fatty acid transport in response to compromised energy stores and is consistent with the other phenotypic similarities between Tor and gbb mutant larvae, as well as wild type larvae that have been nutrient deprived.

Gbb/BMP signaling is required in the fat body cells for proper metabolism

The larval fat body is a central player in coordinating the function of more peripheral tissues for Drosophila energy homeostasis (Leopold and Perrimon, 2007). Our data thus far indicate that the fat bodies of gbb mutants are morphologically abnormal, they exhibit abnormal transport of the Bo-C12 fatty acid, and overall the metabolic stores in gbb mutants are not at the level expected of wild type animals. It is possible that the abnormal functioning of the gbb mutant fat body results from a loss of Gbb signaling within the fat body itself or a loss from distant tissue(s) that impacts fat body morphology and function. In previous studies, we have shown that gbb is broadly expressed the imaginal discs and larval brain (Khalsa et al., 1998). Here, using both in situ hybridizations and semi-quantitative RT-PCR on isolated fat bodies, we show that gbb is also expressed in the third instar larval fat body (Fig. 4A; data not shown). Furthermore, we find that wild-type fat body cells clearly receive BMP signals as indicated by the high levels of phosphorylated Mad (pMad) localized within their nuclei (Fig. 4C). Wild-type levels of gbb are required for this signal, given that little to no pMad accumulates in the nuclei of gbb mutant fat body cells (Fig. 4C).

In order to determine whether gbb endogenously expressed in fat body cells is required for proper metabolism, we knocked down gbb function in the fat body by expressing gbbRNAi (UAS-gbbRNAi) under the control of the Gal4-UAS system, by using the FB-Gal4 or ppl-Gal4 drivers, each of which have been shown to be expressed in the larval fat body (Colombani et al., 2003; Gronke et al., 2003; Gutierrez et al., 2007). In both crosses, the experimental (e.g. FBGal4>UASgbbRNAi) third instar larvae were transparent, and when raised on food containing Bo-C12, their fat bodies displayed higher Bo-C12 fluorescence than those from larvae lacking UAS-gbbRNAi (Figs. 4D, E and data not shown). Given that there are no other tissues in common that express both FB-Gal4 and ppl-Gal4 (see Materials and methods), we conclude that a reduction of gbb within the fat body is primarily responsible for the obtained phenotypes.

In agreement with a role for gbb in the larval fat body, the phenotypes associated with gbb mutants including larval transpar-


cency, high levels of Bo-C12 fluorescence, and lethality are rescued by the expression of wild-type gbb in FBGal4 gbb$^{1}$/UASgbb9.9 gbb$^{b}$ larvae (Figs. 4B, F; Table S1). A similar rescue of gbb mutant phenotypes was achieved with fat body expression of a constitutively active Sax construct (Table S1). Somewhat surprisingly, we found that when BMP signaling is blocked by the overexpression of the inhibitory Smad, Dad (hsFLP; AyGal4 UAS-nGFP/UAS-dad), Bo-C12 uptake is affected in a cell autonomous manner, as evidenced by the increase in fluorescence in fat body cells overexpressing Dad (overexpressing cells are marked by nuclear GFP) (Fig. 4G). This cell autonomous elevation in lipid droplet Bo-C12 fluorescence indicates that the reduction or elimination of BMP signaling within a fat body cell alters the metabolic properties of that cell. Taken together, our findings indicate that Gbb/BMP signaling within the fat body is both necessary and sufficient to maintain normal metabolic physiology.

Finally, we find that nutrient-deprived larvae show a marked reduction or loss of nuclear pMad in their fat body cells (Fig. 4C). This finding indicates that the nutritional status of an animal is not only affected by a reduction in BMP signaling but an external change in nutrient availability can also alter the transduction of BMP signals. Taken together, fat body cells of third instar larvae receive and transduce BMP signals but the metabolic status of these animals both dictates and depends on fat body BMP signaling.

