PERILIPIN-Dependent Control of Lipid Droplet Structure and Fat Storage in *Drosophila*

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SUMMARY

Lipid droplets are intracellular organelles enriched in adipose tissue that govern the body fat stores of animals. In mammals, members of the evolutionarily conserved PERILIPIN protein family are associated with the lipid droplet surface and participate in lipid homeostasis. Here, we show that *Drosophila* mutants lacking the PERILIPIN PLIN1 are hyperphagic and suffer from adult-onset obesity. PLIN1 is a central and Janus-faced component of fat metabolism. It provides barrier function to storage lipid breakdown and acts as a key factor of stimulated lipolysis by modulating the access of proteins to the lipid droplet surface. It also shapes lipid droplet structure, transforming unilocular into multilocular fat cells. We generated flies devoid of all PERILIPIN family members and show that they exhibit impaired yet functional body fat regulation. Our data reveal the existence of a basal and possibly ancient lipid homeostasis system.

INTRODUCTION

Lipid droplets are multifunctional and highly dynamic intracellular organelles that undergo a complex life cycle serving a variety of functions (Ducharme and Bickel, 2008; Farese and Walther, 2009; Murphy et al., 2009). Their most eminent physiological role is the packaging and management of the body fat stores, which consist of neutral lipids, mainly triglycerides. Fat stores are the most important energy reserve of animals and dynamically shaped by alternating mobilization and reaccumulation cycles in response to food availability. Adjustment of fat stores to a genetically determined set point involves a regulatory process described as lipid homeostasis. Impairment of lipid homeostasis in humans causes severe threats to health such as obesity and type II diabetes (Kopelman, 2000).

The lipid droplet surface acts as intracellular compartment boundary of central importance for lipid homeostasis. Regulatory proteins associated with this surface control deposition of storage fat and its mobilization. Members of the PERILIPIN (PLIN) family are the most prominent mammalian lipid droplet proteins (Bickel et al., 2009; Brasaemle, 2007; Londos et al., 1999; nomenclature according to Kimmel et al., 2010). They are named after the founding member Perilipin, referred to as PLIN1 (Greenberg et al., 1991). PLIN1 and the four other mammalian PERILIPINs PLIN2-5 (previously called ADRP, TIP47, S3-12, and OXPAT, respectively) are key regulators of lipometabolism that act at the lipid droplet surface of adipose and nonadipose cells. PERILIPINs are further classified according to their intracellular localization as either constitutive (PLIN1 and PLIN2) or exchangeable (PLIN3, PLIN4 and PLIN5) lipid droplet proteins. They differ in tissue restriction (PLIN1, PLIN4, and PLIN5) or show ubiquitous (PLIN2 and PLIN3) expression patterns (Bickel et al., 2009). The best-characterized PERILIPIN is PLIN1. It plays a dual role in adipocyte lipid mobilization as it acts as barrier in basal lipolysis and also promotes stimulated lipolysis (Martinez-Botas et al., 2000; Tansey et al., 2001). Lipolysis is triggered by β-adrenergic signaling via protein kinase A (PKA)-mediated phosphorylation of PLIN1 (Brasaemle et al., 2000; Souza et al., 2002; Tansey et al., 2003), which releases the adipose triglyceride lipase (ATGL) (Zimmermann et al., 2004) activator ABHD5/CGI-58 (Lass et al., 2006) and recruits hormone sensitive lipase (HSL) to the lipid droplet surface (Sztalryd et al., 2003). PKA phosphorylation of PLIN1 also mediates the dispersion of the unilocular white adipocyte lipid droplets during stimulated lipolysis (Marcinkiewicz et al., 2006). In contrast, PLIN2 is involved in efficient lipid storage. PLIN2 promotes fat storage in lipid droplets, while fatty acid sorting to β-oxidation and VLDL production was suppressed in primary rat adipocytes (Magnusson et al., 2006). Its overexpression causes increased lipid deposition in all cellular systems examined so far.

Polymorphisms at gene loci encoding these two human PLIN genes have been correlated with metabolic diseases, i.e., PLIN1 polymorphisms have been identified as obesity risk factors (Qi et al., 2005) or as modulators of gene–diet interactions that are associated with obesity (Smith et al., 2008), and PLIN2 participates in the pathogenesis of diet-induced insulin resistance (Varela et al., 2008).

