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Characterization of the *Drosophila* lipid droplet subproteome

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Abbreviations

ADRP	Adipose differentiation-related protein
EGFP	Enhanced green fluorescent protein
ER	Endoplasmatic reticulum
GO	Gene Ontology
LSD-1	Lipid storage droplet-1
LSD-2	Lipid storage droplet-2
Nano LC-MS/MS	Nano liquid chromatography tandem mass spectrometry
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TIP47	47kDa tail interacting protein

Summary

Lipid storage droplets are universal organelles essential for the cellular and organismal lipometabolism including energy homeostasis. Despite their apparently simple design they are proposed to participate in a growing number of cellular processes, raising the question to what extent the functional multifariousness is reflected by a complex organellar proteome composition. Here we present 248 proteins identified in a subproteome analysis using lipid storage droplets of *Drosophila melanogaster* fat body tissue. In addition to previously known lipid droplet-associated PAT-domain proteins and homologues of several mammalian lipid droplet proteins, this study identified a number of proteins of diverse biological function, including intracellular trafficking supportive of the dynamic and multi-faceted character of these organelles. We performed intracellular localization studies on selected newly identified subproteome members both in tissue culture cells and in fat body cells directly. The results suggest that the lipid droplets of fat body cells are of combinatorial protein composition. We propose that subsets of lipid droplets within single cells are characterized by a protein "zip code", which reflects functional differences or specific metabolic states.

Introduction

Lipid droplets are ubiquitous intracellular energy storage organelles of organisms as diverse as bacteria and humans (for review see [1]). They are composed of a protein-coated phospholipid monolayer membrane, which encloses a hydrophobic core of neutral storage fats, the triglycerides, or cholesterol esters. The simple design of lipid droplets reflects their predominant role as passive storage depots for fats in energy homeostasis and for membrane and lipid hormone precursors in lipometabolism. However, much in contrast to their plain composition, lipid droplets have a complex life cycle (for review see [2]) and are subject of elaborate differentiation processes as described for mammalian cells that undergo adipocyte maturation [3]. Conversely, stimulated lipolysis induces lipid droplet fragmentation in differentiated adipocytes [4,5], underscoring the dynamic cell biology of these organelles. Moreover, lipid droplets have recently been suggested to represent target compartments for fatty acid scavenging to protect cells from lipoapoptosis [6].

Given the simple overall structure of lipid droplets, it is tempting to speculate that differential occupation of a heterogeneous population of lipid droplets by associated proteins enables the versatility of lipid droplet biology. Along these lines, several lipid droplet proteomic screens were performed in yeast, mammalian tissue culture cells and the mouse mammary gland [5,7-9]. Based on the characteristics of the identified lipid droplet proteins, these organelles have been reported to be interconnected with various cellular compartments including for example the cytoskeleton and the endoplasmic reticulum (ER) [10,11]. In addition, lipid droplets were proposed to be active players in various cellular processes such as vesicular transport and lipid trafficking [9]. However, the full complexity of the lipid droplet proteome awaits identification and subsequent functional characterization of the lipid droplet-associated proteins.

Among the few well-studied members of the lipid droplet proteome are mammalian members of the PAT-domain protein family (composed of Perilipin, ADRP and TIP47), which are crucial for controlling lipid droplet function in organismal energy homeostasis in various ways [4,12,13]. ADRP, for example, is involved in intercellular neutral lipid transport [14], whereas Perilipin modulates the rate of adipocyte lipolysis by acting both as a barrier and attachment site for lipases in a phosphorylation-dependent manner. This way, Perilipin facilitates basal and stimulated lipolysis [15,16]. Recently, this regulatory function of lipid droplet-associated PAT-domain proteins for the organismal energy storage was shown to be evolutionary conserved, since the Perilipin homologue LSD-2 of the fruit fly *Drosophila melanogaster* acts as a Perilipin-like regulator of organismal energy storage [17,18]. Additionally, *Drosophila* LSD-2 has been demonstrated to control directed lipid droplet transport in cooperation with the Klarsicht protein [10]. These findings suggest that lipid droplet-associated proteins empower lipid droplets involvement in various cellular processes in both vertebrates and invertebrates.

In order to gain a more comprehensive view on constitutive lipid droplet resident proteins of *Drosophila* third instar fat body cells and to compare the lipid droplet subproteomes of individuals that are genetically predisposed to obesity or leanness, we employed nano liquid chromatography tandem mass spectrometry (nano LC-MS/MS) to highly enriched lipid droplets. Our findings reveal an unexpected high complexity of the constitutive lipid droplet proteome, implicating this organelle in a variety of biological processes, and we observed only few differences in the global lipid droplet proteomes from individuals predisposed to fat storage abnormalities. Identifications were supplemented by intracellular localization studies of representative lipid droplet proteins both in tissue culture cells and in transgenic animals. The localization pattern of proteins on subsets of lipid droplets suggests a

functional diversification within the lipid droplet population of a cell. These subsets cannot be resolved by global subcellular proteomics but rather require the functional characterization of individual proteins by employing the powerful genetics and cell biological tools that are established for *Drosophila melanogaster*.

