Brummer lipase is an evolutionary conserved fat storage regulator in *Drosophila*

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Summary

Energy homeostasis, a fundamental property of all organisms, depends on the ability to control the storage and mobilization of fat, mainly triacylglycerols (TAG), in special organs such as mammalian adipose tissue or the fat body of flies. Malregulation of energy homeostasis underlies the pathogenesis of obesity in mammals including human. We performed a screen to identify nutritionally regulated genes that control energy storage in the model organism *Drosophila.* The *brummer* (*bmm*) gene encodes the lipid storage droplet-associated TAG lipase Brummer, a homolog of human adipocyte triglyceride lipase (ATGL). Food deprivation or chronic *bmm* overexpression depletes organismal fat stores in vivo, whereas loss of *bmm* activity causes obesity in flies. Our study identifies a key factor of insect energy homeostasis control. Their evolutionary conservation suggests Brummer/ATGL family members to be implicated in human obesity and establishes a basis for modeling mechanistic and therapeutic aspects of this disease in the fly.

Introduction

Providing constant energy supply despite variability in food access and metabolic energy demand is a fundamental property of animals. Key to an individual's survival during food deprivation is the ability to mobilize stored energy resources accumulated during periods of excessive energy supply. In organisms as different as humans and the fruit fly *Drosophila*, energy-rich diet components are converted into glycogen and—to a larger extent—triacylglycerols (TAG), the storage forms of carbohydrate and fat, respectively. Storage fat is deposited in intracellular lipid droplets of specialized organs called the adipose tissue in mammals or the fat body in *Drosophila*. In mammals, adipose tissue cooperates with the digestive tract and the central nervous system to hardwire an integrated molecular communication network ensuring the lifelong integrity of an organism's energy homeostasis under varying environmental conditions (for review, see Flier [2004], Friedman [2004], Spiegelman and Flier [2001]). In the peripheral fat storage tissue, a regulated balance between lipogenesis and lipolysis is believed to continuously match acute energy needs by TAG mobilization and readjust organismal storage fat content to a genetically determined setpoint during periods of excessive energy supply. Chronic imbalance of energy storage control by the lack or malfunction of regulatory genes results in excessive fat accumulation and is causative to the obesity pandemics in human populations as well as to related phenotypes in rodent models.

A key regulator of storage fat lipolysis in mammalian adipocytes is the hormone-sensitive lipase (HSL) (for review, see Holm [2003]). Activated HSL interacts with perilipin at the lipid droplet membrane to eventually mobilize TAG. Acute HSL activation relies on posttranslational modification by protein kinase A (PKA) in response to hormonal β-adrenoceptor stimulation and subsequent activation of the cAMP second messenger signaling pathway (Clifford et al., 2000; Egan et al., 1992; Sztalryd et al., 2003). In addition, extensive fasting causes upregulation of mouse HSL mRNA and protein (Sztalryd and Kraemer, 1994), supporting the enzyme's importance in acute and chronic TAG mobilization control. However, HSL knockout mice are viable and not obese (Osuga et al., 2000), having substantial residual lipolytic activity (Okazaki et al., 2002). Accordingly, additional TAG lipases of the nutrin family (Villena et al., 2004), such as the most recently identified human adipose triglyceride lipase (ATGL) (Zimmermann et al., 2004), have been implicated in mammalian lipolysis. However, the in vivo relevance of this lipase family in fat storage control on the organismal level waits to be analyzed.

In insects, storage fat lipolysis is stimulated by adipokinetic hormone (AKH) in various species including the grasshopper *Locusta migratoria* (Gäde and Beenakkers, 1977), the tobacco hornworm *Manduca sexta* (Ziegler et al., 1990), and the fruit fly *Drosophila melanogaster* (Lee and Park, 2004), suggesting a general role in insect energy balance control. Like in mammalian TAG mobilization, AKH-stimulated lipolysis in the insect fat body relies on signaling via a G protein-coupled receptor (Staubli et al., 2002), increase in intracellular cAMP, and activation of PKA (for review, see Gäde and Auerswald [2003], Van der Horst et al. [2001]). An insect TAG lipase, however, which makes the storage fat metabolically accessible for the energy-demanding target tissues, is currently unknown.

Given the intriguing similarities in the regulatory mechanisms of TAG mobilization between mammals and insects, we performed genome-wide transcriptome profiling in the model or-

ganism *Drosophila* to screen for nutritionally regulated and evolutionary conserved lipolysis effectors. Here we present the functional in vivo analysis of *brummer*, which encodes a TAG lipase of the nutrin family, whose lack causes obesity in the fly.

Results and Discussion

Genome-wide transcriptome profiling identifies the nutritionally regulated gene *brummer*