The function of the other members of the PLIN protein family, PLIN3, PLIN4, and PLIN5, has not yet been addressed by gene knockout studies. However, they localize mainly to small lipid droplets, suggesting a function in early lipid droplet biogenesis. During adipocyte differentiation, they decorate small, nascent droplets. PLIN2 also resides on medium-sized droplets and becomes replaced by PLIN1 on the large droplets in the fully differentiated white fat cells (Wolins et al., 2005).
Multiple perilipin genes in mammalian genomes suggest similar and redundant functions. Thus, their functions are not fully accessible to genetic analysis by individual gene mutations. In fact, recent analysis of PLIN2 knockout in mouse hepatocytes revealed that a mutant phenotype is masked by functional redundancy, i.e., PLIN2 can be replaced by PLIN3 (Bell et al., 2008). It therefore appears to be impossible to identify the basic role of the PERILIPIN system in mammals since the generation of a PERILIPIN-free mammal does not seem to be in reach with the available techniques.

The phylogenetic representation of PERILIPINS in nonmammalian genomes supports an evolutionarily ancient function (Lu et al., 2001). In fact, the single PERILIPIN (MPL1) of the ascomycete *Metarhizium anisopliae*, a lower eukaryote, is a lipid droplet-associated and acts in fat storage control (Wang and St Leger, 2007). To assess the function of PLINs also in higher eukaryotes, we used *Drosophila melanogaster*. Its genome encodes only two PERILIPINS (Miura et al., 2002). Lipid storage droplet-2 (Lsd-2), or perilipin2 (plin2), promotes lipid storage (Fauny et al., 2005; Grönke et al., 2003; Teixeira et al., 2003), whereas the second, Lipid storage droplet-1 (Lsd-1) or perilipin1 (plin1), has been implicated in stimulated lipolysis control (Arrese et al., 2008a).

Here, we report that plin1 encodes a constitutive lipid droplet protein with multiple distinct functions. It modulates the access of proteins to the lipid droplet surface, shapes lipid droplet structure, and acts as obesity-related gene. Animals lacking both PLINs are viable, but fat storage homeostasis is profoundly impaired. The results show that the PLINs act as potentiators of an evolutionarily ancient lipid metabolism system that provides animals with the selective advantage of metabolic flexibility under conditions of discontinuous food supply.

**RESULTS**

**PLIN1 Is a Constitutive Lipid Droplet Protein of Fat Body and Neurosecretory Cells**

Genes encoding members of the PLIN protein family are evolutionarily conserved in species ranging from fungi to mammals (Figure 1A). Mammals possess five Perilipins, whereas insects typically have only two, *plin1* and *plin2* (Figure 1A).

The *plin1* encoded protein PERILIPIN1 has a N-terminal PAT domain, two sequence-conserved regions linked by a variable region and an evolutionarily less conserved C terminus (Figure 1B and Figure S1 available online). The *plin1* gene is expressed during all ontogenetic stages (Figure 1C), but unlike *plin2* (Grönke et al., 2003) it is not maternally expressed. Zygotic expression starts late during embryogenesis in neuroendocrine corpus allatum (CA) cells of the developing ring gland and in fat body, the adipose tissue of the fly (Figure 1D). Both *plin1* transcripts and protein continue to be expressed in fat body cells during postembryonic development, including the adult stage (Figures 1C–1E). PLIN1 associates with the lipid droplet surface in fat body cells (Figures 1D and 1E) (see also Miura et al., 2002) and is exclusively found in the lipid droplet fractions of fat body cell lysates after density gradient fractionation (Figure 1F). The lipid droplet association of PLIN1 and the finding that *plin1* is downregulated upon starvation (Grönke et al., 2005) suggest a lipometabolism function.

Quantitative western blot and qRT-PCR analysis revealed that both *plin1* messenger RNA (mRNA) and PLIN1 levels follow the body fat mobilization/reaccumulation profile in flies subjected to a starvation/refeeding regimen. PLIN1 abundance also correlates with the body fat content of obese brummer (bmm) (Grönke et al., 2005) and lean midway (mdy) mutant flies (Figure 1G). However, *plin1* mRNA levels of obese and lean flies did not match the corresponding PLIN1 abundance, implying control at the posttranscriptional level (Figure 1G). Thus, *plin1* expression is differentially regulated in response to acute and chronic changes in lipid storage, respectively, whereas PLIN1 is a reliable marker for the body fat content.

**Adult-Onset Obesity and Hyperphagia of *perilipin1* Mutant Flies**

Homozygous *plin1* mutants, *plin1<sup>1</sup>* and *plin1<sup>2</sup>* (recovered by imprecise transposon mobilization of *plin1*<sup>194304</sup>, Figure 1B), develop into obese but otherwise normal-looking adults. The fat content is doubled in homozygous *plin1<sup>1</sup>* flies or transheterozygous flies carrying *plin1<sup>1</sup>* in combination with *plin2<sup>2</sup>* or Df(3R) mbc-30, a deficiency that uncovers the *plin1* locus (Figure 2A). The obesity phenotype can be fully rescued by a *plin1* genomic rescue transgene and phenocopied by fat body-targeted *plin1* RNA interference (RNAi) expression (Figures 1B and 2A). These data establish that lack of *plin1* activity in fat body causes the obesity phenotype.

