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# IDENTIFICATION OF PROTEINS REGULATING VLDL SORTING INTO THE VLDL TRANSPORT VESICLE (VTV) AND INVOLVED IN THE BIOGENESIS OF THE VTV

by

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M.S. in Biomedical Sciences
University of Central Florida, 2011

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
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Major Professor: Shadab A. Siddiqi

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# ABSTRACT

Increased secretion of very low-density lipoprotein (VLDL), a triglyceride-rich lipoprotein, by the liver causes hypertriglyceridemia, which is a major risk factor for the development of atherosclerosis. The rate of VLDL-secretion from the liver is determined by its controlled transport from the endoplasmic reticulum (ER) to the Golgi. The ER-to-Golgi transport of newly synthesized VLDL is a complex multi-step process and is mediated by the VLDL transport vesicle (VTV). Once a nascent VLDL particle is synthesized in the lumen of the ER, it triggers the process of VTV-biogenesis and this process requires coat complex II (COPII) proteins that mediate the formation of classical protein transport vesicles (PTV). Even though, both VTV and PTV bud off the same ER at the same time and require the same COPII proteins, their cargos and sizes are different. The VTV specifically exports VLDL to the Golgi and excludes hepatic secretory proteins such as albumin and the size of the VTV is larger (~ 100 -120 nm) than PTV to accommodate VLDL-sized particles. These observations indicate (i) the existence of a sorting mechanism at the level of the ER; and (ii) the involvement of proteins in addition to COPII components. This doctoral thesis is focused on identification of proteins regulating VLDL sorting into the VTV and involved in the biogenesis of the VTV. In order to identify proteins present exclusively in VTV, we have characterized the proteome of VTV, which suggest CideB (cell death-inducing DFF45-like effector b) and SVIP (small VCP/P97 interacting protein) as candidates, present in VTV but excluded from PTV. We further confirmed the finding by performing co-immunoprecipitation studies and confocal microscopy studies. CideB, a 26-kDa protein was found to interact with apolipoprotein

B100 (apoB 100), the structural protein of VLDL. Moreover, CideB interacts with two of the COPII components, Sar1 and Sec24. VTV generation was examined after blocking CideB by specific antibodies and by silencing CideB in rat primary hepatocytes. Knockdown of CideB in primary hepatocytes showed significant reduction in VTV generation, however, CideB was concentrated in VTV as compared with the ER suggesting its functional role in the sorting of VLDL into the VTV.

SVIP, a small (~ 9-kDa) protein was found to interact with Sar1, a COPII component that initiates the budding of vesicles from ER membrane. SVIP has sites for myristoylation and we found increased recruitment of SVIP on ER membrane upon myristic acid (MA) treatment. Sar1 that lacks sites for myristoylation also is recruited more on ER upon myristoylation indicating that SVIP promotes Sar1 recruitment on ER. Additionally, our data suggest that Sar1 interacts with SVIP and forms a multimer that facilitates the biogenesis of VTV. Interestingly, silencing of SVIP reduced the VTV generation significantly. Conversely, incubation with MA increased the VTV budding, suggesting recruitment of SVIP on ER surface facilitates the VTV budding. We conclude that SVIP recruits Sar1 on ER membrane and makes an intricate COPII coat leading to the formation of a large vesicle, the VTV. Overall, the data presented in this thesis, determines the role of CideB and SVIP in regulating VLDL sorting and VTV biogenesis.

Dedicated to Chandan Thomas, my loving Husband

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# LIST OF ABBREVIATIONS

AGPAT: Acyl-CoA: 1-acylglycerol-sn-3-phosphate acyltransferase

APOA1: Apolipoprotein A1

APOB: Apolipoprotein B

APOB100: Apolipoprotein B100

APOB48: Apolipoprotein B48

ARF1: ADP- ribosylation factor 1

ATP: Adenosine triphosphate

BAT: Brown adipose tissue

CETP: Cholesterol ester transfer protein

CIDEB: Cell death-inducing DFFA-like effector b

COP I: Coat protein complex I

COPII: Coat protein complex II

DAG: Diacylglycerol

DFF: DNA fragmentation factor

DFFA: DNA fragmentation factor- alpha

DGAT: Diacylglycerol: acyl-CoA acyltransferase

ER: Endoplasmic reticulum

ERAD: Endoplasmic reticulum associated degradation

FA: Fatty acid

FFA: Free fatty acid

Fsp27: Fat specific protein 27

G3P: Glycerol 3 phosphate

GEF: Guanine nucleotide exchange factor

GTP: Guanosine triphosphate

HCC: Hepatoclellular carcinoma

HDL: High density lipoprotein

HL: Hepatic lipase

Hsp 70: Heat shock protein 70

IDL: Intermediate density lipoprotein

LDL: Low density lipoprotein

LPA: Lysophosphatidate

LPL: Lipoprotein lipase

MAG: Monoacylglycerol

MTP: Microsomal triglyceride transfer protein

NASH: Nonalcoholic steatohepatitis

NEFA: Non esterified fatty acid

NMT: N- myristoyltransferase

PA: Phosphatidate

PAPase: Phosphatidic acid phosphohydrolase

PCTV: Prechylomicron transport vesicle

PDI: Protein disulfide isomerase

PM: Plasma membrane

PTV: Protein transport vesicle

RCT: Reverse cholesterol transport

rER: Rough ER

sER: Smooth ER

SNAP: Soluble NSF attachment protein

SNARE: Soluble N-ethylmaleimide –sensitive factor (NSF) attachment protein receptor

