Visual and biochemical evidence of distinct pathways of cholesterol metabolism in human hepatocytes

Alessandro Pisaturo

Faculty of Medicine
Division of Experimental Medicine
McGill University, Montreal

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Table of Contents

Abstract

The liver regulates the level of plasma cholesterol by altering the rate at which lipoprotein particles are released from the liver and the rate at which they are removed from the blood. Atherosclerosis is caused by the deposition of apolipoprotein B (apoB) lipoprotein particles in the arterial wall. Hepatocytes are the carrefour of cholesterol trafficking from both dietary- and endogenous apoB lipoprotein sources. Preliminary studies suggest that while dietary cholesterol in the form of chylomicron remnants (CR) regulates intracellular cholesterol homeostasis, endogenous low-density lipoprotein (LDL)-derived cholesterol influences the circulating cholesterol pool. In this report, we provide evidence suggesting that there exist multiple specific channels of lipoprotein transport within the human hepatocyte that implicate the compartmentalization of the ER. The LDL-derived cholesterol is esterified by SOAT2 to be secreted within very low-density lipoprotein (VLDL). We characterize juxtanuclear ER-resident sites of VLDL assembly (SOVAs) which integrate the docking of LDL-containing endosomes, cholesterol esterification, and apoB synthesis. Furthermore, we validate the role of the SOAT2 enzyme in the shunt model, whereby the esterification of LDL-derived cholesterol prevents its entry in the ER "regulatory pool". Lastly, we present evidence that early after endocytosis, peripherally localized CRs donate their cholesterol to the plasma membrane which then equilibrates with the "regulatory pool".

Résumé

Le foie régule la concentration du cholestérol dans le plasma en modifiant la vitesse à laquelle les particules de lipoprotéines sont libérées du foie et la vitesse à laquelle elles sont éliminées du sang. L'athérosclérose est causée par l'accumulation de lipoprotéines contenant l'apolipoprotéine B (apoB) dans la paroi artérielle. Les hépatocytes sont le carrefour de l'afflux de cholestérol provenant de sources de lipoprotéines alimentaires et les lipoprotéines d'origine endogène contenant l'apoB. Des études préliminaires suggèrent que même si le cholestérol alimentaire sous forme de restes de chylomicron (CR) régule l'homéostasie intracellulaire du cholestérol, le cholestérol dérivé des lipoprotéines de basse densité (LDL) d'origine endogène influence le pool de cholestérol en circulation. Dans ce travail, nous présentons des preuves de multiples voies spécifiques de transport des lipoprotéines au sein de l'hépatocyte humain qui impliquent la compartimentation du RE. Le cholestérol dérivé des LDL est estérifié par SOAT2 pour être sécrété dans les lipoprotéines de très basse densité (VLDL). Nous caractérisons les sites juxtanucléaires résidant aux RE de l'assemblage des VLDL (SOVA) qui intègrent l'amarrage des endosomes contenant des LDL, l'estérification du cholestérol et la synthèse de l'apoB. De plus, nous validons le rôle de l'enzyme SOAT2 dans le modèle de shunt, par lequel l'estérification du cholestérol dérivé des LDL empêche son entrée dans « le pool de régulation » du RE. Enfin, nous présentons des preuves que peu de temps après le moment de l'endocytose, les CR localisés à la périphérie donnent leur cholestérol à la membrane plasmique favorisant ainsi son équilibre avec « le pool de régulation » de cholésterol.

Acknowledgements

I would like to thank Dr. Anouar Hafiane for his invaluable experimental guidance, training, and advice for radioactive cell culture, lipoprotein preparation, and thin-layer chromatography.

I would also wish to thank Shi Bo Feng for her microscopy training and her willingness to help me develop macros in FIJI to measure fluorescence intensity.

Thank you to He Chen, Ioanna Gianopoulos, and Vatsal Sachan for their training in cell culture, immunofluorescence, western blotting, and apoB immunoprecipitation.

To the lab of Dr. Peter Metrakos and Dr. Nabil Seidah, thank you for providing me with immortalized human hepatocytes and reagents for my experiments.

To mom, dad, Andrea, and Georgia, thank you for your constant love and support!

Lastly, I am grateful for the guidance provided by Dr. Allan D. Sniderman, Dr. Robert Scott Kiss, and Dr. Tommy Nilsson throughout my entire Masters degree. Thank you for your generosity, and allowing me to ask the questions I am most interested in.

This work was supported by the Research Institute at the McGill University Health Centre (RI-MUHC), the RI-MUHC Studentship; the Canadian Institute of Health Research (CIHR), Canada Graduate Scholarships-Master's Program; and the Fonds de la Recherche en Santé du Québec, Formation de maîtrise.

Contribution of Authors

Illustrations presented in **Figure 1**, **Figure 2**, **Figure 3**, and **Figure 12** were prepared in BioRender by Alessandro Pisaturo.

Data used in **Figure 4**, **Figure 5**, **Figure 6**, **Figure 7**, **Figure 8**, **Figure 9**, **Figure 10**, and **Appendix 1** were obtained by Alessandro Pisaturo.

Data used in **Figure 11** and **Appendix 2** were obtained by Dr. Robert Scott Kiss and reproduced with their permission.

List of Figures

List of Abbreviations

ABCA1/ABCG1/ABCG5/ABCG8 – ATP binding cassette transporter A1 / G1 / G5 / G8

ACAT – Acyl-CoA cholesterol acyltransferase

AP-2 – Adaptor protein complex 2

ApoB/ApoE – Apolipoprotein B / E

ARFRP1 – ADP-ribosylation factor-related protein 1

ARH – Autosomal recessive hypercholesterolemia

BSEP – Bile salt export pump

CE – Cholesteryl ester

CETP – Cholesteryl ester transfer protein

CideB – Cell death-inducing DFF45-like effector b

COPII – Coat protein II

CR – Chylomicron remnant

CVD – Cardiovascular disease

CYP7A1 – Cholesterol 7 alpha-hydroxylase

Dab-2 – Disabled-2

DGAT1 – Diacylglycerol acyltransferase 1

EGF-A – Epidermal growth factor-like repeat A

ER – Endoplasmic reticulum

ERAD – ER-associated decay

FC – Free cholesterol

FH – Familial hypercholesterolemia

FXR – Farnesoid X receptor

FYCO1 – FYVE and coiled-coil domain-containing 1

GULP – Engulfment adaptor protein

HDL – High-density lipoprotein

HMGCR – 3-hydroxy-3-methyl-glutaryl-CoA reductase

Hsp70/Hsp90 – Heat shock protein 70 / 90

IDOL – Inducible degrader of LDLR

IHH – Immortalized human hepatocytes

Insig – Insulin-induced gene

LAL – Lysosomal acid lipase

LCAT – Lecithin cholesterol acyltransferase

LDL – Low-density lipoprotein

LDLR – LDL receptor

LEL – Late endosome/lysosome

LPDS – Lipoprotein-deficient serum

LRP1 – LDLR-related protein 1

LXR – Liver X receptor

MTOC – Microtubule organizing centre

MTP – Microsomal triglyceride transfer protein

NPC1/NPC2 – Niemann-pick C1 / C2

ORP1L – Oxysterol-binding protein-related protein 1L

ORP5/ORP6 – Oxysterol-binding protein-related protein 5 / 6

PCSK9 – Proprotein convertase subtilisin/kexin type 9

RILP – Rab7-interacting lysosomal protein

SCAP – SREBP cleavage-activating protein

SHP – Small heterodimer protein

SOAT1/SOAT2 – Sterol *O*-acyltransferase 1 / 2

SOVA – Site of VLDL assembly

SR-BI – Scavenger receptor class B type I

SREBP-1c/SREBP-2 – Sterol regulatory element-binding protein-1c / -2

TG - Triglyceride

VAP-A – Vesicle-associated membrane protein-associated protein A

VLDL – Very low-density lipoprotein

VTV/PG-VTV – VLDL transport vesicle / post-*trans*-Golgi network VTV

1. Introduction

1.1 Atherosclerosis: Etiology and Pathophysiology

1.1.1. Etiology

Atherosclerosis is the most common cause of cardiovascular disease (CVD) in Canada and worldwide, and is the leading cause of death globally [1] [2]. In westernized society, atherosclerosis accounts for approximately 50% of all deaths [3]. Indeed, the factors that elevate one's risk of developing this disease typically include sex, cigarette smoking, diabetes, age, and hypertension [4]. Several cellular and molecular factors including genetic factors and elevated low-density lipoprotein (LDL)-associated cholesterol are equally important. In fact, multiple studies have demonstrated that both sedentary lifestyle and elevated LDL cholesterol levels are paramount for the initiation and progression phases of atherosclerosis [5].

1.1.2. Pathophysiology

Atherosclerotic lesions originate in the arteries and block perfusion of an organ—commonly the brain or heart. Ischemia results from inadequate blood supply to these organs resulting in a shortage of oxygen. For example, ischemic heart disease is the consequence of atherosclerotic lesions within the blood vessels, called coronary arteries, that perfuse the heart muscle. When LDL circulates in the blood over long periods of time, it becomes chemically modified, such as by oxidation or acetylation[6]. The initiating step of atheroma is thought to be retention of modified lipoprotein retention within the arterial wall [6]. Monocytes are then recruited to the arterial wall and differentiate into macrophages[6]. Scavenger receptors expressed on the surface of macrophages bind to modified LDL to promote its internalization within the cell[6]. The LDL derived lipids, primarily cholesterol, are stored within intracellular lipid droplets[6]. This process

contributes to the formation of lipid-laden macrophages called foam cells[6]. Aggregates of foam cells in the intimal layer define the fatty streak: the first sign of atherosclerosis that is visible without a microscope. The arterial wall progressively thickens and hardens upon plaque formation and extracellular lipid in the subendothelial layer of arteries [7]. The fibrous cap is formed when vascular smooth muscle cells proliferate, migrate to the lesion site, and deposit matrix proteins such as fibrin and collagen[6]. In the most advanced stage, the plaque may rupture and cause the formation of a blood clot, or thrombosis, severely blocking blood flow[6].

There is now overwhelming evidence from pathophysiological studies in animals, epidemiological studies, mendelian randomization analyses and randomized clinical trials in humans that LDL causes atherosclerotic cardiovascular disease and that lowering LDL reduces the risk of CVD [8]. Understanding the factors that regulate the concentration of LDL particles in plasma is therefore a critical objective of cardiovascular research.

Under normal circumstances, the liver is the organ responsible for clearing LDL particles from blood. As evidence of this critical role, genetic perturbations of this process are an important cause of atherosclerosis (detailed in Section **1.4.3**). The complex events involved in the pathogenesis of atherosclerosis as well as the chronicity of this disease pose a challenge to its study in humans. What is known for certain is that the regulation of plasma cholesterol levels is paramount in dictating CVD risk.

1.2 Lipoprotein biochemistry and physiology

Cholesterol is an amphipathic molecule and its primary function is to regulate fluidity and impart rigidity in the membranes of eukaryotic cells [9]. Its biological necessity is underscored by the fact that every cell has the ability to synthesize their own cholesterol *de novo*. Additionally, cells may acquire cholesterol from the blood by internalizing lipoprotein particles. The set of

regulatory events that culminate in intracellular cholesterol homeostasis are outlined later in Section **1.3**. Due to the hydrophobic features of the cholesterol molecule, it must be transported in the bloodstream within a vehicle that can accommodate these properties. The typical lipoprotein particle consists of a neutral lipid core containing triglycerides (TG) and cholesteryl esters (CE) surrounded by a phospholipid monolayer in which cholesterol molecules are embedded. The protein component of these particles, called apolipoproteins, are also amphipathic and superficially embedded within the phospholipid monolayer. Apolipoproteins may serve a structural role and are templates for lipoprotein assembly but can also regulate cholesterol uptake from cells as well as lipoprotein metabolism. As an example, a schematic illustrating the protein and lipid components of LDL is shown in **Figure 1**.

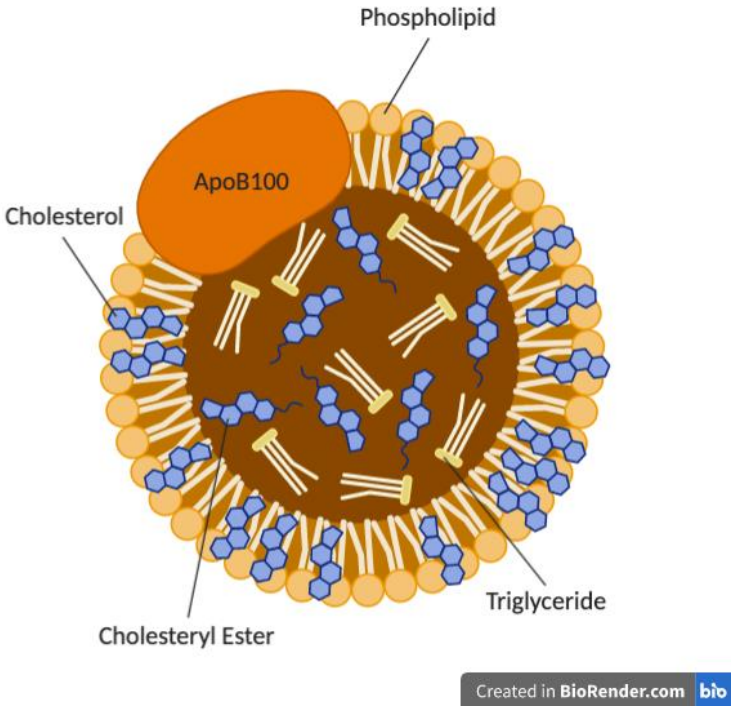


Figure 1. Protein and lipid composition of low-density lipoprotein (LDL).
The typical lipoprotein particle is a combination of lipid and protein components. Within the neutral lipid core of lipoproteins resides cholesteryl esters and triglycerides. The membrane is composed of a phospholipid monolayer in which cholesterol molecules are embedded. Apolipoproteins also associate with the membrane to regulate the metabolism and uptake of their associated lipoprotein. In the example of LDL as shown above, apoB100 is the major irreversible apolipoprotein. Created with BioRender.com.

1.2.1 The exogenous lipoprotein pathway

The different types of lipoprotein particles differ in their lipid content and associated apolipoprotein. They participate in lipid transport throughout the body delivering exogenously obtained or endogenously synthesized lipids. The exogenous pathway begins with intestinally-derived chylomicrons which harbour dietary lipids [10]. Chylomicrons are composed of apoB48—consisting of the first 48% of the full apoB protein—and are the most TG-rich of all lipoprotein classes [10]. In circulation, their TGs are hydrolyzed to deliver free fatty acids to muscle cells for energy and adipose tissue for storage. The resultant chylomicron remnant (CR) particle becomes relatively more enriched in cholesterol than its precursor. The liver is responsible for removing CRs from circulation, thereby marking the end of the exogenous pathway.

1.2.2 The endogenous lipoprotein pathway

The liver secretes very-low density lipoprotein (VLDL) into the circulation (detailed in Section **1.4.2**) which is the first step of the endogenous pathway [10]. VLDL is a TG-rich lipoprotein particle which is composed of an apoB100 protein—the complete apoB protein. The TG are hydrolyzed in the plasma, the freed fatty acids are delivered to peripheral cells, and the remaining cholesterol-rich remnant particle is called LDL. Unlike CRs, apoB100 present on LDL is a ligand for the LDL receptor (LDLR). All peripheral cells can synthesize LDLR, but the liver is the major site that regulates the clearance of LDL particles from plasma. Only a small percent are removed by the adrenal gland and the intestine [11]. Importantly, smaller LDL particles are highly atherogenic as they are denser in CE and have decreased affinity for the LDLR and are therefore retained in the circulation longer [12]. This increases their susceptibility for oxidation, resulting in enhanced uptake by intimal macrophages [12]. However, all LDL particles appear to be more or less equally atherogenic [13]. A separate arm of the endogenous lipoprotein pathway is

responsible for removing peripheral cholesterol and returning it to back the liver—this is called reverse cholesterol transport. Briefly, this pathway begins when the liver synthesizes and secretes apoA-I in the bloodstream. Cells expressing the ATP binding cassette transporter A1 (ABCA1), such as intimal foam cells, are able to activate cholesterol efflux to apoA-I, thereby reducing their excess cholesterol load. The resulting lipidated apoA-I particle is called high-density lipoprotein (HDL). Lipidated HDL particles continue their maturation in the plasma. The HDL particles harboring peripheral cholesterol then deliver cholesterol to the liver through the HDL receptor scavenger receptor class B type I (SR-BI). The cholesteryl ester transfer protein (CETP) mediates the transfer of CE from HDL to VLDL or LDL, and TG from these latter lipoproteins to HDL and LDL [14]. Inhibition of CETP was considered as a measure to reduce CVD, but at least when added to statin therapy, the approach was ineffective[15]. Physiologically, hepatocytes or peripheral cells clear plasma lipoproteins by internalizing them. The lipoprotein-derived cholesterol is released within the cell and raises cellular cholesterol. The set of feedback mechanisms to remove excess cholesterol or increase cholesterol levels are geared towards maintaining intracellular cholesterol homeostasis.

1.3 Traditional model of cellular cholesterol homeostasis

Cellular free cholesterol (FC) levels must be maintained within a narrow range to ensure that the cell's cholesterol needs are met while ensuring viability. Indeed, while cholesterol is crucial in the regulation of membrane fluidity and rigidity, excess cellular cholesterol, especially in the endoplasmic reticulum (ER), is toxic, pro-inflammatory, and pro-atherogenic [16 17]. As a result, lipoprotein uptake and cholesterol biosynthesis are tightly controlled. The mechanism by which cells sense cholesterol and subsequently respond are central to maintaining this homeostasis.

1.3.1 The SREBP-2 and LXR pathways work in concert to achieve homeostasis

While the plasma membrane houses approximately 85% of the cell's FC mass, the level of FC in the ER membrane is maintained at around 0.5%[18]. This constant low level allows the cell to rapidly respond to minute changes in cellular cholesterol in order to regulate cellular FC [19]. These responses are mediated by the sterol regulatory element-binding protein (SREBP) family of transcription factors which are anchored in the ER membrane. Although the SREBPs all have a similar mechanism of action, they differ in which genes they control. SREBP-1c upregulates genes responsible for fatty acid synthesis while SREBP-2 activates transcription of genes that maintain cholesterol homeostasis[20]. These pathways are shut off when cholesterol levels exceed a threshold of 5 mol% of total ER lipids[21]. In the ER, SREBP is complexed to SREBP cleavage-activating protein (SCAP) which itself is bound to insulin-induced gene (Insig). The interaction between SCAP and Insig is dependent on cholesterol binding to the cholesterol sensing domain of SCAP. When ER cholesterol is low, Insig can no longer bind to SCAP and Insig is degraded. Concomitantly, a MELADL sequence on SCAP becomes exposed on the cytosolic surface of the ER which interacts with Sec23, promoting the formation of a coat protein II (COPII) protein coat [20]. The formation of this coat promotes the budding of vesicles from the ER membrane which deliver the SCAP/SREBP complex to the Golgi [20]. In the Golgi, site 1 protease and site 2 protease cleave SREBP at two sites to remove SCAP from SREBP and release the soluble transcription factor domain of SREBP. This transcription factor enters the nucleus and binds to genes with a 10 base pair sterol-regulatory element in their promoter. For SREBP-2, the most important target genes include the LDLR and HMG-CoA reductase (HMGCR) [20]. The LDLR is responsible for binding extracellular LDL and mediating its uptake within the cell. An increase in the number of LDLRs at the cell surface will therefore increase the rate at which cells

internalize LDL-cholesterol from the bloodstream. Additionally, HMGCR is a rate-limiting enzyme of cholesterol biosynthesis. Therefore, SREBP-2 mediated upregulation of HMGCR increases the rate of *de novo* cholesterol synthesis. The net result of the SREBP-2 pathway is to increase cellular cholesterol when it is low, and to prevent this increase when cholesterol levels are high. The SREBP-2 pathway is summarized in **Figure 2**.