Starvation-resistance is linked to the body fat content and serves as a diagnostic marker for storage fat mobilization. Obese *plin1* mutants such as the *plin1<sup>1</sup>* homozygotes (called *plin1<sup>1</sup>* in the following), *plin1<sup>1</sup>*/*plin1<sup>2</sup>* heterozygotes, or *plin1* knockout flies outlive the *plin1<sup>1</sup>* controls under starvation (Figure 2B). Thus, *plin1* is not essential for fat mobilization. However, starvation-resistance strictly depends on the *plin1* dosage. A single *plin1* copy moderately increases the starvation-sensitivity of *plin1<sup>1</sup>* flies, whereas strong expression of *plin1* rescues the *plin1<sup>1</sup>* starvation-resistance phenotype (Figure 2B). Thus, there is a close correlation of PLIN1 and the respective body fat levels (Figure 1G), indicating that PLIN1 does not only hallmark the body fat content but also participates in its regulation.

Obesity of *plin1* mutants progressively manifests starting at day 2 of adult life (Figure 2C). This profile identifies *plin1* as adult-onset obesity-related gene and suggests a causative link to the intermittent, ad libitum feeding mode characteristic of adult flies (Ja et al., 2007; Xu et al., 2008). The *plin1* obesity phenotype is nutrition dependent, implying hyperphagia (Figure 3A). On high-sugar diet, the cumulative food intake of *plin1<sup>1</sup>* flies during the first 6 days of adult life is about one-third higher than that observed with *plin1<sup>1</sup>* flies, and their body fat content is doubled. On low-sugar diet, both *plin1<sup>1</sup>* and *plin1<sup>1</sup>* flies exhibit a similar food intake, but *plin1<sup>1</sup>* flies accumulate up to 50% more body fat than *plin1<sup>1</sup>* controls. Thus, in addition to hyperphagia, other factors contribute to the obesity of *plin1* mutants, possibly metabolic rate reduction. Activity patterns and total cumulative locomotor activity were comparable between mutant and control flies during obesity development (Figures 3B and 3C). Thus, activity-dependent metabolic rate reduction of *plin1<sup>1</sup>* flies does not contribute to obesity, and the obese phenotype does not impair the locomotor activity of the mutants.
PLIN1 Is an Effector of Stimulated Fat Storage Mobilization In Vivo

The PLIN1 ortholog of the tobacco hornworm *Manduca sexta* has been proposed as downstream effector of the adipokinetic hormone (AKH) signaling pathway (Patel et al., 2005). This pathway acts analogous to the β-adrenergic signaling pathway in mammals and is one of two pathways to control fat storage mobilization in flies (Gronke et al., 2007). In this process, the AKH receptor (AKHR) of fat body cells relays the prolipolytic AKH signal via the second messenger cAMP to protein kinase A (PKA), which in turn phosphorylates PLIN1 and thereby stimulates lipolysis of the lipid droplets from *Manduca* fat body ex vivo and of liposomes decorated with recombinant *Drosophila* PLIN1 in vitro (Arrese et al., 2008a; Patel et al., 2005). To gain support for such a PLIN1 function in vivo, we addressed the genetic interaction of *plin1* with AKHR and the BMM lipase, two central components of the dual lipolytic control pathways in the fly. Loss of AKHR or BMM activities caused a *plin1*/*C0*-like obese and starvation-resistance phenotype. The mutant individuals exhibit incomplete storage fat mobilization upon food deprivation as was also observed with the *plin1* mutants (Figures 4A–4C). This finding indicates that *plin1* acts in one or both lipolytic pathways in flies. We addressed this point with double-mutant flies, which lack *plin1* in combination with either *bmm* (*bmm*/*C0* *plin1*/*C0*) or *AKHR* (*AKHR*/*C0* *plin1*/*C0*). Both double mutants are more obese than the single mutants (Figure 4A). Thus, *plin1* participates in...
each of the two lipolytic pathways. bmm\textsuperscript{−} plin1\textsuperscript{−} double mutants fail to mobilize their fat storage and are starvation hypersensitive, as observed with bmm\textsuperscript{−} AKHR\textsuperscript{−} double mutants (Figures 4B and 4C) (Gronke et al., 2007). In contrast, AKHR\textsuperscript{−} plin1\textsuperscript{−} double mutants remain lipolysis competent and outlive the plin1\textsuperscript{+} control flies under food deprivation (Figures 4B and 4C). Thus, the excessive fat accumulation in AKHR\textsuperscript{−} plin1\textsuperscript{−} individuals is likely caused by an impairment of two processes; i.e., lack of either plin1 or AKHR activity reduces fat mobilization in addition to derepression of lipogenesis due to the lack of AKH signaling (Anand and Lorenz, 2008). Overexpression of AKH is known to hyperactivate the AKH/AKHR pathway in wild-type flies (Gronke et al., 2007) and results in strong fat reduction in bmm\textsuperscript{−} plin1\textsuperscript{−} double mutants (Figure S2). These findings establish that plin1 is an essential component of the AKH/AKHR signaling pathway that regulates fat mobilization. However, it is not essential for bmm-dependent lipolysis. This conclusion is consistent with the result that transgene-mediated bmm overexpression in fat body causes lean plin1 mutant flies (Figure 4D).