SM: Sec1/Munc18-like proteins

SVIP: Small VCP/P97 interacting protein

TAG: Triacylglycerol

TGN: Trans Golgi Network

TRLPs: Triglyceride rich lipoproteins

VCP: Valosin- containing protein

VLDL: Very low density lipoprotein

VSV-G: Vesicular stomatitis virus protein

VTV: VLDL transport vesicle

WAT: White adipose tissue

# CHAPTER ONE: GENERAL INTRODUCTION

## Introduction

The understanding of the complex processes the human body carries out to render the dietary and endogenous lipids amenable for their journey to the target site through various interactions with proteins is increasingly gaining importance. This is true because excess of lipids in plasma can cause serious conditions like dyslipidemia. Dyslipidemia is an imbalance in secretion and clearance of triglyceride rich lipoproteins (TRLPs) in plasma, the excess of which can cause atherosclerosis, a serious condition vastly prevailing in the industrialized countries and is the major cause of cardiovascular diseases (CVDs). It is a condition, that results in deposition of fibrocalcific plaque in the inner lining of arteries (1). Along with various risk factors like obesity, tobacco smoking and sedentary lifestyle; dyslipidemia also accounts for high risk of atherosclerosis (2). In order to facilitate the transport of fatty acids in plasma, fatty acids undergo physico-chemical reactions with protein factors forming lipoproteins. Lipoproteins are of different types by virtue of their size and composition (1). High concentration of chylomicrons, very low density lipoprotein (VLDL) and small low density lipoprotein (LDL) along with lower concentration of HDL is the hallmark of dyslipidemia which leads to higher level of triglycerides in the plasma which eventually causes atherosclerosis (3).

Atherosclerosis occurs due to several events in the body. Atherogenic particles upon their secretion into the blood vessels bind to capillary endothelium where they get

hydrolyzed by lipoprotein lipase. This results into formation of much smaller lipoproteins and fatty acids as hydrolyzed products, that can lead to lesion formation damaging the arterial wall (3-5). The small LDL due to their small size penetrates the endothelium and promotes monocyte recruitment on endothelium eventually forming foam cells (6). They also get converted into cholesterol ester rich VLDL and chylomicron remnant by the action of cholesterol ester transfer protein (CETP) and, thus, increases cholesterol in the blood stream. These events lead to formation of atherogenic plaque in the walls of arteries and capillaries eventually leading to coronary heart disease (3,7,8).

**Lipoproteins**

Liver and small intestine are the two major organs involved in lipid metabolism. They convert non-esterified fatty acids (NEFA) into energy rich triacylglycerol (TAG). TAG is transported to peripheral tissue in the form of lipoproteins, which are synthesized exclusively in the liver and small intestine. The lipoprotein synthesized by the liver is called VLDL while the one synthesized by the intestine is called chylomicron. Lipoprotein is made up of both proteins and lipids and is involved in lipid transport. The core of lipoprotein is made up of TAG and cholesteryl ester. TAGs are energy rich lipids, which provide energy to peripheral tissues. Phospholipids, cholesterol and proteins surround the core. Phospholipids are amphipathic and can interact with the non-polar core of lipids and polar surrounding of plasma. Apolipoproteins are the proteins present in lipoprotein. They are of different types depending on the nature of lipoprotein. Few apolipoproteins function to provide structural integrity to lipoproteins while others

function as cofactors to help in metabolism of lipoprotein (7). There are several major classes of lipoproteins based on the lipid content, density, nature and type of apolipoprotein. These are as follows: Chylomicrons, VLDL, IDL (intermediate density lipoprotein), HDL (high density lipoprotein) and LDL respectively. The major sources of TAG rich lipoproteins are chylomicron from the small intestine and VLDL from the liver (9). Both VLDL and chylomicron contain apolipoprotein B (apoB) as apolipoprotein. However, in the intestine only 48% of apoB is present and is called apoB48 while in VLDL a fully mature full length apoB is present which is called apoB100 (9).

ApoB is essential for synthesis, secretion and metabolism of VLDL and chylomicrons. ApoB100 also functions as a receptor for LDL (8). Upon their secretion from the intestine and liver, the TAG present in VLDL and chylomicrons is broken down by lipoprotein lipase (LPL) and hepatic lipase (HL) into fatty acids (FAs) (7). Peripheral tissues later absorb the FA. VLDL and chylomicron can also interchange TAG for cholesteryl esters in HDL and LDL by the action of CETP (7). After losing a considerable amount of TAG, VLDL is converted into VLDL remnant, which is taken up by the liver. Some remain in circulation as IDL, which is made up of cholesteryl ester and cholesterol along with TAG. IDL contains apoB and apoE as apolipoprotein. Eventually by the action of lipases, LDL is formed which contains only apoB100 and are highly atherogenic. VLDL, IDL, LDL and chylomicrons transport TAG from the liver to peripheral tissue and thus involved in the forward lipid transport. In contrast to TAG rich lipoprotein, there is one more class of lipoproteins that transport lipids from peripheral

tissues back to liver. This class contains HDL, an anti-atherogenic lipoprotein that contains apoA1 as a major lipoprotein, along with cholesterol esters, TAG and phospholipids. HDL is involved in cholesterol efflux and transports lipids to the liver, and the process is known as reverse cholesterol transport, and plays a major role in maintaining cholesterol homeostasis (10).

**Hepatic TAG synthesis**

There are three sites of TAG synthesis in humans; the liver, the intestine and adipose tissues (11). TAGs are formed from FAs and serves as energy source (12). The process of conversion of FAs into triglycerides protects the body from toxic effect of excess of FAs which are harmful to the cells (12). TAGs are stored majorly in lipid droplets and also in white adipose tissue (13). They can also be present in other tissues like cardiac and skeletal muscles (13).

