HHS Public Access

Author manuscript

Nat Rev Mol Cell Biol. Author manuscript; available in PMC 2015 August 05.

Published in final edited form as:

Nat Rev Mol Cell Biol. 2013 December ; 14(12): 775–786. doi:10.1038/nrm3699.

The Biophysics and Cell Biology of Lipid Droplets

A. Rachid Thiam[1,2], **Robert V. Farese Jr.**[3,4,*], and **Tobias C. Walther**[1,*]

[1] Yale School of Medicine, Department of Cell Biology, New Haven, Connecticut 06510 USA

[2] Laboratoire de Physique Statistique, Ecole Normale Supérieure de Paris, Université Pierre et Marie Curie, Université Paris Diderot, Centre National de la Recherche Scientifique, 24 rue Lhomond, 75005 Paris, France

[3] Gladstone Institute of Cardiovascular Disease, San Francisco, California 94158 USA

[4] Departments of Medicine and Biochemistry & Biophysics, University of California, San Francisco, California 94158 USA

Abstract

Lipid droplets (LDs) are intracellular organelles that are found in most cells, where they have fundamental and dynamic roles in metabolism. Recent investigations showed the importance of basic biophysical principles of emulsions for LD biology. At their essence, LDs are the dispersed phase of an oil-in-water emulsion in the aqueous cytosol of cells. They function prominently in storing oil-based reserves of metabolic energy and components of membrane lipids. Because of their unique architecture, with an interface between the dispersed oil phase and the aqueous cytosol, LDs require specialized mechanisms for their formation, growth, and shrinkage. Such mechanisms enable cells to use emulsified oil in a controlled manner (e.g., when demands for metabolic energy or membrane synthesis increase). Regulation of the composition of the phospholipid surfactants at the LD surface is crucial for LD growth and catabolism and also modifies protein targeting to LD surfaces. Here, we review new insights into the cell biology of LDs, with an emphasis on concepts of emulsion science and biophysics that apply to this organelle.

Introduction

Living systems are maintained by a constant flux of metabolic energy, and lipids that are rich in reduced hydrocarbons provide a source of energy for many organisms. Because new energy sources are not always available, the ability to store lipids in cells and tissues is often crucial for survival. In addition, cells must be able to buffer and store excess lipids in an inert form. Thus, nearly all cells are capable of storing lipids in partitioned reservoirs. To package lipids efficiently, cells convert them into neutral lipids, such as triacylglycerols (TG) and sterol esters (SE), which exclude water. These lipids are deposited into specialized intracellular organelles called lipid droplets (LDs), also sometimes called adiposomes, lipid

[*] Correspondence should be sent to: Robert V. Farese, Jr., Gladstone Institute of Cardiovascular Disease, 1650 Owens Street, San Francisco, CA 94158, USA, bfarese@gladstone.ucsf.edu or Tobias Walther, Yale University School of Medicine, Department of Cell Biology, SHM-C 425C, 333 Cedar Street, New Haven, CT 06520, USA, tobias.walther@yale.edu.

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bodies, or oil bodies [1-4]. In addition to storing energy, LDs provide reservoirs of lipids for membrane synthesis (e.g., sterols, fatty acids, and phospholipids). Given their diverse functions, LDs lie at the crossroads of membrane biology and energy metabolism and are important organelles in maintaining cell homeostasis. With their role in lipid storage, LDs figure prominently in common pathologies linked to lipid accumulation, including obesity, diabetes, and atherosclerosis [5], and in industrial applications, such as efforts to produce TG for food, and hydrocarbons more generally, as biofuel.

Despite the almost universal presence of LDs in cells of nearly all organisms, surprisingly little is known about the molecular details of the processes underlying their biology. However, recent advances in understanding the cell biological properties and functions of LDs have begun to change this. These advances also have underscored the importance of understanding the biophysical properties of LDs as they relate to cell biology, which we highlight in this review.

Lipid Droplets Lie at the Interface of Emulsion Physics and Cell Biology

An emulsion is a mixture of two immiscible fluids: one dispersed into the other in the form of drops. Examples of emulsions include direct emulsions (i.e. oil drops in water), or inverse emulsions (i.e., water drops in oil). LDs in the cytoplasm provide a biological example of a direct emulsion that is common to most cells. The cytosol represents the continuous aqueous phase, and the dispersed oil phase, LDs, includes neutral lipids, such as TG, SE, retinyl esters, waxes, or ether lipids, depending on cell types and neutral lipids they store [6].

Cells deal with excesses in lipids such as fatty acids (which can act as detergents) by esterifying the potentially toxic lipids to form more inert neutral-lipid oils, such as TG or SE. Formation of these oils occurs within membrane bilayers. However, because bilayers are unsuited for storing large amounts of oil [7, 8], an emulsion of oil droplets forms. The resulting LD emulsion then exists in the cytoplasm, where specific proteins can act on them to regulate their growth or utilization. Increasingly, we are learning that cells have evolved machinery to make and utilize LD/cytosol emulsions in an organized and regulated manner. Concepts in emulsion science are therefore highly relevant to understand LD cell biology. Nevertheless, this paradigm has not yet been well integrated into this field.

To generate stable emulsions, surfactants, such as phospholipids, are required. Artificially generated emulsions are most often formed with surfactants that are soluble in the continuous, but not the dispersed phase, following the Bancroft rule (i.e., the phase in which the emulsifier is soluble tends to be the continuous phase) [9-11]. If the concentration of a surfactant exceeds its critical micellar concentration, micelles form, providing a reservoir of surfactant to buffer surface area fluctuations of emulsion droplets and thus increase their stability.

For cellular LDs, phospholipids at the surface monolayer constitute the main surfactant. In contrast to surfactants used in artificial emulsions, however, phospholipids are soluble neither in the cytoplasmic aqueous phase nor in the oil phase. In cells, therefore, bilayer membranes (e.g. of the ER) rather than micelles serve as phospholipid/surfactant reservoirs. Interestingly, the phospholipid composition differs in ER bilayers and LD monolayers [12].

