The Spatial Organization and Regulation of Triacylglycerol Synthesis

Dissertation zur Erlangung des Doktorgrades der Fakultät für Biologie der Ludwig-Maximilians-Universität München

vorgelegt von

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Thiam A.R., Antonny B., Wang J., Delacotte J., **Wilfling F**., Walther T.C., Beck R., Rothman J.E., Pincet F. COPI buds 60 nm lipid droplets from reconstituted water-phospholipidtriacylglyceride interfaces, suggesting a tension clamp function. *PNAS*, 2013 Aug 13; 110(33): 13244-9.

Krahmer N., Hilger M., Kory N., **Wilfling F.**, Stoehr G., Mann M., Farese R.V. Jr, Walther T.C. Protein correlation profiles identify lipid droplet proteins with high confidence. *Molecular & Cellular Proteomics*; 2013 May; 12(5): 1115-26.

Krahmer N., Guo Y., **Wilfling F.**, Hilger M., Lingrell S., Heger K., Newman H.W., Schmidt-Supprian M., Vance D.E., Mann M., Farese R.V. Jr, Walther T.C. Phosphatidylcholine synthesis for lipid droplet expansion is mediated by localized activation of CTP:phosphocholine cytidylyltransferase. *Cell Metabolism*; 2011 Oct. 5; 14(4): 504-15.

Declaration of Individual Contributions

Publication 1: Wilfling F., Wang H., Haas J.T., Krahmer N., Gould T.J., Uchida A., Cheng J.X., Graham M., Christiano R., Fröhlich F., Liu X., Buhman K.K., Coleman R.A., Bewersdorf J., Farese R.V. Jr, Walther T.C. Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocalizing from the ER to lipid droplets. *Developmental Cell*; 2013 Feb. 25; 24(4): 384-99.

Florian Wilfling designed and performed all experiments except the electron micrographs and the in vivo mouse studies of DGAT1 and DGAT2 overexpression in the intestine. He created all figures and co-wrote all parts of the paper.

Publication 2: Thiam A.R., Antonny B., Wang J., Delacotte J., **Wilfling F**., Walther T.C., Beck R., Rothman J.E., Pincet F. COPI buds 60 nm lipid droplets from reconstituted water-phospholipidtriacylglyceride interfaces, suggesting a tension clamp function. *PNAS*, 2013 Aug 13; 110(33): 13244-9.

Florian Wilfling helped designing experiments and contributed to writing the paper.

Publication 3: Krahmer N., Hilger M., Kory N., **Wilfling F.**, Stoehr G., Mann M., Farese R.V. Jr, Walther T.C. Protein correlation profiles identify lipid droplet proteins with high confidence. *Molecular & Cellular Proteomics*; 2013 May; 12(5): 1115-26.

Florian Wilfling helped to determine the localization of the LD proteins identified in the PCP-LD proteome by fluorescence microscopy.

Publication 4: Krahmer N., Guo Y., **Wilfling F.**, Hilger M., Lingrell S., Heger K., Newman H.W., Schmidt-Supprian M., Vance D.E., Mann M., Farese R.V. Jr, Walther T.C. Phosphatidylcholine synthesis for lipid droplet expansion is mediated by localized activation of CTP:phosphocholine cytidylyltransferase. *Cell Metabolism*; 2011 Oct. 5; 14(4): 504-15.

Florian Wilfling contributed supplement figure 1F and supplement figure 2B to the paper.

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Florian Wilfling Prof. Dr. Stefan Jentsch

Per aspera ad astra

Content

1 Abbreviations

aa	amino acid
ACSL	long-chain acyl-CoA synthetase
ADRP	adipose differentiation-related protein
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
ALG	asparagine linked glycosylation
ApoE	apolipoprotein E
ATGL	adipose triglyceride lipase
cAMP	cyclic adenosine monophosphate
CCT	CTP:phosphocholine cytidylyltransferase
CIDEC	cell death-inducing DFF45-like effector C
CK	choline kinase
CoA	Coenzyme A
COPI	coat protein complex I
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CTP	cytidine triphosphate
DG	diacylglycerol
DGAT	acyl-CoA:diacylglycerol O-acyltransferase
eLD	expanding LD
EM	electron microscopy
ePC	ether-linked phosphatidylcholine
ePE	ether-linked phosphatidylehtanolamine
ER	endoplasmic reticulum
FSP27	fat-specific protein of 27 kDa
GPAT	glycerol-3-phosphate O-acyltransferase

GUV	giant unilamellar vesicles
HCV	Hepatitis C virus
HSL	hormone-sensitive lipase
LD	lipid droplet
LPLAT	lyso-phospholipid acyltransferases
MG	monoacylglycerol
MGL	monoacylglycerol lipase
MTP	microsomal triacylglycerol transfer protein
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PCP	protein correlation profiling
PCTP	phosphatidylcholine transfer protein
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine N-methyltransferase
PI	phosphatidylinositol
PKA	protein kinase A
PLA_2	phospholipase A2
PS	phosphatidylserine
Rab18	Ras-related in brain 18
RNAi	RNA-interference
SE	sterol ester
sLD	static LD
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment receptor proteins
TG	triacylglycerol

2 List of Publications

Wilfling F.*, Thiam A.R.*, Olarte M.J., Wang J., Beck R., Gould T.J., Allgeyer E.S., Pincet F., Bewersdorf J., Robert Farese R.V., Jr. and Walther T.C. Arf1/COPI Machinery Acts Directly on Lipid Droplets and Enables their Connection to the ER for Protein Targeting. In preparation.

Thiam A.R., Antonny B., Wang J., Delacotte J., **Wilfling F.**, Walther T.C., Beck R., Rothman J.E., Pincet F. COPI buds 60 nm lipid droplets from reconstituted water-phospholipidtriacylglyceride interfaces, suggesting a tension clamp function. *PNAS*, 2013 Aug 13; 110(33): 13244-9.

Mejhert N., **Wilfling F.**, Esteve D., Galitzky J., Pellegrinelli V., Kolditz C.I., Viguerie N., Tordjman J., Näslund E., Trayhurn P., Lacasa D., Dahlman I., Stich V., Lång P., Langin D., Bouloumié A., Clément K., Rydén M. Semaphorin 3C is a novel adipokine linked to extracellular matrix composition. *Diabetologia*; 2013 Aug; 56(8): 1792-801.

Wilfling F., Wang H., Haas J.T., Krahmer N., Gould T.J., Uchida A., Cheng J.X., Graham M., Christiano R., Fröhlich F., Liu X., Buhman K.K., Coleman R.A., Bewersdorf J., Farese R.V. Jr, Walther T.C. Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocalizing from the ER to lipid droplets. *Developmental Cell*; 2013 Feb. 25; 24(4): 384-99.

Krahmer N., Hilger M., Kory N., **Wilfling F.**, Stoehr G., Mann M., Farese R.V. Jr, Walther T.C. Protein correlation profiles identify lipid droplet proteins with high confidence. *Molecular & Cellular Proteomics*; 2013 May; 12(5): 1115-26.

Wilfling F, Weber A, Potthoff S, Vögtle FN, Meisinger C, Paschen SA, Häcker G. BH3-only proteins are tail-anchored in the outer mitochondrial membrane and can initiate the activation of Bax. *Cell Death Differentiation*; 2012 Aug; 19(8): 1328-36.

Krahmer N., Guo Y., **Wilfling F.**, Hilger M., Lingrell S., Heger K., Newman H.W., Schmidt-Supprian M., Vance D.E., Mann M., Farese R.V. Jr, Walther T.C. Phosphatidylcholine synthesis for lipid droplet expansion is mediated by localized activation of CTP:phosphocholine cytidylyltransferase. *Cell Metabolism*; 2011 Oct. 5; 14(4): 504-15.

Weber A., Paschen S.A., Heger K., **Wilfling F.**, Frankenberg T., Bauerschmitt H., Seiffert B.M., Kirschnek S., Wagner H., Häcker G. BimS-induced apoptosis requires mitochondrial localization but not interaction with anti-apoptotic Bcl-2 proteins. *Journal of Cell Biology*; 2007 May 21; 177(4): 625-36.

3 Summary

Most cells and organisms store metabolic energy in times of excess supply in the form of triacylglycerol (TG), packaged into cytosolic lipid droplets (LDs). LDs are dynamic organelles consisting of a neutral lipid core bounded by a monolayer of phospholipids into which specific proteins are incorporated (Walther and Farese, 2012). The current model for LD formation posits that they emerge from the ER. There, a TG lens forms between the ER membrane leaflets, eventually budding as a droplet into the cytosol (Walther and Farese, 2012). How proteins and newly synthesized neutral lipids are delivered to nascent LDs is unclear.

In the present thesis I investigated the mechanism of protein targeting from the ER to LDs and the importance of the targeted proteins for LD growth. Most of the enzymes of the TG synthesis pathway (e.g. ACSLs, GPATs, AGPATs, DGATs) are thought to be localized in the endoplasmic reticulum and contain several predicted transmembrane domains (Coleman and Lee, 2004). Fluorescence microscopy combined with high confidence proteomics identified that specific isoenzymes catalyzing each step of *de novo* TG synthesis localize to forming LDs. As a marker protein for this relocalization process I focused on GPAT4, which is the rate-limiting enzyme in the TG synthesis pathway (Coleman and Mashek, 2011). I showed that GPAT4 re-localizes from the ER along ER-LD membrane bridges to the surface of a subset of nascent LDs upon culture with oleate. At the LD surface, these enzymes mediate LD growth of a subpopulation by local TG synthesis. A second LD subpopulation, not targeted by these enzymes, does not expand after formation.

Knockdown of the COPI machinery leads to the lack of expanding LDs and results in smaller, uniformly sized LDs, while depletion of the COPI machinery further impairs targeting of some proteins, including ATGL, to LDs. I show that GPAT4 targeting to LDs is dependent on the COPI machinery. My current model suggests that the COPI machinery modulates the LD surface, allowing the formation of connections between the ER and LDs for targeting of key TG metabolism enzymes, such as GPAT4. Our data suggest that, in addition to the classical role in vesicular trafficking, the COPI machinery also removes surfactant phospholipids from the LD-delimiting monolayer by budding 60 nm nano-LDs from the surface of LDs. Modulation of the LD surface,

in turn, is required and sufficient to allow targeting of GPAT4 even in the absence of a functional COPI machinery.

My findings highlight a general mechanism of how specific proteins re-localize from the ER to LDs, explaining how TG synthesis is coupled with LD growth and identify LD subpopulations based on their capacity for LD localized TG synthesis. The key features of GPAT4 ER-to-LD targeting and function in LD growth are conserved between *Drosophila* and mammalian cells. My results further reveal a new mechanism of protein targeting to LDs, which operates by modification of the target LD surfaces to establish connections that allow proteins to access cytosolic LDs.

4 Aims

The global prevalence in obesity is linked to an increased prevalence of insulin resistance and pandemic type 2 diabetes. In the U.S., more than one-third of the adults (35.7%) are obese (Ogden et al., 2012). At the heart of these diseases lies excessive storage of neutral lipids, such as TG. TGs are stored in cytosolic LDs. LDs consists of a neutral lipid core surrounded by a phospholipid monolayer harboring a set of specific proteins (Walther and Farese, 2012). TG is synthesized by enzymes of the glycerolphosphate pathway (Yen et al., 2008). Many of these enzymes are found in the ER, mitochondria or in mitochondria-associated membranes and occur in multiple isoforms (Coleman and Lee, 2004). Most of the TG synthesis enzymes are predicted multitopic trans-membrane proteins present in the ER. However, a subset of TG synthesis enzymes is strongly enriched in the purified LD fraction analyzed by proteomics. Other proteins, such as Dga1 or METTL7B, first localize to the ER and then concentrate on LDs during their formation (Jacquier et al., 2011; Zehmer et al., 2009; Zehmer et al., 2008). How re-localization of ER proteins to LDs is mechanistically achieved is unknown. Whether and how newly synthesized TG is delivered to nascent LDs after the initial formation is unclear. The current model for LD formation posits that LDs emerge from the ER as a TG lens between the ER membrane leaflets that bud as a LD into the cytosol (Walther and Farese, 2012).

I propose that cytosolic LDs grow by local TG synthesis at the LD surface. I will determine the spatial organization of TG synthesis enzymes and their function for LD formation and growth by completing the following specific aims:

Aim 1: Determine the spatial organization of TG synthesis enzymes in *Drosophila* S2 cells. Our proteome data from *Drosophila melanogaster* S2 cells suggest that a subset of TG synthesis enzymes localizes to LDs. I will test this hypothesis by determining the localization of fluorescently tagged TG synthesis enzymes in respect to known marker proteins.

Aim 2: Determine the mechanism of TG synthesis enzyme re-localization from the ER to LDs. Some ER proteins are able to re-localize from the ER to LDs by (a) yet unknown mechanism(s). Our preliminary data suggest that enzymes of the TG

synthesis pathway, such as GPAT4, follow this pathway. I will determine the topology of GPAT4, the rate-limiting enzyme in the TG synthesis pathway.

Aim 3: Determine the function of the TG synthesis enzyme re-localization in relationship to the growth of LDs. LDs most likely form at the ER. In many cell types, their size and number varies strongly, suggesting that LDs grow after their initial formation. How size and number of LDs are regulated and coordinated is unknown. I will test the hypothesis that LDs grow by locally generating TG, catalyzed by enzymes located on the LD surface.

The proposed work addresses a fundamental question. How is metabolic energy stored in TG during times of excess supply? In particular, I will reveal the mechanism of LD expansion and additionally give insights into trafficking of proteins from the ER to LDs.

5 Introduction

Almost all cells and organisms store metabolic energy in times of excess supply. The most compact storage form of metabolic energy is triacylglycerol (TG) that is packaged in cytosolic organelles called lipid droplets (LDs). These spherical organelles are present in almost all organisms and contain, depending on the cell type, other neutral lipids such as sterol esters (SEs). For a long time LDs were considered inert cytoplasmic inclusions of neutral lipids. In the last decade it has been shown that LDs are not inert structures but that the formation and turnover of these organelles are highly regulated according to cellular needs for energy. Besides storing lipids, LDs are linked to many cellular functions including energy generation, membrane synthesis, viral replication, and protein degradation. Excessive storage of neutral lipids in LDs is a key step to pathogenesis of many metabolic diseases, such as obesity, diabetes and atherosclerosis.

The number and size of LDs can vary greatly. How LDs form and grow is still poorly understood. To understand how LDs grow after their initial formation, I investigated the spatial organization of TG synthesis enzymes and their contribution to LD expansion.

5.1 Structure and composition of LDs

LDs are the main storage organelle for neutral lipids in the cell. They can be found in most prokaryotes and eukaryotes. They consist of a neutral lipid core surrounded by a phospholipid monolayer harboring a set of specific proteins (Figure 1). The number and size of LDs can vary greatly, from 50 nm diameter found in milk-secreting cells to 200 µm diameter found in mature adipocytes (Murphy, 2001). Depending on the energy intake number and size of LDs can change rapidly by several orders of magnitude (Guo et al., 2009).

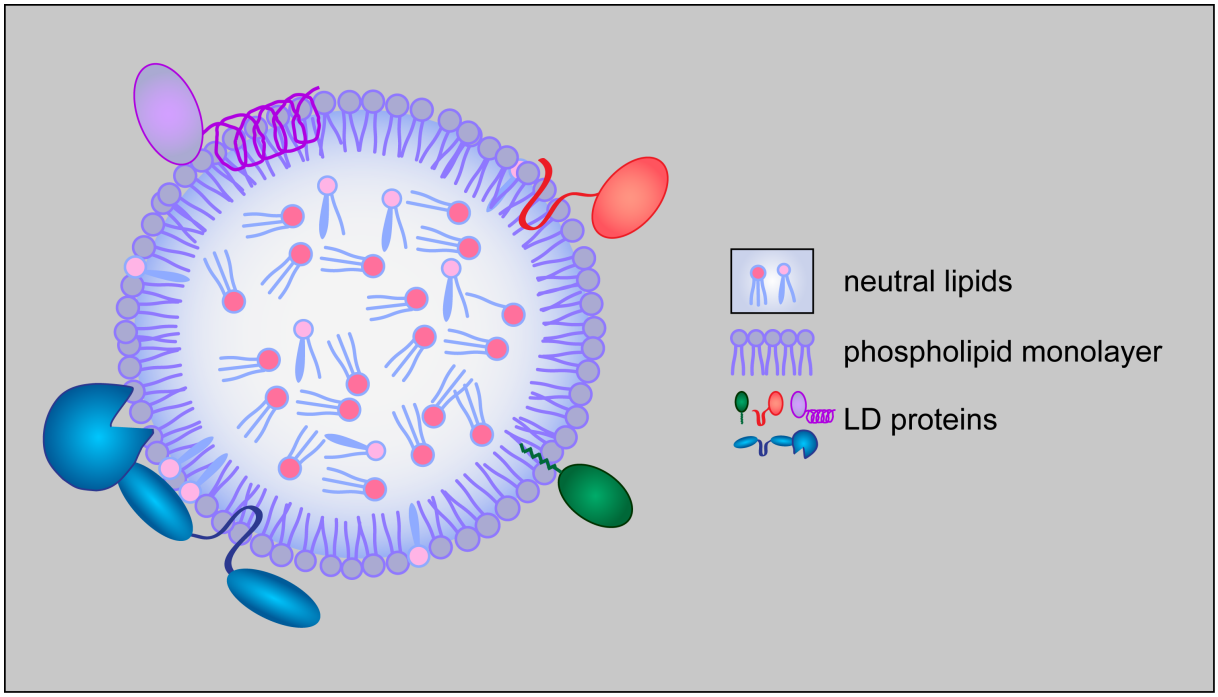


Figure 1: Architecture of a cytosolic lipid droplet. Schematic overview of the structure and composition of a cytosolic lipid droplet.

5.1.1 Phospholipid composition

Although LDs originate from the endoplasmic reticulum (ER) they have a distinct phospholipid composition that is different from that of the ER and other organelles (Tauchi-Sato et al., 2002). The composition of the phospholipid monolayer is dependent on the cell type and includes many diverse phospholipid species. The main phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), ether-linked phosphatidylcholine (ePC), and ether-linked phosphatidylehtanolamine (ePE). The monolayer of LDs is further enriched in lysoPE, lysoPC, free-cholesterol and PC but deficient in sphingomyelin, phosphatidylserine (PS), and phosphatidic acid (PA) when compared to total membranes (Bartz et al., 2007a; Tauchi-Sato et al., 2002). In CHO cells more than 160 phospholipids species with varying head groups and side chains were detected (Bartz et al., 2007a).

The hydrophobic core of LDs consists of neutral lipids, most of them TGs and SEs. Their relative amount varies significantly among cell types. In different cells types, depending on the cell function, various other neutral lipids are found in the core of

LDs. For example retinyl esters are found in LDs of hepatic stellate cells as well as in LDs isolated from bovine retinal pigmented epithelium (Orban et al., 2011; Walther and Farese, 2012). In some mammalian cells LDs can also contain wax esters and ether lipids, which are derived from peroxisomes (Bartz et al., 2007a). The production of these lipids allows storage and detoxification of many neutral lipids.

