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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 knockdown 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 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, $plin1^{1}$ and $plin1^{2}$ (recovered by imprecise transposon mobilization of $plin1^{G4304}$; Figure 1B), develop into obese but otherwise normal-looking adults. The fat content is doubled in homozygous $plin1^{1}$ flies or transheterozygous flies carrying $plin1^{1}$ in combination with $plin1^{2}$ 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 $plin1^{1}$ homozygotes (called $plin1^{-}$ in the following), $plin1^{1}/plin1^{2}$ heterozygotes, or *plin1* knockdown flies outlive the $plin1^{+}$ 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 $plin1^{-}$ flies, whereas strong expression of *plin1* rescues the $plin1^{-}$ 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 $plin1^{-}$ flies during the first 6 days of adult life is about one-third higher than that observed with $plin1^{+}$ flies, and their body fat content is doubled. On low-sugar diet, both $plin1^{-}$ and $plin1^{+}$ flies exhibit a similar food intake, but $plin1^{-}$ flies accumulate up to 50% more body fat than $plin1^{+}$ 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 $plin1^{-}$ flies does not contribute to obesity, and the obese phenotype does not impair the locomotor activity of the mutants.

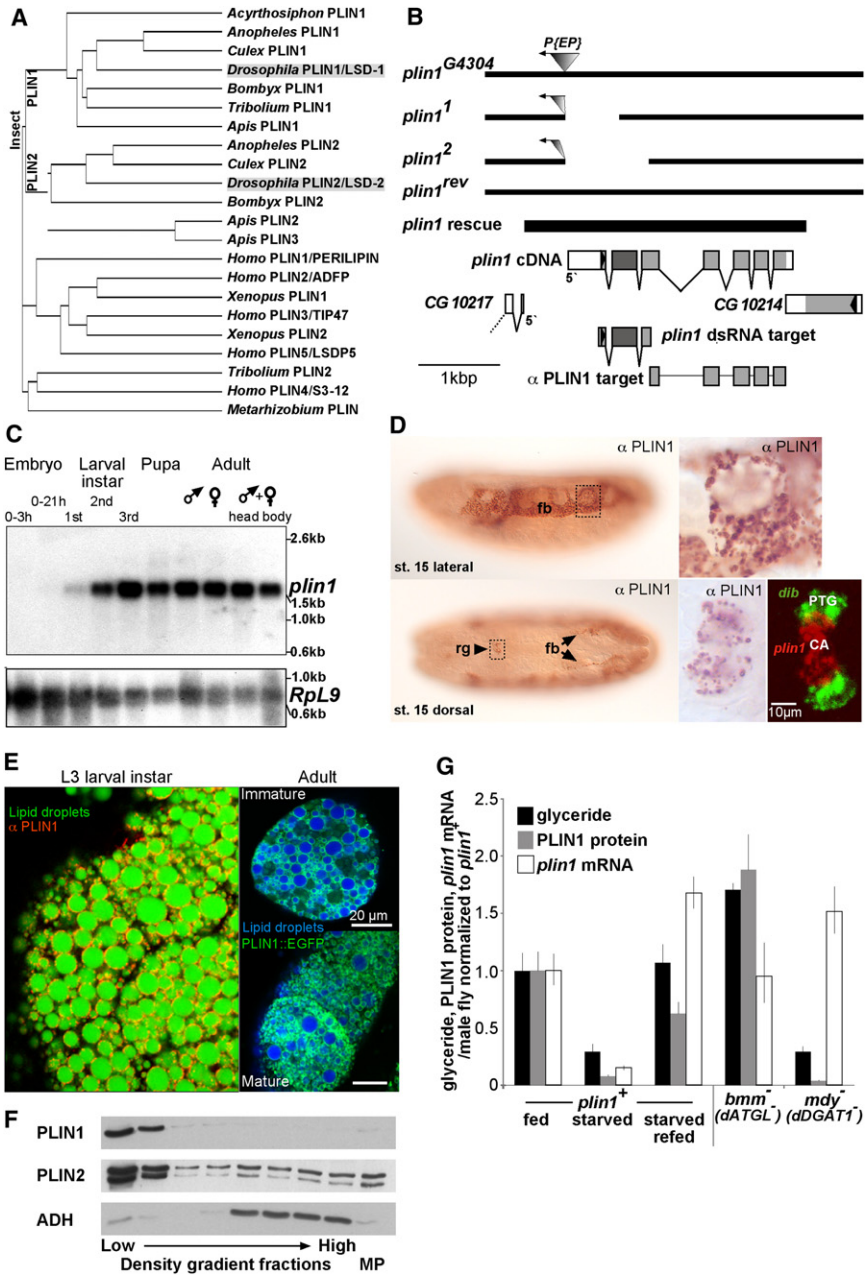

Figure 1. PLIN1 Serves as a Diagnostic Marker of Lipid Storage Levels in *Drosophila*