Although plin1 is not essential for the bmm-dependent lipolytic pathway, it appears to nevertheless modulate bmm activity. This argument is supported by two observations. First, the body fat content of bmm\textsuperscript{−} plin1\textsuperscript{−} individuals only moderately exceeds the obesity of the respective single mutants (Figure 4A), showing that the two activities are not strictly independent. Second, bmm overexpression reduces the fat content of plin1\textsuperscript{+} flies by about one-third and by 80% in plin1\textsuperscript{−} mutants. Thus, flies lacking PLIN1 are more sensitive to bmm-dependent fat mobilization than is the wild-type (Figure 4D and see below), suggesting that PLIN1 affects the availability of lipid droplets for lipases or vice versa, and acts not only in the AKH/AKHR signaling pathway.

In order to further explore this AKH/AKHR-independent function of PLIN1, we employed the larval wing disc as an experimental system. It represents a lipid storing tissue that lacks both PLIN1 and AKH/AKHR pathway activities (Figure 4E and data not shown). Instead, it only depends on PLIN2 (Figure 4E) (see also Fauny et al., 2005). Ectopic expression of PLIN1 in wing discs suppresses PLIN2 expression posttranscriptionally without affecting lipid storage (Figure 4E). Thus, PLIN1 activity can...
both suppress and functionally replace the barrier function of PLIN2 in lipid storage. *Manduca* PLIN1 was shown to represent a PKA-dependent phosphorylation target (Patel et al., 2005). Consistent with a prolipolytic PKA action, the body fat content of *plin1*+ flies that overexpress the catalytic subunit of PKA (PKAc) in the fat body is reduced (Figure 4F). However, PKA hyperactivation also reduces obesity of *plin1* mutants (Figure 4F). Thus, PKA acts at least in part independently of PLIN1 in decreasing the body fat content in vivo.

Since PKA-mediated lipid storage regulation is not strictly PLIN1 dependent, we asked more specifically whether PLIN1 phosphorylation is important for lipid storage regulation in vivo. We therefore generated the PLIN1 mutant PLIN1\textsubscript{D\textsubscript{H}}, in which six serine/threonine residues were replaced by alanines. These sites include the previously in vitro-characterized PKA target serine (Arrese et al., 2008b) and five sites found by PLIN1 mass spectrometry (Figure S3) or by in silico prediction of evolutionarily conserved putative phosphorylation sites (Figures S1 and S4A). Transgenic expression of PLIN1 or PLIN1\textsubscript{D\textsubscript{H}} in fat body of *plin1* mutant flies reverted all aspects of the *plin1* mutant phenotype (Figures S4B and S4C), including the structural change of lipid droplets (see below). Thus, phosphorylation at canonical PKA target sites is not essential for PKA-dependent PLIN1 action in vivo.