5.1.2 Protein composition

The LD is the only compartment in the cell with a phospholipid monolayer instead of a bilayer. This unique structure of LDs and their specific phospholipid composition allow targeting of a specific set of proteins. The first protein identified to be specific for LDs was perilipin1 (Greenberg AS 1991). Today five members of the perilipin family are known (perilipin1-5). Analyses of LD proteomes in different organisms resulted in various lists of LD-associated proteins (Athenstaedt et al., 1999; Beilstein et al., 2013; Beller et al., 2006; Brasaemle et al., 2004; Cermelli et al., 2006; Ding et al., 2012a; Ding et al., 2012b; Fujimoto et al., 2004; Ivashov et al., 2013; Liu et al., 2004; Orban et al., 2011; Sato et al., 2006; Wan et al., 2007; Zhang et al., 2011; Zhang et al., 2012). The overlap of the published proteomes is very low illustrating the difficulty of purifying these organelles to homogeneity since LDs are often found in very close proximity to other organelles (Figure 2). In the case of the ER, the distance between the two compartments can be less than 40 nm (Robenek et al., 2006). A major group of proteins identified by proteomics studies are enzymes of lipid metabolism such as acyl-CoA synthetases, lipases that act on triglyceride, and enzymes of sterol biogenesis. Furthermore, many well-characterized proteins have been found on LDs that previously had been assigned a different subcellular localization. It is possible that some of these proteins have a dual localization, such as histones (Cermelli et al., 2006), but likely most of these proteins are contaminants due to the high sensitivity of the mass spectrometer. The lack of a protocol for purifying LDs to homogeneity further complicates the identification of LD proteins. Ideally, a combination of different methods is used to confirm the lipid droplet localization of a protein.

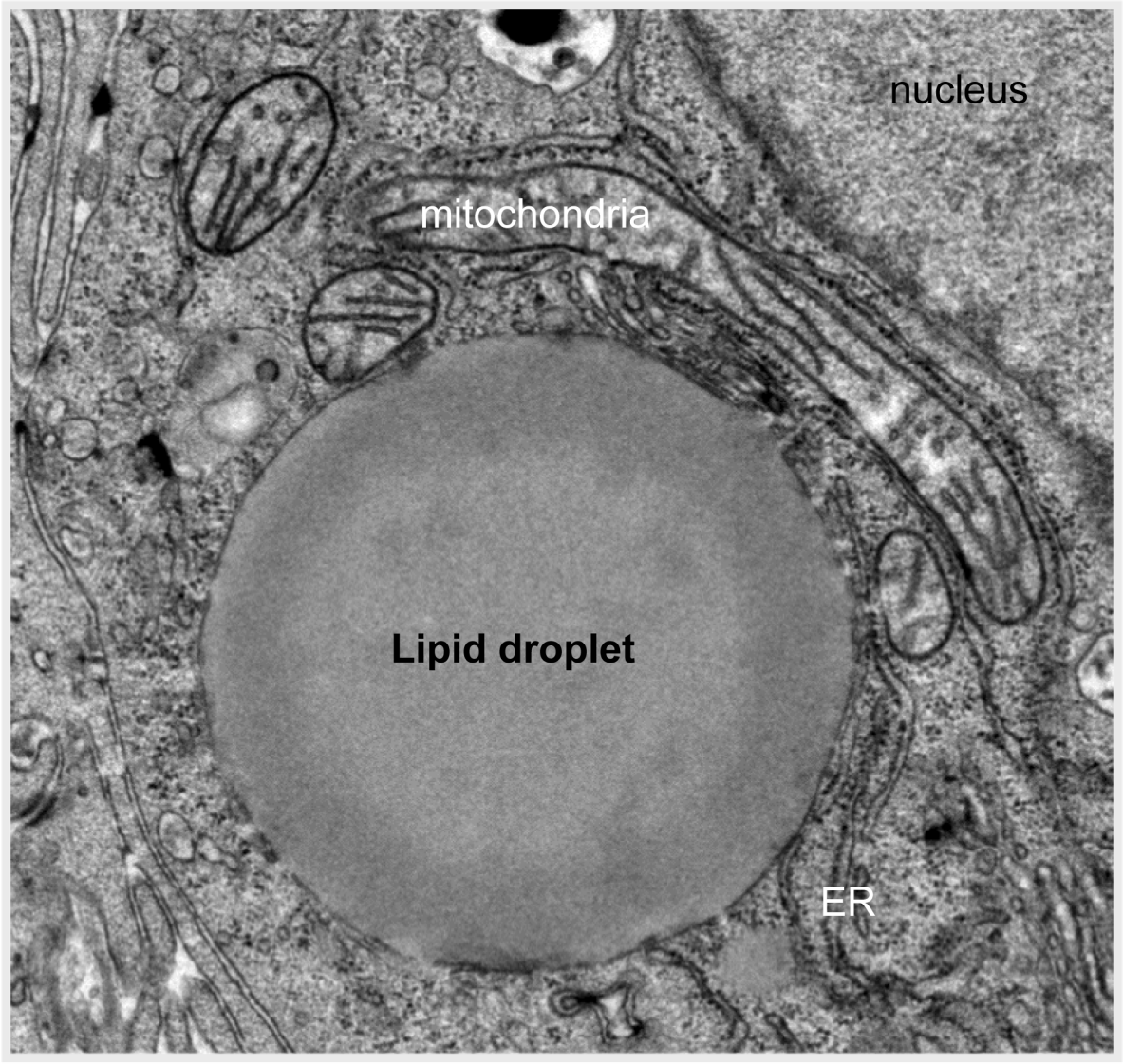


Figure 2: Lipid droplets are in close contact to other organelles. Shown is an electron micrograph of a single lipid droplet from a rat hepatoma cell (image courtesy of S. Stone and J. Wong). Abbreviation: ER, endoplasmic reticulum; LD, lipid droplet; M, mitochondria; N, nucleus

5.2 Protein targeting to LDs

It is poorly understood how proteins are targeted to LDs. Proteins identified in the LD proteome can be divided into at least three different structural classes: peripherally associated proteins such as the perilipin family members, lipid-anchored proteins of the small GTPase type, and monotopic integral membrane proteins. Polytopic membrane proteins have been reported in LD proteome lists, but so far no localization to LDs was confirmed for this group of proteins, suggesting that they represent contamination of the LD fraction with other membranes. Several

mechanism are emerging that mediate direct targeting to the LD surface (Walther and Farese, 2012). An overview of these features is shown in Figure 3.

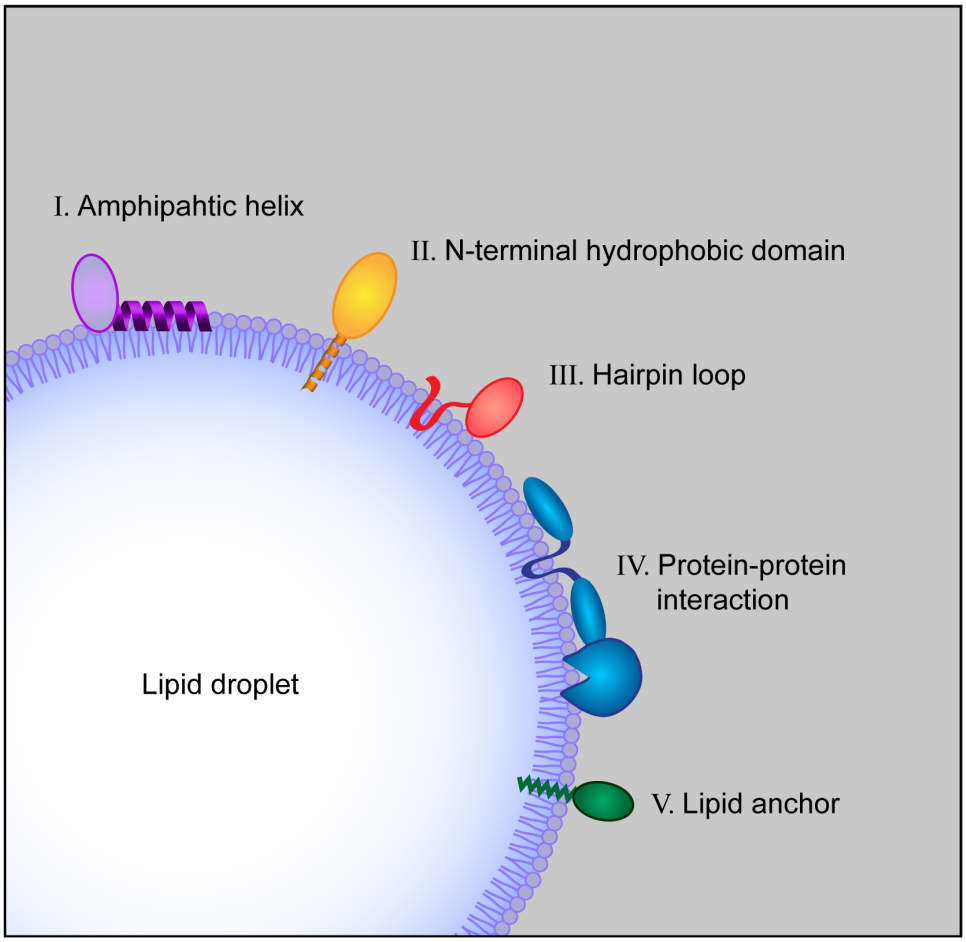


Figure 3: Schematic overview of different binding motives for lipid droplet proteins. (adapted from Walther and Farese, 2012).

5.2.1 Amphipathic α-helix

One structural feature that allows proteins to directly bind to the LD surface is the membrane-binding amphipathic α-helix. The amphipathicity results from the segregation of hydrophobic and polar residues between the two opposite faces of the α-helix (Drin and Antonny, 2010). Several different proteins harboring this motif have been described to target to the LD surface. Among these proteins perilipin3/TIP47 is the best-characterized protein. The crystal structure of the C-terminal portion of perilipin3 reveals four amphipathic helices that form a hydrophilic bundle similar to the one found in apolipoprotein E (ApoE) (Hickenbottom et al., 2004). During lipid binding the amphipathic helix bundle of ApoE opens to expose the hydrophobic faces

of the amphipathic helices. These interact with the surface phospholipid monolayer of lipoproteins (Hatters et al., 2006; Lu et al., 2000). Based on the high structural homology to ApoE, similar properties may allow perilipin3 to bind to the LD surface. It is yet unknown how the binding specificity of amphipathic-helices to the LD surface compared to other membranes is accomplished. For perilipin3 it is suggested that it binds diacylglycerol (DG) rich membranes in the ER during the formation of LDs (Skinner et al., 2009). Other examples for proteins targeted to the LD surface via amphiphatic-helices are the Hepatitis C virus (HCV) core protein, viperin (endogenous inhibitor of HCV), and CTP:phosphocholine cytidylyltransferase (CCT) (Barba et al., 1997; Hinson and Cresswell, 2009).

5.2.2 Short hydrophobic regions localized at the N-terminus

Similar to signal sequence-containing or tail anchor proteins, which possess a transmembrane domain at the N- or C-terminus, proteins have been identified that bind to LDs via a N-terminal short hydrophobic domain. This domain is necessary and sufficient for the targeting of proteins to the LD surface. The most studied proteins of this group are AAM-B and ALDI (Turro et al., 2006; Zehmer et al., 2009; Zehmer et al., 2008). It is notable that these proteins localize to the ER in the absence of LDs. In cells where protein synthesis is blocked the existing protein pool can still target to LDs (Zehmer et al., 2009). How the targeting of these proteins from the ER to LDs is achieved is not clear.

5.2.3 Internal hydrophobic domains that are embedded in the LD

A subset of proteins binds to the monolayer surface of LDs with an internal hydrophobic domain, which likely forms hairpin structures. This topology allows a membrane-embedding of the protein in such a way that the N- and C-terminus of the protein are facing the cytosol with the hydrophobic domain of the hairpin inside the core of the LD. Proteins harboring a hairpin domain could be targeted to a monolayer or bilayer. Examples for such proteins are the plant oleosins. *Arabidopsis* oleosins are integrated into membranes by a cotranslational, translocon-mediated pathway *in vitro*. The long hydrophobic hairpin domain prevents the C-terminal domain from

translocating into the ER lumen (Abell et al., 2002). This feature of the hairpin domain leads to a topology where the protein is embedded into the membrane by the hairpin with the N- and the C-terminus facing the cytosol. After translocation into the ER oleosins are targeted to LDs. Within the hydrophobic hairpin the “proline knot” motif is important for targeting of oleosins to LDs. The proline knot motif of oleosins is a short sequence of 12 amino acids (aa), which contains 3 prolines. These prolines likely induce a kink in the two hydrophobic helix segments leading to the hairpin topology. Examples for proteins targeted to LDs by a hairpin motif in mammalian cells are caveolins and 17-hydroxysteroid dehydrogenases. Caveolins are found in many mammalian cells and contain a similar hairpin motif (Fujimoto et al., 2001; Ostermeyer et al., 2001; Pol et al., 2001). Caveolins are mainly found at the plasma membrane but a fraction of them has been identified to localize to LDs under physiological conditions (Fujimoto et al., 2001; Ostermeyer et al., 2001; Pol et al., 2001).

5.2.4 Interaction with lipid droplet-bound proteins

Another possibility for proteins to localize to LDs is by interacting with LD-bound proteins. An example is the recruitment of hormone-sensitive lipase (HSL) to LDs. Under non-lipolytic conditions HSL is mostly cytosolic. Upon hormonal stimulation perilipin1 as well as HSL are getting phosphorylated. This leads to the interaction between HSL and perilipin1 and to the recruitment of HSL to LDs (Granneman et al., 2007; Sztalryd et al., 2003).

5.2.5 Lipid anchors

Instead of binding to LD-bound proteins, proteins lacking hydrophobic protein segments can bind to cellular membranes through a lipid-anchor. The lipid anchor is a posttranslational modification by which at least one lipid moiety or fatty acid is attached to the protein. Naturally occurring lipid anchors are isoprenyl groups, e.g. farnesyl or geranylgeranyl, and saturated fatty acyl groups e.g. palmitoyl or myristoyl (Magee and Seabra, 2005). The by far most studied class of lipid-anchored proteins is the Ras family of small GTPases. Depending on the lipid anchor these proteins are

sorted and targeted to different membranes with different signaling effects (Mor and Philips, 2006). The small GTPase, Rab18 (Ras-related in brain 18), localizes to LDs by a C-terminal prenyl anchor combined with protein-protein interactions (Martin et al., 2005). This induces close apposition of LDs to ER. Interestingly, Rab18 contains a mono-cysteine prenylation motif rather than the di-cysteine prenylation motif that is present in most other Rab family members (Martin et al., 2005; Ozeki et al., 2005).

5.3 Cellular pathways involved in targeting proteins from other organelles to lipid droplets

Most LD proteins are cytosolic proteins, which are targeted by the described domains to LDs from the cytosol. How this is mechanistically achieved is mainly unknown. A large number of LD-associated proteins identified in the proteomes have been suggested to be membrane-bound proteins of the ER. Recently, a mechanism has been suggested according to which proteins harboring the described hairpin motifs or N-terminal hydrophobic domains are able to diffuse to LDs by ER-LD connection sites. As shown for Rab18, certain proteins can mediate the interaction of LDs with other organelles. Contact sites between LDs and other organelles could allow transfer of membrane bound proteins from one organelle to another (Figure 4). Cytosolic LDs could be either transiently or permanently associated with the ER. Given the mentioned topology features, certain membrane-bound proteins could diffuse along these connections to the surface of LDs. A number of proteins have been identified that are localizing to the ER in the absence of LDs, but when LDs are formed these proteins change their localization to the monolayer of LDs. Examples are the yeast proteins Dga1 and Erg6 as well as the mammalian proteins AAM-B and UBXD8. The movement of the yeast protein Dga1 from the ER membrane to LDs is independent of temperature and energy, suggesting that the relocalization is not mediated by classical vesicular transport routes (Jacquier et al., 2011). Connections of the ER and LDs have been visualized by electron microscopy in *Neurospora crassa*, maturing cotyledons of *Pisum* and *Vicia faba* (Wanner et al., 1981). Proteins might also target to LD-associated membranes. In freeze-fracture electron microscopy studies on lipid-laden macrophages LDs appeared to be wrapped by ER membranes in an “egg-cup” like fashion (Robenek et al., 2006). Given the resolution

limit of light microscopy, targeting to ER subdomains close to LDs could be falsely interpreted as targeting to the surface of LDs. Other interactions of LDs with endosomes, mitochondria, and peroxisomes have been suggested (Murphy et al., 2009). These interactions with other organelles could explain the broad spectrum of proteins identified in many different proteomic studies.

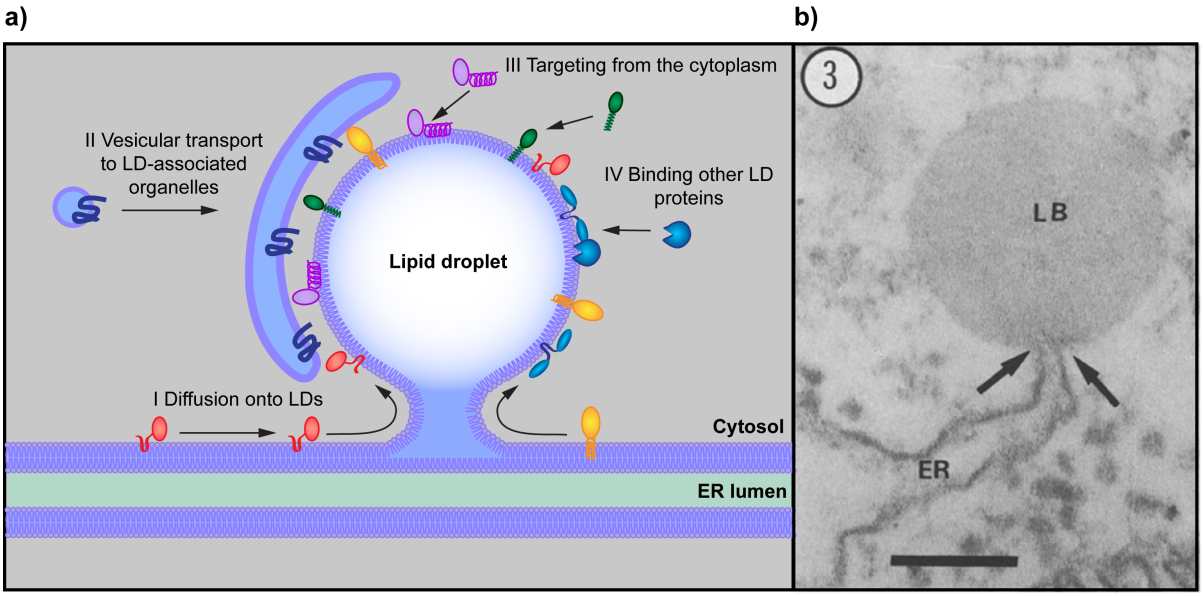


Figure 4: Possible mechanism of proteins targeting LDs. a) Proteins with a hydrophobic hairpin domain like the triacylglycerol synthesis enzyme DGAT2 might diffuse along membrane bridges onto LD. Proteins might target from the cytosol directly to LDs either by binding to the LD surface or by interaction with LD proteins. Some proteins might target to LD-associated membranes and this might be achieved by vesicular trafficking by the COPI machinery (adapted from Walther and Farese, 2012). b) The Electron micrograph shows connectivity between the ER and a lipid droplet (adapted from Wanner et al., 1981). LB, lipid body; ER, endoplasmic reticulum.