(A) Phylogenetic tree analysis characterizes PLIN1 as one of two PERILIPIN family members in insects. Note that sequence similarity does not identify *Dm*PLIN1 as ortholog of a particular mammalian PERILIPIN.

(B) Organization of the *plin1* gene showing the transposon integration $plin1^{G4303}$ used to generate *plin1* mutant alleles and the genetically matched $plin1^{+}$ revertant $plin1^{rev}$. Black box, extent of the genomic *plin1* rescue transgene. Open boxes, *plin1* exons (coding exons in gray; the N-terminal PAT-domain is hatched). CG10217 and CG10214 are the neighboring genes. In boxes, PLIN1 sequences targeted by a *plin1* dsRNA transgene or used as antigens for αPLIN1 antibodies.

(C) Northern blot showing *plin1* transcripts throughout fly ontogenesis starting at late embryonic stages.

(D) Immunohistochemistry detects PLIN1 in fat body (fb) and ring gland (rg) of late embryos (stage 15). *plin1* expression in the corpus allatum (CA) next to *disembodied* (*dib*), which marks prothoracic gland cells (PTG). Note that PLIN1 decorates the surface of vesicles.

(E) Lipid droplet-association of PLIN1 in fat tissue during postembryonic development detected by immunofluorescence (L3 larval stage) or ex vivo by PLIN1::EGFP fluorescence in fat body cells of immature and mature adult flies. Note discontinuous lipid droplet surface coverage by PLIN1.

(F) PLIN1 is a constitutive lipid droplet protein as revealed by density gradient fractionation and western blotting. Alcohol dehydrogenase (ADH) marks cytoplasmic fractions. MP, microsomal pellet.

(G) PLIN1 protein content indicates the fat storage level and is subject to posttranscriptional regulation. PLIN1 abundance follows the acute body lipid store depletion/re-accumulation. PLIN1 but not *plin1* mRNA levels parallel the lipid stores in chronic obese *brummer* (bmm^{-}) and lean *midway* (mdy^{-}) mutants flies. Error bars represent the STDEVP for glyceride and protein and 95% confidence intervals for mRNA. Representative experiment based on triplicate measurements involving a total of 24 (glyceride) or 6–10 (protein; mRNA) male flies per genotype.

See also Figure S1.

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 (Grönke 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 $plin1^{-}$-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^{-} $plin1^{-}$) or *AKHR* ($AKHR^{-}$ $plin1^{-}$). Both double mutants are more obese than the single mutants (Figure 4A). Thus, *plin1* participates in

