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VLDL biogenesis and secretion: it takes a village

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Abstract

The production and secretion of very-low density lipoproteins (VLDL) by hepatocytes have a direct impact on liver fat content as well as the concentrations of cholesterol and triglycerides in the circulation and thus affect both liver and cardiovascular health, respectively. Importantly, insulin resistance, excess caloric intake and lack of physical activity are associated with overproduction of VLDL, hepatic steatosis, and increased plasma levels of atherogenic lipoproteins. Cholesterol as well as triglycerides in remnant particles generated by VLDL lipolysis are risk factors for atherosclerotic cardiovascular disease and have garnered increasing attention over the last few decades. Presently, however, the increased risk of atherosclerosis is not the only concern when considering today's cardiometabolic patients, as they often also suffer from hepatic steatosis, a very prevalent disorder that can progress to steatohepatitis and cirrhosis. This duality of metabolic risk highlights the importance of understanding the molecular regulation of the biogenesis of VLDL, the lipoprotein that transports triglycerides and cholesterol out of the liver. Fortunately, there has been a resurgence of interest in the intracellular assembly, trafficking, degradation, and secretion of VLDL by hepatocytes, which has led to many exciting new molecular insights that are the topic of this review. Increasing our understanding of the biology of this pathway will aid in the identification of novel therapeutic targets to improve both the cardiovascular and the hepatic health of cardiometabolic patients. This review focuses, for the first time, on this duality.

Key points

- VLDL is the “parent” lipoprotein of atherogenic TG-rich remnants and LDL.
- VLDL secretion is increased in the majority of people with atherogenic dyslipidaemia.
- VLDL biogenesis and secretion, which have been characterised in cellular and rodent models, involves approximately 50 molecular entities.
- Early stages of VLDL biogenesis in the ER, including translocation of apoB into the lumen of the endoplasmic reticulum (ER) concomitant with initial lipidation, have been well-studied at the molecular and biochemical level.
- Much less is known about the molecular machinery crucial for additional lipidation of VLDL, transport of VLDL within the ER lumen, and transfer of mature particles from the ER to the Golgi as well as from the Golgi to the plasma membrane for secretion from the liver.
- Extending our knowledge of intracellular hepatic VLDL metabolism is critical to improving our understanding of hepatic as well as plasma lipid homeostasis, which are both abnormal in patients with hepatic steatosis and cardiovascular disease.
- Pharmaceutical targeting of the VLDL secretion is a promising approach to reducing plasma levels of atherogenic lipoproteins but must be achieved without causing steatotic liver disease.

Abbreviations

ASCVD	Atherosclerotic cardiovascular disease
ERAD	ER-associated degradation
ER	Endoplasmic reticulum
LDL	Low-density lipoprotein
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment *protein* receptor
VLDL	Very low-density lipoprotein

Abbreviations of proteins

ApoB	Apolipoprotein B
APOBEC-1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1
BIP/GRP78	Binding immunoglobulin protein / Glucose-regulated protein 78
CIDEB	Cell death-inducing DFFA-like effector b
DERLIN-1	D*egradation in endoplasmic reticulum protein 1*
DGAT	Diglyceride acyltransferase
ERLIN	ER lipid raft-associated protein
ERGIC	ER-Golgi intermediate compartment
ERP57/PDIA3	ER resident protein 57 / Protein disulfide-isomerase A3
ERP72/PDIA4	ER resident protein 72 / Protein disulfide-isomerase A4
GRP94	*Glucose-regulated protein* 94
GP78/AMFR	Glycoprotein 78 / Autocrine motility factor receptor
HSP	Heat shock protein
KLHL12	Kelch-like protein 12
LAP1	Lamina-associated polypeptide 1
LULL1	Luminal domain-like LAP1
LZP	Zona pellucida domain-containing protein
MDM2	Mouse/murine double minute 2 homolog
MIA2	Melanoma inhibitory factor 2
MEA6/cTAGE5C	Meningioma-expressed antigen 6 (Mea6)/cutaneous T cell lymphoma-associated antigen 5C (cTAGE5C)
MTP	Microsomal triglyceride transfer protein
PCSK9	Proprotein convertase subtilisin/kexin type 9
PDI/PDIA1	Protein disulfide-isomerase / Protein disulfide-isomerase A1
PEMT	Phosphatidylethanolamine N-Methyltransferase
PRAP1	Proline-rich acidic protein 1
RTN3	Reticulon 3
SAR1	Secretion associated ras-related GTPase 1
SMLR1	Small leucine rich protein 1
SURF4	Surfeit locus protein 4

SVIP	Small valosin-containing protein-interacting protein
TALI	TANGO1-like (cTAGE5 bound to Mia2)
TANGO1	Transport and Golgi organization protein 1
TGH	Triacylglycerol hydrolase / Carboxylesterase (Ces) 3 or Ces1d in mice / Ces1 in humans
TIAL1	TIA1 Cytotoxic granule associated RNA binding protein-like 1
TMEM41B	Transmembrane protein 41b
TM6SF2	Transmembrane 6 superfamily member 2
UBXD8	Ubiquitin regulatory x domain-containing protein
VMP1	Vacuole membrane protein

Introduction

After several decades of decreasing global rates of atherosclerotic cardiovascular disease (ASCVD), the last two decades have seen a rise toward the very high rates present in the mid-20th century. There are many potential reasons for this change in the direction of the most common cause of death in most countries, but the pandemic of obesity and its sequelae, diabetes, hypertension, and dyslipidaemia, are certainly very significant factors [1]. Dyslipidaemia is not simply high levels of low-density lipoprotein (LDL) cholesterol, which in most people can be reduced significantly with statins. Rather, it is typically a lipid disorder driven by increased hepatic assembly and secretion of very low-density lipoproteins (VLDL), resulting in elevated levels of these triglyceride-rich lipoproteins and their remnants that are, based on genetic evidence (e.g., common genetic variation associated with the efficiency of triglyceride lipolysis), a risk factor for ASCVD [2,3]. Drugs that inhibit the assembly and secretion of VLDL are available for use in the clinic for patients at very high risk of ASCVD concomitant with severe elevations of LDL cholesterol concentration. These drugs, however, lead to the accumulation of hepatic lipids that have deleterious consequences for liver health [4]. The key question is, therefore, whether it will be possible to prevent or treat ASCVD by reducing VLDL secretion without increasing hepatic lipids.

Context

Achieving the latter goal will require a more complete understanding of the complex and highly regulated and inter-related pathways of VLDL biogenesis and hepatic lipid homeostasis. However, even as we acknowledge all the complexity inherent in these pathways, we can conclude that hepatic steatosis, which has been associated with ASCVD, results when triglyceride synthesis is greater than the combination of triglyceride secretion and fatty acid utilization. **Figure 1** provides a simplistic but clear view of the major metabolic pathways involved in the “arithmetic of hepatic lipid health”.

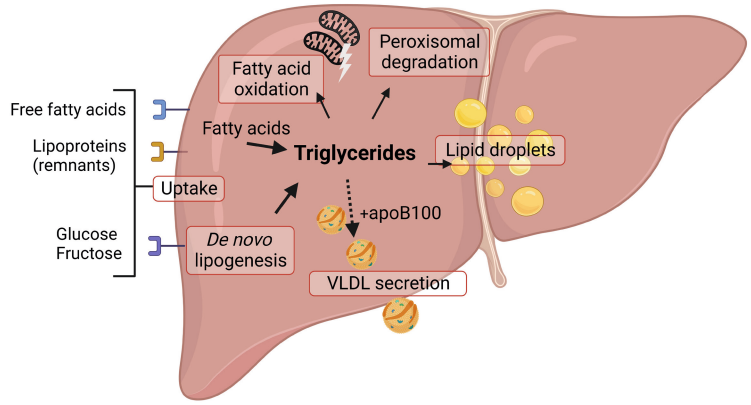

Figure 1. Major metabolic pathways involved in hepatic triglyceride metabolism.
The level of triglycerides that is stored in hepatic lipid droplets is determined by the balance of fatty acids incorporated into triglyceride and fatty acid utilization. Triglyceride synthesis requires fatty acids that can be taken-up as free fatty acids from the plasma; that are synthesized from acetyl-CoA by de novo lipogenesis; and that derive from the breakdown of triglycerides taken up as the major core lipid component of VLDL and chylomicron remnants. Utilization comprises fatty acid oxidization in mitochondria and peroxisomes, and fatty acids used to synthesize triglycerides that are incorporated into VLDL, which are secreted into the circulation. For simplicity, we have not depicted pathways that maintain hepatic homeostasis of cholesterol or phospholipid levels, as they play minor quantitative roles in the development of hepatic steatosis.