Unbiased genome-wide screens in model systems, such as *Drosophila* cells, have started to reveal factors that are required for LD targeting of proteins (Beller et al., 2008; Guo et al., 2008). Specifically, members of the coat protein complex I (COPI) machinery, but not other proteins involved in secretory trafficking (e.g., COPII or clathrin), are required for normal LD morphology and function (Beller et al., 2008; Guo et al., 2008). The COPI machinery is a protein complex important for retrograde trafficking of proteins from the golgi back to the ER (Nickel et al., 2002). The depletion of Arf1/COPI proteins from cells leads to the formation of uniformly sized, lipolysis-resistant LDs that lack the major TG lipase adipose triglyceride lipase (ATGL) (Beller et al., 2008; Guo et al., 2008; Soni et al., 2009). Besides ATGL other proteins have been identified to be dependent on the COPI machinery for targeting to

LDs. Examples are phospholipase D and adipose differentiation-related protein (ADRP) (Nakamura et al., 2005; Soni et al., 2009). In one model the COPI machinery transports certain LD proteins by vesicular transport to LD-associated membranes (Figure 4). Interestingly, depleting the COPII machinery that is important for anterograde trafficking from the ER to the golgi has only a mild effect on the targeting of COPI dependent proteins, such as ATGL, to LDs (Soni et al., 2009). This mild effect on targeting of proteins to LDs suggests that classical trafficking might not be the mechanism that targets specific proteins to LDs or LD-associated membranes. It is currently unknown how the COPI machinery mediates the targeting of certain proteins to the LD surface and further studies are necessary.

5.4 Formation of lipid droplets

In the most cited model of LD formation, newly synthesized TGs accumulate in the lipid bilayer of the ER creating a fat lens between the leaflets of the ER membrane. The formation of TG drives the budding of nascent cytosolic LDs or, in cells that secrete TG, nascent lipoproteins (Figure 5). Lipoproteins are a mixture of proteins and lipids. The proteins serve to emulsify the lipid molecules. This allows the transport of fat through the aqueous environment of the cell and enables the secretion of fat out of the cell. The precise mechanism by which TGs are deposited into LDs is unknown. It is also unknown how the directionality of LD formation is achieved. Likely perilipin proteins such as perilipin3, which have been shown to bind very early to forming LDs, give directionality. Further it is unknown if the newly formed LDs stay connected or get detached from the ER. Surprisingly, genome-wide RNAi screens in *Drosophila* melanogaster or *Saccharomyces* cerevisiae have failed to identify proteins essential for LD formation. This suggests a protein-independent mechanism where LD formation is driven by emulsification. On the contrary, many proteins have been identified to be important for proper LD morphology. One example is seipin, a protein associated to congenital generalized lipodystrophy type 2 in human. The absence of seipin in yeast leads to “super sized” LDs. In addition to the first model, in which the release of TG between the two leaflets of the ER membrane drives LD formation, three alternative models have been proposed. The first model is the bicelle formation model, which predicts that LDs are excised from

both leaflets of the ER membrane bilayer as a bicelle (Ploegh, 2007). The second model is the vesicular budding model, in which LDs are initially formed within small bilayer vesicles harboring TG synthesis enzymes (Walther and Farese, 2009). The third model is the “eggcup” model, in which an ER enclosed LD forms through transport of TG from the ER to the LD (Robenek et al., 2006).

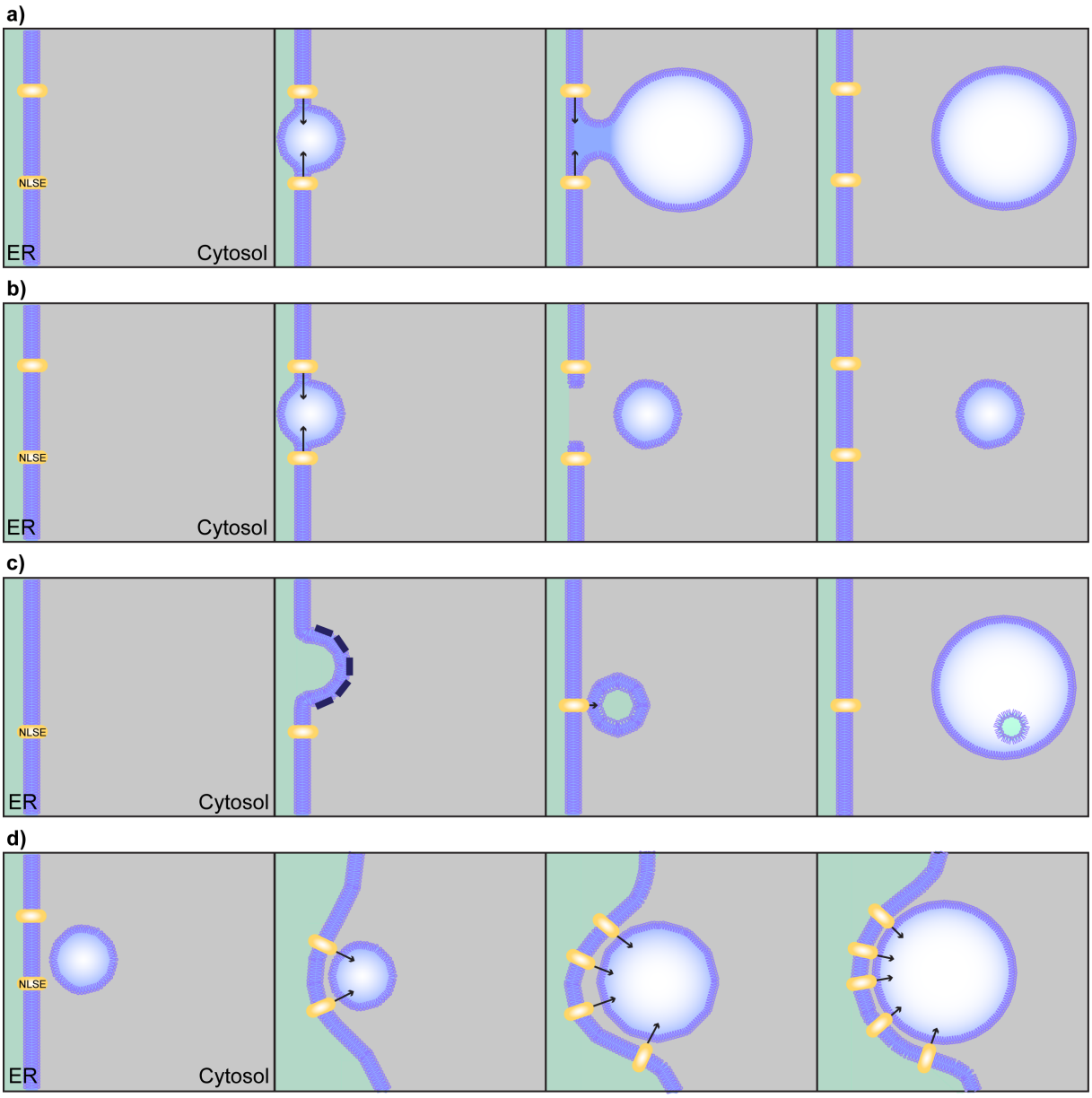


Figure 5: Different models of lipid droplet formation. a) LDs form by releasing TG in between the two leaflets of the ER membrane. b) LDs are formed in the ER similar to a) but are consequently excised from the membrane. c) LDs are formed from small bilayer vesicles. d) Enclosed LDs form by transporting TG and phospholipids from the adjacent organelle to the forming LD.

5.5 TG synthesis pathway

The driving force of *de novo* LD formation is the synthesis of neutral lipids such as TG. TG is synthesized when excess energy in form of fatty acids is available. Cells can absorb these fatty acids by passive diffusion or actively by fatty-acid transport and fatty-acid translocase proteins (Ehehalt et al., 2006; Schaffer and Lodish, 1995). To store the absorbed fatty acids in the form of TG they are activated to allow the esterification with glycerol. This is achieved through conjugation of fatty acids to coenzyme A forming fatty acyl-CoA. This reaction is energy-dependent (one ATP per conjugation) and is catalyzed by long-chain acyl-CoA synthetases (ACSLs) (Ellis et al., 2010). In the ER, *de novo* TG synthesis occurs in four sequential steps catalyzed by glycerol-3-phosphate O-acyltransferase (GPAT), 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP), and acyl-CoA:diacylglycerol O-acyltransferase (DGAT) enzymes (Figure 6). For the four classes of TG synthesis enzymes many isoforms exist with different tissue expression and biological function.

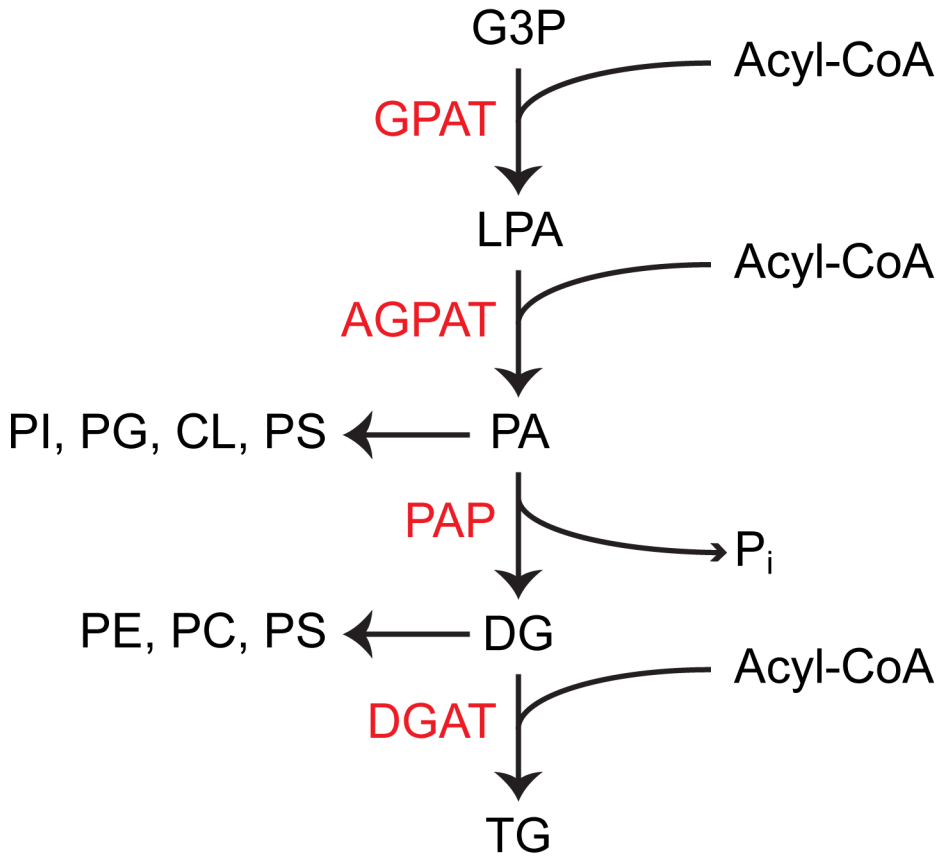


Figure 6: Pathway of TG synthesis. In the first step, catalyzed by GPATs, glycerol-3-phosphate (G3P) is esterified by a fatty acid coenzyme A ester to form lysophosphatidic acid (LPA). This is in turn acylated by AGPAT enzymes to from phosphatidic acid (PA), a precursor of the phospholipids PI, PG,

CL, and in yeast PS. PAP enzymes subsequently remove the phosphate group to form 1,2-diacylglycerol (DG), which then allows acylation by DGAT enzymes and the formation of triacylglycerol (TG). DG is also a precursor for the phospholipids PC, PE, and PS. Abbreviation: PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GPAT, glycerol-3-phosphate O-acyltransferase; AGPAT, 1-acylglycerol-3-phosphate O-acyltransferase; PAP phosphatidic acid phosphatase; DGAT, acyl-CoA:diacylglycerol O-acyltransferase.

5.5.1 GPAT family

The first and rate-limiting step in *de novo* TG synthesis is catalyzed by GPAT enzymes (Coleman and Lee, 2004). This enzyme class converts glycerol-3-phosphate and fatty acyl-CoA to 1-acylglycerol-3-phosphate (lysophosphatidate). In recent studies four mammalian GPAT isoforms have been identified (GPAT1-4). GPAT1 and GPAT2 localize to the mitochondrial outer membrane, whereas GPAT3 and GPAT4 localize to the ER (Wendel et al., 2009). In most tissues microsomal GPAT accounts for 90% of total GPAT activity. In liver, however, both microsomal GPAT (50–80% of total activity) and mitochondrial GPAT (20-50% of total activity) are expressed and active (Coleman and Lee, 2004).

5.5.2 AGPAT family

The second step of *de novo* TG synthesis is catalyzed by AGPAT enzymes, which convert 1-acylglycerol-3-phosphate and fatty acyl-CoA to 1,2-diacylglycerol-3-phosphate (phosphatidic acid). AGPAT activity has been found in mitochondrial and microsomal, as well as in plasma membrane fractions (Coleman and Lee, 2004). Due to sequence homology to known AGPAT enzymes from yeast and *E.coli* six different AGPATs have been identified in humans. The enzymatic activity for AGPAT1 and AGPAT2 has been experimentally confirmed and both these enzymes localize to the ER. In contrast, AGPAT3 localize to the Golgi (Schmidt and Brown, 2009) and AGPAT5 is suggested to localize to mitochondria (Prasad et al., 2011). The enzymes AGPAT3-5 show very low activity when compared to AGPAT2 suggesting that they may have different substrate specificity (Lu et al., 2005).

5.5.3 PAP family

The product of the AGPAT catalyzed reaction, phosphatidate, is a key molecule in lipid biosynthesis that serves as a precursor for all glycerophospholipids (Kent, 1995). It can be converted to CDP-diacylglycerol, a precursor of phosphatidylinositol, phosphatidylglycerol, cardiolipin, and in yeast to phosphatidylserine (Figure 6). Phosphatidate can also be dephosphorylated by PAP enzymes to produce diacylglycerol. Diacylglycerol is a precursor for the synthesis of TG, phosphatidylserine (in higher eukaryotes), phosphatidylcholine and phosphatidylethanolamine (Figure 6). Both soluble as well as membrane-associated fractions contain PAP activity (Kent, 1995). Membrane-associated PAP activity is associated with microsomal and plasma membrane fractions. Two PAP isoforms, known as PAP-1 and PAP-2, have been identified. PAP-1 is involved in TG and phospholipid synthesis whereas PAP-2 activity is important for signal transduction pathways at the plasma membrane. In mammals, three PAP-1 homologues Lipin1, 2, and 3 exist.

5.5.4 DGAT family

The final step of TG synthesis is catalyzed by DGAT enzymes, which covalently attach a third fatty acyl-CoA to a DG molecule to form TG and CoA (Coenzyme A). Two isoforms of DGAT enzymes exist in mammals: DGAT1 and DGAT2, which are different at a sequence level. Due to the localization of both enzymes to the ER it is believed that TG synthesis mainly occurs at the ER (Yen et al., 2008). DGAT2 has a higher affinity for its substrates than DGAT1 and is therefore more active at lower fatty acid concentrations than DGAT1. In contrast to DGAT2, DGAT1 has multiple acyltransferase activities using a broad spectrum of lipids as fatty acyl acceptors such as DG, monoacylglycerol, long-chain alcohols, and retinol. Both enzymes are integral membrane proteins with different membrane topology. DGAT1 is predicted to be a multiple spanning transmembrane domain and forms homotetramers (Yen et al., 2008). Mammalian DGAT2 contains two transmembrane domains or one single long hydrophobic region (aa 66–115) at the N-terminus that is embedded in the membrane bilayer (Yen et al., 2008). The subcellular localization of DGAT enzymes appears to be different. In plant cells DGAT1 and DGAT2 localize to distinct

punctuated regions in the ER suggesting the existence of different ER subdomains for TG synthesis (Shockey et al., 2006). Interestingly, one of the plant homologues of the microsomal GPATs (GPAT9) has been identified to localize to the same ER subdomains as DGAT2. The different localization of DGAT1 and DGAT2 within the ER suggests the existence of different complexes for TG synthesis in the ER (Gidda et al., 2011). The function of the different complexes is currently unknown. DGAT2 has been further identified to localize in close proximity to the surfaces of LDs (Yen et al., 2008). It is currently under debate whether DGAT2 localizes to the LD surface or if it localizes to ER subdomains that are in very close proximity to the LD. The hairpin-like transmembrane domain of DGAT2 would allow targeting of DGAT2 to the surface of LDs. Recent data from the yeast homologue Dga1 support this model (Jacquier et al., 2011).

5.6 Expansion of the lipid droplet core

Cells are able to expand existing LDs to store more TG in LDs. This requires local synthesis or transfer of TG from the ER to LDs. The localization of DGAT2 to LDs during fatty acid loading (Yen et al., 2008) (supplementation of oleate to the culture medium) is a hint that local synthesis of TG might occur on LDs in vivo. It is currently unknown if the proximal steps in TG synthesis also happen on LDs. If LDs are connected with the ER at all time, channeling of TG to LDs through membrane connections could be possible. Alternatively, transport proteins could transfer TG from the ER to the core of LDs. This has been shown in the case of nascent lipoproteins, where the microsomal triacylglycerol transfer protein (MTP) transfers lipids by a shuttle mechanism (Atzel and Wetterau, 1993). First, MTP binds the ER membrane and extracts individual lipids. Then, MTP dissociates from the ER membrane and diffuses to an acceptor membrane to deposit its bound lipid. Such a mechanism would be possible for cytosolic LDs but no lipid transfer proteins have been identified. LDs could expand alternatively or additionally by coalescence of small LDs to bigger ones. Recent work suggests that the fat-specific protein of 27 kDa (FSP27), a member of the cell death-inducing DFF45-like effector C (CIDEC) family, is involved in catalyzing LD fusion by transferring lipids between two adjacent LDs (Gong et al., 2011). This model is supported by the localization of FSP27 to LD-

LD contact sites (Gong et al., 2011). Overexpression of FSP27 in cells leads to increased LD size (Puri et al., 2007). However, CIDE proteins are not expressed in all tissues and FSP27 is mostly restricted to adipocytes. Some organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans,* lack CIDE proteins completely but expansion of LDs has still been observed. This suggests that FSP27 mediated LD fusion cannot provide a general mechanism for LD expansion. Another study has suggested that SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment receptor proteins) proteins mediate homotypic fusion between LDs (Bostrom et al., 2007). Knockdown of genes encoding SNAP23, syntaxin-5, and VAMP4 decreased the rate of LD fusion in mouse embryonic fibroblast NIH 3T3 cells (Bostrom et al., 2007). The classical role of SNARE proteins is to mediate homotypic fusion of bilayer-bounded vesicles with organelles. However, it is unclear how SNARE proteins could mediate fusion of two monolayers, and more mechanistic insights are necessary. In most cell types (besides in adipocytes) fusion of LDs is a rare event and therefore other mechanism are more likely important for LD expansion.