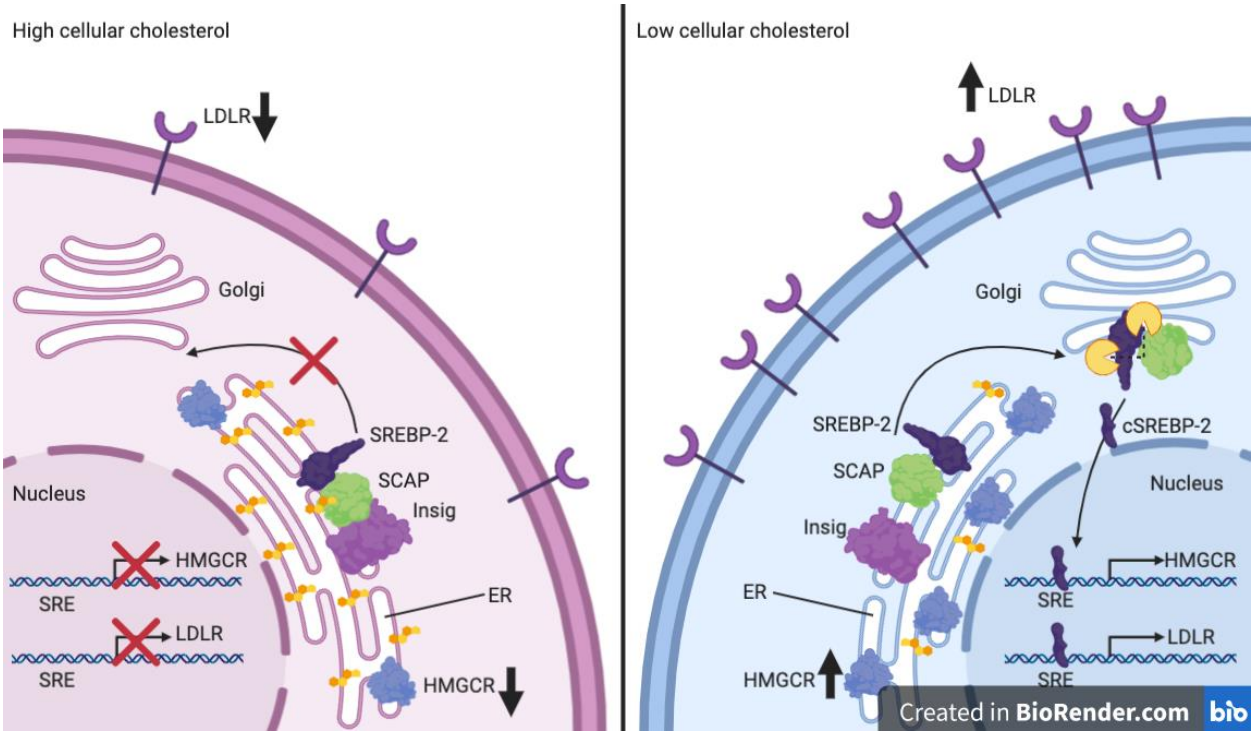


Figure 2. The SREBP-2 pathway controls cholesterogenic genes in the cell. When cellular cholesterol is above 5 mol% of total ER lipids (left panel), SCAP binds to cholesterol which allows Insig to retain the SCAP/SREBP-2 complex in the ER. This interaction abrogates the ER-to-Golgi trafficking of SREBP-2. No SREBP-2 transcription factor is generated. As a result, HMGCR and LDLR are not expressed and protein levels decrease. When cellular cholesterol is below 5 mol% of total ER lipids (right panel), SCAP does not bind cholesterol. The SCAP/SREBP-2 complex is free to migrate to the ER. There, SREBP-2 is cleaved by two proteases, releasing a soluble transcription factor (cSREBP-2, cleaved SREBP-2). cSREBP-2 upregulates levels of HMGCR and LDLR to increase cellular cholesterol. Created with BioRender.com.

While the overall purpose of the SREBP-2 pathway is activate genes responsible for cholesterol synthesis and uptake, cholesterol may also be removed from the cell when it is in excess. When cellular cholesterol levels become excessively high, hydroxylated derivatives of

cholesterol called oxysterols are generated. Oxysterols are approximately 10^6-fold[22] less abundant than cholesterol and have very short half-lives *in vivo* [22]. Moreover, they are potent negative regulators of cellular cholesterol levels. Oxysterols, in particular 25-hydroxycholesterol, are able to bind directly to Insig to promote its binding to SCAP [23]. In this way, cholesterol and 25-hydroxycholesterol act synergistically to abrogate the ER-to-Golgi trafficking of the SCAP/SREBP-2 complex [23]. Oxysterols are also important ligands of another nuclear transcription factor called liver X receptor (LXR). Upon binding oxysterols, LXR will upregulate expression of the genes *ABCA1* and *ABCG1* to promote cholesterol efflux, as well as the inducible degrader of LDLR (*Idol*) to decrease the uptake of LDL cholesterol. IDOL is an E3 ubiquitin ligase able to ubiquitinate the LDLR, thereby targeting it for degradation [24]. Taken together, LXR activates the transcription of genes to prevent cellular cholesterol accumulation. Intracellular cholesterol homeostasis can therefore be viewed as a balance between the activity of two transcription factors: SREBP-2 and LXR. When cholesterol is low, SREBP-2 is activated and LXR is suppressed to increase cellular cholesterol; when cholesterol is high, SREBP-2 is suppressed and LXR is activated to remove cellular cholesterol.

1.3.2 Plasma membrane and lipid droplet storage of cholesterol prevent toxic accumulation

Storage of cholesterol within lipid droplets is a separate mechanism by which cells can reduce the pool of active cholesterol without effectively removing it from the cell. As mentioned earlier, ER cholesterol is purposely maintained at a low level so that small changes in cellular cholesterol can be detected. The plasma membrane, which harbors the majority of the cell's cholesterol by mass, is able to donate some of its cholesterol to the ER membrane so that the ER pool of cholesterol reflects total cellular cholesterol [19]. In fact, cholesterol is in constant flux between these two compartments to maintain this homeostasis. Because of the massive

cholesterol store in connection with the ER, the cell may occasionally become overwhelmed with cholesterol and remain unable to rapidly dispose of it. In this situation, the ER-resident enzyme sterol *O*-acyltransferase (SOAT), also called acyl-CoA:cholesterol acyltransferase (ACAT), is able to esterify cholesterol to prevent its toxic accumulation. The resulting CE is a hydrophobic, inert, molecule, which must be stored within a lipophilic environment. The exact isoform of SOAT will dictate the fate of the CE in question. While SOAT1 is expressed in all tissues, SOAT2 is primarily expressed in the cells of the intestine and liver. SOAT2 will be discussed later in the context of VLDL assembly, trafficking, and secretion. SOAT1 is responsible for esterifying and storing CE within lipid droplets. As a result, lipid droplet biogenesis allows cells to avoid the toxic accumulation of cholesterol without releasing it into the bloodstream. Although lipid droplets are traditionally viewed as fat storage centres within adipocytes, they may be formed in any cell type [17]. Lipid droplets themselves are not passive inert structures and their metabolism is rather dynamic [17]. In fact, the FC that has been esterified and stored as CE within lipid droplets can be reversibly hydrolyzed to release FC to maintain homeostasis [25] [17]. In summary, all cells maintain cholesterol homeostasis by controlling its synthesis, uptake and removal, or reversibly storing it in lipid droplets. Hepatocytes possess a set of additional mechanisms to maintain cellular and systemic homeostasis.

1.4 Hepatic regulation of cholesterol homeostasis

Due to the central role of the liver in both the exogenous and endogenous lipoprotein pathways, there are several unique regulatory mechanisms in addition to those mentioned above that allow hepatocytes to maintain intracellular cholesterol homeostasis. The constant flux of CR, LDL, and HDL into the liver call for mechanisms to deal with this cholesterol [26]. One of the consequences of impaired lipid removal from the hepatocyte includes hepatic steatosis [26].

Therefore, the above model where increased cholesterol influx leads to decreased influx is insufficient for hepatocyte physiology. Two important and unique cholesterol outlets from the hepatocyte include the secretion of bile acids and the secretion of VLDL. The latter process also modulates the systemic cholesterol pool.

The polarization of hepatocytes implicates two surfaces that act as outlets of cholesterol. The basolateral surface of hepatocytes faces the sinusoids, while the apical surface forms the biliary canaliculus along with adjacent hepatocytes [27]. Specialized junctions maintain the lateral organization and attachment between adjacent hepatocytes. The endothelial cells that line the sinusoids are highly specialized and mediate processes such as endocytosis and filtration [28]. The blood that flows along the sinusoids originates from both the hepatic artery and the portal vein. While the hepatic artery supplies oxygen to the parenchyma, the portal vein carries nutrients absorbed in the small intestine to the liver. Fenestrations in the endothelial cell lining allow the exchange of smaller remnant lipoproteins—such as CRs and LDL—between the bloodstream and hepatocytes [29]. This exchange occurs in the space between the sinusoidal endothelial cells and hepatocytes called the space of Disse [29]. The biliary canaliculi, on the other hand, is simply a space between cords of laterally attached hepatocytes. Canaliculi converge into larger ducts and eventually release the bile into the lumen of the small intestine or collect in the gall bladder.

1.4.1 Bile acid synthesis and the enterohepatic circulation

Bile acids are required for the emulsification and absorption of lipids in the intestine. The hepatic synthesis of bile acids begins with FC and requires 17 distinct enzymes located throughout the cell [30]. The end product is secreted into the bile to emulsify fats. Enterocytes absorb emulsified dietary lipids and secrete the remaining bile acids into the portal vein system to be returned to the liver. The recycling of bile acids between the liver and the intestine is called

the enterohepatic circulation [30]. Approximately 5% of the total bile acid pool is able to bypass intestinal reabsorption and is excreted in the feces [30]. Because the cholesterol molecule cannot be degraded, this excretory pathwaya along with cholesterol secretion into the bile remain the only ways by which the body can rid of cholesterol.

Just like cholesterol, there is an intracellular regulation of bile acid synthesis in hepatocytes. When bile acids are present in excess, they bind to the nuclear receptor farnesoid X receptor (FXR) to induce gene transcription. FXR upregulates small heterodimer partner (SHP), bile salt export pump (BSEP), and ABCG5 among many other proteins [30]. When SHP is upregulated, it inhibits the transcription of the rate limiting enzyme of bile acid biosynthesis cholesterol 7 alpha-hydroxylase (CYP7A1) [30]. BSEP pumps bile acids into the bile canaliculi, while ABCG5 forms the heterodimer ABC-half transporter ABCG5/8 required for cholesterol efflux to the bile [30]. Therefore, hepatic bile acid uptake from the portal circulation downregulates its own biosynthesis and increases the secretion of bile acids and cholesterol into the canaliculi.

1.4.2 VLDL assembly, trafficking, and secretion

In addition to biliary cholesterol secretion, cholesterol is also removed from the liver through VLDL, a major secretory product of the liver. The synthesis and release of VLDL from the hepatocyte is a complex process involving a multitude of steps in both the ER and in the Golgi. The very first step in VLDL biogenesis begins with the synthesis and co-translational translocation of a single apoB peptide into the ER lumen. The first 1000 amino acids in the apoB N-terminus form a lipid binding pocket in association with the microsomal triglyceride transfer protein (MTP) [31]. There, MTP transfers TG—and possibly CE—to the nascent VLDL particle [32]. The cellular availability of TG dictates the amount of apoB lipidation. In the event of low cellular TG, apoB lipidation is reduced and this increases the likelihood of ER-associated

degradation (ERAD). Briefly, apoB will be co-translationally ubiquitinated then retrotranslocated into the cytosol [33]. There, heat shock protein 70 (Hsp70) and Hsp90 escort the unfolded polypeptide to the proteasome for degradation [33]. Interestingly, the degradation of apoB100 by the proteasomal pathway is unique as its fate is determined by the lipidation of a wild type protein and not a mutation in the primary sequence. While the synthesis of apoB is relatively constant, it is the degradation of this peptide that determines the amount of VLDL production [34]. Lipidation of apoB100 in the ER is also proposed to occur through the transfer of TG from intracellular lipid droplets to the developing VLDL particle. Cell death-inducing DFF45-like effector b (CideB) localizes to both the lipid droplet and ER surface and mediates the transfer of TG from lipid droplets to immature VLDL through interactions with apoB [35]. Another important component of the VLDL lipid core are CEs. Multiple studies have demonstrated that SOAT2 is required to synthesize the CE within VLDL [36, 37 38 39]. Moreover, SOAT2 is the primary enzyme responsible for cholesterol esterification in human hepatocytes [40]. This ER-resident [40] enzyme generates primarily cholesteryl oleate and cholesteryl palmitate [41]. Unlike SOAT1, the active site of SOAT2 does not face the cytosol, but rather is postulated to face the ER lumen [42]. One study demonstrated that overexpression of both SOAT2 and diacylglycerol acyltransferase 1 (DGAT1), an enzyme catalyzing TG synthesis, in a rat hepatoma cell line significantly increases VLDL secretion [43]. Deletion of liver *Soat2* in mice implicated significant remodeling of the secreted VLDL particle [44]. First, plasma VLDL- and LDL-cholesterol were significantly reduced [44]. Hepatic *Soat2* deletion also decreased the percent of monounsaturated fatty acids and increased the percent of polyunsaturated fatty acids in plasma CE when compared to wild-type [44]. Therefore, both CE and TG are important components of the VLDL core, and their availability are paramount in determining VLDL secretion.

The rate-limiting step of VLDL secretion is its trafficking from the ER to the *cis*-Golgi [45]. Although this trafficking is a COPII-dependent pathway, VLDL transport does not follow the canonical COPII pathway like the aforementioned SREBP-2 does. For one, the vesicle that transports VLDL from the ER to the Golgi, called the VLDL transport vesicle (VTV), is much larger than classical protein transport vesicles. Additionally, some of the proteins required for the budding and fusion of VTVs are different than those required for protein vesicles. One notable example of a VTV interacting protein is CideB [46]. In addition to acting as a putative TG transfer protein, CideB interacts with apoB on the ER to assemble the COPII coat around the budding VTV [46]. It is believed this protein may be required for the budding of the larger VTVs as CideB is not found in classical protein transport vesicles [46]. Moreover, CideB may function as a VLDL-selecting protein for the VTV. Following COPII coat formation and budding from the ER, the VTV traffics through the cytosol where it fuses with the *cis*-Golgi. At this point, VLDL exists as a primordial dense particle requiring further processing. The associated apoB100 peptide becomes glycosylated and phosphorylated in the Golgi lumen. There is strong evidence supporting that the final maturation and bulk lipidation of VLDL occurs in the Golgi part of the secretory pathway [47 48]. This lipidation step may be required to increase the surface area of VLDL particles which allows its reversible association with apoE [48]. One possible mechanism of VLDL lipidation in the Golgi again involves CideB [49]. Briefly, the presence of CideB on the surface of lipid droplets and the Golgi membrane may allow the close apposition of immature VLDL and lipid droplets to promote TG transfer [49]. Evidence also suggests that ADP-ribosylation factor-related protein 1 (ARFRP1) plays a role in the *trans*-Golgi lipidation of VLDL [50]. In the *trans*-Golgi, VLDL is a fully lipidated particle and prepared for secretion. One group determined the existence of post-*trans*-Golgi network VLDL transport vesicles (PG-

VTV)[51]. The PG-VTV is formed by the budding of VLDL from the *trans*-Golgi and transports the mature lipoprotein to the plasma membrane [51]. The fusion of PG-VTVs with the basolateral membrane releases the VLDL particle into the bloodstream [51]. Through this pathway, hepatocytes release large quantities of TG, FC, and CE to maintain homeostasis.

1.4.3 Physiologic and therapeutic control of systemic cholesterol

Not only must the liver maintain intracellular cholesterol homeostasis, but it must also respond to changes in circulating cholesterol levels in the bloodstream. The liver remains the site of up to 90% of LDL uptake and metabolism in the body [52]. The well-documented LDLR is expressed on the basolateral surface of hepatocytes for the binding of plasma LDL and its subsequent clearance from the bloodstream [53]. A higher LDLR expression will therefore increase the rate LDL removal and lower plasma LDL cholesterol. The binding of LDL to the LDLR triggers its internalization by endocytosis (detailed in Section **1.5.1**). As the endosome containing the LDL-LDLR complex acidifies, LDL is released from its receptor and continues down the endocytic pathway for degradation while the LDLR is recycled back to the cell surface[54].

The regulation of hepatic LDLR relies more on proprotein convertase subtilisin/kexin type 9 (PCSK9) than both SREBP-2 and IDOL when compared to other cells [55]. Hepatocytes express and secrete PCSK9, which then binds the LDLR and promotes its post-translational degradation within lysosomes. It was revealed that PCSK9 interacts with the LDLR through association of the PCSK9 catalytic domain with the epidermal growth factor-like repeat A (EGF-A) of LDLR [56]. The PCSK9-LDLR complex is subsequently taken up by endocytosis. Unlike LDLR-mediated LDL uptake, the PCSK9-LDLR is not recycled to the plasma membrane; instead, PCSK9 chaperones LDLR to the lysosome by an unknown mechanism, causing its degradation. Evidence of an intracellular pathway of PCSK9-mediated LDLR degradation was shown when

PCSK9 overexpression reduced LDLR levels in *Arh*$^{-/-}$ mice: these mice are incapable of LDLR endocytosis [57]. It was later shown that PCSK9 may escort the LDLR from the *trans*-Golgi to lysosomes for degradation [58]. The net result is that PCSK9 reduces levels of surface LDLR by intracellular and extracellular routes. Interestingly, expression of the *PCSK9* gene is under the control of the SREBP-2 transcription factor. This implies that the upregulation of cell surface LDLR due to low cellular cholesterol is blunted by the concomitant upregulation of PCSK9. A group led by Dr. Jay Horton proposed that this type of short-term regulation allows newly secreted VLDL to escape the liver parenchyma so that it may enter the periphery [59].

Genetic mutations in the *LDLR* gene cause familial hypercholesterolemia (FH) due to an inability to efficiently clear plasma LDL cholesterol. In addition to elevated blood cholesterol, FH patients have cholesterol deposits in certain areas of the skin (i.e. xanthomas), and develop atherosclerotic lesions much earlier than the general population [53]. Those with single mutations, heterozygotes, acquire a less severe form of FH than those with mutations on each allele, homozygotes [53]. FH mutations in the LDLR may affect its intracellular trafficking, its stability at the cell surface, or block its function [53]. There exist two important types of therapeutics to tackle hypercholesterolemia: statins and PCSK9 antibodies. Statins inhibit HMGCR, thereby depleting the cell of endogenously synthesized cholesterol, and relieving the inhibition of LDLR synthesis [60]. The net result is increased LDLR at the cell surface and increase plasma LDL clearance. There is compelling evidence that statins lower LDL cholesterol substantially in the great majority of individuals[61 62 63 64]. Recently, inhibitors of PCSK9 have emerged as a potent alternative or supplement to lower LDL. These monoclonal antibodies function by binding allosterically to PCSK9 and changing the conformation of the catalytic domain so that it can no longer associate with LDLR, thereby neutralizing circulating PCSK9. As a result, less LDLR

will be degraded, hepatocyte cell surface LDLR expression will be increased, and clearance of plasma LDL cholesterol will be enhanced. The available therapies to treat hypercholesterolemia therefore rely heavily on the physiological role of hepatic LDLR in plasma LDL uptake. The intracellular fate of internalized cholesterol is determined by the endocytic pathway which is tasked with both the metabolism and intracellular delivery of cholesterol.

1.5 Lipoprotein endocytosis and trafficking

Endocytosis is the means by which peripheral cells and hepatocytes internalize and degrade lipoproteins intracellularly [65]. In fact, the molecular machinery involved in this process doubles as a trafficking system to move the degraded contents within the cell.

1.5.1 LDLR-mediated endocytosis of LDL

The binding of LDL particles to the LDLR triggers a sequence of molecular events that lead to clathrin-mediated endocytosis of the LDL-LDLR complex. Upon this binding event, an adaptor protein recognizes the NPxY motif (FDNPVY in LDLR) present on the LDLR cytoplasmic tail to help recruit clathrin to the budding vesicle[53]. The liver expresses two adaptor proteins: Disabled-2 (Dab-2) and autosomal recessive hypercholesterolemia (ARH). However, ARH is expressed in hepatocytes while Dab-2 is mostly expressed in sinusoidal endothelial cells[66]. Their importance in serum cholesterol homeostasis is underscored by the severe hypercholesterolemia that results upon their deletion[66]. In the hepatocyte, ARH bound to the LDLR recruits the multimeric adaptor protein complex 2 (AP-2) which bridges the clathrin lattice to a specific locus of the plasma membrane[53, 65, 67]. Once internalized, the endocytosed vesicle will fuse with an early endosome, decreasing the lumen pH. The increasing acidity causes the ligand-binding domain of the LDLR to fold back on to the EGF-like repeat domain, thereby releasing LDL from its receptor[53, 65]. While the LDLR can either be degraded or recycled back to

the cell surface, the fate of the LDL particle is to be sent to the lysosome for degradation. The pH of the early endosome gradually decreases and eventually adopts a late endosome/lysosome (LEL) character. The acidic environment then activates enzymes responsible for the degradation of proteins and lipids. Importantly, the cholesterol de-esterifying enzyme lysosomal acid lipase (LAL) converts CE to FC [65 68]. Niemann-Pick C2 (NPC2) is a soluble receptor in the LEL that recovers the FC from the degraded LDL and escorts it to NPC1 at the lumenal membrane [69]. There, NPC1 binds FC via its N-terminal domain and transports it to the LEL limiting membrane [69]. Interestingly, these LELs are localized centrally and in proximity to the nucleus, while early endosomes are located near the periphery [70]. The spatiotemporal compartmentalization of the endocytic system allows the cell to sequentially process and degrade internalized cargo.

1.5.2 The endosomal transport system

The movement of endosomes within the cell is dependent on the microtubule network and associated motor proteins. Microtubules originate from the juxtanuclear microtubule organizing centre (MTOC) and stretch out to the extremities of the cell. These rod-like proteins are polarized, meaning they have a minus-end (at the MTOC) and a plus-end (at the periphery). Vesicles move along microtubules with the help of their associated motor protein. Dynein is a minus-end directed protein, while kinesin carry their cargo toward the plus-end. Motor proteins are tethered to the endosomal membrane through effector proteins, which are themselves recruited based on members of the small GTPase family. The Rab5 small GTPase is present on the early endosomal membrane and recruits a dynein to travel along its minus-end directed route [70]. During endosomal maturation, Rab5 is swapped for Rab7 as the endosome becomes a LEL. One of the binding partners of Rab7 includes the Rab7-interacting lysosomal protein (RILP). RILP then recruits dynein to the LEL by interacting with the p150Glued portion of the motor

protein, resulting in minus-end directed movement [71] [70]. A separate adaptor protein of Rab7 includes FYVE and coiled-coil domain-containing 1 (FYCO1) effector. FYCO1 binds directly to kinesin-1 to promote the plus-end directed movement of LELs (i.e. toward the periphery) [70]. Which motor protein is expressed on the LEL surface at a given time determines where in the cell the vesicular contents are targeted.