Triglyceride (triacylglycerol) synthesis results from the esterification of fatty acids to a glycerol backbone. In the liver, fatty acids are derived from the uptake of plasma-free fatty acids released from adipose tissue (accounting for approximately 70-80% of hepatic triglyceride synthesis), from triglycerides that have undergone lysosomal lipolysis after uptake of triglyceride-rich remnant lipoproteins by the liver (accounting for about 5-10% of triglyceride synthesis), and from *de novo* lipogenesis of fatty acids from acetyl-CoA (accounting for about 5-30% of triglyceride synthesis) [5]. Triglyceride utilization includes the oxidation of fatty acids, mainly in mitochondria, and the secretion of VLDL. When synthesis is greater than utilization, lipid droplets emerge from the cytosolic side of the endoplasmic reticulum (ER) membrane and accumulate in the cytoplasm [6,7], a process that has been studied in great detail because it leads to steatotic liver disease, the initial step in the development of steatohepatitis and cirrhosis [8]. Countering the development of steatotic liver disease, however, is the assembly and secretion of VLDL, which can carry significant quantities of triglycerides out of the liver as a means of maintaining hepatic lipid homeostasis. However, the majority of individuals with dyslipidaemia and ASCVD in the 21st century present with a fatty liver despite having increased secretion of triglyceride-rich VLDL [9]. This suggests that the liver has a limited capacity to assemble and secrete VLDL to prevent hepatic lipid accumulation.

Increasing the capacity of the liver to secrete VLDL would be a way to ameliorate hepatic lipid accumulation, but this would exacerbate the existing dyslipidaemia and increase the already high ASCVD risk in patients. Compared to the extensive characterization of the biology of triglyceride and lipid droplet synthesis conducted during the past 60 years, the molecular knowledge of assembly and secretion of VLDL remained at a simple level until the past 1-2 decades, despite the potential therapeutic benefit likely to derive from identifying ways to reduce VLDL secretion without increasing hepatic fat: a “win-win” treatment.

It takes a village

VLDL biogenesis and secretion have been characterized extensively and based on current data, involve approximately 50 molecular entities. However, the way these factors interact with apoB/VLDL, and what determines how a VLDL at any stage of biogenesis is targeted for either degradation or secretion, remains largely unknown. Importantly, it is also unclear how VLDL secretion is co-regulated with lipid synthesis and disposal to prevent hepatic steatosis.

The genesis of VLDL starts with the translation of the *APOB* transcript into apolipoprotein B (apoB), one of the largest proteins in humans (4536 amino acids). This core VLDL protein is continuously synthesized and loaded with lipids in the ER. It is thereafter transported to the Golgi network by dedicated so-called VLDL transport vesicles. Following transport through the Golgi, VLDL is secreted into the circulation to deliver energy, in the form of triglyceride fatty acids, to the peripheral tissues, particularly adipose tissue, and skeletal (including cardiac) muscle.

Focus of review

As highlighted above, VLDL assembly and secretion are critical to the maintenance of hepatic lipid homeostasis, and, therefore, have been the focus of several excellent reviews of this very complex biology during the past 2 decades [10–12]. This review builds upon the foundational knowledge acquired during the end of the 20th and the first decade of the 21st centuries by presenting a detailed examination of the important molecules discovered during the past several years. It thereby provides an up-to-date review of the entire published work on the molecular biology of VLDL biogenesis and secretion with a focus on the impact of these molecules on liver and plasma lipids.

In **Figure 2** we have attempted to give an overview of the current state-of-the-art, integrating old and new working models published by numerous researchers in the field. For clarity, we have not included all molecular players. In **Table 1,** however, we have defined the functions of all molecules known to directly regulate intracellular VLDL metabolism. More detailed cartoons have been generated to better illustrate early events in VLDL biogenesis at the ER leaflets (**Figure 3**), and the transport of nascent VLDL to the ER lumen and its exit from the ER (**Figure 4**). The legends to the figures summarize the main body of knowledge that has been obtained over the past decades.

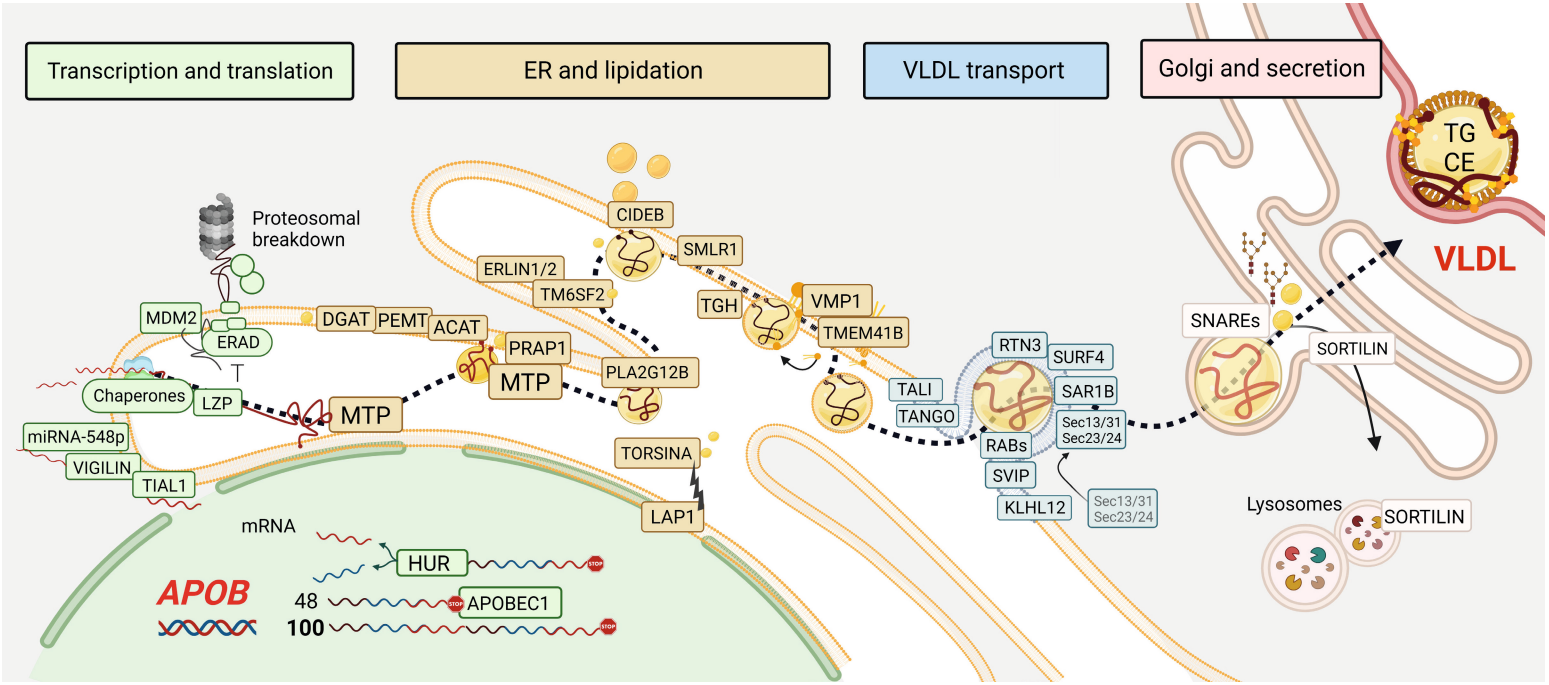

Figure 2. A cellular model of the hepatic VLDL assembly and secretion pathway, with a focus on recently discovered key players.

*For clarity, the proteins are localized to subcellular regions where they have significant effects on apoB/VLDL, although these may not be the only locations where they reside. ApoB, the core protein of VLDL, is widely accepted to be produced constitutively. There is however, increasing evidence that gene expression is also regulated at the mRNA level through e.g., human antigen R (**HUR** [13]), TIA1 Cytotoxic granule associated RNA binding protein like 1 (**TIAL1)** [14], **VIGILIN** [15] and **miRNAs**[16]. Note: In the nucleus we have depicted the apoB100 mRNA editing enzyme (**APOBEC1)** to make clear that in mouse hepatocytes but not human hepatocytes, APOBEC1 generates apoB48 mRNA which encodes for a truncated (48%) portion of the full-length APOB100 protein. This means that wild-type mice produce both apoB48 and apoB100 containing lipoproteins in the liver. Some investigators,*

