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Schlank, a member of the ceramide synthase family controls growth and body fat in *Drosophila*

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Ceramide synthases are highly conserved transmembrane proteins involved in the biosynthesis of sphingolipids, which are essential structural components of eukaryotic membranes and can act as second messengers regulating tissue homeostasis. However, the role of these enzymes in development is poorly understood due to the lack of animal models. We identified *schlank* as a new *Drosophila* member of the ceramide synthase family. We demonstrate that schlank is involved in the *de novo* synthesis of a broad range of ceramides, the key metabolites of sphingolipid biosynthesis. Unexpectedly, *schlank* mutants also show reduction of storage fat, which is deposited as triacylglyerols in the fat body. We found that schlank can positively regulate fatty acid synthesis by promoting the expression of sterol-responsive element-binding protein (SREBP) and SREBP-target genes. It further prevents lipolysis by downregulating the expression of triacylglycerol lipase. Our results identify schlank as a new regulator of the balance between lipogenesis and lipolysis in *Drosophila*. Furthermore, our studies of schlank and the mammalian Lass2 family member suggest a novel role for ceramide synthases in regulating body fat metabolism.

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Introduction

In all animals energy homeostasis is under tight control of evolutionarily conserved nutrient-sensing systems. These include the target of rapamycin (TOR) pathway (Martin and Hall, 2005) and several families of secreted peptide hormones, which regulate and fine-balance carbohydrate and lipid metabolism to match energy requirements. Particularly during growth, a coordinated regulation of the lipid metabolism is important on both the cellular and organismal level. On the cellular level, sterols in mammalian cells or phosphatidylethanolamine, the major phospholipid in *Drosophila*, control the release of sterol-regulatory element-binding protein (SREBP) from cell membranes, exerting feedback control on the synthesis of fatty acids (FAs) and phospholipids (Dobrosotskaya *et al*, 2002; Seegmiller *et al*, 2002; Kunte *et al*, 2006). SREBPs are membrane-bound transcription factors that monitor cell membrane composition and adjust lipid synthesis accordingly. The *de novo* synthesis of sphingolipids is also pivotal since sphingolipids are essential structural components of eukaryotic membranes and play important roles as second messengers regulating apoptosis, survival and differentiation (Spiegel and Milstien, 2000; Hannun *et al*, 2001; Acharya and Acharya, 2005). Misregulation of the sphingolipid metabolism is involved in the aetiology and pathology of a number of human diseases, including neurodegeneration, cancer, immunity, cystic fibrosis, emphysema, diabetes and sepsis (Kolter and Sandhoff, 2006; Lahiri and Futerman, 2007). The enzymes of the sphingolipid pathway are conserved in all genetically studied eukaryotes (Jiang *et al*, 1998; Hannun *et al*, 2001; Acharya and Acharya, 2005). However, *in vivo* information on how these enzymes are regulated in response to growth requirements or during starvation, is either limited by early lethality of the knockout animals reflecting the fundamental necessity of these enzymes (Li *et al*, 2002; Hojjati *et al*, 2005; Mizugishi *et al*, 2005) or by the lack of mutants. The latter includes mutants for key enzymes of the pathway such as ceramide synthases, which produce the precursor metabolites for all sphingolipids. Mammals contain six family members of the ceramide synthase family, also called Lass (longevity assurance homologue-1 of yeast *Lag1*) proteins. Their function has mainly been studied in tissue culture cells since knockout models are not available yet, and it was shown that they control the synthesis of different ceramides (for reviews see Pewzner-Jung *et al*, 2006; Teufel *et al*, 2009).

On the organismal level, energy homeostasis depends on the ability to control the balance between lipid synthesis, storage and lipid mobilization during conditions of energy abundance or deprivation, respectively (Hay and Sonenberg, 2004; Zechner *et al*, 2005). Storage fat is deposited as triacylglycerols (TAGs), in intracellular lipid droplets (Martin and Parton, 2006), which accumulate in specialized organs such as mammalian adipose tissue or the fat body of flies (Canavoso *et al*, 2001; Rosen and Spiegelman, 2006). Lipolysis of TAGs is induced by lipases and leads to release of free FAs or *sn*-1,2-diacylglycerol in mammals and insects, respectively, into the circulatory system (Arrese and Wells, 1997; Gibbons *et al*, 2000; Patel *et al*, 2005). Chronic dysregulation of the balance between lipolysis and lipogenesis may lead to metabolic abnormalities such as obesity, lipodystrophy syndromes or insulin resistance in humans (Kahn *et al*, 2006; Simha and Garg, 2006).

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We have identified a new regulator of growth and lipid homeostasis in *Drosophila*, the *schlank* gene. We show that *schlank* encodes a *Drosophila* member of the Lass/ceramide synthase family required for *de novo* synthesis of ceramides. Unexpectedly, schlank is also involved in the regulation of body fat metabolism and we show that it regulates the balance between lipogenesis and lipolysis during larval growth.

Results

Identification and molecular characterization of schlank

Wild-type larvae hatch after embryogenesis and pass through three larval instar stages until puparium formation, which occurs at about 96–120 h after hatching. During larval development, the animals increase their body size by about 200-fold. In a search for genes controlling larval growth in *Drosophila*, we screened the Göttingen P-element collection (Peter *et al*, 2002) and identified four P-element insertion lines affecting a single gene locus on the X-chromosome, which we had previously named *Drosophila longevitiy assurance gene-1 homologue* (DLag1) due to its sequence homology to the yeast *LAG1* ceramide synthase (for review see D'mello *et al*, 1994; Teufel *et al*, 2009). Since we now show that the P-element insertions into the *DLag1* locus cause defects in larval growth and fat metabolism (see below), we propose to rename the gene *schlank* ('slim' in German) following the *Drosophila* nomenclature in which genes are named according to the phenotype of the respective mutants.

The *schlank* gene locus maps to the X-chromosome (Supplementary Figure S1A). Hemizygous *schlank* mutants show a delay of larval development and pronounced growth defects, which depend on the strength of the alleles (Figure 1A–C). Mutants carrying the stronger $schlank^{G0349}$ allele fail to grow in the larval stages, although they feed as determined by feeding assays. After about 3days, these animals die as small larvae, which morphologically correspond in size to first instar larvae (Figure 1A–C). In contrast, hemizygous animals carrying the weaker $schlank^{G0061}$ allele are developmentally delayed; however, they reach the third instar larval stage and a fraction thereof even pupariates. Quantitative real-time PCR (qRT–PCR) experiments reveal that the level of *schlank* transcripts is reduced to less than 40% in hemizygous $schlank^{G0061}$ larvae and to less than 10% in hemizygous $schlank^{G0349}$ larvae as compared with control animals (Figure 1C). However, using an antibody against the C-terminus of the schlank protein (Supplementary Figure S4), we found that the level of schlank protein is only reduced by about 20% in the $schlank^{G0061}$ and by 40% in the $schlank^{G0349}$ mutants (Figure 1D), indicating a maternal supply of the gene products and/or an enhanced stability of the protein. Consistently, we found that *schlank* mRNA and protein are highly abundant in oocytes and during early embryogenesis (Supplementary Figure S5A and B). In order to generate a 'null' situation, we largely eliminated both *schlank* mRNA and protein expression by generating germline clones using the $schlank^{G0349}$ allele. When both the zygotic and maternal contributions of *schlank* are largely eliminated, we did not obtain any eggs. This demonstrates that *schlank* has both a maternal and a zygotic supply and explains the residual mRNA and protein activity in $schlank^{G0349}$-mutant animals. Furthermore, this is consistent with a fundamental function of schlank in the lipid metabolism (see below) and unfortunately impedes to work with complete *schlank*-null animals in *Drosophila*.