5.7 Expansion of the lipid droplet surface

Within a few hours LDs can expand rapidly and often their diameter increases more than 3 times leading to a more than 10 times increase in surface area. This increase in area needs to be covered by phospholipids to prevent coalescence of LDs. The most abundant phospholipids in the LD monolayer are PC and PE. Between these two phospholipids, PC is key for coating LDs and preventing their coalescence. The expansion of the LDs leads to an increased need of PC on the surface of LDs. In most cells, PC and PE are synthesized by an aminoalcoholphosphotransferase reaction, which uses DG and the activated form CDP-choline or CDP-ethanolamine, respectively. This biosynthetic pathway is called the Kennedy pathway and named after Eugene Kennedy who discovered it in 1956 together with his graduate student Samuel Weiss (Kennedy and Weiss, 1956). Synthesis of PC consists of three enzymatic steps. In the first step, choline kinase (CK) catalyzes the ATP-dependent phosphorylation of choline, forming phosphocholine, and ADP. In the second and rate-limiting step of the Kennedy pathway (Sundler and Akesson, 1975), CCT uses PC and cytidine triphosphate (CTP) to form the high-energy donor CDP-choline with

the release of pyrophosphate. CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) catalyzes the final reaction of the pathway, using CDP-choline and DG to form PC and CMP as byproducts. Depending on the cell type PC can be alternatively synthesized by conversion of PE into PC through three sequential methylations of the ethanolamine head group and phosphatidylethanolamine N-methyltransferase (PEMT) enzymes catalyze this reaction. PE is synthesized in a series of similar reactions except that ethanolamine is used instead of choline (Figure 7). Alternatively PE can be synthesized via decarboxylation of PS (Schuiki and Daum, 2009). The contribution of this pathway is organism- and cell type-dependent (Gibellini and Smith, 2010). Little is known how the need for phospholipids on growing LDs is sensed and how these phospholipids are consequently targeted to LDs. A genome-wide RNAi (RNA-interference) screen in Drosophila S2 cells identified genes important for LD morphology. Depletion of CK and CCT decreased LD number but increased LD size suggesting a fusion of LDs under these conditions due to decreased PC levels (Guo et al., 2008). The rate-limiting enzyme in the Kennedy pathway, CCT, is mainly regulated by translocation of the inactive protein from the cytoplasm to cellular membranes causing its activation. Binding of the amphipathic α-helix to the membrane activates CCT by relieving an inhibitory restraint in the catalytic domain (Friesen et al., 1999). Further it has been shown that CCT binding is increased for anionic membranes or membranes enriched in DG or PA. Binding is also enhanced when cellular membranes are “PC deficient” due to the overexpression of phospholipase D or depletion of choline (Kent, 2005). But the exact mechanism that triggers CCT translocation is still unknown. CCTs exist in two isoforms in most eukaryotes (CCT1 and CCT2 in Drosophila *melanogaster*). The primary structure is made up of four functional domains, the N-terminal nuclear localization signal, a catalytic core, a lipid-binding domain (long amphipathic a-helix) and a C-terminal phosphorylation domain (Gibellini and Smith, 2010). Phosphorylation and dephosphorylation of CCT is suggested to fine tune the membrane affinity of CCT. The soluble, inactive form of CCT is highly phosphorylated, whereas translocation to membranes is followed by dephosphorylation (Kent, 2005). Interestingly, both isoforms localize to the surface of LDs suggesting a role in PC homeostasis on LDs. How CK and CCT are regulated to maintain PC homeostasis on LDs during expansion is unknown.

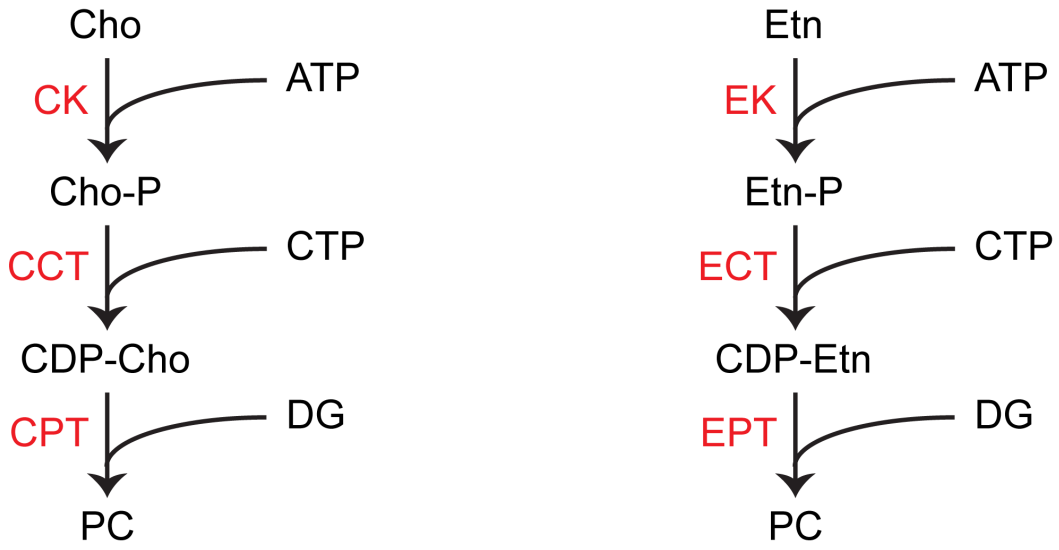


Figure 7: The Kennedy pathway. PC and PE are synthesized in three reactions from choline/ethanolamine. In the first step choline/ethanolamine are phosphorylated which are then used in the second step to form the activated form CDP-choline/ethanolamine. In the final step the activated head groups are bound to DG to form PC or PE. Abbreviation: Cho, choline; Cho-P, phosphocholine; CDP-Cho, cytidine-diphosphocholine; Etn, ethanolamine; Etn-P, phosphoethanolamine; CDP-Etn, cytidine-diphosphoethanolamine; PC, phsphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol; CK, choline kinase; CCT, CTP:phosphocholine cytidylyltransferase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; EK, ethanolamine kinase; ECT, CTP:phosphoethanolamine cytidylyltransferase; EPT, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase.

5.8 Breakdown of lipid droplets - Intracellular lipolysis

In times when energy or lipids are needed cells are able to metabolize the stored energy by hydrolysis of TG into glycerol and fatty acids. The hydrolysis of TG requires three consecutive steps and the activity of at least three different enzymes in mammals (Zechner et al., 2012). The first step is catalyzed by ATGL, converting TG into DG and a fatty acid. HSL catalyzes the second step, in which a second fatty acid is removed to yield monoacylglycerol (MG). MG is subsequently hydrolyzed by MG lipase (MGL) producing glycerol and another fatty acid (Figure 8). Due to low abundance of ATGL and HSL in some non-adipose tissues other lipases might be involved in hydrolysis of TG. Intracellular lipolysis is a strongly regulated process.

Lipolysis has mostly been studied in adipocytes and occurs according to a slightly different mechanism in oxidative tissue, where perilipin1 is absent. In adipocytes, lipolysis is stimulated during fasting by the catecholamine norepinephrine, which activates beta-adrenergic receptors coupled to G-proteins on the plasma membrane.

This stimulates adenylyl cyclase to generate cyclic AMP (cAMP). cAMP binds subsequently PKA (protein kinase A) increasing the activity of the enzyme (Duncan et al., 2007). Activation of PKA is an important step for the regulation of different proteins involved in intracellular lipolysis. PKA catalyzes the phosphorylation of perilipin1 at multiple sites. This causes the release of the bound coactivator protein CGI-58 from perilipin1, fully inducing ATGL's hydrolysis activity (Zechner et al., 2012). ATGL is further regulated on a transcriptional level as well as by the peptide inhibitor G0S2 (Zechner et al., 2012). PKA also phosphorylates HSL directly on multiple sites. This causes activation of HSL and subsequent translocation of the lipase from the cytosol to LDs. Interestingly, recent studies have shown that phosphorylated HSL binds to PKA phosphorylated perilipin1, thereby gaining access to the hydrophobic core of LDs (Miyoshi et al., 2007; Shen et al., 2009; Wang et al., 2009).

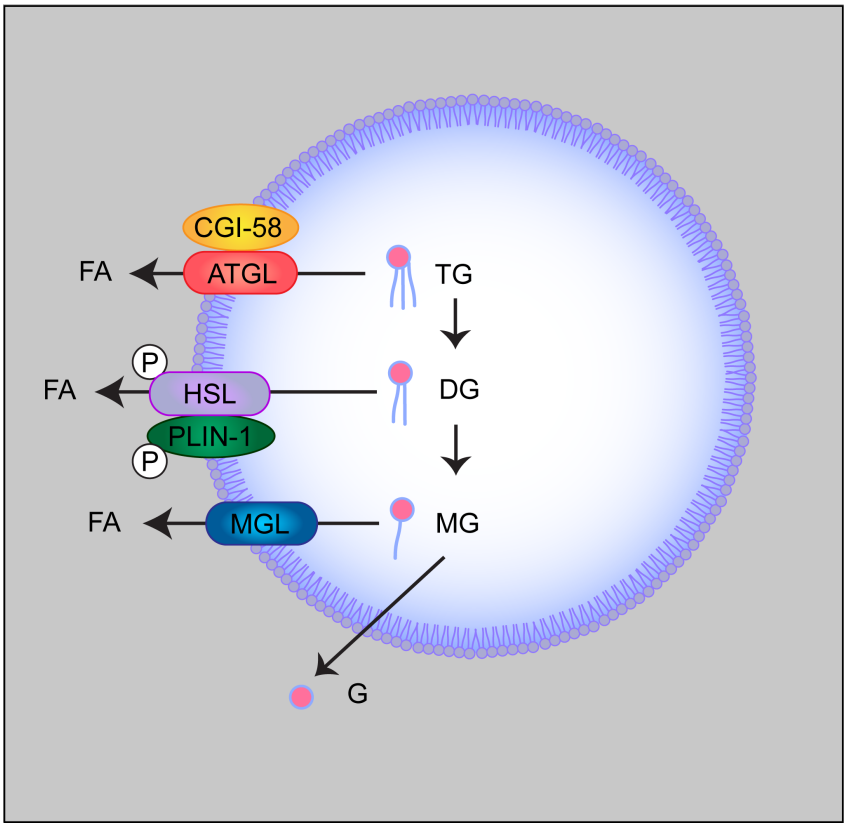


Figure 8: Model for fasting stimulated lipolysis in adipose tissue. The process requires three enzymes: ATGL cleaves the first esterbond in TGs, HSL hydrolyzes DGs, and MGL MGs. ATGL interacts with its coactivator protein CGI-58 for full activity. Phosphorylated HSL translocates to the LD, and interacts with phosphorylated PLIN-1 (adapted from Zechner et al., 2012). Abbreviations: TG, triacylglacerol; DG, diacylglycerol; MG, monoacylglycerol; G, glycerol; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification-58; HSL, hormone-sensitive lipase; MGL, monoglyceride lipase; PLIN-1, perilipin-1.

The free fatty acids are subsequently converted to fatty acid CoA by ACSL enzymes and imported to the mitochondria for beta-oxidation. Activated fatty acids with chain length shorter than 10 carbons can diffuse through the inner mitochondrial membrane. Activated fatty acids with more than 10 carbon atoms are bound to carnitine in a reaction catalyzed by carnitine acyltransferase I and transported across the inner mitochondrial membrane via carnitine-acylcarnitine translocase (Indiveri et al., 2011). In the mitochondrial matrix, acylcarnitine is converted back to fatty acid CoA and carnitine by carnitine acyltransferase II.

A recent study by Singh and colleagues reported compelling evidence linking lipolysis to macroautophagy in starved hepatocytes (Singh et al., 2009). In this alternative lipolytic process, whole LDs can be encapsulated in double-membrane vesicles (autophagosome), which then fuse with lysosomes for degradation. The contribution of autophagy for lipid homeostasis is currently controversial.

6 Abstract of publications

For copyright reasons, full text versions of the publications are not included in the online version of this thesis. Please follow the links to download pdf files directly from the publisher's website. In the printed version reprints of the papers are found in the section 12 at the end of this thesis.

6.1 Publication I

Phosphatidylcholine synthesis for lipid droplet expansion is mediated by localized activation of CTP:phosphocholine cytidylyltransferase

Krahmer N., Guo Y., **Wilfling F**., Hilger M., Lingrell S., Heger K., Newman H.W., Schmidt-Supprian M., Vance D.E., Mann M., Farese R.V. Jr, Walther T.C

Cell Metabolism, 14(4): 504-15 (2011).

Link to pdf

Abstract

Lipid droplets (LDs) are cellular storage organelles for neutral lipids that vary in size and abundance according to cellular needs. Physiological conditions that promote lipid storage rapidly and markedly increase LD volume and surface. How the need for surface phospholipids is sensed and balanced during this process is unknown. Here, we show that phosphatidylcholine (PC) acts as a surfactant to prevent LD coalescence, which otherwise yields large, lipolysis-resistant LDs and triglyceride (TG) accumulation. The need for additional PC to coat the enlarging surface during LD expansion is provided by the Kennedy pathway, which is activated by reversible targeting of the rate-limiting enzyme, CTP:phosphocholine cytidylyltransferase (CCT), to growing LD surfaces. The requirement, targeting, and activation of CCT to growing LDs were similar in cells of Drosophila and mice. Our results reveal a mechanism to maintain PC homeostasis at the expanding LD monolayer through targeted activation of a key PC synthesis enzyme.

6.2 Publication II

Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocalizing from the ER to lipid droplets.

Wilfling F., Wang H., Haas J.T., Krahmer N., Gould T.J., Uchida A., Cheng J.X., Graham M., Christiano R., Fröhlich F., Liu X., Buhman K.K., Coleman R.A., Bewersdorf J., Farese R.V. Jr, Walther T.C.

Developmental Cell, 24(4): 384-99 (2013).

Link to pdf

Abstract

Lipid droplets (LDs) store metabolic energy and membrane lipid precursors. With excess metabolic energy, cells synthesize triacylglycerol (TG) and form LDs that grow dramatically. It is unclear how TG synthesis relates to LD formation and growth. Here, we identify two LD subpopulations: smaller LDs of relatively constant size, and LDs that grow larger. The latter population contains isoenzymes for each step of TG synthesis. Glycerol-3-phosphate acyltransferase 4 (GPAT4), which catalyzes the first and rate-limiting step, relocalizes from the endoplasmic reticulum (ER) to a subset of forming LDs, where it becomes stably associated. ER-to-LD targeting of GPAT4 and other LD-localized TG synthesis isozymes is required for LD growth. Key features of GPAT4 ER-to-LD targeting and function in LD growth are conserved between Drosophila and mammalian cells. Our results explain how TG synthesis is coupled with LD growth and identify two distinct LD subpopulations based on their capacity for localized TG synthesis.

6.3 Publication III

Protein correlation profiles identify lipid droplet proteins with high confidence.

Krahmer N., Hilger M., Kory N., **Wilfling F.**, Stoehr G., Mann M., Farese R.V. Jr, Walther T.C.

Molecular & Cellular Proteomics, 12(5): 1115-26 (2013).

Link to pdf

Abstract

Lipid droplets (LDs) are important organelles in energy metabolism and lipid storage. Their cores are composed of neutral lipids that form a hydrophobic phase and are surrounded by a phospholipid monolayer that harbors specific proteins. Most well-established LD proteins perform important functions, particularly in cellular lipid metabolism. Morphological studies show LDs in close proximity to and interacting with membrane-bound cellular organelles, including the endoplasmic reticulum, mitochondria, peroxisomes, and endosomes. Because of these close associations, it is difficult to purify LDs to homogeneity. Consequently, the confident identification of bona fide LD proteins via proteomics has been challenging. Here, we report a methodology for LD protein identification based on mass spectrometry and protein correlation profiles. Using LD purification and quantitative, high-resolution mass spectrometry, we identified LD proteins by correlating their purification profiles to those of known LD proteins. Application of the protein correlation profile strategy to LDs isolated from Drosophila S2 cells led to the identification of 111 LD proteins in a cellular LD fraction in which 1481 proteins were detected. LD localization was confirmed in a subset of identified proteins via microscopy of the expressed proteins, thereby validating the approach. Among the identified LD proteins were both well-characterized LD proteins and proteins not previously known to be localized to LDs. Our method provides a high-confidence LD proteome of Drosophila cells and a novel approach that can be applied to identify LD proteins of other cell types and tissues.

6.4 Publication IV

COPI buds 60-nm lipid droplets from reconstituted water-phospholipid-triacylglyceride interfaces, suggesting a tension clamp function.

Thiam A.R., Antonny B., Wang J., Delacotte J., **Wilfling F**., Walther T.C., Beck R., Rothman J.E., Pincet F.

PNAS, 110(33): 13244-9 (2013)

Link to pdf

Abstract

Intracellular trafficking between organelles is achieved by coat protein complexes, coat protomers, that bud vesicles from bilayer membranes. Lipid droplets are protected by a monolayer and thus seem unsuitable targets for coatomers. Unexpectedly, coat protein complex I (COPI) is required for lipid droplet targeting of some proteins, suggesting a possible direct interaction between COPI and lipid droplets. Here, we find that COPI coat components can bud 60-nm triacylglycerol nanodroplets from artificial lipid droplet (LD) interfaces. This budding decreases phospholipid packing of the monolayer decorating the mother LD. As a result, hydrophobic triacylglycerol molecules become more exposed to the aqueous environment, increasing LD surface tension. In vivo, this surface tension increase may prime lipid droplets for reactions with neighboring proteins or membranes. It provides a mechanism fundamentally different from transport vesicle formation by COPI, likely responsible for the diverse lipid droplet phenotypes associated with depletion of COPI subunits.

7 Unpublished data

7.1 Results

The COPI machinery modulates LD surfaces to mediate protein targeting

Unbiased genome-wide screens in model systems, such as *Drosophila* cells, revealed factors that are required for LD targeting of proteins (Beller et al., 2008; Guo et al., 2008). Specifically, members of the Arf1/COPI machinery, but not other proteins involved in secretory trafficking (e.g., COPII or clathrin), are required for normal LD morphology and for targeting of some proteins (Beller et al., 2008; Guo et al., 2008; Soni et al., 2009). Depletion of Arf1/COPI proteins from cells leads to the formation of uniformly sized, lipolysis-resistant LDs that lack the major adipose TG lipase (ATGL). How Arf1/COPI proteins function in LD biology and protein targeting is unknown.