Sources of FAs to liver for lipoprotein synthesis

The synthesis of TAGs depends on the availability of free FAs (14). FAs can follow either one of the three possible pathways. First, they can undergo oxidation to generate energy; second, they can be secreted as lipoproteins; or finally they can be stored in adipocytes (14). In the liver there are various sources that supply free FAs; for example, from adipose tissue after lipolysis of TAGs, from the intestine and also from chylomicron and VLDL remnants (14-16). *De novo* synthesis of FA is also one major source of FA to

liver (14). *De novo* synthesis of FA takes place in the liver and adipose tissue and is dependent on the diet of the individual as well as the hormones (14,17). It has been reported that excessive carbohydrate diet along with insulin can lead to regulation of enzymes involved in FA synthesis (14,18). Reduction in lipogenesis may lead to increased oxidation of FA and reduction in VLDL synthesis (14). This is further supported by findings that VLDL secretion is related to the diet of the individual as well as on *de novo* lipogenesis (14,19,20).

Formation of TAG from FAs in the ER

FAs in the liver gets incorporated into TAGs and secreted as lipoproteins while in adipose tissue FAs get deposited as fats (17). Free FAs arrive in the liver by the action of transport proteins like fatty acid transport protein (FATP) or by fatty acid translocase (FAT) (17). FAs can also enter the liver by diffusion upon binding with the liver FA binding protein (LFABP) (17,21). The two most common pathways by which TAGs formation occurs are either from conversion of glycerol 3 phosphate to glyceride moiety followed by esterification to form diacylglycerol (DAG) and finally to TAG (13), or from monoacylglycerol (MAG) pathway which involves re-esterification of DAG obtained from dietary TAG (11,13). Both these pathways can take place in hepatocytes. On the other hand in enterocytes, formation of TAG takes place from FAs and MAG both of which are produced by the action of pancreatic TAG lipase on dietary TAG (21). In hepatocytes, TAG synthesis takes place in the lumen of ER by a series of enzymatic reactions. It starts with conversion of glycerol 3 phosphate (G3P) produced by glycolysis and by

phosphorylation of glycerol (12,17). Next acylation of G3P takes place, which results in the formation of lysophosphatidate (LPA) (12). This reaction is catalyzed by enzyme glycerol-P-acyltransferase (GPAT) (12). The second step is the formation of phosphatidate (PA) by acylation of LPA by the action of enzyme acylglycerol-P acyltransferase (AGPAT) (12,17). Interestingly, formation of LPA and PA can take place in ER as well as mitochondria. However, both PA and LPA moves to ER from mitochondria as the final steps of TAG synthesis occur in the ER (12). PA can either transform into CDP-diacylglycerol; the precursor of acidic phospholipids or can form DAG by hydrolysis of PA by phosphatidic acid phosphohydrolase (PAPase) also called lipin (22). The final step of TAG synthesis results in formation of TAG by the action of enzyme diacylglycerol acyltransferase (DGAT) on DAG (12).

**VLDL Biogenesis**

VLDL biogenesis in the endoplasmic reticulum (ER) is a multi-step, complex yet sequential process that involves interaction between proteins and lipids along with the interaction of different sorting signals that are required for biosynthesis and subsequent secretion of VLDL (23-25). VLDL is composed of a core of TAG that is surrounded by a phospholipid monolayer, unesterified cholesterol and apolipoprotein (26). The structure of VLDL is as shown in Figure 1. Apolipoprotein is the protein component of lipoprotein and contributes in its transport, as TAG alone cannot be transported in hydrophobic environment provided by plasma in the body. It is incorporated as apoB100 in VLDL of hepatic origin and as apoB48 in chylomicron of intestinal origin. Apart from apoB,

another important protein known as microsomal triglyceride transfer protein (MTP) plays a role in assembly and biogenesis of VLDL and chylomicrons (27,28).

MTP

MTP is made up of two subunits, which are strongly associated; as, addition of antibody to either subunit results in the exclusion of both the subunits and also the loss of activity to transfer TAG (27,29). The subunits are called large subunit or 'M' subunit of 97kDa and a small subunit or 'P' subunit of 55kDa molecular weight (29-32). The smaller subunit showed sequence homology with protein disulfide isomerase (PDI), however the isomerase activity of PDI is not required in context of MTP function (31,33). The M subunit plays key role in transferring lipid to apoB and, hence, serves as the functional subunit of MTP complex (33,34). However, PDI is essential for the function of MTP as mild denaturing condition results in the dissociation of both the subunits as well as loss of function of MTP complex (27,34). Separation of both the subunits can lead to aggregation of larger subunits in the absence of PDI, suggesting the association of subunits is a prerequisite for the functionality of MTP complex (27,29).

Mutations in gene encoding MTP results into a serious condition called abetalipoprotienemia, which is characterized by lack of apoB containing lipoproteins along with lower TAG and cholesterol in the circulation (27,33,35). It leads to deficiency in fat-soluble vitamins and FAs, which are required for normal functioning of body (27). Genetic studies of homozygous abetalipoprotienemia individuals suggested that, the mutation in the large subunit of ATP leads to loss of function of MTP which results in

defects in apoB formation as well as in the assembly of lipoproteins and hence secretion (27,36,37). M subunit of MTP is a lipid transfer subunit and has three domains, viz., lipid transfer domain, membrane association domain and apoB binding domain as proposed by Hussain and coworkers (30). Both apoB-binding domain and lipid binding domain function independently and inhibition of either of the domains do not affect the other one and vice versa (30,38,39). ApoB-binding domain and lipid transfer domain also work independently of each other (30,40). The undeniable requirement of MTP in the assembly of lipoproteins has been shown by experiments by transfection of apoB and MTP in cells that do not synthesize lipoproteins. Transfection of apoB alone did not result in secretion of lipoproteins, however, simultaneous transfection of MTP along with apoB resulted in increased secretion of apoB (27,41,42). Furthermore, it has been established that inhibitors of MTP decreased the secretion of apoB thus, supporting the role of MTP in lipoprotein assembly (33,43-45). MTP helps in the transfer of lipids by binding simultaneously with lipids and apoB100 via lipid binding domain and apoB binding domain respectively. It has been shown that binding of lipids with MTP, in turn, increases its binding affinity with apoB (40).