To screen for nutritionally regulated genes, we performed a genome-wide transcriptome analysis comparing gene expression of fed and food-deprived adult *Drosophila* flies. Sorting the total of 223 starvation-responsive genes (see Table S1 with the Supplemental Data available with this article online) according to their predicted function reveals that most of the starvation-induced genes are coding for metabolic enzymes (n = 44). In addition, genes coding for cytochromes (n = 10), metabolite transporters (n = 6), kinases (n = 5), and proteins involved in lipid metabolism (n = 7) are upregulated under starvation. Few metabolic enzymes (n = 7) are downregulated in response to starvation, whereas proteases and protease inhibitors form the largest group (n = 38). Nearly half of the starvation-induced metabolic enzymes are involved in carbohydrate catabolism, including key regulators like hexokinase (encoded by *Hex-C*), transketolase (*CG8036*), and phosphoglucomutase (*Pgm*) or enzymes involved in the breakdown of sugars like an α-Amylase (*AmyD*), a α-Glucosidase (*CG11909*), and six maltases (*CG11669*, *CG8690*, *CG30359*, *CG30360*, *CG14934*, *CG14935*). Protein degradation is reflected by the upregulation of genes involved in amino acid catabolism, including two aminotransferases encoded by *got2* and *spat*, a phenylalanine-4-monooxygenase (*henna*), a 4-hydroxyphenylpyruvate-dioxygenase (*CG11796*), and a homogentisate-1,2-dioxygenase (*hgo*). The starvation-induced metabolic activation is further reflected by the transcriptional upregulation of five regulatory kinases or kinase subunits, which have all been implicated in energy homeostasis control. While the pyruvate dehydrogenase kinase encoded by *pdk* is critical for the regulation of oxidative glucose metabolism, the β subunit of the SNF1/AMP-activated protein kinase (AMPK) acts as a cellular energy sensor (Pan and Hardie, 2002), and the cAMP-activated protein kinase A (PKA) promotes glycogen and TAG catabolism (Londos et al., 1999). The SNF4 γ subunit *loechrig* has been implicated in cholesterol homeostasis control (Tschäpe et al., 2002). In addition, Lk6 kinase mutants have recently been described to have increased organismal TAG content, suggesting a function of the kinase in the control of organismal lipid storage (Arquier et al., 2005; Reiling et al., 2005).

Among the seven upregulated genes involved in lipid metabolism are genes encoding a putative TAG-lipase (*CG5966*), phospholipase A_2 (*CG1583*), low-density lipoprotein receptor (*LpR2*), long chain fatty acid CoA ligase (*CG9009*), and carnitine-O-palmitoyltransferase (*CPTI*). Anabolic reactions of the lipid metabolism are repressed under starvation, as indicated by the transcriptional downregulation of a lipogenic 1-acylglycerol-3-P-O-acyltransferase (*CG4753*) and a long chain fatty acid elongase (*CG6261*). Moreover, the PAT domain containing lipid storage droplet-associated protein *Lsd-1* (Miura et al., 2002) and three TAG lipases are among the nine genes involved in lipid metabolism that are downregulated in response to starvation.

Taken together, genome-wide transcriptome profiling of fed versus food-deprived flies displays various regulatory aspects of the metabolic starvation response in *Drosophila*, including carbohydrate, amino acid, and lipid catabolism. However, no function has been assigned to 25% of the 223 starvation-responsive genes. Among those, we found that the gene *CG5295* in region 70F5 on chromosome 3L (FlyBase Consortium [2003] and Figure 1A), termed *brummer* (*bmm*), is upregulated upon starvation. The single *bmm* transcript, which encodes a 507 amino-acid-long protein (BMM) closely related to TTS-2/ATGL of mouse and human (see below), is expressed during all ontogenetic stages of the fly (Figure 1B). It is highly enriched in the energy storage tissue (Figures 1C–1E) as well as the food-absorbing parts of the digestive tract, i.e., the larval midgut and gastric caeca (Figure 1F). Quantitative Northern blot analysis confirms sustained transcriptional upregulation in response to food-deprivation (Figure 1G; see also Zinke et al. [2002] for data during larval starvation) and downregulation upon refeeding (Figure 1H). The nutritional regulation and the patterns of *bmm* expression suggest that *bmm* participates in the control of energy homeostasis.

brummer encodes a TAG lipase related to human ATGL

BMM contains a patatin-like domain (PLD) including a serine hydrolase motif, originally described in plant acyl-hydrolases, and a so-called Brummer box (BB) of unknown function (Figure 2A). The BB motif is found in a number of PLD-containing proteins, which we refer to as the Brummer/Nutrin subfamily. It includes the *Anopheles* BMM ortholog, a *Drosophila* paralogue called *doppelgänger von brummer* (*dob*; *CG5560*), the human proteins Adiponutrin, GS2-like, TTS-2/ATGL and GS2, *Caenorhabditis elegans* C05D11.7 and D1054.1 as well as *Arabidopsis* NP_174597 (Figure 2B).

PLD proteins are phospholipases in plants (Hirschberg et al., 2001), human (van Tienhoven et al., 2002), and *Pseudomonas* (Sato et al., 2003) or TAG lipases, as recently shown for the human Brummer/Nutrin family members TTS-2.2/ATGL, GS2, and Adiponutrin (Jenkins et al., 2004; Zimmermann et al., 2004). Recombinant BMM exhibits esterase activity on an esterified fatty acid (6,8-difluoro-4-methylumbelliferyl octanoate) as substrate (data not shown) but fails to catalyze the release of fatty acid from either the A2 position of a phospholipid (PAP), the glycosylphosphatidylinositol (GPI) membrane glycolipid membrane anchor of GPI-modified proteins (5′-nucleotidase, Gce1), or monoacylglycerol (MAG) (Figure 2C and data not shown). However, it cleaves TAG in vitro (Figure 2C), whereas the BMM^{S38A} mutant, in which serine residue 38 of the catalytic center had been replaced by alanine, is enzymatically inactive. Thus, *bmm* as its mammalian homologs are candidates for nutritionally regulated in vivo effectors of TAG mobilization.