Experimental Procedures

Fly strains and fly culture

All flies were propagated on a complex cornflour-soyflour-molasse medium (cornflour and barley malt 80g/l each, molasses 22g/l, yeast 18g/l, soyflour 10g/l, agar-agar 8g/l, propionic acid 6,3ml/l and nipagin 1,5g/l), supplemented with dry yeast at 25°C and 20-30% humidity with a 12h/12h light/dark cycle. The following fly stocks were used:

Designation	Genotype	Internal stock #	Source/Reference
wild type	*OregonR*	RKF142	Bloomington stock center, USA
Lsd-2^{51}	*y* Lsd-2*^{51} */ FM7i;* *P{w*$^{+mC}$=ActGFP*}JMR3* or *Dp(1;Y) y*$^{+}$	RKF610	based on [18]
FB-Gal4	*y* w*; P{w*$^{+mW.hs}$*=GawB}FB/SNS*	RKF125	[18]
*Lsd-2:*EGFP	*w* ; + ; P{w*$^{+mC}$ *UAS-LSD-2:*EGFP*}*	RKF437	[18]
adp^{60}	*adp*^{60}	RKF141	[19].
*CG1112:*EGFP	*w* ; P{w*$^{+mC}$ *UAS-CG1112:*EGFP*}#40 / TM3 Sb* e* float*	RKF736	this study
*CG2254:*EGFP	*w* ; P{w*$^{+mC}$ *UAS-CG2254:*EGFP*}#3 / TM3 Sb* e*	RKF738	this study
*CG10691:*EGFP	*w* ; P{w*$^{+mC}$ *UAS-CG10691:*EGFP*}#6 / CyO float*	RKF732	this study

The term “induced *Lsd-2:*EGFP” is used in the text to designate the progeny of the cross between *FB-Gal4* and *Lsd-2:*EGFP flies expressing LSD-2:EGFP fusion protein in the fat body.

Expression of fluorescently labeled lipid droplet proteins

Candidate lipid droplet-associated proteins were PCR amplified from the respective EST-clones *Lsd-1*: GH10767; *Lsd-2*: RE58939; *CG1112*: GH13950; *CG2254*: RH47744; *CG10691*: GH12454 [20]. The following primer sequences were used:

Lsd-2NotI forward	GCTTGCGGCCGCCACCATGGCCAGTGCAGAGCAGAAAC
Lsd-2AscI no stop	GCAAGGCGCGCCCCTGAGACGACACCGCCGGCG
Lsd-1NotI forward	GCTTGCGGCCGCCACCATGGCAACTGCAACCAGCGGC
Lsd-1AscI no stop	GCAAGGCGCGCCCGTAGACGCCGTTGATGTTATTG
CG10691NotI forward	GCTTGCGGCCGCCACCATGGCTGCTCAGTTCTTTAATCGC
CG10691AscI reverse	GCAAGGCGCGCCCCTACTGCGCGATGGTCGATGGC
CG2254NotI forward	GCTTGCGGCCGCCACCATGTCGAAAGTGACGCAAAGTG
CG2254AscI reverse	GCAAGGCGCGCCCGGACTTATCGGTATCCACACC
CG1112NotI forward	GCTTGCGGCCGCCACCATGAATAAGAACCTCGGCTTTG
CG1112AscI reverse	GCAAGGCGCGCCCTTAAAACAATAAATCTTTGTTGTCG

Primer sequences were flanked with 5' *Not*I and 3'*Asc*I restriction enzyme sites for subsequent cloning into the pENTR/TOPO D vector for Gateway recombination sub-cloning (Invitrogen, Karlsruhe, Germany). For tissue culture expression the entry vectors were recombined with a modified pBluescript vector containing the necessary *att* sites and sequences encoding either a C-terminal EGFP- or red fluorescent-protein under the control of an ubiquitin promoter. For germline transformed transgenic *Drosophila* the same entry clones were recombined with the pTWG expression vector obtained from the *Drosophila* Genomics Resource Centre (http://dgrc.cgb.indiana.edu). Transgenic fly stocks were generated by standard germ line transformation.

Lipid droplet fractionation

For each sucrose gradient 60 to 75 fat bodies from wandering late third instar larvae were hand-dissected in phosphate buffered saline (PBS) on ice. The dissected fat bodies were transferred into 100µl fat body buffer (FBB: 10mM HEPES pH 7.6,

10mM KCl, 0.1mM EDTA, 0.1mM EGTA and 1mM DTT) including protease inhibitors (EDTA-free complete protease inhibitors, Roche Diagnostics, Mannheim, Germany). Fat bodies were frozen and kept at –80°C until use. Lysis of the fat bodies was performed by mild bath sonication (Bandelin Sonorex RK100; 3-6 pulses of 10 sec. each in a volume of 30µl FBB / fat body) until dispersion of fat bodies. Lipid droplets were basically purified as described by Yu et al. [21]. In brief, after sonication the cellular debris was pelleted by centrifugation at 3000xg for 8min. The resulting postnuclear supernatant (PNS) was adjusted to a volume of 3ml with FBB, mixed with an equal volume of FBB including 1.08M sucrose, and afterwards transferred into a 12ml polyallomer ultracentrifugation tube (Beckman Instruments, Palo Alto, USA). It was then sequentially overlayed with 2ml of 0.27M and 0.135M sucrose each in FBB and top solution (FBB only). The gradient was centrifuged for 1h 30min at 4°C at 30000rpm (> 100 000xg). After the run eight 1.5ml fractions were collected by pipetting from top to bottom: the buoyant lipid droplets (fractions F1 and F2), the mid-zone (F3 and F4) and the cytosol (F5-F8). The protein content of 50µl of each fraction was subsequently measured using the Pierce BCA assay kit (Perbio Science, Bonn, Germany) according to the manufacturers` instructions.

The desired protein amount of the respective fraction was subsequently precipitated using the method of Wessel and Flügge [22] and protein pellets either frozen at –20°C or solubilized in the respective buffer.

Electron microscopy

Electron microscopy of purified lipid droplets using Epon embedding was carried out as described in [23,24]. In brief, the topmost 500µl of density gradient centrifugation fraction F1 (see above) was used for embedding. The fraction was fixed by 2% glutaraldehyde for 60min at room temperature and immobilized with 2% agarose in

cacodylate buffer at pH 7.4. The agarose was cubed and further fixed with 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.4) at room temperature. After a pre-embedding staining with 1% uranyl acetate, samples were dehydrated with an ethanol series and embedded in Agar 100 (equivalent to Epon). Thin sections (60nm) were again counterstained with uranyl acetate and lead citrate and examined using a Philips CM 120 BioTwin transmission electron microscope (Philips Inc. Eindhoven, The Netherlands). Images were taken with a 1K slow scan CCD camera (GATAN, Inc., Munich, Germany). Tissues were embedded as described above without immobilization in agar prior to the fixation.