2-step model of VLDL assembly

A two-step model of VLDL assembly is widely accepted (2,46-48). According to this model, the first step of VLDL assembly takes place in the rough ER (rER), which involves interaction between apoB100, MTP and lipids (2,33,49). The first step involves incomplete lipidation of apoB 100 along with its translation and translocation (2,46,50).

MTP is required for the lipidation of apoB100 both during its translocation in the rER as well as during and shortly after its translation (2,51-53). The first step leads to formation of incomplete lipidated apoB100 in the form of primordial lipoprotein (2). This step has been further sub-divided in two steps by Hussain *et al*, namely nucleation and desorption (28). Nucleation is a result of association between newly synthesized apoB100 with neutral lipids of inner membrane of ER, resulting in neutral lipid rich nucleation site in the ER, the formation of which is regulated by MTP and lipid load inside ER (28). For the formation of complete VLDL particle and for its subsequent secretion, the nucleation step is not the only requirement (28). Nucleation is followed by desorption of nucleation sites that requires the phospholipid transfer activity of MTP (28). The addition of phospholipids to apoB at the nucleation site results in stabilization of primordial apoB particle (28). As a result of desorption, partially lipidated primordial VLDL is formed that undergoes a second step of maturation in sER (28).

The second step of VLDL maturation is known to occur in the sER as shown by Olofsson and coworkers by utilizing pulse chase experiment followed by oleic acid treatment to study VLDL assembly (2,54). ADP- ribosylation factor 1 (ARF1) promotes synthesis of phosphatidic acid, a key substance in VLDL synthesis. It has been observed that inhibition of ARF 1 by a fungal metabolite Brefeldin, which is known to inhibit transport, halts the synthesis of VLDL (55-58). This metabolite only affects the second step of VLDL maturation and thus supports the idea that the second step of VLDL maturation takes place in sER (2,46). In an elegant study using immune-electron

microscopy Alexander *et al* (47) observed a lipid droplet devoid of apoB in the sER (2,47,59). It has been proposed that the lipid droplet fuse with the product of first step i.e. pre-VLDL to form fully matured VLDL particle (2,46,51,53). The fusion takes place at the intersection of rER containing primordial VLDL and lipid droplet in sER containing TAG and cholesterol ester (47,60).

Whether MTP has a role in the second step of maturation is still not clear, however, it has been suggested that MTP is required for TAG accumulation in the ER and thus, may associate with lipid droplet that is responsible to transfer bulk of lipid to primordial VLDL for its subsequent maturation (30,40,61).

ApoB 100

It is noteworthy that for the assembly of VLDL, folding of apoB is very critical. This is true because if apoB fails to acquire structural configuration for MTP to initiate VLDL synthesis, it will be degraded by ubiquitin-proteasome pathway after interacting with heat shock protein 70 (Hsp 70) (33,62). This finding was further strengthened by an experiment in which Zhou *et al* increased level of Hsp70 by using agonist herbimycin, which resulted in increased degradation of apoB100 (33,63). ApoB100 can undergo post-translational degradation by a reuptake pathway after it leaves ER once encountered by specific receptors on the cell surface which directs it to degradation in proteasome or lysosome (64). Additionally, it can undergo a proteasome independent, post-translational degradation that can be stimulated by n-3 fatty acids (65). The N

terminus of ApoB enters ER lumen through a channel in the ER membrane made up of protein sec61 and simultaneously binds to ribosome to complete translation (64,66-68).

The key factor that determines whether apoB should undergo proteosomal degradation or should be committed to VLDL synthesis is the availability of TAG in the ER lumen (33,66,69). The addition of oleic acid to hepatocytes has been documented to decrease degradation of apoB100 (70,71). MTP lipidates the N terminus of apoB which enters into ER lumen; the rate of which depends on the lipid pool in the ER (66). The role of MTP is very critical here, as in the absence of lipidation apoB can undergo rapid degradation (64,66). It is proposed that the strong affinity between ribosome and sec61 may relax for a short while which leads to exposure of newly made apoB polypeptide to cytosol which results in the formation of a cytosolic loop of apoB (64,72). This arrangement terminates apoB entry inside ER lumen if apoB fails to acquire lipidation status (62,73). The degradation of apoB takes place at the C terminal of apoB, which gets withdrawn from ribosome in absence of lipidation of N terminal (74). ApoB is made up of 3 α helices ($\alpha_1$, $\alpha_2$ and $\alpha_3$) and 2 β sheet domains ($\beta_1$ and $\beta_2$) arranged alternatively (23,75). The two-β domains are known to interact with the lipids of VLDL present in the core, which are TAG and cholesteryl ester (23,76). The lipophilic nature of apoB suggests that a constant supply of lipids is necessary for maintaining proper folding and structural integrity of apoB (23). A 70kDa portion from N terminal of apoB is known to form a globular structure by disulfide bonding and is able to fold without lipidation and gets separated from the rest of the polypeptide in certain conditions

(2,46,77). This is probably the reason why the degradation of apoB takes place at the C terminal since a globular portion is difficult to retract from translocation pore (2,78). If ER lumen has sufficient lipids, MTP transfers lipids to apoB. MTP and apoB are known to interact with strong affinity in the cells (79-81).