brummer controls organismal fat storage in vivo

To test whether *bmm* promotes fat mobilization in vivo, we generated *bmm* loss-of-function mutant alleles (bmm^{1} and bmm^{2}) by mobilization of a transposable P element located in the first exon of *bmm* ($P\{w^{+mC} = EP\}bmm^{EP3174}$). Precise excision of the P element, as obtained with bmm^{rev}, served as genetically matched control for phenotypic analysis (Figure 1A). Embryos lacking both maternal and zygotic *bmm* activity are lethal (Figure 3), indicating that *bmm* carries an essential function. They develop pleiotropic degeneration phenotypes and

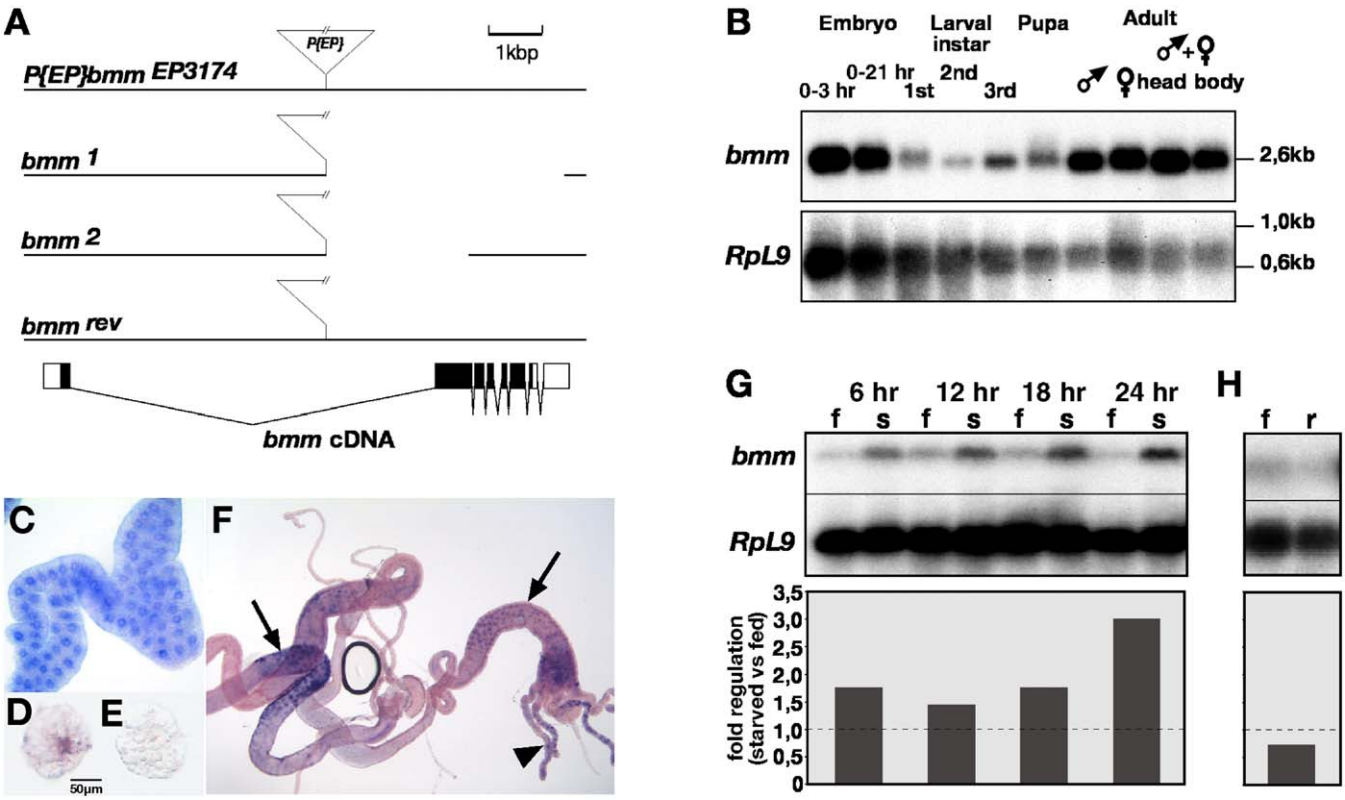


Figure 1. Characterization and nutritional regulation of the *brummer* gene

A) Genomic organization of the *bmm* gene (black boxes, coding exons; open boxes, UTRs). Flies carrying the *P{w*$^{+mC}$ *= EP}bmm*^{EP3174} insertion (Rorth, 1996) were used to generate *bmm* mutants (*bmm*^{1}, *bmm*^{2}) and control flies (*bmm*^{rev}).

B) Developmental Northern blot analysis detects a single *bmm* transcript of 2.6 kb expressed during all ontogenetic stages but highly abundant in early embryogenesis and adult flies of both gender. (Note that *RpL9* has been used as normalization control.)

C–F) *bmm* expression detected by in situ hybridization in fat body cells of third instar larvae (**C**) and adult flies (**D**) (sense control in [**E**]), larval midgut cells (arrows in [**F**]), and gastric caeca (arrowhead in [**F**]).

G and H) Nutritional regulation of *bmm* gene expression detected by quantitative Northern blot analysis. (**G**) Upregulation of *bmm* transcript level in starved flies (s) compared to ad libitum fed (f) control flies. (**H**) Reversion of *bmm* upregulation by poststarvation refeeding (r) compared to continuously fed siblings (f).

have increased TAG levels in late embryogenesis (data not shown). Embryonic lethality can be partially rescued by a paternally provided functional *bmm* gene and almost completely reverted by ubiquitous *bmm* expression from a cDNA-bearing transgene (Figure 3). Similar phenotypes and a reduced embryonic hatching rate have been reported for mutants of the perilipin-like fly gene *Lsd-2* (Teixeira et al., 2003). These results suggest that *bmm* fulfils a vital function in TAG mobilization during embryogenesis.

