Organismal energy homeostasis is continuously challenged by fluctuating environmental and internal conditions, which require a fast and precisely controlled adaptation to energy needs. To maintain this homeostasis, organisms as different as yeast, plants, nematodes, flies, mice, and humans accumulate energy stores during periods of food supply. Whenever energy expenditure exceeds energy intake, these energy stores become mobilized and catabolized to ensure permanent energy balance. Lipids, in particular triglycerides (TGs), are the calorically most important energy depots in eukaryotic organisms. Besides their pivotal function as metabolic fuel, i.e., as a source of FAs for β-oxidation,
intermediates of TG catabolism provide building blocks for a variety of lipids with structural and signaling function. Due to the cross-talk within the lipid metabolism network, a variety of dysfunctions can manifest in excessive body fat accumulation, with human obesity being the most prominent example. However, neutral lipid over-storage is not restricted to tissues dedicated to fat storage, such as mammalian adipose tissue or the so-called fat body of insects. Rather, ectopic lipid storage as a hallmark of uncontrolled local or global lipid homeostasis is widespread in multicellular eukaryotes, as in Drosophila brain glial cells (1) or Arabidopsis leaves (2). Importantly, the physiological consequences of ectopic fat accumulation are only poorly understood.

The universal packaging of storage lipids in unique organelles called lipid droplets (LDs) is a common motive of fat storage in many cell types. The remarkable structural and regulatory similarities of LDs in a wide range of organisms point to evolutionary ancestry of the network in control of the metabolic dynamics of LDs. Indeed, comparative physiological and genetic studies in a variety of organisms revealed that central regulators acting on LDs are evolutionarily conserved. Examples in the fly are the LD-associated perilipins [DmPlins (3–6)] or the key TG biosynthesis proteins (37–39) in Drosophila (40) and Arabidopsis (41) (Fig. 1A). Central to TG lipolysis is the brummer (bmm) gene (9), which encodes the ortholog of the mammalian adipose TG lipase [ATGL (10, 11)].

Comparative gene identification-58 (CGI-58), also known as α/β-hydrolase domain-containing 5 (ABHD5) (12) is one member of the paralogous protein pair, α/β-hydrolase domain-containing 4 (ABHD4) and ABHD5, and acts as co-activator of mammalian ATGL. ABHD5/CGI-58 physically interacts with perilipin 1 on the LD surface (15–16). Upon lipolytic stimulation, ABHD5/CGI-58 dissociates from perilipin 1 and stimulates TG lipase activity of ATGL by a so far unknown mechanism (13). A knockout of mammalian ATGL (17) or ABHD5/CGI-58 (18) causes neutral lipid storage disease in mice and humans. However, knockout mutations in these genes result in different phenotypes (18) [recently reviewed in (19, 20)]. In contrast to ATGL knockout mutants, mice lacking ABHD5/CGI-58 exhibit ichthyosis, skin permeability defects, more severe hepatic steatosis, and altered acyl-ceramide production (21). These data strongly support ATGL-independent functions for ABHD5/CGI-58. It is likely that mammalian ABHD5/CGI-58 cooperates with other enzymes to execute ATGL-independent functions, as the protein is catalytically inactive due to replacement of the active serine within the conserved GxSxG lipase motif by an asparagine (Fig. 1A) (14, 22, 23). Recently, it has been shown that ABHD5 interacts with patatin-like phospholipase domain-containing 1 (PNPLA1), recruiting this enzyme to LDs and modulating its acyl-ceramide synthesis activity (24).

In contrast to ABHD5/CGI-58, the paralogous ABHD4 is an active lipid hydrolase, which is involved in anoikis resistance (25) and endocannabinoid biosynthesis (26). The characteristics of the ABHD4 and ABHD5/CGI-58 protein pair are consistent with the hypothesis that they evolved from an ancestral enzyme, which underwent gene duplication and functional diversification in the mammalian lineage (27).

ABHD4/5-related genes are found in diverse species beyond mammals. In all cases studied, the lack of ABHD4/5 consistently leads to altered neutral and/or phospholipid metabolism as demonstrated in mice (28–31), in the thale cress Arabidopsis thaliana (2), in the yeast Saccharomyces cerevisiae (32), and in the nematode Caenorhabditis elegans (33, 34). Notably, however, C. elegans encodes multiple ABHD4/5-related proteins, two of which, the ABHD4-relative CeLid-1 (33) and the ABHD5/CGI-58-relative Cebhgd5.2 (34), interact with CeATGL-1, determine its localization to LDs, and thereby control lipolysis comparable to their mammalian homologs.

This study presents the functional characterization of CG1882, which we renamed Pummelig (Puml; colloquial German for chubby) in accordance with the mutant phenotype (see below). Pummelig is the single Drosophila melanogaster α/β-hydrolase domain protein, which is sequence-related to the mammalian paralogs, ABHD4 and ABHD5/CGI-58. We showed that pummelig deletion mutants (puml) suffered from defects in overall physical fitness and accumulated extra body fat. These obese flies were still capable of mobilizing storage lipids and exhibited increased lipogenesis. Additionally, abnormal lipid storage could be detected in fly kidneys, called Malpighian tubules (MTs), and the FA profile of these ectopically stored TGs was altered in flies lacking puml function. In contrast to mammalian ABHD5, Puml did not stimulate TG lipase activity of Brummer (DmATGL) in vitro. Besides exhibiting no autonomous TG lipase activity, Puml actually is a functional phospholipase. Tagged Pummelig localized to various intracellular compartments in vivo, i.e., LDs, mitochondria, and peroxisomes.