The type of interaction between apoB and MTP is of ionic nature, which is modulated by the length of apoB as well as lipidation state of apoB (33). The strength of binding is very strong at N terminal of apoB, which corresponds to amino acids (aa) 1-781 (80) and is dependent on the presence of lysine and arginine bearing positive charges (33). Further studies established that aa 430-570 which are part of α-globular domain of apoB have a very important role in binding with MTP (33,82). Finally work done by Bradbury *et al* has shown that MTP binds with apoB at two different places of apoB; aa 1-264 and aa 512-712 (33,83). Interaction between apoB and MTP leads to addition of more lipids to the growing polypeptide chain of apoB which results into the transfer of hydrophilic lipids like phospholipids to the outer surface while accumulation of hydrophobic lipids to the core and thus results into a dense apoB which can get further lipidated in the second stage of VLDL synthesis (84). The correct folding of apoB is imperative for its lipidation and it is achieved by the formation of disulfide bridges at the N terminal domain of apoB (46). Thus, MTP has a dual function; to transfer lipid to apoB polypeptide during its translocation and simultaneous translation and also serves as chaperone, which enables apoB translocation inside the ER lumen (47,53,85-90).

**VTV**

Once apoB assembles into VLDL, the next step is the secretion of VLDL from ER so that it can reach its next destination Golgi apparatus (64,91). Proteins secreted from organelles are packed into vesicles for their subsequent transport to the next destination (64). It has been reported that a set of proteins called coatomers help in the formation of secretory vesicles. For the export of ER derived proteins, the class of coatomer is called coat protein complex II (COPII) (64,92). The size of lipoprotein depends on the amount of lipids incorporated into lipoprotein and can range from 200 nm for VLDL in the liver to as big as 1000 nm for chylomicron in the intestine (91,93). Table 1 summarizes the differences between VTV and PCTV. It is important to understand that the rate-determining step in VLDL secretion is their transport from ER to the Golgi (94,95).

It has been proposed that the large size of VLDL is too big to incorporate in the classic ER derived vesicles that are of size of nearly 50nm (96,97). The fact that VLDL secreted by ER is larger in size than the conventional vesicles that transfer proteins (91,93) suggest for the need of specialized vesicles for transport of VLDL from ER. It has been shown that export of VLDL does not take place in protein transport vesicles termed as PTV by our group and others (98,99). Using radio-isotope labeling technique differentially labeled albumin and oleic acid were shown to be transported in separate vesicles in primary rat hepatocytes (95). Albumin was shown to transport in PTV, while radiolabelled oleic acid containing VLDL was shown to transport in a specialized vesicle termed as VLDL transport vesicle (VTV) (95). In order to generate VTV, PI has

established an *in vitro* budding assay as shown in Figure 2. It was shown that VTV obtained from budding of ER membranes of primary rat hepatocytes differs from PTV in size, density and in proteome. The bigger size of VTV (100-120 nm), as compared to albumin carrying PTV (55-70 nm) accounts for its capacity to carry VLDL particles (80nm). It was shown that for VTV formation cytosol and ATP is a necessary requirement. These unique vesicles were shown to carry apoB100 from ER to Golgi by v-SNARE sec22b. A complete proteomic analysis of VTV has been done to characterize the proteins that might play a key role in VLDL assembly (99).They were shown to fuse with Golgi, which is the next destination of VLDL after its exit from ER (100). In summary, these COPII containing vesicles were successfully shown to carry VLDL from ER to Golgi in primary rat hepatocytes (95). Figure 3 depicts the details of VTV transport from ER to the Golgi apparatus in hepatocytes.

COP II Proteins

It is now well known that before its secretion into the blood stream, it is essential that the VLDL be transported from the ER to the Golgi. However, to date there are no sufficient studies based on which the extensive details of this transport can be delineated. Studies have indicated the involvement of coat protein complexes (COPs) that facilitate the transport of newly synthesized proteins and lipids between ER and Golgi (101,102).

In a series of studies carried out utilizing yeast genetic as well as biochemical experiments, it was shown that COPII were involved in the transport of newly synthesized proteins from the ER (101-104). Coat protein complex I (COPI) are involved in transport from Golgi to ER and such type of transport is called a retrograde transport (105,106). COPII is a set of cytosolic proteins that are capable of generating vesicles carrying VLDL from ER in the presence of ATP and GTP (102). It consists of Sar1, Sec23/24 and Sec 13/31(101,102,107). The first step of VLDL budding/exit from ER is activation of inactive GDP bound Sar1 into active GTP bound Sar1 by a guanine nucleotide exchange factor Sec12. Once activated, Sar1 then gets recruited on ER at specific sites from where budding of vesicles take place and is called as ER exit site (92,107-110). This is followed by binding of cytosolic factors Sec23 and Sec24 complex to Sar1, which results in sorting of VLDL for its packaging into specialized vesicles once the Sec 13/31 complex gets associated (111-113). Sec23 is known as GTPase activating protein while Sec24 helps in cargo selection and binding with SNARE proteins (114). Heterodimer Sec13/31 also helps in cargo selection and vesicle budding respectively (115). COPII complex is made up of these five proteins that help in budding of vesicles from ER (107). COPII vesicle formation is summarized as shown in Figure 4.

There is general consensus today that ER is the site where the first step of initial lipidation involved in VLDL assembly occurs. However, the site of final VLDL maturation, whether it takes place in the ER or post ER has been an area, where there have been contrasting reports. Certain studies have indicated that VLDL maturation

occurs in the ER (47,116-121), while some studies have suggested the Golgi lumen as the final site of maturation (122-125). Using rat hepatoma cells; McA-RH7777, it was shown that the second step of VLDL assembly where the further lipidation of the VLDL takes place occurs post ER (98,116). Cell-free systems were used in order to find out the details of the exit of apoB 100-lipoproteins from ER. These studies further showed that apoB100 enriched lipoproteins exit the ER in a COPII dependent process but independent of the state of lipidation and hence, the final size of the VLDL. This was contrary to the earlier published reports which suggested requirement of either specific factors or a COPII independent mechanism for transport of aopB100 lipoproteins that are larger in size (93). Further, the authors also showed that there was evidence of exposure of the domain/s of aopB100 in ER derived vesicles to the cytosol (98), similar to earlier studies (91), which suggested exposure of apoB100 domains to the cytosol when present in the ER and Golgi (74,126).