Flies lacking only zygotic BMM lipase activity develop normally but show progressive obesity accumulating 17% (immature adults, <1 day old) to 101% (mature adults, 6 days old) more storage fat compared to control flies (Figure 4A). Conversely, transgene-dependent *bmm* overexpression in fat body cells of fed flies, which mimics the effect of starvation-induced upregulation of *bmm* transcription (see above), depletes the TAG content of immature and mature adults by 96% and 46%, respectively (Figure 4A). These effects were not observed upon transgenic expression of the enzymatically inactive *bmm*^{S38A} mutant (Figure 4A), indicating that the TAG mobilization is caused by the lipase activity of BMM. *bmm*-dependent differences of organismal TAG content are also reflected by the lipid storage phenotype of fat body cells showing variously sized storage droplets in *bmm*^{1} mutant fat body cells (Figure 4B) and their reduction in size and number upon overexpression of the gene (Figure 4C). The effect of BMM is specific for the fat-based aspect of energy storage, since the glycogen content is not affected in *bmm* mutant or *bmm*-overexpressing flies (Figure 4D).

Excessive fat storage in flies lacking *bmm* function reduces the median lifespan by only 10% (Figure 4E). Acute TAG mobilization is impaired but not abolished in *bmm* mutants. While controls deplete their storage TAG during starvation, *bmm* mutants are able to consume 73% of their prestarvation fat content (Figure 4F). Accordingly, food-deprived *bmm* mutants outlive controls by 56% (Figure 4G) on the expense of their increased prestarvation fat storage (Figures 4A and 4F). The lipolytic activity present in *bmm* mutants allows fuelling their extended survival under food deprivation by metabolizing in total 65% more TAG than controls (Figure 4F). Thus, as in

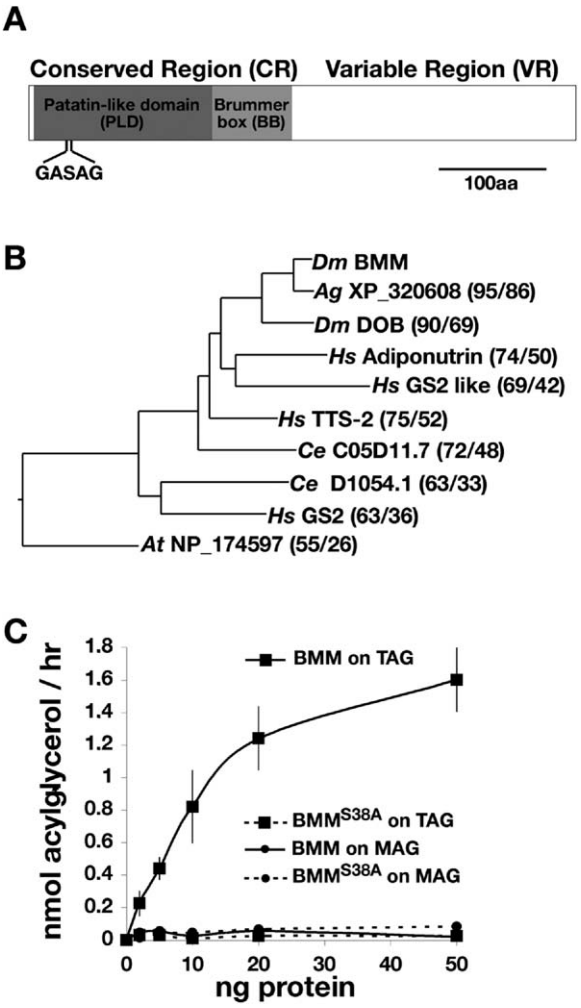


Figure 2. Brummer is an evolutionary conserved triacylglycerol lipase

A) BMM is composed of a conserved (CR) and a variable region (VR). CR contains a patatin-like domain (PLD) including the serine hydrolase motif GASAG and the Brummer box (BB).

B) Phylogenetic analysis of *brummer*/nutrin gene family members on the basis of CR sequences from *Drosophila melanogaster* (*Dm*), *Anopheles gambiae* (*Ag*), *Homo sapiens* (*Hs*), *Caenorhabditis elegans* (*Ce*), and *Arabidopsis thaliana* (*At*) (percent refers to amino acid similarity/identity).

C) Recombinant BMM (negative control, catalytic center mutant BMM^{S38A}) has triacylglycerol (TAG) but no monoacylglycerol (MAG) lipase activity in vitro. Mean values and error bars are calculated as detailed in Experimental Procedures.

Parental genotype (males x females)	# of embryos scored	% hatched embryos
w^{1118}; bmm^1/bmm^1 x w^{1118}/w^{1118};bmm^1/bmm^1	1206	1.7
w*; P{w^{+mC} $bmm^{Scer\UAS}$=UAS-bmm}/ P{w^{+mC} $bmm^{Scer\UAS}$=UAS-bmm}; bmm^1/bmm^1 x y^1 w^*/y^1 w^*; P{w^{+mC}=Act5C-GAL4}25FO1/CyO, y^+ ; bmm^1/bmm^1	269	45.0*
w^{1118}; bmm^{rev}/bmm^{rev} x w^{1118}/w^{1118};bmm^{rev}/bmm^{rev}	1131	93.8
w^{1118}; bmm^{rev}/bmm^{rev} x w^{1118}/w^{1118};bmm^1/bmm^1	907	39.3