The molecular response to a loss in Gbb signaling is not identical to nutrient deprivation

Drosophila larvae respond to their environment by altering the expression of genes involved in the sensing and storage of nutrients (Zinke et al., 2002). A distinct change in the transcript level of genes that control the breakdown of stored lipids and proteins is seen in Drosophila larvae deprived of nutrients (Zinke et al., 1999, 2002). We compared the expression levels of several of these starvation-
responsive genes between wild type larvae both fed and deprived of nutrients, and from gbb mutant larvae, also both fed and nutrient deprived (Fig. 5). In both fed gbb mutants and starved wild-type larvae, a lower level of expression level is seen for the amino acid transporter encoded by minidiscs (mnd), which is reportedly expressed exclusively in the fat body (Martin et al., 2000) (Fig. 5A). In contrast, an increase is seen in the transcript levels of lipase3 (lip3), whose expression appears to be limited to the larval fat body (Pistillo et al., 1998), and Drosophila insulin-like peptide 2 (dilp2), whose expression is abundant in symmetrical clusters of neurons in the larval brain and in the larval salivary glands and at low levels in the imaginal discs (Brogiole et al., 2001) (Fig. 5B and F). Interestingly, other starvation-responsive genes, such as the human adipocytedefined triglyceride lipase (ATGL) orthologue, encoded by brummer (bmm) and a human acid lipase orthologue encoded by lipase4 (Lip4/CG6113) display a significant change in expression levels in wild-type starved larvae but not in fed gbb mutant larvae (Fig. 5E; data not shown). Brummer is expressed at all stages of development and its larval expression is limited to the fat body, midgut, and gastric caeca (Gronke et al., 2005). In addition, the proposed long chain fatty acid transporter (CG4563) and the fatty acid synthase BcDNA:GH07626 have not yet been determined. Taken together these results indicate that gbb mutants do not precisely mimic the transcriptional response induced by nutrient deprivation.

Despite not mimicking a starvation response at the transcriptional level, gbb mutants could suffer from a reduced amino acid uptake through the permease-like Mnd transporter and from alterations in lipid and sugar metabolism due to increased levels of lipase 3 and dilp2 (Zinke et al., 1999, 2002). However, because our data to this point identified some similarity between gbb mutants and nutrient deprived animals with regards to metabolic stores, etc., we next asked if gbb mutants are able to properly mount a starvation response. We find that when deprived of nutrients, gbb mutants show the expected increase (lip3, bmm, and dilp2) or decrease (mnd) of gene expression that is seen in nutrient deprived wild-type larvae (Figs. 5A, B, E and F). Furthermore, nutrient deprived gbb mutants show an increase in CG4563 and a decrease in BcDNA:GH07626 expression levels consistent with their ability to mount a starvation response (data not shown). Thus, the metabolic changes observed in gbb mutants result in a change in the expression of some genes involved in nutrient processing but not all.

Upon nutrient deprivation wild-type larvae do not display a significant change in the level of transcription of some genes required for lipid transport, such as, Retinoid and fatty-acid binding protein (Rfapb), the vertebrate apolipoprotein 1 and 2 orthologue, and CG31217, a low-density lipoprotein (LDL) receptor (Zinke et al.,
However, we find that transcript levels for both of these genes are increased in gbb mutants (Figs. 5C, D), further highlighting the difference between the organismal responses to a loss of Gbb signaling versus the deprivation of nutrients. Another interesting difference between gbb mutant larvae and nutrient-deprived larvae is in the total level of C16:1 fatty acids present in third instar larvae. gbb mutants show similar levels of C16:1 FA as wild type fed larvae and thus, do not seem to metabolize these fatty acids as nutrient-deprived larvae (Fig. 2E). Thus, while gbb mutants exhibit many morphological and physiological similarities to nutrient-deprived larvae, the molecular response to reduced Gbb signaling is different from that observed when nutrient availability is reduced.