**VLDL secretion**

After the packaging of proteins and VLDL into their respective vesicles, the next step in secretory pathways is their transport from their site of synthesis (ER) to *cis*- Golgi apparatus (100,127,128). However, till date there are two schools of thoughts as far as site of VLDL maturation is concerned (123). The first thought suggests Golgi apparatus as the site of VLDL maturation (122-124,129) while the second supports ER as the site of VLDL maturation (47,116,118). Regardless of the site of VLDL maturation, VLDL

particles move to the Golgi apparatus from the ER where apoB100 undergoes further post-translational modifications like phosphorylation and glycosylation (100,129).

VTV fusion with Golgi

It is now well understood that proteins are involved in membrane fusion and that this process is critical for various processes that take place in the cells (130). Once VTV successfully exits the ER, the next phase is its fusion with the Golgi membrane where apoB, the protein component of VLDL, gets glycosylated and phosphorylated (125,131). Since, vesicle fusion is very critical in eukaryotes and the life's important processes are very much dependent on the orderly carrying of these fusion mechanisms by various proteins, it is very important to understand how the VTVs carry out this fusion process with Golgi (16,130,132).

Two different proteins have been identified to be involved in important but complementary functions in membrane fusion; soluble N-ethylmaleimide–sensitive factor (NSF) attachment protein receptor also known as SNAREs and Sec1/Munc18-like proteins (SM). Rothman and his coworkers discovered SNAREs. In a series of studies it has been shown that SNAREs are critical in membrane fusion. Targeting, docking and fusion of transport vesicles with specific acceptor membrane is reported to be facilitated by SNAREs (130,132-137). They are essential for directing the vesicles to their destination for the subsequent delivery of their cargo. In the absence of such machinery vesicles can fuse with other organelles randomly and disturb the orderly trafficking vital

for life (130). Hence, it becomes very imperative that we understand the various fusion mechanisms and the respective proteins involved in the fusion process.

In the study carried out by Rothman and group, which is now widely regarded as the SNARE hypothesis (137), it was postulated SNAREs to be either localized to vesicles as v-SNAREs or on target membranes as t-SNAREs. SNARE proteins are membrane proteins that serve as receptors for the NSF and soluble NSF attachment proteins (SNAP), and it was proposed that it forms a part of a complex that was involved in linking the exocytic vesicle to the plasma membrane (130,137). Based on structural studies carried out, it is known that every SNARE protein has a SNARE motif, which is considered as a characteristic of every SNARE protein. This motif is made of heptad repeats of around 60-70 amino acids (130,134). It is known that different sets of SNAREs located on vesicles and target membrane interact through SNARE motifs to form SNARE complex. For the docking of transport vesicles, one v-SNARE interacts with three t-SNAREs to form SNARE complex (138). Once SNARE complex has formed, individual SNARE motifs transform from a monomer unstructured state to a highly stable bundle of four coiled α helices conformation (132).

Sutton and Fasshauer's group reported an X-ray crystal structure for the SNARE complex. This complex was made up of syntaxin-1A, synaptobrevin-II and SNAP-25B. This was the structure of the SNARE complex involved in synaptic exocytosis (139). Further, Fasshauer and Sutton's group performed sequence alignment analysis studies

(138). The crystal structure studies (139) of the SNARE complex revealed the presence of a “0” layer or sometimes referred to as the iconic layer of amino acids found to occur at the center of the fusion complex embedded in the leucine-zipper layers of the fusion complex (139). A four-helix bundle that is highly twisted and parallel was revealed from this x-ray crystallography study. This layer of amino acids was highly conserved in all of the SNARE proteins investigated (138,140). This iconic or “0” layer at the center of the fusion complex showed the presence of one arginine (R) and three glutamine (Q) residues, based on which the same group further re-classified the SNAREs into: R-SNAREs that is localized to vesicles and Q-SNAREs that is localized to target membranes. The Q-SNAREs based on the presence of highly conserved glutamines are further classified as Qa-, Qb- and Qc-SNARE proteins (132,138,141). When one R-SNARE and three Q-SNAREs (Qa, Qb and Qc) interact, a SNARE complex is formed which is known as SNAREpins (138,142) and helps in the initiation of the fusion process by bringing the two membranes into close proximity. Further, the SNARE complex also can facilitate the fusion between two membranes (130,137). SNARE complex formation is depicted in Figure 5.

Because of the varying nature of the SNARE complex composition, this provides specificity in the variety of intracellular trafficking the SNAREs are involved (16,132). In previous work conducted in our laboratory it was shown that VTV are involved in transport of VLDL from ER to Golgi and its biogenesis and cargo selection occurs independently of other protein transport vesicles that exit the ER (95). It is likely that

VTV maintains the same specificity in choosing their SNARE proteins as compared to other ER derived vesicles. This idea is supported by previous work conducted in our lab, which shows that pre-chylomicrons transport vesicles (PCTV) utilize VAMP7 to form a complex with syn5, rBet1 and vti1a (95,143,144).

