Article

The Lipid-Droplet Proteome Reveals that Droplets Are a Protein-Storage Depot

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Summary

Background: Lipid droplets are ubiquitous organelles that are among the basic building blocks of eukaryotic cells. Despite central roles for cholesterol homeostasis and lipid metabolism, their function and protein composition are poorly understood.

Results: We purified lipid droplets from *Drosophila* embryos and analyzed the associated proteins by capillary LC-MS-MS. Important functional groups include enzymes involved in lipid metabolism, signaling molecules, and proteins related to membrane trafficking. Unexpectedly, histones H2A, H2Av, and H2B were present. Using biochemistry, genetics, real-time imaging, and cell biology, we confirm that roughly 50% of certain embryonic histones are physically attached to lipid droplets, a localization conserved in other fly species. Histone association with droplets starts during oogenesis and is prominent in early embryos, but it is undetectable in later stages or in cultured cells. Histones on droplets are not irreversibly trapped; quantitation of droplet histone levels and transplantation experiments suggest that histones are transferred from droplets to nuclei as development proceeds. When this maternal store of histones is unavailable because lipid droplets are mislocalized, zygotic histone production starts prematurely.

Conclusions: Because we uncover a striking proteomic similarity of *Drosophila* droplets to mammalian lipid droplets, *Drosophila* likely provides a good model for understanding droplet function in general. Our analysis also reveals a new function for these organelles; the massive nature of histone association with droplets and its developmental time-course suggest that droplets sequester maternally provided proteins until they are needed. We propose that lipid droplets can serve as transient storage depots for proteins that lack appropriate binding partners in the cell. Such sequestration

may provide a general cellular strategy for handling excess proteins.

Introduction

Lipid droplets are ubiquitous organelles found in yeast, plants, and animals as well as some prokaryotes [1]. Droplets are storage sites for energy, sterols, and precursors of membrane phospholipids. Among organelles, the structure of lipid droplets is unique: A core of neutral lipid (triglycerides and sterol esters) is surrounded by a surface monolayer of polar lipids (cholesterol, phospholipids, and fatty acids) [2]. Abnormalities in intracellular lipid storage are associated with obesity and fatty liver, cardiovascular disease [3], diabetes [4], neutral lipid-storage disease [5], and Niemann Pick C disease [6].

Despite the importance of lipid droplets in lipid metabolism and disease, their protein content and the function of droplet-associated proteins is not clear. Existing proteomics studies differ greatly in identified droplet-bound proteins. This variation suggests that each study identified only a small part of the total protein content of the droplets or that the proteins on droplets from different sources are tremendously varied. We would like to determine the lipid-droplet proteome in a genetically tractable model organism and thus facilitate future functional analysis of the identified droplet-associated proteins.

We focus on lipid droplets in *Drosophila* embryos. Synthesized by the adult female during oogenesis, droplets are the major energy store for developing embryos. Droplets move bidirectionally along microtubules and are a major model system for studying the regulation of motor-driven transport. Thus, determination of droplet-bound proteins can provide insights into energy metabolism, the regulation of intracellular transport, and droplet biology in general. To date, only three proteins present on these droplets have been identified: the motor dynein [7], the motor regulator Klar [8], and the PAT family member LSD2 [9].

This study identifies a large number of droplet proteins. We find homologs of almost all proteins previously identified in the different mammalian studies, suggesting that the *Drosophila* droplets are a good model system for uncovering fundamental aspects of the biology of lipid droplets. In addition, our study provides a striking example of proteins that associate with droplets in a developmentally controlled manner: histones H2A, H2Av, and H2B. We propose a general role for lipid droplets as transient storage depots for proteins in temporary excess.

Results

Isolation of Lipid Droplets from *Drosophila* Embryos
Drosophila embryos are full of small (approximately 0.5 μm) maternally provided neutral lipid droplets (Figure 1A). Because of their high lipid content, lipid

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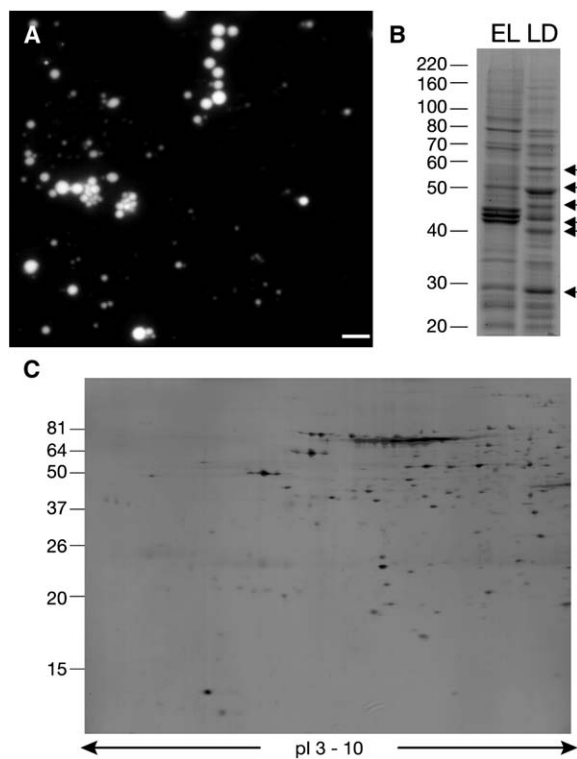
Figure 1. The Proteins of the Lipid-Droplet Fraction

(A) Lipid-droplet fraction stained with Nile Red. The fraction is enriched in lipid droplets of various sizes; the scale bar represents 1 μm.
(B) The protein composition of the lipid-droplet fraction (LD) compared to that of embryo lysate (EL). Equal amounts of total proteins (20 μg/lane) were processed for gel electrophoresis and stained with Coomassie Blue. Arrows indicate bands enriched in the lipid-droplet fraction.
(C) Proteins from the lipid-droplet fraction, separated by 2-D gel electrophoresis and detected by silver-staining. Molecular-weight markers are indicated (left).
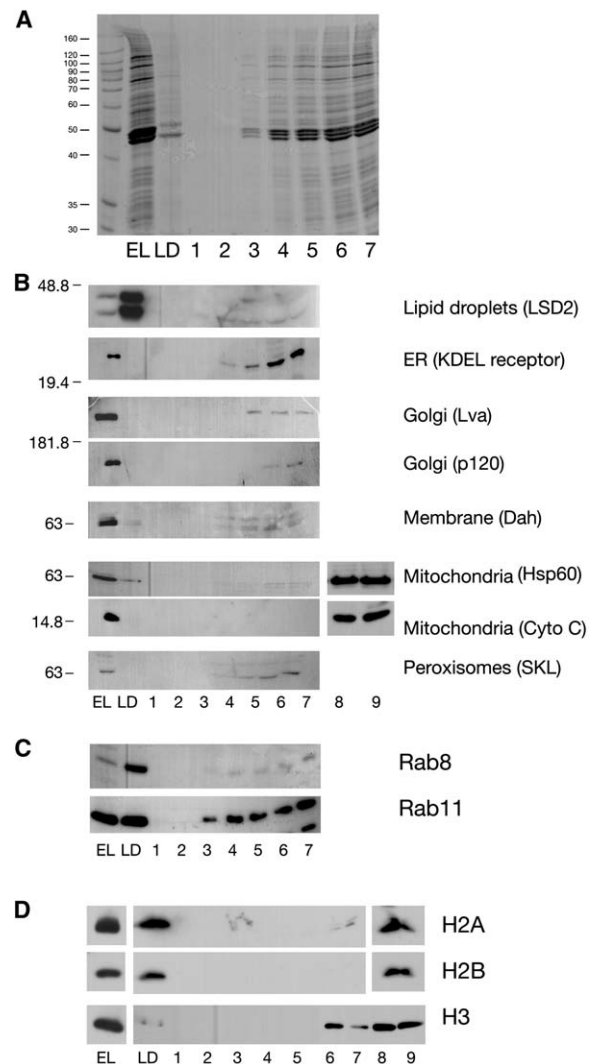

