

Lipid Droplet Formation and Lipophagy in Fatty Liver Disease

Ryan J. Schulze, PhD¹ Mark A. McNiven, PhD¹

¹ Department of Biochemistry and Molecular Biology and the Center for Digestive Diseases, Mayo Clinic, Rochester, Minnesota

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Address for correspondence Ryan J. Schulze, PhD, Department of Biochemistry and Molecular Biology, Mayo Clinic, 200 1st St SW, Guggenheim 16, Rochester, MN 55905
(e-mail: Schulze.Ryan@mayo.edu).

Abstract

Lipid droplets (LDs) are key sites of neutral lipid storage that can be found in all cells. Metabolic imbalances between the synthesis and degradation of LDs can result in the accumulation of significant amounts of lipid deposition, a characteristic feature of hepatocytes in patients with fatty liver disease, a leading indication for liver transplant in the United States. In this review, the authors highlight new literature related to the synthesis and autophagic catabolism of LDs, discussing key proteins and machinery involved in these processes. They also discuss recent findings that have revealed novel genetic risk factors associated with LD biology that contribute to lipid retention in the diseased liver.

Keywords

- autophagy
- NAFLD
- lipid droplet

The leading cause of chronic liver disease in the United States and Europe is nonalcoholic fatty liver disease (NAFLD), a complex manifestation of pathologies that exist on a spectrum ranging from simple steatosis (fat accumulation) to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis.¹ Untreated, NAFLD represents a significant risk factor for the development of other metabolic disorders, including Type 2 diabetes and cardiovascular disease. Globally, NAFLD is estimated to affect nearly one in four adults and represents the second leading indication for liver transplant in the United States,² whereas NASH is predicted to become the primary justification for transplant by 2020.³ Furthermore, NAFLD is increasingly being recognized as a risk factor for the onset of hepatocellular carcinoma (HCC), with various studies reporting that HCC incidence rates of 4 to 22% may be directly attributable to NAFLD.^{4,5} Alcoholic liver disease represents a related disease state characterized by fatty liver at early stages (alcoholic steatosis) with the potential for the onset of severe complications, including alcoholic hepatitis, cirrhosis, and HCC.^{6,7}

A defining pathophysiological hallmark of the steatotic liver is the accumulation of significant amounts of fat within the parenchymal cells of the liver, the hepatocytes. Normally, these cells are especially adept at the routine storage and efflux of fat to facilitate organismal metabolic requirements. However, in the disease state, they are susceptible to the accumulation of supraphysiological levels of fat. There is

considerable evidence that abnormal levels of free fatty acids (FFAs) act as a driving force behind the pathogenesis of the more serious sequelae associated with NAFLD (i.e., NASH). As no approved pharmacological compounds exist for the treatment of the fatty liver, there remains a great deal of interest in understanding the complex relationship between fat accumulation and its effect on hepatic function.

Fatty acids (FAs) derived from peripheral storage in the adipose tissue as well as those synthesized de novo in response to carbohydrate metabolism are the two major pools that flux through the liver. In response to defects in FA oxidation or aberrant accumulation, the hepatocyte can prevent lipotoxic damage by compartmentalizing excessive FAs and sequestering them away from the cytoplasm. In hepatocytes (and indeed, most mammalian cells), the primary reservoir of fat is within a dedicated storage organelle, the cytoplasmic lipid droplet (LD). Initially considered inert depots of fat, LDs have over the course of the past three decades become appreciated to have a unique biology and are now considered to be *bona fide* cellular organelles. As the presence of LDs is intimately linked with the metabolic state of the hepatocyte, their biosynthesis and turnover are tightly regulated. A better understanding of the regulatory processes governing the formation and breakdown of these organelles will help guide the development of novel intervention strategies for the treatment of NAFLD. Recent

insights into the biology of LDs have uncovered key new players governing the cellular manufacture of LDs as well as important roles for the process of autophagy, a lysosome-directed catabolic pathway of cellular self-renewal, in the turnover of LDs. In this review, we will discuss the biogenesis of LDs as well as mechanisms of autophagic turnover within the context of the fatty liver.