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Specifically, LD monolayers have more phosphatidylcholine (PC) and less free cholesterol and sphingomyelin than ER membranes [12]. The mechanisms leading to the differences in ER and LD composition are unclear but are likely important for understanding the biophysical properties of LDs. Additionally, functional implications of the variability of LD surface lipids are particularly intriguing as the surface composition of LDs varies greatly. For example, during LD growth or shrinkage, their surface area changes markedly, and phospholipid surfactants change in amount. Moreover, during those phases, intermediates of metabolic reactions, such as unesterified sterols, diacylglycerol (DAG), or fatty acids (FAs), may accumulate on LD surfaces where they act as fluidifiers or cosurfactants, [13, 14]. This may lead to dynamic interfacial behaviors, in some instances favoring spontaneous LD formation.

LD sizes differ considerably between cells and vary across different emulsion scales, from nano- to macro-emulsion type droplets (i.e., from 100 nm to 100 μm in diameter). For example, LDs in yeast are typically smaller than a micron, and adipocytes often contain a single LD of tens or hundreds of microns. Therefore, LDs may be subject to different kinetic and thermodynamic destabilization forces, depending to the cell type. However, the functional implications of the vastly different size LDs are poorly understood.

Lipid Droplet Stability in Cells

Many emulsions are metastable (i.e., thermodynamically unstable but so long-lived as to be stable for practical purposes). Key to emulsion stability is the presence of interfacial surfactants. When an interface between oil and water is formed, a surface tension γ (expressed in mN/m) is generated due to a lack of cohesive interactions at the interface between molecules belonging to each phase. Generating an interface of area A between the LDs and cytosol results in an energy cost of γA. An emulsion evolves to minimize the contact area between the immiscible fluids and to decrease the energy costs by lowering the surface tension γ. Stabilizing surfactants decrease the surface tension per area and buffer against thermal surface fluctuations, which would otherwise favor emulsion destabilization. The presence of surfactants proffers to the interface a high bending elasticity that dampens such fluctuations [11, 15, 16]. Some basic principles of emulsion physics relevant to LDs, including surfactant properties, are shown in **Figure 1**.

Cells have stable LDs on biological time scales because the oil phase of LDs is covered by a monolayer of surfactant phospholipids [17, 18]. Phospholipids preferentially adsorb at the interface due to their amphiphilic property and satisfy molecular interactions in both phases. They decrease γ, provide high elasticity to the monolayer by dampening deformations, and thus increase LD emulsion stability [15, 16, 19]. For instance, in their presence, the surface tension of LDs can be reduced to the order of 0.1–1mN/m, compared with a exposed interface $\gamma \sim$ 30mN/m [20], providing LDs with kinetic stability under most conditions. LD monolayer elasticity is of the order of 1-10k_BT, mainly through the bending modulus contribution [21, 22]. In addition to phospholipid surfactants, proteins on the LD surface increase the interface elasticity, further contributing to LD stability. However, even with lowered surface tension and increased elasticity in the presence of phospholipids and

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proteins, LD emulsions in cells are *a priori* only metastable and predestined for destabilization in the long term.

The composition of the phospholipid monolayer depends on the cell type, but mostly comprises PC, phosphatidylethanolamine (PE), and lesser amounts of phosphatidylinositol (PI) and lyso-phospholipids [12]. Each of these phospholipids has specific interfacial properties that determine its ability to stabilize emulsions. For example, PC is cylindrical in shape and, therefore, provides excellent coverage of the surface area and greatly lowers surface tension. PC is crucially important for LD stability. PE in contrast is conically shaped, has a smaller head group and is not as good a surfactant for stabilizing oil droplet emulsions as PC. Some unesterified lipids, such as sterols or diacylglycerol, may also localize to the LD surface, where they serve as less optimal surfactants but can fill the space between phospholipids and act as cosurfactants. The surfactant properties of some of the common lipids found at the surfaces of LDs are shown in **Figure 1a**.

Mechanisms and Consequences of Emulsion Destabilization

Two physical processes destabilize LD emulsions: coalescence and ripening (see **Figure 2**). Each process is driven towards minimizing the interfacial surface (energy cost) and generally results in fewer and larger LDs.

Fusion or coalescence of LDs proceeds in two steps (see **Figure 2a**). First, two LDs come in close proximity, and the aqueous film separating them is depleted. Different types of forces including Van der Waals, entropic, or electrostatic interactions, mediated by biochemical or hydrodynamic parameters can drive aqueous film drainage, bringing LDs closer together [15, 23-25]. In a second step driven by thermal fluctuations, a pore, with direct connection of the two oil phases, connects the two LDs. Pore formation occurs when the droplets are so close to each other that the thickness of the aqueous film approaches the nanometric scale. Finally, complete fusion occurs as the pore expands. Of note, with fusion, there is no shrinkage of one of the droplets, as is seen with Ostwald ripening (discussed below).

For two TG drops covered with phospholipids, the time scale of pore expansion and fusion is well below a second at room temperature. However, pore formation does not necessarily lead to fusion; pores can exist transiently and reseal. Whether a pore expands and leads to LD fusion depends on the intrinsic curvature of the surfactants, which determines the monolayer’s intrinsic curvature [9, 11, 16]. During pore formation, the monolayer is highly bent in a small region. The deviation of this curvature from the intrinsic curvature of the monolayer generates an energy barrier for fusion. Coalescence thus depends on the intrinsic curvature of the monolayer: if it is of the same sign as the curvature imposed by the pore formation, the pore is stabilized, can expand, and fusion is favored; otherwise, it closes due to the energy barrier caused by curvature mismatch. For example, if surface lipids such as PE, cholesterol, DAG or FA are present, which due to their small head groups favor bending that stabilizes the junction between the monolayer and the pore, the pore is stabilized and coalescence of oil droplets is favored [21, 22, 26]. The contribution of surfactants to pore

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opening is expressed in the line tension term (also shown in **Figure 2**), *Γ*, which takes into account the bending modulus of the monolayer.

Thus, two properties determine if coalescence occurs, surface tension and line tension of the coalescence intermediate. More specifically, coalescence is an activated process in which a transient pore between two objects forms that leads to complete fusion of both droplets if thermal fluctuations overcome an energy barrier proportional to Γ^2/γ. Stated differently, coalescence is favored if surface tension is high and line tension is low, and less likely to occur if line tension is high and surface tension is low.