Figure 2. Identification of Proteins in the Lipid-Droplet Fraction

(A) Sucrose-gradient fractionation of *Drosophila* embryo lysate. EL: embryo lysate (20 μg). Lysates were first cleared of large organelles by an initial centrifugation step (pellet fraction 9, not shown) and then separated over a sucrose gradient. LD; lipid-droplet fraction (20 μg; 4 μl of 40 μl total). 1–7: sequential fractions from top to bottom (10 μl each of 1.5 ml). Not shown is the pellet (fraction 8). Proteins were separated by 10% SDS-PAGE and stained with Coomassie Blue. First lane: Molecular-weight markers. Yolk proteins (triplet of bands just below 50 kDa) are present in most fractions but are not detectable in the droplet layer.
(B) Western-blot analysis of the same fractions as those in (A). Blots probed for markers for: lipid droplets (LSD2), the Golgi complex (top, Lva; bottom, p120), mitochondria (top, Hsp60; bottom, cytochrome c), the plasma membrane (Dah), the ER (KDEL receptor), and peroxisomes (SKL). LSD2 is highly enriched in the droplet fraction; other markers are absent or present in only small amounts. The mitochondria markers were detected in pellets 8 and 9 (10 μl of 1.5 ml), but other organelle markers were absent (not shown).
(C) Immunoblot analysis of candidate droplet proteins Rab8 and Rab11. Fractions are as in (A). Although not immediately apparent because of saturation of loading, a careful comparison of the amount of Rab 11 versus Rab 8 on the droplets indicates that Rab 11 is present in larger amounts, as expected from Rab 11's appearance in Table 1 versus Rab 8's presence in Table S1 (data not shown).
(D) Immunoblot analysis for histones H2A, H2B, and H3. Fractions are essentially as in (A), but the LD lane represents 2.5 μl of 50 μl total, fractions 1–9 represent 25 μl each of 1.5 ml total, and the EL lane was loaded with 5 μg total protein.

droplets have a low buoyant density; we therefore employed a sucrose-step gradient to purify them from cytoplasm and other organelles. Lipid droplets were recovered from the very top of the gradient and washed repeatedly to remove potential contaminants. This technique is an adaptation of flotation procedures to isolate lipid droplets from mammalian cultured cells and tissues [10–12].

We assessed the quality of our purification by comparing equal amounts of protein from the starting material (embryo lysate) and the droplet fraction for overall protein content (Figure 2A) and for the presence of a range of marker proteins (Figure 2B). On 1D gels, the droplet fraction had a much simpler protein pattern than the embryo lysate, with a few bands strongly enriched in the lipid-droplet fraction (Figure 1B). As assessed by immunoblotting, proteins specific for the ER, Golgi complex, mitochondria, peroxisomes, and plasma membrane were either completely absent or highly depleted from the lipid-droplet fraction. On the other hand, the droplet protein LSD2 [9] was represented prominently. Thus, our lipid-droplet fraction is indeed highly enriched for lipid droplets and is largely free of contamination by other organelles.

The Lipid-Droplet Fraction Has a Complex Protein Profile

On 2D gels, the protein pattern of the droplet fraction was complicated (Figure 1C), reminiscent of studies on mammalian lipid droplets [12]. This pattern was quite reproducible between different purifications (see, for example, [9]). Proteins were identified by digestion with trypsin in solution and processed by capillary liquid chromatography-tandem mass spectroscopy (LC-MS-MS). Compared to in-gel digestion techniques, this procedure can identify a large set of proteins (approximately 40 proteins were reported with the previous techniques [12]). We performed six independent droplet purifications and present the identified proteins in four tables. Table 1 (51 proteins) presents proteins that were identified in five or all six experiments. Table 2 (76 proteins) lists proteins recovered in three or four experiments. Proteins marked with one, two, or three asterisks were independently identified numerous times and are therefore likely present in quite high amounts (see table legend). In the supplement, additional proteins that were found in exactly two (Table S1, 56 proteins) or one (Table S2, 397 proteins) of the proteomic experiments are indicated. Proteins in Tables 1 and 2 are either extraordinarily well represented or highly stably bound to the droplets, whereas those in Tables S1 and S2 are likely less well represented and could reflect contaminants or proteins present in low copy number (see Discussion).

The identified proteins represent a range of functional groups, including proteins implicated in lipid metabolism (e.g., LSD2, lipases), molecules involved in signal transduction (e.g., Protein phosphatase 2A subunits), and proteins involved in membrane trafficking (e.g., Rab 8 and Rab 11). There were also many proteins of unknown function and proteins with previously described functions not obviously connected to known roles of lipid droplets. Among the highest-scoring candidates were three histone proteins (H2A, H2B, and H2Av), proteins that package DNA into chromatin and regulate transcription. A priori, they had not been expected to be associated with lipid droplets.

To confirm that the detected proteins are present in the lipid-droplet fraction, we compared protein levels in embryo lysates and in the lipid-droplet fraction for some of the candidates (Figures 2B–2D). For the candidates LSD2, Rab8, Rab11, H2A, and H2B, we detected substantial amounts in the lipid-droplet fraction. Because of limited antibody availability, only a few of the candidates were tested; however, there was no occasion where an antibody against a candidate protein crossreacted with the embryo lysate but failed to detect the protein in the droplet fraction.

How Pure Are the Droplet Preparations?

The sensitive protein-detection method provides an opportunity to establish a more complete set of the proteins attached to lipid droplets, but contaminants are also more easily detected. Non-droplet proteins might contaminate the droplet fraction through several mechanisms. First, during floatation, other organelles might get trapped between lipid droplets and end up in our "lipid-droplet fraction." Because markers for several organelles were absent or highly depleted in the droplet fraction (Figure 2B), we can rule out gross contamination, a situation similar to published results from other systems [10–13]. The presence of certain mitochondrial proteins (e.g., HSP60) in Tables 1 and 2 nevertheless raised the possibility that whole mitochondria contaminate our preparations. If so, the relative abundance of mitochondrial proteins should be the same in the embryo lysate and our droplet preparations. That was, however, not the case; relative to HSP60, the mitochondrial marker Cytochrome C was greatly depleted (Figure S4).

A second possibility is that the proteins we detect are present throughout the gradient and are not particularly enriched on the lipid droplets. To examine this possibility, we determined the amount of several candidate proteins in each fraction of the purification. In each case, the proteins were enriched in the droplet fraction relative to the fraction right beneath the droplets (Figure 2), suggesting that these proteins are indeed comigrating with the droplets and are thus likely bound to them.