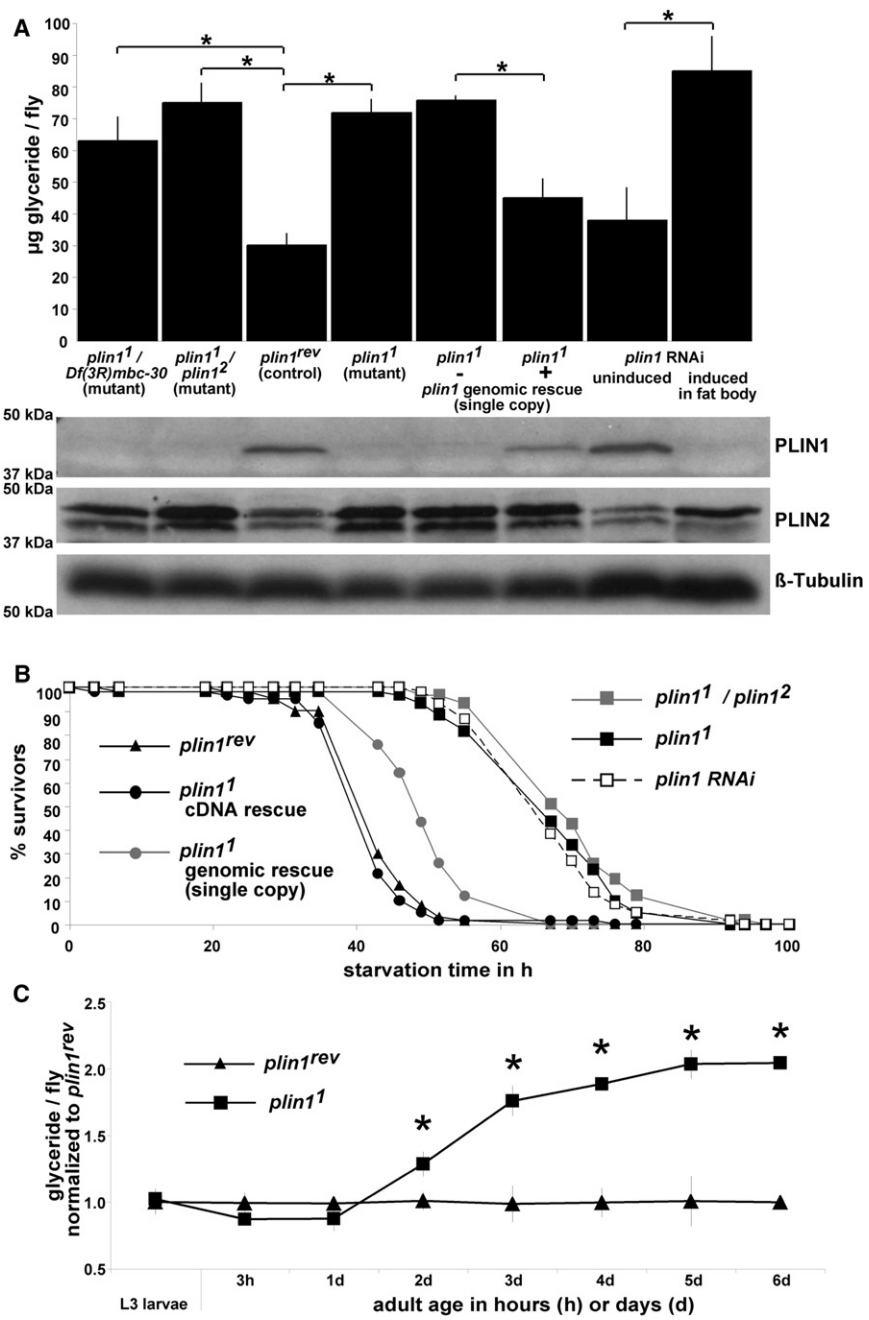

Figure 2. Fly *plin1* Is an Adult-Onset Obesity-Related Gene

(A) Body fat increase in mature adult male *plin1* mutant flies [*plin1*^{1} homozygotes, *plin1*^{1}/*Df(3R) mbc-30* or *plin1*^{1}/*plin1*^{2} transheterozygotes] or flies with a fat body-specific *plin1* RNAi knockdown. *plin1*$^{-}$ obesity phenotype reversion by a *plin1* genomic rescue transgene. Bottom: PLIN1 and PLIN2 protein abundance of the corresponding genotypes by western blot analysis. Note that PLIN2 is upregulated in *plin1* mutants.

(B) Starvation resistance of *plin1* knockout and knockdown mutants. The *plin1*$^{-}$ starvation resistance phenotype is dosage-sensitive and can be partially reverted by a single *plin1* transgene copy.

(C) Adult-onset obesity of *plin1* mutants. Shown are representative experiments based on triplicate measurements involving a total of 24 (A and C) or $\geq$ 45 (B) male flies per genotype.

(A and C) Error bars represent STDEVP; *p < 0.05.

each of the two lipolytic pathways. *bmm*$^{-}$ *plin1*$^{-}$ double mutants fail to mobilize their fat storage and are starvation hypersensitive, as observed with *bmm*$^{-}$ *AKHR*$^{-}$ double mutants (Figures 4B and 4C) (Grönke et al., 2007). In contrast, *AKHR*$^{-}$ *plin1*$^{-}$ double mutants remain lipolysis competent and outlive the *plin1*$^{+}$ control flies under food deprivation (Figures 4B and 4C). Thus, the excessive fat accumulation in *AKHR*$^{-}$ *plin1*$^{-}$ 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 (Grönke et al., 2007) and results in strong fat reduction in *bmm*$^{-}$ *plin1*$^{-}$ 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*$^{-}$ *plin1*$^{-}$ 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*$^{+}$ flies by about one-third and by 80% in *plin1* 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