Since pore expansion or resealing is curvature dependent, several interfacial lipids have strong influences on the stability of oil-in-water emulsions and have different properties with respect to line tension (see **Figure 2a**). Unesterified cholesterol provides one relevant example. In bilayer membranes (e.g., at the plasma membrane), cholesterol decreases line tension and typically increases kinetics of hydrophilic pore closing [27]. However, it has the opposite effect at the surface of monolayers by favoring hydrophobic pore opening, such as in a bilayer hemifusion process [28]. In such configurations, cholesterol decreases the line tension and therefore the energy barrier for coalescence. DAG and PA provide other examples of lipids with strong effects on membrane dynamics. In bilayer membranes, they facilitate closing the hydrophilic pore during vesicle formation at the pinching-off stage, e.g. as in the case of COPI vesicles budding from the membrane [22, 29-31]. Conversely, in the context of LDs, these molecules of negative curvature would favor fusion between LDs or fusion of LDs with bilayer membranes. Other surface lipids, such as PI or lyso-phospholipids, with intrinsic positive curvature, may influence budding and pinching-off of LDs.

A second mechanism of LD destabilization is Ostwald ripening (**Figure 2b**). This destabilization process involves a coarsening mechanism in which small droplets of an emulsion disappear as bigger ones grow. For oil droplets, this occurs when molecules of oil, though relatively insoluble in the aqueous phase, transfer from one droplet to another through the continuous phase. In ripening, molecules from smaller droplets are transferred through the continuous phase to larger droplets (if they have similar surface composition). The direction of transfer is determined by the difference in the Laplace pressure $P = 2\gamma (1/r_1 - 1/r_2)$ between droplets of radius r_1 and r_2. The mismatch of Laplace pressures is the motor of the ripening instability. A simple analogy is the generation of an electric current (TG) from an electrode of high potential (smaller LD of higher Laplace pressure) to an electrode of low potential (bigger LD of lower Laplace pressure) when a conductor (the aqueous phase) links them. During ripening, the cube of the growing drop of radius r increases linearly over time, $r^3 \propto t$. The rate of growth is proportional to the solubility of the transferred molecule in the continuous phase. Therefore, Ostwald ripening is suppressed when the dispersed phase molecules have extremely low solubility in the continuous phase. This is the case for both TG and SE, which are almost completely insoluble in water. Thus, it is difficult to imagine that this type of ripening occurs between LDs *in vivo*.

There are, however, alternative ripening pathways. When non-ionic amphiphilic molecules, such as detergents, are able to form micelles in the continuous phase they may form swollen

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micelles (several nm in size) containing the dispersed phase [32-35]. In the case of LDs, these would be micelles containing TG and/or SE. Under these conditions, the micelles may carry small fractions of oil from small drops to bigger drops. This solubilization of TG into micelles is analogous to the solubilization of hydrophobic protein domains into detergents micelles. Depending on the state of the cell, the presence of small amounts of amphiphilic lipids, such as FAs or DAG, may favor such processes. For example, FAs form micelles in water at neutral pH, which could mediate ripening by swollen micelles [36]. Similarly, micelles forming in the oil could cause tiny water phases in LDs, possibly explaining the observations of proteins that have been localized to within the oil phase of LDs [37].

The stability of clustered LDs for long periods of time in some cells suggests that ripening between LDs is not constitutive but may be a triggered mechanism. LD remodeling that occurs during lipolysis probably illustrates such a mechanism [38, 39]. In this case, lipolysis products might form swollen micelles that facilitate ripening. In addition, surface lipid properties are altered. LD remodeling due to ripening would be expected to occur on a time scale of a few minutes to hours, and drops can be farther apart. This is completely different to the coalescence mechanism for which drops must be close and content mixing occurs within seconds.

A particular case of ripening, permeation, has been observed for LDs and is attributed to the action of specific proteins [40-42]. For this type of ripening, droplets must be very close so that molecules of the dispersed phase (TG or SE for LDs) avoid traveling through the continuous phase [41, 43]. For example, Fsp27/CIDEC is required to form unilocular LDs from smaller LDs during adipocyte differentiation [40, 42, 44]. It has been suggested that Fsp27 creates channeling pores between the droplets that allow ripening by facilitating TG permeation from one LD to another without crossing the aqueous phase. The oil transport occurs in a few minutes. In a permeation process, the square of the growing droplets radius is expected [11, 45] to be linear over time, $r^2 \propto t$. This time frame matches that observed for FSP27-mediated LD coarsening and LD remodeling during lipolysis and growth in adipocytes [38-40, 42, 44].

Specific Proteins Interact with the Lipid Droplet Monolayer Surface

In addition to phospholipids, the surface of LDs is decorated with proteins. Since the identification of perilipins as LD marker proteins important for regulating lipid metabolism [46, 47], proteomic and cell biological analyses have revealed hundreds of candidates for LD proteins in a variety of cell types. However, methodological issues confound the interpretation of some of these studies. The sensitivity of mass spectrometry for proteomics is consistently increasing, leading to ever-longer lists of proteins identified in LD fractions. While the overlap among these studies serves to identify a core set of LD proteins, it is difficult to determine *a priori* which of the proteins identified by mass spectrometry are genuine LD proteins in a specific cell type, and which are low level contaminants of the analyzed LD fraction. A case in point was the identification of histones as LD proteins of the *Drosophila* embryo [48]. Although this finding was initially met with some skepticism, unexpectedly, this association is specific, mediated by a protein receptor, and functionally important to buffer histone levels during early development [48, 49]. Recent

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development of quantitative proteomics approaches (e.g., protein correlation profiling) that measure the abundance and enrichment of proteins in the LD fraction, rather than just their presence, likely will overcome the limitations of mass spectrometry, especially for low abundant or proteins expressed in a specific tissue cell type [50]. We suggest that *bona fide* LD proteins must fulfill two criteria: they are quantitatively enriched in a purified LD fraction, and the endogenous protein localizes to LDs in cells, as assessed by microscopy.

In repeated studies, a couple dozen proteins have been confirmed to be LD proteins. These proteins have a variety of cell functions. Many of them function in lipid metabolism, for example in the synthesis or trafficking of PC, sterols, or TG. Some of the proteins not involved directly in lipid metabolism are involved in controlling LD surface properties. Perilipins for example were proposed to protect LDs from lipolysis by shielding the TG core of LDs from lipases [51].