Investigation of the SNARE complex machinery responsible for VTV fusion with Golgi showed that sec 22b functions as v-SNARE, while syntaxin5, rBet1 and Gos28 protein serves as t-SNARE counterpart of SNARE complex. Interestingly, Ykt6 was not found to be involved as a v-SNARE in VTV, which serves as a v-SNARE in PTV fusion with Golgi along with Gos28, syn5 and rBet1. The Principle Investigator (PI) confirmed these studies by isolating the SNARE complex involved in VTV docking and fusion by developing an *in vitro* docking assay which allowed docking of VTV to Golgi at the same time inhibiting its fusion. After separating the complex by immunoprecipating it with sec22b specific antibodies, it was found that VTV specific SNARE complex comprises sec22b, syntaxin5, rBet1 and Gos28. These studies showed that VTV and PTV use different set of v-SNAREs, which confirms specificity to these vesicles. This set of studies carried out by the PI in hepatocytes and enterocytes established a physiological relevance of v-SNAREs in vesicular docking and fusion, as by inhibiting these SNAREs, vesicle docking and fusion is inhibited (100). Though the physiological relevance of SNAREs is established, clinical significance of SNARE proteins is still in its infancy.

Post Golgi Fusion of Vesicles with Plasma membrane

It has been shown that in enterocytes the post Golgi apparatus transport of chylomicron is mediated by vesicles which transport cargo to plasma membrane (PM) or basolateral surface (145). Cargoes are transported from Golgi apparatus to different destinations like ER, lysosome and PM and the network that helps in sorting and packaging of cargo is known as trans Golgi network (TGN) (146). The transfer of cargo from Golgi to ER is carried out by COPI complex, and the initiator of budding in this case is a small GTP binding protein Arf which gets activated by conversion of GDP to GTP (147,148). The TGN to PM carriers (TPC) are ill defined, and their protein coat has not been studied (146). However, hydrolase enzymes of lysosome are reported to transport in clathrin coat from Golgi body to Lysosome (136,146,149-152). In a study using purified Golgi apparatus and cytosol from HepG2 cells, Simon *et al* have established a cell free system to generate vesicles from Golgi to study the basolateral transport of sialylated VSV-G (vesicular stomatitis virus) protein and have concluded that Arf factor is required for the formation of vesicle along with GTP. They have also indicated role of protein kinase C in vesicle formation (146). However, involvement of specific (if any) vesicle in the export of VLDL from Golgi to PM has not been studied in detail till date (16).

**Dyslipidemia**

High level of TAG is a hallmark of metabolic syndrome and diabetes (153,154). As reported earlier dyslipidemia is a condition in which there is an increased concentration

of plasma lipoproteins like VLDL, LDL and small dense LDL particles (9,154,155). Also there is a reduction in concentration of HDL, which clears a high level of atherogenic lipopoprotein in dyslipidemia. Dyslipidemia is a major cause of insulin resistance (IR) (156) whereas hyperinsulinemia leads to excess production of VLDL (157-160). In IR state, insulin fails to supress producton of FA by lipolysis resulting in increased concentration of FAs (157,160). This leads to an increase in VLDL production. Increase in VLDL in turn increases TAG, which is a major risk factor of type 2 diabetes (T2D). Insulin also suppresses degradation of apoB, while in IR state more of apoB is secreted which leads to increased VLDL in plasma (153,154,161). IR also leads to increased lipogenesis which in turn increases VLDL production (162,163). In this way, this viscious cycle continues which results in metabolic syndrome. It is noteworthy that IR can also lead to liver steatosis (154,156,164). It can occur due to two reasons. First, in IR state, an increase in TAG translates into increased VLDL secretion (156). However, the amount of TAG in IR state cannot be balanced by VLDL secretion after a certain limit. This is true because an increase in TAG can lead to biogenesis of larger VLDL as compared to more VLDL and, since the size of VLDL cannot exceed a certain limit, excess of TAG remains in the liver (156,165,166). Secondly, increased amount of FAs in the liver can initiate an ER stress response which leads to degradation of apoB100 (156,167-169). These imbalances due to FA overproduction can lead to hepatic steatosis which can lead to serious conditions like nonalcoholic steatohepatitis (NASH) and ultimately leads to liver failure and hepatoclellular carcinoma (HCC) (170,171).

In order to control dyslipidemia one of the approach is to control hypertriglyceridemia. Higher secretion of atherogenic particles like VLDL accounts for hypertriglyceridemia. Understanding of the proteins and molecular signals involved in the secretion of VTV would offer an attractive target to control its secretion. In order to understand proteins involved in the biogenesis, secretion and transport of VTV, our lab has carried out a thorough proteomic profile of VTV (99). Two of the proteins revealed in this proteomic study that were found to be present in VTV are CideB and SVIP.

**CIDEB**

Cell death-inducing DNA fragmentation factor-alpha (DFFA)-like effector known as CIDE protein is a family of proteins that includes the following three molecules; CIDEA, CIDEB and CIDEC also known as fat specific protein 27 (Fsp27) (172). DFFA is a 45-kDa subunit and is one of the two subunits of the DNA fragmentation factor (DFF), the other being a 40-kDa subunit known as DFF40.They are involved in the caspase dependent apoptosis. DFF40 is involved in activating the caspase while the DFF45 is the subunit involved as an inhibitor of caspase (173-175). Cide proteins show sequence homology to the N-terminal of the DFF (174). Additionally, all the proteins in the Cide family also have the unique CIDE-C domain (172,176).

The involvement of CIDE proteins in apoptosis is well documented (175). In the recent years, the CIDE super family of proteins is reported to play a critical role in lipid metabolism (16,175,176). The tissue distribution of each of these protein varies (176).

In rodents Cidea is expressed in brown adipose tissue (BAT) (172,177), while in humans mRNA expression is found in white adipose tissues (WAT) (178), whereas Cideb is reported to be expressed predominantly in the liver, with lower levels in the kidney, small intestine and colon (172,179). Cidec is reported to be expressed in both BAT and WAT (177,180,181).