MATERIALS AND METHODS

Fly husbandry and stocks

Flies were propagated on a complex medium as described (35). For further details, see the supplemental Experimental Procedures. The fly stocks used in this study are listed in supplemental Table S1 in the supplemental Experimental Procedures. A puml deletion mutant was generated by a conventional P-element mobilization scheme using the PSUPor-P CG1882[KG09852] (36) fly line, that carried a P-element insertion in the puml 5′UTR (Fig. 1B). Subsequent molecular characterization by sequencing the relevant part of the genome revealed an imprecise excision with 12 bp residual P-element sequence remaining between the deletion breakpoints and puml gene locus being devoid of coding DNA sequence from position 188-2369 relative to the transcription start of puml RA cDNA. For appropriate and biologically comparable controls, the obtained puml mutant allele was backcrossed several generations into w1118 fly stock (referred to as control stock) to get genetically matched flies. For other transgenic fly stocks, additional information can be found in the supplemental Experimental Procedures. Transgenic fly stocks were obtained from the Vienna Drosophila Resource Center (VDRC, www.vdrc.at). Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study.
Physiological assays

Lipid analysis. Fly body fat content and total body neutral lipids (TGs, DGs) were quantified by a coupled colorimetric assay (37) and by thin-layer chromatography as described (35). For further details, see the supplemental Experimental Procedures.

Glycogen analysis. Stored glycogen of flies was quantified as described (35). For further details, see the supplemental Experimental Procedures.

Food intake. Food intake of ad libitum-fed flies was determined as described (38). For further details, see the supplemental Experimental Procedures.

Weight measurements. Empty 1.5 ml vials were labeled and weighed. Thirty flies per replicate were collected and anesthetized with CO2. After weighing (wet weight) flies were snap-frozen in liquid nitrogen and dried overnight (65°C, 5% humidity). Then flies were weighed again (dry weight). The fly water content was calculated by subtracting dry weight from wet weight.

Starvation, desiccation, and locomotor activity assay. Six-day-old male flies were briefly anesthetized with CO2. After weighing (wet weight) flies were snap-frozen in liquid nitrogen and dried overnight (65°C, 5% humidity). Then flies were weighed again (dry weight). The fly water content was calculated by subtracting dry weight from wet weight.

Locomotor activity was calculated from the total accumulated activity per fly measured in the DAM2 system during the first 24 h of starvation.

Energy expenditure. The metabolic rate of 6-day-old flies was estimated by the manometric measurement of CO2 production as described in (39). For further details, see the supplemental Experimental Procedures.

Lipogenesis assay. Lipogenesis in adult flies was traced by incorporation of D-[14C(U)]glucose into neutral lipids as described in (40). For details, see the supplemental Experimental Procedures.

Enzymatic assays. TG hydrolase activity was measured as described in (41). For further details, see the supplemental Experimental Procedures. The substrate screen on Puml was performed as described in (42). Tested substrates and their suppliers are listed in supplemental Table S3. Substrate saturation, pH, protein, and time dependence of the hydrolase activity were measured as essentially described in (43). For details on cloning and protein expression, see the supplemental Experimental Procedures.

Imaging

Images from adult fat body tissue were obtained as described in (38). To acquire images from MTs, 6-day-old male flies were dissected in ice-cold 1× PBS. Flies were grabbed with forceps at the thorax and the anal plate. Then both forceps were gently pulled apart. Along with the intestine, the MTs were pulled out from the abdomen. The MTs (together with the intestine) were then embedded in 1× PBS containing Bodipy493/503 (38 μM; Invitrogen, D3922) or LipidTOX DeepRed (2×; Invitrogen, H34477) for LD staining. DAPI (3.6 μM; Invitrogen, D1306) for nuclei staining, and CellMask™ Deep Red (5 μg/ml; Invitrogen, C10046) for plasma membrane staining. Images were acquired with a Zeiss...
LSM-780 microscope (at 20°C) in 16-bit mode using a C-Apochromat 40×/1.20 W Korr PCS M27 objective and the Zeiss ZEN software. For fluorescence detection, the following settings were used: DAPI [excitation (Ex): 405 nm; emission (Em): 410–468 nm]; Bodipy493/503 (Ex: 488 nm; Em: 490–534 nm); and Cell-Mask (Ex: 561 nm; Em: 585–747 nm). For the detection of fluorescent proteins, the following settings were used: eGFP (Ex: 488 nm; Em: 490–540 nm); ECFP (Ex: 405 nm; Em: 450–540 nm); and mCherry (Ex: 561 nm; Em: 570–712 nm). Mitochondria were stained by Mitotracker Orange CMTMRos (75 nM; Invitrogen, M7510; Ex: 561 nm; Em: 570–590 nm). Images were subsequently processed in Image J v1.49m and Adobe Illustrator CS6.

**Lipid staining with Oil Red O.** LDs were stained with Oil Red O as described in (38). For further details, see the supplemental Experimental Procedures.

**Electron microscopy.** Ultrastructural analyses of MTs were performed as described for fat body tissue (38). For further details, see the supplemental Experimental Procedures.

**LD size quantification.** An improved protocol for LD size quantification based on (35) was used. Various optimizations were introduced to detect especially small and weakly stained LDs and balance out inhomogeneous LD signals and, therefore, get a more reliable detection of LDs by the particle analyzer plugin of Image J v1.49m. For detailed information see the supplemental Experimental Procedures.