The unique properties of the LD surface, compared with those of other organelles, have important consequences for targeting of proteins specifically to LDs. For bilayer membranes, the defined thickness and hydrophobicity of the FA side chains in the bilayer restricts membrane proteins to those with specific domains, such as transmembrane α-helices and β-barrels. Such transmembrane segments with hydrophilic regions of proteins on either side of the bilayer cannot exist as LD proteins, as this would place at least one of the hydrophilic segments in the oil phase, which would be energetically unfavorable. Instead, LD-associating proteins must interact with the surface monolayer lipids, be embedded in the hydrophobic core, or both.

Currently, targeting mechanisms for LD proteins are largely unknown. However, at least two types of LD protein targeting signals are emerging from a collection of studies: amphipathic α helices and hydrophobic hairpins (see **Figure 3**, see also [4]). LD proteins containing one or more amphipathic α helices, such as CTP:phosphocholine cytidylyltransferase (CCT, the rate limiting enzyme of PC synthesis) or viperin (an antiviral protein), are synthesized in the cytoplasm and target to the LD monolayer presumably through binding on the hydrophobic side of the amphipathic helix [18, 52]. Other proteins, such as hepatitis virus core protein, have amphipathic helices that bind the ER membrane bilayer before targeting the LD monolayer. What causes some amphipathic helix–containing proteins, but not others, to target specifically to LDs and accumulate there instead of other organelles is unknown. As yet, no specific LD lipids are known to be involved in protein recruitment, in contrast to other examples, such as the endocytic system, where phosphoinositides are involved in protein targeting.

Alterations in membrane surface tension may serve to restrict protein targeting to LDs. Whereas bilayer membranes, with relatively vast and fluid continuous surfaces, have ultra low surface tensions, LD monolayers are delimited entities that can have much higher surface tension. At higher surface tension, portions of the oil phase underlying the monolayer may be dynamically exposed, which could generate strongly hydrophobic patches that enable protein binding. For CCT, this model is supported by *in vitro* protein binding data to artificially generated emulsion droplets. Specifically, CCT binding to artificial LDs occurs when little PC is present, and thus, surface tension is likely high [18]. For

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CCT, binding to PC-poor LDs or bilayer membranes activates the protein [18, 53, 54] and increases PC synthesis to balance this deficit, providing the cell with a homeostatic mechanism for maintenance of PC levels. Consistent with this model, amphipathic helices in other oil-binding proteins may have a similar binding mode. For instance, amphipathic helices are also found in apolipoproteins (apo) that associate with the monolayer surfaces of extracellular lipoprotein particles, such as chylomicrons or very low-density lipoprotein (VLDL), which can be thought of as smaller, extracellular LDs. An amphipathic helix, the C-terminus of apoA-I, senses exposed TG. It dissociates from PC-covered oil emulsions during PC monolayer compression [55], **Figure 3**. In contrast, the amphipathic helices of apoE or apoC-I bind more efficiently to densely packed PC monolayers than sparsely covered ones [56], **Figure 3**. These examples provide precedence for proteins detecting differences in surface properties of oil-water interfaces, which likely will be an important factor for determining the targeting of LD proteins.

Another sequence motif found in many LD proteins is a hairpin of two alpha helices that dips into and out of a bilayer membrane, such as the ER, without completely spanning it. Proteins containing this type of signal include specific isoenzymes of the TG synthesis pathway, caveolins, and plant oleosins [57-61]. These proteins are co-translationally inserted into the ER membrane. When LDs form, hairpin-containing proteins migrate to LDs along membrane bridges connecting the ER membrane with the LD-delimiting monolayer [61, 62]. Although membrane bridges provide the path for proteins to LDs, it is unclear which energy and mechanism leads to accumulation of proteins on LDs. One possibility is that this targeting is also regulated by surface properties of the LD, with the targeted proteins reducing surface tension of the oil/water interface, thereby providing the energy changes that lead to accumulation.

Lipid Droplet Formation and Growth

In eukaryotic organisms, LD form from the ER. Although direct visualization and knowledge for the initial stages of the formation process are lacking, many lines of evidence support an ER derivation model. For example, most of the enzymes involved in TG or SE synthesis are localized to the ER (in the absence of LDs). Moreover, electron microscopy data reveal close apposition between LDs and the ER [37, 47, 61, 63]. Also, many proteins, particularly those containing hydrophobic hairpins, show dual localization between the ER and LDs. Recent model systems of inducible LD formation in yeast provide more direct evidence that newly formed LDs originate from the ER in this organism [62]. While this does not rule out additional origins from other organelles under some conditions, the available data strongly suggest that LD formation is a function of the ER.

The steps of initial LD formation are still unknown. Most models posit the initial accumulation of TG (or SE) in a lipid lens within the bilayer. At some point, the growing lens is predicted to bud off the ER, forming a LD. From a physical standpoint, we suggest a model based on a dewetting mechanism (**Figure 4a**). When a liquid is deposited on a surface, it spreads to either fully wet the surface, i.e. it forms a thin film, or it partially wets (i.e., it forms a contact angle with the substrate surface). In the extreme case of complete dewetting (for example water on a waxed car surface), the liquid nearly forms a spherical

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drop. This dewetting process has been studied extensively [64] and aspects of it might correspond to the budding of a LD from a bilayer membrane[65, 66]. In this manner, a lipid lens would be converted to a nascent LD by gradually decreasing the contact angle, θ (See **Figure 4a**). The oil dewetting process from a bilayer is influenced by the phospholipid composition of the bilayer membrane and the forming monolayer. Budding of LDs is thermodynamically favored when monolayers and bilayer surface tensions are lowered [43, 66-70] (**Figure 4b**). During LD formation, FA and DAG might be present in elevated levels and act as cosurfactants to phospholipids, lowering surface tension and bending moduli, and favoring LD budding [14, 26, 71-73]. A model for LD formation based on spontaneous emulsification, where surface tension is lowered approach values close to zero, predicts that the size of the formed droplet ranges from 100 to 300nm[74, 75]. The budding size is a function of the wetting properties of the oil with the monolayer, tensions and moduli [7, 67, 76]. Such a model would also posit that LD formation occurs spontaneously when sufficient TG accumulates and adequate surfactants are available to lower surface tension and elastic moduli. It also predicts that the size of the budded LD depends on the surfactant type. Such a purely physical process would help to explain why no single gene products have been identified as required for LD formation.