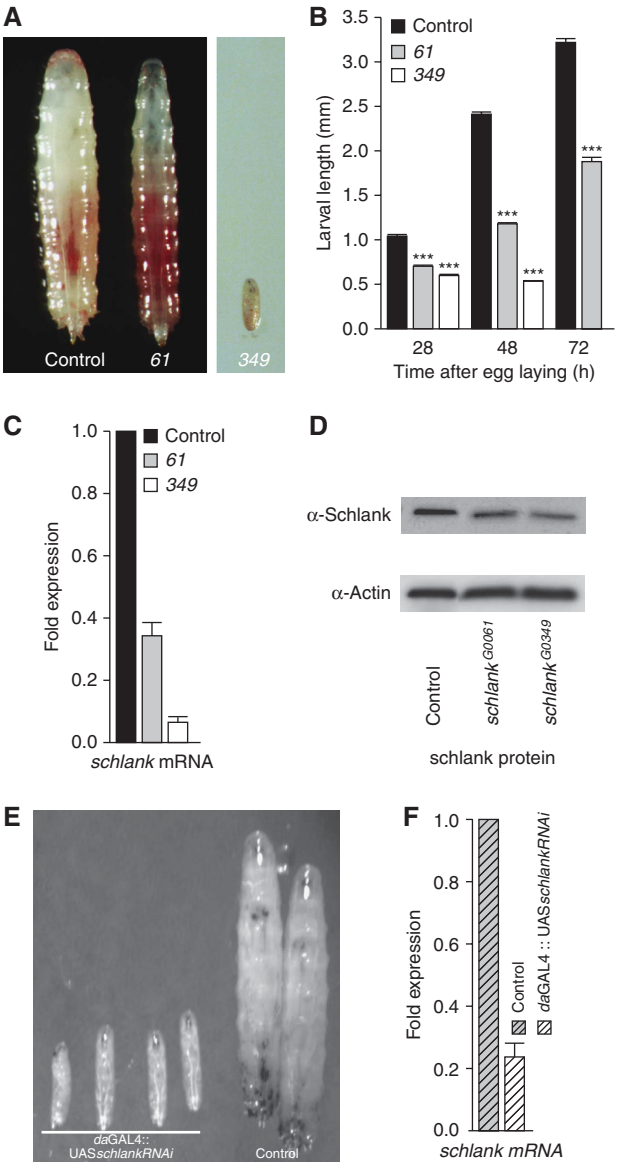

Figure 1 *Schlank* is essential for larval growth. (**A**) Larval growth of $schlank^{G0061}$ (*61*) mutants compared with wild-type w^{1118} larvae (control) at late L3 stage. $schlank^{G0349}$ (*349*) hatch as first instar larvae and die after about 3 days as morphological first instar larvae. Food intake in mutants was controlled by feeding red-coloured yeast. (**B**) Average length of control [w^{1118}] ($n=123$, $n=81$, $n=48$), $schlank^{G0061}$ ($n=54$, $n=187$, $n=63$) and $schlank^{G0349}$ ($n=14$, $n=106$) larvae after 24–25, 48–49 and 72–73 h after egg laying. (**C**) Reduced *schlank* mRNA expression in *schlank* mutants as compared with that in w^{1118} controls. (**D**) Determination of residual schlank protein (predicted size of about 46 kDa) in *schlank* mutants; reduction to 80% in $schlank^{G0061}$ and 60% in $schlank^{G349}$ mutants as compared with that in wild-type controls (100%; w^{1118}) using schlank antibody (Supplementary Figure S4). An actin antibody was used to determine the loading control. (**E**, **F**) *schlank* knockdown using *schlank* RNAi (UAS*schlankRNAi*) in combination with the *daughterless*-GAL4 (*da*GAL4) driver line phenocopies the *schlank* larval growth phenotype (E). Quantification of mRNA levels by qRT–PCR in panel F. Control: *da*GAL4::w^{1118}. Asterisks in panel B indicate significant differences to the wild type ($P<0.001$). (**B**–**G**) Error bars indicate s.e.m.

To further provide evidence that the lethality and the growth phenotypes of the schlank P-alleles are caused by downregulation of the *schlank* gene, we used RNAi-mediated knockdown. Expression of a UAS*schlankRNAi* transgene in combination with the ubiquitous *daughterless*-GAL4 (*da*GAL4) driver line phenocopied the growth defects observed in the *schlank*-mutant alleles (Figure 1E and F). Furthermore, transgenic flies carrying the full-length *schlank* cDNA were able to rescue *schlank*^{G0349}-mutant animals to the third larval instar stage and some of the animals even to the pupal stage (Supplementary data and Supplementary Figure S1C). Together, molecular analysis of the *schlank* alleles, reversion of the phenotype by perfect excision of the P-elements (Supplementary data from Fly strains, and data not shown), RNAi-mediated knockdown and genetic rescue experiments demonstrate that the lethality and the growth phenotype of the *schlank* alleles are linked to the *schlank* gene function.

Schlank encodes the Drosophila homologue of the Lass/ceramide synthase family

The *schlank* gene locus codes for two transcripts, which differ in their 5′ UTR; however, they encode the same open reading frame (ORF; Supplementary Figure S1A). The *schlank* ORF codes for a transmembrane protein with high homology to ceramide synthases and a phylogenetic analysis indicates that schlank is closely related to the mammalian Lass family members both in sequence and protein domain structure (Figure 2A; Supplementary Table and Supplementary Figure 1B).

Ceramide synthases use long-chain bases, sphinganine or sphingosine, and FA-CoAs with varying chain length to produce (dihydro)ceramide, which is a precursor metabolite for all sphingolipids. Sphingolipids are structural components of most cellular membranes and can act as signalling molecules in cell growth, differentiation and apoptosis (Spiegel and Milstien, 2000; Hannun *et al*, 2001; Acharya and Acharya, 2005). Lass family members contain four to seven predicted transmembrane domains (Venkataraman and Futerman, 2002), a catalytic Lag1 motif and most an N-terminal domain showing sequence homology to DNA-binding homeodomains (Hox domain) (Gehring *et al*, 1994; Venkataraman and Futerman, 2002). Structure predictions indicate that the putative schlank protein contains six transmembrane domains, a Lag1 motif and a Hox domain, which are highly conserved (Supplementary Figures 1B and 2C). The Lag1 motif of Lass proteins, which are found in organisms ranging from yeast to mammals, is functionally required