therefore, use liver-specific Apobec1$^{-/-}$ mice to simplify their studies. In the majority of studies with active APOBEC1, however, manipulation of the VLDL biogenesis machinery affects apoB100 to a much greater extent than apoB48. During translation, apoB crosses the ER membrane, facilitated by numerous chaperones (co-translational translocation). During this process, apoB is lipidated by microsomal triglyceride transport protein (**MTP**) (co-translocational lipidation).*[17,18] *When folding of apoB is abnormal or when lipidation is inadequate, apoB will be ubiquitinylated followed by proteasomal degradation. This process is facilitated by numerous proteins involved in ER-associated degradation** (ERAD). Newly identified factors in this process include liver-specific zona pellucida domain-containing protein (**LZP** [19]) and murine double minute 2 (**MDM2** [20]). MTP is essential for stabilization and initial lipidation of apoB to form nascent VLDL. The supply of lipids, mostly triglycerides, for the initial lipidation of apoB derives from de novo lipogenesis in the ER membrane. The figure illustrates that many other proteins are implicated in the biogenesis of nascent lipid-poor VLDL and its conversion to more mature, well-lipidated VLDL. Newly proposed players in the first steps of this pathway are an AAA+ ATPase called **TORSINA** (activated by lamina-associated polypeptide 1, **LAP1** [21]), proline-rich acidic protein 1 (**PRAP1** [22]), phospholipase A2, group XIIB (**PLA2G12B** [23]), ER lipid raft proteins 1 and 2 (**ERLIN1/2** [24]), and transmembrane 6 superfamily member 2 **(TM6SF2)** [25]. Triglycerides added during the later or second stage of lipidation of nascent are believed to derive from both the ER membrane and from lipid droplets in a process involving triglyceride hydrolase **(TGH** [26,27] **)**. Vacuole membrane protein 1 (**VMP1** [28,29]) and transmembrane protein 41b (**TMEM41B** [30]), have recently been identified as key molecules in the budding off of VLDL from the ER membrane into the ER lumen. For the subsequent transport of VLDL from the ER to the Golgi, a long list of proteins of the COPII trafficking machinery (**SAR1B, SEC13/31 and SEC23/24**), and those needed for the packaging of larger cargos (**TANGO1** and **TALI** [31], **KLHL12**[32]) have been described, as well as small valosin-containing protein-interacting protein (**SVIP** [33]); reticulin 3 (**RTN3** [34]), and several small RAB GTPases (**RABs** [35,36]), with surfeit locus protein 4 (**SURF4** [37]) as the most recent addition. The docking of VLDL transport vesicles at the Cis-Golgi is reported to be mediated through interactions with **SNAREs** [38]. The Golgi is the site where VLDL may be subjected to sorting, glycosylation, and phosphorylation, as well as additional/final lipidation, but only very few investigations have been conducted with a focus on the role of the Golgi in VLDL maturation and secretion. **SORTILIN1**, which has been localized to several post-ER sites, including the Golgi, appears to regulate VLDL secretion by re-directing some particles for auto-lysosomal degradation* [39,40]. *While reviewing the literature, the scarcity of information on VLDL processing in the Golgi, and how VLDL leaves the Golgi and is transported to the cell membrane for eventual secretion into the circulation was striking.*

**Chaperones involved in APOB stabilization/folding: = BIP, PDI, ERP57, ERP72, HSP110, Calreticulin, Calnexin, GRP94, Cyclophilin B, HSP70 and HSP90.*

*** Proteins involved in ER associated degradation (ERAD) of APOB: DERLIN-1, GP78/AMFR, HRD1, HSP40, P97,* P58^{IPK}*, SEC61, UBXD8.*

***Table 1**. Established and new players in the VLDL assembly, trafficking, and secretion pathway*

Protein name	Role in VLDL biogenesis	Studies performed in	Ref.
Previously described players in VLDL biogenesis			
VIGILIN	*ApoB* RNA-binding protein	Liver-specific KO in mice,	[15]
BIP, HSP110, Calreticulin, Calnexin, ERP57, ERP72, GRP94, Cyclophilin B.	ER-chaperones, mediating correct folding of apoB.	HepG2 and McArdle RH-7777 cells, yeast.	[41–44]
HSP70, HSP90	Cytosolic chaperones, participate in proteasomal targeting of apoB.	HepG2 and McArdle RH-7777 cells	[45,46]
GP78 (=AMFR)	E3 ligase that targets apoB for proteasomal degradation	HepG2 cells	[47,48]
HSP40, P58^{IPK}, P97, GP78, HRD1, UBXD8, BIP, DERLIN-1, SEC61	Involved in proteasomal degradation of apoB	Yeast, HepG2 and HuH7 cells, whole body KO mice (P58)	[49–54]
PDI	Subunit of MTP, ER-chaperone	Bovine liver, Sf21 cells.	[55,56]
MTP	Lipidation and translocation of apoB across the ER membrane	HepG2 and McArdle-RH7777 cells, rodent models, human genetics/drug	[17,18]
DGAT1 and DGAT2	Lipid supply for VLDL via *de novo* triglyceride synthesis in the ER	Rodents, primates, and Phase 2 trials in humans	[57–59]
TGH	Lipid supply for VLDL assembly	Whole body KO mice, inhibition of TGH in McArdle-RH7777 cells	[26,60]
CIDEB	Lipidation and VLDL transport	Whole body KO mice and both mouse and rat primary hepatocytes	[61,62]
KLHL12	VLDL transport vesicle	McArdle RH-7777 cells	[32]
TANGO1, TALI	VLDL transport vesicle	Caco-2 and HepG2 cells	[31]
SVIP	VLDL transport vesicle	siRNAs for rat primary hepatocytes or McArdle RH-7777 cells.	[33]
SEC13/31, SEC23/24,	VLDL transport vesicle	See review	[63]
SAR1B	VLDL transport vesicle	Intestinal and liver-specific KO mice, CMRD patients, McArdle RH-7777 cells	[64,65]
SNARES; SEC22b, GOS28, Syntaxin5 and RBET1	Cis-Golgi docking of VLDL transport vesicles	Rat hepatocytes	[38]
SORTILIN1	Deviation from VLDL secretion to lysosomal degradation	Rodent models, McArdle RH-7777 cells	[39,40,66]

***Table 1**. Continued*

Protein name	Role in VLDL biogenesis	Studies performed in	Ref.
New players in VLDL biogenesis			
HUR	ApoB mRNA splicing	Liver-specific KO in mice, Hepa1-6 cells	[13]
MicroRNA-548p	*ApoB* RNA binding microRNA	Overexpression and inhibition in human primary hepatocytes, HuH7 and HepG2 cells	[16]
TIAL1	*ApoB* RNA binding protein	Liver-specific KO in mice, Hepa1-6 cells	[14]
LZP	ERAD	Whole body KO mice, HepG2 cells	[19]
MDM2	E3 ligase that targets apoB for proteasomal degradation	Liver-specific KO mice, HepG2 cells	[20]
PRAP1	Facilitates apoB lipidation	Whole body KO mice	[22]
PLA2G12B	Facilitates apoB lipidation	Zebrafish, liver-specific KO mice, Caco-2 and HepG2 cells	[23]
ERLIN1/2	Stabilize TM6SF2	Liver-specific KO and KD in mice, HuH7 cells	[24]
TM6SF2	Lipidates and/or traffics apoB	Whole body and liver-specific KO in rodents, HuH7, HepG2, McArdle RH-7777 cells, humans	[24,67–69]
LAP1, TORSINA	Affects apoB secretion	Whole body and liver-specific KO mice	[21]
SMLR1	Affects apoB secretion	Liver-specific KO mice	[70]
TMEM41B, VMP1	Involved in the budding of VLDL into the lumen of the ER	Liver-specific KO mice, HEK293 and HuH7 cells	[28,30]
RAB1b, GP73	VLDL transport	HuH7 cells and liver-specific overexpression in mice (GP73)	[35,36]
RTN3	VLDL transport vesicle	HepG2 cells	[34]
SURF4	VLDL transport vesicle	Liver-specific KO mice and HepG2 cells	[37,71,72]

Proteins are ordered by the place in the VLDL biogenesis pathway. Abbreviations: KO; knockout, KD; knock down, CMRD; chylomicron retention disease, Rodents; both mouse and rat models.

New insights into intracellular VLDL metabolism

Following the overall depiction of the many proteins involved in apoB/VLDL biogenesis, the next sections provide a focused presentation of several older, but mostly newly identified, key players with special attention on their effects on liver and plasma lipid homeostasis.

Transcription and translation

The *Apob* gene in mice or the *APOB* gene in humans are generally thought to be constitutively expressed, suggesting minimal involvement of transcriptional or translational regulatory mechanisms [12,73]. Several studies, however, have shown that transcriptional and translational regulation can play important roles in apoB/VLDL metabolism. An example is a study that identified an RNA-binding protein called **VIGILIN** (upregulated in patients with steatotic liver disease) as a positive regulator of translation of apoB mRNA. VIGILIN was shown to control VLDL secretion and hepatic triglyceride content in mice [15]. While short-term and strong silencing of VIGILIN in the liver caused mild steatosis, hepatic downregulation of VIGILIN with GalNAc-conjugated siRNAs in *Ldr-/-* mice resulted in decreased plasma lipids (cholesterol and triglycerides) and atherosclerosis without alterations in liver lipids [15]. The latter was suggested to be associated with other targets of VIGILJN such as *ApoC3* mRNA.

The same investigators very recently showed that T-cell restricted **TIAL1 Cytotoxic Granule Associated RNA Binding Protein Like 1 (TIAL1)** [14], another RNA-binding protein, could affect VLDL metabolism. Loss of TIAL1 in mice markedly reduces apoB synthesis by 50% with a 31% reduction in VLDL secretion but increases in hepatic triglycerides and cholesterol [14]. The authors suggest that TIAL1 may recruit apoB to translationally active polysomes or prevent translational repressors of other RNA-binding proteins or microRNAs.

Another recent study showed that **human antigen R** (**HUR**) is required for splicing of *Apob* pre-mRNA [13]. HUR, encoded by the *Elavl1* gene, is an RNA-binding protein implicated in various biological processes including tumorigenesis and inflammation, lipid transport, and ATP synthesis. Bioinformatic prediction models identified HUR-binding sites in intronic *Apob* pre-mRNA. Hepatic *Elav1* knockout in mice (*Elav1*-LKO) was shown to increase *Apob* pre-mRNA levels and reduce *Apob* mRNA levels. Concordantly, chow-fed *Elav1*-LKO mice have reduced plasma levels of apoB (≈43%) despite unaltered plasma lipid levels implying increased lipidation of VLDL. After a high-fat diet for four weeks, these mice showed increased liver triglyceride and cholesterol and impaired liver function compared to controls. Loss of hepatic HUR, however, also increased levels of other genes in the liver, indicating that HUR not only promotes lipid transport but also ATP synthesis.