Theoretically, new LDs can form from existing LDs. If the surface tension of existing LDs is sufficiently lowered (e.g., below a threshold (~0.01 mN/m) [77-79]), new LDs might form spontaneously [80]. In this process, the oil and surface lipids are present in a ratio energetically favoring surface generation or the spontaneous formation of new LDs. More generally, any molecule that efficiently cycles between TG and aqueous phases favors spontaneous droplet formation. For example, particularly short chain FAs or alcohols may favor this process.

Proteins also likely influence the surface tension and the budding process as already observed for bilayer vesicle formation [81, 82]. Perilipins, for example, bind to regions of the ER in some, but not all, cells during LD formation [83], suggesting they modulate the budding process. Additional proteins that might aid in the formation of LDs are BSCL2/seipin and FIT proteins, which have unclear molecular functions but are ER proteins whose deficiency dramatically alters LD size [84, 85].

It is unknown whether LD formation occurs similarly for different neutral lipids (e.g., SE, TG, or retinyl esters). Different types of LDs with either preferentially SE or TG exist in cells, suggesting different origins [86]. We speculate that some TG may be needed in SE-containing LDs to maintain a liquid phase at physiological temperatures (where pure SE would be solid). In addition, among TG-containing LDs, a subpopulation expands after their initial formation [61]. This process is mediated by the re-localization of a subset of TG synthesis enzymes catalyzing the successive steps of TG synthesis, including GPAT4, AGPAT3, and DGAT2 (**Figure 2c**). Fatty acyl CoA synthetases have been localized to LDs [87-89] and likely serve to generate the fatty acyl CoAs needed. On LDs, these enzymes together locally generate TG, leading to linear volume expansion over time of specifically these LDs [61].

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Lipid Droplet Disappearance and Shrinkage

Cells break down TG, or SE, from LDs to generate metabolic energy and to liberate lipids for membrane synthesis. Most information on the LD breakdown process, or lipolysis, has been derived from studies in adipocytes. In these cells, FAs are released from cells as fuel for tissues, such as skeletal muscle and heart. At LDs, the sequential hydrolysis of TG, or lipolysis, is catalyzed by the sequential action of three lipases [90] (see **Figure 5a**). PNPLA2/ATGL (adipose tissue TG lipase) removes a fatty acid preferentially from the *sn2*-position of TG [91] to yield diacylglycerol (DAG). Subsequently, hormone sensitive lipase (HSL) hydrolyzes DAG to monoacylglycerol (MAG) and a fatty acid. In the final step, MAG is hydrolyzed by MGL (MAG lipase) to glycerol and a fatty acid. Sterol esters are also hydrolyzed by HSL. However, other lipases of the carboxylesterases (such as Ces1 or Ces3) have been suggested to catalyze this reaction. The relative contribution of these enzymes *in vivo* is still under debate [92, 93].

ATGL and HSL are constitutively localized to LDs. In addition to their lipase domains, they have hydrophobic stretches that mediate LD targeting. In HSL, the first 300 amino acids are responsible for LD binding [94]. A hydrophobic stretch in ATGL is located in its C-terminus, mediating its association with LDs. It is unknown how lipases, such as ATGL, access the oil phase of LDs and TG substrate. Intriguingly, ATGL localization to LDs requires the Arf1/COPI machinery. From the canonical function of Arf1/COPI proteins in retrograde vesicular trafficking from the Golgi apparatus to the ER, these proteins might mediate transport of ATGL in vesicles from a donor membrane to LDs [95]. Alternatively, since Arf1/COPI have been observed on LDs, these proteins might act directly at LD surfaces, altering the surface properties and indirectly regulating ATGL targeting. Arf1/COPI proteins form nano-LDs (~60 nm) from a phospholipid monolayer interface [20], supporting a possible function for these proteins in regulating the surface properties of the LD.

Lipolysis is strictly controlled in adipocytes (see [96-98] for reviews). Hormonal stimulation (e.g., through β-adrenergic receptors) mediates cAMP- and PKA-dependent phosphorylation and activation of HSL. These kinases also phosphorylate perilipin1 on LDs. Phosphorylation of perilipin1 releases an interaction with CGI-58, which becomes available to activate ATGL on LDs. The mechanism of CGI-58 activation of ATGL is unknown, but could be mediated by altering the surface properties of LDs, making the TG substrates available for the lipase. This hypothesis might explain why ATGL specifically requires a cofactor to gain access to TG. In contrast, the substrates for other lipases, such as HSL and MGL (DAG and MAG, respectively), partition well into a monolayer. MAG partitions more to the monolayer than DAG, which is TG soluble, and this may explain why MGL does not need to bind LDs and instead exists in the cytosol, **Figure 5a**.

Unlike in adipocytes, much less is known about TG mobilization in other tissues. In cells with high energy demands, mobilized fatty acids can be directly oxidized for ATP generation. For example, muscle cells generate a lot of metabolic energy, and in them, contact sites between LDs and mitochondria are important for funneling FAs for oxidation. Recently, a LD protein, perilipin5/OXPAT has been implicated in establishing the close

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contacts between mitochondria and ER [99]. How this protein binds to mitochondria and how the contact sites are established or regulated are unknown.

Many tissues express ATGL, HSL, and MGL, but they are often present at much lower levels than in adipocytes. With the identification of homologous lipases of the PNPLA family, this suggests that other lipases have important lipolytic roles in different cell types. Intriguingly, a polymorphism in one of these lipases, PNPLA3/adiponeutrin, is strongly associated with the development with hepatic steatosis in humans [100]. However, it is unclear whether it is due to the enzyme's acting as a lipase, an acyltransferase or yet another, unidentified function.