**Lipidomics (dataset 1)**

Five replicates of 10 MT pairs (per genotype) were obtained from 6-day-old male animals. Fly dissection was performed in ice-cold Ringer solution. Samples were collected in 1.5 ml SAFE-lock Eppendorf tubes (T2795-1000EA) and buffer was removed as much as possible. Then samples were snap-frozen in liquid nitrogen. Lipids were extracted from tissue by a modified protocol from (44). In detail, 1 ml of precooled (~20°C) extraction solvent [methanol:methyl tertiary butyl-ether (1:3, v/v)] was added to the tissue and well mixed. The solvent-homogenate solution was incubated for 30 min (4°C) under constant shaking, followed by a 10 min incubation in an ultrasonication bath filled with an ice-water mix. For phase separation, 650 µl of water:methanol (3:1, v/v) were added, mixed, and centrifuged for 5 min at 4°C at 21,000 g in a tabletop Eppendorf centrifuge. Six hundred microliters of the resulting upper lipid-containing phase were transferred to a new 1.5 ml Eppendorf tube, dried in a speed-vac concentrator, and stored at ~80°C. As control for the extraction and for the analysis, PC (17:0/17:0) (Avanti Polar Lipids, 850360C) was introduced to detect especially small and weakly stained LDs and for the analysis, PC (17:0/17:0) (Avanti Polar Lipids, 850360C) was used as an internal standard at a concentration of 0.1 g/ml.

Liquid chromatography was performed following the protocol of Hummel et al. (44). Dried lipid extracts were resuspended in 300 µl 7:3 (v/v) acetonitrile:2-propanol. For separation of lipids, a Waters Acquity UPLC system with a Waters C8 reversed-phase column (100 × 2.1 mm, 1.7 µm particle size) was used. Flow rate was set to 400 µl/min, column temperature was set to 60°C, and samples were kept cooled at 10°C in the autosampler. Of this sample, 5 µl were loaded onto the C8 column. For elution, a gradient of two mobile phases (A and B) was used. Phase A was water and phase B consisted of acetonitrile:isopropanol (7:3, v/v); both phases contained 1% (v/v) 1M ammonium acetate and 0.1% (v/v) acetic acid. The elution gradient was 1 min 45% A, in 4 min from 45% A to 25% A, in 12 min from 25% A to 11% A, in 15 min from 11% A to 0% A (=100% B), for 4.5 min 100% B, and back to 45% A, equilibrating for 4.5 min before the next elution, giving the total of 24 min for one run.

Mass spectrometric analysis was performed using an LTQ-Orbitrap XL instrument using the following parameters: mass range, normal; resolution, 60,000; scan type, full; data type, profile; scan range, m/z 150–1,500. We used a heated ES source with the following settings: heater temperature, 350°C; gas flow rates (sheath gas, 20; aux gas, 16; sweep gas, 0). Spray voltage was set to 3.2 kV in negative mode and 3.5 kV in positive mode. Capillary was set to ±35 V and heated to 275°C, and tube lens to ±110 V for (+) positive and (−) negative mode.

**RESULTS**

**Pummelig controls organismal energy stores**

In order to characterize the *puml* gene function in vivo, a deletion mutant (*puml<sup>Δ</sup>*; was generated, covering the complete *puml* coding region (Fig. 1B). Homozygous *puml<sup>Δ</sup>* flies were viable and fertile. However, compared with control flies, *puml<sup>Δ</sup>* flies were significantly short-lived (Fig. 2A). Additionally, startle-induced climbing activity in *puml<sup>Δ</sup>* flies was significantly lower compared with controls (Fig. 2B) and *puml<sup>Δ</sup>* flies weighed less due to reduced body water content (Fig. 2C). On the other hand, critical physiological parameters such as the respiratory rate under fed and starved conditions (Fig. 2D), spontaneous locomotor activity under starvation (Fig. 2E), and food intake (Fig. 2F) were normal in *puml<sup>Δ</sup>* flies compared with controls.

Collectively, these data demonstrate that *puml* is not an essential gene but controls selective physical fitness parameters in flies.

Proper energy homeostasis is critical for physical fitness, and therefore we analyzed energy storage in *puml<sup>Δ</sup>* flies. *puml<sup>Δ</sup>* flies accumulated less glycogen compared with control flies, which could be properly mobilized during starvation (Fig. 3A). Consistent with glycogen being an important metabolic water reservoir and with the finding that *puml<sup>Δ</sup>* flies had overall lower body water content (Fig. 2C), flies lacking Puml were desiccation sensitive (Fig. 3B).

Due to the high similarity of Pummelig to mammalian ABHD5, we investigated whether *puml* flies also had altered lipid metabolism. Indeed, *puml* flies of both sexes accumulated excess body fat from early adult stages onwards (Fig. 3C; supplemental Figs. S2, S3A, B) similar to *bmm*/*DmAETGL<sup>Δ</sup>*/ mutant flies (9). Organ-specific *puml* function in the fly fat storage tissue is critical because the obese phenotype of the deletion mutant (*puml<sup>Δ</sup>*; could be phenocopied by a tissue-specific *puml* gene knockdown exclusively in the fat body (Fig. 3C). In contrast to *bmm* (9), in vivo overexpression (gain-of-function) of *puml* in the fat body had no significant effect on body fat stores (Fig. 3C).