Non-coding RNAs have also been shown to play a role in regulating the metabolism of apoB-containing lipoproteins. MiRNA-mediated regulation can occur via the binding of miRNAs to messenger RNA in the cytoplasm, which can prevent or delay protein translation. Other miRNAs have been implicated in transcriptional control of gene expression. A recent study showed that non-coding RNAs affected the expression of *MTTP, PCSK9, and SORT1,* which can alter the assembly, secretion, and/or re-uptake of VLDL [74]. One published study indicated a role for **miR-548p** in regulating *APOB/Apob* expression in human primary hepatocytes but not mouse primary hepatocytes [16]. Mechanistic studies showed that miR-548p interacts with human *apoB* mRNA to enhance posttranscriptional degradation resulting in decreased apoB secretion from human hepatoma cells. Although additional miRNAs have been linked to *Apob* regulation [75], studies showing causality are still lacking.

Translation of the 16kb transcript of *Apob100* into an appropriately folded full-length protein requires multiple chaperones (listed in **Table 1**). Misfolding and/or inadequate lipidation of apoB result in its co-translational ubiquitinylation and subsequent degradation by the proteasome during translocation [76]. ApoB100 degradation is a type of ER-associated degradation (ERAD). The ERAD of apoB requires several proteins listed in **Table 1** that are regulated by microsomal triglyceride transfer protein (MTP) and *sterol regulatory element-binding protein 1c which* regulates lipogenesis, but also cytosolic chaperones like HSP70 and HSP90 (for a review see [12]). In addition, post-ER, pre-secretory proteolysis affect intracellular apoB100 degradation [77].

Liver-specific zona pellucida domain-containing protein (LZP), encoded by the *Oit3* gene, can be regarded as a new member of this group of proteins that mediate apoB100 degradation. LZP was initially studied in the context of hepatocarcinogenesis [78], but *Oit3*$^{-/-}$ mice were also found to exhibit hepatic lipid accumulation and reduced plasma on chow and high-fat diet. This study also shows decreased hepatic apoB levels and a 70% decrease in VLDL secretion compared to controls but without an indication of the diet that was used [19]. It was furthermore shown that LZP interacts with apoB in immunoprecipitation experiments, while proteasome inhibition restores intracellular apoB levels in primary hepatocytes of *Oit3*$^{-/-}$ mice. The investigators show that LZP is in the ER and Golgi and that it stabilizes apoB in the ER by preventing ubiquitinylation of the nascent protein by the ER transmembrane E3 ubiquitin ligase, GP78/autocrine motility factor receptor (AMFR)[47].

Despite the high energy demand to generate the very large apoB protein, it is always being synthesized and degraded. Inhibition of proteins facilitating this breakdown might lead to increased apoB secretion. This was recently shown after hepatic deletion of **murine double minute 2 (MDM2)**, a protein that acts as an E3 ubiquitin ligase and targets apoB for proteasomal degradation [20]. MDM2 is well known for its function as a negative regulator of the p53 tumor suppressor protein, and this was the first study showing that MDM2 also targets apoB100 for proteolysis. Liver-specific deletion of *Mdm2* in mice on chow

did not alter hepatic triglyceride levels, but on a high-fat, high-cholesterol diet hepatic triglycerides were reduced by 50%. This was in line with increased circulating apoB and plasma lipids in a context of a 25% elevation of VLDL secretion measured by inhibition of VLDL lipolysis with Tyloxapol. The potential clinical significance of this finding was provided through pharmacological inhibition of MDM2, which alleviated non-alcoholic steatohepatitis (NASH) in choline-deficient amino acid-defined high-fat diet fed mice. The authors also showed that the opposite is true; overexpressing MDM2, but not an inactive MDM2 mutant, increased ubiquitinylation of apoB in HepG2 cells. Whether this leads to excess hepatic lipids in mice was not shown. Taken together, blocking MDM2 may be useful as a means of reducing hepatic lipid accumulation, although this will be associated with increased levels of atherogenic plasma lipids.

ER and lipidation

ER-resident proteins, including MTP, are essential for the stabilization of apoB. MTP was identified as a microsomal lipid transfer protein in 1985 [79]. It was only after the identification of abetalipoproteinemia patients (#OMIM 200100) with loss-of-function mutations in the *MTTP* gene, that MTP was recognized for its essential role in the assembly of apoB-containing lipoproteins. MTP exists in a heterodimeric complex with protein disulfide isomerase [80]. MTP transfers phospholipids, and cholesterol esters from the ER membrane into the apoB lipid-binding pocket, i.e., the N-terminal domain of approximately 1000 amino acids. Initial lipidation comes from the ER bilayer lipids as shown in **Figure 3**. MTP also mediates the translocation of apoB across the ER membrane [17] (detailed information about the interactions between apoB and MTP can be found in [18,81]).

The lipids for apoB/VLDL lipidation are in part synthesised *de novo* within the ER membrane as described in the introduction and the legend to Figure 3. It has previously been suggested that lipolysis of triglycerides in lipid droplets also provides substrates for VLDL-triglycerides synthesis [89]. Additional studies have shown that **triacylglycerol hydrolase (TGH)** may mediate this reaction. More recent studies now show that hepatic ablation of *Tgh* in mice decreases plasma lipids but only moderately increases liver triglycerides on a chow diet [27]. Of note, whole-body knockout of TGH can attenuate NASH [90]. These data together show that this lipase is involved in the mobilization of triglycerides in lipid droplets for the secretion of VLDL, but it is not clear whether these lipid droplets are in the ER lumen or the cytosol. In contrast, triglycerides from lipid droplets in the cytosol can also be made available for VLDL maturation without being lipolyzed as shown by the studies of CIDEB [61] that are summarized below.

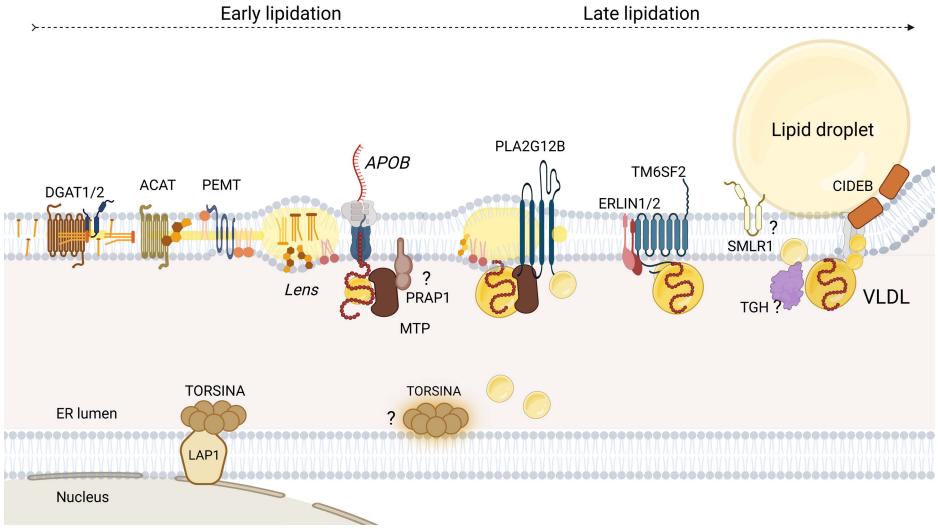

Figure 3. A cellular model of ApoB lipidation in the ER.

*In the outer leaflet of the hepatocyte ER, diacylglyceride acyltransferase 1 and 2 (**DGAT1** and **DGAT2**), acyl acyl-CoA cholesterol acyltransferase (**ACAT**), and phosphatidylethanolamine N-methyltransferase (**PEMT**) synthesize the main lipid components of VLDL, e.g., triglycerides, cholesteryl esters, and phospholipids, respectively. Triglycerides are the most abundant lipid in VLDL and modulation of DGAT1 or DGAT2 has been shown to affect VLDL biology* [58,82]. *More details and a working model of how these proteins synthesize and incorporate triglycerides in the ER can be found elsewhere* [83]. *Changes in cholesterol esterification by ACAT are also known to affect apoB lipoprotein assembly and secretion by the liver (for review see* [84]*). The importance of the synthesis of phospholipids via the PEMT pathway for VLDL synthesis and secretion was described by Vance and colleagues* [85–87]. *The newly synthesized lipids are proposed to be present in lens-like structures between the inner and the outer leaflets of the ER membrane. In this position, they are accessible as substrates for the lipidation of apoB by MTP to form nascent VLDL. Recent studies have demonstrated roles for additional proteins, including **PRAP1*** [22], ***PLA2G12B*** [23], ***ERLIN1/2*** [24]***, TM6SF2*** [67,88], ***TORSINA*** [21] *and **SMLR1*** [70] *that we have tried to capture in one scheme. In the figure, we have depicted these new factors in an arbitrary order as it is not known what the actual sequence of events is and whether these proteins act individually or in concert. Little is known about the role of proline-rich acidic protein 1 **(PRAP1),** a new interacting partner of MTP* [22]. *Phospholipase A2, group XIIB **(PLA2G12B)** is a catalytically inactive phospholipase that appears to play an important role in VLDL lipidation and secretion* [23]. *The lipidation of VLDL in the ER has previously been shown to also require transmembrane 6 superfamily member 2 **(TM6SF2)*** [67,88]*; loss of TM6SF2 function in humans results in steatotic liver disease and lower plasma lipids* [25]. *More recently it has been shown that for TMSF62 to stabilize apoB it needs **ER lipid raft-associated proteins (ERLIN) 1** and **2*** [24]. *Another factor which has previously been shown to play a role in making TG in cytosolic lipid droplets available for further lipidation of VLDL is cell death-inducing DFFA-like effector b **(CIDEB)*** [61]. *CIDEB has also been described to regulate the transport of VLDL transport vesicles* [62] *and we therefore also display this factor in Figure 4. Triglyceride hydrolase (**TGH**) has more recently*