When TGs are hydrolyzed from the core of an LD, the LD shrinks. Unless phospholipids are removed from the interface, their packing likely becomes increasingly dense on the surface of shrinking LDs. Highly packed phospholipid monolayers may transition to a solid-like phase, leading to a buckling interface. As an example, during ripening or permeation processes observed by adding "fusogens" to LDs [101], the situation for the shrinking LD (loosing TGs) is similar to that of lipolysis where TGs are removed enzymatically. Phospholipids on the shrinking LDs are compressed over time and probably form a buckled monolayer, which appears as a black line visible by interference contrast microscopy. The compression of proteins on the surface of LDs could also lead to the appearance of such staining due to the same buckling effect.

Little is known about how phospholipids and proteins are removed from shrinking LDs. As the surface area per phospholipid decreases during LD shrinkage, excess phospholipids could be expelled from the interface, but this mechanism is unlikely due to the extremely low solubility of amphipathic phospholipids in either TG or cytosol. Several LD proteins, such as oxysterol binding proteins or STARD-proteins, contain lipid transfer domains, which could mediate phospholipid transport from LDs to other organelles, but evidence for such a process is lacking. Phospholipids also may be removed from LDs interfaces enzymatically, cleaving them to more water/TG-soluble products, such as fatty acids and DAG. Consistent with this possibility, several phospholipases localize to LDs [102, 103]. However, as yet phospholipases have not been implicated as required for lipolysis, which would be expected if this process were crucial for LD shrinkage.

Phospholipids also could be removed from LDs in the form of swollen micelles. During lipolysis, large amounts of DAG and FAs are transiently generated. These lipids can access the monolayer surface, decreasing the LD surface tension. As a result, small micelles (10–50 nm[77] and below the resolution limit of light microscopy) containing TG and phospholipids at their surface, as well as FAs/DAG, could form from LDs towards the cytosol. During lipolysis, reverse micelles, containing water, phospholipids at their surface and FAs/DAG, might form inside the oil. No evidence supports or contradicts the formation of small micelles. However, lowering of surface tension on LDs and spontaneous emulsification during lipolysis might explain the LD fission observed in *Schizosaccharomyces pombe* during cell division [104].

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In addition to phospholipids, proteins need to be removed from shrinking LDs. For proteins that bind to LDs via amphipathic helices, changes in the surface lipids might alter the affinity, and some proteins might dissociate. For other proteins, such as those with hairpin topology, removal from LDs presents a process that likely requires energy for extraction of the hydrophobic regions. Of note, several proteins functioning in proteasome-mediated protein degradation have been localized to LDs [105-107]. It remains to be determined whether these proteins function in LD protein turnover. At least one such protein, Ubxd8, has a function on LDs distinct from protein turnover. Ubxd8 interacts with p97/VCP to block the interaction between CGI-58 and ATGL, thus inhibiting lipolysis [105].

In addition to lipolysis, autophagy has been proposed to mediate LD turnover [108] (**Figure 5b**). In hepatocytes, autophagy proteins are observed in the vicinity of LDs. In livers of mice with dysfunctional autophagy, TG accumulates and the amount of autophagic intermediates depends on the metabolic states of cells. Similarly, autophagy is required for normal LD metabolism in other cell types, such as neurons and stellate cells [109, 110]. Thus, a specialized form of macroautophagy, termed lipophagy, might mediate the trafficking of LDs to lysosomes where LD lipids and proteins are degraded (**Figure 4b**). The relative contribution of lipolysis and lipophagy for LD turnover in different tissues is unclear. It is also unclear how LDs are recognized by the autophagic machinery or how autophagy of LDs is regulated.

Non-Canonical LD Functions and Outlook

The unique structural features of LDs provide cells with variable amounts of a phase-interface and a bulk organic phase. Several processes appear to have evolved to capitalize on these unique LD features. For example, LD surfaces might temporarily store hydrophobic proteins destined for degradation, such as the transmembrane domain containing HMG-Co reductase [111]. The LD surface more generally might function as a sequestration platform for proteins that otherwise might be toxic for cells, such as histones [48]. LDs also appear to serve as platforms for the transient storage of some viral proteins containing amphipathic helices, such as the core protein of hepatitis C virus, which can be used subsequently for viral assembly [112].

The bulk organic phase might also provide space to store and synthesize large and bulky lipid metabolism intermediates that would otherwise disrupt bilayer membranes. For example, several enzymes involved in the synthesis of dolichol, which can contain a hundred carbons in its isoprenoid chain, have been found at LDs [113]. However, the significance of LD localization of these enzymes is not understood. The hydrophobic cores of LDs also likely provide an important reservoir for hydrophobic drugs, which partition into this phase, and fat-soluble vitamins (A, D, E, and K). Indeed, such partitioning has inspired the design of TG emulsion droplets as carriers for various drugs, and motivated research in the phase behavior of mixtures of TG, water, phospholipids, and derivatives.

For many of these and other biological processes involving LDs, understanding their unique behavior as defined by biophysical principles is essential. Viewing cells containing LDs as an oil-in-water emulsion is an emerging concept that colors the interpretation of LD biology.

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Soft matter physics knowledge is only now being integrated into this field, but promises to rapidly expand our understanding of LD cell biology and related physiological processes.

ACKNOWLEDGMENTS

We thank Frederic Pincet, Florian Wilfling and Nicolas Bremond for critical discussions and Gary Howard for editorial assistance. Work on lipid droplets in the Walther and Farese laboratories is supported by NIH grants R01GM097194 (to T.C.W.), and RO1GM099844 (to R.F). A.-R.T. is a fellow of the Marie Curie BFLDs IOF within the 7th European Community Framework Program grant to a Partner University Funds exchange grant between Yale and Ecole Normale Supérieure laboratories.