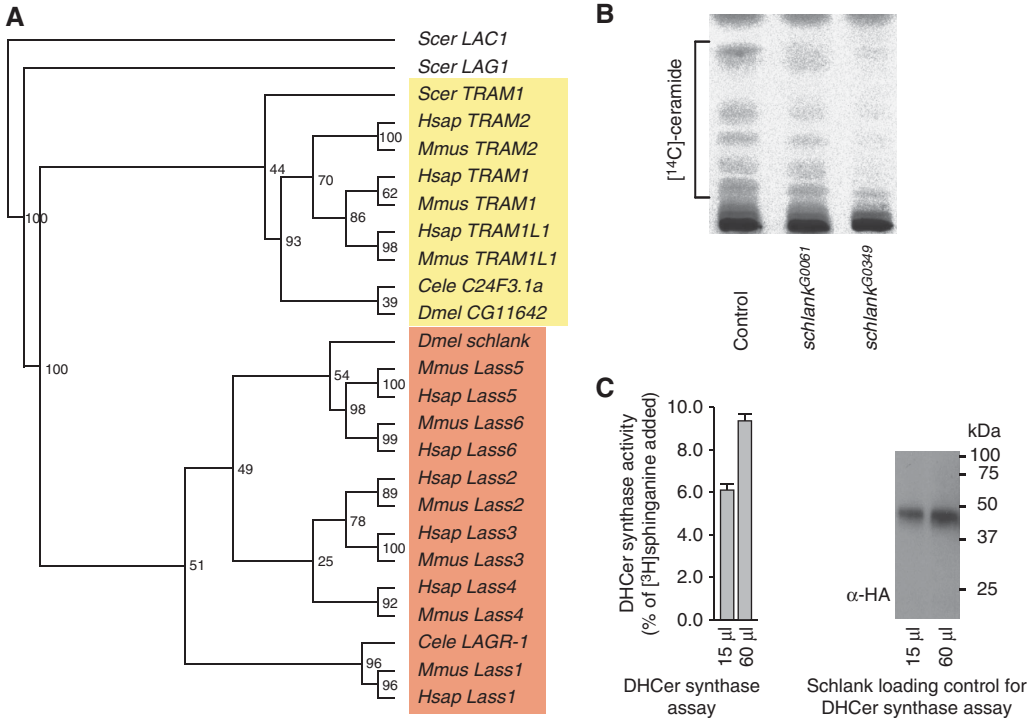

Figure 2 Schlank is involved in *de novo* ceramide synthesis. (**A**) Phylogenetic tree of Lass family members and TRAM proteins. Protein sequences were derived from the Ensembl database. If more than one protein variant existed, the version with all domain features was used (for accession numbers see Supplementary Table SI). Sequences were aligned using EMBL-EBI ClustalW2 online service using standard settings. The alignment file was used to generate 100 bootstrapped data sets with seqboot (PHYLIP 3.68 package; see also Felsenstein, 1989). The output was analysed using maximum likelihood (proml, PHYLIP package). An unrooted consensus tree was generated with consense (PHYLIP package). Bootstrap values are marked above each branch. The ceramide synthase family is highlighted in red and the TRAM family in yellow. Note that schlank falls into the Lass family and CG11642 into the TRAM family. (**B**) Biosynthesis of ceramides is significantly reduced in first instar *schlank*^{G0061} and *schlank*^{G0349} larvae as compared with that in w^{1118} wild-type controls. Sphingolipids were labelled by feeding larvae with radiolabelled L-[3-^{14}C]-serine for 12 h. After lipid extraction equal amounts of radioactivity were applied to TLC plates, developed with chloroform/ methanol/ glacial acetic acid (190:9:1) and quantified. The total ceramide content in *schlank*^{G0061}- and in *schlank*^{G0349}-mutant larvae was reduced to 89 and 60%, respectively, as compared with that in the wild-type (100%). (**C**) The dose-dependent increase in the expression of immunoreactive schlankHA product correlates with an increase in ceramide synthase activity. Different amounts of eluted fractions after binding to a HA affinity matrix were used for immunoblotting with an HA antibody or determination of ceramide synthase activity (see also Supplementary data).

for ceramide synthesis and is contained within a stretch of 52 amino acids (Pewzner-Jung *et al*, 2006; Spassieva *et al*, 2006). The Lag1 motif of schlank is highly conserved and shows sequence identity to the Lag1 consensus motif of 82.3% (Supplementary Figure S2A). Furthermore, it contains all of the conserved amino acids that were shown to be crucial for the catalytic function of Lag1 domains in ceramide synthesis (Spassieva *et al*, 2006 and Supplementary Figure S2A). In addition, schlank contains a putative homeodomain, which is also found in most vertebrate Lass proteins (Supplementary Figures S1B and S2C), but not in yeast, worms and plants (Venkataraman and Futerman, 2002). The function of Lass homeodomains is unknown. In addition to *schlank*, a second gene (CG11642) with some homology to Lass/ceramide synthase family members was identified in the *Drosophila* genome by sequence similarity searches (Acharya and Acharya, 2005). However, the sequence similarity of the putative Lag1 motif of the CG11642 gene product is much lower as compared with schlank, and it is of note that most of the amino acids, which were shown to be crucial for the function of the Lag1 motif in ceramide synthesis (Spassieva *et al*, 2006), are not conserved and changed in the CG11642 gene product (Supplementary Figure S2B). Rather, the gene product of CG11642 seems more homologous to members of the translocating chain-associated membrane (TRAM) protein family (Figure 2A), which are not involved in ceramide synthesis (Jiang *et al*, 1998; Winter and Ponting, 2002; Spassieva *et al*, 2006). This is further supported by RNAi-mediated knockdown of CG11642 showing no phenotype in ceramide synthesis (Supplementary Figure S3B). Together, these data suggest that *schlank* may encode the only ceramide synthase family member in *Drosophila*.

Schlank is involved in de novo ceramide synthesis in Drosophila

Since schlank appears to be a member of the Lass/ceramide synthase family, we first tested whether schlank is involved in ceramide metabolism and found that the total ceramide content in $schlank^{G0061}$ and in $schlank^{G0349}$-mutant larvae was significantly reduced as compared with wild-type animals (Figure 2B). To specifically address whether *de novo* synthesis of ceramides is affected in the mutant animals, we fed $schlank^{G0061}$ and $schlank^{G0349}$-mutant larvae for 12 h with radiolabelled L-[3-^{14}C]-serine, a precursor of sphingolipid biosynthesis, and we analysed the incorporation of this label into *de novo* generated ceramide. $schlank^{G0061}$ and $schlank^{G0349}$ mutants as well, as the wild-type controls, incorporated the same amount of total radioactivity per dry weight ($schlank^{G0349}$ 19 882 cpm/mg, $schlank^{G0061}$ 18 930 cpm/mg and wild-type controls 20 685 cpm/mg; s.e.m. ± 5%). After the extraction of lipids, ceramides were separated by thin-layer chromatography (TLC) and identified and quantified using commercially available reference standards (Supplementary Figure S3A and Supplementary data). We found in our metabolic labelling studies that the levels of *de novo* generated ceramides were significantly decreased in $schlank^{G0061}$ (69.25%) and $schlank^{G0349}$ (39%) mutants as compared with wild-type controls (100%; Figure 2B), in line with the reduced *schlank* protein levels in the mutants (Figure 1D).

To further establish a role of schlank in the *de novo* synthesis of ceramides, we generated transgenic flies expressing a C-terminally HA-tagged, full-length *schlank* protein (UAS*schlankHA*). We induced the tagged protein in larvae using a *heat shock*-GAL4 (*hs*GAL4) driver line, prepared extracts to purify it partially (Materials and methods) and performed standard *in vitro* ceramide synthase assays (Wang and Merrill, 1999). We found that increasing amounts of purified schlank led to a dose-dependent increase in ceramide synthase activity and increased ceramide levels (Figure 2C).