The directionality of endosomal movement may in part be determined by the cholesterol content harbored within the migrating vesicle. The oxysterol-binding protein-related protein 1L (ORP1L) is an important multidomain effector protein of Rab7. Under conditions of high endosomal cholesterol, ORP1L binds cholesterol and adopts a closed conformation. This allows RILP to bind to dynein and promote minus-end transport [69] [71]. When endosomal cholesterol drops, ORP1L now adopts an open conformation which kicks off $p150^{Glued}$ from RILP, thereby arresting minus-end movement [70] [69]. In this state, FYCO1 may bind to protrudin on the ER and load a kinesin to promote plus-ended movement towards the periphery [72]. Additionally, ORP1L exposes an FFAT domain which binds to the ER-resident protein vesicle-associated membrane protein-associated protein A (VAP-A), effectively forming a membrane contact site [70] [69]. Under these conditions, ORP1L may also mediate the transfer of cholesterol from the LEL membrane to the ER membrane [69]. Zhao and Ridgway demonstrated that knockout of ORP1L in HeLa cells blocks LEL-to-ER cholesterol transport, as cholesterol esterification was inhibited by 60-80% and ER cholesterol biosynthesis increased [69]. The ER is therefore a logistic support while endosomes are travelling through the endo-lysosomal system. Endocytosed lipoproteins harbor large amounts of cholesterol which may eventually enter the ER. Yet, in hepatocytes, the post-lysosomal and metabolic fate of this cholesterol differs across lipoprotein species.

1.6 Hepatic responses to lipoprotein uptake

Brown and Goldstein's model of cholesterol homeostasis in the cell posits that any lipoprotein exerts regulatory effects through SREBP-2. In fact, Brown and Goldstein were accredited for discovering both the SREBP-2 [73] and the lipoprotein uptake pathways [74]. Evidence for these pathways have been validated many times. On the other hand, in the late 1980's, it was shown that LDL does not exert a significant regulatory effect in hepatocytes [75] [76]. However, this data appears to have been overlooked by Brown and Goldstein's discovery. Around the same time, evidence pointed toward the existence of distinct pools of lipids in the ER for lipoprotein assembly [77]. In 2013, our laboratory identified and outlined distinct metabolic pathways for cholesterol derived from LDL, CR, and HDL within primary hamster hepatocytes [78].

1.6.1 Hepatic response to LDL: the "shunt" pathway

In hepatocytes, LDL-derived cholesterol does not exert a regulatory effect through SREBP-2 but is instead preferentially esterified and secreted as VLDL. There are several indications demonstrating that LDL uptake does not follow the traditional model of cholesterol homeostasis outlined in Section **1.3** [55]. For one, LDL does not appreciably downregulate the LDLR [75] and HMGCR[79] in hepatocytes. These results have been verified in different hepatic cell culture models. Our laboratory has demonstrated that this effect is also observed in primary hamster hepatocytes, where the levels of LDLR and HMGCR transcripts are the same for LDL and serum-free media treated cells [78]. Secondly, in primary hamster hepatocytes it was shown that LDL was the only lipoprotein that was able to significantly upregulate expression of SOAT2 [78]. Indeed, LDL-derived cholesterol was shown to be esterified, and this CE is packaged within VLDL and secreted from the hepatocyte [78]. In the presence of a general SOAT inhibitor, LDL cholesterol now exerted a regulatory effect on LDLR and HMGCR [76]. Lastly, in patients with

severe FH, hepatic LDL uptake continues even in the presence of elevated concentrations of serum cholesterol[55]. Together, this evidence argues in favor of a shunt pathway in the hepatocyte, whereby LDL-derived cholesterol does not enter a "regulatory pool" but is instead shunted away by its immediate esterification and packing within VLDL.

1.6.2 Hepatic response to CR: the regulatory pathway

On the other hand, CR-derived cholesterol in hepatocytes enters an ER "regulatory pool". The "regulatory pool" is the pool of ER cholesterol that is free to interact with SREBP-2 to downregulate cholesterol synthesis and uptake. By contrast, in the shunt pathway, LDL-derived cholesterol does not appear to enter the "regulatory pool". In 1991, it was shown that LDL particles are arrested in a perinuclear region of the cell, while intestinally-derived mouse β-VLDL—similar to human CR—remains in the periphery[80]. The intracellular separation of these lipoprotein particles suggested that there exist distinct processes governing their metabolism. Indeed, primary hamster hepatocytes treated with CR exhibited a significant decrease in both LDLR and HMGCR transcripts—and effectively reduced *de novo* cholesterol biosynthesis—while LDL treatment had no significant effect on these parameters [78]. Other lipoprotein species that exert a similar regulatory effect in this cell type include chylomicrons and β-VLDL[78]. The regulatory effect of CRs is consistent with early studies demonstrating the effect of dietary cholesterol on reducing HMGCR activity and inhibiting *de novo* cholesterol synthesis in liver slices from dogs and rat microsomes (outlined in [81]). To exert this regulatory function, dietary cholesterol must remain within the hepatocyte and not be secreted as VLDL.

Another indication that CR-cholesterol is processed differently from LDL-cholesterol is the receptor responsible for their hepatic uptake. CR treatment was shown to induce the LDLR-related protein 1 (LRP1) receptor via insulin [82]. This suggests that LRP1 is responsible for the

postprandial clearance of lipoproteins [82]. LRP1 is also an important receptor for the uptake of apoE containing lipoproteins. CRs may acquire apoE prior to reaching the liver parenchyma. One documented adaptor protein of the LRP1 is the engulfment adaptor protein (GULP), although these studies were carried out in non-hepatic cell lines [83]. Influx of dietary cholesterol in the hepatocyte through CR uptake enters a "regulatory pool" to control intracellular cholesterol homeostasis. A model illustrating the differences in LDL and CR metabolism within the hepatocyte based on our current knowledge is shown in **Figure 3**.

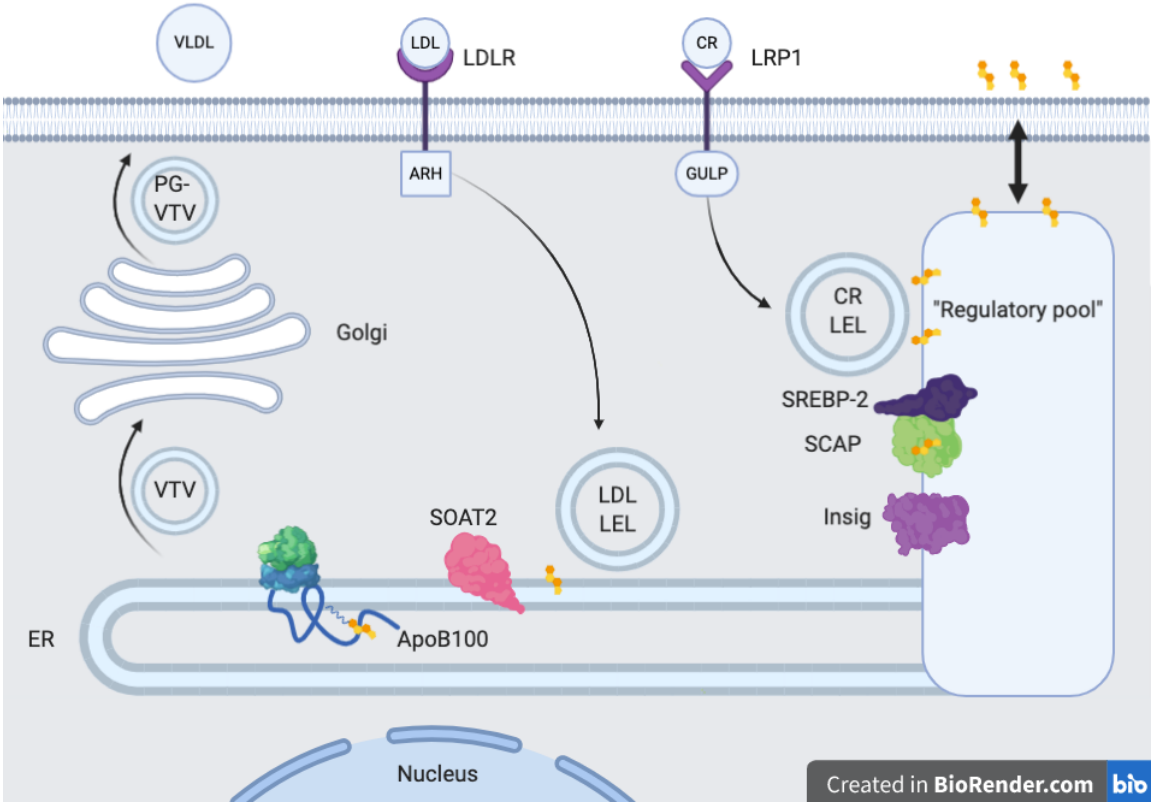


Figure 3. Model of separate channels of LDL and CR metabolism in the hepatocyte. Extracellular LDL is taken up by the hepatocyte upon interacting with the LDLR. The LDLR associated adaptor protein, ARH, aids in endocytosis of LDL. Cholesteryl ester (CE) harbored within the LDL-containing late endosome/lysosome is de-esterified and released to a region of the ER where it is immediately esterified and packaged with apoB100 to be secreted as apoB. As such, LDL-derived cholesterol never interacts with the hepatocyte ER "regulatory pool". Extracellular CR is taken up by endocytosis upon binding to a separate receptor, the LRP1, which is bound to its adaptor protein GULP. The cholesterol derived from CR ends up in the ER "regulatory pool" where it is free to interact with SCAP and SREBP-2 to decrease cholesterogenic activity in the cell. This "regulatory pool" is also in equilibrium with the plasma membrane. Created with BioRender.com.

1.6.3 Hepatic response to HDL: the biliary pathway

The hepatic uptake of HDL results in the transcytosis of this cholesterol to the apical membrane where it is destined to be secreted into the bile [84]. Two types of receptors that mediate the uptake of HDL-derived cholesterol include the SR-BI and ecto-F1-ATPase both expressed on the basolateral membrane. The SR-BI mediates the selective uptake of HDL-derived CE. This CE is de-esterified and the FC is trafficked to the apical membrane for secretion into the bile [84 85]. Ecto-F1-ATPase simply binds HDL then activates a separate receptor called P2Y13 [86]. This activation results in the endocytosis of the HDL particle and its migration across the cell. Once the endosome reaches the apical membrane, ABCG5/8 promote the efflux of cholesterol to the bile. Although little is known about this transcytosis pathway, it is well-accepted that this channel works independently from the uptake of other lipoprotein species.

1.7 Significance of study and hypothesis

Much of what is known and well-accepted about cellular cholesterol homeostasis today is based on the model outlined by Brown and Goldstein—a model which was developed in the fibroblast. However, it appears that in the hepatocyte, LDL, CR, and HDL particles appear to undergo separate metabolic processing. Strikingly, both LDL and CR-derived cholesterol enter the ER, yet the regulatory response is drastically different between them. The shunt pathway posits that the cholesterol derived from LDL is immediately esterified to be packaged within nascent VLDL particles, thereby shunting it away from accessing the ER "regulatory pool".

Herein, we aim to understand the intracellular fate of cholesterol from different lipoprotein sources within the human hepatocyte through immunofluorescence (IF), and through radiolabeling experiments. We hypothesize that there exist multiple specific channels of lipoprotein transport within the hepatocyte that implicate the compartmentalization of the ER for

the processing of LDL and CR-derived cholesterol. Specifically, SOAT2 is localized to a region of the ER whereby the docking of LDL-containing endosomes is accompanied with cholesterol esterification and VLDL assembly. This study bolsters the hypothesis that LDL-derived cholesterol is the preferential substrate for SOAT2 by the use of a specific SOAT2 inhibitor. Moreover, we underscore the dual role of SOAT2 in 1) controlling VLDL secretion, and 2) shunting LDL-cholesterol away from the "regulatory pool". In the short term at least, the egress of CR-derived cholesterol from the hepatocyte within VLDL is minimal when compared to LDL. Instead, we find that CR-cholesterol rapidly enters the plasma membrane and equilibrates with the ER "regulatory pool" to control cellular cholesterol homeostasis. Taken together, these findings demonstrate how human hepatocytes utilize SOAT2 to separate feedback regulation of intracellular cholesterol homeostasis from that of systemic cholesterol homeostasis.

2. Materials & Methods

2.1 Antibodies

Antibody	Company, catalog number	IF dilution	WB dilution	IP dilution
ApoB	EMD-Millipore, 178467	N/A	1:2000	1:200
ApoB	Novus Biologicals, NBP2-38608	1:500	N/A	N/A
ORP1L	Abcam, ab113530	1:500	N/A	N/A
RILP	Abcam, ab140188	1:500	N/A	N/A
SOAT2	Santa Cruz, sc-69837	1:100	N/A	N/A
SREBP2	Santa Cruz, sc-5603	1:100	N/A	N/A

2.2 Cell Culture

IHH cells were cultured in Dulbecco's modified eagle medium (DMEM) (Wisent, 319-005-CL) supplemented with 10% heat-inactivated FBS (Wisent, 080-150) and 1% penicillin-streptomycin (Wisent, 450-200-EL). Cells were grown in a humidified incubator set to 37°C and 5% CO_2.

2.3 Lipoprotein Preparation

2.3.1 Chylomicron Isolation

Healthy human blood was drawn approximately 90 minutes after consuming a fatty meal, and the serum was immediately isolated by spinning whole blood at *2000g* for 15 minutes. Serum was transferred to Beckman Coulter ultracentrifugation (UTC) tubes and spun at 48000rpm for 15 minutes in a Type 70.1 Ti rotor. The top white layer of chylomicrons was collected.

2.3.2 Chylomicron Remnant Preparation

Chylomicrons were treated with 10 µg of reconstituted hepatic lipase (Creative BioMart, LIPC-268H) and incubated at 37°C for 5 hours with inversion every hour. The resulting chylomicrons were treated with 2 mg of lyophilized ApoE3 (courtesy of the lab of Dr. Paul Weers, California State University Long Beach) and incubated overnight at room temperature. The resulting CRs were isolated by spinning at 48000 rpm for 90 minutes in a Type 70.1 Ti rotor by UTC. Finally, CR was dialyzed against 4 L of 0.9% NaCl for 30 minutes, 1 hour, 4 hours, followed by 16 hours. Lipoprotein mass was determined by a modified Lowry protein assay. Chylomicron remnants were aliquoted and stored at 4°C until use.

2.3.3 LDL Isolation

The density of chylomicron-deficient serum was changed from 1.006 g/mL to 1.030 g/mL with KBr and spun at 48000rpm for 21 hours in a Type 70.1 Ti rotor by UTC to remove VLDL and IDL. The top lipoproteins were removed, and the density was changed again to 1.063 g/mL. The resulting solution was spun at 48000 rpm for 21 hours in a Type 70.1 Ti rotor by UTC—the top yellow layer consisted of LDL. To remove any remaining KBr, LDL was dialyzed against 4 L of

0.9% NaCl for 30 minutes, 1 hour, 4 hours, followed by 16 hours. Lipoprotein mass was determined by a modified Lowry protein assay. LDL was aliquoted and stored at 4°C until use.

2.3.4 Lipoprotein-deficient serum isolation

The density of serum was changed to 1.21 g/mL with KBr and spun at 48000rpm for 21 hours in a Type 70.1 Ti rotor by UTC to remove all HDL species. The top layer of HDL was removed, and the bottom layer consisting of total LPDS was collected. LPDS was dialyzed against 4 L of 0.9% NaCl for 30 minutes, 1 hour, 4 hours, followed by 16 hours. LPDS was aliquoted and stored at -20°C until use.

2.4 Microinjection for Fluorescent/Radioactive Lipoprotein Labelling

Lipoproteins were labelled by microinjection. Labelling media was prepared with DMEM, 1% penicillin-streptomycin, and either 50 µg/mL LDL or 12 µg/mL CR. These concentrations were determined elsewhere as they allow uptake of equal loading of total mass of cholesterol to the cells[78]. Using a syringe, DiO (Invitrogen, D275), ^{3}H-cholesterol (Perkin-Elmer, NET139005MC), or ^{3}H-cholesteryl oleate (Perkin-Elmer, NET746L250UC) was rapidly injected in the media at a final concentration of 25 µg/mL DiO or 5 µCi/mL ^{3}H-labelled species. Media was incubated at 37°C for 1 hour with frequent inversion to allow incorporation of labels in lipoprotein particles.

2.5 Immunofluorescence

Cells were fixed with 3% PFA at room temperature for 20 minutes and washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS). Coverslips were then incubated with 50 mM NH_4Cl for 10 minutes, washed with CMF-PBS, then incubated in a blocking solution (0.1% saponin, 0.2% FSG diluted in CMF-PBS) for 30 minutes. After three more washes in blocking

solution, cells were incubated with primary antibody diluted in blocking solution for 1 hour at room temperature. Coverslips were again washed three times, then incubated with fluorescent secondary antibodies at 1:1000. Lastly, coverslips were washed, dipped in milliQ H_2O, then mounted on glass microscope slides with Mowiol mounting media (25% glycerol, 10% Moviol 4-88, 1% n-propyl gallate, 0.2 M tris HCl, pH 8.5) for 1 hour at room temperature. Images were acquired using an Olympus IX81 inverted microscope (Olympus Corporation, Tokyo, Japan) equipped with a 60x oil immersion PlanApo N 1.42 objective and a QuantEM:512SC electron-multiplying CCD (EMCCD) camera operated by the MetaMorph software. Images were then processed in ImageJ/FIJI, by adding color to different emission channels, creating composite images, and adjusting contrast and brightness.

2.6 FIJI macro for SREBP-2 localization

Images for both DAPI and SREBP2 channels were opened on ImageJ/FIJI, and the DAPI channel image was selected. The following macro was then run on the images:

```
run("Images to Stack", "name=Stack title=[]");
run("Stack to Images");
run("Duplicate…", " ");
selectWindow("Stack-0001");
setAutoThreshold("Default dark");
//run("Threshold…");
setAutoThreshold("Default dark");
run("ROI Manager…");
run("Create Selection");
roiManager("Add");
selectWindow("Stack-0002");
roiManager("Select", 0);
roiManager("Measure");
selectWindow("Stack-0002-1");
run("Gaussian Blur…", "sigma=2");
setAutoThreshold("Default dark");
//run("Threshold…");
setAutoThreshold("Default dark");
run("Create Selection");
```

```
roiManager("Add");
selectWindow("Stack-0002");
roiManager("Select", 1);
setKeyDown("alt");
roiManager("Select",0);
setKeyDown("none");
roiManager("Add");
roiManager("Select", 2);
roiManager("Measure");
```

The first measurement acquired corresponds to nuclear intensity of SREBP2, while the second measurement corresponds to the cytoplasmic intensity of SREBP2.

2.7 Live cell imaging of DiO-LDL/CR

To prepare cells for live cell imaging, IHH grown on glass-bottom dishes were incubated in serum-free media supplemented with DiO-loaded LDL (50 µg/mL) or CR (12 µg/mL) at 4°C for 1 hour to allow binding of lipoproteins to the cell surface. Cells were washed, and media was changed for live cell imaging media prepared according to ThermoFisher (140mM NaCl, 2.5mM KCl, 1.8mM $CaCl_2$, 1.0mM $MgCl_2$, 20mM HEPES, pH 7.4, mOsm=300). LysoTracker Red DND-99 (Invitrogen, L7528) was added to media at 1:1000, and lipoprotein uptake was visualized at room temperature under the microscope. IHH were immediately fixed with 3% PFA when LDL or CR movement stopped in preparation for immunofluorescence.

2.8 *De novo* cholesterol biosynthesis assay

IHH were incubated with unlabeled LDL (50 µg/mL) or CR (12 µg/mL) for 24 hours in the presence of 5 µCi ^{3}H-mevalonolactone (Perkin-Elmer, NET602000MC). Lipids were extracted using hexane:isopropanol (3:2, v/v), dried under nitrogen gas, then separated by TLC. The free cholesterol (FC) and cholesteryl ester (CE) band from each sample were separately subjected to scintillation counting, and values were normalized to cellular protein.

2.9 Cholesteryl Ester Secretion Assay

Lipoproteins were labelled with ^{3}H-cholesterol by microinjection explained above. IHH were starved for 1 hour with 7.5 µM pyripyropene A (PPPA) (Cayman Chemical, 11896) or DMSO (0.001%), then incubated with pulse media (DMEM with 10% LPDS, 1% P/S, 50 µg/mL LDL or 12 µg/mL CR, 5 µCi ^{3}H-cholesterol) for 24 hours again in the presence of PPPA or DMSO. Cells were washed then incubated for 8 hours in chase media (DMEM with 0.2 mM DTNB—an LCAT inhibitor—and either PPPA or DMSO). Lipids were extracted from chase media by Folch method. The Folch solution (2:1, v/v, chloroform:methanol) was added directly to harvested media, vortexed, then shaken vigorously overnight at 4°C. Cold 0.9% NaCl was then added to each sample and spun at 3000 rpm for 10 minutes. The whole chloroform (organic) phase was dried under nitrogen gas. Lipid species were separated by TLC, and the CE was subjected to scintillation counting. Counts of secreted CE were expressed as %*100 of total cellular radioactivity.