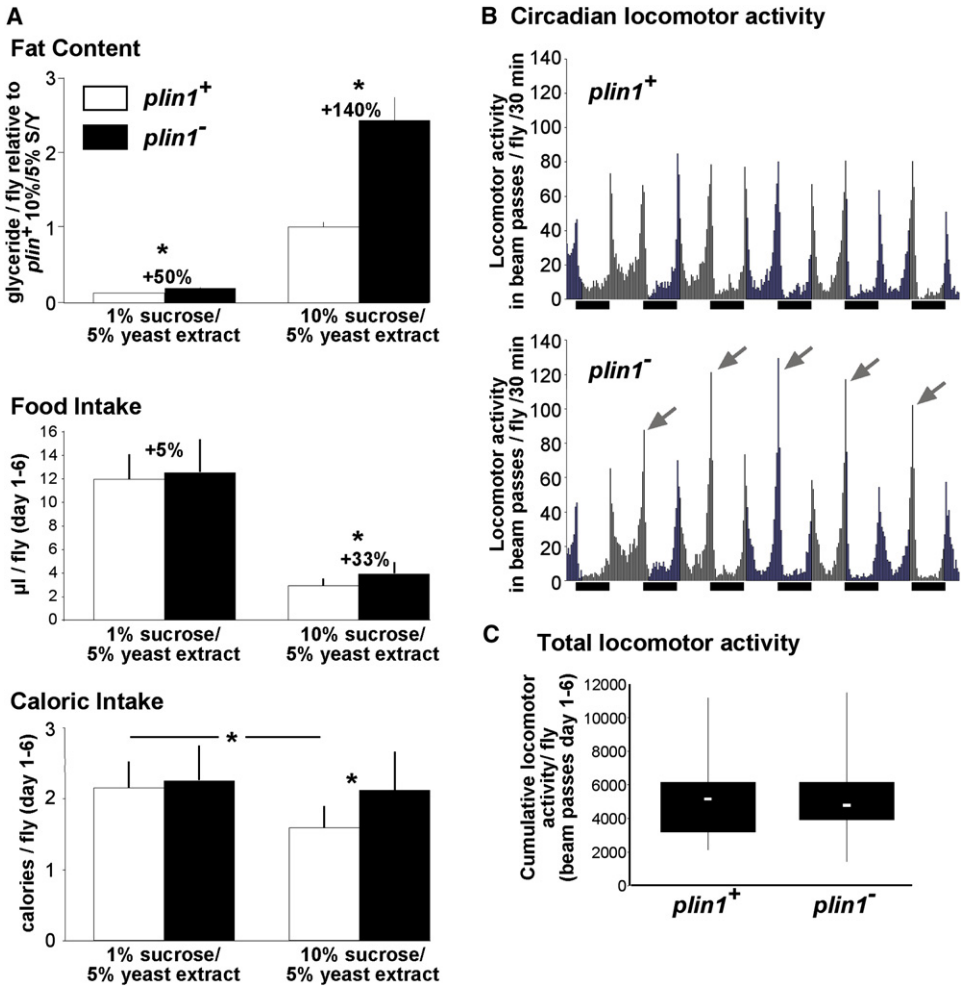

Figure 3. Hyperphagia but Not Activity Impairment Contributes to *plin1* Mutant Obesity

(A) Body fat storage is strongly sugar dependent in *plin1*$^{+}$ and *plin1*$^{-}$ flies. On a high-sugar diet, hyperphagia and surplus calorie intake contribute to *plin1* mutant obesity. Shown is a representative experiment based on triplicate measurements involving a total of 24 (glyceride) or ≥16 (food/calorie intake) male flies per genotype (and food condition). Error bars represent STDEVP; *p < 0.05.

(B) Normal biphasic circadian locomotion pattern with an altered evening peak profile (arrows) of *plin1* mutants.

(C) Comparable total locomotor activity during obesity progression *plin1*$^{-}$ and *plin1*$^{+}$ flies. n = 32 male flies per genotype. Box plot illustrations represent minima, maxima, and median values with lower and higher quartiles.