Manipulation of schlank activity in loss- and gain-of-function experiments

To further support a role of schlank as ceramide synthase, we reduced or elevated its activity in loss- and gain-of-function experiments and analysed the effects on ceramide levels. To this end, we fed radiolabelled L-[3-^{14}C]-serine to larvae carrying transgenic UAS*schlankRNAi* or UAS*schlankHA* in combination with the *hs*GAL4 driver line. Upon a short heat shock (1 h) to induce *schlank* RNAi knockdown or *schlank* overexpression, we observed a decrease or increase of ceramide levels, respectively (Figure 3A and B). Most interestingly, overexpression of the murine Lass2 homologue for which ceramide synthase activity has been shown previously (Mizutani *et al*, 2005), resulted in a similar increase in ceramide levels (Figure 3A and B). In contrast, an increase in ceramide levels could not be observed when we overexpressed a schlank protein variant, schlankH215D (Supplementary Figure S3B), which contains a point mutation in the Lag1 motif shown to inhibit ceramide synthase function in Lass1 and 5 (Spassieva *et al*, 2006; change of a highly conserved histidine at position 215 into glutamate; Supplementary Figure S2A).

To further test whether the effects on larval lipid composition were due to the influence of schlank on the metabolism and catabolism of endogenous lipids, rather than, for example, on an impaired uptake from the yeast food, we also analysed *de novo* synthesis of ceramide in SL-2 cells. Upon RNAi-mediated knockdown of *schlank* (dihydro)ceramide levels were significantly downregulated, whereas they were upregulated upon *schlank* overexpression (Figure 3C and D), consistent with the results obtained in larvae. Together, the analysis of *schlank* mutants, knockdown animals and overexpression studies *in vivo* and *in vitro* strongly support a role of schlank as a ceramide synthase in *Drosophila*.

TAG levels are reduced in schlank mutants

While hemizygous animals carrying the stronger $schlank^{G0349}$ allele fail to grow in the larval stages and die with a morphology of first instar larvae, the $schlank^{G0061}$ mutants are developmentally delayed; however, some of them pass through the third instar larval stage and die later after eclosion. When analysing third instar larvae of $schlank^{G0061}$ mutants, we noticed that they appeared much slimmer and somewhat transparent, indicating loss of storage fat in the fat body (Figure 1A). A function in regulating organismal fat storage or mobilization has previously not been observed for Lass/ceramide synthase family members due to lack of animal models. We, therefore, investigated this phenotype in more detail.

The larval period is critical for the control of animal growth. Regulation of larval growth by fat body has been demonstrated previously (Britton *et al*, 2002; Colombani *et al*, 2005). The larval stage is characterized by extensive

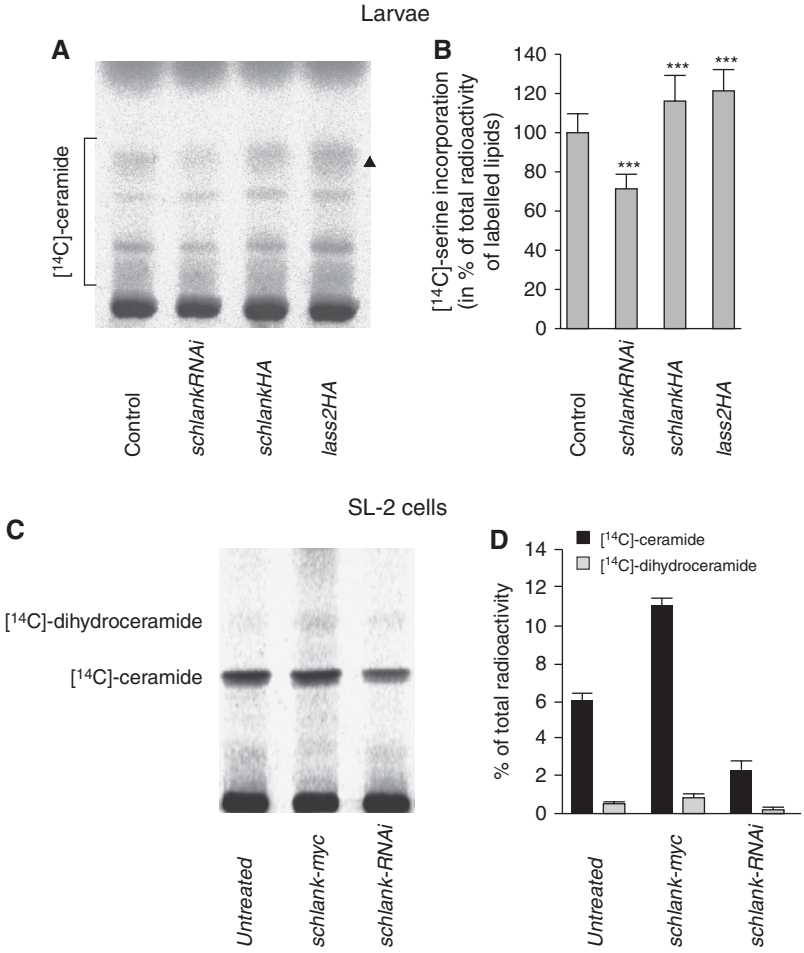

Figure 3 Modulation of schlank activity correlates with the rate of ceramide *de novo* synthesis (**A**, **B**) Larvae carrying *hs*GAL4 and either UAS*schlankRNAi (schankRNAi)*, UAS*schlankHA (schlankHA)* or UAS*lass2HA (lass2HA)* were heat shocked for 1 h and subsequently fed L-[3-^{14}C]-serine. Its incorporation of L-[3-^{14}C]-serine into *de novo* ceramide was analysed in larvae 12 h after heat shock by TLC with chloroform/methanol/glacial acetic acid (190:9:1) (A) and quantified (B). Asterisks in panel B indicate significant differences to wild-type controls [*hs*GAL4::w^{1118}] ($P<0.001$). (**C**, **D**) Treatment of SL-2 cells in the presence of [^{14}C]serine with *schlank* dsRNA or *schlank* overexpression after transfection of SL-2 cells shows significant downregulation or upregulation of (dihydro)ceramide, respectively. Ceramides and dihydroceramides were separated on TLC plates impregnated with borate with chloroform/methanol 9:1. The main (dihydro)ceramide bands depicted in panel C correspond to the main ceramide marked with an asterisk in panel A (see also Supplementary Figure S2A). Error bars indicate s.d.

feeding, which supports rapid growth of the animal and allows accumulation of energy stores, primarily in the larval fat body (Aguila *et al*, 2007). Immunohistochemical analysis indicates that schlank is strongly expressed in the fat-body cells (Supplementary Figure S5C–F). To study whether schlank may play a role in fat metabolism, we analysed TAG, diacylglycerol (DAG) and FA levels in *schlank*^{G0061}- and *schlank*^{G0349}-mutant larvae of the same developmental stage and compared them with those in wild-type animals. Both *schlank*^{G0061} and *schlank*^{G0349} mutants showed significantly reduced TAG levels to 63 and 13%, and FA and DAG levels were also altered (Figure 4A; Table I for quantification). The specificity of this phenotype was further confirmed by induction of *schlank* RNAi knockdown showing also strong TAG reduction (Supplementary Figure S3C). In contrast, we observed an increase of TAG, DAG and FA levels upon overexpression of schlank and the murine Lass2 homologue (Figure 4B; Table I for quantification), suggesting a conserved role for Lass proteins in lipid homeostasis. When we overexpressed the schlankH215D variant containing a mutation, which severely affects ceramide synthase activity (Supplement Figure S3B; Materials and methods), we observed a similar increase of TAG, DAG and FA levels (Figure 4B and Table I). This effect was comparable to the increase seen when overexpressing wild-type schlank or murine Lass2, suggesting that there are also effects of schlank on fat metabolism that may be independent of its ceramide synthase function. In summary, these data indicate an important role of schlank in regulating TAG levels and body fat metabolism, consistent with the strong expression of schlank protein in the larval fat body. To study how this effect occurs, we analysed the expression of key regulators of lipogenesis and lipolysis in *schlank* mutants and in animals overexpressing schlank.