Structure and Biogenesis of Lipid Droplets

The LD has a unique architecture befitting a dedicated lipid-storage organelle. Generally, LDs are simple structures surrounded by an endoplasmic reticulum (ER)-derived phospholipid monolayer and decorated by a limited but dynamically changing proteome.⁸ The proteins on the surface of the LD are thus essential for coordination with various cytoplasmic machineries to promote further expansions in the size of the droplet, or as detailed below, the catabolism of the droplet under appropriate conditions. Within the interior of the hepatocellular LD is a neutral lipid core consisting primarily of triacylglycerol (TAG) and cholesteryl ester (CE) or retinyl ester (RE). These lipid species are each synthesized by dedicated ER-localized acyltransferases—attractive targets for interfering with aberrant lipid accumulation in fatty liver disease.

Neutral Lipid Biosynthesis

The terminal step of triglyceride biosynthesis is catalyzed by an esterification reaction between fatty acyl-CoA and diacylglycerol by two acyl-CoA:diacylglycerol acyltransferases (DGAT1 and DGAT2). Evolutionarily distinct, these enzymes catalyze the same reaction but are thought to play differing contributions to the biogenesis of LDs. DGAT1 appears to be responsible for only a minor contribution to LD biogenesis, perhaps playing a more important role in the repackaging of excess FFAs in an effort to thwart lipotoxicity and possibly a larger role in intestinal TAG synthesis.^{9–11} An examination of DGAT1 null mice found decreases in adipose tissue levels, increased insulin sensitivity, and resistance to diet-induced hepatic steatosis.^{12,13} These promising findings resulted in the subsequent development of DGAT1-targeted pharmacological inhibitors for use in humans; however, owing to the onset of intolerable side effects, these treatments are currently thought to be of questionable utility.¹⁴ Mutations in DGAT1 have since been identified in humans that result in reduced protein levels and are correlated with congenital diarrhea, suggesting that alterations in the function of DGAT1 result in much different phenotypes between mice and humans.^{10,15} The second isoform, DGAT2, is highly expressed in the liver and is thought to be the predominant isoform with regard to storage of triglycerides. Owing to a unique membrane topology that is very different from DGAT1, this isoform is able to simultaneously associate with both the ER and the LD.¹⁶ Whole-animal knockouts of DGAT2 are lipopenic and die shortly following birth, likely due to insufficient triglyceride stores.¹⁷ Studies in mouse models of obesity have found that reductions in DGAT2 expression by antisense oligonucleotide treatment result in improvements to liver steatosis but also exacerbate the

onset of hepatic fibrosis.^{18,19} Like studies with DGAT1, however, the translatability of DGAT2 inhibition from rodent studies to higher mammals has had inconclusive results, with a recent study in nonhuman primates showing that acute or chronic DGAT2 inhibition had no significant effect on triglyceride metabolism.²⁰ Clearly, more work will be required to understand how each of these enzymes central to LD biogenesis cooperates to promote hepatic TAG synthesis and how they might be targeted to stem lipid accumulation in NAFLD.

Cholesteryl esters represent a second important type of neutral lipid stored within LDs. The acyl-CoA:cholesterol acyltransferases (ACAT1 and ACAT2) catalyze the conversion of free cholesterol to CE, and are central to cellular cholesterol metabolism. These two enzymes are currently being investigated as drug targets for the treatment of dyslipidemias.^{21,22} Both isoforms are expressed in the liver, with ACAT1 showing distribution in other tissues and ACAT2 possibly being more specific to the liver. Inhibition of ACAT2 was shown to prevent dietary cholesterol-associated hepatic steatosis by promoting TAG hydrolysis and mobilization from LDs into nascent very-low-density lipoprotein particles for secretion.²³ REs are a third species of neutral lipid commonly localized in LDs of the liver; however, these are found almost exclusively within hepatic stellate cells (HSCs), where they serve as the major storage form of vitamin A.²⁴ The synthesis of RE is mediated by lecithin:retinol acyltransferase, an enzyme that appears to be essential for LD formation in HSCs. In response to liver injury, these RE-enriched LDs are rapidly mobilized and reduced in size as HSCs are activated; as a consequence, fibrosis is enhanced.^{25,26} How these stored REs contribute to the activation of HSCs remains unclear.