As the extra body fat is metabolically accessible, obese *puml* flies were more starvation resistant than control flies (Fig. 3D). Notably, compared with controls, significantly more neutral lipids were mobilized in *puml* flies during the first 24 h of starvation (Fig. 3E). Whereas *puml* flies mobilized 18.6 ± 2.1 µg TG, control flies utilized 14.3 ± 0.4 µg storage lipids (one-way ANOVA F<sub>1,24</sub> = 61.11, P = 6.36E-8). This might be a consequence of low glycogen stores (Fig. 3A), as carbohydrates represent the preferred energy...
source during early starvation. Interestingly, in spite of its increase during early fasting, the starvation-induced body fat mobilization was incomplete in puml1 flies (Fig. 3E), thus we cannot exclude an impairment of TG catabolism in puml1 flies.

As shown above, flies lacking puml function had normal food intake and reduced glycogen but increased fat storage under ad libitum feeding. Therefore, we hypothesized that in puml1 flies, dietary sugars might have a divergent channelling into the various energy stores compared with control flies. To address this question, we monitored in vivo lipogenesis in a pulse-chase experiment using radioactively labeled glucose. Pulse-feeding puml1 flies with food spiked with D-[1-14C(U)]glucose for 24 h resulted in a significantly higher tracer incorporation into neutral lipids compared with controls (Fig. 3F), suggesting an increased lipogenesis rate. The difference between puml1 and control flies was even more pronounced after a 60 h chasing period on tracer-free food (Fig. 3F), indicating a slower turnover rate for storage lipids in puml1 flies. Consistent with increased lipogenesis in puml1 flies, the mRNA expression of the lipolytic genes, FASN1 and acetyl-CoA carboxylase (ACC) (but not of midway/DmDgat1), were slightly increased compared with controls (Fig. 3G), notwithstanding that the encoded enzymes are subjected to functionally important posttranscriptional regulation, which was not addressed in this study.

Collectively, the organismal phenotypes of puml1 flies support a complex function of the puml gene in lipid metabolism and nutrient channelling to body energy stores.

**pummelig mutant flies store ectopic LDs in the MTs**

puml gene expression is not restricted to the fat body, the main energy storage tissue in fruit flies, but is widely expressed in adult Drosophila, including the fly kidneys, called MTs (45, 46). In contrast to MTs of adult control flies, which had very few LDs (Fig. 4A–C), puml1 flies showed...
strong lipid accumulation in this tissue (Fig. 4D–F). Lipidomic profiling of TGs in MTs revealed an increase of many TG species in pumld flies compared with controls (Fig. 4G).

Ubiquitous (Fig. 4I) or MT-specific (Fig. 4J) but not fat body-specific (Fig. 4K) puml expression rescued LD over-storage in pumld flies compared with controls. This demonstrates that ectopic LD accumulation in pumld flies is due to the tissue-autonomous lack of Puml function in MTs. Consistent with this tissue-autonomous function and the limited lipid storage capacity of the fly kidney, MT-specific puml expression caused only a moderate reduction of the total body fat content of pumld flies (Fig. 4L). In contrast, ubiquitous or fat body-specific pumld expression reverted the obesity phenotype of the mutants to control levels (Fig. 4L).

MTs serve various functions in Drosophila, with osmoregulation being the most prominent. Because pumld flies store less body water, we hypothesized that LD accumulation impairs the function of MTs in water balance. To address their osmoregulatory capacity, we monitored the survival of pumld flies under salt stress. Control and pumld
flies on a salty diet (food supplemented with 4% NaCl) out-lived food-deprived flies but both comparably deceased within a week (Fig. 4M), suggesting that lipid accumulation in the MTs in puml flies has no major negative impact on osmoregulation.

Remarkably, lack of Puml function in MTs not only increased LD abundance but also altered the size distribution of the LD population. Compared with MT LDs of control flies, the droplets of puml flies showed a smaller average diameter and a more uniform size distribution (Fig. 4N), suggesting a role of Puml in the modeling of the LD structure.

Pummelig does not stimulate Brummer/DmATGL TG hydrolase activity in vitro

A key role of the mammalian Puml ortholog, ABHD5/CGI-58, is the activation of ATGL (12). In support of a putative ABHD5-like function of Pummelig, all currently known amino acids critical for the ABHD5/CGI-58 interaction with ATGL are sequence conserved in Drosophila Pummelig (Fig. 1A, supplemental Fig. S1). Therefore, we tested to determine whether Pummelig acts as a part of a Pummelig+Brummer lipid mobilization complex comparable to ABHD5/CGI-58+ATGL in mammals. To this aim, we recombinitely expressed Pummelig, Brummer, ABHD5/CGI-58, and mammalian ATGL, and determined TG hydrolase activity of Pummelig+Brummer in the absence or presence of Pummelig or ABHD5/CGI-58 (Fig. 5A). Whereas ABHD5 and Brummer showed basal TG hydrolase activity, lysates from cells expressing Pummelig+Brummer did not leak the lipolytic activities of cells expressing β-galactosidase (negative control). Combinatorial assays (Fig. 5B) confirmed the well-established activation of ABHD5 by ABHD5/CGI-58 (12). As negative control, ABHD5 was coexpressed with Pummelig. In contrast, basal TG activity was not potentiated by the analogous coinubation with Pummelig (Fig. 5B). Cross-species combinations of ABHD5 with Pummelig or Brummer with ABHD5/CGI-58 did not indicate any increase in TG lipase activity (Fig. 5B). Collectively, these in vitro data do not support an evolutionarily conserved TG mobilization module consisting of ATGL and ABHD4/5 proteins in flies.