Western blot analysis

Equal amounts of protein from the sucrose gradient were precipitated using the method of Wessel and Flügge [22]. Protein separation was carried out using standard SDS-PAGE prior to transfer of the proteins onto PVDF membrane (ImmobilonP, Millipore, Schwalbach, Germany). The membrane was washed with PBS including 0.1% Tween-20 (PBT) and blocking was carried out over night with 5% BSA in PBT at 4°C. Primary antibodies detecting LSD-2 (dilution 1:3000; [18]) and eIF-4A (dilution 1:5000; [25]) were used in PBT including 2.5% BSA. Secondary antibodies conjugated to POD (Perbio Science, Bonn, Germany) were used in a dilution of 1:8000 under otherwise identical conditions. Results were visualized using the Super signal West Pico ECL system (Perbio Science, Bonn, Germany) and Kodak BioMax XAR-films (Kodak, Stuttgart, Germany). For re-probing, bound antibodies were removed from the membrane by Restore Western blot stripping solution (Amersham biosciences, Freiburg, Germany) according to the manufacturers` instructions.

LC-MS/MS of precipitated lipid droplet proteins

For mass spectrometry analyses the precipitated proteins were resolved in SDS-sample buffer, separated by Mini-SDS-PAGE and proteins were stained with Coomassie. Each gel lane was cut reproducibly into three sections (>80kDa, 50-80kDa and <50kDa) in order to decrease the complexity of the individual samples.

Individual gel sections were sliced in small cubes and washed with Milli-Q water for 5min followed by two-step dehydration in 50% and 100% acetonitrile (ACN), respectively. Subsequently, the gel pieces were rehydrated in 100mM NH_4HCO_3 (3x vol of rehydrated gel) and the dehydration procedure was repeated. The gel pieces were then completely dried in a SpeedVac concentrator and rehydrated with digestion buffer (20µg/ml sequencing grade modified porcine trypsin (Promega, Madison, WI, USA), 50mM NH_4HCO_3, 10% ACN) followed by overnight digestion at 37°C. Peptides were extracted from the gel pieces as previously described in Wehmhöner et al. [26]. Extracted peptides were purified using ZipTip C18-microcolumns (Millipore, Billerica, MA, USA), following the manufacturers` instructions. Digests were lyophilised in a SpeedVac concentrator and resolubilized in 0.1% TFA. The RP-HPLC separation of the peptide samples was performed using a bioinert Ultimate nano-HPLC system (Dionex, Sunnyvale, CA, USA). 10µl of each sample (up to 500ng) was injected and peptides were purified and concentrated on a C18-PepMap precolumn (0.3mm i.d. x 5mm, 100Å pore size, 3µm particle size, Dionex) at a flowrate of 30µl per min 0.1% TFA. Subsequently peptides were separated on an analytical 75µm i.d. x 150mm C18-PepMap column (Dionex, 100Å pore size, 3µm particle size) using a 120 minute gradient at a column flowrate of 250nl per min. The acetonitrile gradient (Solution A: 0.1% formic acid, 5% ACN; Solution B: 0.1% formic acid, 80% ACN) started at 5% and ended at 60% B.

MS and MS/MS data were acquired using a tandem mass spectrometer (Q-TOF II ™ Waters, Milford, MA, USA). Doubly and triply charged peptide-ions were

automatically chosen data-dependent by the MassLynx software (Waters) and fragmented for a maximum of 18 seconds for each component. MS-data were automatically processed and peak lists for database searches were generated by the MassLynx software (MassLynx 4.0, Mass Measure All, 2 x Savitzky Golay Smooth Window 5, minimum peak width at half height 5). Database searches were carried out with an in-house MASCOT server (Version 2.1; Matrix Science) using a *Drosophila* protein database (FlyBase version 4.2; 19178 sequences, 10826103 residues; http://flybase.bio.indiana.edu). The assessment of predicted protein identifications was based on the MASCOT default significance criteria (score at least 28 calculated for $p<0.05$). To avoid false positive identifications that can occur by the cumulation of low-scoring peptides we exclusively accept rank-one peptide database matches that itself achieve the default significance criteria. Furthermore, protein identifications with total scores less then 56 were either verified manually or rejected. Iterative calibration algorithms were applied on the basis of significantly identified peptides to achieve an average absolute mass accuracy of better than 50ppm.

Three independent preparations were carried out designated as I, II and III in Table 1 and Supplemental Tables. The Supplemental Tables provide detailed information of all individual LC-MS/MS experiments and the derived protein identifications. Results obtained from the different gel-sections are indicated as “upper” (>80kDa), “middle” (50-80kDa) and “lower” (<50kDa). The first entry of each section hyperlinks the supplementary data of each investigated gel-section with the corresponding copy of the original MASCOT result report comprising also links to labeled MS/MS spectra from all peptides.

Protein data retrieval

Protein specific information was obtained from FlyBase (version 4.2, http://flybase.bio.indiana.edu) using the batch query interface resulting in a tab-separated flat file. Swissprot identifiers were subsequently used to batch retrieve corresponding FASTA sequences from the Swissprot database (version November 2005) [27] applying a BioPerl (version 1.4) script [28]. Sequence information was further processed to determine the amino acid length of the specific proteins by a custom Perl script as well as to retrieve pI-value information using a BioPerl (version 1.4) script and the EMBOSS pK set [29]. All information was manually curated to avoid redundancies before adding to the results tables.

Tissue culture transfection

Tissue culture experiments for the investigation of the localization of selected lipid droplet candidate proteins were performed using Schneider S2 cells [30] in a 25°C incubator using standard procedures.