been described to play a significant role in VLDL biogenesis in mice [26,27]. *It is proposed to make triglycerides from lipid droplets available for VLDL lipidation but it is not clear whether the lipid droplets are in the ER lumen or the cytosol. Small leucine-rich protein 1* ***(SMLR1)*** *is the most recent addition to this scheme* [70]. *This protein of unknown function is localized in the ER but also the Golgi, and loss of hepatic SMLR1 results in hepatic steatosis and reduced VLDL secretion in mice, but its exact role has yet to be determined* [70].
The loss of hepatic ***TORSINA*** *has also been shown to induce the latter phenotype* [21]. *It is a soluble ER protein that localizes to co-activators at the inner nuclear envelope and possibly the ER membrane, but how TORSINA affects the VLDL biogenesis machinery is as of yet not known.*

A role for proteins in the nuclear envelope in the production of VLDL was only established recently[21]. It was shown that hepatic deletion of **lamina-associated polypeptide 1 (LAP1)** results in 20% lower VLDL secretion and mild steatosis, including intra-nuclear accumulation of lipid droplets, in chow-fed mice. Previous research has revealed that LAP1, as well as luminal domain-like LAP1 (LULL1), binds to and activates the **ER-resident AAA+ ATPase TORSINA**, encoded by *Tor1a* [91]. Whereas *Tor1a*$^{-/-}$ mice exhibit perinatal lethality, some mutations in human *TOR1A* are implicated in primary dystonia (OMIM#605204), a severe movement disorder. Hepatic loss of TORSINA in mice causes profound hepatic steatosis and a marked reduction in VLDL secretion with 64% lower plasma triglyceride levels on a chow diet. Unexpectedly, this is a more severe phenotype compared to the hepatic deletion of LAP1 [21]. It is not clear if LAP1 and LULL1 affect apoB secretion other than via TORSINA, nor is it known how and where in the cell TORSINA regulates VLDL biogenesis, hence we placed a question mark with TORSINA in Figure 3. A link between *TOR1A* and lipid metabolism has also been established by genetic studies that listed regulators of lipid metabolism after mapping 107 genetically distinct mouse strains (Suppl. table 11 of [92]).

Proline-rich acidic protein 1 (PRAP1), was recently identified to facilitate MTP-mediated lipid transfer in the intestine [22]. These investigators performed transcriptomic analysis on the murine intestine to identify novel cytoprotective genes induced by probiotics. *Prap1* was a top candidate gene that protected the intestinal barrier from oxidative insult [93]. To delineate its function, they used pull-down assays and found MTP as the main interacting partner of PRAP1, which prompted studies into lipid metabolism [22]. Isolated intestinal epithelial cells from *Prap1*$^{-/-}$ mice displayed almost 50% lower triglyceride and phospholipid transfer activities, and near absent apoB48 secretion in pulse chase experiments. Oil-Red-O staining of enterocytes revealed marked lipid accumulation in the *Prap1*$^{-/-}$ mice compared to controls 2 hours after receiving an intragastric bolus of lipids. While plasma triglycerides were lower in *Prap1*$^{-/-}$ mice (on both chow and a high-fat diet), total cholesterol levels were unaffected. This phenotype resulted mainly from a defect in lipid absorption, as indicated by increased faecal lipid content with similar food intake in the *Prap1*$^{-/-}$ mice compared to controls. On a high-fat diet, *Prap1*$^{-/-}$ mice appeared leaner with reduced increases in body weight compared to controls [22]. PRAP1 levels are low in the liver and fractionation of

plasma by density gradient ultracentrifugation revealed only a slight reduction in VLDL levels. Additional studies into liver-specific ablation of PRAP1 are needed to study its role in the VLDL pathway.

Phospholipase A2, group XIIB (PLA2G12B) has also been reported to have marked effects on apoB/VLDL lipidation. The gene is a target of HNF4alpha, a liver-specific transcription factor[94] and has also been found to be co-expressed with *MTTP* and *APOB* in mice and humans [95]. Livers from chow-fed whole body *Pla2g12b*$^{-/-}$ mice have a 2- and 3-fold increase in liver cholesterol and triglyceride content, respectively, which was associated with an approximate 50% reduction in VLDL-triglyceride secretion as determined by Tyloxapol injection. Interestingly, plasma cholesterol and triglyceride levels were more profoundly reduced by 92% and 79%, respectively [95]. Phospholipase A2 (PLA2) catalyses the lipolysis of glycerophospholipids to lysophospholipids and fatty acids but the subtype PLA2G12B lacks this catalytic phospholipase activity [96] and a mutation in *Pla2g12b* has previously been shown to be associated with a 98% decrease in HDL cholesterol and a 58% reduction in plasma triglyceride levels in mice, which interestingly did not affect atherosclerosis [97]. The molecular mechanism underlying these observations was recently studied in detail. Zebrafish larvae with complete loss of *Pla2g12b* were shown to accumulate large lipid droplets in the lumen of the ER associated with abnormally small lipoproteins in the circulation, suggesting a defect in the "second stage" formation of mature, larger apoB-containing lipoproteins [23]. *Pla2g12b*$^{-/-}$ mice were moreover shown to be protected against hyperlipidaemia and atherosclerosis, but at the expense of hepatic steatosis. Additional studies in HepG2 and Caco-2 (intestinal) cells implicated a role for PLA2G12B in calcium and MTP recruitment to the ER membrane to enhance lipid transfer and expansion of triglyceride-rich lipoproteins [23]. These results led the authors to propose PLA2G12B as a key molecule for transferring triglycerides from a luminal lipid droplet to nascent VLDL. This work provides support for predictions and questions first raised in an early review on luminal lipid metabolism. [98]

TM6SF2 is an example of the contribution of human genetic studies as a starting point to identify novel genes involved in intrahepatic VLDL metabolism. *TM6SF2*, encoding for transmembrane 6 superfamily member 2, was found to affect the development of steatotic liver disease in humans [99]. TM6SF2 is a transmembrane protein of the ER, the ER-Golgi intermediate compartment (ERGIC) and the Golgi [67–69]. There are several studies demonstrating that TM6SF2 is involved in VLDL lipidation although the role of TM6SF2 in apoB secretion is less clear based on studies in mice, rats, and humans [67,100]. A recent study shows that TM6SF2 stabilizes apoB through complex formation with **ER lipid raft proteins 1** and **2** (**ERLIN1** and **ERLIN2**) [24]. These proteins were found in pull-down assays of TM6SF2 in a rat hepatoma cell line. The ERLINs were formerly known as KE04p and C8orf2 and were later defined as lipid-raft like domains in the ER [101]. Short hairpin RNA-mediated silencing of *Tm6sf2* or both *Erlins* in mouse liver has been shown to reduce plasma

cholesterol (≈80%, ≈70%) and triglyceride (≈50%, ≈35%) levels, but resulted in a ≈3-fold or ≈1.4-fold increase in liver triglycerides in mice on chow diet, respectively [24].

Cell death-inducing DFFA-like effector b (CIDEB) is another protein involved in VLDL lipidation and transport. Sixteen years ago, *Cideb*$^{-/-}$ mice were reported to be resistant to high-fat diet induced obesity and diabetes while exhibiting increased liver triglycerides and reduced VLDL secretion [102]. A later intriguing study showed that CIDEB is a lipid droplet protein that interacts with apoB, possibly facilitating the availability of triglycerides in cytosolic lipid droplets for further lipidation of VLDL in the ER. These findings highlight the complex relationship between VLDL and lipid droplet biology. CIDEB was later also found to regulate the secretion of VLDL transport vesicles in primary hepatocytes [62] and we therefore also included this protein in Figure 4.

Small leucine-rich protein 1 (SMLR1) is one of the latest novel players in intracellular VLDL metabolism. Upon its identification, there was no literature in the public domain on SMLR1. The encoding gene was identified upon contextual co-expression analysis with *MTTP/Mttp* in human and murine transcriptome datasets [70]. Liver-specific ablation of *Smlr1* in mice has been shown to reduce VLDL secretion by 45% while increasing hepatic triglyceride levels 7-fold on a chow diet compared to controls. SMLR1 was found to be located in the ER membrane and Cis-Golgi, and a role for VLDL transport was implicated, but the underlying molecular mechanisms remain to be elucidated. While we have positioned SMLR1 in the section 'ER and lipidation', it could e.g., also play a role in the transport from the ER to the Golgi.