**Lipid Droplet Structure Control by perilipin1**

*Drosophila* adipose tissue is composed of multilocular fat body cells, characterized by heterogeneously sized lipid droplets (Figures 5A and 5B). In response to physiological stimuli or mutations, the number and size distribution of the lipid droplets is altered in both differentiated fat body (Grönke et al., 2005; Güttierrez et al., 2007) and tissue culture cells (Beller et al., 2008; Guo et al., 2008). PLIN2 has no structural effect on lipid droplets...
(Grönke et al., 2003), whereas the number and size heterogeneity of the lipid droplets in *plin1* mutants is changed into a giant lipid droplet phenotype typical for unilocular fat cells (Figure 5A). Analysis of chimeric animals, whose fat body is composed of *plin1* and *plin1* cells, shows that this phenotype is both cell-autonomous and PLIN1 dependent (Figure 5B). The phenotype develops during larval stages, prior to the onset of obesity of *plin1* mutants (see Figure 2C). Therefore, it represents a reallocation phenotype and is not a consequence of progressive lipid overstorage due to the lack of PLIN1. This conclusion is supported by PLIN1-dependent giant lipid droplets in fat cells of lean animals such as lipogenesis-impaired *mdy* (*dDGAT1*) mutants. These mutants accumulate only one-fourth of the body fat of *plin1* mutants than in control flies. (E) PLIN1 barrier function stabilizes AKHR-independent fat stores. Lipid storage in the wing imaginal disc is dependent on PLIN2 (but not on PLIN1 or AKHR), as shown by transgenic *plin2* knockdown under control of the *pto*-Gal4 driver. Ectopic PLIN1 expression suppresses PLIN2 posttranscriptionally but stabilizes lipid storage in the wing blade region of the disc. (F) The AKHR effector kinase PKA decreases body fat stores independent of *plin1*. Conditional overexpression of the PKA catalytic subunit (PKAc) in the adult fat body reduces the body fat content of *plin1* and *plin1* flies.

Fat differences observed in all single- and double-mutant combinations (A) and in fed versus starved animals (B) are statistically highly significant *p* < 0.01 (except for n.s.). Shown are representative experiments based on triplicate measurements involving a total of 24 (A, B, D, and F) or 45 (C) male flies per genotype. Error bars represent STDEVP; *p* < 0.05 (D, and F). See also Figures S2, S3, and S4.
Figure 5. PLIN1 Controls Lipid Droplet Structure

(A) Ontogenetic development of unilocular plin1− fat body cells compared to multilocular adipocytes of control flies (first instar larval stage to mature adults).

(B) Cell autonomy of plin1 effects on lipid droplet structure and derepression of PLIN2 shown in plin1− adipose cells in chimeric larval fat tissue composed of plin1− and plin1+ cells as revealed by immunofluorescence.

(C) PLIN1 control of lipid droplet structure is independent of the cellular fat content. Large lipid droplets in lean mdy− plin1− but not in mdy− bmm− double-mutant fat cells shown by bright-field images.

(D) PLIN1 reciprocally controls the lipid droplet structure and body fat content. plin1 transgene activation in adult plin1− flies reduces lipid droplet size (compare I to II) and body fat content of adult plin1− mutants. Conversely, PLIN1 depletion in plin1− flies by a plin1 RNAi transgene slightly increases lipid droplet sizes (compare III to IV) and body fat content.

Glyceride assays in (C) and (D) are representative experiments based on triplicate measurements involving a total of 24 male flies per genotype. Error bars represent STDEVP; “p < 0.05.
In order to address whether PLIN1 reciprocally controls the lipid droplet structure, we expressed a plin1 complementary DNA (cDNA)-based transgene in the fat body of ad libitum-fed, mature plin1−/− adults and, conversely, we depleted PLIN1 by a plin1 RNAi transgene in plin1+ controls. PLIN1 expression in adult plin1 mutants reduced the average lipid droplet size, whereas the PLIN1 depletion moderately increased the lipid droplet size (Figure 5D). Both processes were paralleled by corresponding body fat changes; i.e., the obese phenotype of plin1 mutants is rescued upon PLIN1 induction and the body fat content of plin1+ flies increases upon PLIN1 depletion (Figure 5D). These results establish a dual role of PLIN1 as a regulator of the lipid droplet structure and the body fat content.

A single large droplet has a smaller surface area than several smaller droplets with the same total volume. The giant lipid droplet phenotype of plin1−/− mutants could therefore be caused by an early droplet fusion to counteract an altered ratio of the lipid droplet surface area to the surface-covering proteome. Alternatively, the phenotype could reflect a specific plin1 control function in the lipid droplet biogenesis and/or turnover. To address these possibilities, we expressed lipid droplet-associated proteins such as human PLIN1a and EGFP-tagged lipid droplet-associated proteins CG2254 (Beller et al., 2006) or PLIN2 (Figure 6 and Figure S5) in plin1−/− fat body cells to increase the lipid droplet surface occupation by proteins. They associate with lipid droplets as PLIN1 does, but neither the giant lipid droplet phenotype nor the obesity of plin1 mutant flies was rescued (Figures 6A–6C). However, PLIN2 overexpression resulted in extra body fat accumulation (Figure 6B). Thus, the plin1 mutant phenotype is likely not due to reduced protein coverage, suggesting a role of PLIN1 in directly or indirectly modulating the accessibility of the lipid droplet surface to regulatory factors. Notably, expression of human PLIN1a does not affect the plin1 mutant phenotype. To address a role of PLIN1 in modulating the access of proteins to the lipid droplet surface, we examined the distribution of BMM, an established regulatory factor at the lipid droplet surface in plin1−/− mutants. The BMM::EGFP fusion protein, which normally localizes to few spots on the surface of lipid droplets (Figure 6C) (Gronke et al., 2005) was altered in plin1 mutants by showing numerous BMM::EGFP spots that speckle the surface of the giant lipid droplet and by
covering the surface of medium-sized droplets (Figure 6C). This localization pattern correlates well with the extreme leanness of plin1− flies in response to bmm overexpression (Figure 4C), supporting the argument that PLIN1 participates in lipometabolism homeostasis by directly or indirectly mediating the access of regulatory factors such as BMM to the lipid droplet surface.