Cells almost reaching confluence were diluted to a final cell density of 2–5 x 10^6 cells per ml. 1.5ml of this cell suspension was distributed in each well of six-well plates (Nunc, Wiesbaden, Germany) and incubated for 18–24 hours in order to recover. Transfections were performed using the lipofectamine derivate Effectene (Qiagen, Hilden, Germany) according to the manufacturers` instructions. After transfection, the cells were incubated for 48 hours prior to feeding with 400μM oleic acid (Sigma-Aldrich, Seelze, Germany), to promote lipid droplet generation [31]. After incubation for additional 12-18 hours the cells were stained and imaged.

Confocal Microscopy

Cells or tissue was fixed using buffer B (5% PFA pH 6.8, 16.7mM KH_2 PO_4/ K_2HPO_4, 75mM KCl, 25mM NaCl, 3.3mM $MgCl_2$) for 5 to 10 minutes. After washing with PBT, the specimens were stained for lipid droplets by adding 1µl/ml PBT of the diluted Nile Red stock solution (Molecular Probes, Leiden; The Netherlands; 1mg/ml stock solution 1:500 diluted in PBT). Staining was performed for three minutes before the specimen were mounted using Prolong Antifade solution (Molecular Probes, Leiden, The Netherlands). Imaging was done using a Leica TCS SP2 confocal microscope (Leica Microsystems, Bensheim, Germany). Images were processed using the Macintosh version of Adobe Photoshop 5.0.

Results

Lipid droplet subproteome of *Drosophila* third instar larval fat body cells

In *Drosophila* lipid droplets are most prominent in organismal energy storage tissue such as the fat body cells of larvae (Fig. 1A) in particular at wandering third instar stage when the individuals are ready to undergo metamorphosis. These fat body cells are densely packed with lipid droplets of variable sizes ranging from 0.5-10µm in diameter (Fig. 1B). In order to initiate a systematic analysis of the proteome of these lipid droplets we applied the experimental strategy outlined in Figure 1C. Briefly, fat bodies from third instar larvae were manually dissected, lipid droplets released from the tissue and subsequently purified by sucrose-gradient density centrifugation. Lipid droplet-associated proteins were shed from the core lipids by delipidation and precipitation, size-fractionated by SDS-PAGE and identified by nano LC-MS/MS (for details see Experimental Procedures). {Suggested location of Fig.1}

Purity of lipid droplet preparations is critical for the assignment of the identified proteins to the lipid droplet proteome. Therefore, we controlled the purity by the following examinations (Fig. 1D-F). First we assayed for the relative protein abundance of sucrose-gradient density fractions. The profile showed a small protein peak in the most buoyant lipid droplet fractions (F1+F2) that was separated from the bulk of cytoplasmic proteins (F5-F8) by a midzone (F3+F4) low in protein (Fig. 1D). This distribution is in accordance with established protein profiles [18,21]. Secondly, we confirmed the correct representation of cellular compartments after density fractionation by examination of equivalent protein amounts of the fractions for the presence of cytoplasmic eIF-4A [25] and lipid droplet-enriched LSD-2 protein [18], respectively (Fig. 1E). Thirdly, the most buoyant density fraction was examined by transmission electron microscopy to show that lipid droplets are of the same size as

observed for larval storage lipid droplets *in situ* (compare Fig. 1F to Fig. 1B). As a possible source of impurities we infrequently observed membrane fragments of unknown origin, suggesting that preparations might still be contaminated with low amounts of proteins of a different source than lipid droplets (data not shown).

Fractionation by 1D SDS-PAGE predicts a high complexity of the lipid droplet proteome represented by more than 80 separable protein bands (Fig. 1G). Nano LC-MS/MS measurements on triplicate samples from fat bodies of larvae of four different genotypes identified a total of 248 proteins (Supplemental Tables; for details of the analysis see Experimental Procedures). In addition to lipid droplet preparations from wild type larvae, samples from mutant larvae with genetic predisposition for obesity (*adp*^{60}, [19]; induced *Lsd-2:*EGFP; [18]) or leanness (*Lsd-2*^{51}; [18]) were examined. Individual measurements with independent samples showed that about 68% of the identified proteins were reproducibly found and that the remaining 32% of identified proteins varies most likely due to differences in their abundance and dependence on the detection sensitivity. Based on the latter, we also consider single protein detections as potentially meaningful.

Of the 248 proteins identified, 127 proteins were found in wild type larvae, 137 in *adp*^{60} mutant larvae, 153 in induced *Lsd-2:*EGFP mutant larvae and 159 in *Lsd-2*^{51} mutant larvae. Of those proteins two subclasses (class A and class B) could be formed. Class A contains 168 proteins that were reproducibly identified in separate lipid droplet preparations (see Table 1; for the complete list of proteins see Supplemental Tables). This class includes 113 proteins obtained from wild type larvae, 116 proteins of *adp*^{60} mutants, 135 proteins of *Lsd-2*^{51} mutants and 132 proteins of induced *Lsd-2:*EGFP mutant larvae. Class B consists of the 80 proteins that were identified only once (see Supplemental Tables). {Suggested location of Table 1}

Figure 2A depicts the distribution of the identified proteins for each genotype analysed. A total of 60 proteins were common to the lipid droplet proteome of the larvae, irrespective of their genotype. We refer to them as members of the constitutive lipid droplet proteome. Most other proteins were present in the subproteomes obtained from larvae of at least two different genotypes and only few proteins were reproducibly identified in the proteome of larvae with a distinct genotype (see Supplemental Tables). They include the Regucalcin protein (Table 1), which was detected in the lipid droplet proteome of *adp*^{60} mutant larvae only. Interestingly, a vertebrate homologue of Regucalcin, called Senescence marker protein-30 (SMP30), was recently shown to affect cellular lipid droplets, organismal lipid storage, body weight and lifespan in mice [32]. Taken together, the identified proteins imply a high complexity of the lipid droplet proteome. In addition, the findings suggest that genotype-specific differences, which lead to obesity or leanness, are not reflected in qualitative differences in the respective lipid droplet proteomes.