Pummelig is a phospholipase

The Pummelig protein contains an α/β-hydrolase domain with an active catalytic lipase motif (Fig. 1A, supplemental Fig. S1B) and was previously reported to be a functional esterase in the adult Drosophila fat body (47).

To characterize the enzymatic function of Pummelig, we expressed wild-type Pummelig and Pummelig variants with point mutations (Fig. 5C) in the predicted catalytically relevant motifs in High Five™ insect cells, and performed a substrate scan using cell lysates on various ester bond-containing lipids in comparison to control cells expressing β-galactosidase. Pummelig lipase released FAs from phosphatidic acid (PA), Nα-cyolphosphatidylethanolamine (NAPE), and phosphatidylglycerol (PG) (Fig. 5D). In contrast, glycerolipids like TGs with various FA side chain lengths as well as diolein and monoolein were not hydrolyzed by Pummelig (Fig. 5D). Point mutations in any one of the two predicted catalytically relevant motifs (i.e., the catalytic center nucleophile GxSxG and the Hx3D motif, Fig. 5C) of Pummelig abrogated the lipolytic activity of Pummelig on all substrates tested. Collectively, these data identify Pummelig as a phospholipase with a substrate spectrum that overlaps with mammalian ABHD4 (48). Pummelig exhibited a pH optimum at ~7 (Fig. 5E). Substrate saturation measurements with PA revealed a K_m of ~0.78 mM (V_max = 1.49 µmol/h/mg protein) and a drastically lower activity on sn-1-oleoyl-lysoPA (Fig. 5F).

Further experiments revealed that Pummelig degrades PA in a dose- and time-dependent manner (Fig. 5G, H).

Pummelig protein localizes to LDs, mitochondria, and peroxisomes

In support of Pummelig being a bona fide LD-associated protein, the endogenous protein had been found on embryonic LDs (37), and protein correlation profiling in Drosophila S2 cell culture identified overexpressed Pummelig as an exclusive LD resident (49). We generated a genomic rescue transgene construct composed of a C-terminally mCherry-tagged Pummelig under the endogenous promoter control (Fig. 1C) to demonstrate that the fusion protein localizes on the LD surface of adult fat body cells (Fig. 6A–C). This finding leaves the possibility that the Pummelig phospholipase acts directly on the phospholipid monolayer covering the LD surface. Importantly, however, next to LDs, Pummelig localized to additional cellular compartments (Fig. 6C). Overexpression of Pummelig::mCherry in MTs not only detected the fusion protein in ring-like structures characteristic for LDs (blue arrow in Fig. 6F), but also a substantial fraction of Pummelig::mCherry colocalized with a mitochondria marker in fly kidney cells (Fig. 6D–F; yellow arrow in Fig. 6F). Importantly, the average size of MT mitochondrial pummelig flies was significantly increased compared with control flies (Fig. 6G). Notably, mitochondrial anomalies have also been reported in ABHD5/CGI-58 mutant mice (50). Yet, another fraction of Pummelig::mCherry did not exhibit a ring-like pattern and was not colocalized with mitochondria (magenta arrow in Fig. 6F). Collectively, Pummelig localized to various intracellular organelles, including LDs and mitochondria. This localization is important for their structural integrity and might be of functional relevance for these organelles.

Analysis of transgenic flies coexpressing pummelig::mCherry and an eYFP reporter targeted to peroxisomes revealed that a small fraction of Pummelig::mCherry (Fig. 6K) localized to the majority of peroxisomes detectable in MT cells (Fig. 6L). As mitochondria and peroxisomes are sites of cellular FA β-oxidation, we performed TG FA profiling of MTs on two different lipidomic platforms to address a possible implication of Pummelig in this process. Next to the general increase of all TG species in pummelig flies (Fig. 4G, supplemental Fig. S5), relative TG species abundance analyses indicated a trend to TG species with longer and more unsaturated FA in pummelig flies (supplemental Figs. S4, S5), which deserves future research attention. Interestingly, these changes in the TG FA profile are reminiscent of peroxisomal biogenesis mutants such as pex3 (32, 51).
Fig. 4. Ectopic lipid storage and LD structural changes in the MTs of pummelig mutant flies. A, D: Ectopic lipid storage in fixed MTs of pum" mutant flies compared with controls as detected by Oil Red O staining. B, E: Electron microscopy detects the presence of bona fide LDs as spherical electron-lucent structures in MTs of pum" mutant and control flies. C, F: Confirmation of excess LDs in pum" mutant MTs compared with control MTs by the lipophilic dye Bodipy493/503 (green). G: General increase of TG species in pum" mutants compared with controls detected by TG FA profiling (AU, arbitrary units). Tissue-autonomous function of pum for storage lipid control in the MTs. Shown is the reversion of ectopic lipid storage in MTs of pum" mutant flies (H) by expressing pum ubiquitously (Act5c>pum) (I) or specifically in the MTs (UO>pum) (J), but not by expression in the fat body (FB-SNS>pum) (K). L: Ubiquitous and fat body-targeted expression of pum in pum" flies restores body fat storage to control levels (blue line). In contrast, the reversion of MT-specific
Taken together, our data suggest that Puml serves a variety of functions in lipid metabolism of flies due to its enzymatic substrate spectrum and due to the association of the protein with various organelles like LDs, mitochondria, and peroxisomes. Collective lack of these functions resulted in an excess of TG storage in the fat body and ectopic lipid storage in MTs. These profound changes in *Drosophila* lipid metabolism correlate with severe physiological phenotypes in *puml* flies, such as shorter lifespan and reduced physical performance.