Schlank negatively regulates the expression of lipases in the fat body, downregulating lipolysis

Lipolysis in response to fasting is accompanied by upregulation of lipases, the rate-limiting enzymes regulating TAG mobilization in insect fat body cells (Arrese and Wells, 1997; Grönke *et al*, 2005). As schlank is highly expressed in the larval fat body and TAG levels were reduced

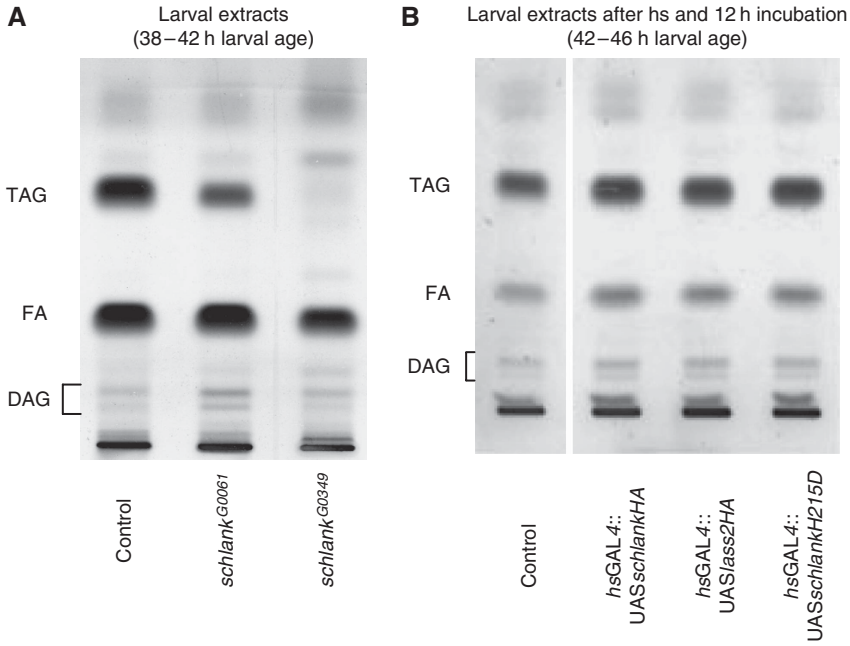

Figure 4 A role for schlank in TAG regulation. (**A**) Comparison of *in vivo* TAG levels in 38- to 42-h-old $schlank^{G0061}$- and $schlank^{G0349}$-mutant animals of the same age with w^{1118} controls. In both mutants TAG and FA levels were reduced to a different extent correlating well with the severity of the mutant *schlank* alleles. (**B**) TAG and FAs are elevated in 42- to 46-h-old larvae upon overexpression of either *schlankHA (hs*GAL4::UAS*schlankHA)*, murine *lass2HA (hs*GAL4::UAS*lass2HA)* or *schlankH215D (hs*GAL4::UAS*schlankH215D)*, which cannot upregulate ceramide synthesis (see Supplementary Figure S3B). Triacylglycerol (TAG), diacylglycerol (DAG), fatty acids (FA). For photodensitometric quantification, see Table I.

Table I Quantification of TAG, FA and DAG in *schlank*-mutants (Figure 4A)

	TAG	FA	DAG
Control[a]	100.0	100.0	100.0
$schlank^{G0061}$[a]	63.41	98.06	139.57
$schlank^{G0349}$[a]	13.40	73.25	105.36
Control[b]	100.0	100.0	100.0
UAS-*schlankHA*[b]	109.0[c]	121.8	116.4
murine UAS-*lass2*[b]	106.6[c]	114.6[c]	120.0[c]
UAS-*schlank*H215D[b]	106.8	106.5	117.4[c]

[a]Relative lipid content of TAGs, FAs and DAGs in 38–42 h *schlank*-mutant larvae (Figure 4A) compared to w^{1118} larvae as control.
[b]Relative lipid content of TAGs, FAs and DAG (Figure 4B) after heat shock (for 1 h at 37°C) followed by 12 h incubation at 20°C to exclude further induction by the heat shock promoter. Larval age was 42 to 46 h; w^{1118} was used as control.
[c]$P < 0.05$.

in $schlank^{G0349}$ and $schlank^{G0061}$ animals, we first asked whether lipases are misregulated in *schlank* mutants. Using quantitative RT–PCR we found that both mRNA levels of *brummer* encoding an ATGL lipase (Grönke *et al*, 2005; Haemmerle *et al*, 2006), and of *lipase3* (*lip3*), which encodes a TAG lipase (Zinke *et al*, 2002; Fuss *et al*, 2006), were upregulated (Figure 5A) in hemizygous $schlank^{G0349}$ and $schlank^{G0061}$ mutants, fitting well with the decreased TAG levels in these animals (Figure 4A and Table I). Likewise, upon RNAi-mediated knockdown of the *schlank* gene, the mRNA levels of both lipases were also increased (Figure 5A). A detailed analysis by ESI-MS of first instar $schlank^{G0349}$ animals demonstrated that particularly myristic acid (C14:0), myristoleic acid (C14:1) and palmitic acid (C16:1) were reduced (Figure 5C and Table II). In contrast, the mRNA level of *lipase3* was decreased upon ubiquitous expression of *schlank* in larvae with the *hs*GAL4 driver (Figure 5B).

We next addressed the question whether schlank is also functionally required in the fat body. Therefore, we expressed UAS*schlankHA* under the control of the fat body-specific driver line *pumpless*-GAL4 (*ppl*Gal4; Zinke *et al*, 2002) in the background of hemizygous $schlank^{G0349}$ mutants. We found that $schlank^{G0349}$ larvae, which normally die as small morphologically first instar larvae, were now rescued. A couple of these rescued animals generated a fat body, showed an increase of TAG, DAG and FA levels (Supplementary Figure S3D and E), and grew to the second or third larval instar stage. Some of these animals even pupariated and showed a phenotype similar to the weaker $schlank^{G0061}$ mutants (Supplementary Figure S1C). Noteworthy, expression of the murine Lass2 in the fat body of $schlank^{G0349}$ mutants led also to rescue of the small $schlank^{G0349}$ mutant larvae to the second and third larval instar stages (Supplementary Figure S1C). However, when we expressed the schlankH215D variant in $schlank^{G0349}$ mutant larvae no rescue could be observed (Supplementary Figure S1C).