Following the genesis of nascent VLDL at the ER membrane, recent studies have shown that there are specific requirements for VLDL to bud off the membrane and enter the ER lumen (see **Figure 4**, left panel) prior to its transfer to the Golgi. These recent studies highlighted the roles of **vacuole membrane protein 1 (VMP1)** and **transmembrane protein 41B (TMEM41B)**, proteins known to function in autophagy and lipid droplet biology. Both are ER integral transmembrane proteins and have phospholipid scramblase activity [103].

Initial studies in zebrafish and mice [29] have shown that loss of VMP1 causes lipoprotein accumulation in the intestine and liver, while in VMP1-depleted cells neutral lipids accumulate within lipid bilayers of the ER membrane which affects lipoprotein secretion. More recently, hepatic loss of VMP1 in mice was shown to result in NASH in the context of disturbances in phospholipid homeostasis and mitochondrial beta-oxidation [28]. Similar to MTP [107], overexpression of VMP1 in mice was shown to result in increased VLDL secretion and reduced hepatic steatosis without significant changes in hepatic levels of MTP or PDI. The anticipated effect of this intervention is likely dyslipidaemia, but this was not studied. As in the previous study, the lack of VMP1 was shown to cause the retention of large neutral lipid cores surrounded by an ER membrane.

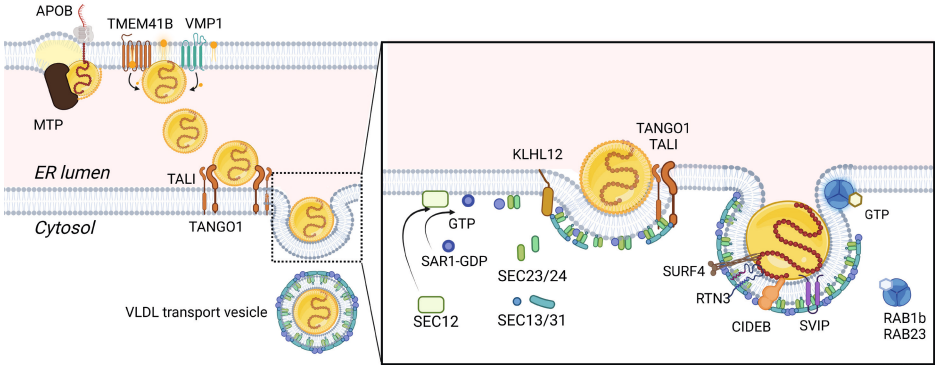

Figure 4. A cellular model of VLDL recruitment to the ER exit site for VLDL transport vesicle formation.

Nascent VLDL is suggested to first bud off from the inner leaflet of the ER membrane to reach the lumen, of the ER after which it exits the ER to move to the Golgi and then the cytoplasm. For clarity, we show these steps in different regions of the ER membrane, but the exact sites of these steps in VLDL trafficking are unknown. We have also left out all the lipidation steps to make the depiction of these trafficking processes easier to visualize. Vacuolar protein 1 ***(VMP1)*** *and transmembrane protein 41B* ***(TMEM41B)****, have recently been shown to mediate this step in VLDL biogenesis* [29,30]*. Trafficking of VLDL from the ER lumen to the Golgi requires the insertion of VLDL into COPII vesicles (for review see* [104]*). A key role for* ***Ras related GTPase1B (SAR1B)*** *in this process is illustrated by patients suffering from chylomicron retention disease but who also have reduced VLDL secretion and hepatic steatosis* [105]*. The large size of the VLDL cargo, however, requires specialized, large vesicles that are formed with the help of* ***TANGO*** *and* ***TALI*** [31]*. These proteins recruit VLDL to ER exit sites and allow the fusion of VLDL with the ERGIC membrane to generate an export pathway* [31]*. Kelch-like protein 12* ***(KLHL12)****, another protein known to assist the transport of larger cargos in COPII vesicles, has previously also been shown to affect apoB/VLDL in McArdle RH-7777 cells* [32]*.* ***SURF4*** *has most recently been shown to also be involved in the exit of VLDL from the ER. The absence of SURF4 in the liver of mice was shown to result in hepatic steatosis with accumulation of VLDL in the ER lumen*[37]*. In addition, Reticulon 3* ***(RTN3)****, has been shown to be involved in the transport of VLDL transport* vesicles [31]*. Several RABs such as* ***RAB1b, RAB23, and RAB24*** *have been shown to affect apoB/VLDL in both cell culture and experimental mouse studies* [35,106]*.* ***GP73*** *is a Golgi-resident protein but is thought to affect VLDL metabolism via RAB23* [36].

Support for a model where VLDL needs to first bud off from the ER membrane into the ER lumen comes from a series of studies of TMEM41B. Loss of TMEM41B in Hela or HuH7 cells has been shown to impair the cellular distribution of phosphatidylserine and cholesterol in the ER in association with the accumulation of very large cytosolic lipid droplets [103]. Other investigators subsequently showed that liver-specific ablation of *Tmem41b* in mice on chow led to depletion of plasma triglycerides due to reduced lipidation of apoB and lipoprotein trafficking [30], which was seen in the context of depleted intracellular apoB and rapid development of NASH, as seen for hepatic loss of VMP1.

The authors of this last study suggested that TMEM41B depletion was associated with the trapping of lipids between the bilayers of the ER membrane (as well as in the increased cytosolic lipid droplets) indicating that TMEM41B is necessary for the movement of triglycerides together with phospholipids from the inner layer of the ER membrane to integrate into VLDL. Presumably, this is made possible by a lipid scrambling process which could make phospholipids available to the growing VLDL within the lumen as illustrated in **Figure 4**.

VLDL transport

Prior to the transport of VLDL to the Golgi, it must first exit the ER. Several studies have provided evidence for the involvement of the COPII machinery, which has mostly been studied for its role in the transport of protein cargos from the ER to the Golgi (for review see [104]) in this step in the transport of VLDL. Larger sized cargo's such as VLDL, however, require help from several proteins. **Golgi organization protein 1 (TANGO1)** and **TANGO1-like or TALI** [31,108] assist in the recruitment of VLDL to ER exit sites and possibly allow fusion with the ERGIC membrane to generate an export pathway as previously suggested [31]. **Kelch-like protein 12 (KLHL12),** another protein known to assist the transport of larger cargos in COPII vesicles, has also been shown to affect apoB/VLDL secretion in McArdle RH7777 cells [32].

While many of these studies have been conducted *in vitro*, there are several intriguing examples of the involvement of this type of intracellular trafficking of apoB-containing lipoproteins *in vivo*. For example, a mutation in **melanoma inhibitory activity 2** (***MIA2)*** (a gene homologous to **melanoma inhibitory activity** *MIA* as well as *MIA3/TANGO1*), a liver protein that localizes to ER exit sites and interacts with COPII proteins has been shown to markedly reduce plasma triglycerides and cholesterol but not apoB in *Mia2*$^{cpto/cpto}$ mice [109]. Interestingly this did not cause changes in liver cholesterol and only a mild, non-significant increase in liver triglycerides. The hepatic loss of **meningioma-expressed antigen 6 (Mea6)/ cutaneous T cell lymphoma-associated antigen 5C (cTAGE5C**), another protein that interacts with components of COPII and TANGO1, induces severe fatty liver and hypolipidemia in mice with the hepatic accumulation of apoB [110]. MIA2 and Mea6/cTAGE5c are not shown in our figures because TANGO1 and TALI are chimeric proteins resulting from the fusion of MIA2 and cTAGE5 gene products. The role of the COPII machinery in humans is clearly illustrated by loss-of-function mutations in secretion associated **Ras related GTPase 1B** (**SAR1B**), which cause chylomicron retention disease and hepatic steatosis (OMIM#246700) [64].

To identify novel receptors that can bind cargos and recruit them to nascent vesicles, like the cargo receptors TANGO1 or SAR1b, a proximity-dependent proteomics approach with SAR1B has been conducted [37]. This study led to the identification of **surfeit locus protein**

4 (SURF4), which was previously linked to the exit of vitellogenin 2 (also synthesised as a lipoprotein complex) from the ER in yeast [111]. They showed that apoB interacts with SURF4 in HepG2 cells [111], and subsequently studied its role in mice [37]. Like *Sar1b*, liver-specific loss of *Surf4* results in a drastic reduction of VLDL secretion (>85%) and 90% lower plasma lipids in mice. This was accompanied by increased hepatic lipid accumulation as assessed by oil-red-O staining. In contrast to the results from this acute CRISPR-mediated knockout model, other studies using *Surf4*$^{fl/fl;}$Alb-Cre mice describe no changes in liver lipid levels compared to controls [72]. SURF4 has been shown to be involved in many other processes including the regulation of the transport of PCSK9 [71], erythropoietin [112] and growth hormones [113] (summarized in [114]), while it also plays a role in maintaining the architecture of the ERGIC and the Golgi [115]. Possibly, the diverse ways of targeting hepatic SURF4 in various mouse models affect the numerous functions of SURF4 differentially.