In line with this conclusion, we find that while the oenocytes of wild-type larvae accumulate lipids during periods of nutrient deprivation (Gutierrez et al., 2007), the oenocytes of fed gbb mutants do not show an accumulation of neutral lipids (Fig. S4). Upon starvation, the oenocytes of gbb mutant larvae accumulate neutral lipids, consistent with the ability of gbb mutants to elicit a starvation response.

**Discussion**

The nutritional status of an organism dictates its growth and maturation during development. How the balance of nutrient uptake, storage, and metabolism is coordinated with growth and developmental progression is not thoroughly understood. More recently, specific genetic and physiological pathways responsible for the coordination of growth, development and metabolism are being identified in a number of metazoan systems (Edgar, 2006; Leopold and Perrimon, 2007; Saltiel and Kahn, 2001). Here, we show that the Gbb/BMP signaling pathway regulates the metabolic status of Drosophila larvae, in addition to its role in cell fate specification. Previous studies have shown that BMP2 and BMP4, as well as a BMP transcriptional cofactor, Schnurri, can influence adipocyte differentiation (Jin et al., 2006) and that BMPs can promote the accumulation of lipids in mammalian brown pre-adipocytes (Tseng et al., 2008). However, the data presented here indicate that BMP signaling can also have a significant impact on nutrient uptake and metabolic status of the organism well after fat cell differentiation, during its progression through larval development.

gbb mutant larvae resemble nutrient deprived larvae in that they are transparent, exhibit growth defects, and utilize stored energy sources, especially short chain fatty acids (Figs. 1, 2). When deprived of food, wild-type larvae mount a starvation response that results in the uptake of nutrients remaining in the gut, as indicated by Bo-C12 pulse-chase experiments. Interestingly, gbb mutants show a higher level of Bo-C12 uptake despite the fact that they are not deprived of food. This indicates that while gbb mutants ingest food and take up nutrients, they are unable to maintain energy stores and thus, share physiological similarities with starved larvae. However, despite physiological and morphological similarities, nutrient-deprived wild-type larvae and fed gbb mutant larvae exhibit somewhat different gene expression profiles, based on the transcript levels of several starvation-responsive genes (Fig. 5). Such dissimilarities in molecular responses suggest fundamental differences in the mechanisms responsible for “environmental starvation” and “genetically-induced gbb mutant starvation.”

Larval transparency is perhaps the most striking aspect of the “starvation” phenotype, and changes in the appearance of the larval fat body are largely responsible for this phenotype. Given that all regions of the fat body are present in gbb mutant animals, albeit exhibiting smaller cells with lipid droplets of varying sizes, and especially since these defects can be rescued by the expression of wild type gbb in the larval period, there is no indication that gbb mutants are defective in the specification and differentiation of the fat body in general, or in specific regions of this organ. Furthermore, defects in gbb mutant larvae are not evident until the late second/early third larval instar (S. Ballard and K. Wharton, personal observation). Thus, the role for Gbb signaling appears to be a later function that influences metabolism, and not one in the specification of a fat body fate or in the specific differentiation of fat body cells.