Schlank can act as a positive regulator of SREBP-dependent lipogenesis in Drosophila larvae

Since TAG levels were increased upon overexpression of schlank (Figure 4B and Table I), we studied its putative role in lipogenesis in more detail. Lipid homeostasis is controlled in all metazoans studied by a regulatory pathway involving the SREBP protein. SREBPs are transcription factors required for synthesis of cholesterol and unsaturated FAs in mammals and saturated FAs in *Drosophila* (Sato *et al*, 1994; Seegmiller *et al*, 2002). SREBPs are generated as precursors containing two membrane-spanning helices inserted into ER membranes. In response to cellular lipid needs, SREBP exits

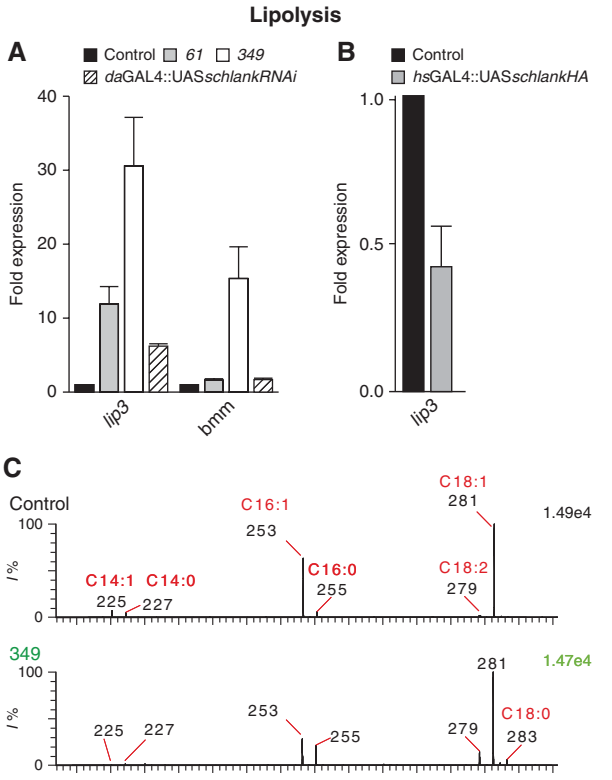

Figure 5 Schlank affects lipolysis. (**A**) Transcript levels of *lip3* and *bmm* lipases are upregulated in first instar *schlank*^{G0061} and *schlank*^{G0349} mutants and upon *schlank* knockdown using a *da*GAL4 driver line, as quantified by qRT–PCR. *schlank* mutants were compared with wild-type w^{1118} larvae, and *schlankRNAi* was compared with *da*GAL4::w^{1118}) (**B**) Overexpression of UAS*schlankHA* using a *heat shock*-GAL4 (*hs*GAL4) driver line decreases *lip3* transcript levels (*hs*GAL4::w^{1118} were used as control). (**C**) Analysis and quantification of FAs in 38- to 42-h-old w^{1118} control and *schlank*^{G0349} mutants. Quantification of FAs was performed by ESI-MS using an internal standard (Table I and Supplementary data). Error bars indicate s.e.m.

Table II ESI-MS quantification of FAs in first instar *schlank*^{G0349} mutants (see Figure 4A)

FA	*Control*	*schlank* ^{G0349}	Ratio (*349/control*) (%)
	(pmol/1 mg dry weight)		
C14:0	27.87	8.56	30.7
C14:1	9.64	1.32	13.7
C16:0	48.35	21.31	44.1
C16:1	38.74	11.94	30.8
C18:0	21.48	10.67	49.6
C18:1	40.59	17.29	42.6

For the quantitative MS analysis of fatty acids we used 1 μg 18,18,18-D_3-stearic acid as internal standard and lipid extract from 100 or 200 μg dry larvae. Fatty acids were identified by their *m/z* values $[M\text{-}H]^-$ and their relative concentrations were calculated on the basis of peak intensity; w^{1118} was used as control. Values represent means ($n = 2$); s.d. was in the range of 10 to 15%.

the ER and travels to the Golgi apparatus, where it is subject to sequential cleavages. A transcriptionally active domain is then released from the membrane, enabling it to travel to the nucleus and activate the transcription of target genes required for FA synthesis. Since *schlank*^{G0349} mutants die as first instar larvae, we analysed *schlank*^{G0061} mutant larvae in which the fat body, the storage organ for lipid droplets, is well developed. Furthermore, using feeding assays, we made sure that *schlank*^{G0061}-mutant larvae feed properly. We found that in *schlank*^{G0061} hemizygous animals the level of SREBP mRNA is markedly decreased (Figure 6A) and accompanied by reduced SREBP protein level of both the membrane-bound precursor protein and the transcriptionally active nuclear protein (Figure 6B). Consistently, transcription of the SREBP-target genes (Seegmiller *et al*, 2002) *acetyl-CoA synthetase* (*ACS*), *acetyl-CoA carboxylase* (*ACC*), *fatty acid synthase* (*FAS*), encoding enzymes of FA biosynthesis, and *fatty acyl-CoA synthetase* (*FCS*), encoding an enzyme involved in synthesis of phospholipids, are strongly reduced (Figure 6A).

In contrast, overexpression of schlank in larvae using transgenic UAS*schlank* effector lines and *hs*GAL4 driver lines led to an increase of SREBP transcript levels and of SREBP-dependent target gene expression, including *FAS* (Figure 6C). These data are in agreement with the observed downregulation of TAG and FA levels in *schlank*-mutant larvae and elevated levels of TAG and FAs in larvae overexpressing *schlank* (Figures 4A and B; Table I).

Data from mutant and overexpression suggest that schlank may act as a positive regulator of SREBP transcript levels and of SREBP-dependent FA and phospholipid synthesis. This is consistent with the similarity of the phenotypes found in *dSREBP* and *schlank* mutants, which both show growth and metabolic defects, including reduction of the FA levels (Kunte *et al*, 2006). Additional evidence for a link between SREBP and schlank was provided by a dietary supplementation assay. For *SREBP* mutants it was shown that they suffer from FA deficiency due to a transcriptional deficit in genes needed for lipid synthesis, and that addition of soy lipids to the food improved their survival (Kunte *et al*, 2006). Similarly, feeding *schlank* mutants with yeast paste supplemented with soy lipids led to significant increase of *schlank*^{G0061} mutant animals surviving to adulthood (Figure 6D). These results further indicate that SREBP is downstream of schlank and regulated by schlank activity.

Discussion

Our results provide strong evidence that schlank is a *Drosophila* member of the highly conserved Lass/ceramide synthase family and is involved in the synthesis of a broad range of ceramides, key metabolites of the *de novo* biosynthesis of sphingolipids. Although ceramide synthases are highly conserved in all eukaryotes, their role in development and growth is poorly understood due to the lack of animal models.

Mutants of the Drosophila Lass homologue schlank show severe growth defects

Genetic experiments and our mRNA and protein expression studies indicate that schlank is provided both maternally and zygotically, reflecting the importance of the enzyme for cellular metabolism and survival. We found that zygotic mutants of the stronger *schlank*^{G0349} allele still have considerable amount of schlank protein, although mRNA levels are strongly reduced. This reflects the maternal supply of the