2.10 ApoB Immunoprecipitation

Pulse and chase were identical to the CE secretion assay experiment with non-radiolabelled lipoproteins. To 400 µL of harvested media was added 400 µL lysis buffer (6.1 mM Na_2HPO_4, 4.5 mM NaH_2PO_4, 88.4 mM NaCl, 36.58 mM LiCl, 36.6 mM SDS, 24.1 mM sodium deoxycholate, 1% Triton X-100, 4 µL/mL PMSF, 40 µL/mL protease inhibitor, pH 7.4), 152 µL milliQ-H_2O, 240 µL 5X NET buffer w/o SDS (0.75 M NaCl, 25 mM EDTA, 250 mM Tris pH 7.5, 5% Triton X-100), 8 µL complete protease inhibitors (1 tablet dissolved in 1.5 mL water) (Roche, 11836153001), 5 µL antibody (EMD Millipore, 178467-1ML), and 50 µL protein G sepharose beads (GE Healthcare, 17-0618-01). The resulting mixture was allowed to rotate

overnight at 4°C. Lastly, bound beads were washed three times NET buffer 1X (0.15M NaCl, 5mM EDTA, 50 mM Tris pH 7.5, 1% Triton X-100, 1% SDS).

2.11 ApoB western blot

ApoB-bound beads were boiled in 10 µL SDS loading buffer for 10 minutes at 95°C, and supernatants were resolved on a 6% polyacrylamide gel at 80 V for 30 minutes, followed by 150 V until the 100 kDa marker ran off the gel. Protein was transferred to a PVDF membrane (EMD Millipore, IPVH00010) using a discontinuous buffer system in the semi-dry Trans-Blot Turbo blotting system at 0.3 A constant for 45 minutes. The membrane was then blocked in 5% BSA (Sigma, A6003) dissolved in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 hour, followed by a primary antibody solution dissolved in 5% BSA incubated overnight at 4°C. The membrane was washed, then incubated with an anti-goat HRP-conjugated secondary antibody for 1 hour at room temperature. Lastly, the membrane was thoroughly washed, developed in chemiluminescent substrate (Thermo Scientific, 34580), and bands were visualized using ImageQuant LAS 4000. Arbitrary units of band intensity were measured using the ImageJ/FIJI program. Values were reported as mean intensity and normalized to cell protein.

2.12 Protein assay

Cell dishes placed on ice were washed with PBS then incubated in RIPA lysis buffer (1X RIPA, 1 mM $NaVO_3$, 50 mM NaF) supplemented with 50X protease inhibitor solution (1 tablet in 1.5 mL water) for 45 minutes. The 10X RIPA stock was prepared as follows: 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA. Cells were scraped to collected lysates, which were centrifuged at 13 000 rpm for 10 minutes at 4°C.

The protein concentration in the supernatant was tested using the *DC* protein assay (Bio-Rad, 5000111) in triplicates according to the manufacturer's instructions.

2.13 Thin Layer Chromatography (TLC)

The TLC chamber was prepared by enclosing the solvent mixture consisting of heptane/ethyl ether/methanol/acetic acid (80:30:3:1.5, v/v/v/v) with a Whatman paper, allowed to incubate for 30 minutes to ensure proper vapour equilibration. Lipids dried under nitrogen gas were re-dissolved in 100 µL chloroform, then carefully applied to a silica gel TLC plate (Analtech, 34101-00). A FC and TG standard were also applied to the plate properly identify lipid species. Once the plate dried, it was incubated in the TLC chamber until the solvent front of the mobile phase was 1 cm from the top of the silica gel plate. The plate was then dried once more then transferred to an iodine gas chamber to visualize the lipid species. Individual lipid bands were scraped into separate liquid scintillation vials, to which was added 5 mL Eco-Lite (ICN Biomedicals, IC88247501). The counts per minute for β (CPMA) was determined for each sample by counting emission for 5 minutes with a beta counter (Perkin Elmer).

2.14 Plasma membrane cholesterol extraction

^{3}H-Cholesteryl oleate was incorporated into 50 µg/mL LDL or 12 ug/mL CR by microinjection at a concentration of 5 µCi/mL, as described in Section **2.4**. IHH cells were treated with the appropriate lipoprotein media and incubated at 37°C for up to 6 hours. Throughout the 6 hour time course of the experiment, plasma membrane cholesterol was extracted at different time points. To do so, at the timepoint cells were transferred to 4°C for 30 minutes and then incubated with 10 mM methyl-β-cyclodextrin (MβCD) for 10 minutes. Supernatants were collected and subjected to scintillation counting. Values were reported as cpm normalized to cellular protein.

2.15 Statistical Analysis

Statistically significant differences were determined by a student's t-test in RStudio. Statistical significance was determined at a p-value < 0.05.

RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA

URL *http://www.rstudio.com/*

3. Results

3.1. The location of endocytosed LDL and CR differ in the hepatocyte

The metabolism of endocytosed lipoproteins likely begins with their delivery to specialized compartments within the hepatocyte responsible for their differential processing. Based on this model, lipoprotein particles which are being actively degraded are found in a late endosomal/lysosomal compartment and are localized to a region of the cell where their cholesterol content is to be delivered. One group demonstrated that in mouse macrophages, lysosomes containing intestinally derived β-VLDL—which are similar to human CRs—are delayed in the periphery of the cell while those containing LDL are perinuclear in localization [80]. Despite these findings, no one has yet tracked the uptake and intracellular migration of the LDL and CR species in human hepatocytes.

To investigate these pathways, the real-time movement of lipoprotein particles within IHH cells was tracked by live-cell imaging. LDL and CR were labelled with the lipophilic fluorescent dye DiO by microinjection then added to IHH cells at 4 °C to allow lipoprotein binding to the cell surface. After a 1 hr incubation, cells were washed with live-cell imaging media and moved to the microscope stage at room temperature to restart cellular functions. A Lysotracker Red dye was also supplemented in the live-cell imaging media in order to visualize acidic compartments within the cell. The endocytosis and movement of lipoprotein particles within individual cells were tracked until they stopped moving, at which point a picture was taken **(Figure 4**). It was observed that DiO-CR particles were distributed peripherally throughout the entire hepatocyte and co-localized with acidic compartments of the cell. In contrast, DiO-LDL were localized to a juxtanuclear region of the cell and also co-localized with acidic compartments. Qualitatively,

these results suggest that the cholesterol content of CR particles is processed peripherally in the human hepatocyte, while that of LDL particles is localized in close proximity to the nucleus.

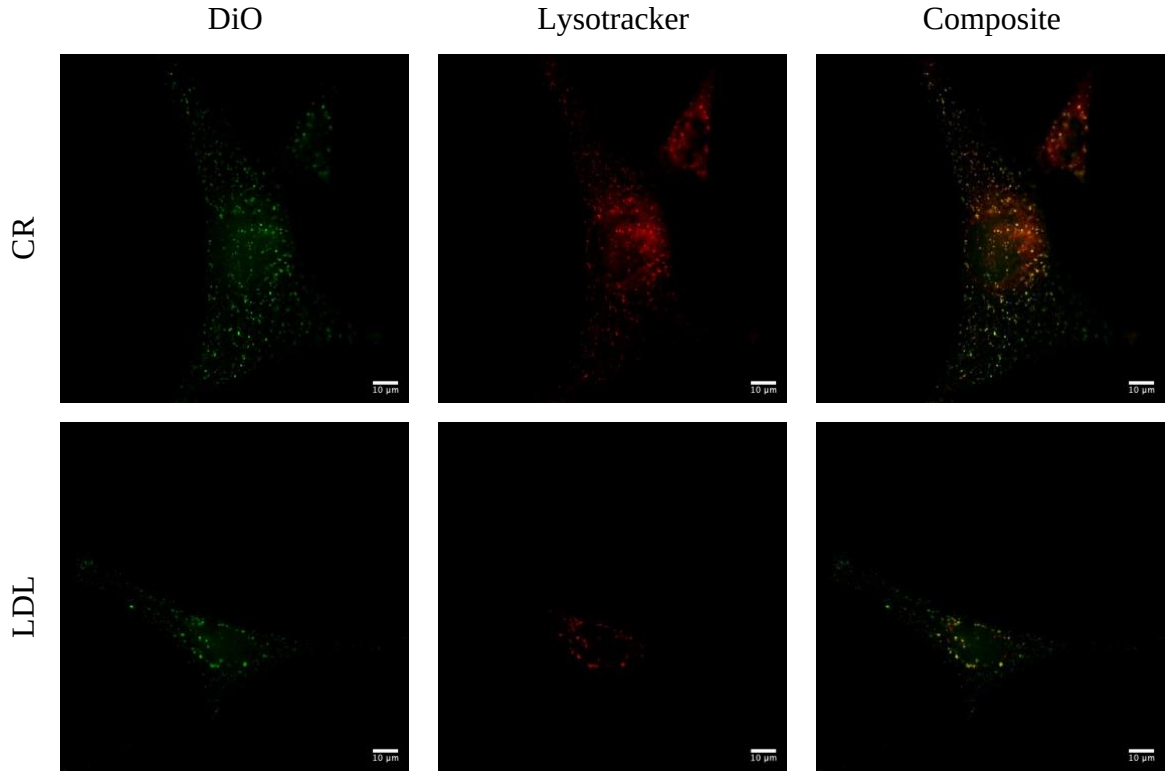


Figure 4. Endocytosed LDL and CR are differentially distributed in the hepatocyte. LDL and CR were isolated from human serum then loaded with the lipophilic tracer DiO by microinjection. IHH cells were fed with DiO-loaded lipoproteins for 1 hr on ice, washed, then replenished with live cell imaging media supplemented with Lysotracker at 1:1000. Cells were allowed to readjust to room temperature under the microscope to monitor lipoprotein uptake, and pictures were taken after 30 minutes. Images were processed in ImageJ/FIJI. Scale bars represent a length of 10 µm.

3.2. SOAT2 adopts a juxtanuclear distribution in the hepatocyte

The shunt pathway model posits that LDL-derived cholesterol is quickly esterified upon entry to the ER, thereby preventing its interaction with the "regulatory pool". This model would agree with the observations of **Figure 4**, in that the immediate processing of LDL-derived cholesterol could occur in a specialized juxtanuclear region of the cell, distinct from CR-derived cholesterol. In 2013, our group demonstrated that the cholesterol esterifying enzyme SOAT2 is

significantly upregulated upon treating primary hamster hepatocytes with LDL relative to CR treatment[78]. We therefore proposed that this enzyme is responsible for esterifying LDL-derived cholesterol and therefore also adopts a juxtanuclear distribution in the hepatocyte.

To test this hypothesis, the intracellular localization of SOAT2 was observed in human hepatocytes by immunofluorescence. IHH cells grown on coverslips were fixed, then stained for SOAT2 and the nucleus using the DAPI stain (**Appendix 1**). It was observed that SOAT2 adopts a punctate and juxtanuclear distribution in IHH cells. These results suggest that SOAT2 likely clusters in specific domains of the ER in proximity to the nucleus to exert its function.

In order to test whether LDL is delivered to SOAT2 domains, the uptake and migration of DiO-LDL was observed as previously described. Once DiO-LDL stopped moving, cells were fixed and stained for SOAT2 and the nucleus via the DAPI stain (**Figure 5A**). DiO-LDL was observed to partially co-localize with punctate SOAT2 adjacent to the nucleus, demonstrated by yellow structures in the composite image. Given the previous result in **Figure 4**, LDL particles are likely degraded in a juxtanuclear region of the cell, where their cholesterol content is then in close proximity to SOAT2 for esterification.

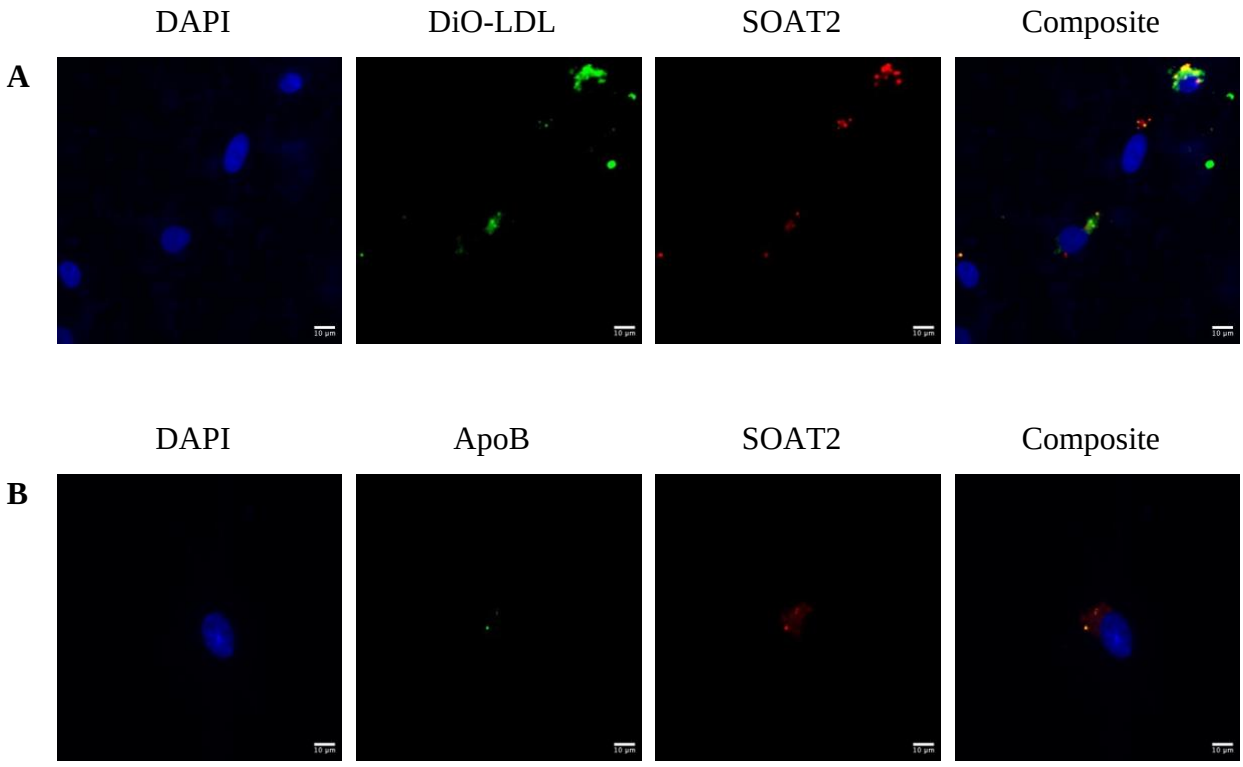


Figure 5. LDL is delivered to sites of VLDL assembly (SOVAs) in IHH cells.
(A) IHH were incubated with DiO-LDL (green) and observed under microscope at room temperature. Cells were fixed when DiO-LDL particle movement arrested, then stained for SOAT2 (red). Co-localization of DiO-LDL and SOAT2 is indicated by yellow structures in the composite image. **(B)** IHH were fixed and stained for both apoB (green) and SOAT2 (red). Co-localization of apoB and SOAT2 is indicated by yellow structures in the composite image. Images were processed in ImageJ/FIJI. Scale bars represent a length of 10 µm.

Previous work has shown that SOAT2 is the principal enzyme responsible for the generation of CE associated with secreted apoB-containing lipoprotein particles[37]. Moreover, there is increasing evidence showing that nascent apoB peptide must be associated with a neutral lipid species to prevent its degradative fate and promote its secretion [32 87 88]. Given this information, the shunt model posits that SOAT2 forms specialized domains whereby esterification of LDL-derived cholesterol is coupled with apoB synthesis and VLDL assembly. To determine whether the localization of SOAT2 and apoB reflect this, immunofluorescence was used to determine the co-localization of these proteins. An apoB antibody reactive to human but not bovine apoB was used to ensure that cell-derived apoB was detected. It was observed that

apoB partially co-localized with SOAT2, indicated by yellow juxtanuclear structures **(Figure 5B**). All in all, **Figure 5** is suggestive of the existence of SOVAs, whereby the docking of LDL-containing late endosomes to the ER is coupled to VLDL assembly. In support of these observations, in a separate experiment it was found that DiO-LDL partially co-localized with the minus-end associated motor protein RILP, as well as the endosomal-ER cholesterol transfer protein ORP1L (**Appendix 2**). Taken together, the necessary components of a theoretical SOVA co-localize: LDL-LELs traffic to a juxtanuclear region of the hepatocyte, where its cholesterol content is delivered to the ER and quickly esterified by SOAT2 and coupled to apoB synthesis.

3.3. LDL stimulates VLDL secretion from the hepatocyte via SOAT2

One of the major outlets of cholesterol from the hepatocyte is through VLDL secretion. As mentioned earlier, SOAT2 esterifies FC and packages it with apoB for VLDL assembly and secretion. We show evidence suggesting that LDL-derived cholesterol is the substrate for SOAT2, while CR-cholesterol is not (**Figure 4** & **Figure 5**). In order to quantitatively measure this phenomenon, the proportion of secreted CE derived from either LDL or CR was measured by radiolabelling. IHH cells were treated with ^{3}H-cholesterol-loaded LDL or CR in the presence of DMSO or a SOAT2 inhibitor (7.5μM PPPA) for 24 hours. Cells were washed, then incubated with serum-free media in the presence of DMSO or PPPA for 8 hours. Lipids were extracted from the media by a Folch extraction, then run on a TLC to separate the different lipid species. The band corresponding to CE from each sample was then subjected to scintillation counting in order to determine the amount of secreted CE that was derived from LDL or CR. The counts were finally normalized to total cellular radioactivity, as described in Sniderman et al. (2013) [78], and reported in **Figure 6**. Inhibition of SOAT2 by PPPA was shown to significantly reduce secretion of CE by 30% when IHH were challenged with LDL (yellow and gray bars, $p<0.001$),

but not CR (orange and blue bars, p>0.05). As a control, LDL-derived cholesterol was preferentially esterified and secreted over CR-cholesterol by over 250% (yellow and orange bars, p<0.0001). The inhibition of SOAT2 did not appear to completely abolish LDL-cholesterol esterification as there was still significantly more esterification and secretion of LDL-cholesterol over CR-cholesterol in the presence of PPPA (gray and blue bars, p<0.0001). These results further highlight that SOAT2-mediated esterification of LDL-cholesterol controls the secretion of CE from the hepatocyte.

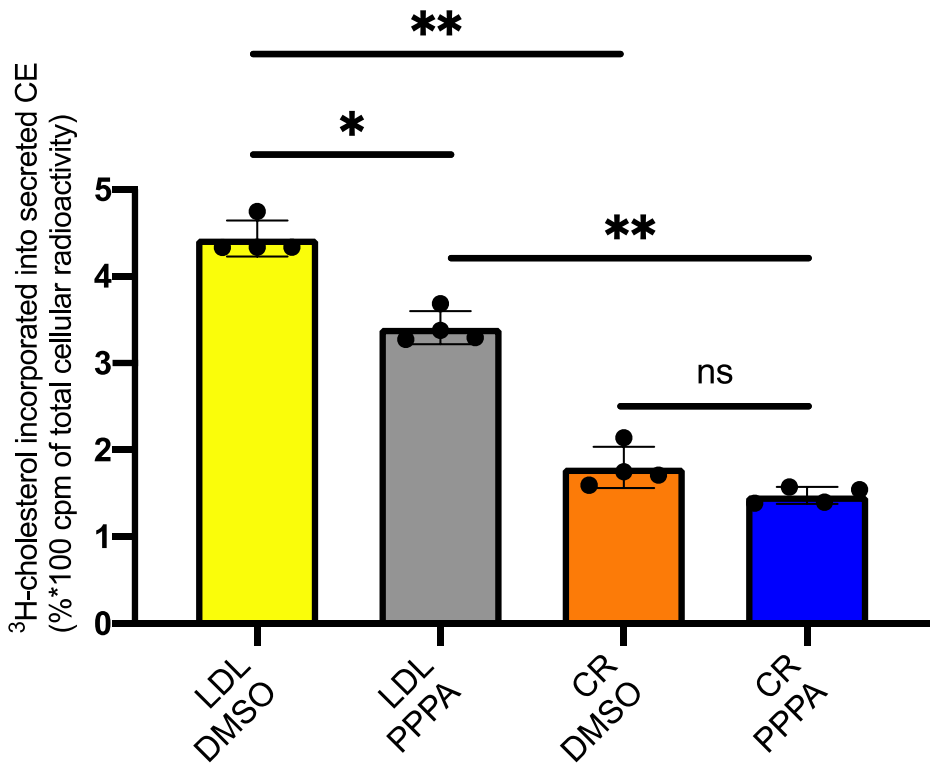


Figure 6. LDL-derived cholesterol is selectively esterified by SOAT2 and secreted. IHH cells were incubated with LDL (50 µg/mL) or CR (12 µg/mL) labelled with ^{3}H-cholesterol (5 µCi) in the presence of DMSO (0.001%) or PPPA (7.5 µM) for 24 hours. Cells were washed, then incubated with serum-free media for 8 hours with DMSO or PPPA. Lipids were extracted from harvested media by Folch extraction (2:1, chloroform:methanol), and separated by TLC. The CE band from each sample was subjected to scintillation counting. Values represented as %*100 cpm of total cellular radioactivity. Mean values were calculated from n=4 replicates ± SD. Student's t-test was used to determine significance. Ns: not significant (p>0.05). * significant (p<0.001). ** very significant (p<0.0001).