To investigate the mechanism of Arf1/COPI-mediated LD protein targeting, I tested the scope of proteins requiring this machinery. Consistent with previous reports (Beller et al., 2008; Soni et al., 2009), I found that *brummer* (*Drosophila* ATGL homologue) localization to LDs was greatly reduced and shifted to the cytoplasm in cells lacking Arf79F (*Drosophila* Arf1) or COPI proteins (Figure 9a). The Arf1/COPI machinery is not required for all proteins to access to LDs: I found no difference in the targeting of Lsd1 (a perilipin orthologue) to LDs in Arf1/COPI-depleted cells (Figure 9b). Specific enzymes involved in TG synthesis, including the rate-limiting enzyme glycerol-phosphate acyltransferase 4 (GPAT4), contain a membrane-embedded hairpin motif that mediates their localization to surfaces of expanding LDs (Wilfling et al., 2013). Similar to *brummer*, GPAT4 required Arf1/COPI and *garz* (*Drosophila* GBF1 homologue, a GTP exchange activator of Arf1) to target a subset of LDs. Without them, GPAT4 remained in the ER (Figure 9c). In agreement with previous reports (Beller et al., 2008; Guo et al., 2008), depletion of each subunit of COPI except εCOPI had a similar effect on GPAT4 LD targeting and LD morphology. GPAT4 targeting to LDs is required to convert smaller LDs into expanded LDs (Wilfling et al., 2013). In agreement with this function of GPAT4 targeting, I found that LDs in Arf1/COPI-depleted cells had monodisperse size and lacked large, expanded

LDs. LDs of Arf1/COPI depleted cells were slightly larger than the smaller, non-expanded population of LDs in control cells (Figure 9d), likely due to the lack of *brummer* and a consequent reduction of lipolysis rate at those LDs (Beller et al., 2008; Guo et al., 2008).

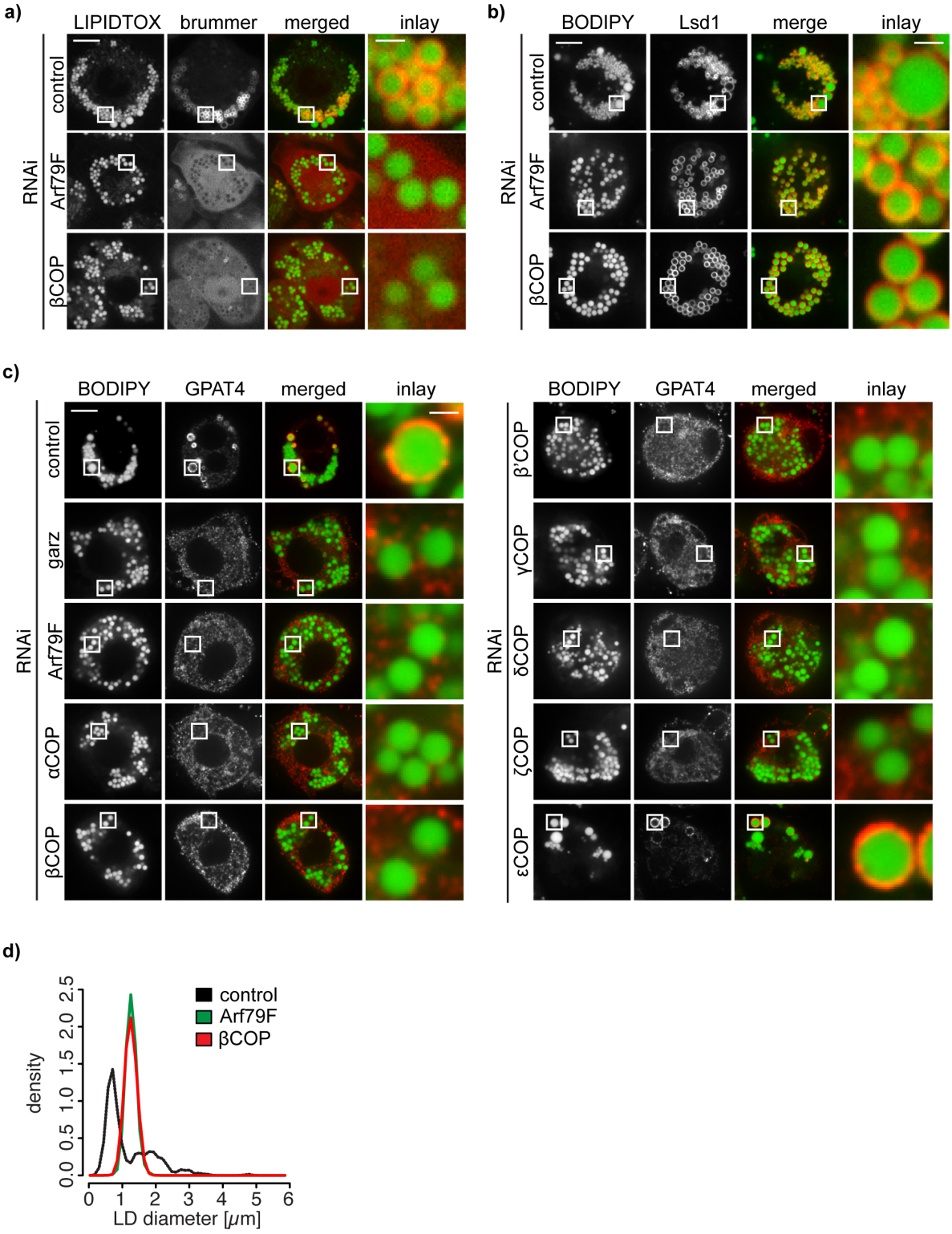


Figure 9: The COPI machinery is required for LD targeting of specific proteins. a) *brummer* LD targeting in S2 cells is dependent on Arf1/COPI. GFP-*brummer* localizes to LDs stained with lipidtox (top panel). GFP-*brummer* LD targeting is abolished in the absence of either Arf79F (middle panel) or βCOP (bottom panel). **b)** Lsd1 LD targeting is independent of Arf1/COPI. *Cherry*-Lsd1 localizes to LDs stained with BODIPY in the absence of Arf179F (middle panel) or βCOP (bottom panel). **c)** Targeting of GPAT4 from the ER to LDs depends on Arf1/COPI. Endogenous GPAT4 detected by immunofluorescence localizes to LDs (stained by BODIPY) in control treated cells, but not in the absence of COPI machinery subunits, except eCOP. **d)** The bimodal size distribution of control cells (black line) with few large LDs and many small LDs is changed to a monodisperse size in Arf1/COPI-depleted cells (orange and red line). Scale bars are 10 µm (overview) or 1 µm (inlay).

How do Arf1/COPI proteins act in targeting proteins to LDs? The canonical function of Arf1/COPI in mediating vesicle trafficking at the Golgi suggests these proteins might form vesicles that carry protein cargos to LDs. Alternatively, Arf1/COPI might act directly at the LD monolayer surface, budding off a “nano-LD”, thus removing primarily phospholipids and allowing for protein access to LDs. These models can be distinguished by determining the site of Arf1/COPI action (i.e., donor membrane bilayers or the LD surface). I thus localized COPI with a specific antibody against βCOP, β‘COP, and GBF1 (the mammalian *garz* homologue GTPase exchange factor activating Arf1) with respect to LDs in mammalian NRK cells, for which more antibodies are available than for *Drosophila* cells (Figure 10a and Suppl. Fig. 1a). As expected, I found strong βCOP, β‘COP, and GBF1 signals in the perinuclear region of cells, likely representing the Golgi apparatus (Suppl. Fig 1c). Additionally, I consistently found βCOP, β’COP, or GBF1 foci in close proximity to LDs and colocalized with the LD marker protein perilipin3 (Figure 10a,c and Suppl. Fig. 1b). LD colocalization was specific for βCOP and GBF1, as the Golgi marker GM130 was not detected at LDs (Figure 10b). To further determine whether colocalization was specific to LDs or due to overlapping signals at LDs and peripheral Golgi-derived vesicles, I compared the experimental distributions of βCOP around LDs with the random distributions of foci around LDs. I found significantly more COPI spots colocalized with LDs in the experiment than expected from a random distribution (Figure 10a). This effect was specific for COPI; other trafficking complexes of the Golgi apparatus, such as clathrin, showed a decreased number of foci around LDs. Importantly, I also found the KDEL receptor cargo of the Arf1/COPI machinery excluded from LD-localized β‘COP foci (Figure 10b). Similar localization results were obtained for the localization of αCOP and *garz* in *Drosophila* S2 cells (Figure 10d). Together with previous proteomic and cell biological results (Bartz et al., 2007b;

Ellong et al., 2011; Nakamura et al., 2005), these data show localization of the COPI machinery on LDs.

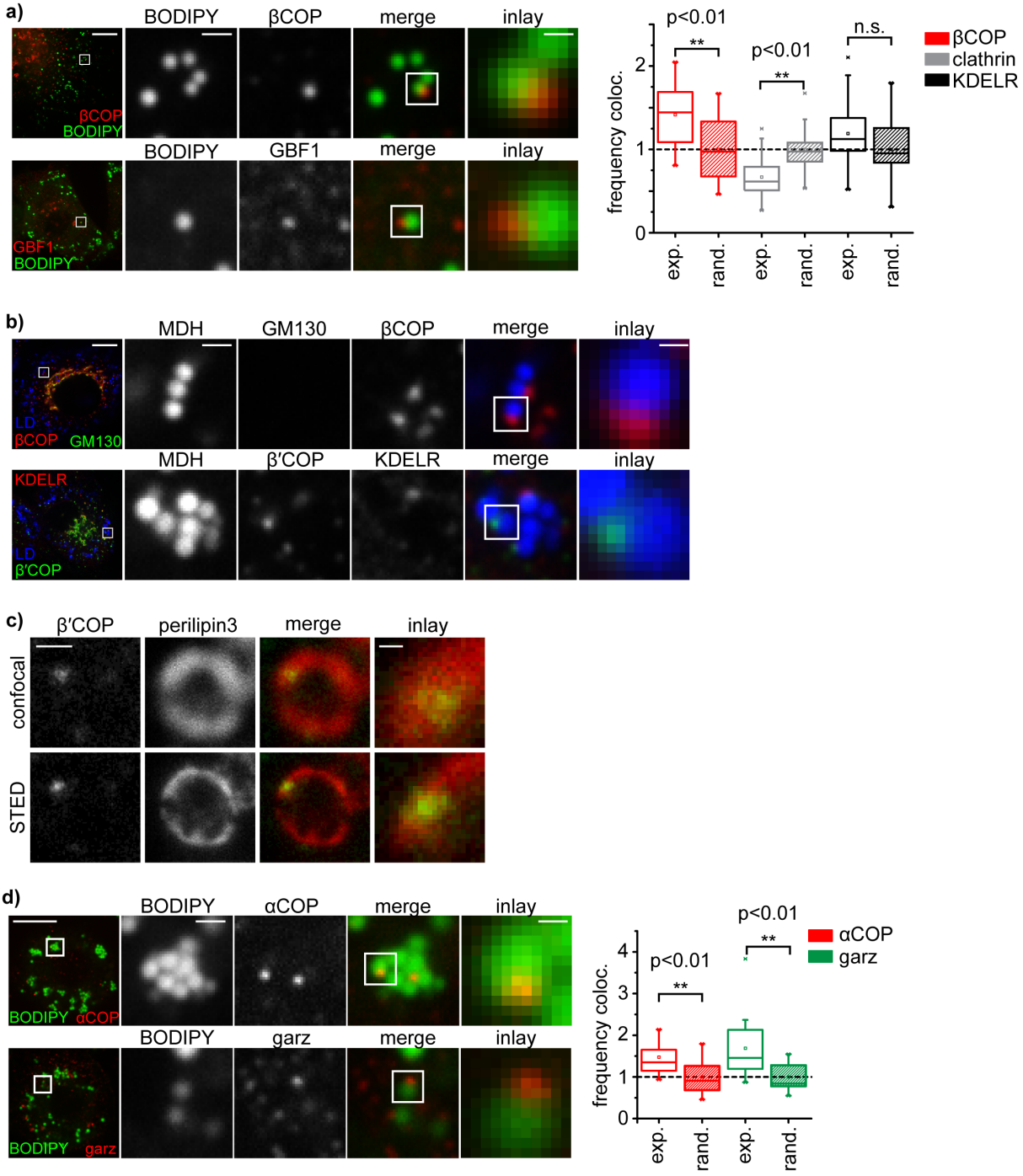


Figure 10: The COPI machinery localizes to the LD surface. a) The endogenous COPI machinery localizes to LDs in NRK cells. NRK cells stained for βCOP or GBF1 by immunofluorescence (red) show partial colocalization with LDs stained with BODIPY (green). βCOP colocalization with LDs in NRK cells is not random. Relative frequencies of βCOP, KDEL receptor and clathrin spots colocalizing with LDs in experiments are determined and respectively compared to the frequencies of colocalization from a binomial random distribution. From the two frequencies (experiment vs. simulation) a significant overrepresentation of βCOP on LDs is observed, whereas clathrin and KDEL receptor (KDELR) are not found on LDs. **b)** Golgi GM130 or KDELR (green) are excluded from LD

localized COPI (red). **c)** β'COP (green) localizes to the surface of (perilipin3, red) by confocal (upper panel) and super-resolution STED microscopy (lower panel). Scale bar=500nm (overview) or 100nm (inlay). **d)** The endogenous COPI machinery stained with αCOP or *garz* (red) antibodies localizes to LDs in S2 cells. Frequencies of colocalization of αCOP and *garz* spots with LDs from experiments are higher than expected from a random distribution. Statistical significance was tested by a student t-test with $P<0.01$ (n=30). Scale bars in (a), (b), and (d) are 10µm (overview) or 1µm (first inlay) or 250nm (second inlay)

If Arf1/COPI acts directly on LDs to remove primarily phospholipids, reducing LD coverage by another method should rescue the defect of GPAT4 targeting in cells missing Arf1/COPI. I tested this strong prediction by co-depleting Arf1/COPI proteins and CCT1 from cells. CCT1 is the rate-limiting enzyme of PC synthesis, required for PC synthesis to cover LDs during their expansion. In its absence, LDs become deficient in PC, leading them to coalesce over time and to yield few very large LDs (Krahmer et al., 2011). As expected, depletion of Arf1 or βCOP, but not CCT1, led to the absence of GPAT4 from LDs (Figure 11a). As predicted from the model that Arf1/COPI proteins act to remove phospholipids from LDs, efficient co-depletion of these proteins with CCT1 rescued the GPAT4 targeting defect (Figure 11a and Suppl. Fig. 2). Importantly, this effect was not due to a general restoration of Arf1/COPI function. Either Arf1/COPI depletion or co-depletion of Arf1/COPI and CCT1, but not CCT1 depletion alone, greatly reduced the secretion of a horseradish peroxidase containing a signal sequence that was used previously to unravel the secretory pathway of *Drosophila* cells (Figure 11b) (Bard et al., 2006). These data reveal a function of Arf1/COPI in controlling LD phospholipid levels, separate from their function in secretory trafficking. To further investigate this possibility, I assayed the time course of LD targeting of CCT1, which we previously found to respond to PC deficiency (Krahmer et al., 2011). I found a significant delay of CCT1 targeting to LDs in βCOP-depleted cells (Figure 11c), indicating that under these conditions, LDs become PC-deficient later during expansion, likely due to relatively larger amounts of phospholipids on their surface.

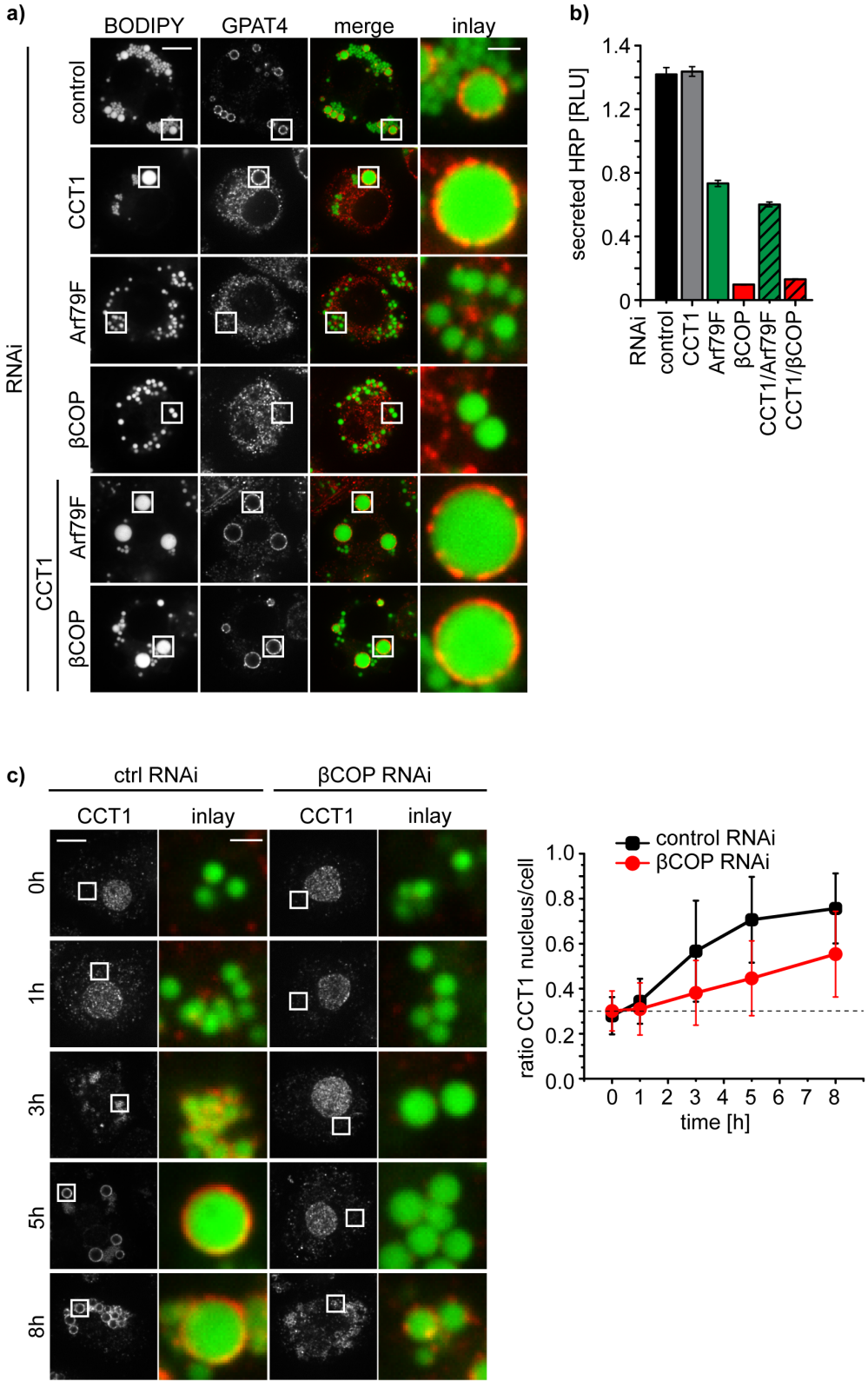


Figure 11: Reduction of cellular PC levels rescues GPAT4 LD targeting in the absence of the COPI machinery. a) Endogenous GPAT4 (red) localizes to LDs (green) in control and CCT1, but not Arf1/COPI depleted cells. Efficient co-depletion of CCT1 and Arf1 or βCOPI restores targeting to LDs. **b)** Arf1/CCT1 or βCOP/CCT1 co-depletion blocks HRP secretion. Error bars represent the s.d. of triplicate measurements **c)** Endogenous CCT1 (red) targeting to LDs (green) is delayed in cells depleted of bCOP. Ratios between nuclear and LD CCT1 signals are shown. Error bars represent the s.d. of the mean ratio from 100 cells.