Functional diversity of *Drosophila* lipid droplet proteins

To gain insight into the cellular processes that are associated with the lipid droplet surface, the identified proteins of both class A and class B were classified on the basis of their assigned gene ontology (GO) terms (category "Biological process"; [33]). The vast majority of the proteins (154 class A and 66 class B proteins) can be assigned to established biological processes, leaving only about 9% of class A and 19% of class B proteins with still unpredicted functions (for the GO-classification of all identified proteins see Supplemental Tables).

The majority of lipid droplet-associated proteins are involved in cellular metabolism (GO:0044237; class A: 67%, class B: 51%; Fig. 2B and Supplemental Tables). In addition, cellular functions such as transport processes (GO:0006810, class A: 8%,

class B: 16%), cell organization and cell biogenesis (GO:0016043 class A: 4% class B: 1%) are well represented, reflecting the active character of these organelles in addition to lipid metabolism (GO:0006629; class A: 3%, class B: 1%). This observations suggest that the surface of lipid droplets participates in various and diverse metabolic as well as cellular processes. {Suggested location of Fig. 2}

The view of lipid droplets as compartments with functional specialization on particular although diverse biological processes is supported by GOstat algorithm analysis. This type of analysis allows for the identification of over- and under-represented GO-terms in a test set of proteins as compared to the whole *Drosophila* proteome [34]. Significantly enriched and depleted GO-terms could be identified ($p<0.05$; Fig. 2C). In addition to the previously mentioned general metabolism-associated GO-term 0044237 (=cellular metabolism) class A contains several overrepresented lipid metabolism-associated GO-terms (0044255 = cellular lipid metabolism, 0006631 = fatty acid metabolism, 0006084 = acetyl-CoA metabolism, 0006629 = lipid metabolism, 0008610 = lipid biosynthesis and 0006633 = fatty acid biosynthesis). Few GO-terms are significantly underrepresented in class A, including regulatory proteins that are likely to be present only in small amounts and might be localized specifically at the site of action (e.g. GO:0051244 = regulation of cellular physiological processes; Fig. 2C).

In accordance with the role of lipid droplets as fat storage compartment, various enzymes involved in fatty acid/lipid metabolism were found. They include an acetyl-CoA carboxylase (CG11198), ATP citrate lyase (CG8322), esterases/lipases and enzymes modifying short as well as long chain fatty acids (for details see Table 1 and Supplemental Tables). Other enzymes of annotated function, such as several short chain dehydrogenases, cannot be assigned to a particular biological process. However, identification of multiple proteins with predicted lipid-binding function such

as CG9342, CG5958 or the sterol carrier protein 2 (SCP2) emphasize a possible role of lipid droplets in intra- or intercellular lipid trafficking (Table 1).

The dynamic character of the lipid droplet compartment is further supported by the identification of several members of the Rab protein family (Rab 2, 5 and 6; Supplemental Tables).

Moreover, the identification of proteins implicated in the trafficking and protein insertion into the ER membrane suggests that, like in plants, a portion of the lipid droplet-associated proteins are localized to the outer leaflet of the ER membrane co-translationally [35]. This hemimembrane is proposed to provide the monolayer surface of the lipid droplets [1,36]. Such proteins could support the persisting close vicinity of some lipid droplets to the endoplasmic reticulum (data not shown). Furthermore, several proteins with predicted chaperone function, including protein disulfide isomerase (PDI), calreticulin and members of the heat shock protein family, were identified. Functional relevance of chaperone proteins on lipid droplets remains elusive. However it is interesting to note that homologues of several of these proteins were also identified in recently characterized mammalian lipid droplet proteomes [5,8]. A total of 30 of the identified lipid droplet proteins (12%) did not allow classification based on a GO-annotation in the category “Biological process” and the molecular function of 17 among them (7% of all proteins) is completely unknown. These proteins will be subject of future studies.

Functional analysis of selected lipid droplet proteins *in vivo*

In order to confirm the lipid droplet association of some of the identified proteins, we tested the localization of fusion proteins containing an enhanced green fluorescent protein (EGFP) tag that were expressed either in transfected *Drosophila* Schneider S2 cells [30] or in the fat body of transgenic flies.

S2 cells exposed to oleic acid in the culture medium accumulate intracellular lipid storage droplets. Control experiments indicated that they are able to bind fluorescently tagged variants of the PAT-domain proteins LSD-1 and LSD-2 (Fig. 3A) as previously shown in transgenic flies [18,37]. We therefore used this experimental system to examine the subcellular localization of three proteins, the alpha esterase CG1112, the stomatin-like protein CG10691 and the short chain dehydrogenase CG2254. CG1112 was classified as constitutively lipid droplet-associated protein, CG10691 was identified in three out of four genotypes analysed, whereas CG2254 was found in *adp*^{60} mutant and in induced *Lsd-2:*EGFP mutant larvae only (Table 1; for gene/protein predictions see [38]). Fig. 3B and C show that in tissue culture cells, CG1112 and CG10691 reside in the cytoplasm but also associate with a subset of lipid droplets. In contrast, CG2254 is solely associated with lipid droplets (Fig. 3D) and, as observed with CG1112 and CG10691, it also labels only a subset of the droplets (Fig. 3E). This observation suggests that the composition of the protein coat of lipid droplets within a given cell involves different proteins. {Suggested location of Fig. 3}

We also generated transgenic *Drosophilae* in which the EGFP fusion proteins were expressed specifically in the fat body cells of larvae and immature adults (for details see Experimental Procedures). Fig. 4A-C indicate a similar fusion protein distribution in fat body *in vivo* as demonstrated in tissue culture cells. CG1112 localizes in larval fat body cells along membranes, in cytoplasmic patches and appears to be enriched around some but not all lipid droplets (Fig. $4A_{1-3}$). The latter observation is more pronounced in the fat body cells of immature adults (Fig. $4A_4$). In contrast, CG10691 decorates larval lipid droplets and additionally accumulates in patches close to their surface (Fig. $4B_{1-3}$). This pattern is also clearly visible on lipid droplets from isolated fat body cells of immature adults (Fig. $4B_4$). Finally, as in tissue culture, CG2254

specifically accumulates at the rim of a subset of cellular larval lipid droplets *in vivo* (Fig. $4C_{1\text{-}3}$), a pattern that is also observed with isolated lipid droplets (Fig. $4C_4$). Taken together, these results indicate that the three proteins examined are associated with lipid droplets. They mark distinct subsets of droplets either by exclusive localization (CG2254) or by enrichment of proteins around them (CG1112, CG10691). {Suggested location of Fig. 4}