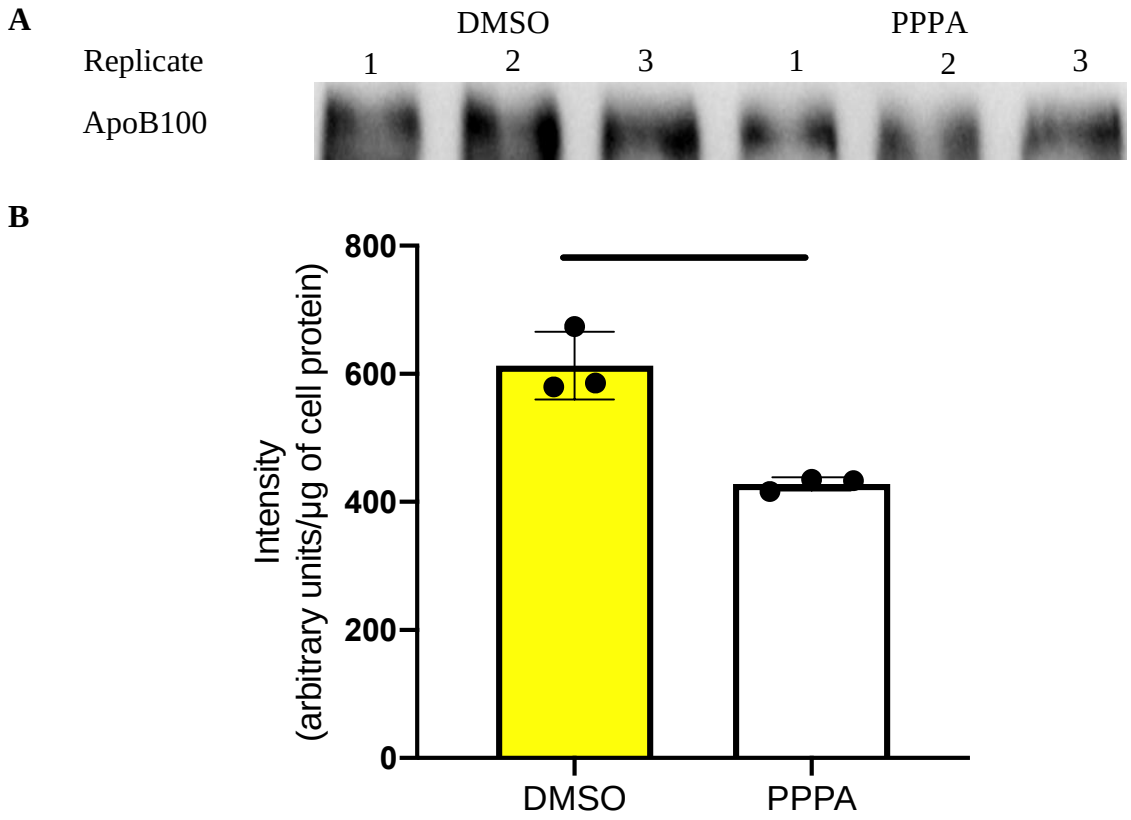


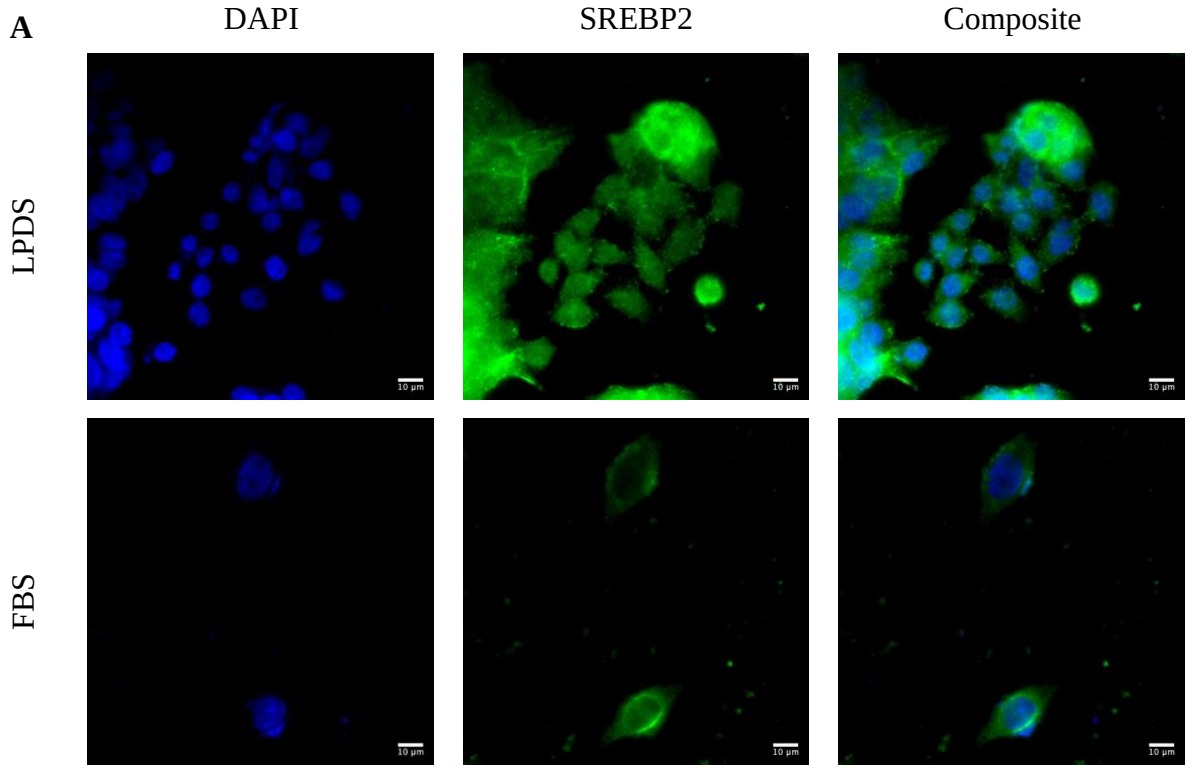
Figure 7. SOAT2 controls VLDL secretion in response to a hepatic LDL challenge. IHH cells were incubated with LDL (50 µg/mL) in the presence of DMSO (0.001%) or PPPA (7.5 µM) for 24 hours. Cells were washed, then incubated with serum-free media for 8 hours with DMSO or PPPA. ApoB was immunoprecipitated from harvested media, run on an SDS-PAGE, and **(A)** detected by immunoblot with an anti-apoB antibody. **(B)** Band intensity was measured in ImageJ/FIJI and normalized to cellular protein. Mean values were calculated from n=3 replicates ± SD. Student's t-test was used to determine significance.
* significant ($p<0.01$).

The only direct outlet of CE from hepatocytes is through VLDL secretion. To determine whether SOAT2 controls VLDL secretion or the CE content of individual VLDL particles, apoB particles secreted in the media were quantified by immunoprecipitation followed by immunoblotting. The quantity of apoB is a direct reflection of the number of VLDL particles [89]. IHH cells were treated with LDL in presence of PPPA or DMSO as described in the previous experiment. Media was harvested and apoB was selectively immunoprecipitated. The resulting precipitates were subjected to immunoblotting to quantify secreted apoB. In the absence of PPPA, apoB100 levels appeared slightly higher in each replicate than in the SOAT2 inhibitor

condition **(Figure 7A**). To quantify statistical differences, band intensity was determined in FIJI and normalized to total cellular protein. It was found that PPPA caused a 40% decrease in apoB levels when compared to non-treated hepatocytes ($p<0.01$) **(Figure 7B**). This decrease approximates the 30% decrease of CE when IHH are treated similarly with LDL (**Figure 6**, yellow and gray bars). Taken together, these results are suggestive that SOAT2 controls VLDL secretion when hepatocytes are challenged with LDL.

3.4. SOAT2 inhibition directs LDL-cholesterol to the "regulatory pool"

We next sought to confirm that SOAT2 is also required to shunt LDL-derived cholesterol away from the "regulatory pool". While a nuclear localization of SREPB2 indicates that there is little cholesterol in the regulatory pool, a cytoplasmic localization means that there is plenty. IHH cells were challenged with LDL or CR in the presence of DMSO or PPPA, and the localization of SREBP2 was determined by immunofluorescence (**Figure 8**).



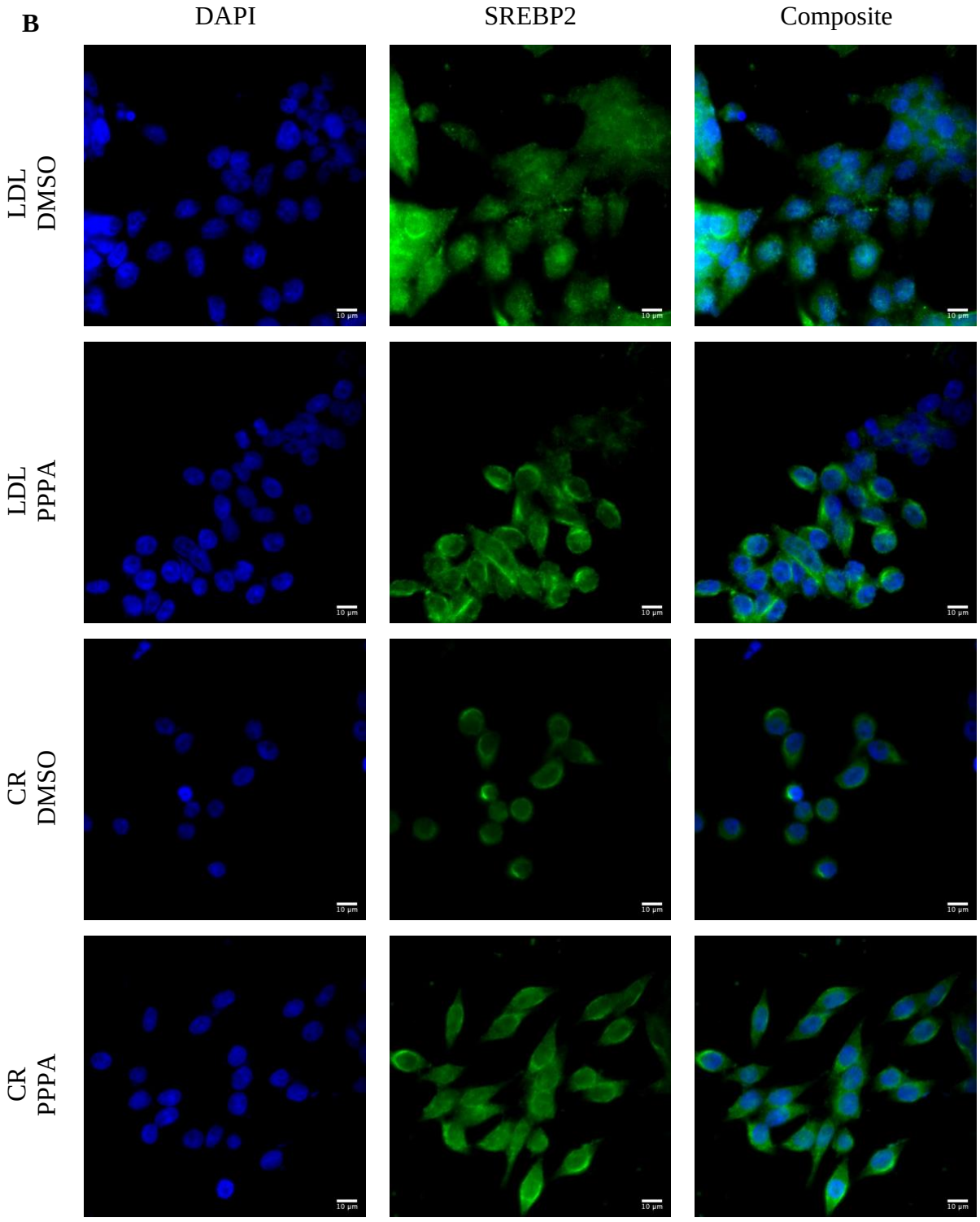


Figure 8. Nuclear SREBP2 is dependent on SOAT2 upon LDL treatment.
IHH cells were starved in serum-free media for 4 hours in the presence of DMSO or 7.5 µM PPPA, washed, then treated with **(A)** 10% FBS or 10% LPDS as a control, and **(B)** 50 µg/mL LDL or 12 µg/mL CR for another 4 hours with DMSO or PPPA. Coverslips were fixed with 3% PFA and stained for SREBP2 by immunofluorescence. Scale bar indicates a distance of 10 µm.

As expected, SREBP2 was present in the nucleus of serum-free—LPDS—treated hepatocytes, and remained primarily cytoplasmic in FBS-treated hepatocytes (**Figure 8A**). In response to LDL, SREPB2 was present in the nucleus (LDL DMSO, **Figure 8B**). However, SOAT2 inhibition by PPPA drove SREBP2 out of the nucleus in LDL-treated hepatocytes, as indicated by the green outline (LDL PPPA, **Figure 8B**). As another control, SREBP2 was always primarily cytoplasmic in CR-treated hepatocytes whether SOAT2 was inhibited or not (CR DMSO and CR PPPA, **Figure 8B**). In order to quantitatively compare these different conditions, the nuclear-to-cytoplasmic ratio of SREBP2 intensity was determined in ImageJ/FIJI. The application of a SOAT2 inhibitor was shown to significantly reduce the nuclear-to-cytoplasmic ratio of SREBP2 in LDL-treated hepatocytes (yellow and gray bars $p<0.01$, **Figure 9**). In CR-treated hepatocytes, no significant difference was observed in the nuclear-to-cytoplasmic ratio when PPPA was used. These results suggest that LDL-derived cholesterol is directed away from the "regulatory pool" unlike CR-cholesterol, and this shunt is dependent on SOAT2.

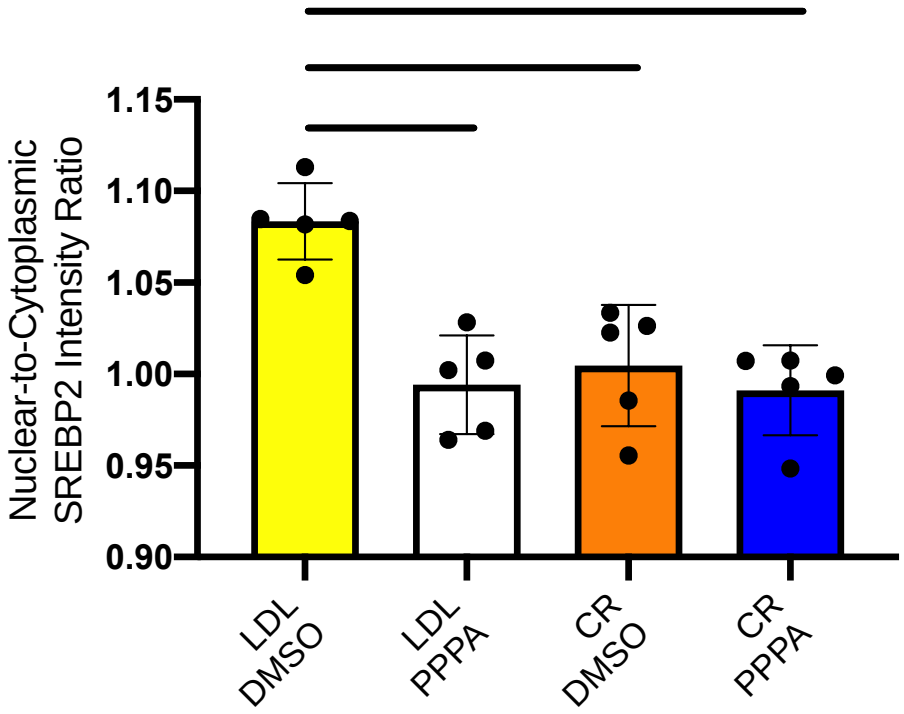


Figure 9. Nuclear-to-cytoplasmic ratio of SREBP2 intensity.
The intensity of SREBP2 fluorescence in the nucleus and cytoplasm of IHH cells was determined using the ImageJ/FIJI macro described in "Materials & Methods". The intensities from representative images of each condition were quantified, and the nuclear intensity divided by the cytoplasmic intensity. Values are plotted as a mean of n=5 images ± SD. Student's t-test was used to determine significance. * significant $p<0.01$.

Next, we sought to determine whether the observed localization of SREBP2 was artefactual or if it is truly functional. When SREBP2 senses low cholesterol, it enters the nucleus and acts as a transcription factor to upregulate HMGCR and the LDLR to increase cholesterol biosynthesis and uptake, respectively [21]. Therefore, the rate of cholesterol biosynthesis is a reflection of the amount of HMGCR, which is dependent on the nuclear localization of SREBP2 and low "regulatory pool" cholesterol. Given this information, the *de novo* biosynthesis of cholesterol was monitored when LDL-treated hepatocytes were presented with a SOAT2 inhibitor. IHH cells were incubated with 5 µCi ^{3}H-mevalonate—the substrate of HMGCR—50 µg/mL LDL, and incremental doses of PPPA (0 µM, 2.5 µM, 5.0 µM, 7.5µM) for 24 hours.

Lipids were extracted, then separated by TLC. The FC and CE bands were subjected to liquid scintillation, and counts were normalized to cellular protein (**Figure 10**).

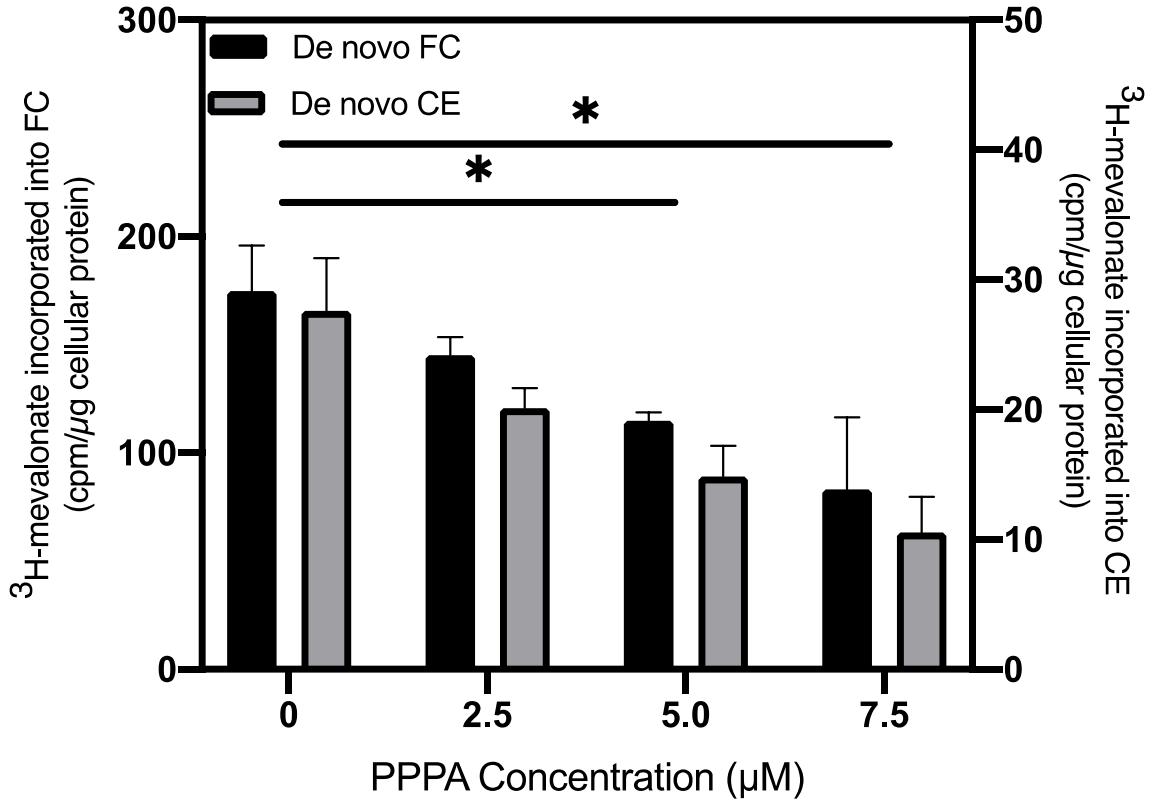


Figure 10. Cholesterol biosynthesis is blocked by SOAT2 inhibition of LDL-treated IHH. IHH cells were incubated in DMEM supplemented with 50 µg/mL LDL, 5 µCi/mL ^{3}H-mevalonate, and doses concentrations of PPPA for 24 hours. Lipids were extracted and separated by TLC. The FC and CE species for each condition were separately subjected to scintillation counting. Black bars indicate *de novo* FC synthesis and measured on the left vertical axis, while gray bars indicate *de novo* CE synthesis and measured on the right vertical axis. Values are reported as a mean of n=3 replicates ± SD. Student's t-test was used to determine significance. * significant $p<0.05$.

The relative amount of *de novo* FC biosynthesis achieved a significant decrease at concentrations of 5.0 µM (black bars, 0 µM compared to 5.0 µM PPPA p=0.03, **Figure 10**) and 7.5 µM (black bars, 0 µM compared to 7.5 µM PPPA p=0.02, **Figure 10**) when compared to non-inhibited cells. Therefore, PPPA dose-dependently reduced *de novo* cholesterol synthesis, suggesting that LDL-cholesterol is being directed to the regulatory pool. For further validation,

the subsequent esterification and synthesis of CE exhibited a similar trend (gray bars, **Figure 10**). Taken together, these results demonstrate that SOAT2 is the keystone of the shunt pathway—when it is non-functional both LDL-cholesterol and CR-cholesterol are regulatory.

3.5. CR-derived cholesterol is delivered to the hepatic plasma membrane

The fate of CR-derived cholesterol is to enter the "regulatory pool" of hepatocytes, but its exact intracellular itinerary remains unknown. According to **Figure 4**, CR-containing endosomes are degraded in the periphery of the cells, which suggests that its cholesterol content is entering the plasma membrane. To test this hypothesis, plasma membrane cholesterol was monitored from hepatocytes treated with radiolabelled LDL and CR by scintillation counting. IHH were incubated with equal masses of either LDL or CR, both loaded with ^{3}H-cholesteryl oleate (a form of CE). Plasma membrane FC was then extracted with MβCD at 4 °C at different time points and measured by scintillation counting (**Figure 11**). Because the radioactivity in FC and not CE is measured in this experiment, the lipoprotein particle must have been endocytosed and its CE de-esterified by CE hydrolases, which function only at acidic pH *[68]*.

