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Loss of hepatic VMP1 trapped VLDL in the bilayer of endoplasmic reticulum membrane☆

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Non-alcoholic fatty liver disease (NAFLD) is a highly prevalent form of liver disease associated with obesity, diabetes, and other metabolic diseases.[1] NAFLD is characterized by hepatocyte lipid accumulation. Left untreated, this disease can progress to non-alcoholic steatohepatitis (NASH), typified by increased hepatocyte death, hepatocyte ballooning, inflammation, and fibrosis.[2,3] The molecular basis of NAFLD/NASH development and progression is still poorly understood; thus, no effective treatments currently exist to combat this burgeoning health problem.

Broadly, triglyceride (TG) metabolism in the liver involves at least four processes: (i) Liver TG uptake from very low-density lipoprotein (VLDL) remnants and chylomicrons within the plasma; (ii) Hepatic *de novo* lipid synthesis; (iii) Mitochondrial fatty acid beta-oxidation-mediated TG and fatty acid turnover for energy generation; (iv) Excess TG storage within lipid droplets (LDs) or through delivery to other tissues in the form of VLDL in an apolipoprotein B100 (APOB100)-dependent process.[4,5] Alteration of one or more of these processes may induce hepatic steatosis.

To maintain hepatic lipid homeostasis, hepatocytes increase the formation of either LDs on the cytoplasmic face of the endoplasmic reticulum (ER) or VLDL on the luminal face of the ER, dependent on the ER-derived phospholipid coating of the growing neutral lipid cores.[6]

VLDL biogenesis and assembly take place within the lumen of the ER (Fig. 1). APOB100, which is synthesized within the rough ER, translocates to the ER lumen where it recruits

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Authors' contributions

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Declaration of competing interest

The authors declare that they have no conflict of interest.

neutral lipids including TG, cholesterol esters, and phospholipids to form pre-VLDL.[7] These neutral lipids are synthesized within the lipid bilayer of the ER membrane and released into the ER lumen to complex with APOB100 for secretion.[8,9] Early VLDL assembly requires microsomal triglyceride transfer protein (MTP) for APOB100 lipidation and protein disulfide isomerase (PDI) to increase MTP activity for hepatic VLDL assembly.[5,7,10,11] The pre-VLDL particle continues to recruit neutral lipids, eventually forming nascent VLDL. The nascent VLDL must be transported from the ER to the Golgi to ultimately be secreted from hepatocytes. The formation of specialized transport machinery, the VLDL transport vesicle (VTV), is necessary for the delivery of newly synthesized VLDL particles from the ER exit site (ERES) to the Golgi.[12] Biogenesis of VTV is mediated by the orderly recruitment of a specific set of cytosolic proteins to the ER membrane to form the protein complex known as coat protein complex II (COPII). The formation of COPII starts at the ERES with the Sec12-facilitated conversion of Sar1-GDP to Sar1-GTP on the ER membrane. Sar1 subsequently inserts into the ER membrane and recruits Sec23 and Sec24 to the ER membrane as a heterodimer. The Sec23/Sec24 complex forms the inner layer of COPII, recruiting Sec13 and Sec31 to the ER membrane as a second heterodimer. The Sec13/Sec31 complex forms the outer layer of COPII and triggers membrane deformation leading to vesicle formation. APOB100 and COPII are essential components involved in VLDL assembly and VTV formation.[5,9] Several ER proteins, including Sec16A and the Tango1/cTAGE5 family located at the ERES, bind to COPII components and regulate COPII assembly to facilitate protein and VLDL secretion.[13–15] The COPII subunits are mainly regulated at the post-translational level through phosphorylation (casein kinase II, Unc-51-like autophagy activating kinase 1), O-GlcNAcylation (O-GlcNAcase), and ubiquitination (cullin3), which have been implicated in altering VLDL secretion.[16–21] Conversely, little is known about how the transcription of COPII genes is regulated.