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$^{\Delta H}$, 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$^{\Delta H}$ 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; Gutierrez et al., 2007) and tissue culture cells (Beller et al., 2008; Guo et al., 2008). PLIN2 has no structural effect on lipid droplets

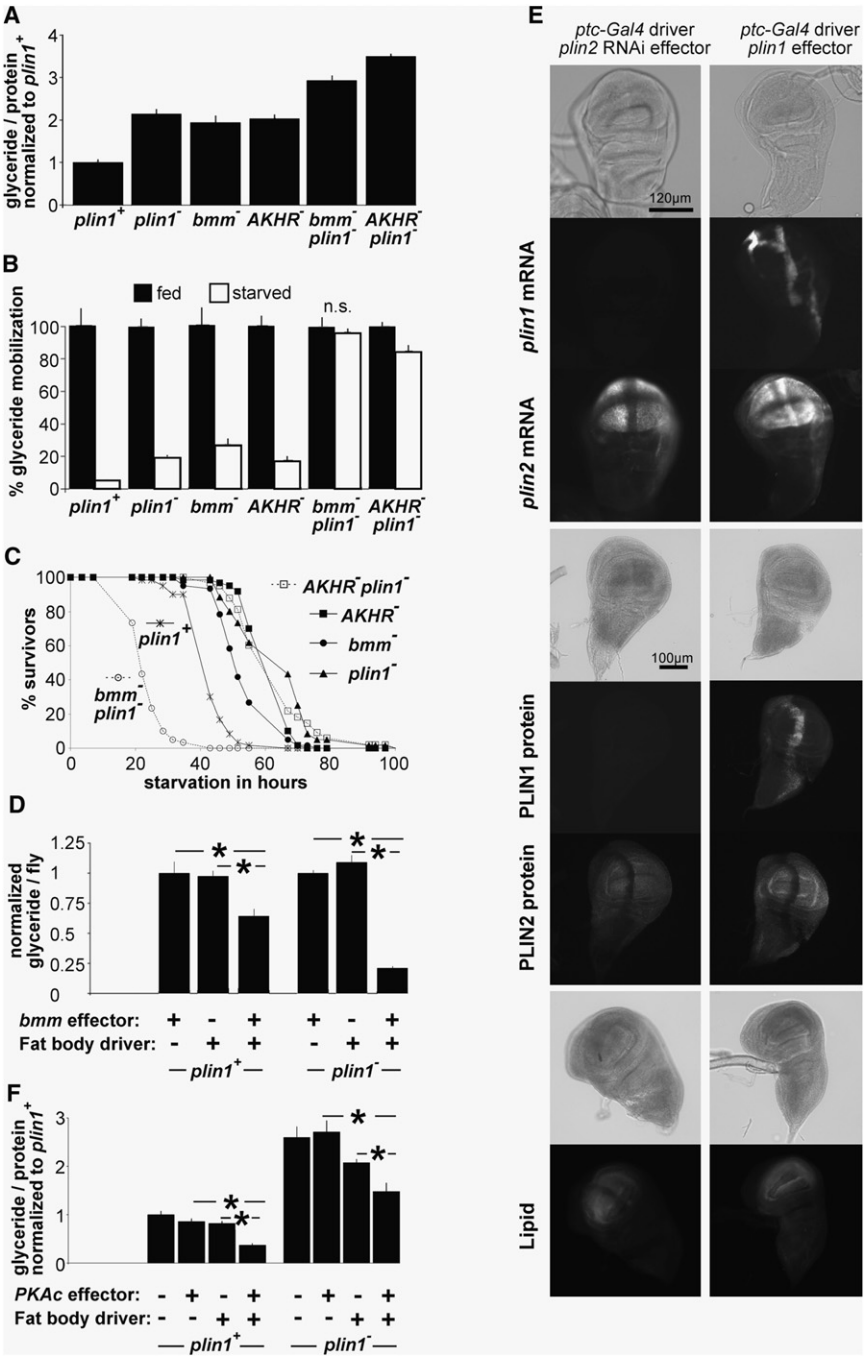

Figure 4. PLIN1 Acts as Lipolytic Gatekeeper at the Lipid Droplet Surface

(A–C) Body fat accumulation (A), impaired fat mobilization (B), and starvation resistance (C) of *plin1* mutants equals the phenotypes of flies mutant for *brummer* (*bmm*$^-$) and the *adipokinetic hormone receptor* (*AKHR*$^-$).

(A) *plin1* interaction with the *bmm* and *AKHR* lipolytic pathways as revealed by incomplete additive obesity of *bmm*$^-$ *plin1*$^-$ and *AKHR*$^-$ *plin1*$^-$.