**DISCUSSION**

Our study demonstrates that *pumelig* (*puml*), the single *Drosophila* representative of the ABHD4/5 lipase gene family, has versatile functions in flies. *puml* flies are viable but short-lived, exhibit excessive body fat accumulation, and suffer from impaired physical fitness. Despite its structural similarity to mammalian ABHD5/CGI-58, Puml does not stimulate TG lipase activity of Bmm/DmATGL in vitro. Puml acts as a phospholipase localized on LDs, mitochondria, and peroxisomes. Longevity in flies is a complex trait, and currently it is unknown what causes lifespan reduction in *puml* flies. One known longevity-promoting factor is spermidine, which extends lifespan in a number of species, including flies, by an autophagy-dependent mechanism (52). Notably, the plant ABHD4/5 family member, *A. thaliana* CGI-58, acts in spermidine biosynthesis (53). Accordingly, an evolutionarily conserved function of *puml* in *Drosophila* polyamine metabolism or a possible involvement in autophagy should be addressed in the future.

The prominent phenotype of *puml* flies is excessive body fat accumulation. It is likely that increased channeling of ingested sugars toward lipogenesis, at the expense of carbohydrate storage, contributes to this phenotype. However, food intake and energy expenditure are normal in *puml* flies. This imbalance of energy stores has up-and downsides for the survival of *puml* flies under environmental challenges, such as starvation or desiccation. Whereas the higher body fat storage warrants extended starvation survival of *puml* flies, desiccation resistance is reduced. The body water content of *Drosophila* is controlled by the insect renal tissue called MTs (54), which express high levels of *puml* (45, 55). We showed that MTs store abnormal amounts of neutral lipids in a cell-autonomous manner in flies lacking *puml* function. Accordingly, a potential impairment of the MTs’ osmoregulatory function due to the absence of *puml* deserves future research attention, although the salt stress response of *puml* flies was normal.

Ectopic neutral lipid accumulation in various tissues is the hallmark of mammals missing ABHD5/CGI-58 gene function (18, 21). The mechanistic basis for this phenotype is the lack of ABHD5/CGI-58 interaction with ATGL (and possibly other lipases), which reduces lipolysis (12). Recently, two conserved amino acids (R299, G328) in the C-terminal region shared by vertebrate ABHD5 homologs, but not by ABHD4 proteins (supplemental Fig. S1B), were shown to be crucial for the functional interaction with ATGL (27). Remarkably, substitution of just these two amino acids is sufficient to capacitate rat ABHD4 as an ATGL activator similar to ABHD5 (27). Although these two amino acids are sequence-conserved in *Drosophila* (supplemental Fig. S1B), Puml did not stimulate ATGL hydrolase activity in vitro, neither of mammalian ATGL nor of the DmATGL-ortholog Bmm. Conversely, mammalian ABHD5 also failed to stimulate Bmm lipase activity. Collectively, these data suggest that, in contrast to the evolutionary ancient role of ATGL family lipases in storage lipid mobilization, ABHD family proteins are a more recent addition to ATGL regulation. This hypothesis gains further support by the fact that a critical tyrosine residue in the Hx4D-motif of mammalian ABHD5/CGI-58 is not conserved in Puml (supplemental Fig. S1B). In rat ABHD5/CGI-58, this tyrosine residue (Y330) is essential to bind long-chain acyl-CoAs (56), which in turn promotes the interaction with LD-associated perilipins to negatively regulate lipolysis (16, 57).

While Puml is a LD-associated protein, our data do not support an accessory function of Puml in Bmm-mediated lipolysis on the LD surface comparable to ABHD5/CGI-58 stimulating ATGL. In vivo, a broad range of TG species, including TG 54:3 (largely representing triolein), accumulated in the MTs of *puml* flies (Fig. 4G). Comparably, the TG composition of *bmm* flies resembles the changes observed in *puml* flies (supplemental Fig. S6). This finding equally supports a cooperative interaction of Puml and Bmm in a CGI-58/ATGL-like manner, or a Bmm-independent function of Puml, such as remodeling of the LD phospholipid layer.

A weak lysosphatic acid acyl transferase (LPAAT) had been reported for murine (58) and plant (59) ABHD5/CGI-58. However, this LPAAT activity, presumably mediated by the Hx4D-motif, has been dismissed (60, 61). Consistently, unlike mammalian ABHD5/CGI-58, Puml contains a functional catalytic site Gx(Sx)G-motif characteristic for active lipases (Fig. 5C, supplemental Fig. S1B). Indeed, our earlier study identified Puml (at that time called CG1882) as a fat body lipase by functional proteomics using an activity-based esterase probe (47).