Figure 3. *brummer* is an essential gene during embryogenesis

Hatching rate counts of maternal plus zygotic *bmm*^{1} mutants compared to *bmm*^{rev} control reveal that loss-of-*bmm* activity causes almost complete embryonic lethality. Embryonic lethality of *bmm*^{1} mutants is either partially rescued by a paternally supplied zygotic copy of the gene or rescued to 90% by ubiquitously expressed zygotic *bmm* supplied via a *bmm* transgene under indirect control of an *Actin 5C* promotor using the UAS/Gal4 System (*expected hatching rate is 50%).

mammals, mobilization of TAG storage in flies is controlled by more than one TAG lipase. Candidate effectors of *bmm*-independent TAG mobilization are the *bmm* paralogue *dob* and the genes *CG5966* and *CG11055*, which code for a starvation-induced putative TAG lipase (Table S1) and a *Drosophila* HSL homolog, respectively (FlyBase Consortium, 2003).

Brummer localization on adipocyte lipid storage droplets

To possibly extend the functional similarity between mammalian and *Drosophila* TAG lipases, we asked whether BMM localizes at the surface of lipid droplets. Transgenic flies expressing BMM:EGFP fusion protein variants (Figure 5A) in their fat body cells allow examination of BMM intracellular localization and lipolytic activity in vivo. Ubiquitous expression of BMM:EGFP or BMM reverts the obese phenotype of *bmm* mutant flies (data not shown). Targeting of BMM:EGFP but not BMM^{S38A}:EGFP expression to the fat body of otherwise wild-type flies depletes the organismal TAG storage (Figure 5B; see also Figure 4A) and reduces both the number and size of lipid droplets in fat body cells. BMM:EGFP localizes at islands on the droplet surface, often at interdroplet contact sites (Figure 5C). In contrast, nonfunctional BMM^{S38A}:EGFP distributes homogenously over the droplet surfaces (Figure 5D). Figures 5E and 5F indicate that the evolutionary conserved part of BMM including the Brummer box is sufficient to properly localize the protein on lipid droplets, likely to represent active sites of BMM-dependent TAG mobilization. Other BMM-related lipases, such as hamster desnutrin (Liu et al., 2004) and human TTS-2.1/ATGL (Umlauf et al., 2004), also localize on lipid droplets, but their localization sequences are presently unknown.

brummer antagonizes perilipin-related *Drosophila Lsd-2*

The results indicate that the surface of lipid droplets is an evolutionary conserved intracellular compartment boundary for organismal TAG storage control, as has been suggested for mammalian adipocytes where perilipin modulates activity of HSL (Zhang et al., 2003) and possibly non-HSL lipases such as ATGL. Lack of perilipin results in lean mice with increased lipolysis and reverses the obese phenotype of leptin receptor-deficient mutants (Martinez-Botas et al., 2000; Tansey et al., 2001). The perilipin-like LSD-2 of fly localizes to lipid droplets of fat cells and adjusts organismal TAG content in a dosage-dependent manner, suggesting that it functions as an evolutionary conserved modulator of lipolysis (Grönke et al., 2003). In fact, *bmm*$^-$ *Lsd-2*$^-$ double mutants have wild-type TAG levels (Figure 6), indicating that loss of *Lsd-2* activity compensates for the lack of *bmm*. Conversely, combined overexpression of *bmm* and *Lsd-2* in the fat body can partially revert the complementary phenotypes caused by the overexpression of