Lipid Droplet Assembly

Irrespective of the neutral lipid species generated within the ER lumen, its gradual accumulation is thought to result in a distention of the bilayer as an oil ‘lens’ begins to form (**►Fig. 1**), with the two phospholipid leaflets of the bilayer eventually separating from each other at sites where LDs are formed.^{27,28} Live-cell imaging experiments demonstrated that these nascent sites of LD biogenesis are highly mobile within the ER network but can be stabilized at fixed contact sites delimited by the protein BSCL2/seipin.^{29–31} Seipin can therefore be considered a key governor of the initial flux of neutral lipid into the maturing LD. It may accomplish this function partly by assisting in the destabilization of the ER bilayer to permit the formation of the oil lens in the first place.²⁸ Continued funneling of lipid into the stabilized droplet results in a directional ‘budding’ of the LD outward into the cytoplasm.³² Like seipin, FIT2 is a second protein that may participate in the orchestration of these initial steps in LD biogenesis, by promoting this vectorial budding process.²⁷

Through unknown mechanisms, the LD remains either stably associated with the ER or is released freely into the cytoplasm. It remains unclear exactly at which point in this process that LD-specific proteins (e.g., the PLIN family of proteins) begin to accumulate on the growing droplet. As these early ER-LD contact sites represent critical settings for

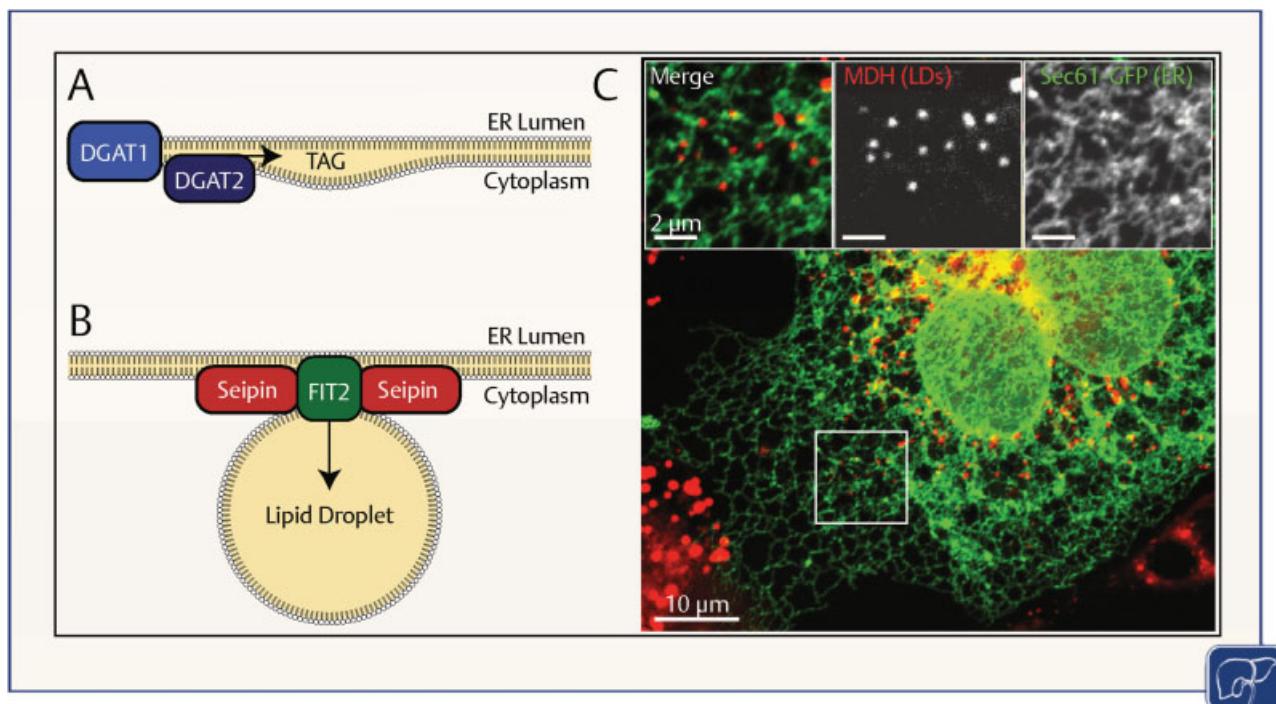


Fig. 1 Biogenesis of LDs. (A) The accumulation of neutral lipid (i.e., triglyceride synthesis by DGAT1 or DGAT2) within the ER results in a cytoplasmic distention of the outer leaflet of the ER bilayer and subsequent formation of an oil 'lens.' (B) These nascent sites of LD biogenesis can be stabilized at fixed LD-ER contact sites delimited by the proteins BSCL2/seipin and FIT2, which govern the vectorial flux of neutral lipid into the maturing LD. (C) Fluorescence microscopy of nascent LD biosynthesis as visualized by expression of Sec61-GFP (to label the ER network, green) and staining with a LD-specific dye (monodansylpentane [MDH], labeled in red). LD, lipid droplet; ER, endoplasmic reticulum.