The fat body tissue is the central metabolic organ within the Drosophila larva that communicates the nutritional status of the organism to other tissues to influence their growth and function. At the same time, various tissues communicate to the fat body to promote proper storage and mobilization of energy. The defects in
both the morphology and function of the fat body in gbb mutants can be at least in part accounted for by a loss or reduction in BMP signaling within the fat body itself. gbb is expressed in the fat body, and gbb is required for the nuclear localization of pMad within these cells (Fig. 4), suggesting that Gbb acts within the fat body to provide active BMP signaling. A loss of Gbb/BMP signaling due to the expression of either gbb-RNAi or dad, within fat body cells compromises the nutritional status of the fat body in an autonomous manner. Expression of wild type gbb within the fat body can partially rescue larval transparency, increased levels of Bo-C12 fluorescence, and lethality caused by loss of Gbb signaling (Figs. 4B and F; Table S1). Not all gbb mutant phenotypes are rescued when gbb is expressed in the fat body. The synapses at the larval neuromuscular junction (NMJ) are reduced in size in gbb mutants, with a decrease in overall bouton number (McCabe et al., 2003 and Fig. S6). This undergrowth is not rescued when gbb is expressed in the fat body (Fig. S6). In addition, the patterning defects observed in the adult gbb mutant wing were not rescued (S. Ballard and K. Wharton, personal observation). Furthermore, we do not find that when gbb expression is increased in the fat body of wild type larvae (FB-Gal4>UASgbb) that a concomitant increase is seen in the size of wing discs (Fig. S5), an effect we observe when gbb is specifically expressed in the wing imaginal disc (Khalsa et al., 1998). If Gbb were solely influencing cell/tissue growth through secretion into, and circulation via, the hemolymph, then we would expect increased expression of Gbb from tissues such as the fat body to influence distant tissues, such as the NMJ and the growth of imaginal discs. This is not what is observed, as discs from FB-Gal4/UASgbb larvae are comparable in size to wild-type discs.

However, we do find that the reduced size of gbb mutant wing imaginal discs can be partially rescued by gbb expression within the fat body (Fig. S5). While it is possible, albeit unlikely as discussed above, that elevated levels of Gbb secreted into the hemolymph could be responsible for rescue, our results more strongly support the likelihood that the role of Gbb signaling in regulating metabolism via its effect on the physiology of fat body cells is rescued and thus, nutrients and energy are available for the growth of distant tissues. Our results point to an essential role for Gbb/BMP signaling within the fat body itself for regulating metabolism, however, they do not completely rule out the possibility that Gbb/BMP could also signal from another defined site to impact fat body function.

The hepatocyte-like oenocytes have been implicated in lipid metabolism based on their starvation induced accumulation of lipids following fat body depletion of lipid stores (Gutierrez et al., 2007). In gbb mutants, we did not detect an accumulation of lipids in the oenocytes as seen for starved wild-type animals which could have suggested that loss of Gbb signaling affected the communication between oenocytes and the fat body. Rather, we found that when gbb mutants were deprived of nutrients they showed an accumulation of neutral lipids, albeit not at the level of wild-type larvae, presumably because gbb mutants have lower total triglycerides than wild-type (Fig. S4). We did not find that when a potential function of gbb in oenocytes was disrupted by the expression of gbb-RNAi by BO-Gal4 (Gutierrez et al., 2007) (BO-Gal4 gbb'/UASgbbRNAi) that there was any effect on the number of oenocytes or oenocyte clusters. Nor did we find that this manipulation induced a transparent larval phenotype or an increase of Bo-C12 in fat body lipid droplets (data not shown). Finally, we did not observe nuclear pMad in oenocytes nor a change in pMad distribution in gbb mutant or nutrient deprived larvae. Thus, the loss of gbb function does not appear to impact the specification or function of oenocytes in lipid uptake.

The larval brain is another important player in maintaining energy homeostasis and it communicates with the fat body through the secretion of insulin-like peptides (dilps) and adipokinetic hormone, AKH (glucagon), to either promote storage of nutrients or the breakdown of metabolic stores, respectively (Ikeya et al., 2002; Lee and Park, 2004; Van der Horst, 2003). We found that gbb mutant larvae have elevated levels of dilp2 expression but despite these changes we were unable to detect a synergistic genetic interaction between mutations in Gbb signaling and the insulin receptor (InR) or the insulin receptor substrate chico (M. Psotka, K. Wharton, unpublished). It is of interest, however, that an increase in insulin is associated with a mobilization of stored carbohydrates. In Drosophila, Broughton et al. (2007) have shown that a specific decrease in dilp2 expression within the mNSCs (medial neurosecretory cells) of the brain results in increased levels of whole body trehalose, and interestingly, in decreased levels of dilp-2 and -5. Furthermore, it is interesting to note that profound changes in foraging behavior is associated with animals deprived of nutrients (Sokolowski, 2001), and recent work has shown that these behaviors are elicited by elevated levels of dilp2 and dilp4 (Wu et al., 2005). Consistent with these findings and the increased level of dilp2 expression observed in gbb mutants, we have observed that late third instar gbb mutant larvae forage for long periods (data not shown).