If Arf1/COPI proteins function to remove phospholipids from LDs and thus allow proteins to bind, modulating the LD surface properties should similarly alter protein targeting to LDs. To test this hypothesis, I added PC to cells. Consistent with the hypothesis, PC addition was sufficient to prevent GPAT4 targeting to the LD surface (Figure 12a). In this instance, it presumably shields the TG core of LDs and lowers surface tension. I next determined if adding a surfactant with a low potential to shield TG and inducing higher surface tension would restore the GPAT4 targeting effect to LDs in the setting of Arf1/COPI depletion. I reasoned that cholesterol, with its small head-group and pronounced cone shape, would increase the surface tension of the LD oil-cytosol interface. In vitro measurements confirmed the cholesterol effect on the surface tension of TG-buffer interface in presence of phospholipids mimicking the LD surface (PC and PE) (Figure 12c), as did measurements of LD emulsion stability in the presence of cholesterol (Figure 12d). When cholesterol was added to Arf1/COPI-depleted cells, the cholesterol reached the LD surface (Suppl. Fig. 3a and b). Importantly, adding cholesterol to cells is sufficient to restore targeting of GPAT4 to LDs in Arf1/COPI depleted cells (Figure 12a), and the number of GPAT4-positive LDs depends on the ratio of cholesterol and PC added to cells (Figure 12a). To test whether the effect is due to cholesterol's physical properties or alternatively some physiological change in the cells, I repeated these experiments with the artificial lipids SR59230A and stearylamine. SR59230A is an amphiphilic molecule that normally does not occur in cells, but which induces LD fusion (Murphy et al., 2010), likely by increasing their surface tension in vivo. Stearylamine on the otherhand is an aliphatic amine and decreases surface tension but leads to fusion of membranes likely by increasing the line tension. In agreement with findings with added cholesterol, adding SR59230A or sterylamine efficiently restored GPAT4 targeting to Arf1/COPI-depleted LDs (Figure 12b).

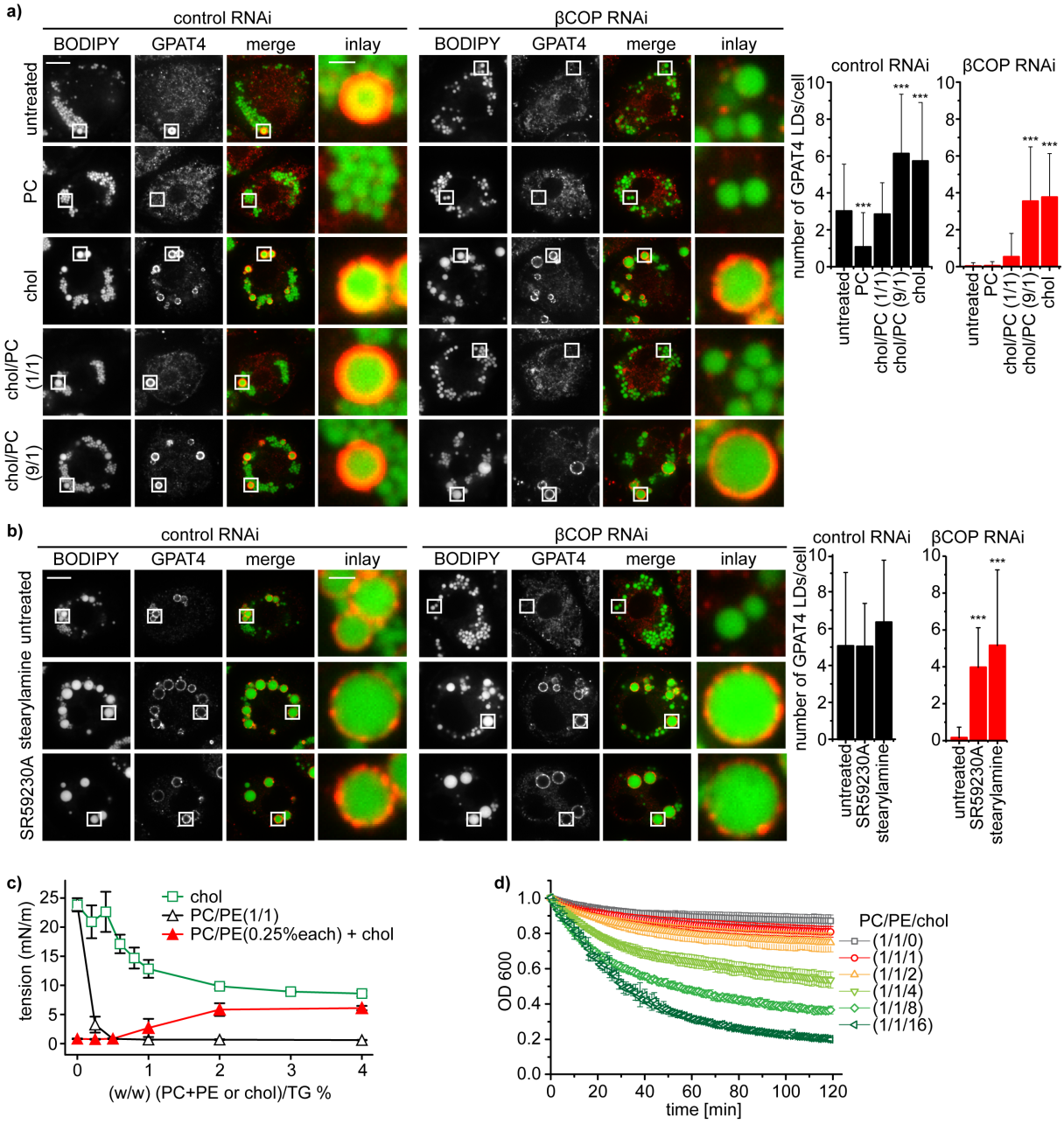


Figure 12: LD surface properties modulate GPAT4 LD targeting. a) Addition of exogenous PC to S2 cells inhibited GPAT4 LD targeting in βCOP or control RNAi treated cells. Cholesterol (chol) addition to cells restored GPAT4 LD targeting in βCOP-depleted cells. **b)** Targeting efficiency depends on the ratio of added cholesterol and PC in βCOP or control RNAi-treated cells. The artificial lipids SR59230A and sterylamine also rescued GPAT4 LD targeting in βCOP depleted cells. The numbers of GPAT4-targeted LDs per cell are shown. Error bars represent the s.d. from the mean number of GPAT4-targeted LDs in 40 cells. Statistical significance was calculated using ANOVA followed by a Dunnett test with a 99% confidence interval (p=0.01). **c)** Cholesterol increases the surface tension of PC/PE monolayer at a TG/buffer interface. Surface tension was measured by a drop weight method for the indicated phospholipid/cholesterol ratios. Error bars represent the s.d. of the mean from a minimum of 15 experiments. **d)** Cholesterol decreases the stability of artificial oil microdroplets in buffer. Time course of the optical density evolution of TG droplets with PC/PE and increasing amounts of cholesterol is shown. Error bars represent the s.d. of the mean from six independent experiments.

To further test whether changes of LD surface properties are sufficient to control GPAT4 targeting to LDs, I reconstituted this reaction in vitro with a microfluidic device (Suppl. Fig. 4). I introduced microsomes harboring GPAT4 fused to fluorescent GFP protein into buffer-in-oil micro-reactors (Figure 13). Mixing the content of the micro-reactors by flow through zig-zagging micro-channels led to localization of some GPAT4 to the monolayer delimiting the TG phase. The amount of GPAT4-GFP at the monolayer depended on its composition and varied according to the surface tension. Like the situation in cells, monolayers rich in cholesterol and having higher surface tension, bound GPAT4-*GFP* more efficiently than control monolayers composed of PC/PE (Figure 13).

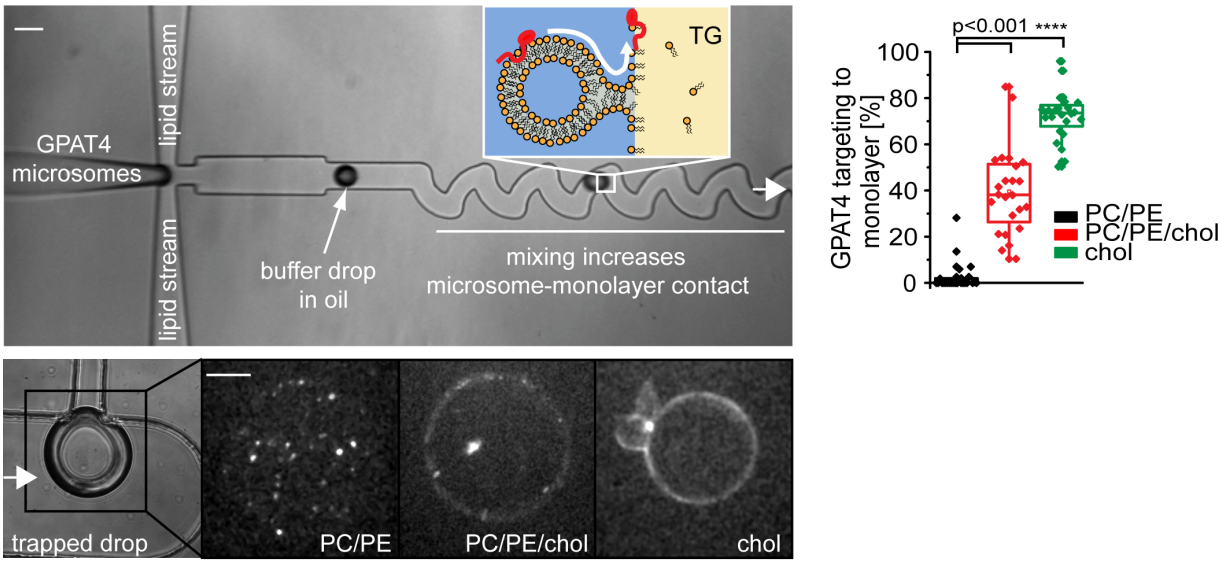


Figure 13: Cholesterol rich surfaces mediate fusion with ER microsomes. GPAT4 targeting to phospholipid monolayers depends on the surface tension. Buffer drops containing GPAT4-GFP-labeled microsomes are formed in a microfluidics device by flow focusing. The buffer micro-reactors are surrounded by oil of different composition (TG containing PC/PE (0.25% ea.) or PC/PE (0.25% ea.) + 2% cholesterol, or cholesterol only (0.5%); concentrations are w/w compared to TG). Each formed buffer drop pass through a zigzag region where microsomes inside the buffer drop are constantly brought into contact with the monolayer at the oil interface (Richmond et al., 2011). Drops are arrested in a network of trapping chambers. In the presence of PC/PE, little GPAT4-GFP is targeted to the monolayer but stays in microsomes. Adding 2% cholesterol or cholesterol alone significantly increased the GPAT4-GFP signal on the monolayer. Shown is a quantification of the efficiency of relocalization of GPAT4 from microsomes to the monolayer interface. Bar=100 µm (device) or 25 µm (drop).

My data suggest a previously unrecognized function for the Arf1/COPI machinery at LDs. I propose that the COPI machinery in cells localizes to LDs and operates to remove excess phospholipids from the surfaces of LDs, thereby increasing LD

surface tension and allowing for the recruitment of proteins. Consistent with our microfluidics experiments, LDs activated by COPI machinery could fuse with the ER establishing a continuous bridge between the ER and LD monolayer that allows protein targeting, as has been observed in the case of GPAT4 (Wilfling et al., 2013).

7.2 Material and Methods

Antibodies

Rabbit polyclonal antibodies used: anti-GPAT4 (Wilfling et al., 2013), anti-CCT1 (Wilfling et al., 2013), anti-GBF1 (BD Biosciences), anti-KDEL-receptor (KDELR; gift from Dr. J.E. Rothman; Yale University), anti-βCOP (gift from Dr. J.E. Rothman; Yale University), anti-perilipin3 (TIP47; Novus Biologicals), anti-αCOP (Abcam), anti-GRP78/BiP (ET-21) (Sigma-Aldrich) and anti-garz (Wang et al., 2012) (gift from Dr. A. Paululat; University of Osnabrück). Mouse monoclonal antibodies used: anti-GM130 (BD Biosciences), anti-tubulin (Sigma-Aldrich), anti-β'COP (gift from Dr. J.E. Rothman; Yale University), and anti-clathrin heavy chain (x22) (Thermo Scientific) antibody. The following secondary antibodies were used: Alexa Fluor® 568 goat anti-rabbit (Invitrogen), Alexa Fluor® 488 goat anti-mouse (Invitrogen), ATTO 647N (STED) goat anti-rabbit (Active Motif), and goat anti-mouse STAR470SX (Abberior).

Plasmid DNA Construction

Full-length cDNA encoding CG5295 (*brummer*) and CG10374 (Lsd1) were obtained from the DGRC (https://dgrc.cgb.indiana.edu/) and subcloned into the pENTR™/SD/DTOPO vector (Invitrogen) and indicated destination expression vectors (actin promoter). The destination vectors used in this study are part of the Drosophila Gateway™ Vector Collection and are available from the DGRC (https://dgrc.cgb.indiana.edu/).

Cell Culture and Transfection

WT *Drosophila* S2 or stably transfected cells (pAGW-*brummer* or pA*Cherry*W-Lsd1) were cultured, treated with oleate, transfected and depleted by RNAi as described (Krahmer et al., 2011). Cells were analyzed four days after RNAi treatment. Table S1 contains a list of primers to generate dsRNAs for RNAi. A segment of pBluescript backbone was used as the template for control RNAi. Expression of the ss-HRP construct was induced and the secretion assay was performed as previously described (Bard et al., 2006). If not otherwise indicated, cells were treated after RNAi treatment with 1mM oleate for 8h. Exogenous lipids (PC, or cholesterol, or PC/cholesterol) were added to *Drosophila* S2 cells at the second day of the RNAi treatment. The final concentration of these lipids in the growth medium was 5mM. On

the fourth day, medium was replaced by fresh medium containing 1mM oleate and LD formation was induced for 8h before cells were fixed. The artificial lipid SR59230A was added to RNAi treated cell during the last hour of oleate treatment to a final concentration of 100µM. NRK cells were cultured in DMEM with 10% FBS and antibiotics (100 units of penicillin and 100 µg of streptomycin per ml). Cells were split onto glass bottom plates and incubated in the culture media the day before imaging. LDs were induced by treatment with 0.5mM oleate for 2h prior to fixation and imaging.

Lipid Droplet Size Measurements

Cells were treated with 1mM oleate, stained with BODIPY, and subsequently imaged and measured as previously described (Wilfling et al., 2013). Density plots were computed using R (http://www.r-project.org/).

Light Microscopy

For live cell imaging and immunostaining, cells were prepared and imaged as previously described (Wilfling et al., 2013). The antibody dilution buffer used for immunostaining of perilipin3 in NRK cells did not contain detergent. The permeabilization buffer used for immunostaing of CCT1 in *Drosophila* S2 cells had a final concentration of 0.1% NP-40. Also, the buffer for first and secondary antibody dilution was detergent free. LDs were stained with 1µg/ml BODIPY (Invitrogen) or LipidTOX (Invitrogen) or 10mM of MDH(Yang et al., 2012).

Surface tension measurements

The surface tension of different lipids or lipid mixtures was measured using a drop weight method. A buffer solution (25mM HEPES-KOH at pH 7.4, 100mM KCl, 10mM $MgCl_2$) containing different concentrations of phospholipids and/or cholesterol was formed in a TG oil phase. Buffer drops were slowly formed in the oil (at a flow rate of 20µl/h) to allow dynamic interfacial equilibrium. At a critical size the drop detaches. For each concentration, movies of this process were taken using a 1394 Unibrain camera. From the inner diameter *d* of the injection tube (d=250µm), the surface tension is determined by $mg/(\pi*d*f)$ where *f* is a Wilkinson geometric parameter correction that depends on the ratio between *d* and the radius of the detached drop and *g* is the gravity constant. The mass *m* of the drop was calculated according to

$m=v*\Delta\rho$ (v is the volume of the drop and $\Delta\rho$ is the volume mass difference between oil and buffer phases).

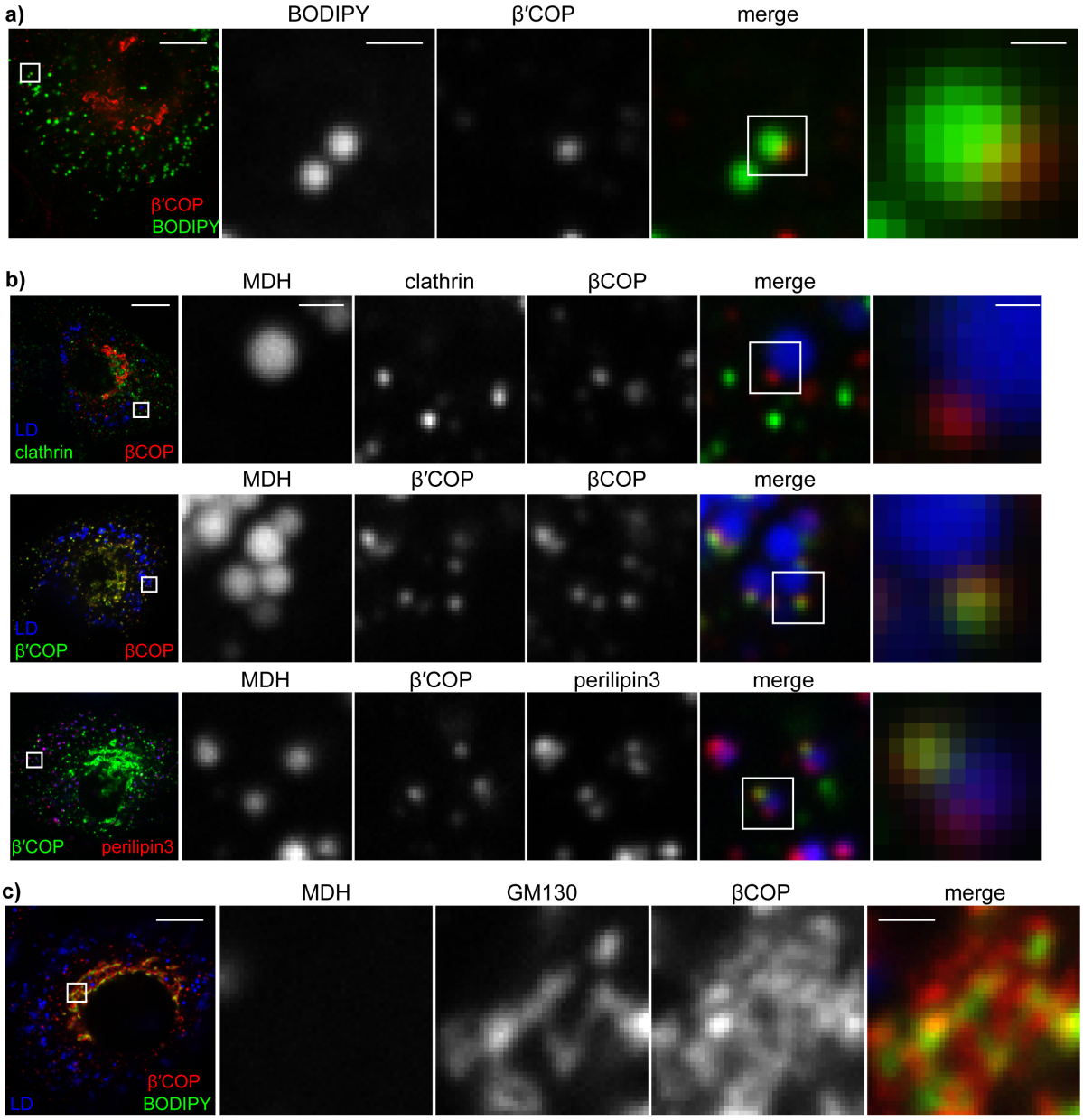
Stability assay of oil microdroplets in aqueous solution

2.5mg DOPE and 2.5mg DOPC (Avanti Polar Lipids) were solubilized in 250mg TG (Sigma-Aldrich) by sonication. Lipids were then mixed with buffer (25mM HEPES-KOH at pH 7.4, 100mM KCl, 10mM $MgCl_2$) in a ratio of 1/16 (oil/buffer) by vortexing and sonication for 5min using a Branson 3510 sonicator water bath. The emulsion was added to the indicated amounts of cholesterol and sonicated for 2min. The optical density of the emulsion was monitored over a time course of 2h in 1min intervals using a TECAN infinite M200.