the initial expansion of LDs and hepatic TAG accumulation, identification of the machinery involved at these cellular locations will be critical for understanding how liver steatosis might be prevented at early stages.

Catabolic Pathways Mediating Lipid Droplet Breakdown

In contrast to enhanced synthesis, decreased catabolism of LDs is another potential root cause of hepatic steatosis. Two major pathways exist for the turnover of LDs in the hepatocyte: conventional lipolysis and autophagy. Lipolysis involves a cytoplasmic cascade of enzymes (of which some directly interact with the LD) that sequentially remove one FA at a time from the glycerol backbone of the TAG stored inside the LD. Much of our understanding regarding lipolysis has been defined in adipocytes and involves three key lipases, adipose triglyceride lipase (ATGL), hormone-sensitive lipase, and monoacylglycerol lipase, which catalyze the rapid lipolysis central to adipose tissue biology. These same proteins also play important roles in the liver. The regulation of these enzymes is complex and requires the participation of many hormones and growth factors across numerous signal transduction pathways.³³ For recent reviews on lipolysis, please refer to Duncan et al and Zechner et al.^{34–36}

In addition to the soluble lipases described above, hepatocytes also utilize the autophagic pathway to catabolize LDs. This lysosome-centric process involves the construction of a specialized membrane (known as a phagophore) around the perimeter of organelles destined for degradation. Once

enclosed within this autophagic membrane, in a structure referred to as an 'autophagosome,' the downstream recruitment of lysosomes to the autophagosome results in cargo degradation within a hybrid compartment known as an autolysosome (►Fig. 2). Autophagy requires the coordinated activity of nearly 30 proteins (Atg cascade) and can be either nonselective (bulk autophagy) or selective for particular organelles such as LDs, a process referred to as 'lipophagy.'^{37,38} A seminal study by Singh and colleagues found that lipophagy plays an important role in hepatic lipid metabolism³⁹ and that the genetic or pharmacological inhibition of autophagic flux significantly increased TAG and LD content, resulting in a concomitant decrease in hepatic FA oxidation.

Recent progress has been made toward understanding the mechanisms underlying the process of selective autophagy for other organelles such as the mitochondrion (mitophagy) or peroxisome (pexophagy) and has resulted in the identification of organelle-specific receptors that guide the assembly of the phagophore around these organelles.^{40–43} These receptors often contain key motifs which mediate the interaction with LC3, a protein found on the phagophore and autophagosomal membranes.⁴⁴ Although no specific receptor has been identified as being present on the LD, clues from proteomic analyses have provided insights into proteins that permit lipophagy to proceed.

Because the autophagic process utilizes select components of the endocytic and lysosomal pathways, it is not surprising that a significant number of membrane trafficking proteins have been identified on the surface of the LD. While