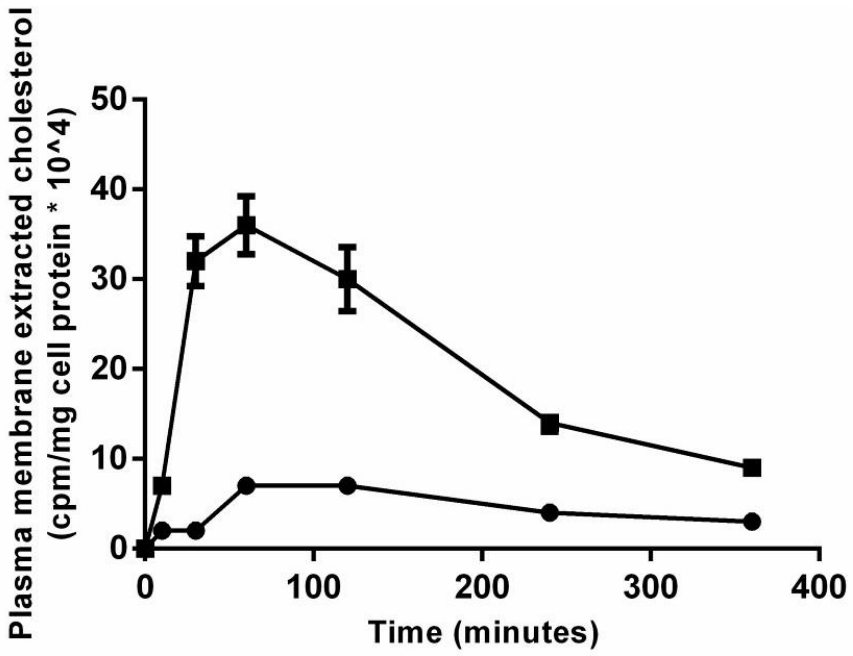


Figure 11. CR-derived cholesterol is delivered to the plasma membrane in hepatocytes. IHH cells were treated with 50 µg/mL LDL or 12 µg/mL CR loaded equally with 5 µC ^{3}H-cholesteryl oleate for up to 6 hours. Throughout the time course, plasma membrane cholesterol was extracted by transferring cells to 4°C, then incubated with 10 mM MβCD for 10 minutes. Supernatant were subjected to scintillation counting and recorded as cpm/mg of cell protein *10_4. SD error bars are shown for statistically significant data $p<0.05$. Square (■) data are from CR-treated hepatocytes, while circle (●) are from LDL-treated hepatocytes. Data obtained by Dr. Robert Scott Kiss.

After 1 hour of lipoprotein treatment, there was approximately 5-fold more CR-derived cholesterol at the plasma membrane than in LDL-treated hepatocytes. At approximately 4 hours after initial treatment, the difference in plasma membrane cholesterol content was no longer statistically significant. Indeed, the level of LDL-derived cholesterol at the plasma membrane never appeared to peak relative to CR-treated hepatocytes. These results suggest that CR-derived cholesterol is delivered to the plasma membrane and LDL-derived cholesterol is not.

Taken together, SOAT2 is the keystone of the shunt pathway, whereby LDL-derived cholesterol is selectively removed from the hepatocyte as secreted VLDL. Without this enzyme, the fate of LDL-derived cholesterol mimics CR-cholesterol and enters a “regulatory pool”. Furthermore, CR-cholesterol accumulates at the plasma membrane early after endocytosis.

4. Discussion and Conclusion

4.1. Discussion

The Brown and Goldstein model of cholesterol homeostasis, while thorough in explaining regulatory mechanisms in non-hepatic cells, is incomplete in the hepatocyte. Indeed, the mechanisms governing cholesterol homeostasis in the liver not only depend on the SREBP-2/LXR balance and lipid droplet storage, but also on biliary and VLDL secretion. These efflux channels are in turn regulated by the availability of cholesterol. Importantly, the secretion of VLDL influences the systemic cholesterol pool. The level of LDL-cholesterol in plasma is determined by the rate at which LDL-cholesterol is added to the LDL pool as well as the rate of LDL removal from the plasma. The rate of LDL cholesterol formation is determined to an important degree by the rate of secretion of VLDL cholesterol[90]. The data in this thesis add to the evidence that an important portion of the cholesterol secreted within VLDL apoB particles is derived from the cholesterol within LDL particles that were taken up by the hepatocyte. That is, at the level of the hepatocyte, the shunt pathway connects LDL influx with VLDL efflux. Influx of CR cholesterol, however, principally regulates cholesterol homeostasis within the hepatocyte. In this study, we use a combination of immunofluorescence and radiolabeling techniques to demonstrate that in the human hepatocyte there exist separate channels responsible for the metabolism of LDL and CR-derived cholesterol. These specific channels point to specialized compartmentalization of the ER with regard to cholesterol homeostasis.

We report that endocytosed LDL and CR particles are catabolized in different regions of the hepatocyte. Treatment of hepatocytes with fluorescently labelled lipoproteins demonstrated that LELs containing LDL are identified in a juxtanuclear region of the cell 30 minutes after endocytosis, while CR-containing LELs are distributed peripherally in the hepatocyte (**Figure 4**).

Our laboratory previously discovered that in primary hamster hepatocytes simultaneously treated with DiO-CR and DiD-LDL, "the vast majority of LDL and CR particles take separate endocytic routes" after 15 minutes [78]. Our findings corroborate studies in mice, where 20 minutes after injection of BODIPY-cholesteryl oleate loaded chylomicrons, remnants were evenly distributed in hepatocytes of liver sections [91]. Reports of LDL accumulation in juxtanuclear regions of the cell have been demonstrated multiple times, but in non-hepatic cell lines [80 92 93 94]. Although we observe a similar distribution of CR and LDL in the human hepatocyte, simultaneous loading of DiD-CR and DiO-LDL could demonstrate that they follow separate endocytic pathways. Moreover, incorporation of fluorescently tagged CE in lipoproteins would allow the tracking of the exact location of hydrolysis and be a more specific indicator of lipid metabolism. To determine the candidate proteins involved in the differential trafficking on LDL and CR endosomes, we could employ BioID of their receptor's adaptor protein, ARH and GULP, respectively. Shotgun proteomics of biotinylated proteins would allow the identification of potential candidates involved in the early stages of metabolism. Nevertheless, the pathways outlined in this report agree with the biochemical evidence of LDL and CR-specific cholesterol.

In the human hepatocyte, LDL catabolism occurs in proximity to juxtanuclear SOAT2 puncta required for VLDL secretion. In the SOVA model, the docking of LDL-containing LELs results in immediate cholesterol esterification, VLDL assembly, and VTV exit. One advantage of using IHH cells in localization studies is that they are polarized and therefore more a more reliable cell model [95]. We show that LDL-partially co-localized with RILP and ORP1L, indicating that the intracellular transport of endocytosed LDL is minus-end directed in the hepatocyte (**Appendix 1**). The presence of RILP indicates that LDL is within a LEL. Moreover, co-localization of RILP and ORP1L indicates that ORP1L is in a closed conformation, and

therefore the cholesterol content within the LEL is high, as expected for an LDL endosome. Based on these results, ORP1L is a likely candidate for the endosomal-ER transfer of cholesterol at the SOVA in the hepatocyte. It could be that in the hepatocyte, ORP1L is required to deliver cholesterol to both the regulatory pool and the juxtanuclear SOVAs. It would be interesting to see if ER protrudin, an indicator of plus-end directed movement of LELs, is a marker of the ER regulatory pool [72]. Other candidates for LEL-to-ER transfer of cholesterol include oxysterol-binding protein-related protein 5 (ORP5) and ORP6. ORP5 is found at many membrane contact sites of the ER and is shown to interact with NPC1 at the limiting membrane of the LEL [96]. On the other hand, ORP6 is likely localized to the early endosome and mediates transfer of cholesterol with the ER [97]. More recently, evidence has emerged demonstrating that NPC1 tethers LELs to the ER through interactions with Gramd1b and this promotes lysosomal cholesterol egress [98]. This finding does not exclude the role of ORP1L and in fact they may both be crucial in mediating LEL-ER tethering and LEL cholesterol egress [98]. We also find that LDL partially co-localizes with SOAT2, which overlaps with cell-synthesized apoB (**Figure 5**). This data provides evidence that the docking, esterification, and VLDL assembly likely occur in succession. Importantly, the punctate SOAT2 fluorescence is also an indicator that this enzyme clusters in the cell to form a domain composed of many enzymes.

In 1997, Cartwright et al. discovered that in rabbit hepatocytes, small quantities of TG, FC, and CE co-localize with apoB in the rough ER, while the bulk of VLDL-associated lipids were found in the smooth ER, suggesting a physical separation for a successive VLDL assembly [99]. SOAT2 may be involved in synthesizing CE in the former step to stabilize apoB and prevent its degradation. The last step in this pathway would be the release of the VTV to the Golgi. In 2012, Rahim et al. published a proteomic analysis of the VTV in primary rat hepatocytes [100]. In

stark contrast to the findings published in rabbit hepatocytes, the results suggested that proteins involved in LDL cholesterol import in the ER and VTV export to the Golgi occur in the same location [100]. This would mean that the intermediate step—apoB lipidation—also likely occurs in one physical location of the ER. Notable hits from the VTV proteomic analysis included SOAT2, apoB100, MTP, tubulin, CideB, and VAP-A [100]. Taken together, this data supports the existence of juxtanuclear SOVAs within the human hepatocyte, which couple LEL docking, esterification of LDL cholesterol, VLDL assembly, and VTV release to the Golgi **Figure 12**.

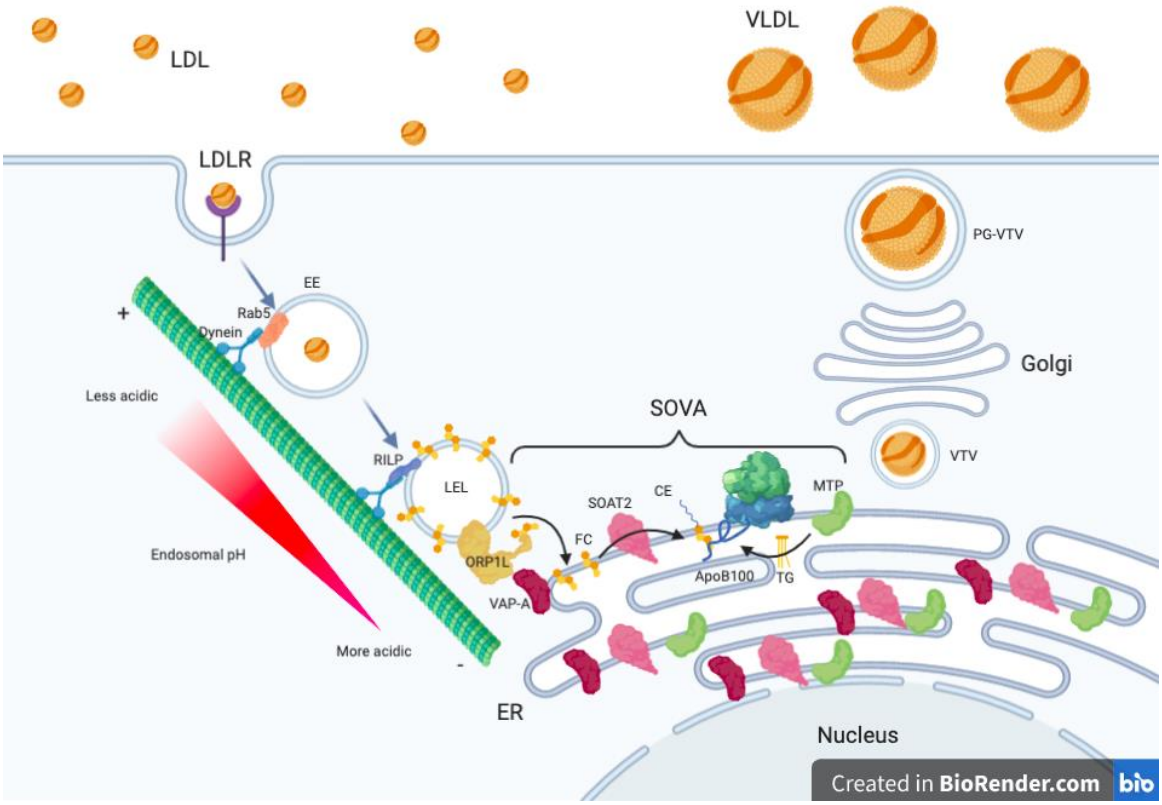


Figure 12. Model of a theoretical site of VLDL assembly (SOVA) in the hepatocyte. In the hepatocyte, the endocytosed endosomes containing the LDL particle are trafficked in a minus-end direction towards a SOVA in the ER. The progressive decline in endosomal pH prompts the activation of CE hydrolases, thereby increasing the FC content in the endosomal membrane. ORP1L docks the LEL to the ER upon binding to VAP-A and transfers FC to the ER membrane. In the ER, LDL-derived FC is immediately esterified by SOAT2 and the resulting CE associated with the nascent apoB100 peptide. MTP also transfers TG associated with apoB100. The primordial VLDL particle is trafficked to the Golgi in a VTV. The bulk lipidation of VLDL occurs in the Golgi (not shown). In the final step, the mature VLDL particle is trafficked from the Golgi to the plasma membrane in a PG-VTV and VLDL is released in the blood. Created with BioRender.com.

Next, we showed that SOAT2-mediated esterification of LDL cholesterol stimulates VLDL secretion. Selective inhibition of SOAT2 with PPPA reduced CE secretion by 30% and apoB secretion by 40% in LDL-treated IHH (**Figure 6** & **Figure 7**). The similarity between these decreases indicates that the CE content of individual VLDL particles remained the same, while the overall number of secreted particles decreased. Therefore, a decreased rate of apoB lipidation by PPPA reduced the number of mature VLDL particles generated and secreted by the hepatocyte. This result is in agreement with the function of SOAT2 in generating CE associated with apoB lipoproteins [37]. Moreover, overexpression of SOAT2 in rat hepatoma McA-RH7777 cells had a significantly greater impact on VLDL secretion than did SOAT1 ovexpression[43]. It would be interesting to repeat the experiment with lipoproteins loaded with ^{3}H-CE to determine the proportion of CE within VLDL that was de-esterified then re-esterified for secretion.

There is little concern for off-target effects in our experiment, as work performed in the lab of Dr. Lawrence L. Rudel demonstrated that the specificity of PPPA against SOAT2 is 2000-fold higher than it is for SOAT1 [101]. These results also call into question whether TGs or CEs form the initial neutral lipid core to prevent apoB degradation in the hepatocyte. In one study, the use of the general SOAT inhibitor Sandoz 58035 (S-58035) in HepG2 cells reduced CE synthesis, increased TG synthesis, but reduced the mass of apoB secreted[102]. This finding contrasted with another study where it was found that HepG2 cells treated with incremental doses of S-58035 increased apoB secretion in a dose-dependent manner by decreasing apoB degradation[103]. One publication even indicates that inhibition of CE synthesis by S-58035 does not have any effect on apoB secretion [104]. A few years later, it was shown that a separate SOAT inhibitor CI-1011 decreased apoB secretion in HepG2 cells by causing enhanced apoB degradation [105]. Differences in these results could be due to the fact that HepG2 cells secrete

lipid-poor VLDL particles that are less buoyant than those secreted *in vivo* by the mammalian liver and are therefore a less reliable model for studying VLDL secretion [106]. The IHH cells used in this study retain several properties of differentiated hepatocytes, unlike hepatoma cells [95]. However, using primary human hepatocytes, which secrete apoB particles in the VLDL density range [95], would be a more reliable model to study the effect of SOAT2 inhibition. Additionally, higher doses of PPPA may be required to verify if our findings hold at complete inhibition of SOAT2. The optimal non-toxic dose of PPPA to completely inhibit SOAT2 needs to be determined. Rats and miniature pigs treated with statins resulted in significantly lower VLDL production and secretion [107] [108]. It is postulated that a decrease in the hepatic FC pool lowers CE synthesis to the point where apoB translocation and VLDL assembly are impeded [108].

The inhibition of SOAT2 directed internalized LDL-cholesterol to the "regulatory pool". The relative amount of cholesterol in the "regulatory pool" was indirectly measured by localizing SREBP-2 by immunofluorescence. **Figure 8** & **Figure 9** demonstrate that while SREBP-2 localization in CR-treated hepatocytes is unaffected by PPPA, the nuclear-to-cytoplasmic intensity ratio of SREBP-2 decreased by 8.9% in LDL-treated hepatocyte in the presence of PPPA. This relatively small change could be due to the fact that very little SREBP-2 is required to enter the nucleus and activate transcription of target genes. This is because one transcript of LDLR or HMGCR could go through multiple rounds of translation to generate many polypeptides. It would also be interesting to see if a higher dose of PPPA could decrease the nuclear-to-cytoplasmic ratio of SREBP-2 even further. In **Figure 10**, incremental doses of PPPA lead to a decrease in *de novo* FC biosynthesis in LDL-treated hepatocytes. This result validates the findings from **Figure 8** & **Figure 9** that SOAT2 is required to direct LDL cholesterol away from the regulatory pool. Taken together, we identify two pools of cholesterol within the human

hepatocyte: one that is a substrate for SOAT2 and destined for VLDL secretion and the other is a regulatory pool. The idea that there exist separate endocytic channels that deliver cholesterol to a SOAT and an SREBP-2 accessible pool was proposed in Chinese Hamster Ovary (CHO) cells [109]. It was found that when LDL cholesterol was unable to reach the ER upon NPC1 inhibition, "cholesterol transport is delayed more to ACAT…than to the SREBP-SCAP system" [109]. This difference in kinetics is also observed in this study, as PPPA directed more cholesterol away from the SOAT2 compartment and to the SREBP-2 compartment. While both compartments ensure intracellular cholesterol homeostasis, the former influences systemic cholesterol homeostasis. These two functions must be physically separated or else the ER would become flooded with FC from LDL and cause cell toxicity.

It is important to note that this shunt pathway does not describe the fate of all LDL cholesterol entering the hepatocyte. That is, not all LDL-derived cholesterol will be packaged within VLDL and the same applies to CR-derived cholesterol entering the "regulatory pool". Increased dietary cholesterol intake in mice increases plasma VLDL cholesterol, and this effect is abolished in the liver-specific *Soat2*$^{-/-}$ mouse [110]. In terms of the LDL pathway, it is possible that the shunt may only be bypassed when cellular SOAT2 is saturated. However, in primary hamster hepatocytes LDL treatment was shown to upregulate SOAT2, potentially to cope with increased ER cholesterol and prevent saturation [78]. Another point for consideration is that there exist two methods of LDL uptake by the hepatocyte: the LDLR pathway, which is high affinity and saturable, and the non-specific pathway, which is low affinity and non-saturable[90]. In the LDLR pathway, the number of LDL particles cleared per unit time will not increase when all the receptors are saturated (at moderate plasma LDL concentrations). The only way to increase LDL uptake in this pathway is to increase the number of LDLRs at the cell surface. However, LDL

will still enter the hepatocyte via the non-specific pathway in hypercholesterolemic conditions. At higher LDL concentrations, the non-specific pathway will continuously take up more LDL. The uncontested example of this pathway is in patients with FH; *Ldlr* homozygous or severe heterozygous mutations exhibit drastic reductions in hepatic LDL internalization, yet cholesterol-rich apoB secretion continues unabated[55]. Work from the laboratory of Dr. John Dietschy demonstrates that the balance between the specific and non-specific pathways can be affected by treating hepatocytes with either saturated or polyunsaturated free fatty acids[111]. Hamsters fed a diet rich in polyunsaturated fatty acids increased the rate of LDLR-mediated uptake and did not change LDL production (an indirect measurement of VLDL secretion), while saturated fatty acids enhanced non-specific uptake of LDL and increased LDL production and secretion[111]. Interestingly, unsaturated fatty acids are a preferred substrate of SOAT2 over saturated fatty acids[112]. Taken together with the shunt model, SOAT2-mediated esterification of LDL cholesterol is low in the presence of saturated fatty acids, and it is re-directed to the regulatory pool. This would lead to the downregulation of the LDLR and enhanced non-specific uptake of LDL with increased apoB secretion. However, polyunsaturated fatty acids enhance SOAT2 esterification, reduce FC in the regulatory pool, relieve the inhibition on LDLR synthesis, and increase the number of LDLR at the cell surface. In this case, plasma LDL cholesterol remains constant. Given this information, internalized LDL cholesterol may only be trafficked directly to SOAT2 in the non-specific pathway (i.e. when plasma LDL is elevated and LDLR is saturated). Specific uptake by the LDLR, the predominant pathway when plasma LDL is low, directs LDL cholesterol to the regulatory pool. One way to monitor lipid metabolism in the non-specific pathway would be to modify LDL particles—through methylation or acetylation—such that they

can no longer bind the LDL receptor. It would then be interesting to compare VLDL secretion in IHH cells treated with LDL and modified LDL.