Glossary of Terms

Bending Modulus — The bending modulus represents the energy needed to bend a monolayer from its spontaneous curvature

Buckling Interface — A collapse of the linear interface of two fluids due to transition to a solid-state monolayer reached by crowding of phospholipids or surfactants

Cosurfactants — A surfactant that acts in combination with another primary surfactants and can lower surface tension. Generally smaller than the primary surfactant, cosurfactants partition easily between the different phases. They fill the space between primary surfactants and therefore decrease the surface tension

Contact Angle θ — The angle where a liquid interface meets a solid surface. It is also applicable to the angle between an LD and the ER bilayer

Dewetting — The rupture of a thin film on a substrate to form a droplet. The counterpart to dewetting is spreading. Dewetting depends on the surfactant concentration. Dewetting of an oil droplet within a bilayer occurs when the monolayers of the bilayer zip or wet together. This process is favored by lowering surface tension

Intrinsic Curvature — The intrinsic curvature of a surfactant is its spontaneous curvature. It reflects the hydrophilic and lipophilic balance of the molecules. If the mean area of the hydrophilic part is larger than of its hydrophobic part, the curvature of the molecules is considered positive, and it tends to form direct micelles. In the opposite case, the curvature is negative. The intrinsic curvature of surfactants is dependent on properties such as pH, length of acyl chains, and temperature

Laplace Pressure — The pressure difference between the inside and outside of a curved liquid surface. Surface tension normally tends to compress the disperse liquid to minimize the interfacial area and the energy of the system. This leads to a spherical shape of the dispersed liquid or a drop. The contraction is arrested when a relative positive pressure called Laplace pressure builds up inside the drop. Decreasing the surface tension minimizes the Laplace pressure

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Line Tension, Γ Line tension is the energy cost per unit length at the boundary line between different phases. Among many parameters, line tension is a function of surfactant acyl chains length and bending modulus. Decreasing these parameters decreases line tension

Permeation Permeation in the context of emulsions is the process by which one type of molecule (e.g., TG), present in one compartment crosses a membrane barrier or a liquid film by diffusing through it, and thus reaching another compartment. A misbalance of chemical potentials of a solute present in different compartments generally triggers permeation. The rate of permeation is a function of the diffusion coefficient of the molecule through the barrier, the thickness of the barrier and the solubility of the molecule in the barrier

Surface Tension γ Surface tension is the energy required to increase the surface area of a liquid by a unit area, and can be thought of as the energy cost per unit area generated between two immiscible fluids. The presence of phospholipid surfactants minimizes the energy cost by shielding the interface

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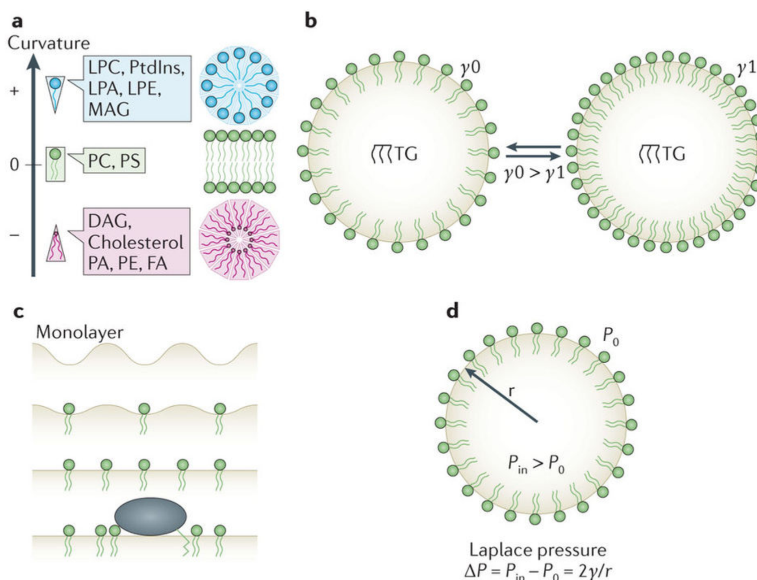

Figure 1. Basic principles of emulsion physics relevant to lipid droplets

a. Surface lipids on LDs and their curvatures. Curvature of typical surfactants is defined according to the difference between the area occupied by their hydrophilic head and the area of their lipophilic tail. Positive curvatures correspond to a predominant hydrophilic part. Positively curved lipids, lyso-phospholipids, PI, and MAG, tend proffer a monolayer a positive curvature. Monolayers mainly formed by DAG, PA, or cholesterol on the other hand have a negative curvature. PC is cylindrical in shape and has almost no curvature. PC therefore generally assembles into lamella and is the main component of bilayer membranes.

b. Influence of surfactants on emulsion stability. More surfactant at the interface tends to lower surface area A and increase stability. Less surfactant, or less effective surfactants (e.g., PE vs. PC) tend to do the opposite.

c. Elasticity of surface monolayers. A loose monolayer, such as an oil-water interface, has wrinkles associated to thermal fluctuations. The presence of phospholipids dampens the fluctuations by creating an energy barrier to surface deformation. They also increase the elasticity of the monolayer. Phospholipids with longer acyl chains are more efficient for dampening fluctuations (left). Likewise, higher concentrations of phospholipids result in a higher barrier to induce deformation by thermal fluctuation (right). The presence of proteins also increases the elasticity.

d. Laplace pressure is the pressure that builds up inside the drop to counterbalance the compression effect of surface tension. P_0 is pressure in the continuous phase. The surface tension is denoted γ. The drop radius is r. The Laplace pressure of the drop is the difference between the pressures inside and outside the drop and corresponds to $2\gamma/r$. If surface tension is similar, smaller drops have higher Laplace pressures than larger drops

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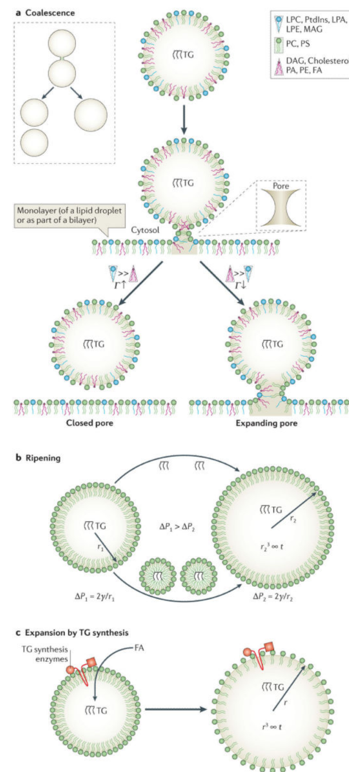