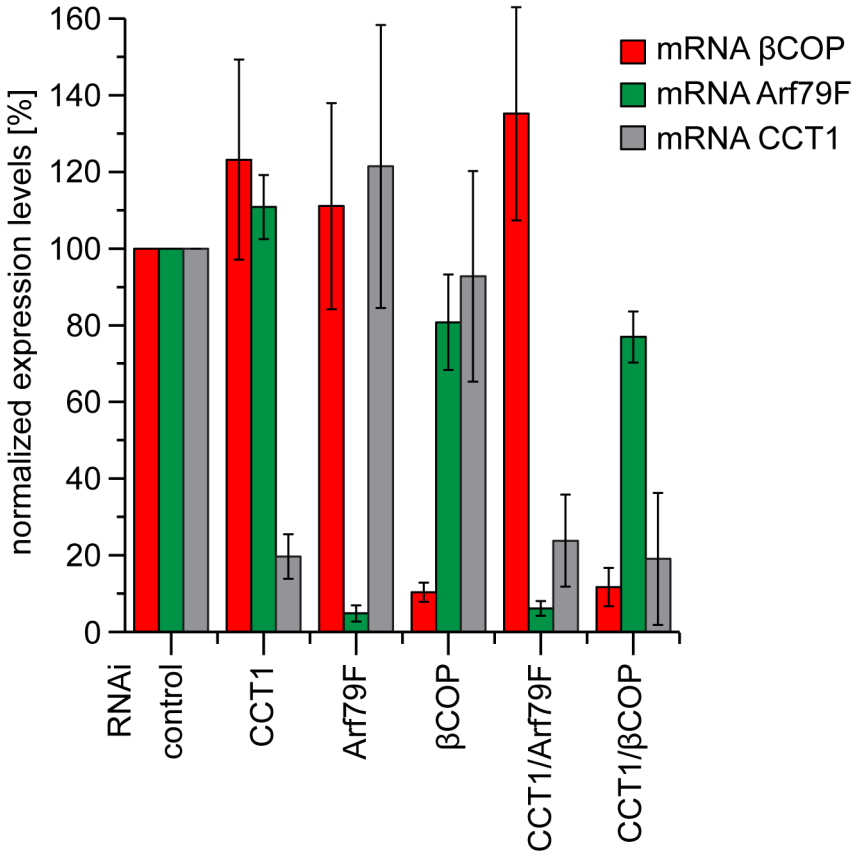
Microfluidic experiments

The microfluidics device was fabricated following well-established soft lithography techniques. A wafer mold was made by lithography using a negative resin (SU8-2035). The device was made of a poly-dimethylsiloxane polymer (PDMS), used to replicate the pattern on the mold and stuck on a glass cover slip. The height of the device is 58±5 µm. A buffer and oil stream are generated using a syringe pump. By flow focusing, defined buffer drops are generated in the oil stream. These buffer micro-reactors contained GFP-GPAT4 microsomes. The oil used was a mixture of TG with phospholipids and/or cholesterol (PC/PE each 0.25% (w/w); PC/PE/cholesterol 1/1/4 with 2% (w/w) cholesterol; cholesterol 0.5% (w/w); indicated lipid concentrations are compared to TG). To ensure the same frequency of interaction of the microsomes with the monolayer interface, the flow rate of the buffer and oil stream (150µl/h and 30µl/h) was kept constant for all experiments.

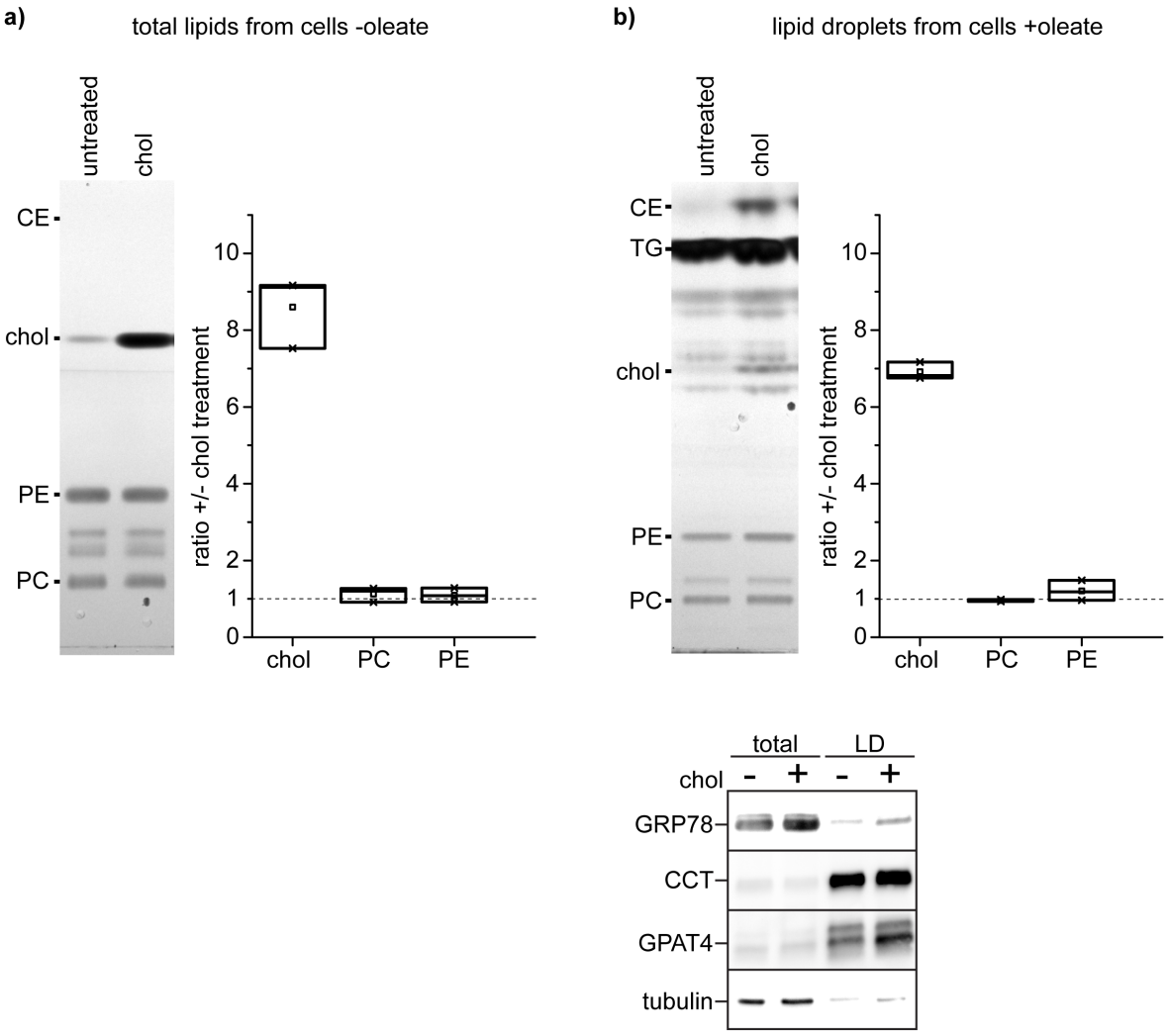
7.3 Supplementary Information



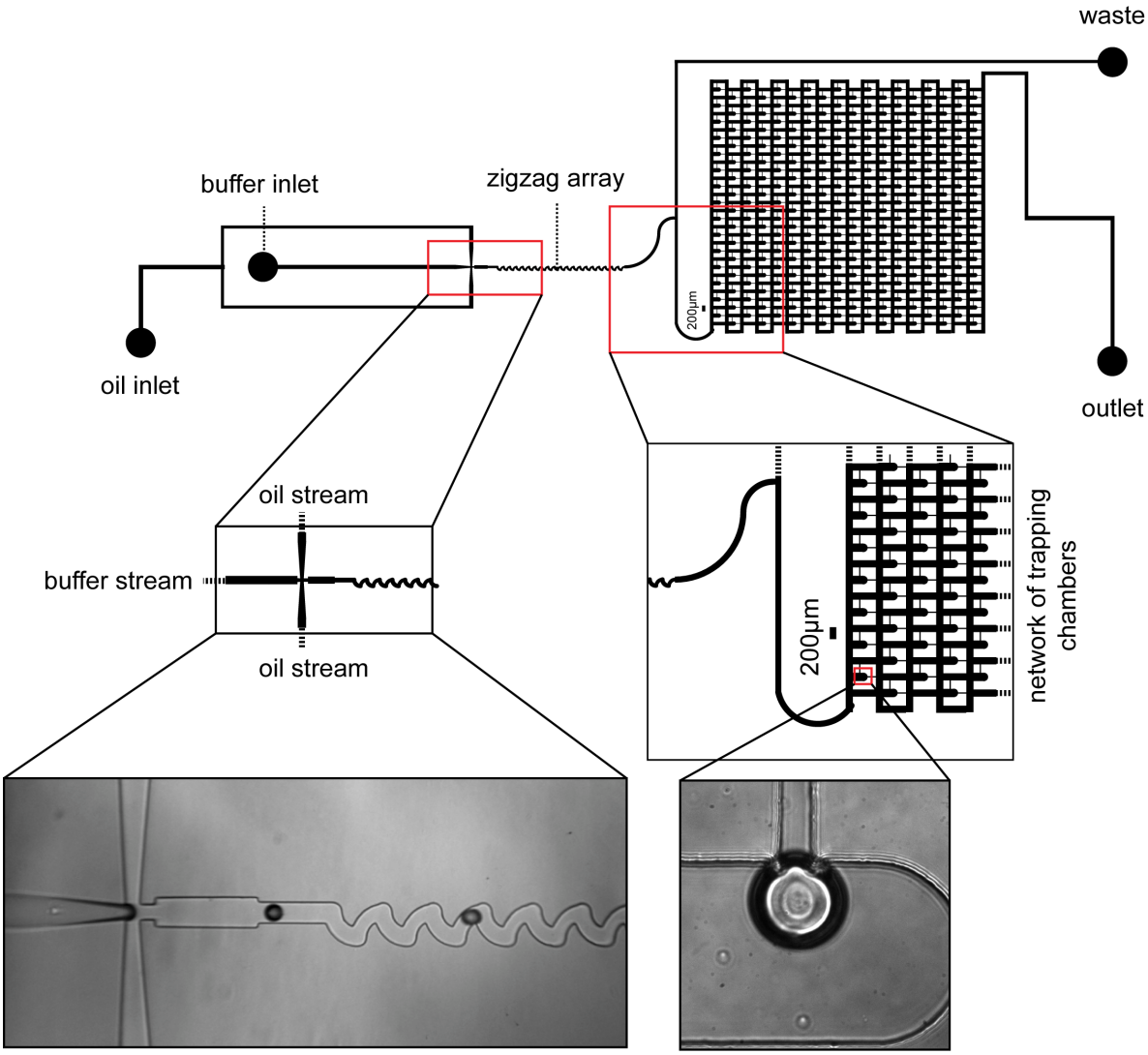
Supplementary Figure 1: **Endogenous COPI subunits specifically localize to LDs in NRK cells. a)** NRK cells stained for β'COP by immunofluorescence (red) show partial colocalization with LDs stained with BODIPY (green). **b)** NRK cells co-stained for endogenous clathrin (green) and βCOP (red) show no colocalization (upper panel). NRK cells costained for endogenous βCOP (red) and β'COP (green) show colocalization in the same structures on LDs. Endogenous β'COP (green) colocalizes with the LD marker protein perilipin3 (red). LDs are visualized by MDH (blue). **c)** NRK cells co-stained for endogenous GM130 (green) and βCOP (red) show colocalization in the perinuclear region of cells, representing the Golgi apparatus.



Supplementary Figure 2: Depletion of COPI machinery components is efficient. Expression of indicated subunits were measured by quantitative real-time PCR. Primers used are listed in Table S2. The Mean ± s.d. of three experiments is shown.



Supplementary Figure 3: Exogenously added cholesterol is incorporated into cellular membranes and LDs. Total lipids from control or cholesterol-treated cells normalized to 200 µg of protein **a)** or isolated LD fraction normalized to 50 µg of protein **b)** were separate by TLC and stained using Hanessian's stain. The band intensity for PE, PC and cholesterol was measured from three biological replicates. Western blots show the purity of the LD fraction



Supplementary Figure 4: Schematic overview of the microfluidic device used for the reconstitution of GPAT4 targeting to LDs. The initial oil stream (containing TG, cholesterol and/or phospholipids) is split into two streams that flow perpendicularly to a buffer stream with GPAT4-GFP containing microsomes. “Flow focusing” the oil streams continuously pinches small buffer micro-reactors with defined size into the oil stream. After formation, the micro-reactor enters a zigzag pattern that creates a chaotic flow inside the micro-reactor. This increases the contact of the encapsulated microsomes with the interfacial monolayer formed at the buffer micro-reactor phase and the surrounding oil. Micro-reactors are arrested in a network of trapping chambers after the zigzag, allowing imaging and analysis of the monolayer targeted GPAT4 population.

8 Discussion

The visualization of the cell interior by electron microscopy (EM) has advanced the field of cell biology significantly. The structure of the cell revealed by EM is unexpectedly rich and surprisingly uniform for practically all eukaryotic cells. Eukaryotic cells are subdivided into functionally distinct, membrane-enclosed compartments called organelles. Each organelle contains its own set of proteins and specialized molecules important for their function. The fine structure of organelles like ER, mitochondria, or the golgi-complex was defined by Albert Claude, Christian de Duve, and George E. Palade, who were awarded with the Nobel Prize in 1974 for their findings. To understand the eukaryotic cell, it is important to know the function of each compartment, how molecules and proteins move between them, and how the compartments themselves are created and maintained. Proteins play an important role in creating and maintaining these compartments. Subcellular fractionation and mass spectrometry-based proteomics allow the characterization of the protein composition of organelle-enriched fractions. Today, protein catalogs are available for almost all cytoplasmic and nuclear organelles (Mann et al., 2013).

In the last 10 years, LDs have received a lot of attention as a hallmark of metabolic disease and insulin resistance. Proteomic analysis of lipid droplets has yielded a comprehensive catalog of lipid droplet proteins and many LD proteomes in different organisms or cell lines have been described (Athenstaedt et al., 1999; Beilstein et al., 2013; Beller et al., 2006; Brasaemle et al., 2004; Cermelli et al., 2006; Ding et al., 2012a; Ding et al., 2012b; Fujimoto et al., 2004; Ivashov et al., 2013; Liu et al., 2004; Orban et al., 2011; Sato et al., 2006; Wan et al., 2007; Zhang et al., 2011; Zhang et al., 2012). Due to the high sensitivity of mass spectrometers and the difficulties inherent in purifying organelles to homogenity, the overlap between published proteomes is often very low and proteins likely to be contaminants have been identified (like ribosomes). Therefore it has been challenging to distinguish *bona fide* organellar proteins from those that are contaminating. To address this problem, new methods had to be developed. In 2003 protein correlation profiling (PCP) was described for the first time (Andersen et al., 2003). PCP is a quantitative approach for determining the purification profiles of proteins through a high-density sucrose centrifugation gradient. By comparing the correlation of profiles with known organelle

markers, the subcellular localization of proteins can be determined (Andersen et al., 2003). Therefore contaminants, which have a different profile, can be distinguished from *bona fide* organellar proteins.

We described a high confidence LD-PCP proteome with a set of just over 100 LD proteins from *Drosophila melanogaster* S2 cells. For most of the tested proteins the localization to LDs could be confirmed by fluorescence microscopy, increasing our confidence in the methodology. This approach led to the identification of novel proteins with a possible function at LDs as well as previously known LD proteins, such as ATGL. Comparison of our LD-PCP proteome with published LD proteomes isolated from *Drosophila* larval fat body or total larvae LDs shows little overlap between identified proteins, most likely due to differences in cell types or the different amount of contaminants.

Besides the well-characterized LD proteins, we found several interesting proteins with unknown LD function that have been identified before. One protein identified is a to this point uncharacterized protein with homology to lipases (CG9186), which has been recently described to be important for positioning of LDs and fat storage (Thiel et al., 2013). Other proteins identified are the lipid metabolism enzymes prenyltransferase (involved in dolichol synthesis) and fatty acid desaturase for which their function in LD biogenesis has to be determined. Also several proteins, previously not known to localize to LDs, have been identified. Among them is the uncharacterized protein CG17292 that has a lipase motif and is expressed in *Drosophila* oenocytes.

Unexpectedly, many enzymes catalyzing the early steps of the N-linked protein glycosylation pathway were identified in the LD-PCP. Lipid-linked oligosaccharide biosynthesis is performed by a series of glycosylation events, catalyzed by glycosyltransferases of the ALG (asparagine linked glycosylation) family. The process is initiated by ALG7 N-acetylglucosamine-phosphate transferase adding GlcNAc-P to a dolichol-phosphate. The next GlcNAc residue is added by the ALG13 and ALG14 protein complex. After that five mannose residues using GDP-Man are added. The first addition of mannose is catalyzed by ALG1. Subsequently the addition of the two branching mannose residues is catalyzed by ALG2, followed by elongation of the $Man_3GlcNAc_2$ by ALG11. This results in a $Man_5GlcNAc_2$ oligosaccharide representing the final product of the cytoplasmic lipid-linked

oligosaccharide biosynthesis. In the following steps the glycolipid flips across the membrane in a yet poorly characterized reaction to function as a substrate for oligosaccharyltransferase, which transfers the glycan to asparagine residues of ER-lumen exposed proteins. We have identified all the enzymes of the cytoplasmic pathway as specific LD proteins in the LD-PCP, except ALG7 (not identified). We also detected the suggested flippase Rft1, which may flip the glycolipid from the cytosol into the lumen, only in the lipid droplet fraction suggesting a strong enrichment. Taken all the localizations of all the proteins of this pathway into account, it suggests that both dolichol synthesis, as well as the later assembly of the lipid-linked oligosaccharide happens on the surface of LDs. The very extended dolichol isoprenoid sidechains (with up to 90 carbons for Dol-18) might be much easier accommodated in the hydrophobic core of the LD then in the thinner ER bilayer.