The cholesterol derived from CRs rapidly appears at the plasma membrane upon endocytosis, while LDL-derived cholesterol does not. After 1 hour of lipoprotein treatment, there was significantly more CR-derived cholesterol at the plasma membrane than LDL-derived cholesterol (**Figure 11**). We ensure that this cholesterol is taken up by an endocytic mechanism through the loading of ^{3}H-labelled CE in LDL and CR and monitoring the radioactivity in FC at the plasma membrane. Moreover, CR and LDL were loaded with equal masses of cholesterol to ensure there was no bias in the results [78]. These results also agree with the timeframe from **Figure 4**, where LELs containing metabolized lipoproteins were identified only after 30 minutes post-endocytosis. However, there is no indication as to whether CR cholesterol arrives first at the plasma membrane, or the regulatory pool in the ER. We recognize the similarity between the CR pathway in hepatocytes and the LDL pathway in non-hepatocytes—both sources of cholesterol arrive in the regulatory pool. In the literature, there are two schools of thought regarding what the post-lysosomal fate of LDL-cholesterol in non-hepatic cells is. On the one hand, some researchers argue that it moves directly from lysosomes to the ER [113, 114]. Thereafter, the bulk of cholesterol is rapidly mobilized to the plasma membrane. The other side of the argument, for which the most recent work argues for, posits that LDL-derived cholesterol is first delivered to the plasma membrane before it enters the ER regulatory pool [19]. Upon LDL endocytosis, CE is de-esterified, and FC is shuttled directly to the plasma membrane to raise cholesterol to the optimal levels. There must be no LEL-to-ER delivery of cholesterol during the intermediate trafficking step. When the plasma membrane is replete with cholesterol, excess cholesterol is equilibrated with the ER to determine the optimal cholesterol level [19]. The pathway taken by CR

in hepatocytes resembles this LDL pathway in non-hepatocytes. Evidence for this latter pathway has been outlined in A431 carcinoma cells [115]. Briefly, LDL is endocytosed, its CEs are de-esterified, and NPC1 transports this cholesterol to the limiting membrane. NPC1 is required to recruit Rab8a, which then promotes the motility of these specialized organelles containing LDL cholesterol back to the cell periphery, while lysosomes continue towards the cell interior [115]. The LDL cholesterol-containing organelles are then anchored to cortical actin via a Rab8a and Myo5b dependent mechanism [115]. These vesicles then slowly make their way to focal adhesions where cholesterol is delivered to the plasma membrane [115]. In the hepatocyte, Rab8a has been implicated in lipid droplet fusion and potentially fatty liver formation [116]. However, the possible role of Rab8a in mediating the delivery of CR-derived cholesterol to the plasma membrane has not yet been ruled out. Further studies using dominant negative and fluorescent analogs of Rab8a in hepatocytes could help elucidate its function in lipoprotein trafficking.

The LDL particle is not a physiological particle, but rather a pathological particle. All cells express HMGCR to make their own cholesterol, and therefore LDL is not required to deliver cholesterol to peripheral cells. Due to the liver's ability to prepare cholesterol-loaded apoB particles for secretion, it is interesting to see how SOAT2 is also purposed to divert the influx of apoB-associated cholesterol from decreasing further lipoprotein uptake. The liver can be thought of as a selfish organ, as it continuously re-secretes LDL—when plasma LDL is already excessive—to the detriment of the organism. This would therefore initiate or aid in the progression of the atherosclerotic process.

Our current knowledge regarding the shunt pathway in hepatocytes could guide therapeutic strategies to tackle atherosclerosis and CVD. The most obvious approach would be to inhibit hepatic SOAT2 in order to decrease VLDL secretion. Mutations in human *Soat2* are not well

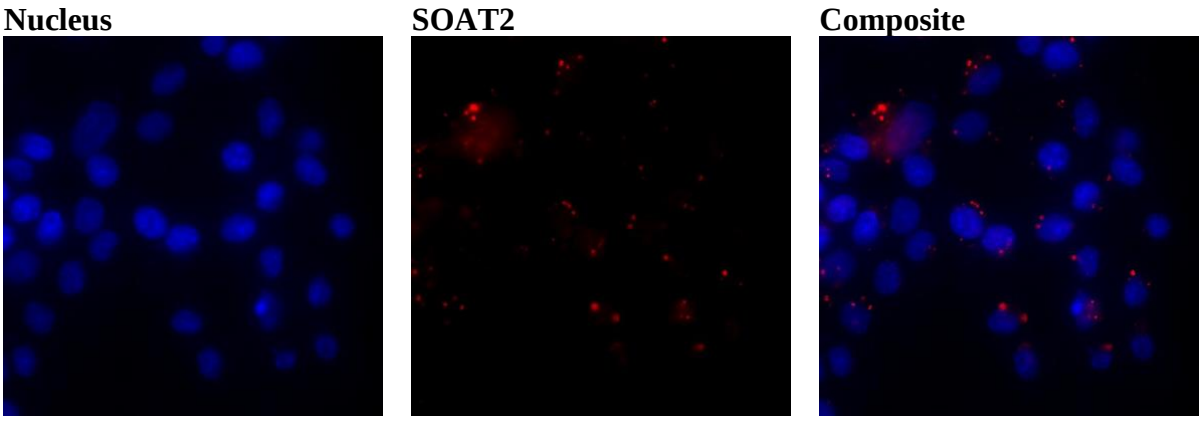
documented in the literature. For those single nucleotide polymorphisms in *Soat2* that have been discovered (E14G, T254I, IVS7 -35G→A, and IVS1 -8G→C), none have any significant effect on plasma concentrations of lipids [117] [118]. Interestingly, it was found that T254I heterozygotes have a higher concentration of plasma apoC-III than individuals without the mutation [117]. Despite this evidence, there is no documented human mutation that would suggest long-term benefits of SOAT2 inhibition. In the atherosclerotic *Apoe*$^{-/-}$ murine model, PPPA was shown to reduce total plasma cholesterol by 55.6 ± 9.3% and atherosclerotic lesion areas were also reduced by 62.2 ± 13.1%[39]. Atherosclerotic lesions were also decreased in the *Ldlr*$^{-/-}$ mouse[119]. The whole-body *Soat2*$^{-/-}$ mouse exhibits reduced cholesterol absorption, due to the inefficient packaging of CE within nascent chylomicrons in intestinal cells [120]. As a result of hepatic *Soat2*$^{-/-}$, secreted VLDL is more TG rich and CE depleted than the wild type mouse [120]. Interestingly, plasma LDL cholesterol remains unchanged despite the reduction in atherogenicity [120]. It is speculated that this effect is due to the depletion of cholesteryl oleate in circulating LDL particles, thereby decreasing the LDL binding affinity to arterial proteoglycans [120]. It would be interesting to see if this effect can be mimicked by treating FH and other types of hyperlipidemia with PPPA. One important biochemical aspect of PPPA is that it noncovalently binds to a specific site in SOAT2 and does not decrease its protein levels, meaning that the inhibitory effect can be reversed [119]. These benefits trump the effects of general SOAT inhibitors, avasimibe and pactimibe, which have previously failed in clinical trials[119]. Pactimibe may even promote atherogenesis as its inhibitory effect on SOAT1 in foam cells may increase FC in the cell without making it available for efflux to HDL, thereby increasing cytotoxicity[119]. Acute hepatic knockdown of SOAT2 has also been done using antisense nucleotide treatment [120]. This model of SOAT2 knockdown suggested newly synthesized lipoproteins feed into a non-biliary pathway that ultimately leads in

fecal excretion [120]. Another therapeutic avenue towards the treatment of atherosclerosis would be to implement a combination of SOAT2 inhibitor and statins. Statins would reduce cholesterol biosynthesis, thereby further relieving the break on LDL receptor synthesis; allowing LDL uptake to occur. Subsequently knocking down of SOAT2 by antisense oligonucleotide or PPPA would direct this incoming cholesterol for fecal excretion.

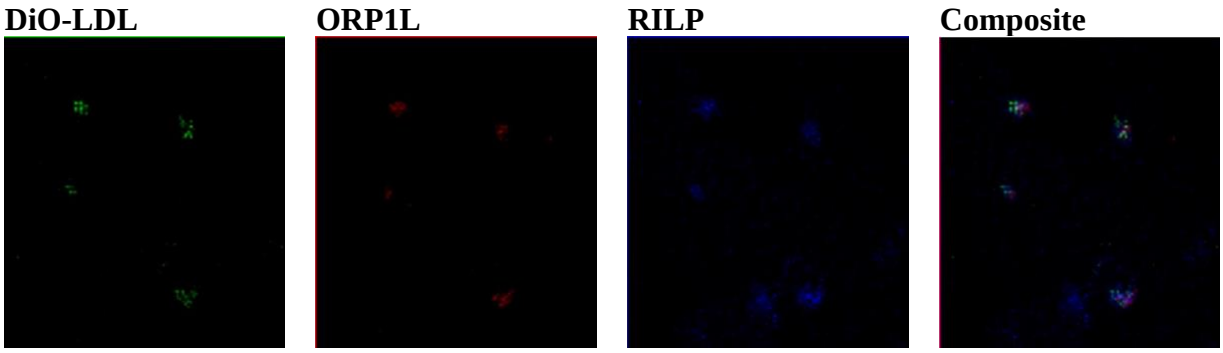
4.2. Conclusion

The plasma levels of cholesterol change as a consequence of the liver's response to influx of LDL or CR. These responses are a direct reflection of the different channels of lipoprotein trafficking and metabolism within the hepatocyte. Increased delivery of dietary cholesterol in CRs leads to the downregulation of hepatic LDLR, elevation of plasma LDL, and increased risk of CVD. The kinetics of LDL internalization in the hepatocyte depend on either the LDLR or non-specific pathways. It is unclear whether one or both of these pathways contribute to the shunt pathway. Furthering our understanding of how LDL cholesterol is shunted in the hepatocyte will help us comprehend cholesterol homeostasis in the liver, and how it is a determinant of plasma VLDL levels and CVD risk. Nonetheless, it remains undisputed that increased delivery of cholesterol to the liver increases the production of LDL cholesterol. The adaptions of the liver in response to LDL and CR are therefore designed to protect the organ and not the individual. Further work is required to understand how LDL is selected for the shunt pathway, the full composition of SOVAs, and the mechanism of delivering CR-derived cholesterol to the plasma membrane.

4. Appendix



Appendix 1. SOAT2 is localized to a punctate and juxtanuclear region in IHH cells.



Appendix 2. LDL-endosomes partially co-localize with ORP1L and RILP.
Data was obtained by Dr. Robert Scott Kiss.

References

[1] Manuel, D. G., Leung, M., Nguyen, K., Tanuseputro, P., Johansen, H., and Canadian Cardiovascular Outcomes Research, T. (2003) Burden of cardiovascular disease in Canada, *Can J Cardiol 19*, 997-1004.

[2] Ismaiel, A., and Dumitrascu, D. L. (2019) Cardiovascular Risk in Fatty Liver Disease: The Liver-Heart Axis-Literature Review, *Front Med (Lausanne) 6*, 202.

[3] Pahwa, R., and Jialal, I. (2020) Atherosclerosis, In *StatPearls*, Treasure Island (FL).

[4] Fruchart, J. C., Nierman, M. C., Stroes, E. S., Kastelein, J. J., and Duriez, P. (2004) New risk factors for atherosclerosis and patient risk assessment, *Circulation 109*, III15-19.

[5] Lichtenstein, A. H., Appel, L. J., Brands, M., Carnethon, M., Daniels, S., Franch, H. A., Franklin, B., Kris-Etherton, P., Harris, W. S., Howard, B., Karanja, N., Lefevre, M., Rudel, L., Sacks, F., Van Horn, L., Winston, M., and Wylie-Rosett, J. (2006) Summary of American Heart Association Diet and Lifestyle Recommendations revision 2006, *Arterioscler Thromb Vasc Biol 26*, 2186-2191.

[6] Hafiane, A. (2019) Vulnerable Plaque, Characteristics, Detection, and Potential Therapies, *J Cardiovasc Dev Dis 6*.

[7] Linton, M. R. F., Yancey, P. G., Davies, S. S., Jerome, W. G., Linton, E. F., Song, W. L., Doran, A. C., and Vickers, K. C. (2000) The Role of Lipids and Lipoproteins in Atherosclerosis, In *Endotext* (Feingold, K. R., Anawalt, B., Boyce, A., Chrousos, G., Dungan, K., Grossman, A., Hershman, J. M., Kaltsas, G., Koch, C., Kopp, P., Korbonits, M., McLachlan, R., Morley, J. E., New, M., Perreault, L., Purnell, J., Rebar, R., Singer, F., Trence, D. L., Vinik, A., and Wilson, D. P., Eds.), South Dartmouth (MA).

[8] Boren, J., Chapman, M. J., Krauss, R. M., Packard, C. J., Bentzon, J. F., Binder, C. J., Daemen, M. J., Demer, L. L., Hegele, R. A., Nicholls, S. J., Nordestgaard, B. G., Watts, G. F., Bruckert, E., Fazio, S., Ference, B. A., Graham, I., Horton, J. D., Landmesser, U., Laufs, U., Masana, L., Pasterkamp, G., Raal, F. J., Ray, K. K., Schunkert, H., Taskinen, M. R., van de Sluis, B., Wiklund, O., Tokgozoglu, L., Catapano, A. L., and Ginsberg, H. N. (2020) Low-density lipoproteins cause atherosclerotic cardiovascular disease: pathophysiological, genetic, and therapeutic insights: a consensus statement from the European Atherosclerosis Society Consensus Panel, *Eur Heart J*.

[9] Kessel, A., Ben-Tal, N., and May, S. (2001) Interactions of cholesterol with lipid bilayers: the preferred configuration and fluctuations, *Biophys J 81*, 643-658.

[10] Feingold, K. R., and Grunfeld, C. (2000) Introduction to Lipids and Lipoproteins, In *Endotext* (Feingold, K. R., Anawalt, B., Boyce, A., Chrousos, G., Dungan, K., Grossman, A., Hershman, J. M., Kaltsas, G., Koch, C., Kopp, P., Korbonits, M., McLachlan, R., Morley, J. E., New, M., Perreault, L., Purnell, J., Rebar, R., Singer, F., Trence, D. L., Vinik, A., and Wilson, D. P., Eds.), South Dartmouth (MA).

[11] Meddings, J. B., and Dietschy, J. M. (1986) Regulation of plasma levels of low-density lipoprotein cholesterol: interpretation of data on low-density lipoprotein turnover in man, *Circulation 74*, 805-814.

[12] Carmena, R., Duriez, P., and Fruchart, J. C. (2004) Atherogenic lipoprotein particles in atherosclerosis, *Circulation 109*, III2-7.

[13] Sniderman, A. D., Thanassoulis, G., Glavinovic, T., Navar, A. M., Pencina, M., Catapano, A., and Ference, B. A. (2019) Apolipoprotein B Particles and Cardiovascular Disease: A Narrative Review, *JAMA Cardiol*.

[14] Barter, P. J., Brewer, H. B., Jr., Chapman, M. J., Hennekens, C. H., Rader, D. J., and Tall, A. R. (2003) Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis, *Arterioscler Thromb Vasc Biol 23*, 160-167.

[15] Miller, N. E. (2014) CETP inhibitors and cardiovascular disease: Time to think again, *F1000Res 3*, 124.

[16] Zhang, K., and Kaufman, R. J. (2003) Unfolding the toxicity of cholesterol, *Nat Cell Biol 5*, 769-770.

[17] Sorci-Thomas, M. G., and Thomas, M. J. (2016) Microdomains, Inflammation, and Atherosclerosis, *Circ Res 118*, 679-691.

[18] Lange, Y., Ye, J., and Steck, T. L. (2004) How cholesterol homeostasis is regulated by plasma membrane cholesterol in excess of phospholipids, *Proc Natl Acad Sci U S A 101*, 11664-11667.

[19] Infante, R. E., and Radhakrishnan, A. (2017) Continuous transport of a small fraction of plasma membrane cholesterol to endoplasmic reticulum regulates total cellular cholesterol, *Elife 6*.

[20] Howe, V., Sharpe, L. J., Alexopoulos, S. J., Kunze, S. V., Chua, N. K., Li, D., and Brown, A. J. (2016) Cholesterol homeostasis: How do cells sense sterol excess?, *Chem Phys Lipids 199*, 170-178.

[21] Radhakrishnan, A., Goldstein, J. L., McDonald, J. G., and Brown, M. S. (2008) Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance, *Cell Metab 8*, 512-521.

[22] Lange, Y., Ye, J., Rigney, M., and Steck, T. L. (1999) Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol, *J Lipid Res 40*, 2264-2270.

[23] Radhakrishnan, A., Ikeda, Y., Kwon, H. J., Brown, M. S., and Goldstein, J. L. (2007) Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig, *Proc Natl Acad Sci U S A 104*, 6511-6518.

[24] Zhang, C. P., Tian, Y., Zhang, M., Tuo, Q. H., Chen, J. X., and Liao, D. F. (2016) IDOL, inducible degrader of low-density lipoprotein receptor, serves as a potential therapeutic target for dyslipidemia, *Med Hypotheses 86*, 138-142.

[25] Naslavsky, N., Rahajeng, J., Rapaport, D., Horowitz, M., and Caplan, S. (2007) EHD1 regulates cholesterol homeostasis and lipid droplet storage, *Biochem Biophys Res Commun 357*, 792-799.

[26] Gluchowski, N. L., Becuwe, M., Walther, T. C., and Farese, R. V., Jr. (2017) Lipid droplets and liver disease: from basic biology to clinical implications, *Nat Rev Gastroenterol Hepatol 14*, 343-355.

[27] Krishna, M. (2013) Microscopic anatomy of the liver, *Clin Liver Dis (Hoboken) 2*, S4-S7.

[28] Shetty, S., Lalor, P. F., and Adams, D. H. (2018) Liver sinusoidal endothelial cells - gatekeepers of hepatic immunity, *Nat Rev Gastroenterol Hepatol 15*, 555-567.

[29] Cooper, A. D. (1997) Hepatic uptake of chylomicron remnants, *J Lipid Res 38*, 2173-2192.

[30] Chiang, J. Y. (2013) Bile acid metabolism and signaling, *Compr Physiol 3*, 1191-1212.

[31] Dashti, N., Gandhi, M., Liu, X., Lin, X., and Segrest, J. P. (2002) The N-terminal 1000 residues of apolipoprotein B associate with microsomal triglyceride transfer protein to create a lipid transfer pocket required for lipoprotein assembly, *Biochemistry 41*, 6978-6987.

[32] Hussain, M. M., Shi, J., and Dreizen, P. (2003) Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly, *J Lipid Res 44*, 22-32.

[33] Fisher, E. A., Khanna, N. A., and McLeod, R. S. (2011) Ubiquitination regulates the assembly of VLDL in HepG2 cells and is the committing step of the apoB-100 ERAD pathway, *J Lipid Res 52*, 1170-1180.

[34] Fisher, E. A. (2012) The degradation of apolipoprotein B100: multiple opportunities to regulate VLDL triglyceride production by different proteolytic pathways, *Biochim Biophys Acta 1821*, 778-781.

[35] Ye, J., Li, J. Z., Liu, Y., Li, X., Yang, T., Ma, X., Li, Q., Yao, Z., and Li, P. (2009) Cideb, an ER- and lipid droplet-associated protein, mediates VLDL lipidation and maturation by interacting with apolipoprotein B, *Cell Metab 9*, 177-190.

[36] Lee, R. G., Shah, R., Sawyer, J. K., Hamilton, R. L., Parks, J. S., and Rudel, L. L. (2005) ACAT2 contributes cholesteryl esters to newly secreted VLDL, whereas LCAT adds cholesteryl ester to LDL in mice, *J Lipid Res 46*, 1205-1212.

[37] Temel, R. E., Hou, L., Rudel, L. L., and Shelness, G. S. (2007) ACAT2 stimulates cholesteryl ester secretion in apoB-containing lipoproteins, *J Lipid Res 48*, 1618-1627.

[38] Rudel, L. L., Lee, R. G., and Parini, P. (2005) ACAT2 is a target for treatment of coronary heart disease associated with hypercholesterolemia, *Arterioscler Thromb Vasc Biol 25*, 1112-1118.

[39] Ohshiro, T., Ohtawa, M., Nagamitsu, T., Matsuda, D., Yagyu, H., Davis, M. A., Rudel, L. L., Ishibashi, S., and Tomoda, H. (2015) New pyripyropene A derivatives, highly SOAT2-selective inhibitors, improve hypercholesterolemia and atherosclerosis in atherogenic mouse models, *J Pharmacol Exp Ther 355*, 299-307.

[40] Parini, P., Davis, M., Lada, A. T., Erickson, S. K., Wright, T. L., Gustafsson, U., Sahlin, S., Einarsson, C., Eriksson, M., Angelin, B., Tomoda, H., Omura, S., Willingham, M. C., and Rudel, L. L. (2004) ACAT2 is localized to hepatocytes and is the major cholesterol-esterifying enzyme in human liver, *Circulation 110*, 2017-2023.

[41] Parini, P., Jiang, Z. Y., Einarsson, C., Eggertsen, G., Zhang, S. D., Rudel, L. L., Han, T. Q., and Eriksson, M. (2009) ACAT2 and human hepatic cholesterol metabolism: identification of important gender-related differences in normolipidemic, non-obese Chinese patients, *Atherosclerosis 207*, 266-271.

[42] Joyce, C. W., Shelness, G. S., Davis, M. A., Lee, R. G., Skinner, K., Anderson, R. A., and Rudel, L. L. (2000) ACAT1 and ACAT2 membrane topology segregates a serine residue essential for activity to opposite sides of the endoplasmic reticulum membrane, *Mol Biol Cell 11*, 3675-3687.