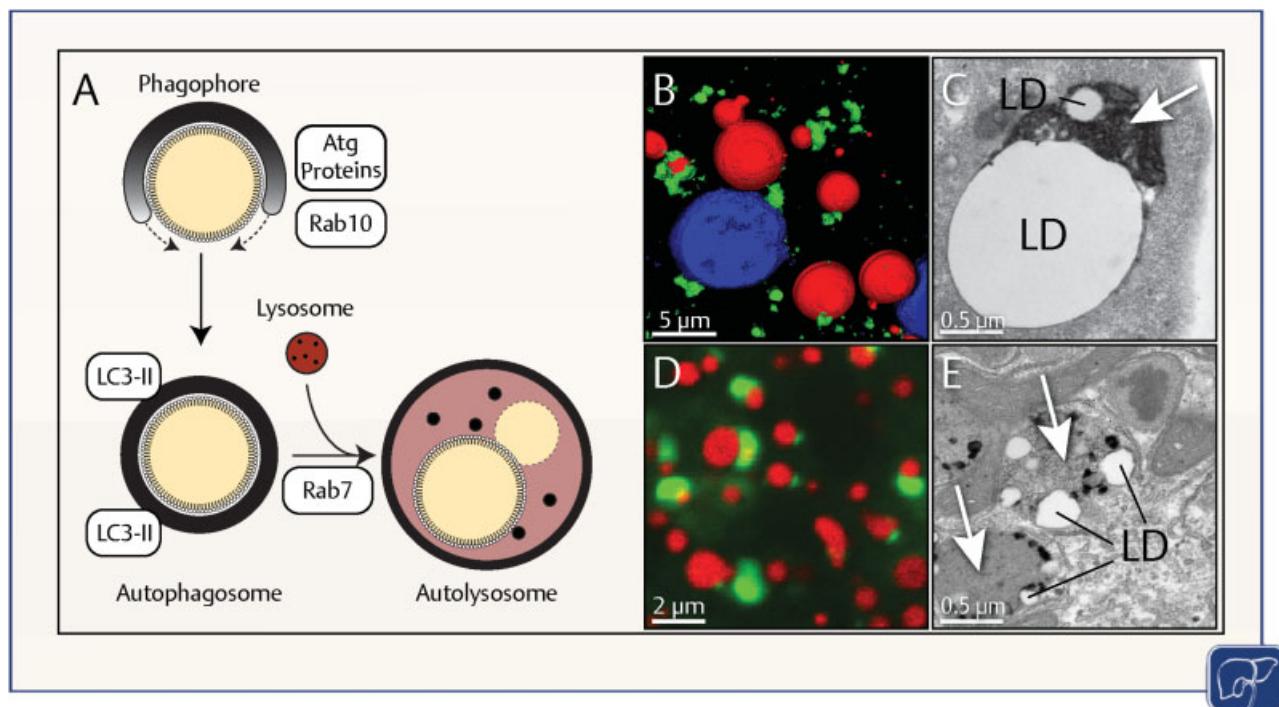


Fig. 2 Catabolism of LDs by lipophagy. (A) The process of lipophagy involves the construction of a specialized membrane (known as a phagophore) around the perimeter of LDs destined for degradation. Once enclosed within this autophagic membrane, in a structure referred to as an ‘autophagosome,’ the downstream recruitment of lysosomes to the autophagosome results in turnover of LDs within a hybrid compartment known as an autolysosome. (B) Maximum-intensity projection of Oil Red O-stained LDs (red) targeted by LC3-positive autophagic membranes (green) in primary porcine hepatocytes. (C) Electron micrograph of a large LD in close apposition to an autolysosome or lysosome (arrow) containing an internalized LD in HuH-7 human hepatoma cells.⁵⁰ (D) Fluorescence microscopy reveals the association of a constitutively active GFP-Rab10 with LDs (red) in HuH-7 human hepatoma cells cultured in serum-starvation conditions.⁵⁰ (E) Electron micrograph of a primary rat hepatocyte cultured under basal conditions and exhibiting autolysosomal structures (arrows) containing numerous enclosed LDs (these images are provided courtesy of Eugene W. Krueger, Mayo Clinic). LD, lipid droplet.

as many as 30 members of the Rab family of small GTPases have been identified to associate with LDs, the functions of just a few of these have been resolved.^{45–47} Among these small GTPases, Rab7 has been shown to be activated in response to nutrient depletion and to promote the association of LDs with components of the late endocytic pathway (i.e., multivesicular bodies and late endosomes/lysosomes).⁴⁸ In agreement with a potential role in mediating lipophagy, inhibition of Rab7 activation results in an impairment in the turnover of hepatic LDs.^{48,49} Rab7 was recently shown to be a target of alcohol-induced steatosis, with activity found to be dramatically reduced in rats chronically fed an ethanol-containing diet.⁴⁹ An additional Rab GTPase, Rab10, was also shown to be activated under autophagy-stimulating conditions and to participate in the physical engulfment of LDs by the expanding phagophore.⁵⁰ Rab32, a mitochondrial protein with reported LD localization, has been shown to play a key role in LD storage in larval adipocytes from flies, with impaired autophagy upon genetic depletion.^{51,52} A similar role for Rab32 in LD metabolism was observed in a subsequent study.⁵³ Another Rab GTPase, Rab18, is thought to be localized primarily to the LD as well as the ER.^{54–56} Although this localization suggests a direct role for this GTPase in LD catabolism, recent studies demonstrated that Rab18 depletion has no effect in several mammalian nonadipose cell types,^{57,58} indicating a potential