A third possibility is that during lysis certain proteins freed from their usual compartments stick to the droplets. This possibility must be investigated on a case-by-case basis; we addressed it specifically for the histones because they are known to function in the nucleus. However, although unexpected, the large number of independent identifications of histones in the proteome argues that they are quite abundant on the lipid droplets.

Histone Enrichment on Droplets Is Not an Artifact of Embryo Lysis

To test whether histones are associated with lipid droplets in embryos before lysis, we centrifuged living embryos to enrich droplets locally in a "droplet layer." The known droplet-associated proteins Klar and LSD2 are highly enriched in this layer [8, 9], and Klar proteins that lack Klar's droplet-targeting domain fail to accumulate in the droplet layer [8]. We modified this assay such that the anterior ends of the embryos pointed toward the center of rotation, resulting in consistently stratified embryos, with lipid droplets at the anterior and yolk granules near the posterior end (Figure 3A). Other organelles, such as ER, Golgi vesicles, and mitochondria, accumulate at intermediate positions, indicating that the droplet layer is not grossly contaminated with other organelles. Embryos stained for histone H2A had signal in nuclei as expected but also had abundant signal in the lipid-droplet layer (Figure 3B). The same was true for histone H2B. Histone H3, however, was not enriched in the droplet layer but was instead present throughout the embryo and depleted from the regions taken up by lipid droplets and yolk. This distinct behavior of histones was also observed for isolated droplets: levels of H2A and H2B in our lipid-droplet fraction were substantially higher than that of H3 (Figure 2D). In the mass-spectroscopy analysis, H2A and H2B were independently identified multiple times, but H3 was found minimally.

Histone variant H2Av was also identified multiple times in our proteomic analysis (Table 1). H2Av functions in chromatin-mediated regulation of transcription and in DNA repair; it combines the roles of the histone H2AX and H2AZ variants in mammals. We monitored H2Av distribution by using an H2Av-GFP fusion that is fully
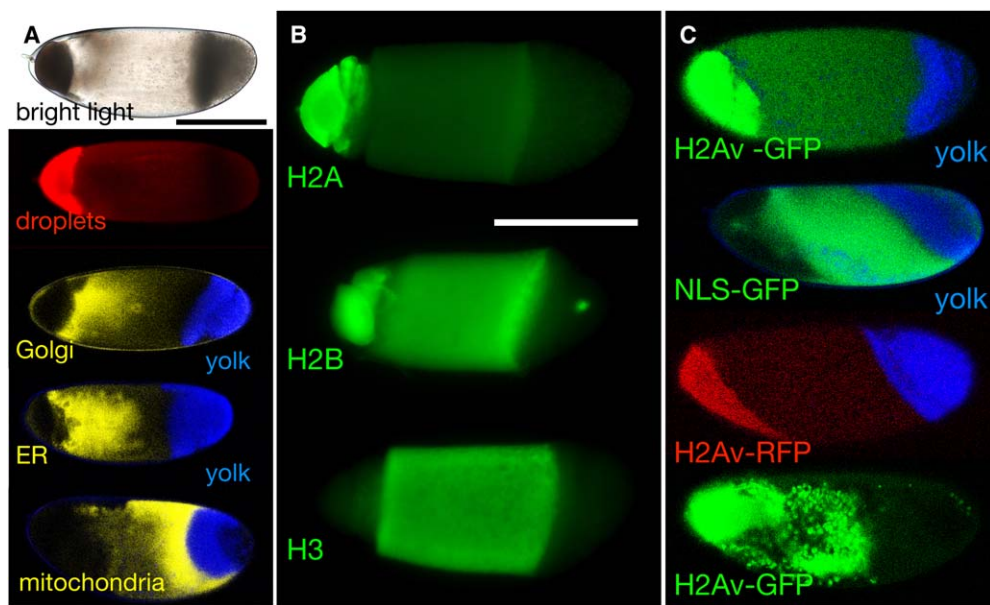
Figure 3. In Vivo Centrifugation Assay

Embryos oriented and centrifuged so that lipid droplets accumulate at the anterior. Distribution of organelles was assessed in live embryos or after fixation.

(A) Centrifugation results in distinct layering (bright light). Lipid droplets (detected in fixed embryos by Nile Red) accumulate at the anterior. ER, Golgi, and mitochondria (shown by the distribution of YFP proteins targeted to these organelles) accumulate in distinct locations. Yolk (detected by autofluorescence) is near the posterior. Thus, the anterior lipid layer is free of gross contamination by other organelles.

(B) Endogenous histones are enriched in the lipid-droplet layer. Cleavage-stage embryos were centrifuged as in (A), fixed, and stained for histones H2A, H2B, or H3 (green).

(C) Embryos expressing fluorescently tagged proteins were centrifuged. In early embryos, H2Av-GFP and H2Av-mRFP are localized to the droplet layer, whereas NLS-GFP is not. In embryos with many nuclei, H2Av-GFP is partitioned between lipid layer and nuclei (bottom). Scale bars represent 200 μm.

functional and can rescue H2Av null mutations [14]. In centrifuged early embryos, the major GFP signal comes from the lipid-droplet layer (Figure 3C, top). In later embryos with many nuclei, the signal was partitioned between the droplet layer and nuclei (Figure 3C, bottom). Recruitment of the fusion protein to the droplet layer is not due to the GFP sequences, as evidenced by our testing of ten other fly lines in which a GFP fusion protein is highly expressed in the early embryo; in each case, GFP signal was excluded from the droplet layer (Figure 3C, and data not shown). Instead, it is the histone portion that is responsible for the recruitment because an H2Av-mRFP fusion protein was also highly enriched in the droplet layer (Figure 3C). We conclude that the association of histones H2Av, H2A, and H2B with lipid droplets in early embryos is not an artifact of embryo lysis but occurs in vivo.

Histones Are Abundant on Lipid Droplets In Vivo

To exclude the possibility that the act of centrifugation induced binding of histones to droplets, we examined uncentrifuged embryos expressing the H2Av-GFP fusion protein. Similar results were obtained with the RFP fusion (not shown). GFP signal was present not only in nuclei but also in small puncta (Figure 4A). By several independent criteria, these GFP-positive puncta are lipid droplets. First, double staining with the lipid-droplet-specific dye Nile Red shows that GFP signal colocalizes with lipid droplets (Figure 4E). Second, lipid droplets undergo a characteristic, developmentally controlled

redistribution along the apical-basal axis. Originally found all over the embryo periphery, they accumulate basally just before cellularization [15]. GFP-histone puncta underwent the same redistribution at the same stage of embryogenesis (Figures 4C and 4D). In embryos lacking the Halo protein, lipid droplets—but no other organelles—are specifically mislocalized just before cellularization [16]. GFP-histone puncta showed the same apical mislocalization in embryos lacking Halo (Figure S5). Finally, direct observation showed that the GFP puncta move rapidly in radial paths during interphases and stop during mitoses (see Movie S9), with motion very similar to the microtubule-based motion of lipid droplets [15]. In summary, we conclude that even in undisturbed early embryos, histones are associated with lipid droplets. Under favorable imaging conditions, the H2Av-GFP puncta resolved into rings the size of lipid droplets (Figure 4B), suggesting that the fusion protein is present on the droplet surface.