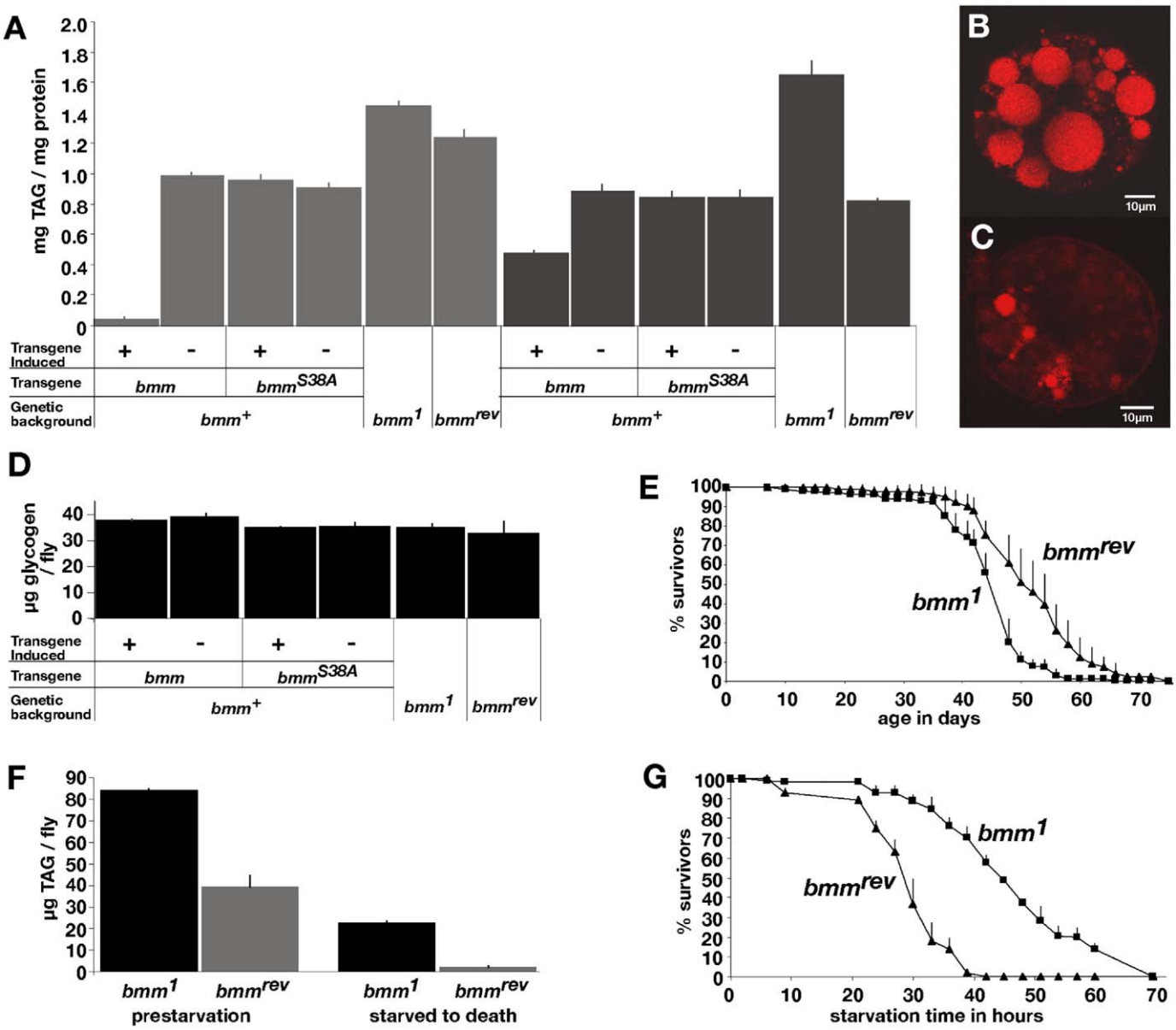


Figure 4. Physiological characterization of *brummer* mutants

A) Adiposity of immature (light gray) and mature (dark gray) adult flies lacking *bmm* (genetically matched control, bmm^{rev}). Leanness of immature and mature flies overexpressing *bmm* but not the catalytic center mutant bmm^{S38A}.

B and C) Cellular phenotype of *bmm*-dependent fat storage regulation visualized by confocal sections of Nile Red-stained lipid storage droplets in single fat body cells of (**B**) obese bmm^{1} and (**C**) lean *bmm*-overexpressing immature adult flies.

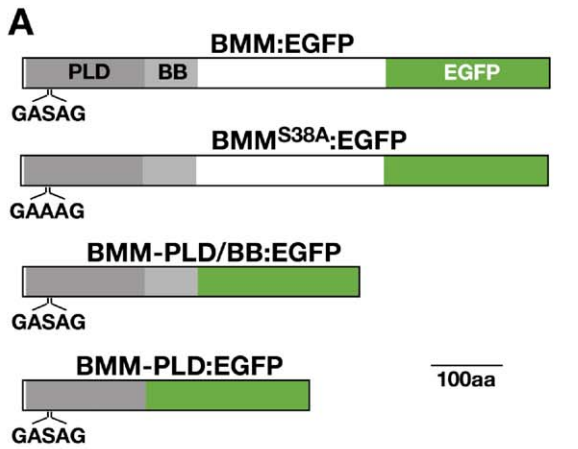
D) Organismal carbohydrate storage is unaffected by *brummer* activity. Glycogen content of fat bmm^{1} mature adult mutant males equals the one of lean flies chronically overexpressing *bmm* in the fat body and is comparable to various control flies with normal TAG storage (compare to [**A**]).

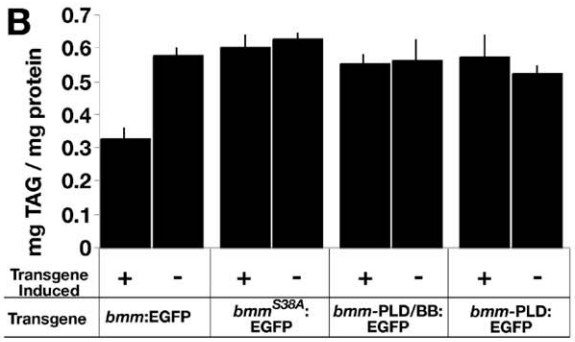
E) Moderately decreased lifespan of bmm^{1} flies (■) compared to bmm^{rev} control flies (▲).

F and G) Incomplete TAG mobilization (**F**) but lifespan extension (**G**) of bmm^{1} flies under starvation (for details, see text). Mean values and error bars are calculated as detailed in Experimental Procedures.

each of the two genes (Figure 6). These data demonstrate that the lipid droplet-associated factors Brummer and LSD-2, which have opposite roles in organismal fat storage, act in an antagonistic manner.

This first in vivo analysis of any insect lipase demonstrates a remarkable conservation of effectors controlling organismal fat storage in mammals and flies, emphasizing the value of *Drosophila* for research in energy homeostasis. On the basis of our results in the fly, we speculate that mammalian members of the *brummer*/nutrin gene family like ATGL play an essential role in organismal fat mobilization and that malfunction of Brummer-homologous TAG lipases might contribute to mammalian obesity. Accordingly, stimulating Brummer-like lipase activity is a potential therapeutic approach to control TAG re-