ER-to-Golgi transport is a two-step process involving short-range transport from the ERES to the ER-Golgi intermediate compartment (ERGIC), followed by long-range transport from the ERGIC to the cis-Golgi.[22] ER-ERGIC-Golgi trafficking is important for nascent protein and lipoprotein secretion. Coat protein complex I (COPI) and COPII vesicles are essential parts of the trafficking machinery involved in conventional protein secretory pathway cycling between the ER and Golgi. COPI vesicles mediate proteins and lipids transport from the Golgi to the ER, whereas COPII vesicles mediate cargo transport from the ER to the Golgi.[23] Recent studies have reported that ERGIC and Golgi fragmentation due to the deletion of lissencephaly 1 (LIS1) or TMEM199 in the liver cause impaired VLDL secretion, steatosis, and NASH.[24,25] Once a VLDL particle is delivered to the Golgi lumen, VLDL-APOB100 is either glycosylated or phosphorylated to further facilitate post-Golgi transport of VLDL.[26,27] The current understanding of APOB100-COPII and Golgi in VLDL secretion has been summarized in Fig. 1.

Vacuole membrane protein 1 (VMP1) and TMEM41B are ER-resident proteins that have phospholipid scramblase activity and are critical in regulating autophagosome closure.[28] Recent studies have shown that genetic deletion of *Vmp1* or *Tmem41b* in the livers of mice leads to impaired VLDL secretion resulting in NASH.[29,30] Ultrastructure studies from electron microscopy (EM) analysis of *vmp1* knockout (KO) zebrafish livers and human

HepG2 cells revealed that VLDL particles may be stalled within the lipid bilayers of the ER membrane.[31]

To visualize and further confirm intracellular nascent lipoprotein assembly and location in the ER of VMP1-deficient hepatocytes, we used liver-specific *Vmp1* KO and wildtype (WT) mouse livers, primary cultured VMP1 KO hepatocytes, and treated WT hepatocytes with oleic acid (OA, 200 μmol/L, 6 h), followed by transmission EM (Fig. 2). In overnight fasted WT mouse livers (**a**) and OA-treated hepatocytes (**c**), LDs appear as round structures consisting of a phospholipid monolayer (white arrows) enclosing a homogenous translucent area, which exemplifies typical cytosolic LDs. In *Vmp1* KO mouse livers (**b**) and cultured hepatocytes(**d**), "LDs" are enclosed by membranes (ER phospholipid bilayer, black arrows) with an additional clear electron dense "edge" (white arrows) surrounding the lipid structure. The ER lumen (denoted by stars) represents the space between the ER membrane and the electron dense-edged lipid structure, suggesting VLDL blockage within the lipid bilayer of VMP1 KO hepatocyte ER. The mechanisms involved in LD stalling within ER bilayers remain unclear, a potential explanation being that lack of VMP1 or TMEM41B may decrease phospholipid content on the ER membrane and affect ER membrane curvature. Future studies are needed to dissect the role of VMP1 and TMEM41B in regulating mitochondria-associated membrane (MAM) assembly and its impact on phospholipid synthesis. All mice received humane care, and all procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center and described previously (ACUP #2020–2593).

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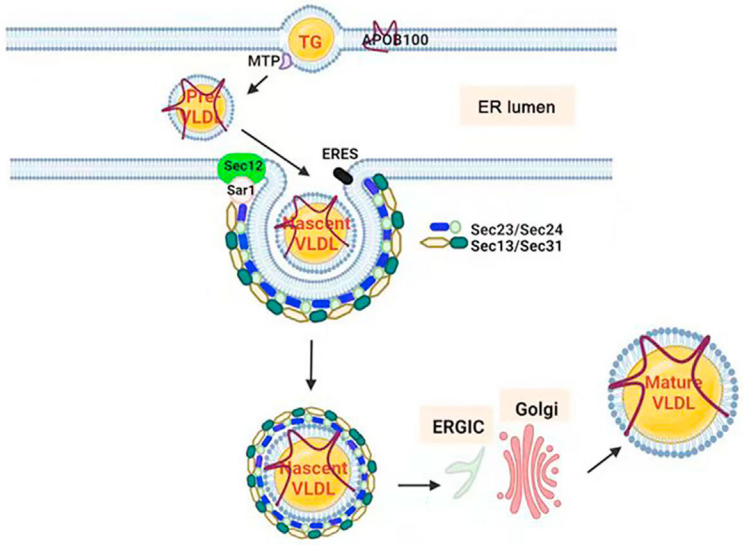

Fig. 1. Summary of APOB100-COPII, ER, ERGIC, and Golgi in VLDL assembly and secretion.