The balance between energy stored and energy expended is critical for animal growth and survival. Our results have clearly shown that a reduction in Gbb/BMP signaling impacts nutrient stores and metabolism and accordingly, we observe an up-regulation of some “starvation-response” genes, indicating that Gbb/BMP signaling acts to promote nutrient storage. Interestingly, when animals are deprived of an external source of nutrients, we in turn observe a loss of BMP signaling (pMad) in fat body cells, consistent with the organism’s need to mobilize nutrients and to postpone nutrient storage (Fig. 6). The TOR pathway has been shown to be an important player in metabolism and Tor also appears to act in the Drosophila fat body. The similarities in larval phenotypes between Gbb/BMP mutants and TOR pathway mutants are striking. The fact that Tor mutants also show lower levels of short chain FAs and total lipids as well as differences in lipid transport is consistent with a central role for TOR in nutrient sensing and homeostasis. Interestingly, we find that the phenotypes of Gbb/BMP pathway mutants are affected by alterations in TOR function and vice versa (S. Ballard and K. Wharton, unpublished). Our preliminary data

Fig. 6. Gbb/BMP signaling regulates energy homeostasis. The model schematizes the proposed action of Gbb/BMP signaling in the fat body to regulate energy homeostasis. gbb is expressed in fat body cells and Gbb/BMP signaling is active within this tissue through the phosphorylation and translocation of pMad to the nucleus. Gbb signaling influences the transcription of genes involved in amino acid uptake (mnd), sugar metabolism (dilp2), and lipid hydrolysis (lipase3) and transport (RfaBp and CG31217 (LDL receptor)). Interestingly, in addition to its role in promoting nutrient storage BMP signaling is itself responsive to the level of metabolic stores. When nutrient levels are low the accumulation of pMad in fat body nuclei is lost.
indicate that there is a feedback mechanism in place involving these two pathways such that the organism is able to achieve homeostasis. While the specifics of the molecular mechanisms underlying this feedback await further characterization, our observations have revealed an intimate relationship between BMP signaling and a developing organism's metabolic status, whereby Gbb/BMP signaling is critical for attaining energy homeostasis with BMP signals likely acting to influence the fat body's ability to coordinate nutrient uptake, storage, and energy availability with tissue growth. Further studies that uncover the molecular details of how BMP signaling impacts the balance of energy storage and mobilization will be critical for tackling many diseases, not only obesity and metabolic syndromes, but those that affect other tissues such as neural (Huntington's, Parkinson's, Alzheimer's, Tuberous sclerosis) and bone (fibrodysplasia ossificans progressiva) (Baggio, 2002; Farooqui et al., 2007; Shore et al., 2006) where it is clear that an individual's metabolic state impacts the presentation and progression of disease.

Acknowledgments

We thank Dr. Tun-Li Shen for his expertise in GC-MS analysis, Mitch Potska and Sarah Bowman for their contributions to the early stages of this project, especially to Fig. 1. We are grateful to Peter ten Dijke, Sean Oldham, Ronald Kuhnlein, Michael Pankratzik, and Alex Gould for generously sharing reagents. Many thanks to Troy Shringari, Michael McKeanow, Kate O'Connor-Giles, and members of the Wharton lab for discussions and comments on the manuscript. This work was supported in part by funds from the Salomon Faculty Research Fund (Brown University), NIH GM068118 and a research grant from the Center for Research in Fibrodysplasia Ossificans Progressiva from The University of Pennsylvania awarded to K.A.W. S.L.B. was supported in part by NIH T32 GM07601.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.11.011.

References


Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.11.011.

References