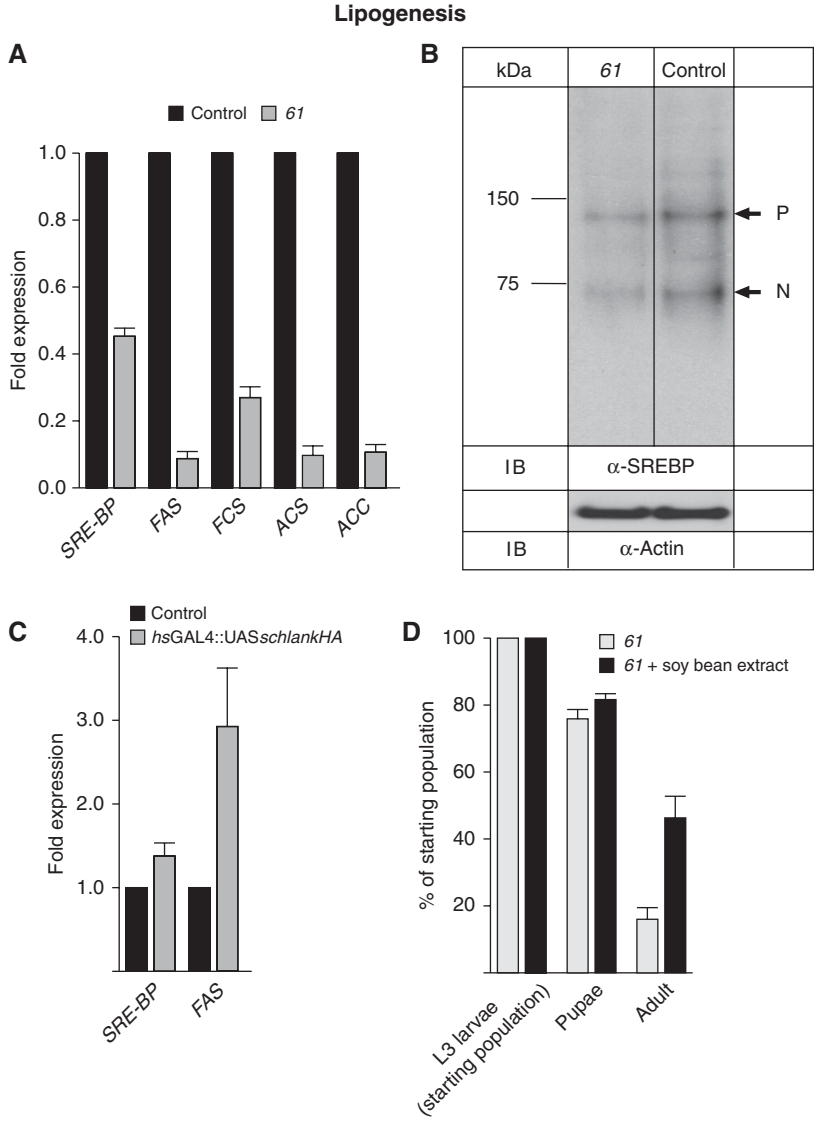

Figure 6 Schlank is a positive regulator of lipogenesis. (**A**) Reduced mRNA expression of *SREBP*, *FAS*, *FCS*, *ACS* as well as *ACC* in $schlank^{G0061}$ as compared with w^{1118} control. Quantification by qRT–PCR. (**B**) Immunoblot analysis of $schlank^{G0061}$ (*61*) and w^{1118} (control) whole-fly lysates (60 μg/lane) shows reduction in SREBP protein levels in $schlank^{G0061}$ mutants. The blot was probed with monoclonal antibody against the NH2-terminal fragment of dSREBP (IgG-3B2; Seegmiller *et al*, 2002). The membrane was then stripped and reprobed with anti-actin antibody used as loading control (lower panel). (**C**) Increased *dSREBP* and *FAS* transcript levels in larvae overexpressing UAS*schlankHA* in combination with an *hs*GAL4 driver line after 1 h of heat shock. (**D**) Soy lipid extract can rescue $schlank^{G0061}$-mutant animals to adulthood while the percentage of larvae and pupae is not altered. Error bars indicate s.e.m.

protein and its stability, and it explains the residual ceramide synthase activity found in the zygotic $schlank^{G0349}$ mutants. When we eliminated both the zygotic and the maternal contributions of schlank to obtain a null situation (in $schlank^{G0349}$ germline clones), oogenesis failed to occur and no embryos could be obtained for further studies. These data also indicate that the *CG11642* gene, which is the only other *Drosophila* gene showing some sequence homology to Lass family members (Acharya and Acharya, 2005), cannot compensate for the loss of schlank and further suggest that *schlank* most likely encodes the only ceramide synthase family member in *Drosophila*. The *CG11642* gene seems more homologous to members of the TRAM protein family (Figure 2A and Supplementary Figure 2B), which are not involved in ceramide synthesis.

Both $schlank^{G0349}$*and* $schlank^{G0061}$ mutants show delay of larval development and pronounced growth defects depending on the strength of the respective allele. Determination of the ceramide synthase activity in both *schlank*-mutant alleles, in RNAi-knockdown animals, in tissue culture cells and in animals overexpressing schlank demonstrate that it is involved in the synthesis of a rather broad spectrum of ceramides (Figure 3A). In contrast, the six known members of the mammalian Lass family each seem to possess characteristic substrate specificity (Mizutani *et al*, 2005; Laviad *et al*, 2008). This may explain why murine Lass2 is not able to rescue the $schlank^{G0349}$ mutants to the pupal stage, but rather causes partial rescue to the second/third larval instar stages (Supplementary Figure 1C). On the other hand, the fact that we do obtain a rescue of *schlank* mutants with Lass2 further

strengthens our conclusion that *schlank* encodes a ceramide synthase.

Interestingly, our analysis of *schlank* mutants and overexpression of schlank and murine Lass2 revealed an additional phenotype and function of Lass family members that has not been observed in the previous studies in mammalian tissue culture cells. We identified schlank as an important regulator of the balance between lipogenesis and lipolysis. We found that *schlank*-mutant larvae or larvae analysed upon downregulation of *schlank* by RNAi are largely devoid of storage fat (Figure 4 and Supplementary Figure S3C), which is deposited as TAGs in the fat body, whereas larvae overexpressing schlank showed increased TAG levels, as determined by TLC analysis (Figure 4A and B; Table I). Even more, overexpression of schlank in $schlank^{G0349}$ mutants could restore the fat body development and lead also to increasing TAG, DAG and FA levels (Supplementary Figure S3D and E). The loss of storage fat in *schlank* mutants or upon downregulation of *schlank* by RNAi in *Drosophila* larvae is partly due to upregulation of mRNA levels of lipases (Figure 5A), including *brummer* encoding an ATGL lipase (Grönke *et al*, 2005; Haemmerle *et al*, 2006), and of *lip3*, which encodes a TAG lipase (Zinke *et al*, 2002; Fuss *et al*, 2006). When schlank or murine Lass2 were overexpressed, TAG, DAG and FA levels were increased and expression of lipolytic lipases was decreased, confirming the negative regulatory effect on lipases (Figure 5B).

Schlank regulates the balance between lipogenesis and lipolysis during growth

Our data suggest that the role of schlank in lipogenesis can be mediated, at least in part, through positive regulation of SREBP, a key regulator required for synthesis of cholesterol and unsaturated FAs in mammals and saturated FAs in *Drosophila* (Sato *et al*, 1994; Seegmiller *et al*, 2002). The decrease of SREBP mRNA levels in *schlank* mutants (Figure 6A) is in line with the reduced transcription of the SREBP-dependent enzymes of FA biosynthesis and with the reduced TAG levels in these animals (Table I). In particular, we found downregulation myristic acid (C14:0), myristoleic acid (C14:1) and palmitic acid (C16:1) in *schlank*-mutant larvae, as demonstrated by ESI-MS analysis and quantification of FA (Figure 5C). In contrast, we found an increase of the level SREBP mRNA and of SREBP-dependent target gene expression, including FAS, upon overexpression of *schlank* (Figure 6C). In line with these data, we found elevated levels of TAG in larvae overexpressing *schlank* (Figure 4 and Table I). Interestingly, ubiquitous expression of the murine Lass2 in larvae also lead to increase of TAG, DAG and FA levels. This suggests a conserved function of Lass proteins in TAG lipid homeostasis. It is of note that ceramide synthesis is dependent on the availability of long-chain saturated FAs, which participate in the initial rate-limiting reaction involving the condensation of a FA-CoA and serine (Adachi-Yamada *et al* 1999; Batheja *et al*, 2003), and in the conversion of (dihydro)sphingosine to (dihydro)ceramid, which involves amino acylation with a long-chain FA at carbon-2 of sphingosine. schlank may, thus, act as a metabolic sensor linking sphingolipid homeostasis with FA metabolism in cells. Together, these data identify schlank as a positive regulator of SREBP and are consistent with phenotypes of *dSREBP* and *schlank* mutants both showing similar growth and metabolic defects and the fact that both mutants can be rescued by supplementation of food with soy lipids (Figure 6D).