Ectopic neutral lipid storage by MT-targeted *puml* expression in *puml* flies has only a minor effect on the global body fat storage. Plotted are the means ± SEM of fold change of body fat compared with the genetically matched flies (Student’s t-test; ***P < 0.001; **P < 0.01). N: M: No osmotic stress sensitivity caused by ectopic lipid storage in MTs of *puml* mutant flies. Shown is survival of *puml* mutants versus control flies under dietary osmotic challenge (4% NaCl; continuous lines), starvation stress (2% agarose; dashed line), and on regular food (dash-dot lines) (flies per condition and genotype (n = 120); log rank-Ptest; P < 0.001 for starved flies). N: The MT LD population of *puml* flies is of smaller mean size and of more uniform size distribution compared with the MTs from control flies [Mann-Whitney test; number of LDs analyzed by genotype: n*puml*1] = 1,715, n*puml*2 = 306; ***P < 0.001].
The substrate profiling presented here identifies Puml as a phospholipase without significant hydrolase activity on neutral lipids. Therefore, the excessive storage lipid accumulation in puml1 flies is unlikely caused by an autonomous TG hydrolysis defect, but is rather an indirect consequence of lipid metabolism imbalance in flies lacking Puml function.

Our current understanding on how the absence of Puml causes neutral lipid accumulation is limited by two factors: on the one hand by the unknown or pleiotrophic effects in vivo of the lipids, which are substrates for Puml in vitro, and on the other hand by the multifaceted intracellular localization of the Puml protein.

Interestingly, similar to mammalian ABHD4 (26, 48), Puml hydrolyzes the endocannabinoid precursor NAPE at neutral to slightly acid pH (supplemental Fig. S7). However, its converted form N-acylethanolamine (NAE) 20:4 is not endogenously present in Drosophila. As the endocannabinoid receptor CB1 is not present in Drosophila either, it is surprising that endocannabinoids like NAE 16:0, 18:0, and 18:1 can be found in flies (62). Additionally, these endocannabinoids in Drosophila act as hedgehog ligands and directly interact with Smoothened (Smo) at physiological concentrations (62, 63). As hedgehog signaling modulates energy metabolism in flies (64), endocannabinoid

Fig. 5. Structural and enzymatic characterization of Pummelig. A: Basal TG lipase activity of recombinant MmATGL and Bmm/DmATGL, but not of MmABHD5 and Puml, shown by FA release from triolein substrate. Note that β-galactosidase (β-gal.) serves as negative control. B: Stimulated TG lipase activity of MmATGL by MmABHD5, but not of Bmm/DmATGL by Puml. No cross-species stimulation of TG lipase activity upon combining MmATGL plus Puml or Bmm/DmATGL plus MmABHD5. Note that two-component mixtures contain only half of the amount of MmATGL and Bmm/DmATGL compared with the activity measurements of MmATGL and Bmm/DmATGL alone. C: Schematic of the recombinant protein Pummelig, the predicted catalytic nucleophile motif mutant PummeligS190N, and the predicted acyltransferase motif mutant PummeligD371A. Black boxes represent the α/β-hydrolase fold domain, gray boxes the N- and C-terminal regions of Pummelig, and white boxes the N-terminal His-tag of the fusion proteins. D: Pummelig is a phospholipase with no activity on neutral lipids. Substrate screening with WT Pummelig and Pummelig catalytic motif mutants (D371A and S190N) expressed in insect cells. Data show mean fold increase of FA release from diverse lipid substrates in comparison to cells expressing β-galactosidase (lower cut-off = 2). Pummelig hydrolyzes PA, NAPE, and PG (for description of all substrates tested; see the supplemental data). Note the lack of hydrolase activity for both of the catalytic motif mutants on any of the tested substrates. E: pH dependence of Pummelig tested with PA reveals an optimum at neutral to slightly acid pH. F: Substrate saturation assay of Pummelig using PA and lyso-PA shows a clear preference toward DG-lipid PA. Saturation of Pummelig with PA was achieved at ~2 mM (K_m = 0.71 mM, V_max = 1.49 μmol/h/mg protein). Time-dependent (G) and dose-dependent (H) hydrolysis of PA by cell lysates overexpressing Pummelig. Note that values were normalized to lysates with overexpressed glutathione S-transferase (GST). A, B, E–H: Plotted are the means ± SEM; Mann-Whitney test; ***P < 0.001. Statistical tests were performed using following controls: β-gal in D; MmATGL or Bmm/DmATGL + β-gal in A and B.
metabolism in \textit{pum}^{-1} flies might be disturbed. Although, in mice, NAPE-PLD provides an alternative pathway to generate and compensate for NAE and anandamide synthesis in ABHD4 knockout mice (48), it is currently not known whether this pathway is evolutionarily conserved in flies. In mice, NAPE 16:0 is produced and secreted from the gut, especially after high-fat meals, lowering locomotor activity and food intake (65). Presuming an evolutionarily conserved endocannabinoid-based mechanism for sensing the nutritional value and composition of food, one would expect alterations in the behavior of \textit{pum}^{-1} flies. However, \textit{pum}^{-1} flies are normophagic and display no obvious locomotion phenotype. Additionally, overall hedgehog signaling (62, 63, 66) appears not to be impaired in \textit{pum}^{-1} flies, as no obvious developmental defects can be observed, and \textit{pum}^{-1} flies are fertile. The question of whether Puml affects NAPE and consecutively NAE metabolism in flies should be addressed in further studies.

The lipid mediator PA is a possible link between the \textit{pum}^{-1} phenotype and the enzymatic activity of the protein, as Puml hydrolyzes PA in vitro. High levels of PA cause increased generation of TGs in mammals (28, 67). Additionally, PA negatively regulates the cellular energy sensor, AMPK, which subsequently causes reduced FA import into mitochondria and decreased mitochondrial biogenesis (68, 69). At the same time, PA stabilizes the TORC1-complex (67). The possible AMPK and TORC1 modulation mediated by changes in PA levels in \textit{pum}^{-1} flies could explain the observed higher lipogenesis and lower carbohydrate storage. Notably, ABHD5/CGI-58 knockdown mice suffering from hepatic steatosis have increased levels of some PA species and accumulate (~10-fold) PG (28), another in vitro substrate of Puml.