Droplets Are Associated with a Large Fraction of Total Embryonic Histones

The images of centrifuged and stained embryos suggest that droplet-associated histones are a significant fraction of the total embryonic histone pool. We therefore compared histone levels in various fractions from our droplet-isolation procedure. H2A and H2B were only present in fractions containing droplets or nuclei (Figure 2D). The histones on droplets accounted for a large percentage of total embryonic histones (62% or

Table 1. Proteins Represented Abundantly in the Lipid-Droplet Fraction

Lipid Droplet

***	CG9057	LSD2/lipid storage droplet 2	

Cytosolic/Vesicular/Unknown

**	CG5112		Fatty acid amide hydrolase
**	CG4264	Hsc70-4/heat shock cognate protein 4	chaperone
***	CG15092		unclassified
**	CG5119	pAbp/polyA-binding protein	RNA binding
*	CG1882	CGI-58 homolog	Triacylglycerol lipase activity
*	CG9186		Triacylglycerol lipase activity
*	CG7113	scully	fatty acid metabolism
*	CG9412	rin/rasputin	GTP binding/ GTPase activity
*	CG5771	Rab11	GTP binding/ GTPase activity
*	CG4799	pendulin/importin alpha2	protein carrier activity
	CG11055		Triacylglycerol lipase activity
	CG8839		Fatty acid amide hydrolase
	CG1404	ran	GTP binding/ GTPase activity
	CG1956	R/roughened	GTP binding/ GTPase activity
	CG3320	Rab1	GTP binding/ GTPase activity
	CG18290	actin 87E	cytoskeleton
	CG2604		unclassified
	CG18811		unclassified
	CG3752	Aldh/Acetaldehyde dehydrogenase	Aminoacid metabolism

Nucleus

**	CG5499	His2Av/histone H2A variant	chromatin
***	CG17949	His2B	chromatin
***	CG31618	His2A	chromatin

ER

**	CG1291	glycosyl transferase homology
*	CG4147	Hsc70-3/heat shock cognate protein 3
	CG5474	SsRbeta/Signal sequence receptor beta

Ribosome

**	CG8280	Efalpha48D/Elongation factor 1 alpha 48D
*	CG6779	RpS3
*	CG3922	RpS17
*	CG10423	RpS27
	CG7434	RpL22
	CG4046	RpS16
	CG2033	RpS15A
	CG12324	RpS15Ab
	CG8332	RpS15

Yolk

**	CG2979	yolk protein 2
**	CG11129	yolk protein 3
*	CG2985	yolk protein 1

Mitochondria

**	CG11154	ATPsyn-β/ATP synthase-β
**	CG3612	blw/bellwether
*	CG12101	Hsp60/Heat shock protein 60
*	CG4307	Oscp/Oligimycin sensitive-conferring protein
*	CG6647	porin (voltage-dependent channel)
*	CG16944	sesB/stress sensitive B
*	CG7998	(L-malate dehydrogenase activity)
	CG10691	l(2)37Cc
	CG5389	
	CG17280	
	CG14724	CoVa/cytochrome c oxidase subunit Va
	CG4600	yippee interacting protein 2
	CG15093	(dehydrogenase activity)

Proteins identified by mass spectrometry in the lipid-droplet fraction and recovered in at least five of six independent purifications. Underlined proteins were recovered in all six experiments. *** = Protein was identified more than 100 times in the six experiments. ** = Protein was identified 50–99 times. * = Protein was identified 20–49 times. Typically, more identifications correspond to more of the protein present in the sample under study. Independent identifications can come about in three different ways: (1) different tryptic peptides corresponding to the same protein; (2) the same tryptic peptide found in independent purifications; or (3) multiple signals from the same tryptic peptide when it was sent to the MS/MS identification step multiple times due to its being extended across several chromatography fractions. The proteins are grouped by expected cellular distribution based on information available on FlyBase. Proteins identified less frequently are listed in Tables 2, S1, and S2.
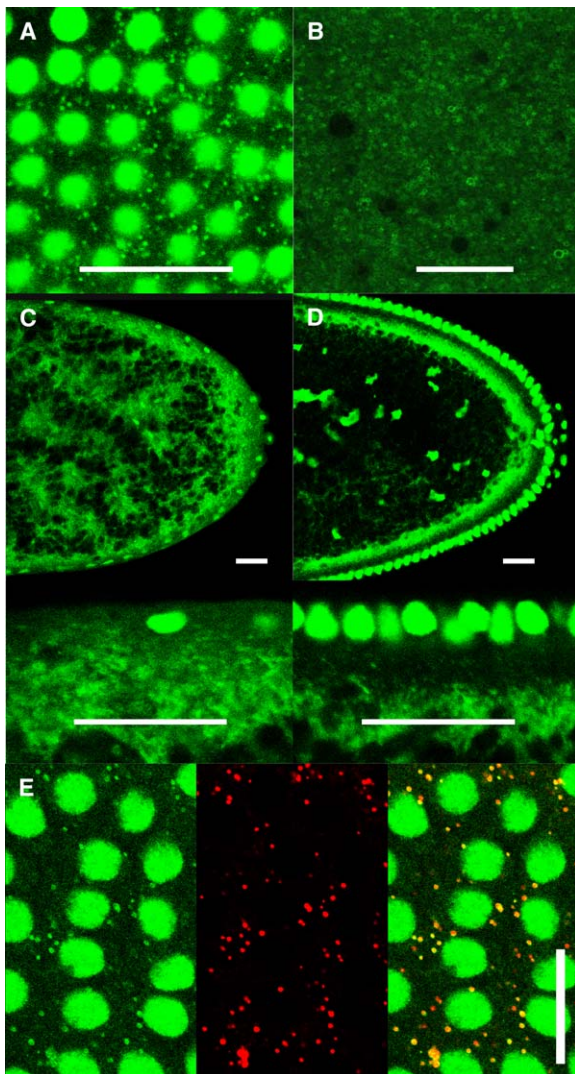
Figure 4. H2Av-GFP Localizes to Lipid Droplets in Uncentrifuged Embryos

(A) H2Av-GFP is detected in nuclei and small cytoplasmic puncta.
(B) Close to the embryo surface, the GFP puncta resolve into rings the size of lipid droplets.
(C and D) H2Av-GFP distribution in developing embryos, phase I (C), and phase II (D). The fusion protein is present in the nuclei at the periphery and in puncta between the nuclei and the yolk (dark central area) (top, low magnification; bottom, high magnification). In (C), cytoplasmic GFP puncta are present throughout the periphery; in (D) they accumulate basally like lipid droplets.
(E) Grazing view of an embryo expressing His2Av-GFP; embryos are labeled with the droplet-specific dye Nile Red. Left, His2Av-GFP; middle, Nile Red; right, merge. H2Av-GFP is present in nuclei and on lipid droplets. Scale bars represent 20 μm.

32% of total H2A and H2B, respectively; Figure S1). Thus, lipid droplets might act as a storage site to supply significant amounts of histones for later development.