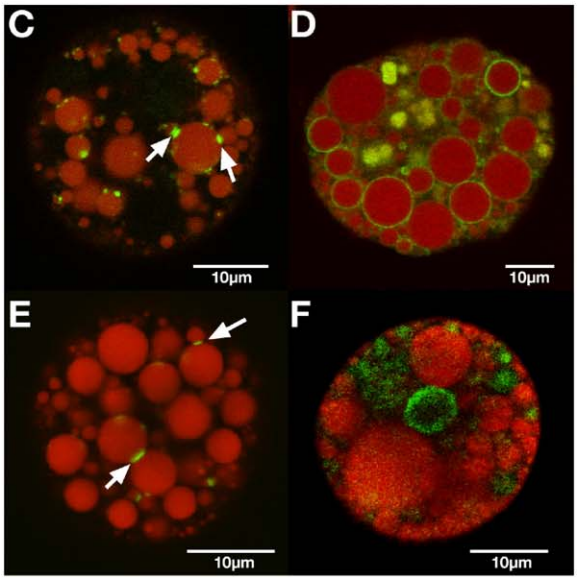


Figure 5. In vivo localization of BMM:EGFP fusion proteins on lipid droplets

A) Schematic representation of transgene-expressed protein variants BMM:EGFP, BMM^{S38A}:EGFP (catalytic center mutant), BMM-PLD/BB:EGFP (deleting the BMM variable region), and BMM-PLD:EGFP (deleting BMM variable region and BB). Abbreviations: PLD (dark gray boxes), Patatin-like domain; BB (light gray boxes), Brummer box; GASAG (intact) and GAAAG (mutated) serine hydrolase motif.

B) Conditional expression of BMM:EGFP but no other chimeric protein in the fat body decreases TAG content of immature male flies.

C–F) Localization of BMM:EGFP fusion proteins (green) relative to Nile Red-stained lipid droplets (red) in single fat body cells from flies in (**B**) visualized by confocal microscopy optical sections. Lipid droplet surface island localization, frequently at interdroplet contact sites (arrows) of (**C**) BMM:EGFP and (**E**) BMM-PLD/BB:EGFP. (**D**) BMM^{S38A}:EGFP distributes homogenously over the lipid droplet surface. (Note: granular yellow signal between lipid droplets represents an autofluorescence signal sporadically observed in fat body cells.) (**F**) BMM-PLD:EGFP is not associated with lipid droplets. (Note: coarse imaging due to high signal amplification necessary to visualize the diffuse distribution.) Mean values and error bars in (**B**) are calculated as detailed in Experimental Procedures.

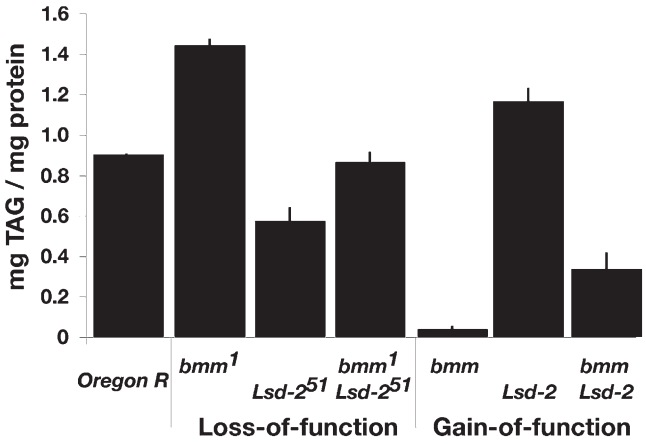


Figure 6. Antagonistic control of fat storage by *brummer* and the *Drosophila* perilipin-like *Lsd-2*

TAG content of immature adult male flies with varying *bmm* and *Lsd-2* activities. Note that the fat storage of flies lacking both *bmm* and *Lsd-2* activities (loss-of-function double mutant *bmm*^{1} and *Lsd-2*^{51}) is comparable to *Oregon R* wild-type and that cooverexpression (gain-of-function) of both *Lsd-2* and *bmm* compensates for effects caused by single gene overexpression. Mean values and error bars are calculated as detailed in Experimental Procedures.

lease from adipose tissue in obese patients, and lipase activators could be tested in the fly model.

Experimental procedures

Fly techniques

All flies were propagated on a complex corn flour-soy flour-molasses medium (corn flour and barley malt 80 g/l each, molasses 22 g/l, yeast 18 g/l, soy flour 10 g/l, agar-agar 8 g/l, propionic acid 6.3 ml/l, and nipagin 1.5 g/l), supplemented with dry yeast at 25°C and 20%–30% humidity with a 12 hr/12 hr light/dark cycle. *Oregon R* wild-type flies as well as *y*^{1} *w*; P{w*$^{+mC}$ *= Act5C-GAL4}25FO1/CyO, y*$^{+}$ (BL4414) and *w*^{1118} *; P{w*$^{+mC}$ *= EP}bmm*^{EP3174} (previously *w*^{1118} *; P{w*$^{+mC}$ *= EP}CG5295*^{EP3174} ; SGF528) flies were obtained from the Bloomington and Szeged *Drosophila* stock centers. A background lethal in the *w*^{1118} *; P{w*$^{+mC}$ *= EP}bmm*^{EP3174} stock was recombined through crosses against *w**. *bmm*^{1} and *bmm*^{2} deletion mutants (missing *bmm* DNA sequences from positions 5006–9479 and 5006–7651 relative to putative start ATG causing lack of BMM aa positions 52–507 and 52–249) as well as flies carrying the precise excision allele *bmm*^{rev} were generated by a conventional P element mobilization scheme and molecularly characterized by sequencing the relevant part of the *bmm* gene (note that *bmm*^{1}, *bmm*^{2}, and *bmm*^{rev} contain residual P element sequences). To generate transgenic fly stocks allowing conditional expression of *bmm* (*w*; P{w*$^{+mC}$ *bmm*$^{Scer\UAS}$ *= UAS-bmm}*; SGF532, 533) and *bmm*^{S38A} (*w*; P{w*$^{+mC}$ *bmm*$^{S38A.Scer\UAS}$ *= UAS-bmm*^{S38A}*}*; SGF537), a *bmm* cDNA was cloned into vector pUAST (Brand and Perrimon, 1993). *bmm* cDNA was PCR amplified from *Drosophila* embryonic cDNA using primer Bmm 5′-ACA CCGCGCCGCAATGAATG and Bmm 3′-TAAACACAGATGGGGATTTGG ATG and subsequently cloned into pCRII-Topo vector (Invitrogen, Carlsbad, USA). In the *bmm*^{S38A} construct, the serine in position 38 was mutated to