Cholesterol in mammals or unsaturated FAs in mammals and in *Drosophila* are known regulators of transcriptional and posttranscriptional processing of SREBP. Recent findings demonstrated that modification of ceramide synthesis can also contribute to SREBP regulation (Worgall *et al*, 2004). It has been shown that leptin-induced reduction of the expression of the ceramide synthases Lass2 and 4 resulted in downregulation of SREBP-1c mRNA and reduced lipogenesis in lean tissue (Gallardo *et al*, 2007), whereas proteolytic maturation of SREBP-1c was reduced in white adipose tissue (Bonzón-Kulichenko *et al*, 2009). These observations are in line with our data using *schlank* mutants showing reduced ceramide synthase activity and reduced lipogenesis, which may be exerted, at least in part, through downregulation of SREBP mRNA. How this occurs mechanistically is not yet known. It is of note that no rescue of $schlank^{349}$ mutants was obtained when we expressed the mutated schlankH215D protein in which the ceramide synthase function is severely affected in the fat body of $schlank^{349}$ mutants. This is consistent with the idea that ceramide biosynthesis is essential for tissue growth and survival of the animals. In contrast, overexpression of the schlankH215D variant did not alter ceramide levels; however, it caused an increase in TAG, DAG and FA levels comparable to that seen when overexpressing wild-type *schlank* or murine Lass2 (Figure 4 and Table I). This suggests the possibility that some effect of schlank on TAG metabolism may be independent of its ceramide synthase function. In this context, the N-terminally located homeobox (Hox) domain, which is contained within many of the Lass proteins of higher organisms including Lass 2, may be relevant. Future studies will have to reveal whether the homeodomain is involved in some of the regulatory effects exerted by schlank.

How schlank expression is regulated is not known. Lipid and carbohydrate metabolism are regulated by a variety of hormones, including insulin and glucagon in mammals or insulin-like peptides and the glucagon-like adipokinetic hormones in insects (Brogiolo *et al*, 2001; Van der Horst *et al*, 2001; Fuss *et al*, 2006; Hafner *et al*, 2006). However, mechanisms by which cellular lipid metabolism might be interlaced with hormone-dependent body fat regulation are unknown at present.

Together, our results identify schlank as a *Drosophila* member of the Lass family of ceramide synthases. Furthermore, our data provide evidence that schlank acts as a regulator of the balance between lipogenesis and lipolysis during larval growth, suggesting a novel role for ceramide synthases in regulating body fat metabolism.

Materials and methods

Fly strains, temperature shift and rescue experiments

Fly stocks were obtained from the Bloomington stock centre. *schlank* alleles $schlank^{G0370}$, $schlank^{G0349}$, $schlank^{G0163}$ and $schlank^{G0061}$ were isolated by screening the Göttingen X-chromosome collection of P-lines (Peter *et al*, 2002). $schlank^{G0370}$, $schlank^{G0349}$ and $schlank^{G0163}$ are strong alleles and $schlank^{G0061}$ is a weak allele. For phenotypic analysis, *schlank* alleles were balanced over *FM7c*, P{*Kr*GAL4} and P{UAS-*GFP*}. For rescue experiments, flies of the genotype $schlank^{G0349}$/*FM7c*, P{*Kr*GAL4}, P{UAS-*GFP*}; +; UAS*schlankHA* or UAS*lass2HA* were crossed with the *ppl*GAL4 transgene (gift from I Zinke) to express full-length

schlank or the murine *lass*2 cDNA in a *schlank*-mutant background. Heterozygous progeny, males and females expressing *Krüppel* (*Kr*; Hoch *et al*, 1990, 1991) GFP can thereby be distinguished from the hemizygous males and heterozygous females carrying the $schlank^{G0349}$ mutation.

Cloning and transgene production

schlankHA or *lass2HA* was generated by PCR reaction using specific *schlank or lass2* primer pairs. The pP{UAS*schlankHA*}and pP{UAS*lass2HA*}were cloned by standard procedures and constructs were integrated into w^{1118} fly genomes by P-element-mediated transformation (Rubin and Spradling, 1982).

Site-directed mutagenesis

Site-directed mutagenesis for pP{UAS*schlankH215D*} was performed with the GeneTailor site-directed mutagenesis system (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's recommendation.

Real-time RT-PCR

Isolation of mRNA and quantification by real-time RT–PCR were performed as described by Fuss *et al* (2006). All template reactions were performed in parallel and repeated with independently isolated RNA samples from different larval or cell collections.

Antibody generation

Polyclonal antiserum was generated in guinea pig (BioGenes, Berlin, Germany) against a schlank-specific oligopeptide (SRAGARVATTERREE) (Supplementary Figure S4A).

Purification of schlankHA

For partial purification of schlankHA cell lysate of larvae expressing UAS*schlankHA* were subjected to a 0.5 ml anti-HA affinity matrix column and schlankHA was purified using the anti-HA affinity matrix kit (Roche, Germany) according the manufacturer's instructions.

Immunoblotting and immunohistochemistry

Immunoblotting or immunohistochemistry experiments were performed as described earlier (Bauer *et al*, 2006). The following antibodies were used for immunoblotting: anti-HA (rat, 1:500; clone 3F10; Roche) or anti-schlank (guinea pig, 1:400). For immunohistochemical staining we used anti-schlank (1:40), anti-HA (rat, 1:100; clone 3F10; Roche) and anti-Spectrin (mouse, 1:40; Developmental Studies, Hybridoma Bank).

De novo ceramide synthase activity

Ceramide synthase activity was determined by measuring *de novo* ceramide generation after 12-h labelling of larvae with L-[3-^{14}C]-serine. For metabolic labelling, larvae were fed with heat inactivated yeast paste containing L-[3-^{14}C]-serine (54 mCi/mmol) as a precursor for sphingolipids (GE, Germany). Ceramides were separated by TLC as described above by loading the same amounts of radioactivity to each line on the TLC plates. Radioactive bands were visualized with a Bio Imaging Analyser 1000 (Fuji, Japan) and quantification was performed with the image analysis software Tina (Raytest, Staubenhardt, Germany). Ceramides were identified and quantified using commercially available reference standards (ceramide (C18:1/C18:0) (Sigma, Germany), phytoceramide (Cosmoferm, Delft, Netherlands), and stearic acid (Fluka)). In order to rule out that any yeast ceramides are included in our determination of *Drosophila* ceramides, yeast ceramides of a yeast control were analysed for every experiment in parallel. Ceramide synthase assay was performed as described previously (Wang and Merrill, 1999), with minor modifications.

Lipid analysis in larvae

Lipids were of larvae were extracted and separated as described by Reichelt *et al* (2004). Unpolar lipids (FA, TAG) were separated by TLC with n-hexane/diethylether/glacial acetic acid (70:30:1, vol/vol/vol) and ceramides with chloroform/methanol/glacial acetic acid (190:9:1, vol/vol/vol). Detail information is provided in the Supplementary data. For ESI-MS we used a ESI-Q-Tof 2 mass spectrometer equipped with a nanospray source (MicroMass, Manchester, UK) and FA quantification we used D_3-stearic acid (Sigma, Germany). Details about methods and primers are provided in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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