cell-type specific role for this particular Rab. Further work is clearly required to dissect the many roles for these small GTPases in LD biology. In addition, large GTPases may also play critical roles in the autophagic turnover of LDs. Following degradation of LDs in the autolysosome, the autolysosomal membrane is recycled to generate nascent lysosomes in a process termed autophagic lysosome reformation (ALR).⁵⁹ Long-membrane tubules are extruded from the autolysosomal membrane and undergo scission by the large GTPase dynamin-2 to regenerate lysosomes and maintain lipophagic flux.⁶⁰ In the absence of dynamin-2, lengthy ALR tubules are observed in hepatocytes and LDs accumulate due to a lack of lipophagy.

Despite the differences in the mechanisms employed by the lipolytic and lipophagic pathways, there is likely to be significant crosstalk between these two processes.^{36,39,61,62} It was recently demonstrated that PLIN2, a major surface protein on hepatic LDs, may be selectively recognized by the chaperone-mediated autophagy (CMA) system for targeted turnover.⁶³ CMA is a quality-control process whereby proteins (such as PLIN2) harboring a specific chaperone (Hsc70)-recognition motif can be selectively imported directly into the lysosome for turnover.⁶⁴ This therefore may represent a key first step in initiating downstream mechanisms of LD turnover. Removal of LD coat proteins like PLIN2 by CMA might allow for enhanced access by cytoplasmic lipases such as ATGL to act on the droplet,

where release of FFAs could be a prerequisite for the onset of macroautophagy. A recent study provided strong evidence showing that ATGL activity was required for the downstream expression of hepatic autophagy gene expression.⁶¹ Importantly, this lipase activity also positively regulated flux through the autophagic pathway and promoted associations between the LD and components of the autophagic pathway, namely lysosomes and LC3. The pharmacological or genetic inhibition of conventional macroautophagy or lysosome-specific lipid hydrolysis was able to negate the effects of adenoviral-mediated overexpression of ATGL in the hepatocyte. This research provides further evidence that ATGL action may be crucial for initiating the autophagic cascade but that lipophagy plays a more significant role than previously appreciated in the lipid turnover occurring in the hepatocyte. Other factors, such as the diameter or lipid composition of individual LDs, may also dictate the specific type of machinery used for LD catabolism.

Roles for Lipophagy in Fatty Liver Disease

Several studies have begun to address the role played by autophagy in the development of fatty liver. Insulin resistance associated with fatty liver is likely to result in the accumulation of serum FFAs available to be taken up by the liver, but the exact mechanisms leading to the accumulation of significant quantities of LDs within the fatty liver are not well understood.⁶⁵ Whether the autophagic defects associated with NAFLD are a result of the excessive lipid accumulation or are themselves the actual cause is still unclear.^{65–67} Recent studies have examined links between autophagic defects and liver disease, although only a few have actually looked at the effects of NAFLD on the selective targeting of LDs. The transcriptional regulator transcription factor EB (TFEB), which integrates signaling and lipid homeostasis through PPAR α and PGC-1 α , may itself be adversely affected in NAFLD, as mice with liver-specific TFEB knockout have significant accumulations of hepatic LDs while viral-mediated hepatic overexpression of TFEB was sufficient for preventing weight gain and metabolic syndrome in mice.⁶⁸ Adenoviral overexpression of TFEB in mice fed a high-fat diet interfered with the oscillating nature of an mTORC1-TFEB regulatory circuit and was able to significantly improve liver function, likely due to enhanced lysosomal function during lipophagy.⁶⁹ Another possibility is that NAFLD patients may have reduced levels of glycine N-methyltransferase, an enzyme that participates in methionine and S-adenosylmethionine metabolism.⁷⁰ Defects in expression of this enzyme result in elevated levels of both serum methionine and S-adenosylmethionine and consequently negatively impact hepatic lipophagy.⁷⁰ Patients with NAFLD were also shown to have slightly elevated levels of rubicon, a protein with known antiautophagic properties.⁷¹ Electron microscopy performed on hepatocytes of rubicon-knockout mice showed defective hepatic lipophagy, manifested as an accumulation of double-membrane structures around the surface of numerous LDs. Mice with a liver-specific defect in macroautophagy (L-Atg5 knockout) were deficient in their adaptation to fasting-induced hepatic steatosis—the resulting liver injury and activation of nuclear factor (erythroid-derived 2)-

like 2 (Nrf2) may be responsible for decreased accumulation of LDs in autophagy-deficient livers in response to fasting.⁷² There is also emerging evidence to suggest that hepatic cholesterol and bile acid metabolism may be linked to hepatocellular autophagic activity, with interactions potentially involved in the pathogenesis of fatty liver disease.⁷³