Histones Can Leave the Lipid Droplets

Many eggs, those of *Drosophila* included, are loaded with maternally provided proteins to ensure rapid development even with limited transcription and translation in the zygote itself. To estimate how much excess histone is present in early embryos, we compared histone levels

between unfertilized eggs and embryos of various ages (Figure S6). During the first few hours of development, the number of nuclei increases from one to several thousand, but levels of H2A and H2B increased only a few fold (Figure S6)—at fertilization, then, the embryo has sufficient H2A and H2B to package the chromatin corresponding to thousands of nuclei. A large fraction of these histones are sequestered on the surface of lipid droplets.

What is the fate of the histones on lipid droplets? Both H2A and H2B levels on the lipid droplets decreased as a function of developmental stage (Figures 5A and 5B). By 12 hr of embryogenesis, histones were almost entirely absent from the droplets. Histones are not irreversibly trapped on droplets but can leave, possibly to be incorporated into chromatin.

To test whether such a droplet-to-chromatin transfer is possible, we centrifuged early embryos expressing H2Av-RFP to generate the distinct lipid-droplet layer. This layer was taken up into a microneedle and injected into embryos with GFP-marked nuclei. Over tens of minutes, strong RFP fluorescence appeared in the nuclei near the injection site, demonstrating that transfer of H2Av-RFP from the lipid-droplet fraction to nuclei was possible (Figure 5C; also Figure S2). This result also implies that the H2Av-RFP from the lipid droplets was functional to the extent that it could be recruited to package chromatin. A similar transfer was observed with H2Av-GFP (not shown).

To determine whether this sequestration of histones is functionally relevant, we examined whether zygotic histone expression (monitored with a H2Av-GFP construct) depends on the ready availability of the histones sequestered on the lipid droplets. After gastrulation, lipid droplets are found throughout the cellular layer in the wild-type, but in embryos from *klar* mutant mothers a defect in microtubule-based transport leaves most of the droplets trapped in the central yolk [7, 15]. Because histones cannot diffuse across cell membranes, the stored maternal histones should therefore be largely unavailable to the cells of the *klar* embryos. Remarkably, *klar* mutant embryos expressed H2Av-GFP more strongly than wild-type embryos of the same age (Figures 5D and 5E; also Figure S7); we detected this difference for three independently isolated *klar* alleles (*klar^1*, *klar^YG3*, and *klar^B* [8]). This unanticipated connection between lipid-droplet transport and histone expression suggests that the zygote compensates with additional histone production when the source of maternal histones is unavailable.

Mechanism of Histone Association with Lipid Droplets

To test the nature of histone association with lipid droplets, we treated purified droplets under various conditions and tested for the presence of H2B and LSD2 (Figure S3). Reagents that disrupt hydrophobic interactions significantly reduced the amount of droplet-bound LSD2, so hydrophobic interactions dominate LSD2's recruitment to lipid droplets, just as for its mammalian counterpart Perilipin [17]. These treatments did not detach H2B from the droplets. Conversely, when droplets were purified in the presence of alkaline carbonate to minimize electrostatic interactions, levels of H2B, but not of LSD2, decreased drastically. Thus, charge
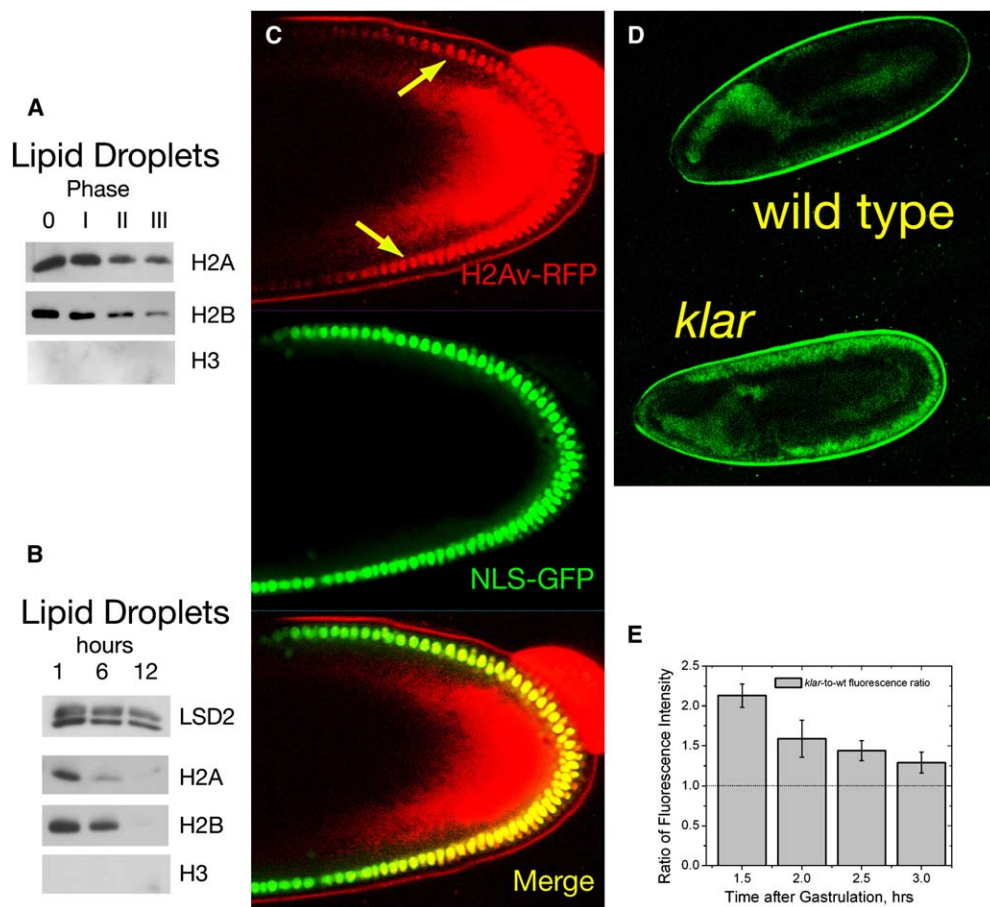
Figure 5. Stored Histones Can Leave Droplets and Are Functionally Relevant

(A and B) Equal amounts of lipid droplets from embryos of different developmental stages were processed for western-blot analysis. Levels of histones H2A and H2B in the droplet fractions decrease somewhat from phase 0 to III (B) and dramatically by 12 hr of development. Histone H3 is not present.

(C) Droplets carrying H2Av-RFP were isolated from centrifuged embryos and transplanted into embryos expressing NLS-GFP, which marks the nuclei of the host embryo. Lipid droplets were injected on the right. H2Av-RFP from donor lipid droplets translocates locally to nuclei. Yellow arrows point to RFP-positive nuclei that are far away from the bolus of injected droplets (see also Figure S2).