Taken together, our proteomics approach identified the composition of the lipid droplet subproteome of the larval fat body of *Drosophila*, showing that different subsets of lipid droplets can be distinguished on the basis of specific or highly enriched protein species. Furthermore, and most importantly, it appears that the protein coat of the droplets represents a versatile biochemical machinery attached to these organelles. The results pave a way for a subsequent *in vivo* functional analysis of the hitherto uncharacterized lipid droplet proteins using genetic, molecular and biochemical tools with the fly.

Discussion

The study presented here was set up to systematically identify the lipid droplet-associated proteome of *Drosophila* third instar larval fat body cells by applying the nano LC-MS/MS technique in combination with a gel-based pre-fractionation of proteins obtained from highly enriched lipid droplets. We found a total of 248 droplet-associated proteins from wild type fat body cells and from fat body cells of three different mutants, which develop either increased or decreased fat storage phenotypes. The isolated lipid droplets, therefore, were specific for the organismal fat storage tissue and, in addition, define a distinct stage during the life cycle of *Drosophila*.

The control experiments showed no apparent contaminations (Fig. 1D-F) except for traces of unidentified membrane structures that could have derived from disrupted mitochondria or endoplasmic reticulum during the purification procedure. The SDS-PAGE showed a high degree of complexity of the larval lipid droplet-associated proteome (Fig. 1G) as has been observed for the embryonic lipid droplet subproteome [10]. Comparative proteomics was used in order to reveal qualitative differences between the droplet proteome compositions of wild type and mutant larval fat body cells, which give rise to lean and obese individuals, respectively. Increasing the stringency criteria to a point where only repeatedly identified proteins were considered (class A), only few genotype-specific proteins could be identified. This finding suggests that the mutant phenotype, i.e. storing high or low amounts of fat, cannot be defined through distinct droplet-associated marker proteins but rather by their relative abundance or through posttranslational modifications of such proteins not addressed in our present study. We note, however, that the SMP30 homologue Regucalcin, identified only in the adp^{60} mutant larvae, could possibly serve as marker protein for *adipose* mutant fat body cell lipid droplets. This assumption seems

reasonable in view of the fact that both SMP30 knock-out mutant mice and *adp*^{60} mutant flies accumulate fat and become obese [19,32]. The lack of more pronounced differences in the pattern of proteins from wild type and mutant lipid droplets could also be attributed to the fact that during the larval stage examined, the mutants under study are only predisposed for fat accumulation abnormalities which become fully apparent during adulthood. Genotype-specific differences in the lipid droplet subproteome composition might therefore be more pronounced during the adult stage.

Previous lipid droplet proteomic screens with other cellular systems such as yeast [7], mammalian mammary epithelial tissue [11] and several mammalian tissue culture cells [5,8,39] revealed in total some hundred proteins with surprisingly little overlap concerning the protein compositions revealed by the different screens. Comparing the outcome of the analysis presented here reveals nevertheless a total of 38 proteins of which homologues were also identified in these earlier screens. They include the PAT-domain protein family members LSD-1 and LSD-2, various lipid-metabolizing proteins such as acetyl-CoA carboxylase, several esterases and vesicle trafficking-associated proteins of the Rab family and chaperones (see Table 1 and Supplemental Tables).

A set of common proteins among the lipid droplet proteomes of cells as different as yeast, *Drosophila* fat body and several types of mammalian cells, suggest that lipid droplets contain a constitutive subproteome which can be complemented by a varying number of species-, stage- and/or cell-specific proteins. Furthermore, even within a given cell, different lipid droplet populations might reflect different metabolic states and/or subpopulations of droplets that even may serve other functions than fat storage. The latter speculation is consistent with the recent finding that the PAT-domain protein LSD-2 of *Drosophila* not only serves a Perilipin-like function in adult

fat body cells but also operates in the control of embryonic lipid droplet transport along the cytoskeleton [10]. An active role in vesicle trafficking is also supported by the identification of Rab proteins in the lipid droplet subproteome (Supplemental Tables, [9,40,41]) as well as the GO-term analysis, predicting 10% of the identified proteins to be implicated in cellular transport (Fig. 2B).

We also identified the cytoplasmic membrane-derived receptor fat body protein 1 (FBP-1) together with its internalized ligand, the storage protein complex consisting of the α, β and γ subunits of the larval serum protein 1 (LSP1) (Table 1). The storage protein complex is internalized in response to the ecdysone pulse in late third instar larvae and serves as a reservoir for amino acids and energy during metamorphosis [42]. It is, indeed puzzling to find that the cytoplasmic membrane-derived FBP1 receptor should be associated with lipid droplets. However, several recent studies on lipid droplet-associated proteins from different vertebrate cell lines also identified lipid droplet-associated receptors and transmembrane proteins such as stomatin [5] and the Big Stanniocalcin hormone receptor [43]. Collectively, these findings support the proposal that intracellular transport processes as well as different storage factors and storage compartments are functionally connected within the cell by their adherence to lipid droplets. It will be interesting to learn how their association is mechanistically possible.