(B and C) Fat mobilization incompetence (B) and starvation hypersensitivity (C) of *bmm*$^-$ *plin1*$^-$ double mutants identifies PLIN1 as essential member of the AKHR-dependent lipolysis pathway.

(D) PLIN1 protects lipid stores against BMM lipase-mediated fat mobilization. BMM overexpression causes about 50% more leanness in *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 *ptc*-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.

(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 control flies, which contain multilocular cells with small lipid droplets (Figure 5C). The fat content of lean *mdy*$^-$ *plin1*$^-$ double mutants is about 60% of control flies similar to *mdy*$^-$ *bmm*$^-$ double mutants (Figure 5C). However, their lipid droplet phenotypes differ significantly. While fat cells of *mdy*$^-$ *bmm*$^-$ mutants are multilocular, fat cells of the *mdy*$^-$ *plin1*$^-$ mutants contain mainly giant lipid droplets (Figure 5C). Thus, lack of *plin1* causes the structural lipid droplet phenotype irrespective of the body fat content. However, fat cells of *mdy*$^-$ *plin1*$^-$ double mutants typically contain more than one large lipid droplet, whereas the *plin1* mutants contain unilocular fat cells only. Therefore, lack of PLIN1 causes giant lipid droplet formation and this effect can be modulated by the fat content.