An intrinsic lysophosphatidylglycerol acyltransferase (LPGAT) activity had been reported for murine ABHD5/CGI-58 (70). However, this is currently under debate, as PGs are actually increased in hepatocytes of ABHD5 knockdown mice (28), and a more recent publication could not confirm this activity for mouse and plant ABHD5 (61).

PA and PG are precursors for cardiolipin (CL), a highly important lipid nearly exclusively found on mitochondria. CL has multiple roles on these organelles, as it stabilizes proteins needed for oxidative phosphorylation, is a proton trap within mitochondrial membranes, is involved in mitochondria-induced apoptosis, and shapes mitochondrial membrane dynamics [for review see (71)]. As Puml localizes on mitochondria as well, it might be involved in modulating the generation of CL by limiting the locally available PA and PG pool for mitochondria, changing their properties. This also might provide a link in the observation that a knockdown of ABHD5/CGI-58 in SW620 and HCT116 cells suppresses the AMPKα-p53 pathway and leads to a metabolic shift toward aerobic glycolysis (Warburg effect) and to lipid accumulation (72). Additionally, CL serves as a substrate for Mito-PLD locally generating PA, critical for mitochondrial fusion. As Puml hydrolyzes PA, LPA, and PG, and is localized on mitochondria, this might provide an explanation for the overall enlarged mitochondria found in \textit{pum}^{-1} flies. Missing \textit{pum}^{-1} gene function may alter local PA and PG on mitochondria, subsequently leading to changed CL levels, and missing PA hydrolysis activity of Puml may cause increased PA levels in mitochondrial membranes leading to increased fusion.

Furthermore, future attention should focus on a possible interaction of Puml with lipin and AGPAT3. All three enzymes are LD residents (73), with the latter two directly involved in the lipogenesis pathway. While AGPAT3 is required for PA production from LPA, lipin is consecutively needed for DG synthesis from PA (PA-phosphatase; PAP activity). Lipin is of especially high interest here, as it regulates lipid metabolism directly by generating DG via its PAP activity and also by acting as a transcription factor in the
nucleus (74). Interestingly, downstream targets of lipin (as a transcription factor) are lipolytic (like PPARα) and oxidative phosphorylation genes, whereas lipogenic genes (like FASN) are suppressed (75). Additionally, lipin interacts and is activated by TORC1, preventing it from translocation into the nucleus (74). As the TORC1 complex is stabilized by PA (76), this might provide a positive feedback loop under nutrition, stimulating lipogenesis. Therefore, Puml might limit the locally available amount of PA for lipin and thereby indirectly modulate the activity of lipin. Hence, absence of puml would enhance the lipogenic feedback loop leading to increased amounts of TGs, an effect observed in puml flies. This would also explain why lipolysis is not affected in puml flies; as without PA, lipin would be able enhance lipolysis as a transcriptional cofactor. Therefore, a possible interaction of Puml with lipin should be addressed in future studies.

Besides possible negative effects on mitochondrial function, the peroxisomal localization of Puml::mCherry, a feature shared with the ABHD4/5 homolog from A. thaliana (77), might be of functional relevance for TG turnover. In support of this, the TG FA composition of puml flies is shifted toward longer and more unsaturated FAs, reminiscent of flies defective in peroxisomal biogenesis (32, 78). Also, both puml and peroxisomal mutant flies share decreased lifespan and impaired startle-induced climbing activity (32, 78). Finally, flies mutant for the core peroxisomal genes, pex2 and pex16, store less glycogen (51), one additional phenotype shared by puml flies. Collectively, these data support a peroxisomal function of Puml, regardless of the protein having no prototypic peroxisomal targeting signal (79), which predicts the localization of Puml to these organelles to be mediated by a currently unknown binding partner.

Key to the understanding of the Puml intracellular localization complexity might be the fact that puml encodes different predicted protein isoforms, which differ in their N termini (80). For example, the longest Puml isoform (Puml-PA) has an extended N terminus that contains multiple phenylalanine (F) and tryptophan (W) residues. This is reminiscent of the tryptophan-rich N-terminal peptide of ABHD5/CGI-58, which is essential for the LD anchoring of the protein (81, 82).

Taken together, our data suggest that Puml serves a variety of functions in lipid metabolism of flies due to its enzymatic substrate spectrum and due to the association of the protein with various organelles like LDs, mitochondria, and peroxisomes. Collective lack of these functions results in an excess of TG storage in the fat body and ectopic lipid storage in the MTs. These profound changes in Drosophila lipid metabolism correlate with severe physiological phenotypes in puml flies, such as shorter lifespan and reduced physical performance. Future research efforts addressing the underlying molecular and physiological mechanisms are necessary. TG storage in the insect kidney indicates ancestral metabolic functions of this gene family with relevance for a more comprehensive understanding of mammalian ABHD4 and ABHD5/CGI-58 proteins.

The authors thank Drs. J. E. Faust, J. Dow, and J. A. McNew for providing fly stocks; Drs. A. Lass, A. Hildebrandt, and I. Berger for providing plasmids; and R. Schuh for support during the writing phase of the manuscript.

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