Mitochondrial proteins have been described as members of the lipid droplet proteome in this study and others [5]. However, it cannot be excluded that they represent false positive identifications, as these organelles are (i) highly abundant in fat storage tissue, (ii) found in close association with lipid droplets and (iii) get easily disrupted during the purification procedure [44,45]. Along those lines of arguments, also the identified ribosomal proteins might be retained from the endoplasmatic reticulum (ER), where at least some lipid droplet-associated proteins seem to become inserted

via the SRP/translocon machinery. The mechanism has already been demonstrated for plant oleosin proteins [35]. On the other hand, our study identified components of the SRP/translocon machinery [46] among the lipid droplet-associated proteins, as well as other typical ER-bound proteins such as protein disulfide isomerase (PDI) and calreticulin that were also identified in other lipid droplet proteome screens [1,8]. Thus, our preparations may also contain material of ER origin. Whether this finding supports the hypothesis that the lipid droplets derive from the ER [2], reflect the tight association of droplets with the ER or whether these proteins are eventually due to undetected ER contaminations within the droplet fraction remains to be shown.

It is worth mentioning, however, that ribosomes and associated mRNA were previously shown to associate with the surface of lipid droplet like “lipid bodies” of mast cells [47]. Thus, it might well be that a subpopulation of the lipid droplet-associated proteins are synthesized at their site of action. This proposal would also be compatible with the identification of chaperone proteins among the lipid droplet subproteome. This proposal is highly speculative as long as no putative signal sequence for lipid droplet targeting and domain structures of proteins for their association with the droplet surface have been identified.

Using green fluorescent protein fusions, we confirmed the co-localization of a few of the identified proteins with lipid droplets of tissue culture cells as well as larval and adult fat body cells. In addition to LSD-1 and LSD-2, which were already shown to associate with lipid droplets [18,37], we found that the three proteins examined were associated with lipid droplets. The putative dehydrogenase CG2254 localizes exclusively in a restricted pattern on lipid droplets. Its localization pattern is reminiscent to the one of Brummer, a lipase recently shown to be central in the control of organismic fat storage of adult flies [48]. Most importantly, we noted that the proteins examined were not associated with all lipid droplets, but each of them

associates with a subset of droplets only. This differential localization suggests the existence of the above-discussed complexity of possible functions and/or different metabolic repertoires of lipid droplets and may reflect part of a “zip code” for functionally different lipid droplets. Future studies will test this proposal by using double labeling experiments combined with fat cell-specific mutations of proteins that define different subpopulations of lipid droplets. This way the question whether different mutations affecting specific subpopulations of droplets result in separable, non-overlapping cellular and organismal phenotypes could be answered.

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Figure Legends

Figure 1: Isolation of the *Drosophila* lipid storage droplets and identification of the lipid droplet proteome.

(A) Extent of the *Drosophila* larval fat body imaged by light microscopy (upper panel) and highlighted by tissue-specific EGFP expression in fluorescence microscopy (lower panel). (B) Transmission electron microscopy image of a larval fat body cell densely packed with lipid droplets (LD). (C) Strategy of fat body lipid droplet isolation and lipid droplet proteome identification (for details see text). (D-F) Lipid droplet purity control by (D) protein profiling of density fractions, (E) Western blot analysis using cytoplasmic (eIF-4A) and lipid droplet-enriched (LSD-2) marker protein detection and (F) transmission electron microscopy inspection. (G) High complexity of lipid droplet proteome revealed by 1D SDS PAGE.

Figure 2: Genotype-specificity and classification of the lipid droplet subproteome.

(A) Graphical representation of the number of presumptive constitutive lipid droplet proteome members identified in all genotypes analysed, compared to the proteome shared by two to three genotypes and the rare genotype-specific proteins. (B) Functional classification of class A and class B lipid droplet proteome members (for definition see text) on the basis of selected GO-terms. The majority of lipid droplet proteins are predicted to operate in cellular metabolism but 9% of class A and 19% of class B proteins are functionally unknown. (C) Biological processes significantly over- or underrepresented on lipid droplets as compared to the global proteome predict various processes of lipid metabolism and storage to operate on lipid droplets (for details see text).

Figure 3: Subcellular localization of representative lipid droplet-associated proteins in *Drosophila* S2 cells.

(A) Lipid droplet association of fluorescently tagged PAT-domain proteins (A_1 LSD-1:EGFP; A_2 LSD-2:red fluorescent protein; A_3 merged channels A_1+A_2; A_4 differential interference contrast (DIC)). (B, C) Localization of CG1112:EGFP (B_1) and CG10691:EGFP (C_1) at a subset of lipid droplets (B_2, C_2) and in the cytoplasm (merged channels B_3, C_3; DIC images B_4, C_4). (D, E) Specific CG2254:EGFP localization (D_1, E_1) around lipid droplets (D_2, E_2; merged channels D_3, E_3; DIC image D_4, E_4). Note that CG2254:EGFP decorates a subset of lipid droplets in panel E. Lipid droplets visualized by Nile Red stain in panel B to E.

Figure 4: Subcellular localization of representative lipid droplet-associated proteins in fat body of transgenic flies.

(A) CG1112:EGFP localizes at membranes and at most lipid droplets both in the larval (A_1, arrows in merged image A_3) and immature adult fat body (A_4). (B) Ubiquitous distribution of CG10691:EGFP in the larval fat body (B_1; merged channels B_3) including the lipid droplet surface as exemplified in an immature adult fat body cell (B_4). (C) Specific localization of CG2254:EGFP at larval fat body lipid droplets (C_1 and C_3 (merged image) intact fat body; C_4 isolated lipid droplets). Lipid droplets visualized by Nile Red stain in panel A to C.

Supplemental Tables:

Supplemental Tables contain the full set of lipid proteome class A and class B identifications along with relevant biochemical and functional data of the identified proteins as well as the details of the corresponding mass spectrometry analysis.

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Table 1: Selected class A *Drosophila* lipid droplet proteome members.

Listed are biological independent identifications (I to III) of the respective proteins in lipid droplet preparations from the four different genotypes analysed. Highlighted are members of the constitutive lipid droplet proteome (light grey) and genotype-specific lipid droplet proteins (dark grey). For details see text.