Neutral lipids including TG, cholesterol esters and phospholipids are synthesized within the lipid bilayer of the ER membrane and transferred to APOB100 by MTP to form pre-VLDL. The pre-VLDL particle is further lapidated by recruiting neutral lipids, eventually forming nascent VLDL. The nascent VLDL is exit from the ER and transported to the ERGIC and the Golgi which is mediated by COPII and ultimately is secreted from hepatocytes. Abbreviations: APOB100, apolipoprotein B100; COPII, coat protein complex II; ER, endoplasmic reticulum; ERES, ER exit site; ERGIC, ER-Golgi intermediate compartment; LDs, lipid droplets; MTP, microsomal triglyceride transfer protein; TG, triglyceride; VLDL, very low-density lipoprotein.

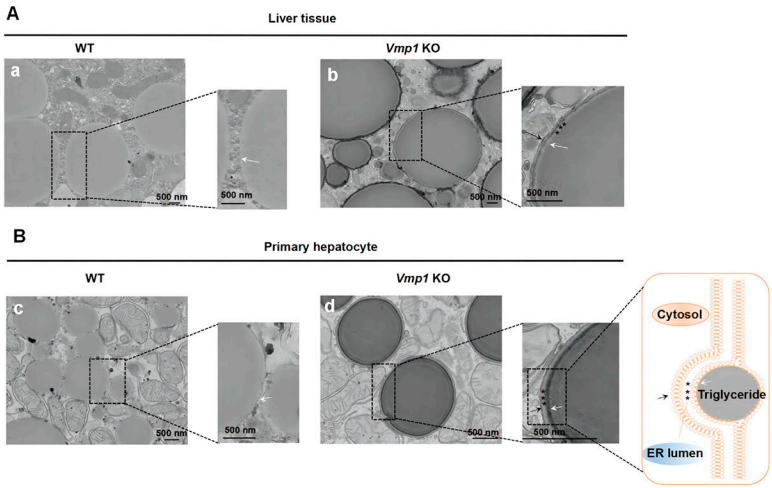

Fig. 2. EM analysis of hepatic VLDL and LDs in VMP1 in *Vmp1* KO mouse livers and primary cultured hepatocytes.

(**i**). Eight to ten-week-old *Vmp1*$^{flox/flox}$ mice (the European Mouse Mutant Archive, electron microscopy (EM):05506) were injected with Adeno-associated virus (AAV) 8-TBG-Cre (*Vmp1* KO) or AAV8-TBG-null (WT) for two weeks. Mouse livers were perfused *in situ* with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 5 min. Livers were excised and fixed in the same buffer overnight at 4 °C. Samples were washed 3 times with 0.1 mol/L sodium cacodylate buffer. The liver tissues and cells were postfixed with 1% osmium tetroxide in 0.1 mol/L sodium cacodylate or a fresh mix of 1% osmium tetroxide and 0.15% potassium ferrocyanide in 0.1 μmol/L cacodylate, respectively. (**ii**) Primary cultured hepatocytes isolated from liver-specific *Vmp1* KO and WT mice were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) overnight at 4 °C. Tissues and cells were dehydrated in 50%, 70%, 80%, 95% and 100% (2 ×) ethanol and were embedded in 100% EMbed 812 resin, and polymerized at 60 °C for 24 h. Ultrathin sections were picked up on 250 mesh copper grids and poststained with 2% uranyl acetate (7 min), and lead citrate for 5 min. The images were taken by Leica EM UC7 Ultramicrotome (Leica-Microsystems, Vienna, Austria) and JEM 1011CX electron microscope (JEOL, Peabody, Massachusetts, United States) with an AMT XR111 8 Megapixel scintillated CCD camera. Panels a & c, white arrows denote the cytosolic LDs with phospholipid monolayer. Panels b & d, white arrows denote phospholipid monolayer of ER membrane; black arrows denote phospholipid bilayer of ER membrane; stars denote ER lumen.