Additional studies done over a decade ago identified several other proteins involved in VLDL trafficking by specialized VLDL transport vesicles, including Sec22b, SVIP, ApoC-1, Reticulon 3 and CIDEB [116] of which CIDEB was already shown to markedly affect VLDL biogenesis in mice as discussed above [62]. **Reticulon 3 (RTN3)**, is a member of a large family of proteins that have been shown to have multiple cellular actions, including affecting the regulation of VLDL secretion by controlling the transport of VLDL transport vesicles from the ER to the Golgi. The latter is compatible with the role of RTN3 in the formation and maintenance of ER tubules [117]. The protein was found to interact with apoB100 in hepatocytes [34]. Blocking RTN3 in an *in vitro* VLDL transport vesicle budding assay resulted in a decreased appearance of radiolabelled triacylglycerol in the cytosolic media, which was used as read-out for the generation of VLDL transport vesicles. To attain the impact of RTN3 silencing on VLDL secretion, secreted triglyceride levels were measured in the media of cultured cells. Although it is unclear whether they used HepG2s or primary rat hepatocytes, triglyceride levels were reduced by ≈30% compared to controls. There are no published studies of the *in vivo* deletion of RTN3.

RAB GTPases are small guanosine triphosphate (GTP)--binding proteins that regulate intracellular trafficking of vesicles [118], in which several Rabs GTPases have also been shown to affect apoB/VLDL [35]. In a screen to identify factors that control lipoprotein secretion in Huh-7.5 derived cells, it was e.g., shown that **RAB1b**, a major regulator of transport from the ER to the Golgi, affects the secretion of apoE and apoB [35]. Another RAB GTPase-activating protein **GP73** was recently shown to affect apoB/VLDL export [36]. GP73 expression is low under normal conditions but increased in patients with acute or chronic liver disease. Adeno-associated virus-mediated overexpression of GP73 in wild-type mice fed a chow diet increased hepatic apoB, and reduced VLDL secretion measured after administration of Tyloxapol [36]. At 6 months on chow, increased GP73 was furthermore shown to cause increased hepatic triglycerides (35%) and reduced plasma triglycerides (30%), as well as a marked reduction in body weight. On a high-fat diet (12 months), NASH

was observed accompanied by a decrease in fatty acid oxidation. GP73 is a Golgi-resident protein, but this study [36] suggests that its effects on intracellular VLDL are mediated via **RAB23,** a protein that regulates ER to Golgi transport and possibly the secretion of lipoproteins [35,36]. The hepatic loss of **RAB24** in mice was recently also shown to decrease liver steatosis as well as plasma lipids [106]. This factor is involved in mitochondrial fission and activation and is upregulated in the livers of obese patients with steatotic liver disease and may also affect VLDL trafficking, but this was not studied.

Altogether, these studies emphasize the need for the involvement of numerous proteins for normal VLDL transport from the ER to the Golgi.

Golgi and secretion

Thus far, little is known about the docking of VLDL transport vesicles to the Cis-Golgi. A role for a unique set of **soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)** proteins has been suggested for fusion-complex formation and includes Sec22b, GOS28, Syntaxin5, and rBet1 [38]. However, no recent studies have been published.

Three decades ago, a series of studies showed that posttranslational modifications of apoB/VLDL occur in the Golgi [119–121]. In a recent review on the glycosylation of apolipoproteins it was reported that ApoB100 has 19 potential N-glycosylation sites, of which 17 sites are glycosylated in regions including the MTP and lipid binding domains [119]. Using McArdle RH-7777 cells, it has been shown that defective N-glycosylation within apoB-37 can affect the secretion of VLDL [122]. Reduced apoB100 secretion can also be due to enhanced proteasomal degradation as a result of glucosamine induced N-linked glycosylation defects in McArdle RH-7777 cells [123]. Which of the numerous glycosyltransferases are responsible for the decoration of apoB is, however, largely unknown, but it has been shown that GLT8D2, encoding for glycosyltransferase 8 domain containing 2, positively regulated apoB levels in HepG2 cells [124]. The *in vivo* impact of glycosylation, following ectopic expression of one of the involved enzymes, N-acetylglucosaminyltransferase III, was demonstrated to disrupt apoB secretion and cause steatotic liver disease in transgenic mice [125]. Only a few studies have focused on the role of phosphorylation of apoB [126,127] but these have not studied whether defective phosphorylation affects apoB secretion. There are to our knowledge, no recent studies that have focused on post-translational modification of apoB/VLDL in the Golgi in preclinical models. There are several congenital disorders of glycosylation that are associated with changes in apoB, triglycerides and cholesterol, but this phenotype is often seen in the context of a broad range of clinical manifestations and is not discussed here (see [128] for a recent review).

Although many studies of VLDL biogenesis have focused on the ER as the site of bulk lipidation and the formation of mature VLDL [129], others, have demonstrated further lipidation in the Golgi [130,131] However, this issue has not been the focus of recent studies and the differences remain unresolved. Indirect evidence for specific cellular VLDL secretion routes (from the ER directly to the cell membrane and from the trans Golgi to the cell membrane) comes from human kinetic studies in which smaller triglyceride-poor $VLDL_2$ particles and larger triglyceride rich-$VLDL_1$ are studied [130,132,133]. In such studies, however, it cannot be excluded that these VLDL subpopulations are a product of peripheral lipolysis.

An interesting player in intracellular VLDL metabolism is SORTILIN1. This protein is encoded by ***SORT1***, which has been identified as a regulator of apoB levels in genome-wide association studies [134]. One of the proposed functions of this multi-ligand receptor includes protein transport from the Golgi to lysosomes or the cell surface. Although SORTILIN1 is also recognized as a plasma membrane receptor for VLDL, LDLR, and PCSK9, the role of SORTILIN1 in apoB secretion remains incompletely understood due to conflicting results on apoB secretion in mice [135]. Evidence on the role of SORTILIN1 in cardiovascular and metabolic diseases, and how it regulates LDL levels has been summarized in a recent review [66]. This review did not include the latest insights that loss-of-function of SORTILIN1 under basal non-stressed conditions has little effect on apoB secretion whereas, under ER stress or lipid loading conditions, absence of SORTILIN1 leads to increased apoB secretion [40]. This suggests that hepatic SORTILIN1, under stress conditions, may direct apoB towards lysosomal degradation, whereas it targets apoB for secretion in the absence of stress. The dual function of SORTILIN1 is supported by a recent report which suggests that mutating two different binding sites on SORTILIN1 either increases or decreases VLDL secretion in McArdle RH-7777 cells [136], suggesting allosteric conformational changes depending on the activated binding site.

To our knowledge, the export of VLDL from the Golgi and subsequent transport to the plasma membrane has only been studied by one group. [137]. These investigators developed a trans-Golgi network budding assay in primary rat hepatocytes to examine post-trans Golgi VLDL transport vesicles. Besides cytosol, ATP, GTP hydrolysis and incubation at 37°C, no other conditions were needed for vesicle formation from the Golgi. Electron microscopy revealed vesicle sizes ranging from 300-350nm. The reaction mixture of this assay was subsequently resolved on a sucrose gradient and showed apoB, apoA4, apoA1, and apoE, but no albumin or transferrin was present on the vesicles. Whereas the acquisition of apoA1 by chylomicrons has been described [138], it is unclear whether apoA1 is secreted together with VLDL. The same investigators also identified two post-Golgi SNARE proteins on the post-trans Golgi VLDL transport vesicles named VAMP7 and SNAP23, which suggests that SNARE proteins facilitate the particle from the trans-Golgi network to the plasma membrane [137].

Summary

The VLDL biogenesis pathway has been studied continuously for five decades, but over the last few years, there has been a new surge in interest. This may be related to a series of studies indicating that the molecular regulation of this pathway has profound effects on both hepatic and plasma lipid homeostasis, both of which, when disrupted, can negatively affect cardiovascular health. The technological advances allowing for fast and efficacious silencing or ablation of genes in the liver (which has now also become clinical practice [139–141]) is probably another reason for the recent increase in publications focusing on this area. Genes already targeted include *APOC3* and *ANGPTL3,* whose protein products affect the lipolysis of VLDL triglycerides in the periphery. These drugs are in various stages of development or approved for limited use (*APOC3* antisense oligonucleotide (ASO) is approved in Europe for reducing triglycerides in patients with patients with familial chylomicronaemia syndrome and *ANGPTL3* monoclonal antibody is approved for the treatment of homozygous familial hypercholesterolemia; for a review see [142]). Of note, hepatic lipids have not been the primary focus of these studies, although a recent post-hoc analysis of three studies with the first-generation *APOC3* ASO suggested a treatment-associated but very modest reduction in steatosis measured by magnetic resonance imaging [143]. Finally, ANGPTL4 is also being targeted to modulate VLDL triglyceride hydrolysis [144,145] but there are thus far no published human data.

This review integrates the new insights from the recent literature with what had been learned previously into an extended, detailed working model of VLDL biogenesis and secretion (Figure 2). It has been well-established that VLDL is regulated at the post-translational level (reviewed in [146]), but we here list several studies that have provided evidence for additional regulation at the transcriptional level (HUR [13]) and strong evidence for regulation at the translational level at the outer ER membrane (VIGILIN, TIAL1 [14,15]). On the other hand, LZP [19] and MDM2 [20] can be added as new members of a large number of proteins that were previously shown to affect apoB/VLDL at the early post-translational level (Table 1).