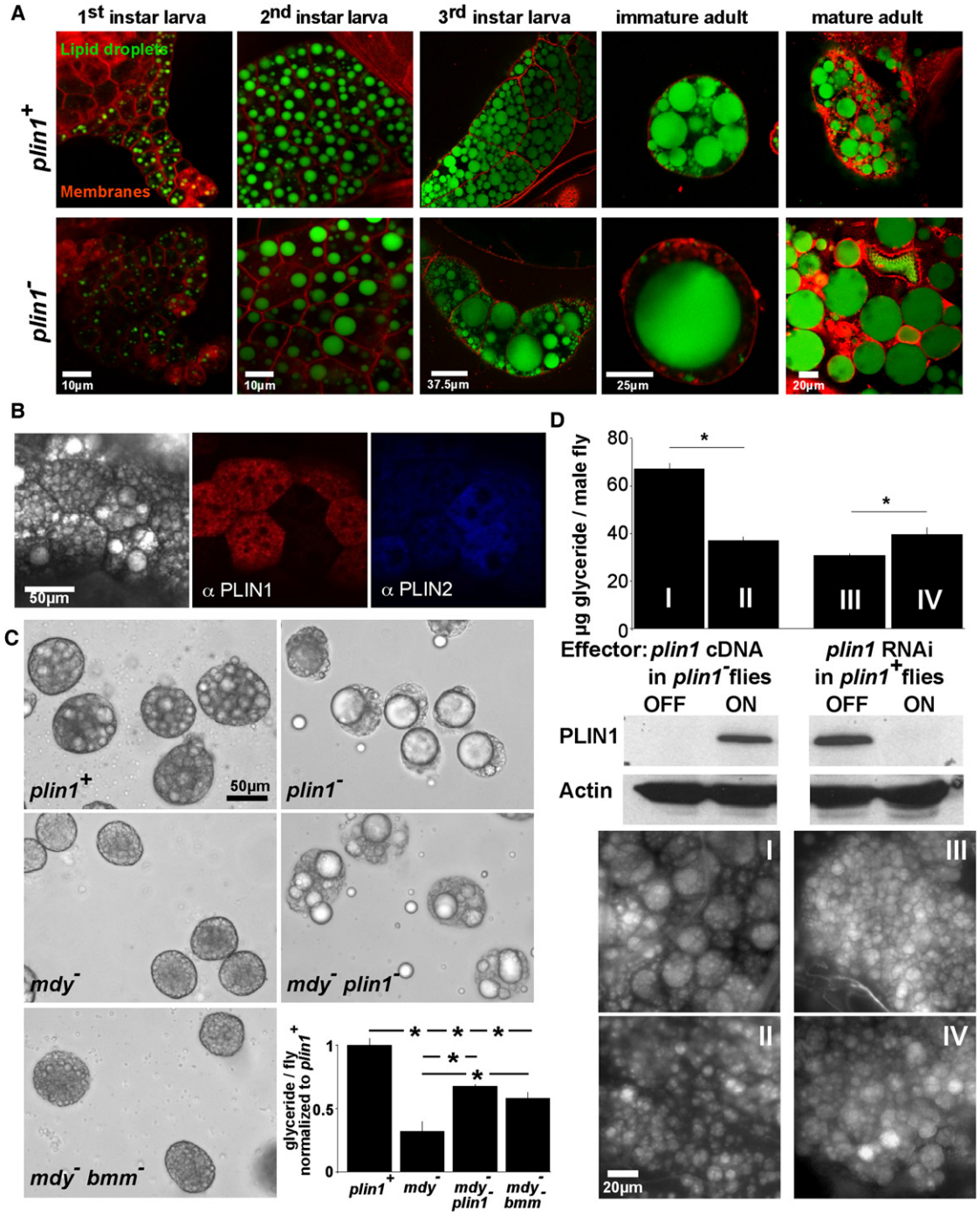

Figure 5. PLIN1 Controls Lipid Droplet Structure

(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.

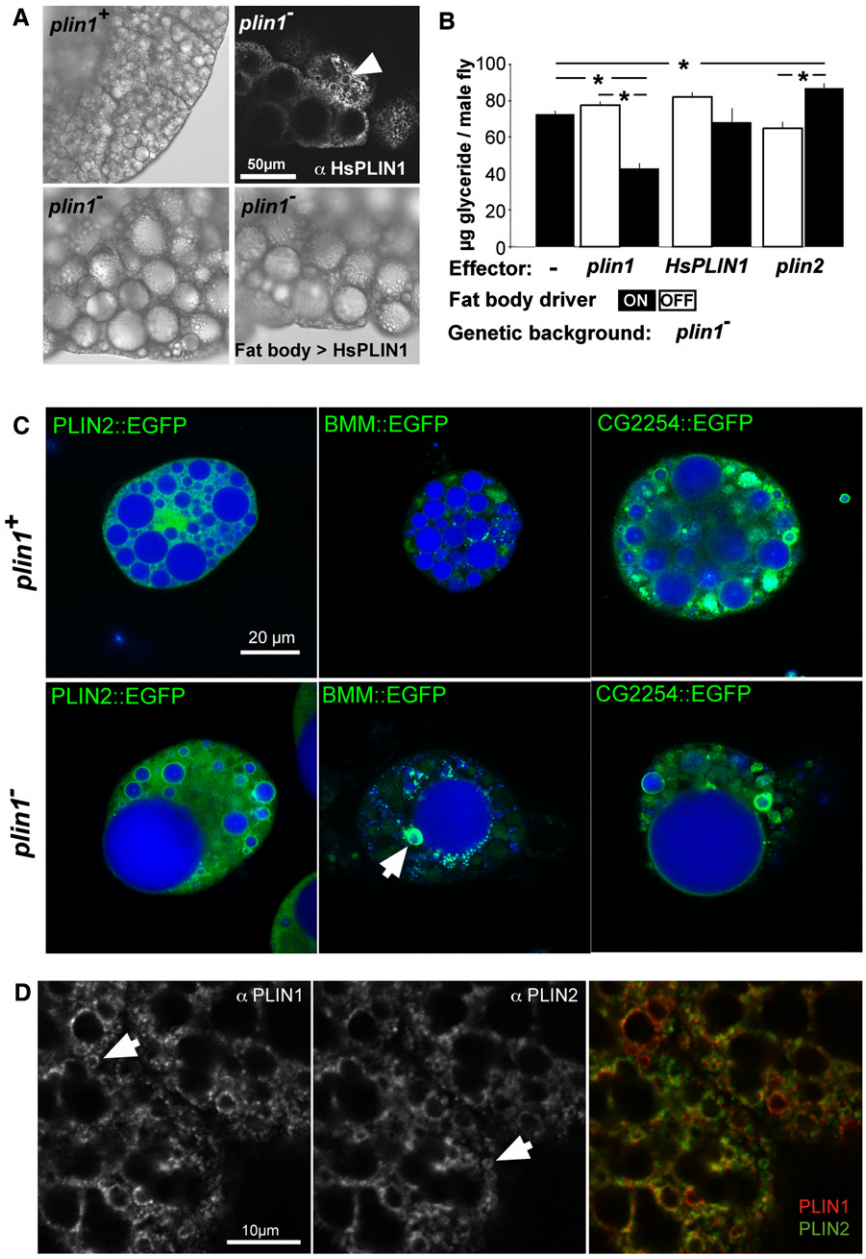

Figure 6. PLIN1 Modulates Protein Access to the Lipid Droplet Surface

(A–C) Transgenic human PLIN1 localizes to fly fat body lipid droplets (arrowhead) but does not revert the larval giant lipid droplet phenotype (A) and the adult obesity (B) of *plin1* mutants, while PLIN2 localizes to the lipid droplets (C) and increases the obesity effect (B). The glyceride assay is a representative experiment based on triplicate measurements involving a total of 24 male flies per genotype. Error bars represent STDEVP; *$p < 0.05$.