**Body Fat Homeostasis in Flies that Lack PERILIPINS**

Multiple PERILIPINS and functional interactions between them make it impossible to establish the possibly fundamental role of PERILIPINS in animal fat storage regulation by single-gene analysis. PLIN1 and PLIN2 are associated with the vast majority of droplets in flies (Figure 6D), and single-mutant analysis revealed opposing functions on body fat storage. However, as a result of the observed cross-regulation, the key question, whether body fat homeostasis can be achieved in the absence of PERILIPINS, could not be answered. To address this question, we generated individuals that lack both PLIN1 and PLIN2, representing PERILIPIN-free organisms. In contrast to the normal body fat levels of newly hatched plin1 and plin2 mutants, young PERILIPIN-free adults have strongly reduced body fat stores (Figure 7A). However, they recover within the first 6 days up to the level of lean plin2 mutants (Figure 7A) (Grönke et al., 2003) and are capable of mobilizing the fat stores upon starvation. The cellular phenotype of the double mutants resembles the unilocular phenotype of plin1−, but the size of the predominant large droplets is reduced (Figure 7B). During aging, PERILIPIN-free flies adjust their body fat stores to a level between the lean plin2 and obese plin1 single mutants (Figure 7C). Thus, PERILIPIN-free flies are capable of sustaining their lipometabolism homeostasis system. Under a starvation/refeeding regime, however, PERILIPIN-free flies show both attenuated lipid mobilization and reaccumulation compared to control flies (Figure 7D). PERILIPIN-dependent body fat control is therefore not essential for lipometabolism per se, but might serve as an add-on system to assure rapid and effective adjustments of the fat storage/mobilization system to the actual energy requirement and food availability. Whether this adjustment of the fat storage/mobilization system is of selective advantage to wild Drosophila populations remains to be shown.

**Figure 7. PERILIPIN-Free Flies Show Defects in Lipometabolism Homeostasis**

(A) Fat-depleted PERILIPIN-free flies at adult hatching accumulate storage lipid during maturation, similar to plin1 mutants, which become obese whereas plin2 mutants become lean during early adult life. PERILIPIN-free flies show incomplete fat mobilization in response to starvation, which is characteristic for plin1 mutants (*p < 0.05).

(B) Unilocular fat storage cells of adult PERILIPIN-free fat tissue show the structural phenotype of plin1 mutants combined with the lipid understorage observed with plin2 mutants.

(C) Intact lipometabolism control adjusts body fat content of aging PERILIPIN-free flies to levels below obese plin1 mutants and above lean plin2 mutants.

(D) A starvation/refeeding regimen discloses the impaired storage fat mobilization/reaccumulation efficiency of PERILIPIN-free flies. Glyceride assays shown are representative experiments based on triplicate measurements involving a total of 24 (A) or ≥15 (C and D) male flies per genotype and point in time. Error bars represent STDEV; *p < 0.05 (A, C, and D).
DISCUSSION

Our results establish that Drosophila PLIN1 is a constitutive lipid droplet protein that is expressed from late embryonic stages onward predominantly in the fat body. It has a dual function in fat storage control as an essential component of the stimulated AKH/AKHR lipolysis pathway and by mediating the localization of lipid droplet-associated proteins such as the BMM lipase. PLIN1 also determines the size of lipid droplets in fat body cells. Its activity is dynamically regulated both at the transcriptional and posttranscriptional level to regulate the body fat content of the organism.