(D and E) H2Av-GFP fluorescence in *klar* and wild-type embryos. Females of two different genotypes (wild-type or *klar^B*) were crossed to males homozygous for the H2Av-GFP transgene. Embryos of near identical age were picked at gastrulation (they underwent gastrulation movements within 5 min of each other) and inspected by confocal microscopy for GFP expression. (D) A matched pair imaged about 70 min after gastrulation. The *klar* embryo shows distinct nuclear GFP expression in the periphery, whereas the wild-type embryo has much less pronounced signal. Signal in the central regions is due to yolk autofluorescence. In repeated matched pairs of such embryos, prominent GFP signal appeared first in the *klar* embryo. (E) The relative difference in H2Av-GFP fluorescence was quantified (see Supplemental Data) as a function of time after gastrulation. The bars represent the average ratio of *klar* to wild-type GFP-signal; the error bars are the standard error of the mean. This graph summarizes results from four matched pairs of embryos. If the genotypes showed no difference, the expected ratio would be 1 (dotted line). The precocious H2Av-GFP expression in the *klar* embryos is unlikely to be due to a general increase in the rate of development of *klar* embryos because the age-matched embryos hatch at very similar times.

interactions play an essential role in localizing histones to the droplets.

Histones and Lipid Droplets in Other Cells

To determine whether histones generally localize to lipid droplets, we expressed either H2Av-GFP or H2Av-RFP in *Drosophila* S2 cells. Neither localized to droplets (Figure 6A and data not shown). Similarly, endogenous H2A was not found on lipid droplets isolated from untransfected S2 and S3 cells (Figure 6B and data not shown).

In contrast, in animals expressing the H2Av-GFP construct ubiquitously, there was punctate cytoplasmic H2Av-GFP signal in ovaries (not shown). Centrifugation of ovaries enriches lipid droplets on one side of the tissue (Figure 6C, top). A fraction of H2Av-GFP colocalized with

the lipid droplets both in nurse cells, where lipid droplets are initially synthesized, and in oocytes (Figure 6C, middle). Thus histone association with lipid droplets begins during oogenesis, consistent with a role in storing maternally provided histones for early embryonic development.

Histone Association with Droplets Is Evolutionarily Conserved

We employed the centrifugation assay to investigate histone distribution in early embryos of other fly species as well. Just as for *D. melanogaster*, H2B was present in the droplet layer of early embryos of all nine additional *Drosophila* species examined (*D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. virilis*, *D. cardini*, *D. pseudoobscura, D. saltans*, and *D. lebanonensis*; data not shown).
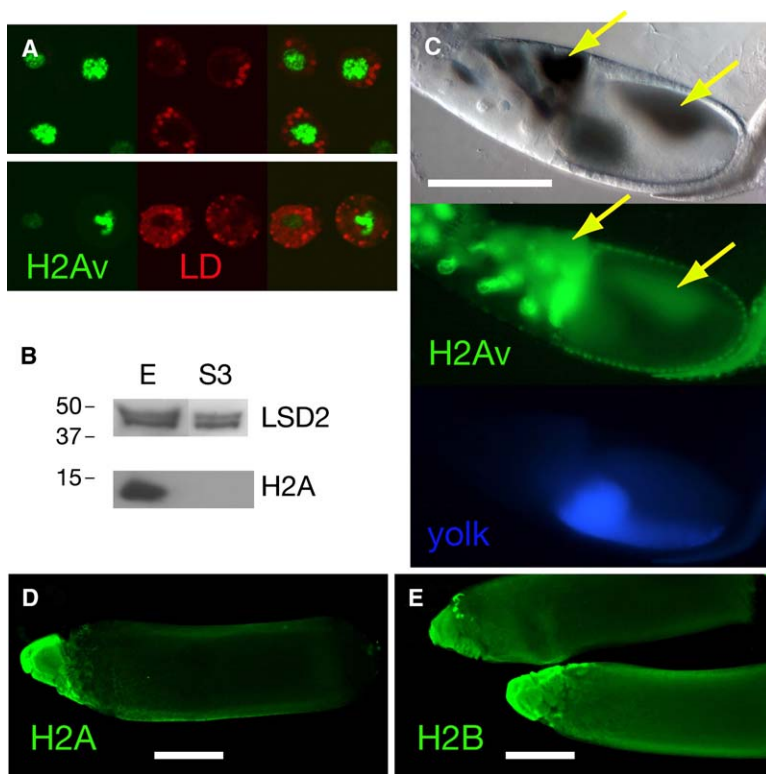
Figure 6. How General Is Histone Localization to Droplets?

(A) In cultured cells, Histone2Av-GFP does not localize to lipid droplets. H2Av-GFP (left) was expressed in S2 cells. Lipid droplets were marked with Klar-LD-RFP (middle) [8]. The merged images (right) demonstrate that there is little, if any, Histone2Av-GFP associated with lipid droplets.

(B) Lipid droplets were purified from early embryos (E) or from S3 cultured cells (S3). Western analysis (equal protein levels) indicates LSD2 on all droplets but H2A only on embryonic droplets.

(C) Histones associate with lipid droplets during oogenesis. Females expressing H2Av-GFP were centrifuged so that lipid droplets accumulated on one side of individual egg chambers. Droplet-rich regions (yellow arrows; left, nurse cells; right, oocyte) appear brownish in bright light images (top). H2Av-GFP (middle) is found in nuclei, but also in the cytoplasm of nurse cells and in the ovary (yellow arrows). Autofluorescence reveals the position of yolk (bottom). (D and E) Early embryos from houseflies (*Musca domestica*) were oriented and centrifuged so that lipid droplets accumulated at one end. Embryos were then fixed and stained for H2A (D) or H2B (E). Scale bars represent 200 μm.

In embryos of the housefly *Musca domestica*, we similarly detected both H2A and H2B in the lipid-droplet layer (Figures 6D and 6E). These fly species diverged 50–100 million years ago [18]. The fact that histone localization to droplets has been retained in all eleven lineages, despite tremendous divergence in the ecology and lifestyle of these animals, provides independent support for the notion that histone recruitment to lipid droplets serves an important adaptive function.

Discussion

We purified lipid droplets from early *Drosophila* embryos and used LC-MS-MS to identify a surprisingly diverse group of droplet-associated proteins, including most of those previously identified in more limited mammalian droplet studies. This proteomic similarity suggests the *Drosophila* droplets share fundamental properties with mammalian lipid droplets and are a good model system for understanding droplet-based cellular processes important for human health and disease. We found a new class of droplet proteins, exemplified by histones H2A, H2B and H2Av: proteins that ultimately function elsewhere in the cell but are transiently stored on the lipid droplets. Below we suggest that, more generally, droplets may temporarily store proteins that are in excess, to protect the proteins from aggregation, degradation, or both and also to protect the organism from detrimental effects of uncontrolled protein activity.

Histone Storage on Lipid Droplets

We found that droplets contain a large fraction of the total embryonic H2A, H2B, and H2Av but not of H3. This finding was confirmed with numerous independent approaches. Droplet association is not the result of overexpression: for H2A and H2B, it is the endogenous histones expressed at wild-type levels that associate with lipid droplets. For H2Av, we observed robust droplet localization of both GFP and RFP fusions even with a single copy of the tagged transgene (data not shown); because expression of the transgene is driven from the endogenous promoter, total H2Av levels are likely only marginally increased relative to wild-type levels. Although such massive sequestration of core histones outside the nucleus is unprecedented, macroH2A, a minor histone variant in mammals, is associated with centrosomes and was proposed to be stored there [19]. The functional importance of that storage is unknown.