Independently confirmed lipid droplet localization of identified *Drosophila* proteins (D) or non-*Drosophila* homologues (ND) are referenced as follows: 1 this study, 2 [37], 3 [7], 4 [11], 5 [8], 6 [9], 7 [39], 8 [5], 9 [48], 10 [41], 11 [18].

Genotypes analysed				Gene name	Protein name or predicted function / GO-term (Biological Process)	Reference
wild type	*adp*^{60}	*Lsd-2*^{51}	induced *Lsd-2*:EGFP			
PAT-Domain Proteins (2 of 2)						
II III	I II III	I II III	I II III	***Lsd-1 (CG10374)***	LSD-1 / lipid storage	D: 1, 2
II III	I II III		I II III	***Lsd-2 (CG9057)***	LSD-2 / lipid storage	D: 1, 2, 11
Metabolic Proteins (13 of 46)						
I II III	I II III	I II III	I II III	***CG3523***	Fatty acid synthase / fatty acid biosynthesis	ND: 4
II III	I II III	II III	I II III	***CG11198***	Acetyl CoA carboxylase / fatty acid biosynthesis	ND: 5, 6
III		III	I III	***desat1 (CG5887)***	Fatty acid desaturase / fatty acid biosynthesis	
I II III	I II III		I II III	***ATPCL (CG8322)***	ATP citrate lyase / acetyl-CoA biosynthesis; citrate metabolism; TCC	
III	II	II		***v(2)k05816 (CG3524)***	[acyl-carrier protein] S-acetyltransferase activity (EC:2.3.1.38) / fatty acid biosynthesis	
I II III	I II III	I II III	I II III	***CG1516***	Pyruvate Carboxylase / fatty acid biosynthesis; gluconeogenesis; pyruvate metabolism	ND: 4
II	I	I II III	I	***Gapdh2 (CG8893)***	Glyceraldehyde-3-P-DH / glycolysis	ND: 3, 7
	I III	III		***CG2254***	Short chain DH / metabolism; visual perception	D:1 ; ND: 5
			I III	***CG5590***	Oxidoreductase short chain DH / metabolism	ND: 5
III	I II III	I II	I II III	***Adh (CG3481)***	ADH / ethanol oxidation	ND: 5
III	III		I II III	***Cyp4d1 (CG3656)***	Cytochrom P450 4D1 / electron transport; steroid metabolism	
III		III	III	***Cyt-b5 (CG2140)***	CytB5 / electron transport; steroid metabolism	
			II III	***CG7430***	Dihydrolipoyl DH / lipoamide metabolism; TCC; electron transport; glycine catabolism; glycolysis	ND: 7
Storage Proteins (5 of 6)						
II III	I II III	I II III	I II III	***Fbp2 (CG3763)***	FBP2 / lipid metabolism	
I II III	I II III	I II III	I II III	***Fbp1 (CG17285)***	FBP1 / fat body storage protein uptake	
I II	I II	I II III	I II	***Lsp1alpha (CG2559)***	LSP1alpha / transport	
I II III	I II	I II III	I II	***Lsp1beta (CG4178)***	LSP1beta / transport	
I II III	I II III	I II III	I II III	***Lsp1gamma (CG6821)***	LSP1gamma / transport	
Lipid binding and transport Proteins (3 of 5)						
III	I	I II	I III	***ScpX (CG17320)***	SCP-X / phospholipid transport	
	I II	I III	I III	***CG9342***	Triglyceride binding / lipid metabolism; lipid transport; trIcylglycerol metabolism	
	I II	III	I II	***CG5958***	Retinol / fatty acid binding / coenzyme metabolism; prosthetic group metabolism; transport	
Cell biology / intracellular transport Proteins (9 of 18)						
I		I	I	***CG11642***	SRP cotranslational membrane targeting / SRP-dependent cotranslational protein-membrane targeting	
	II III	I	I II	***Tapdelta (CG9035)***	TAP delta / protein-ER retention	
III	III	II III	II III	***l(1)G0320 (CG32701)***	SRP binding / protein biosynthesis; protein targeting; vesicle-medIted transport	
III	I II	III	I III	***SsRbeta (CG5474)***	SSR beta / protein-ER retention; vesicle-medIted transport	
	I	I II	I	***l(2)37Cc (CG10691)***	SPFH (band7) domain / DNA replication; larval or pupal development; regulation of cell cycle	D:1 ; ND:5
			I II III	***l(2)03709 (CG15081)***	nothing predictable SPFH (band 7) domain / cell proliferation; regulation of cell cycle and others	ND: 5
II	II			***Actin5C (CG4027)***	Actin 5c / cytoskeleton organization and biogenesis; sperm individualization	ND: 6
I	III	I III	I III	***Rtnl1 (CG33113)***	Rtnl1 / intracellular protein transport	
	I II			***regucalcin (CG1803)***	Regucalcin / anterior-posterior axis specification; calcium medIted signaling	
Proteins without GO-term (biological function) (3 of 14)						
II III	I II	I II III	I II III	***CG5167***	NAD binding / no GO-term (Biological process)	ND: 7
II III	III	II III	II III	***alpha-Est7 (CG1112)***	alpha esterase 7 / no GO-term (Biological process)	D:1 ; ND: 4
	II III			***pastrel (CG8588)***	Pastrel / no GO-term (Biological process)	

Chaperone Proteins (4 of 15)

II	I II III	I II	I II III	***Hsc70-4 (CG4264)***	HSC70-4 / protein complex assembly; protein folding; protein refolding; vesicle-medIted transport and others	ND: 6
II III	I II III	I II III	I II III	***Pdi (CG6988)***	PDI / electron transport; protein folding; protein modification	ND: 5, 6
II III	I II III	I II III	I II III	***Hsc70-3 (CG4147)***	HSC70-3 / defense response; protein complex assembly; protein folding; response to heat; sleep	ND: 4
II	II	II III	III	***Hsp60 (CG12101)***	HSP60 / "de novo" protein folding; protein folding and refolding and others	ND: 6