A second group of proteins enriched in the LD fraction were enzymes of the TG synthesis pathway. We found enzymes catalyzing the first three steps, GPAT, AGPAT and PAP, enriched in the LD fraction. Previously, these enzymes have been described to localize to the ER where they synthesize TG (Coleman and Lee, 2004; Nimmo, 1979). How TG, synthesized in the ER, is incorporated into the core of growing cytoplasmic LDs is unknown. The localization of a subset of these enzymes to LDs suggests the possibility of local TG synthesis on LDs.

To test this hypothesis, we investigated the role of TG synthesis enzymes in LD formation and growth. We showed that cells form two classes of LDs during oleate loading. The first class contains expanding LDs that grow by addition of locally synthesized TG. For this reason, a subset of de novo TG synthesis enzymes moves along membrane bridges to the LD surface (Figure 14). Fluorescence microscopy confirmed that GPAT4 (CG3209), AGPAT3 (CG4729), and DGAT2 (CG1942) are localized to LDs in *Drosophila* S2 cells. These proteins share a hydrophobic domain structure that likely forms a hairpin and allows the re-localization of these enzymes from the bilayer of the ER to the monolayer of LDs. Enzymes of the TG synthesis pathway that are exclusively localized to the ER are missing this feature. In contrast, LDs of a second class that do not acquire these TG enzymes remain small after their initial formation (Figure 14). ER-only localized enzymes are likely important for the formation of this class of LDs.

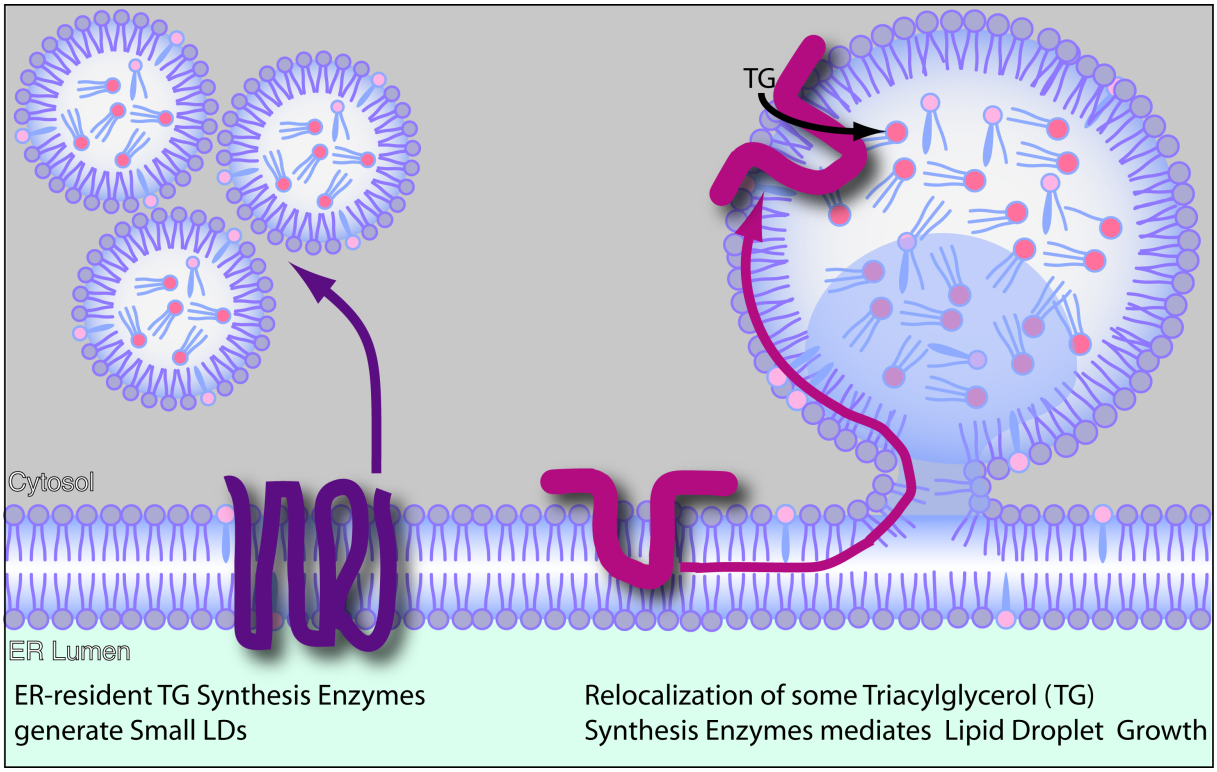


Figure 14: Model for the generation of the two different LD populations. ER-resident TG synthesis enzymes generate small *static* LDs (sLDs). Re-localization of a subset of TG synthesis enzymes mediates LD growth by local TG synthesis and leads to the formation of *expanding* LDs (eLDs).

Under basal growing conditions the LD-localized TG synthesis enzymes are found in the ER. During formation, a subset of LDs is targeted by these enzymes and consequently grows. This observation leads to two main questions: How do these proteins localize to LDs and how is this process coordinated? Focusing on GPAT4, catalyzing the first and rate-limiting step in TG biosynthesis, we investigated the process of re-localization in more detail. Instead of re-localizing GPAT4 from the ER to LDs it is possible that newly synthesized GPAT4 is directly targeted to LDs whereas the ER pool of GPAT4 is degraded. Blocking protein synthesis did not affect the targeting of GPAT4 to LDs suggesting that the ER pool of GPAT4 can be re-localized to LDs even in the absence of newly synthesized proteins.

Unbiased genome-wide screens in *Drosophila* cells have revealed that the COPI machinery is required for LD targeting of certain proteins (Beller et al., 2008; Guo et al., 2008). In an alternative model GPAT4 is transported from the ER to LDs by vesicular trafficking. We found that in the absence of the COPI machinery, GPAT4, similar to ATGL, does not re-localize to LDs. Based on our *in vitro* studies, the COPI

machinery acts directly at the surface of the LD where it buds “nano-LDs” (~60-nm diameter) from a phospholipid covered TG-buffer surface. In mammalian cells as well as in *Drosophila* S2 cells we found βCOP, β’COP, or GBF1 foci in close proximity to LDs and colocalized with the LD marker protein perilipin3 suggesting a similar function *in vivo*. The direct action of COPI at the LD surface is further supported by previous work showing that (a) Arf1, as well as other members of the COPI machinery, were found on LDs in proteomic and cell biological studies (Bartz et al., 2007b; Ellong et al., 2011; Nakamura et al., 2005); (b) expression of dominant-negative Arf1 T31N localizes to LDs (Guo et al., 2008); and (c) Arf1Q71L that cannot hydrolyze GTP (and hence acts as a dominant negative in vesicular trafficking) activates lipolysis on LDs (Guo et al., 2008). The formation of “nano-LDs” by the COPI machinery leads to a decrease of phospholipids on the LD surface compared to the TG volume. This decrease in phospholipid levels suggests that the COPI machinery operates to remove excess phospholipids from the surface of LDs, thereby increasing LD surface tension and allowing for the recruitment of proteins. Modulating the lipid composition of the LD surface can, even in the absence of functional vesicular trafficking, restore the targeting of GPAT4. This proposes a function of COPI independent of its canonical function in retrograde vesicular trafficking. Electron microscopy revealed bridges between ER and LD, which suggested that GPAT4 re-localization to expanding LDs occurs through these connections. This re-localization of GPAT4 along membrane bridges is in agreement to the tubular GPAT4 signal found during FRAP experiments, where the signal on LDs recovered by re-localization of GPAT4 along tubular structures from the ER to LD. Most likely, these bridges are continuities of the outer ER membrane leaflet with the surface of the LD monolayer. Importantly, such structures between the ER and LDs have been observed before in other organisms, suggesting they are a principal feature of LDs (Ohsaki et al., 2008; Wanner et al., 1981). Interestingly these bridges between the ER and LDs are missing when βCOP is depleted (unpublished observation). During oleate loading of cells some LDs rapidly acquired GPAT4 after their initial formation. This suggests the establishment of a pathway for the re-localization of these proteins and supports the idea that direct action of the COPI machinery leads to targeting of GPAT4. Mechanistically and consistent with our microfluidics experiments, LDs activated by COPI action could fuse with the ER establishing a continuous bridge between the ER and LD monolayer that allows

protein targeting, as it has been observed for GPAT4. This is in agreement with the rescue of GPAT4 targeting in a βCOP-depleted cell by the addition of stearylamine. Sterylamine decreases the surface tension and the line tension (Teixeira et al., 2000) making it easier for two membranes to fuse. Alternatively, protein targeting after action of the COPI machinery at LDs could be mediated by allowing proteins, such as ATGL, to bind exposed TG not covered by phospholipids. Intriguingly, the Arf1/COPI mechanism appears to operate particularly for proteins with a need to access the TG bulk phase of the LD, such as the TG lipase ATGL or the TG synthesis enzyme GPAT4. But also other membrane proteins, such as UBXD8 or METTL7B, initially localize to the ER and then concentrate on LDs in a similar fashion then GPAT4 (Turro et al., 2006; Zehmer et al., 2009). This suggests an evolutionarily conserved pathway of ER-to-LD localization by membrane bridges, which is fundamental for targeting of these proteins to LDs. It will be interesting in the future to determine the set of proteins that are dependent on the COPI machinery on their targeting to LDs. Also the fate of the small LDs formed by the COPI machinery is unknown. One function of these "nano-LDs" *in vivo* could be to allow fast remodeling of LDs by Oswald ripening. Oswald ripening is the phenomena in which smaller particles in solution dissolve and deposit on larger particles in order to reach a more thermodynamically stable state wherein the surface to area ratio is minimized. *In vitro* studies have shown that generated "nano-LDs" are able to fuse with other LDs or bilayer membranes of giant unilamellar vesicles (GUVs).

Once GPAT4 is localized to LDs, it does not leave this compartment in our experimental conditions. The mechanism mediating unidirectionality of LD targeting is unknown. One possibility is a unidirectional "gating" mechanism, perhaps mediated by a protein machinery. Another option is that GPAT4 has a higher preference for the monolayer than for the bilayer due to for example hydrophobic mismatching. Hydrophobic mismatching describes the difference between the length of the hydrophobic domain of an integral membrane protein and the hydrophobic thickness of the membrane (de Jesus and Allen, 2013). In the case of *Drosophila* GPAT4 the two transmembrane domains are unusually long for an ER-spanning α-helix (21 and 22 aas), which leads to a structural strain as long as it is confined to a bilayer membrane of defined thickness. This strain is released once the protein localizes to the LD monolayer. The conformational change could result in a new folding state with a new and lower energy minimum, which could provide the energy

necessary for the enrichment of GPAT4 on LDs. This could also explain the trapping of GPAT4 on LDs since energy is needed to bring GPAT4 back into the bilayer conformation to allow re-localization to the ER bilayer. Supporting this hypothesis our data indicate that the hydrophobic domain of GPAT4 likely forms a hairpin loop rather than two separate transmembrane domains. Based on the sequence structure similar topologies are plausible for other enzymes of the *de novo* TG synthesis pathway. For example the topology of DGAT2 has been described to have similar features with the N- and the C-terminus facing the cytosol and a long hairpin loop for membrane targeting (Stone et al., 2006). Whether the loop domain between the two membrane domains is exposed to the luminal site is unknown. Further studies are needed to exactly map the topology of these proteins. Hydrophobic hairpins might be a common LD-targeting motif and highly similar topology has been described for oleosins, the major LD proteins of plants (Abell et al., 2002), or for caveolins (Thiele and Spandl, 2008).

Segregation of TG synthesis to LDs and away from the ER may have several important physiological functions. In a simple scenario cells could just form more LDs rather than expand existing LDs, which would only need one set of enzymes, namely the ER enzymes involved in the formation of LDs. This can be demonstrated by the LD morphology in adipocytes, which are the most specialized cells for lipid storage. In these cells only one giant LD is found with a size up to 100 µm in diameter that occupies 90% of the cell interior. Assuming a diameter of 100 µm, the volume of the LD is about $5.2x10^5$ μm^3. If a cell would not be able to expand LDs but rather would form LDs with a diameter of 1 µm, it would need to form the incredible number of 10^6 LDs in order to enclose the same amount of fat. This would mean that 100 times more surface phospholipids are needed to cover the fat. Also the space occupied by these amounts of LDs would be much higher and therefore the cell size would need to be enormous. Expansion of existing LDs allows the cell to save space and phospholipids for efficient storage of neutral lipids. Colocalizing enzymes of the TG synthesis pathway on LDs might facilitate the efficient handover of substrates from one enzyme to the next in a localized multi-enzyme complex. Consistent with this possibility, biochemical fractionations (e.g., of intestinal microsomes possibly containing LDs) demonstrated a complex containing several of the activities needed for TG synthesis (Lehner and Kuksis, 1995; Rao and Johnston, 1966), and recent data in *C. elegans* provide evidence for an interaction of DGAT2 and ACSL (Xu et al.,

2012). In further support of this notion, we found ACSL1, ACSL3, GPAT4, and AGPAT3 physically associated when LDs are present and AGPAT3 and GPAT4 colocalizing together to a subset of LDs. Moreover, depletion of a single LD-localized enzyme impaired localization of other enzymes and TG synthesis. All these data suggest the possibility of multi-enzyme complex formation, which might facilitate the efficient handover of substrates from one enzyme to the next and drive TG synthesis by making PA and DG unavailable for phospholipid synthesis. But segregation of TG synthesis to LDs may also serve to protect the ER from stress due to the accumulation of lipid synthesis intermediates, such as PA or DG. Accumulation of PA or DG could lead to an imbalance between phospholipid and neutral lipid synthesis. To achieve balance between phospholipid and neutral lipid synthesis, cells stringently regulate the availability of DG and PA for the different biosynthetic pathways. In yeast, a key enzyme for the regulation of this is the PA phosphatase Pah1, which regulates the availability of PA for phospholipid synthesis. The PCP-LD proteome revealed that the *Drosophila* homolog of Pah1 CG8709 localizes to LDs. Together with the segregation of GPAT and AGPAT activities to LDs, which would separate TG synthesis from ER-based phospholipid synthesis, this could provide a mechanism of how phospholipid and neutral lipid synthesis are coordinated.

Expansion of the core of existing LDs is highly co-regulated with the expansion of the surface of these LDs. We found that CCT1, the rate-limiting enzyme in the Kennedy pathway, targets strongly to expanding LDs at early time points of LD formation. We further show that in general binding of CCT1 occurs on LDs with relatively low PC levels and that the binding leads to activation of CCT1. Activated rate-limiting CCT1 increases the flux through the Kennedy pathway. This generates more PC to coat growing LDs and prevent their coalescence. It is unknown how PC enters expanding LDs since the final step of PC synthesis catalyzed by CPT occurs in the ER. A simple possibility is that PC is replaced when droplets connect to the ER and phospholipids can freely diffuse along the ER-LD connection sites. In another model proposed by Thiele et al., phospholipid fatty acids are remodeled by the Lands cycle (Penno et al., 2013). Phospholipase A2 (PLA_2) removes the sn-2 fatty acid of the phospholipid followed by re-acylation by lyso-phospholipid acyltransferases (LPLAT). Lyso-PC has a higher CMC in chloroform then PC and is therefore more water-soluble. This could allow relocation of lyso-PC to droplets, where LPLAT forms PC creating a sink-source situation to drive PC formation on LDs. Interestingly, others and we have

found LPLAT on expanding LDs (Moessinger et al., 2011). Alternatively, PC can be targeted to LDs by transfer of PC from the ER to LDs via lipid transfer proteins. We found phosphatidylcholine transfer protein (PCTP) enriched in the LD fraction and confirmed the localization by fluorescence microscopy. Knockdown of PCTP does not mimic the CCT1 phenotype on LDs (Krahmer N., Wilfling F., and Walther TC. unpublished data). In *Drosophila* many lipid transfer proteins exist suggesting redundant function. But further experiments are necessary to determine the contribution of these proteins on LD PC homeostasis.

Besides expanding LDs, a second class of LDs exists that does not contain GPAT4 or any of the other TG synthesis enzymes. This class of LDs is much smaller in size and shows a uniform size distribution. Possibly, these LDs separate from the ER once they reach a particular size. The budding of these LDs from the ER prevents targeting of TG synthesis enzymes and their further growth and explains their relatively uniform size. Alternatively proteins might gate the connection between these droplets and the ER allowing only certain proteins to enter the LD surface. In plant cells, DGAT1 and DGAT2 have been described to localize to different subdomains within the ER (Shockey et al., 2006). Depletion of DGAT1, which leads to a strong decrease in formation of small LDs in our experiments, dramatically reduced the amount of stored TG. This decrease in overall TG suggests that LD-localized TG synthesis enzymes need the initial formation of LDs by DGAT1 for efficient expansion. This hypothesis is further supported by the fact that initially formed LDs can acquire GPAT4 at much later time points after their formation. TG synthesis enzymes might exist in a low activity state when they are bound to the ER bilayer; re-localization and binding to the monolayer leads then to a switch from the low activity to the high activity state.

The two different LD populations of *static LDs* (sLD) and *expanding LDs* (eLDs) are evolutionary conserved between flies and mice. *Static LDs* (sLD) are abundant, relatively small LDs of fairly uniform size, and their production is closely linked with TG synthesis in the ER. *Expanding LDs* (eLDs) are larger, more heterogeneous LDs that grow in the presence of excess fatty acids due to the relocalization of TG synthesis enzymes from the ER to the surfaces of specific LDs. Their relative contribution to TG storage is unknown, as are their functions in physiology. Mammalian genomes encode two enzymes with high similarity to *Drosophila* GPAT4,

GPAT3 and GPAT4, which constitute in most tissues about 90% of total GPAT activity (Coleman and Lee, 2004). So far, only GPAT4 function has been analyzed in knockout mice (Beigneux et al., 2006; Chen et al., 2008; Nagle et al., 2008; Vergnes et al., 2006). Absence of GPAT4 leads to subdermal lipodystrophy, as well as a strong reduction of TG content in adipose tissue and in liver, where GPAT4 is highly expressed. GPAT4 is further crucial for the production of milk fat by the mammary gland. The absence of GPAT4 leads to a resistance to diet-induced obesity, which suggests that it might be an interesting drug target (Cao et al., 2012). Besides GPAT4 some other mouse models have been studied for the absence of TG synthesis enzymes. The best-studied examples are DGAT1 and DGAT2. The absence of DGAT1 leads to reduced adiposity and resistance to diet-induced obesity (Smith et al., 2000). Further, *dgat* 1-/- mice have dry fur and hair loss (Smith et al., 2000). In the absence of DGAT2, mice had a strong reduction in triglyceride synthesis and died at birth (Stone et al., 2004). It will be interesting to test the physiological role of the different types of droplets in an *in vivo* model, for example to determine the fate of the two different populations during lipolysis. The finding of the different LD populations shows that lipid droplets are more then just a depot of fat but in fact are dynamic organelles interacting with many cellular processes and might have a key role in certain pathways.

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