Why are histones sequestered on lipid droplets in oocytes and early embryos? Droplet association is not a means of irreversibly inactivating these histones. During later development, histones H2A and H2B are released from the lipid droplets (Figures 5A and 5B) and are competent to be transferred to nuclei and presumably to assemble into chromatin (Figure 5C). The specificity of sequestration, its massive nature, and its evolutionary conservation all suggest that it serves an important biological role.

The elaborate regulation of histone levels in most cells provides a tantalizing clue. In somatic cells, the synthesis of histones and DNA is typically tightly coupled, so that just enough nucleosomes are generated to package newly replicated DNA. Because excess histones can cause chromatin aggregation in vitro [20] and slow growth, DNA damage sensitivity, and chromosome loss in vivo [21], cells employ active surveillance mechanisms to detect the accumulation of free histones and induce their degradation [21]. Early embryos, however,

face a special problem. During oogenesis, large amounts of H2A, H2B, and H2Av are deposited into *Drosophila* eggs as protein so that the fertilized embryo has enough H2A and H2B to assemble the chromatin of thousands of nuclei (Figure S6). Because the embryo starts out with but a single diploid nucleus, this great imbalance of histones to DNA has the potential to interfere with normal development. Overexpression of histones can indeed cause embryonic lethality [22]. We propose that sequestration on lipid droplets keeps the maternally provided histones in an inactive state until they are needed; lipid droplets thus serve as a "buffer" to limit the concentration of free histones. Sequestration on lipid droplets may also protect these histones from the surveillance mechanism that destroys histones not packaged into chromatin [21]. Because H3 also seems to be in excess in early embryos (Figures 2D and 3B) but is not associated with lipid droplets, there may exist alternative mechanisms to keep it inactive (e.g., binding to H3/H4-specific chromatin-assembly factors).

In *Drosophila* embryos, the time-course of histone levels on lipid droplets (Figures 5A and 5B) suggests that the stored histones are not utilized at the very beginning of embryogenesis (phase 0 and phase I), but between 2 and 12 hr of development. The number of nuclei goes up exponentially during the initial cleavage and syncytial blastoderm stages, so the demand for histones increases tremendously. S phase 14, at the beginning of phase II, requires more new histones than all previous S phases combined. Generating enough histones quickly may tax the protein synthesis machinery, especially because zygotic gene expression sets in massively in phase II (midblastula transition), requiring translation of many new proteins. Using stores of presynthesized housekeeping proteins such as histones likely allows for quicker development. Also, because transcription is associated with the loss of H2A/H2B heterodimers from chromatin [23], the lipid droplets may provide the extra H2A and H2B needed to replace them.

Lipid Droplets as Protein-Storage Organelles

The proteome of *Drosophila* embryonic lipid droplets (Tables 1 and 2 also Tables S1 and S2) contains many proteins with known functions elsewhere in the cell. Although these proteins may be multi-functional, with new roles in droplet biology, the histone precedent raises the exciting possibility that some of these proteins are stored on droplets for later use. This hypothesis—that lipid droplets generally act as storage sites for proteins in temporary excess—helps unify a number of disparate observations from previous studies of lipid droplets. For example, the yeast protein Erg1p, an enzyme involved in steroid metabolism, is present on lipid droplets in an inactive form. It was proposed that under certain conditions Erg1p could translocate to the ER and become active [24], as if this enzyme were stored on droplets until it was needed elsewhere. In mammalian cells, an enzyme responsible for purine biosynthesis (IMPDH) translocates to the surface of lipid droplets under certain conditions [25], as does α-Synuclein, a protein linked to Parkinson's disease [26]. The droplet localization of two Hepatitis C virus proteins [27] is consistent with the hypothesis that proteins can be stored on droplets to avoid the degradation machinery.

In some cases, localization to droplets is induced by excessive cellular levels of the protein; stomatin (a lipid-raft-associated integral membrane protein) localizes to lipid droplets when it is overexpressed [11], and caveolins relocate to droplets [28, 29] when their concentration in the ER is increased by overexpression, by retention at the ER via an ER retrieval signal, or by the inhibition of vesicular transport from ER by brefeldin A. As with histones, droplet association is reversible: When protein synthesis is blocked, stomatin redistributes to vesicles [11], and after brefeldin A washout, caveolins are removed from the droplets [30].

Individually, these observations might have appeared to be simply curiosities. But in the light of the controlled sequestering and release of histones in *Drosophila* embryos, they point to a more general role of lipid droplets as storage depots, controlling the concentration and availability of a range of proteins. We propose that lipid droplets serve a general "buffering" role that makes it possible to sequester proteins that might otherwise be prematurely degraded or whose uncontrolled presence could cause trouble. Such functions are likely to be especially important in early embryos because eggs are loaded with large amounts of maternally provided proteins needed for later embryonic development.

This protein sequestration hypothesis provides an intriguing explanation for the previously described correlation between stress and lipid droplets. The number of lipid droplets per cell increases in a wide range of different disease states, from osteoarthritis to liver degeneration and cartilage overproliferation [31–36]. Higher droplet number may increase buffering capacity when the cell struggles with environmental challenges resulting in improperly folded proteins or imbalanced quantities of proteins involved in stress responses. For example, certain point mutants in the GTPase Arl8b, presumed to be improperly folded, relocalize to lipid droplets [37].

In many cell types from insects to mammals, lipid droplets move actively along microtubules (discussed in [9]). Such motion could facilitate the delivery of droplet-stored proteins. Our results suggest that this indeed happens for histones in *Drosophila* embryos. Lipid droplets are located basally just before cellularization (Figure 4D), and subsequent apical transport ensures that they are present in the cells of the blastoderm and not outside, trapped in the central yolk [7, 15]. In the absence of this apical transport, zygotic histone production is enhanced (Figure 5D), presumably because the cells are deprived of the maternal supply of histones. Assessing the organismal consequences of the loss of storage of histones or other proteins (e.g., robustness or speed of development) is an exciting avenue for future investigations, but in principle it could profoundly affect overall fitness.

Thus, droplet-bound proteins may be "passengers" that hitch a ride on droplets to get to their ultimate destination; protein availability might in part be controlled by the droplets' location relative to the protein's destination. Intriguingly, lipoprotein particles, which resemble lipid droplets structurally but are found in the extracellular space, function as vehicles for the movement of lipid-bound morphogens and glycophosphatidylinositol-linked proteins [38].