Together, these studies all point to defects in hepatic lipophagy as a critical factor for the onset of NAFLD; therefore, the targeted upregulation of lipophagy may represent a viable therapeutic opportunity to promoting the resolution of fatty liver. Indeed, several autophagy inducers are under investigation as potential treatments for NAFLD.⁷⁴ In one study, mice administered rapamycin exhibited an alleviation in both diet- and alcohol-induced hepatic steatosis.⁷⁵ Other small molecules with potential proautophagy characteristics have also been shown to have positive effects on liver steatosis. Compounds such as celecoxib were found to alleviate NAFLD by restoring autophagic flux both *in vitro* and *in vivo*.⁷⁶ Administration of minerals such as zinc, with known proautophagy properties, has been shown to promote hepatic lipophagy and reduce liver steatosis.⁷⁷ Even alcohol, at least when administered acutely, appears to upregulate lipophagy in both livers of mice as well as cultured hepatocyte cell models, likely as a cytoprotective mechanism geared toward the removal of accumulated LDs.^{78,79}

Other aspects related to the progression of fatty liver disease may also be aided by a better understanding of lipophagy. For example, it was recently shown that the vitamin A-rich LDs found in HSCs are largely catabolized in an autophagic process during HSC activation, ultimately resulting in the onset of fibrosis in NASH and cirrhosis. As HSCs are activated, autophagic flux appears to be elevated and blocking this flux *in vivo* has pronounced effects on liver fibrosis.^{80,81} Therefore, an understanding of the role for lipophagy at multiple aspects of NAFLD progression will be important.

Genetic Risks for NAFLD and Connections to LDs

A promising link to factors predisposing an individual to the onset of NAFLD was recently identified to be a regulator of hepatocellular LD metabolism. A single-nucleotide polymorphism (I148M) in a gene encoding a putative LD lipase, *PNPLA3*, was shown to accumulate on the surface of LDs, perhaps impeding the accessibility of the droplet to normal cytoplasmic regulatory proteins.^{82–84} This accumulation appears to be the result of an inherent resistance of *PNPLA3* I148M to ubiquitination, suggesting that it evades normal proteasomal degradation, ultimately resulting in hepatic lipid retention and steatohepatitis.⁸² Patients with obesity were especially susceptible to the onset of NASH if also carrying this mutation. A search for alternative genetic links to NAFLD recently identified a mutation in the gene *HSD17B13*, which encodes a hydroxysteroid dehydrogenase that is also localized to the surface of LDs.⁸⁵ Patients with a specific truncation variant of *HSD17B13* appear to be significantly less susceptible to chronic liver disease including alcoholic and nonalcoholic cirrhosis. Furthermore, this loss-of-function variant appears to mitigate the negative effects characteristic of the above *PNPLA3* I148M allele.

Conclusions and Future Perspectives

Lipid droplets represent a defining feature of fatty liver disease. These unique organelles have a biology that is only now coming into full view. From a therapeutic standpoint, a better understanding of the machinery involved in the synthesis and catabolism of LDs will offer exciting new strategies for intervention in the development of complications arising from liver steatosis.

Main Concepts and Learning Points

- A defining characteristic of fatty liver disease is the abnormal accumulation of lipid droplets (LDs), *bona fide* cellular organelles with unique biochemical and biophysical properties.
- LDs are synthesized in the endoplasmic reticulum (ER) by resident acyltransferases and catabolized by conventional cytoplasmic lipases (lipolysis) or within the lysosome (lipophagy).
- Several recently identified genetic links to fatty liver and chronic liver disease involve LD-associated proteins, implicating the LD itself as a target for therapeutic intervention.

Conflicts of Interest

None declared.

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