(C) The lipid droplet marker protein CG2254 is unaffected, but BMM lipase (arrowhead) is enriched on *plin1*$^-$ lipid droplets. Note that PLIN2, BMM, and CG2254 are represented by transgenic EGFP fusion proteins.

(D) Both PLIN1 and PLIN2 coreside on the majority of lipid droplets with incomplete lipid droplet surface area coverage. In addition, they mutually exclusive decorate small lipid droplet subpopulations (arrowheads).

See also Figure S5.

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) (Grönke 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

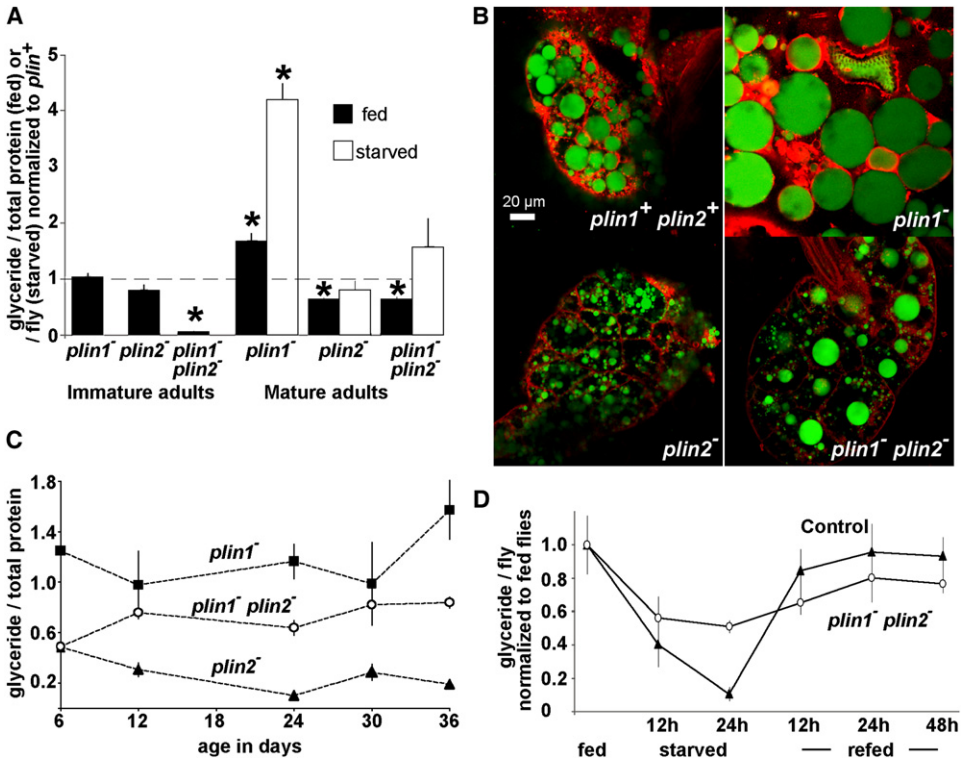

Figure 7. PERILIPIN-Free Flies Show Defects in Lipometabolism Homeostasis

(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 $\geq$15 (C and D) male flies per genotype and point in time. Error bars represent STDEVP; *$p < 0.05$ (A, C, and D).

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.

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 (Géminard 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 (Martinez-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 (Welte 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

Studies Hybridoma Bank), and rabbit anti-*Hs*PLIN1a ("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 ^{32}P-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/). Genetic crosses set up at 18°C and shifted to 25°C 36 hr prior to imaging were used for targeted expression of EGFP-fusion proteins. Fat body cells were manually released from immature adult male flies, embedded in 30% glycerol/PBS supplemented with a neutral lipid dye (see above), and imaged ex vivo with a Leica TCS SP2 AOBS.

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 (STDEVP) 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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