Table 2. Proteins Represented Significantly in the Lipid-Droplet Fraction

Cytosolic/Vesicular/Unknown

*	CG6203	Fmr1	RNA binding
	CG8732	l(2)44DEa	fatty acid metabolism
	CG5915	Rab7	GTP binding/ GTPase activity
	CG9575	Rab35	GTP binding/ GTPase activity
	CG3664	Rab5	GTP binding/ GTPase activity
	CG7073	sar1	GTP binding/ GTPase activity
	CG13388	AKAP200/A kinase anchor protein 200	Protein kinase binding
	CG13383	protein phosphatase 2A at 29B	Protein phosphatase activity
	CG8231	l(1)G0022	chaperone
	CG5525		chaperone
	CG7033		chaperone
	CG2512	alpha-tubulin at 84D	cytoskeleton
	CG33106	mask/multiple ankyrin repeats single KH domain	cytoskeleton
	CG2637	FS(2)Ket/importin beta	protein carrier activity
	CG8947	26-29-p/26-29kDa proteinase	protein modification
	CG5014	Vap-33-1	synaptic vesicle
	CG6540		unclassified
	CG5726		unclassified
	CG7630		unclassified
	CG32694		unclassified
	CG5167		unclassified
	CG5175		unclassified
	CG18212		unclassified
	CG14309		unclassified
	CG5295		unclassified
	CG12055	glyceraldehyde 3 phosphate dehydrogenase1	Aminoacid metabolism
	CG7070	Pyk/pyruvate kinase	glycolysis
	CG9748	bel/belle	Nucleic acid binding
	CG31299	nocturnin	Nucleic acid binding
	CG15081	l(2)03709	Nucleic acid metabolism
	CG1259		Structural constituent of cuticle

Nucleus

	CG11856	Nup358	nuclear pore
	CG6944	lam/lamin	cytoskeleton
	CG31612		

ER

	CG5520	gp93/glycoprotein93 (BiP homolog)
	CG8983	ERp60
	CG9022	Ost48/oligosaccaryltransferase 48kDa subunit
	CG2522	GTP-bp/GTP binding protein
	CG5885	
	CG3725	Ca-P60A/Calcium ATPase at 60A

Ribosome

	CG5920	sop/string of pearls
	CG13389	RpS13
	CG4651	RpL13
	CG15693	RpS20
	CG12775	RpL21
	CG7014	RpS5b
	CG2168	RpS3A
	CG4111	RpL35
	CG10944	RpS6
	CG4464	RpS19
	CG5502	RpL4
	CG7808	RpS8
	CG1883	RpS7
	CG8900	RpS18
	CG11276	RpS4
	CG3195	RpL12

Mitochondria

	CG5012	mRpL12/mitochondrial ribosomal protein L12
	CG11267	
	CG6030	ATPsyn-d/ATP synthase, subunit d
	CG7610	ATPsyn-gamma chain
	CG11015	
	CG7580	
	CG3731	

Table 2. *Continued*

Mitochondria		
	CG7834	
	CG8996	wal/walrus
	CG4169	
	CG1683	Ant2/Adenine nucleotide translocase 2
	CG4581	thiolase
	CG4389	
	CG10932	(acetylCoA C-acetyl transferase activity)
	CG6543	
	CG12262	(acyl-CoA dehydrogenase activity)
	CG9244	Acon/aconitase
	CG4233	Got2/Glutamate oxaloacetate transaminase 2
	CG12233	l(1)G0156
	CG5214	

Proteins identified by mass spectrometry in the lipid-droplet fraction and found in three or four of the six independent experiments, but not in more. * = Protein was identified 20–49 times. See the legend of Table 1 for more details.

Mechanisms of Storage

We do not yet understand how proteins localize to the droplets in a reversible manner, but a broad range of proteins do so: chromatin components (histones), integral membrane proteins (caveolin, stomatin), soluble enzymes (IMPDH), and viral proteins (Hepatitis C core protein). Our results indicate that for the highly positively charged histones, a charge interaction is critical. Thus, posttranslational modifications, e.g., phosphorylation and acetylation, that bring in negative charges could in principle be used to control histone-droplet localization.

Other proteins might be stored on lipid droplets in a partially unfolded state so that hydrophobic residues normally buried in the protein interior or at protein-protein interfaces are exposed and able to interact with the lipid-droplet core. Such a mechanism would make it possible to use a common strategy to deposit quite distinct proteins on the droplet surface. Unfolding proteins for storage and refolding them for release might require the assistance of chaperones, and there are indeed heat-shock proteins present in significant amounts in the lipid-droplet fraction (Tables 1 and 2).

The Lipid-Droplet Proteome

The proteomics approach can identify many droplet-bound proteins, but unavoidable contaminants will also be detected. Distinguishing between bona-fide droplet proteins and contaminants requires validation on a case-by-case basis, as we did with the histones. For relatively clean purifications, proteins present in high abundance are less likely to be contaminants. The converse is not true. For example, molecular motors are expected to be present in only a few copies per droplet and will be in relatively low abundance in any droplet preparation. As a compromise between completeness and purity, we present four proteomes. Combined, Tables 1 and 2 present repeatedly identified proteins highly likely to be on the droplets. Tables S1 and S2, which list additional, less frequently identified proteins, are likely to contain more contaminants but can also reflect proteins with a lower copy number. Overall, we identified more droplet proteins than previous studies, but known *Drosophila* droplet-associated proteins dynein and Klar [7, 8] were not found. Thus, our proteome(s) are not complete.

Some themes emerge from the proteins we recovered. The 29 most frequently isolated proteins (one, two, or three asterisks in Table 1) fall into just a few classes. (1) Five proteins known or implicated in lipid metabolism: the PAT family protein LSD2, the putative lipase CG9186, proteins linked to fatty acid and steroid metabolism (CG5112, scully), and the homolog of CGI-58, a protein mutated in a human neutral lipid-storage disease and known to reversibly associate with lipid droplets [39]. (2) Three histones. (3) Two ER proteins; other proteomic studies have found ER proteins associated with lipid droplets, a localization that may reflect the origin of lipid droplets from the ER. (4) Two chaperones. (5) Three yolk proteins and seven mitochondrial proteins: these might simply represent contamination of our preparations with other major embryonic organelles. However, given the histone example, they should not be dismissed without careful future analysis, especially in light of the fact that other mitochondrial markers appear to be absent from the droplet fraction (Figure 2B; also Figure S4). Lipid droplets and mitochondria cooperate in lipid metabolism [40], and mitochondria often surround lipid droplets in clusters [41], raising the possibility that these organelles dynamically exchange proteins. Mitochondrial proteins have also been reported in some other droplet proteomes [42, 43]. (6) Three ribosomal proteins and the translation factor EF1α; this might again indicate contamination with the abundant ribosomes or specific association of translating ribosomes with droplets, as observed previously [44]. (7) Among the five remaining proteins are two GTPases (Rab11, rasputin) that might be involved in signaling or membrane traffic to droplets. Rab proteins are similarly prominently represented in several mammalian droplet proteomes [10, 11, 45]. One of the challenges for the future will be to determine which of these proteins function at the lipid droplets and which ones are passenger proteins that have roles elsewhere. Comparing the recently determined proteome of droplets from the *Drosophila* larval fat body [43] with the proteome(s) reported here may provide a first inroad into this problem because passenger proteins are likely to be cell-type specific.

Conclusions

Having established the protein similarities between mammalian and *Drosophila* droplets, we can now use

the plethora of tools in this model system—biochemistry, genetics, and biophysical measurements [7, 9, 15, 16]—to dissect the precise function of droplet proteins and thus clarify the roles of their mammalian homologs.

Our analysis revealed a new class of droplet proteins that are transiently associated with droplets but eventually function elsewhere in the cell. We propose that histone sequestration protects the large excess of maternally provided histones from degradation and prevents the vast imbalance between histones and DNA from disrupting development. Lipid droplets may therefore not only provide a new developmental mechanism in early embryos but also play a general cellular role as transient depots to store or sequester proteins that are either misfolded or in temporary excess.

Supplemental Data

Supplemental data include Experimental Procedures, two tables, eight figures, and a movie and are available online at http://www.current-biology.com/cgi/content/full/16/18/1783/DC1/.

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