PLIN1 mutant flies show increased fat storage and hyperphagia. These effects are not unique for PLIN1 mutants but are characteristic for AKH/AKHR signaling pathway impairment. Downregulation of the AKHR-dependent cAMP-responsive transcription factor dCREB2 in fat body causes adiposity and increased food intake (Iijima et al., 2009). The mechanism of how the structural and physiological defects in the fat body are communicated to the central nervous system (CNS) to increase food intake is currently unknown. CNS neuron populations that participate in fat storage and food intake control have been identified (Al-Anzi et al., 2009). Moreover, a yet uncharacterized humoral signal of the larval fat body that triggers insulin-like peptide release from CNS has been described (Gémardin et al., 2009). These studies suggest that communication between fat body and CNS is a prerequisite for lipohomeostatic regulation. In this view, impaired storage lipid mobilization in PLIN1 mutants may interfere with an afferent fat body signal (e.g., an adipokine or metabolite), which is read out in the CNS to incessantly match food intake to energy demand.

Mammalian PLIN1 is largely restricted to adipocytes and subject to posttranscriptional regulation (Brasaemle et al., 1997; Kovsan et al., 2007; Xu et al., 2006) and regulation by altered physiological conditions (Bertile et al., 2003; Blanchette-Mackie et al., 1995; Greenberg et al., 1991; Servetnick et al., 1995). It executes a barrier function in basal lipolysis and serves as platform for the assembly of protein complexes that mediate stimulated lipolysis in a phosphorylation-dependent manner (Brasaemle et al., 2000; Souza et al., 1998; Souza et al., 2002; Tansey et al., 2003). Fly PLIN1 acts in the AKH/AKHR signaling pathway as mammalian PLIN1 does in the corresponding β-adrenergic pathway (Greenberg et al., 1991). These intriguing parallels suggest that functional aspects of the PERILIPIN system are evolutionarily ancient and that PLIN1 acts as a conserved surface-associated module of lipid droplets that promotes stimulated lipolysis in response to cAMP/PKA signaling. Our in vivo data on PLIN1 confirm in vitro and ex vivo studies showing that PKA-phosphorylation of PLIN1 enhances lipase activity on artificial and native lipid droplets (Arrese et al., 2008b; Patel et al., 2005). These data argue that PLIN1 can directly interact with recruit TG lipase(s) and may act as a phosphorylation-dependent regulator of a lipase activator just as mammalian Perilipin 1 acts on of the ATGL activator CGI-58 (Lass et al., 2006). In fact, the Drosophila genome encodes a functionally uncharacterized CGI-58 homolog. Both mechanisms, inappropriate lipase recruitment and failure of lipase activator interaction, would contribute to the increased adiposity of PLIN1 mutants. However, a structural change from multi- to unilocular fat cells, might also influence lipolysis and contribute to the fat storage increase of plin1 mutants.

Lipid droplet association of the BMM lipase is increased in plin1 mutant flies, which are also more sensitive to fat mobilization when challenged by targeted BMM expression. This phenomenon was also observed in murine AML12 hepatocytes, when the two PERILIPINs of this cell type (PLIN2 and PLIN3) were cotargeted by RNAi (Bell et al., 2008). Their loss resulted in fewer and enlarged lipid droplets (Bell et al., 2008). The first engineered PERILIPIN-free organism, as presented here, shows that PERILIPINs, at least in flies, are dispensable for lipid droplet biogenesis but responsible for regulating lipid droplet size in vivo.

PLIN1 knockout mice have a severe lipometabolism phenotype (Martínez-Botas et al., 2000; Tansey et al., 2001) and loss of PLIN2 activity causes triglyceride storage reduction in liver and resistance to diet-induced hepatic steatosis (Chang et al., 2006). Similarly, Mpl1 mutants of the ascomycete Metarhizium anisopliae as well as plin2−/−flies show lipid storage defects. These results underline a distinct role for PERILIPINs in lipometabolism control as shown here for plin1. However, other eukaryotic model systems for fat storage control such as the yeast S. cerevisiae or the nematode C. elegans have no PERILIPIN genes. This notion is consistent with our finding that PERILIPINs are not essential for basal lipometabolic activity but rather to increase its efficacy and to improve the effectiveness of lipometabolism management in some lineages that is not required in others. The existence of multiple and in part functionally redundant PERILIPINs in mammals and insects reflects therefore a positive selection of the ancestral PERILIPIN, followed by gene duplication and functional diversification events. The notion that Drosophila PLIN2 also serves as an adaptor protein for lipid droplet transport during early embryogenesis (Wetle et al., 2005) exemplifies that PERILIPINs can indeed adopt novel cellular functions.