an alanine using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with primer BmmS38A 5′-GAAGATCGGAGGCGCT GCGGCCGGTTCCC and BmmS38A 3′-GGGAACCGGCCGCAGCGCC TCCGATCTTC and the bmm cDNA clone as template. EGFP fusion constructs of full-length and truncated *bmm* variants were generated by PCR amplification of the corresponding parts of the bmm cDNA (coding for aa positions 1–507 for Bmm and BmmS38A, aa positions 1–172 for BmmPLD, and aa positions 1–247 for BmmPLD/BB) and cloned into pEGFP-N2 (Clontech, Palo Alto, CA). Subsequently, the Bmm:EGFP, BmmS38A:EGFP, BmmPLD:EGFP, and BmmPLD/BB:EGFP cassettes were introduced into pUAST (details of the cloning are available upon request). Transgenic fly stocks were established by P element-mediated germ line transformation as described, and a FB-Gal4 strain was used for fat body-targeted expression (Grönke et al., 2003).

Starvation assay

For each genotype, triplicate batches of 40 male flies each (<36 hr of age) were transferred to vials providing water supply only. Mortality rates were determined by regularly counting the number of dead flies as diagnosed by the lack of sit-up response. Plotted are average survival rate values and the corresponding standard deviations of a representative experiment.

Longevity assay

Newly emerged bmm^{1} or bmm^{rev} adult males were collected over a 24 hr period and divided into batches of 30 flies per vial. Flies were maintained at 25°C, constant humidity, and a 12 hr/12 hr light/dark cycle environment and were transferred to fresh food vials and scored for survival every 2–4 days. The survivorship curve of bmm^{1} mutants represents data from 180 flies and, from bmm^{rev}, 450 flies, respectively. Error bars in Figure 4E represent standard deviations of 6 and 15 vials, respectively.

Hatching rate

Drosophila eggs from each genotype were collected on apple juice agar plates supplemented with yeast over an 8–10 hr period. Eggs were aged for 48 hr at 25°C and then the number of hatched embryos determined by counting empty chorion membranes and unhatched embryos.

TAG, glycogen, and lipase substrate specificity assays

Organismal TAG and protein content was quantified as described (Grönke et al., 2003). Glycogen content of fly homogenates was determined using an enzymatic starch bioanalysis kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's guidelines. TAG and glycogen contents of a representative experiment are depicted as mean values of triplicate measurements with corresponding standard deviations. Experiments were repeated at least twice.

Recombinant protein for in vitro substrate specificity tests was produced by cloning Bmm and BmmS38A full-length ORF into vector pGEX-4T3 (Amersham Pharmacia Biotec) and expressing these constructs in *E. coli* BL21 cells. Large-scale cultures transformed with GST-Bmm or GST-BmmS38A were incubated at 37°C, induced with 1 mM IPTG at OD 0.5 for 90 min, and subsequently harvested by centrifugation. Cell pellets were extracted by lysozyme and freeze-thaw treatment in HEMG buffer (25 mM HEPES KOH [pH 7.6], 0.5 M NaCl, 0.1% NP40, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, protease inhibitor [Complete EDTA-free, Roche]) and then centrifuged at 30,000 × g for 30 min at 4°C. The supernatant was adsorbed to glutathione Sepharose 4B (Amersham Biosciences) and eluted with HEMG 15 mM glutathione. Protein concentration was assessed by SDS-PAGE analysis in comparison to a BSA standard. Purified proteins were tested in GPI-specific PLC/D (in the absence or presence of 1% Nonidet P-40), MAG-lipase, TAG-lipase, and phosholipase A2 activity assays as described (Fredrikson et al., 1986; Low and Huang, 1991; Mensa-Wilmot et al., 1995; Tornqvist and Belfrage, 1981) using monooleoyl[^{3}H]glycerol, glycerol-tri-[9,10(n)-^{3}H]oleate and 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzodiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphocholine (PAP) as substrates. In vitro lipase activities are expressed as mean values and standard deviations of duplicate experiments.

Fluorescence microscopy

Fat body cells from adults were prepared as follows for ex vivo confocal laser scanning microscopy. The abdomina of male flies (aged 0–24 hr) were manually opened and the floating fat body cells released into mounting medium (50% glycerol/PBT, Nile Red 1:55,000 [Molecular Probes, Eugene, OR] [Nile Red stock 10% in DMSO]). Cells were analyzed within 2 hr after mounting using a Leica TCS SP2 LSM with 543 nm excitation/600–657 nm emission or 488 nm excitation/500–541 nm emission for visualization of lipid droplets and EGFP fusion proteins, respectively.

Supplemental data

Supplemental data include one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://cellmetabolism.org/cgi/content/full/1/5/323/DC1/.

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