Figure 2. Processes that govern changes in lipid droplet size

a. Coalescence and influence of monolayer curvature. A TG droplet covered with phospholipids forms a pore with another monolayer that can be of another LD or the outer monolayer of a bilayer. At site of the pore, the monolayer is bent, and monolayer curvature, depending on the types of lipids, becomes important. If the spontaneous curvature of the monolayer is positive (e.g., in excess presence of positively curved lipids), this results in a "frustrated" situation, with high line tension, and the pore closes. If the monolayer's spontaneous curvature is negative, e.g. in excess presence of negatively curved lipids, the curvature of the lipids matches the bending, and the line tension is low. Therefore the pore is stable and can open further. In the case of two LDs, this results in fusion or coalescence, generating one larger LD (inlay). In the case of a LD and a membrane, fusion results in a transiently stable connection of LDs with bilayers. Pore opening and fusion occur in a millisecond scale.

b. Ripening of LDs. In ripening, molecules from one LD diffuse to another. The direction is determined by the difference in Laplace pressures of the two LDs, with TG molecules traveling from smaller LDs to bigger LDs. In the case of TGs and LDs, diffusion might occur in swollen micelles, which are micelles containing small amounts of TG. In contrast to coalescence, ripening takes several minutes. The volume increase of the bigger drop is linear over time, $r^3 \propto t$. Ripening leads also fewer and bigger LDs; however, one droplet shrinks while the other one grows.

c. Growth of LDs by new TG synthesis in situ. Enzymes mediating TG synthesis, GPAT4, AGPAT3 and DGAT2, can directly localize to LDs and synthesize TG at the surfaces of LDs. Acyl CoA synthetases localize to LDs and likely provide the fatty acyl CoA substrates. As recently observed [61], the volume of the drop increases linearly over time, $r^3 \propto t$.

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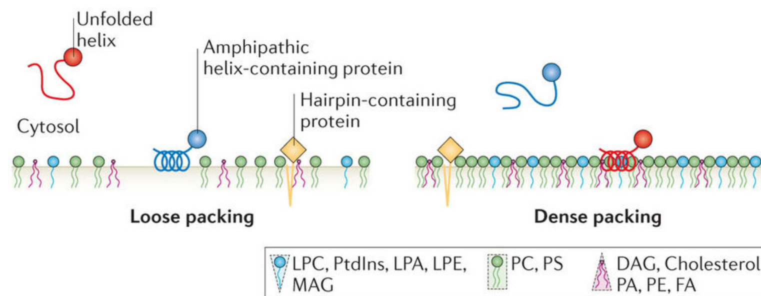

Figure 3. Binding mode of proteins

Illustration of amphipathic helices and hairpin contained proteins binding to LDs. (left) A loose monolayer of higher surface tension is bound by one type of helix (CCTα or ApoA's C-terminus for example) and a hairpin (of GPAT4 for example). A helix, typically of ApoE, prefers staying in the cytosolic phase. (right) The compressed monolayer could be still bound by the hairpin. The helix that bound the loose monolayer is now expulsed. The protein free in the cytosol could prefer folding its amphipathic helix by interacting with the head group of phospholipid and bind.

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Figure 4. Models for mechanisms of lipid droplet formation

a. Illustration of dewetting transitions. A liquid deposited on a surface can wet completely the surface and form a liquid film on top of it. The liquid can dewet and form a drop with a contact angle with the substrate. Wetting is controlled by surface tension.

b. Model for spontaneous budding of a TG droplet from a bilayer based on dewetting transition. Accumulation of TG inside a bilayer can lead to spontaneous emulsification of a TG drop in a low-surface tension environment. The expulsion of the TG LD corresponds to a dewetting state that is energetically favored. Different surface lipids can modulate such process.

c. Origins of different LD populations. Evidence suggests that two populations of basic LDs exist. Smaller LDs bud from the ER (e.g., from the enzymatic products of ER enzymes such as DGAT1). Such LDs pathway has a characteristic size that likely depends on the surface surfactants. A second population of cytosolic LDs, called expanding LDs, acquires enzymes of the GPAT4/DGAT2 TG synthesis pathway. These enzymes can locally synthesize TG at the LD from local substrates.

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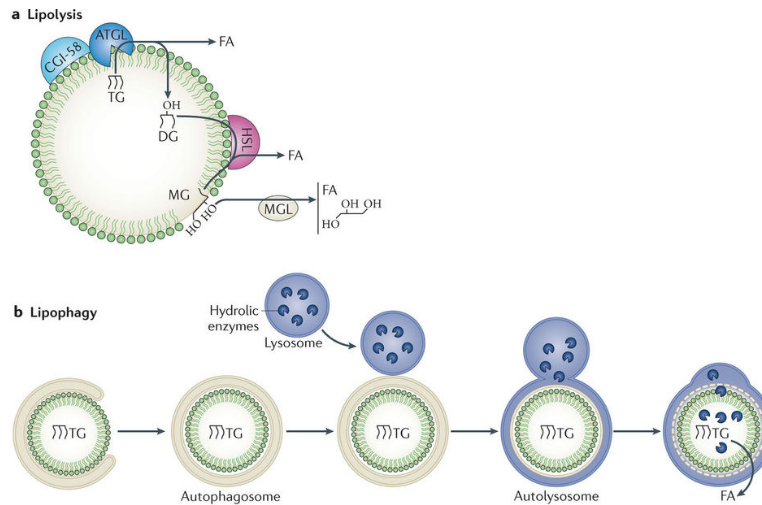

Figure 5. The utilization of consumption of lipid droplets

a. Scheme of lipolysis. ATGL catalyzes the first step of lipolysis and is recruited onto LDs by a co-factor CGI58. TG hydrolysis by ATGL leads to a release of free FA and mainly 1,3-DAG. HSL, also bound to LDs, hydrolyses DAG into FFA and MAG. The latter is in turn hydrolyzed to FFA and glycerol by MGL, which is soluble in the cytosol. The volume of the LD decreases over time, probably linearly. As a result, the surface phospholipids and proteins become more crowded. Mechanisms must exist to facilitate the catabolism or removal of these surface components (discussed in text).

b. Scheme of LD autophagy, or lipophagy. Autophagy is proposed to also regulate LDs degradation and TG utilization. An autophagosome forms in the cytosol and encapsulates a LD. It subsequently fuses with a lysosomal organelle containing hydrolytic enzymes that hydrolyze TG from the encapsulated LD and proteases that degrade LD proteins.