Our finding that PERILIPINs are not essential for survival under ad libitum feeding supports their role as potentiator of lipometabolism. In a natural environment, however, where food access for flies is variable or even limited, impairment of the PERILIPIN system might entail a substantial selective disadvantage. This speculation can be tested with the PERILIPIN-free plin1−/−plin2−/−double mutants, which also provide access to the conserved control system underlying basic lipid homeostasis, and thereby might reveal novel therapeutic targets for the treatment of human lipopathologies.

EXPERIMENTAL PROCEDURES

Fly Techniques

Fly husbandry, the generation of plin1 mutant and plin1 chimeric animals and the plin1 genomic, cDNA, and RNAi transgenes are described in the Supplemental Experimental Procedures.

Lipid Droplet Purification and Western Blot Analysis

Sucrose gradient fractionation followed by western blot analysis of the fractions adjusted to equal protein amounts was performed as described (Grönke et al., 2003). The following primary antibody/antisera were used: rabbit anti-PLIN1 (dil. 1:3000; this work), rabbit anti-PLIN2 (formerly anti-LSD-2; dil. 1:3000) (Grönke et al., 2003), goat anti-ADH dG-20 (dil. 1:200; Santa Cruz Biotechnology), mouse anti-β-Tubulin E7 (dil. 1:500; Developmental Studies Hybridoma bank), mouse anti-Actin JLA20 (dil. 1:500 Developmental
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Studies Hybridoma Bank), and rabbit anti-HisPLIN1a (“PREK”; dil. 1:2000) (Souza et al., 1998). Primary antibodies were combined with anti-mouse HRP (dil. 1:40000) or anti-rabbit HRP (dil. 1:40000) (http://www. piercenet. com/) secondary antibodies. Signals were detected by the Pierce Super Signal West pico system. For semiquantitative western blots, images were acquired with a Fuji LAS-1000 imaging system equipped with an Intelligent Dark Box II and quantified with the Fuji ImageGauge software (for details, see the Supplemental Experimental Procedures).

Immunohistochemistry and Whole Mount In Situ Hybridization

Immunohistochemistry (embryos and larval tissue) and whole-mount in situ hybridization (embryos) were performed as described (Grönke et al., 2003). Wing imaginal discs of migrating L3 larvae were hand dissected in PBS and fixed for 20 min in 4% paraformaldehyde in PBS prior to immunohistochemistry. Specimens were mounted in 30% glycerol/PBS or Mowiol488. For details on double fluorescent RNA in situ hybridizations on imaginal discs, see the Supplemental Experimental Procedures.

Physiological Assays

Low-sugar (1% sucrose/5% yeast extract) or high-sugar (10% sucrose/5% yeast extract) liquid food intake was measured with a modified CAFE system, and caloric food composition was calculated as described (Ja et al., 2007). Fat (glyceride) content measurements and starvation assays were performed as described (Grönke et al., 2003). Locomotor activity of flies on complex food was assayed with the TriKinetics DAM2 system (http://www.trikinetics.com/). For details, see the Supplemental Experimental Procedures.

Molecular Biology

Developmental northern blots (Grönke et al., 2005) were probed with 32P-labeled plin1 and RpL9 DNA. qRT-PCR reactions were performed using FAST Sybr Green Master Mix on a StepOnePlus System (http://www.appliedbiosystems.com/). For details, see the Supplemental Experimental Procedures.

Microscopy

Embryos and fat body cells were imaged with a Zeiss Axiophot under bright-field or fluorescence light conditions. Lipid droplets were stained with BODIPY 493/503 (dil. 1:2000), LipidTOX 637/655 (dil. 1:500), or Nile Red as described (Grönke et al., 2005). Plasma membranes were stained with FM4-64 (dil. 1:300 in 30% glycerol/PBS) (all dyes from http://www.invitrogen.com/). Lipid droplets were stained with BODIPY C14 (Gronke et al., 2005). Plasma membranes were stained with FM4-64 (dil. 1:300 in 30% glycerol/PBS) (all dyes from http://www.invitrogen.com/). (Gro¨ nke et al., 2005). Plasma membranes were stained with FM4-64 (dil. 1:300 in 30% glycerol/PBS) (all dyes from http://www.invitrogen.com/).

In Silico Methods

PERILIPIN family members were aligned with the ClustalW2 service at the EBI (http://www.ebi.ac.uk/Tools/clustalw2/index.html) with the standard settings for sequence identities and details, see the Supplemental Experimental Procedures. Note that the phylogenetic tree shown in Figure 1A is based on the first 300 amino acids.

Statistics

Standard deviations of the entire population (STDEV) are given for replicate measurements. Pair-wise comparisons were subjected to an unpaired Student’s t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/ j.cmet.2010.10.001.

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REFERENCES


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