The list of proteins required for actual VLDL biogenesis in the ER has been expanded with LAP1/TORSINA, PLA2G12B, SMLR1, ERLIN1/2, and TM6SF2 [21,23,24,70]. While hepatic ablation of TORSINA profoundly affects hepatic and plasma lipids by affecting VLDL biogenesis [21], the molecular mechanism remains to be elucidated. Much more is known about PLA2G12B; A series of studies in *in vivo* and *in vitro* models make clear that PLA2G12B interacts with MTP and mediates the expansion of VLDL in the ER through regulating lipid partitioning between luminal lipid droplets and nascent VLDL [23]. Instead, CIDEB has previously been shown to make triglycerides already residing in cytosolic lipid droplets available for apoB lipidation [61]. Interestingly, the loss of both PLA2G12B or CIDEB ultimately results in the secretion of less lipidated apoB indicating that their functions in

VLDL biogenesis are not redundant and that both luminal and cytosolic lipid droplets may be needed for normal VLDL biogenesis and secretion. In the case of TM6SF2, another well-studied gene and protein, it is still not yet clear whether it solely affects the lipidation of apoB or whether it has additional functions [147]. The latest studies show that TM6SF2 interacts with ERLIN1/2 to stabilize apoB [24].

Importantly, although Figure 3 suggests a series of consecutive molecular events in early VLDL biogenesis, the current literature does not allow for a definitive statement regarding the actual sequence of events or the extent to which the proteins involved function independently or together. What can be appreciated, however, is that in every published study, hepatic loss of an individual factor caused various degrees of hepatic steatosis and reduced plasma triglycerides, suggesting non-redundancy.

Interactions of MTP, PLA2G12B, CIDEB, TM6SF2/ERLIN1,2 (and possibly TORSINA) with apoB at the inner ER leaflet may still not be sufficient for VLDL to reach the level of maturation required to exit the ER. Support for this concept is provided by studies of VMP1 and TMEM41B, which suggest that VLDL must first enter the ER lumen, a step requiring these two phospholipid scramblases as detailed above and illustrated in Figure 4. The loss of either of these factors results in the accumulation of neutral lipids in the ER leaflets, steatotic liver disease, and severe reductions in plasma lipoproteins. The authors of the first VMP1 study [29] made the point that this initial step in VLDL transport into the lumen of the ER is distinct from the exit of VLDL from the ER, as the loss of VMP1 results in a different phenotype compared to loss of TANGO1, TALI, and SURF4, which are required for VLDL to exit from the ER. Studies into the loss of hepatic TMEM41B support this idea as it results in the same distinct accumulation of neutral lipids in the ER leaflets. In addition, loss of VMP1 and TMEM41B both cause NASH which has not been reported for the other factors. Caution is, however, warranted when comparing the outcomes of these investigations by different labs. It often concerns diverse animal models on different diets with limited, non-quantitative analyses of transmission electron microscopy images that are carried out without discriminating lipid droplets from nascent lipoproteins. Only head-to-head studies, best done with improved techniques enabling specific detection of lipoproteins at the subcellular level, such as those used in the latest PLA2G12B study [23], will allow for drawing firm conclusions regarding this important step in VLDL biogenesis.

Figure 4 illustrates the need for numerous proteins to allow VLDL as a large cargo (relative to the majority of proteins) to leave the ER via the COPII machinery for transport to the Golgi. SURF4 has now also been shown to affect this pathway [37]. The authors of that study present SURF4 as a cargo receptor and show that loss of SURF4 almost completely abolishes VLDL secretion, associated with hepatic steatosis and reduced plasma lipid levels. Those authors show that this causes VLDL to accumulate in the ER but as already mentioned above, this is an arguable result when considering the number of immunolabeled apoB

particles compared to controls. In addition, others also showed a severe defect in hepatic lipoprotein secretion but without hepatic steatosis after the ablation of *SURF4* in mice [72]. These studies highlight the need for improved tools to study these molecular mechanisms.

Only a few groups have studied the fate of VLDL after it has left the ER. Some recent studies have shown that several RABs affect VLDL trafficking but the specificity for VLDL transport is often not clear in the context of altered vesicular trafficking of other cargos. A single *in vitro study* provides evidence that VLDL docks to ERGIC and the cis-Golgi in a process involving the SNARE complex [38].

In the Golgi, it has been proposed that VLDL undergoes additional lipidation, but the data are conflicting [131,132,148] and further research is warranted. VLDL undergoes extensive glycosylation of apoB (for review see [119]) but there are almost no studies on how apoB/VLDL is transported across the Golgi, with little if anything known about the steps needed for VLDL to exit the Golgi, how it is transported to the cell membrane, or how VLDL is secreted from the cell[137]. *In vivo* studies of VLDL secretion have not added to this paucity of knowledge in this component of cellular VLDL biology. The latter contrasts with the knowledge gained during the past five decades about VLDL biogenesis in the ER.

It is important to note that the above summary of VLDL biogenesis comes with constraints. In many instances, molecular studies have been conducted in hepatocarcinoma cell lines of human (HepG2, IHH), murine (Hepa1-6), and rat (McArdle) origin while some investigators also used primary hepatocytes. Comparisons of the effects of genetic manipulations in mice (viral transduction, transgenesis, whole body knockout, conditional knockout, and more recently somatic gene editing) are often confounded by the use of diverse diets, which have strong effects on the development of steatotic liver disease. Finally, in most cases, key factors have not been studied in both cell and animal models or demonstrated to carry out the same function in humans.

Translation

The key question is what the relevance of all the above-described molecular events is to hepatic and cardiovascular health in humans. To set the stage, we here highlight the phenotypes of three Mendelian disorders with a focus on liver and cardiovascular health. Losing the capacity to produce VLDL in the ER due to loss of function mutations in *APOB* and *MTTP* results in hypobetalipoproteinaemia, and abetalipoproteinemia (OMIM#615558, #200100). Follow-up studies have illustrated protection against atherosclerosis [149]. These individuals also present with steatotic liver disease, but this does not appear to develop into severe liver pathologies in the majority of the cases [149,150]. The very low levels of apoB-containing lipoproteins in these patients actually spurred the development of drugs to reduce the risk of ASCVD despite the fact they might induce steatotic liver disease [151–153]. SAR1B deficiency is a third Mendelian disorder highlighting the impact of attenuated

trafficking of apoB-containing lipoproteins (OMIM#246700). In this case, low plasma lipids are also accompanied by steatotic liver disease, but these patients primarily suffer from the consequences of a lack of fat-soluble vitamins due to defective absorption of dietary lipids in the gut as discussed elsewhere [105].

Human genetics studies have furthermore led to the identification of *TM6SF2* [25], and *SORT1* [154] as common or rare genetic variants in these loci that affect lipid levels in liver and plasma (*TM6SF2*) or plasma (*SORT1*), respectively. Several of the genes identified recently affect VLDL metabolism in humans. For example, single nucleotide variants modulating the transcription of *PLA2G12B* are associated with plasma triglyceride levels [155,156]. Also, genome-wide association studies have identified variants in the *VMP1* gene that are associated with circulating levels of LDL [157,158]. In several cases, including *VIGILIN* [15], *MDM2* [20], and *GP73* [36] it is clear that the encoding genes are upregulated in humans with NASH or steatosis compared to normal livers. In the majority of cases, however, the biology of the new factors discussed in this review has not been studied in humans. Most of the data summarized here derive from basic research and, therefore, translational studies are warranted.

Conclusion and perspectives

In the introduction, we raised the key question of whether it will be possible to prevent or treat ASCVD by reducing VLDL secretion without increasing hepatic lipids. This is possible as we noted for VIGILIN [15], MDM2 [20], TGH [26,27], MIA2 [109], and SURF4 (in [159] but not in [37]). In addition, in mice, hepatic steatosis is absent when microRNA-30c reduces plasma lipids [160] or when differential inhibition of triglyceride and phospholipid transfer activities of MTP dissociate impaired VLDL secretion from hepatic steatosis. [161] It has also been shown that induction of autophagy in mice reduces steatosis under conditions of decreased VLDL secretion [162] while murine G-protein coupled receptor 146 (GPR146) deficiency reduces VLDL secretion, and atherosclerosis without hepatic steatosis [163]. Further support comes from a recent study in humans showing that variation in the *GPR146* gene locus is associated with reductions in both plasma levels of liver enzymes and cholesterol[163]. Taken together, there is considerable evidence for ‘win-win’ opportunities. This review highlights, however, the need to increase our basic knowledge of VLDL biogenesis to identify and select the best targets for the design of optimal strategies that will reduce ASCVD as well as steatotic liver disease. Answering the questions that we have formulated in the box below may help this effort.

Key questions for future research on VLDL biogenesis

- What are the actual subcellular locations of the molecules that affect VLDL biogenesis in the ER?
- Do these molecules only affect VLDL or also other pathways?
- Can we further improve methods to distinguish ER-lipid droplets from apoB-containing lipoproteins?
- Are triglycerides in lipid droplets (whether in the lumen of the ER or the cytosol) transferred directly to VLDL or do they undergo lipolysis to fatty acids that are then re-incorporated into triglycerides targeted for addition to VLDL?
- Is there an *in vivo* role of the Golgi in intracellular VLDL metabolism?
- How does VLDL transit the Golgi and then move to the plasma membrane for secretion into the circulation?
- What are the underlying mechanisms for favourable or non-favourable outcomes when the VLDL production machinery is targeted?
- Can progress in basic VLDL research be translated to human physiology and treatment of the cardiometabolic patient?

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Disclosures

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