[43] Liang, J. J., Oelkers, P., Guo, C., Chu, P. C., Dixon, J. L., Ginsberg, H. N., and Sturley, S. L. (2004) Overexpression of human diacylglycerol acyltransferase 1, acyl-coa:cholesterol acyltransferase 1, or acyl-CoA:cholesterol acyltransferase 2 stimulates secretion of apolipoprotein B-containing lipoproteins in McA-RH7777 cells, *J Biol Chem 279*, 44938-44944.

[44] Zhang, J., Sawyer, J. K., Marshall, S. M., Kelley, K. L., Davis, M. A., Wilson, M. D., Brown, J. M., and Rudel, L. L. (2014) Cholesterol esters (CE) derived from hepatic sterol O-acyltransferase 2 (SOAT2) are associated with more atherosclerosis than CE from intestinal SOAT2, *Circ Res 115*, 826-833.

[45] Tiwari, S., and Siddiqi, S. A. (2012) Intracellular trafficking and secretion of VLDL, *Arterioscler Thromb Vasc Biol 32*, 1079-1086.

[46] Tiwari, S., Siddiqi, S., and Siddiqi, S. A. (2013) CideB protein is required for the biogenesis of very low density lipoprotein (VLDL) transport vesicle, *J Biol Chem 288*, 5157-5165.

[47] Higgins, J. A. (1988) Evidence that during very low density lipoprotein assembly in rat hepatocytes most of the triacylglycerol and phospholipid are packaged with apolipoprotein B in the Golgi complex, *FEBS Lett 232*, 405-408.

[48] Gusarova, V., Seo, J., Sullivan, M. L., Watkins, S. C., Brodsky, J. L., and Fisher, E. A. (2007) Golgi-associated maturation of very low density lipoproteins involves

conformational changes in apolipoprotein B, but is not dependent on apolipoprotein E, *J Biol Chem 282*, 19453-19462.

[49] Li, X., Ye, J., Zhou, L., Gu, W., Fisher, E. A., and Li, P. (2012) Opposing roles of cell death-inducing DFF45-like effector B and perilipin 2 in controlling hepatic VLDL lipidation, *J Lipid Res 53*, 1877-1889.

[50] Hesse, D., Radloff, K., Jaschke, A., Lagerpusch, M., Chung, B., Tailleux, A., Staels, B., and Schurmann, A. (2014) Hepatic trans-Golgi action coordinated by the GTPase ARFRP1 is crucial for lipoprotein lipidation and assembly, *J Lipid Res 55*, 41-52.

[51] Hossain, T., Riad, A., Siddiqi, S., Parthasarathy, S., and Siddiqi, S. A. (2014) Mature VLDL triggers the biogenesis of a distinct vesicle from the trans-Golgi network for its export to the plasma membrane, *Biochem J 459*, 47-58.

[52] Spady, D. K. (1992) Hepatic clearance of plasma low density lipoproteins, *Semin Liver Dis 12*, 373-385.

[53] Gent, J., and Braakman, I. (2004) Low-density lipoprotein receptor structure and folding, *Cell Mol Life Sci 61*, 2461-2470.

[54] Brown, M. S., and Goldstein, J. L. (1986) A receptor-mediated pathway for cholesterol homeostasis, *Science 232*, 34-47.

[55] Scott Kiss, R., and Sniderman, A. (2017) Shunts, channels and lipoprotein endosomal traffic: a new model of cholesterol homeostasis in the hepatocyte, *J Biomed Res 31*, 95-107.

[56] Zhang, D. W., Lagace, T. A., Garuti, R., Zhao, Z., McDonald, M., Horton, J. D., Cohen, J. C., and Hobbs, H. H. (2007) Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation, *J Biol Chem 282*, 18602-18612.

[57] Park, S. W., Moon, Y. A., and Horton, J. D. (2004) Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver, *J Biol Chem 279*, 50630-50638.

[58] Poirier, S., Mayer, G., Poupon, V., McPherson, P. S., Desjardins, R., Ly, K., Asselin, M. C., Day, R., Duclos, F. J., Witmer, M., Parker, R., Prat, A., and Seidah, N. G. (2009) Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation: evidence for an intracellular route, *J Biol Chem 284*, 28856-28864.

[59] Peterson, A. S., Fong, L. G., and Young, S. G. (2008) PCSK9 function and physiology, *J Lipid Res 49*, 1595-1599.

[60] Brown, M. S., Faust, J. R., Goldstein, J. L., Kaneko, I., and Endo, A. (1978) Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated

with compactin (ML-236B), a competitive inhibitor of the reductase, *J Biol Chem 253*, 1121-1128.

[61] Pedersen, T. R., Kjekshus, J., Berg, K., Haghfelt, T., Faergeman, O., Faergeman, G., Pyorala, K., Miettinen, T., Wilhelmsen, L., Olsson, A. G., Wedel, H., and Scandinavian Simvastatin Survival Study, G. (2004) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994, *Atheroscler Suppl 5*, 81-87.

[62] Sacks, F. M., Pfeffer, M. A., Moye, L. A., Rouleau, J. L., Rutherford, J. D., Cole, T. G., Brown, L., Warnica, J. W., Arnold, J. M., Wun, C. C., Davis, B. R., and Braunwald, E. (1996) The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators, *N Engl J Med 335*, 1001-1009.

[63] Long-Term Intervention with Pravastatin in Ischaemic Disease Study, G. (1998) Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels, *N Engl J Med 339*, 1349-1357.

[64] Heart Protection Study Collaborative, G. (2002) MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial, *Lancet 360*, 7-22.

[65] Zanoni, P., Velagapudi, S., Yalcinkaya, M., Rohrer, L., and von Eckardstein, A. (2018) Endocytosis of lipoproteins, *Atherosclerosis 275*, 273-295.

[66] Tao, W., Moore, R., Meng, Y., Smith, E. R., and Xu, X. X. (2016) Endocytic adaptors Arh and Dab2 control homeostasis of circulatory cholesterol, *J Lipid Res 57*, 809-817.

[67] Boucrot, E., Saffarian, S., Zhang, R., and Kirchhausen, T. (2010) Roles of AP-2 in clathrin-mediated endocytosis, *PLoS One 5*, e10597.

[68] Li, F., and Zhang, H. (2019) Lysosomal Acid Lipase in Lipid Metabolism and Beyond, *Arterioscler Thromb Vasc Biol 39*, 850-856.

[69] Zhao, K., and Ridgway, N. D. (2017) Oxysterol-Binding Protein-Related Protein 1L Regulates Cholesterol Egress from the Endo-Lysosomal System, *Cell Rep 19*, 1807-1818.

[70] Neefjes, J., Jongsma, M. M. L., and Berlin, I. (2017) Stop or Go? Endosome Positioning in the Establishment of Compartment Architecture, Dynamics, and Function, *Trends Cell Biol 27*, 580-594.

[71] van der Kant, R., Fish, A., Janssen, L., Janssen, H., Krom, S., Ho, N., Brummelkamp, T., Carette, J., Rocha, N., and Neefjes, J. (2013) Late endosomal transport and tethering are coupled processes controlled by RILP and the cholesterol sensor ORP1L, *J Cell Sci 126*, 3462-3474.

[72] Raiborg, C., Wenzel, E. M., Pedersen, N. M., Olsvik, H., Schink, K. O., Schultz, S. W., Vietri, M., Nisi, V., Bucci, C., Brech, A., Johansen, T., and Stenmark, H. (2015) Repeated ER-endosome contacts promote endosome translocation and neurite outgrowth, *Nature 520*, 234-238.

[73] Brown, M. S., and Goldstein, J. L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor, *Cell 89*, 331-340.

[74] Brown, M. S., and Goldstein, J. L. (1979) Receptor-mediated endocytosis: insights from the lipoprotein receptor system, *Proc Natl Acad Sci U S A 76*, 3330-3337.

[75] Havekes, L. M., Verboom, H., de Wit, E., Yap, S. H., and Princen, H. M. (1986) Regulation of low density lipoprotein receptor activity in primary cultures of human hepatocytes by serum lipoproteins, *Hepatology 6*, 1356-1360.

[76] Havekes, L. M., de Wit, E. C., and Princen, H. M. (1987) Cellular free cholesterol in Hep G2 cells is only partially available for down-regulation of low-density-lipoprotein receptor activity, *Biochem J 247*, 739-746.

[77] Vance, J. E., and Vance, D. E. (1990) Lipoprotein assembly and secretion by hepatocytes, *Annu Rev Nutr 10*, 337-356.

[78] Sniderman, A. D., Qi, Y., Ma, C. I., Wang, R. H., Naples, M., Baker, C., Zhang, J., Adeli, K., and Kiss, R. S. (2013) Hepatic cholesterol homeostasis: is the low-density lipoprotein pathway a regulatory or a shunt pathway?, *Arterioscler Thromb Vasc Biol 33*, 2481-2490.

[79] Cohen, L. H., Princen, H. M., Kwekkeboom, J., Havekes, L. M., and Kempen, H. J. (1987) Regulation of cholesterol metabolism in the liver in vivo and in vitro, *Biochem Soc Trans 15*, 339-340.

[80] Tabas, I., Myers, J. N., Innerarity, T. L., Xu, X. X., Arnold, K., Boyles, J., and Maxfield, F. R. (1991) The influence of particle size and multiple apoprotein E-receptor interactions on the endocytic targeting of beta-VLDL in mouse peritoneal macrophages, *J Cell Biol 115*, 1547-1560.

[81] Brown, M. S., and Goldstein, J. L. (2009) Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL, *J Lipid Res 50 Suppl*, S15-27.

[82] Laatsch, A., Merkel, M., Talmud, P. J., Grewal, T., Beisiegel, U., and Heeren, J. (2009) Insulin stimulates hepatic low density lipoprotein receptor-related protein 1 (LRP1) to increase postprandial lipoprotein clearance, *Atherosclerosis 204*, 105-111.

[83] Kiss, R. S., Ma, Z., Nakada-Tsukui, K., Brugnera, E., Vassiliou, G., McBride, H. M., Ravichandran, K. S., and Marcel, Y. L. (2006) The lipoprotein receptor-related protein-1 (LRP) adapter protein GULP mediates trafficking of the LRP ligand prosaposin, leading to sphingolipid and free cholesterol accumulation in late endosomes and impaired efflux, *J Biol Chem 281*, 12081-12092.

[84] Wang, J., Bie, J., and Ghosh, S. (2016) Intracellular cholesterol transport proteins enhance hydrolysis of HDL-CEs and facilitate elimination of cholesterol into bile, *J Lipid Res 57*, 1712-1719.

[85] Harder, C. J., Meng, A., Rippstein, P., McBride, H. M., and McPherson, R. (2007) SR-BI undergoes cholesterol-stimulated transcytosis to the bile canaliculus in polarized WIF-B cells, *J Biol Chem 282*, 1445-1455.

[86] Rai, A. K., Spolaore, B., Harris, D. A., Dabbeni-Sala, F., and Lippe, G. (2013) Ectopic F0F 1 ATP synthase contains both nuclear and mitochondrially-encoded subunits, *J Bioenerg Biomembr 45*, 569-579.

[87] Benoist, F., and Grand-Perret, T. (1997) Co-translational degradation of apolipoprotein B100 by the proteasome is prevented by microsomal triglyceride transfer protein. Synchronized translation studies on HepG2 cells treated with an inhibitor of microsomal triglyceride transfer protein, *J Biol Chem 272*, 20435-20442.

[88] Higashi, Y., Itabe, H., Fukase, H., Mori, M., Fujimoto, Y., and Takano, T. (2003) Transmembrane lipid transfer is crucial for providing neutral lipids during very low density lipoprotein assembly in endoplasmic reticulum, *J Biol Chem 278*, 21450-21458.

[89] Devaraj, S., and Jialal, I. (2020) Biochemistry, Apolipoprotein B, In *StatPearls*, Treasure Island (FL).

[90] Sniderman, A. D., De Graaf, J., Couture, P., Williams, K., Kiss, R. S., and Watts, G. F. (2009) Regulation of plasma LDL: the apoB paradigm, *Clin Sci (Lond) 118*, 333-339.

[91] Mortimer, B. C., Beveridge, D. J., Martins, I. J., and Redgrave, T. G. (1995) Intracellular localization and metabolism of chylomicron remnants in the livers of low density lipoprotein receptor-deficient mice and apoE-deficient mice. Evidence for slow metabolism via an alternative apoE-dependent pathway, *J Biol Chem 270*, 28767-28776.

[92] Keyel, P. A., Mishra, S. K., Roth, R., Heuser, J. E., Watkins, S. C., and Traub, L. M. (2006) A single common portal for clathrin-mediated endocytosis of distinct cargo governed by cargo-selective adaptors, *Mol Biol Cell 17*, 4300-4317.

[93] Kraehling, J. R., Chidlow, J. H., Rajagopal, C., Sugiyama, M. G., Fowler, J. W., Lee, M. Y., Zhang, X., Ramirez, C. M., Park, E. J., Tao, B., Chen, K., Kuruvilla, L., Larrivee, B., Folta-Stogniew, E., Ola, R., Rotllan, N., Zhou, W., Nagle, M. W., Herz, J., Williams, K. J., Eichmann, A., Lee, W. L., Fernandez-Hernando, C., and Sessa, W. C. (2016) Genome-wide RNAi screen reveals ALK1 mediates LDL uptake and transcytosis in endothelial cells, *Nat Commun 7*, 13516.

[94] Sokol, J., Blanchette-Mackie, J., Kruth, H. S., Dwyer, N. K., Amende, L. M., Butler, J. D., Robinson, E., Patel, S., Brady, R. O., Comly, M. E., and et al. (1988) Type C Niemann-Pick disease. Lysosomal accumulation and defective intracellular mobilization of low density lipoprotein cholesterol, *J Biol Chem 263*, 3411-3417.

[95] Schippers, I. J., Moshage, H., Roelofsen, H., Muller, M., Heymans, H. S., Ruiters, M., and Kuipers, F. (1997) Immortalized human hepatocytes as a tool for the study of hepatocytic (de-)differentiation, *Cell Biol Toxicol 13*, 375-386.

[96] Du, X., Kumar, J., Ferguson, C., Schulz, T. A., Ong, Y. S., Hong, W., Prinz, W. A., Parton, R. G., Brown, A. J., and Yang, H. (2011) A role for oxysterol-binding protein-related protein 5 in endosomal cholesterol trafficking, *J Cell Biol 192*, 121-135.

[97] Ouimet, M., Hennessy, E. J., van Solingen, C., Koelwyn, G. J., Hussein, M. A., Ramkhelawon, B., Rayner, K. J., Temel, R. E., Perisic, L., Hedin, U., Maegdefessel, L., Garabedian, M. J., Holdt, L. M., Teupser, D., and Moore, K. J. (2016) miRNA Targeting of Oxysterol-Binding Protein-Like 6 Regulates Cholesterol Trafficking and Efflux, *Arterioscler Thromb Vasc Biol 36*, 942-951.

[98] Hoglinger, D., Burgoyne, T., Sanchez-Heras, E., Hartwig, P., Colaco, A., Newton, J., Futter, C. E., Spiegel, S., Platt, F. M., and Eden, E. R. (2019) NPC1 regulates ER contacts with endocytic organelles to mediate cholesterol egress, *Nat Commun 10*, 4276.

[99] Cartwright, I. J., Higgins, J. A., Wilkinson, J., Bellavia, S., Kendrick, J. S., and Graham, J. M. (1997) Investigation of the role of lipids in the assembly of very low density lipoproteins in rabbit hepatocytes, *J Lipid Res 38*, 531-545.

[100] Rahim, A., Nafi-valencia, E., Siddiqi, S., Basha, R., Runyon, C. C., and Siddiqi, S. A. (2012) Proteomic analysis of the very low density lipoprotein (VLDL) transport vesicles, *J Proteomics 75*, 2225-2235.

[101] Das, A., Davis, M. A., Tomoda, H., Omura, S., and Rudel, L. L. (2008) Identification of the interaction site within acyl-CoA:cholesterol acyltransferase 2 for the isoform-specific inhibitor pyripyropene A, *J Biol Chem 283*, 10453-10460.

[102] Cianflone, K. M., Yasruel, Z., Rodriguez, M. A., Vas, D., and Sniderman, A. D. (1990) Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge, *J Lipid Res 31*, 2045-2055.

[103] Ooyen, C., Zecca, A., Zanelli, T., and Catapano, A. L. (1997) Decreased intracellular degradation and increased secretion of apo B-100 in Hep G2 cells after inhibition of cholesteryl ester synthesis, *Atherosclerosis 130*, 143-152.

[104] Wu, X., Sakata, N., Lui, E., and Ginsberg, H. N. (1994) Evidence for a lack of regulation of the assembly and secretion of apolipoprotein B-containing lipoprotein from HepG2 cells by cholesteryl ester, *J Biol Chem 269*, 12375-12382.

[105] Wilcox, L. J., Barrett, P. H., Newton, R. S., and Huff, M. W. (1999) ApoB100 secretion from HepG2 cells is decreased by the ACAT inhibitor CI-1011: an effect associated with enhanced intracellular degradation of ApoB, *Arterioscler Thromb Vasc Biol 19*, 939-949.

[106] Meex, S. J., Andreo, U., Sparks, J. D., and Fisher, E. A. (2011) Huh-7 or HepG2 cells: which is the better model for studying human apolipoprotein-B100 assembly and secretion?, *J Lipid Res 52*, 152-158.

[107] Khan, B., Wilcox, H. G., and Heimberg, M. (1989) Cholesterol is required for secretion of very-low-density lipoprotein by rat liver, *Biochem J 258*, 807-816.

[108] Burnett, J. R., Wilcox, L. J., Telford, D. E., Kleinstiver, S. J., Barrett, P. H., Newton, R. S., and Huff, M. W. (1997) Inhibition of HMG-CoA reductase by atorvastatin decreases both VLDL and LDL apolipoprotein B production in miniature pigs, *Arterioscler Thromb Vasc Biol 17*, 2589-2600.

[109] Kristiana, I., Yang, H., and Brown, A. J. (2008) Different kinetics of cholesterol delivery to components of the cholesterol homeostatic machinery: implications for cholesterol trafficking to the endoplasmic reticulum, *Biochim Biophys Acta 1781*, 724-730.

[110] Zhang, J., Kelley, K. L., Marshall, S. M., Davis, M. A., Wilson, M. D., Sawyer, J. K., Farese, R. V., Jr., Brown, J. M., and Rudel, L. L. (2012) Tissue-specific knockouts of ACAT2 reveal that intestinal depletion is sufficient to prevent diet-induced cholesterol accumulation in the liver and blood, *J Lipid Res 53*, 1144-1152.

[111] Woollett, L. A., Spady, D. K., and Dietschy, J. M. (1992) Saturated and unsaturated fatty acids independently regulate low density lipoprotein receptor activity and production rate, *J Lipid Res 33*, 77-88.

[112] Seo, T., Oelkers, P. M., Giattina, M. R., Worgall, T. S., Sturley, S. L., and Deckelbaum, R. J. (2001) Differential modulation of ACAT1 and ACAT2 transcription and activity by long chain free fatty acids in cultured cells, *Biochemistry 40*, 4756-4762.

[113] Underwood, K. W., Jacobs, N. L., Howley, A., and Liscum, L. (1998) Evidence for a cholesterol transport pathway from lysosomes to endoplasmic reticulum that is independent of the plasma membrane, *J Biol Chem 273*, 4266-4274.

[114] Neufeld, E. B., Cooney, A. M., Pitha, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., and Blanchette-Mackie, E. J. (1996) Intracellular trafficking of cholesterol monitored with a cyclodextrin, *J Biol Chem 271*, 21604-21613.

[115] Kanerva, K., Uronen, R. L., Blom, T., Li, S., Bittman, R., Lappalainen, P., Peranen, J., Raposo, G., and Ikonen, E. (2013) LDL cholesterol recycles to the plasma membrane via a Rab8a-Myosin5b-actin-dependent membrane transport route, *Dev Cell 27*, 249-262.

[116] Wu, L., Xu, D., Zhou, L., Xie, B., Yu, L., Yang, H., Huang, L., Ye, J., Deng, H., Yuan, Y. A., Chen, S., and Li, P. (2014) Rab8a-AS160-MSS4 regulatory circuit controls lipid droplet fusion and growth, *Dev Cell 30*, 378-393.

[117] Katsuren, K., Tamura, T., Arashiro, R., Takata, K., Matsuura, T., Niikawa, N., and Ohta, T. (2001) Structure of the human acyl-CoA:cholesterol acyltransferase-2 (ACAT-2) gene and its relation to dyslipidemia, *Biochim Biophys Acta 1531*, 230-240.

[118] Katsuren, K., Fukuyama, S., Takata, K., and Ohta, T. (2003) Effects of a new single-nucleotide polymorphism in the Acyl-CoA:cholesterol acyltransferase-2 gene on plasma lipids and apolipoproteins in patients with hyperlipidemia, *J Atheroscler Thromb 10*, 32-36.

[119] Ohshiro, T., Matsuda, D., Sakai, K., Degirolamo, C., Yagyu, H., Rudel, L. L., Omura, S., Ishibashi, S., and Tomoda, H. (2011) Pyripyropene A, an acyl-coenzyme A:cholesterol acyltransferase 2-selective inhibitor, attenuates hypercholesterolemia and atherosclerosis in murine models of hyperlipidemia, *Arterioscler Thromb Vasc Biol 31*, 1108-1115.

[120] Marshall, S. M., Gromovsky, A. D., Kelley, K. L., Davis, M. A., Wilson, M. D., Lee, R. G., Crooke, R. M., Graham, M. J., Rudel, L. L., Brown, J. M., and Temel, R. E. (2014) Acute sterol o-acyltransferase 2 (SOAT2) knockdown rapidly mobilizes hepatic cholesterol for fecal excretion, *PLoS One 9*, e98953.