Ping Li and coworkers have done extensive studies regarding the role of both Cidea and Cideb in lipid metabolism and their possible involvement as therapeutic targets for the treatment of obesity, diabetes and liver steatosis (177,179,182-186). They reported that Cidea null mice exhibit lean phenotype and were resistant to diet induced obesity and diabetes (177). In this study it was reported that Cidea null mice showed enhanced lipolysis in BAT. When compared to wild type mice the null mice also showed higher energy expenditure and higher metabolic rate. Li and group also showed critical role of Cidea in development of hepatic steatosis (182). Another study showed that in adipose tissues Cidea is involved in the regulation of triglycerides (187).

Cideb, which is the focus of our study; as mentioned earlier, is expressed in higher levels in the liver. A detailed study was carried out to understand the role of Cideb in lipid metabolism and energy expenditure by establishing Cideb knockout or null mice (179). The adiposity index was found to be higher in Cideb knockout mice that were fed with high fat diet. Further, plasma TAG and NEFA levels in Cideb knockout mice were found to be lower both in the fed and fasting state compared to the wild type mice.

Additionally, it was also found in this set of studies that Cideb knockout mice showed improved insulin sensitivity. Overall, this study showed that Cideb plays an important role in regulating high fat diet induced obesity, and liver steatosis. This was reported by the authors to be achieved by reduced lipogenesis and increased energy expenditure (179).

In order to find out how lipid homeostasis is influenced by Cideb, the same group carried out further studies, which showed increased TAG and reduced VLDL secretion in Cideb null mice (183). Cideb-/- mice have been reported to show reduced secretion of VLDL particles, which are also small in size. Interaction of Cideb with apoB100 and apoB-48 was reported and was found to be important for enhanced VLDL lipidation (183). Further, the role of Cideb in the control of cholesterol homeostasis was shown using Cideb null mice, indicating a possible role of Cideb in the future management of cardiovascular and metabolic diseases (183). In a very recent study it was shown that though Cideb is detected in Golgi apparatus, in livers of Cideb knockout mice there was reduced accumulation of VLDL particles in Golgi apparatus (186). Further when another lipid droplet associated protein perilipin 2 (Plin2) was studied it showed higher levels in Cideb knockout mice livers. When Plin2 was knocked down in Cideb null mice this lead to a decrease in VLDL-TAG secretion as well as reduced lipid storage in livers of cideb null mice. It also led to restoration of VLDL lipidation. Plin2 was found to be expressed significantly higher in Cideb null mice livers and specifically in lipid droplets only

compared to Cideb which is expressed in lipid droplets, ER and Golgi fractions (186). However, involvement of cideB in VTV budding has not been studied yet.

**SVIP**

SVIP is a small (~ 9kDa) protein first studied by Masami *et al* as a novel adaptor protein of VCP/P97 a member of AAA (ATPase associated with diverse cellular activities) family (188). VCP/P97 takes part in various processes like formation of organelles, fusion of membranes and ubiquitin-dependent degradation of proteins (189,190). It has multiple adaptors to carry out diverse processes. GP78 is one of the adaptor protein of VCP/P97 and together they carry out an important function like ERAD (188). SVIP is a cytosolic protein and known to inhibit ERAD (190). It forms a complex with VCP/P97, thus rendering VCP/P97 unavailable for the binding with GP78 to undergo ERAD (190). SVIP possesses sites for myristoylation, a process that promotes recruitment of cytosolic proteins on surface of organelle membranes (188). Recent study in our lab has shown the presence of SVIP in VTV (99). However, role of SVIP in VLDL transport has not been studied till date.

**Rationale for the current study**

Higher concentration of VLDL is commonly associated with dyslipidemia that plays a key role in development of CVDs. Overproduction of VLDL attributes to higher concentration of LDL in plasma, which leads to atherosclerosis a condition that opens the door for CVDs. VLDL is synthesized in the ER of hepatocytes and then it gets

transported to Golgi apparatus for its eventual secretion from hepatocytes. The transport of VLDL from ER to Golgi apparatus is a very crucial step and considered as the rate-limiting step in the secretion of VLDL from hepatocytes. Our group has established that novel vesicles that we have identified as VTV mediate the transport of VLDL from ER. VTVs are unique and differ from PTV, the vesicles that transport protein. Both PTV and VTV utilize COPII complex; however, they differ in their size, cargo and proteome that suggest some crucial differences between the two vesicles. In order to attain a unique proteome, VTV must differ from other ER derived vesicles in its sorting machinery to pack cargo into VTV and also in the composition as the larger size of VTV suggests involvement of additional proteins along with COPII. VLDL sorting into VTV and VTV biogenesis are the two important steps for VLDL secretion. VLDL sorting involves signals/proteins inside the ER while the biogenesis of the VTV involves cytosolic proteins like COPII complex. Therefore, some additional and unique proteins must be involved in the processes, which make VTV a unique vesicle. Understanding the proteins involved in VLDL sorting and VTV biogenesis will offer an attractive tool to control VLDL secretion. In order to identify the proteins involved in VLDL sorting the approach we followed was to find proteins interacting with apoB 100.

We found that CideB interacts with apoB100 which made it a candidate to study VLDL sorting. Furthermore, our lab has recently carried out a detailed analysis of the proteins present in VTV and the report supported the findings and stated CideB and SVIP as important proteins in VTV biogenesis. Also myristic acid (MA) is known to increase

apoB100 secretion from hepatocytes. Interestingly, we found that SVIP has sites for myristoylation. We also found that SVIP interacts with Sar1, the protein involved in the initiation of COPII based vesicle biogenesis. By identifying proteins interacting with Sar1 it will give us an idea of the proteins involved in VTV biogenesis and attributing VLDL a larger size as compared to COPII dependent PTV.

**Overall hypothesis**

We hypothesize that different proteins facilitate the sorting and packaging of VLDL into the VTV and are involved in the biogenesis of VTV.

CideB protein is required for the biogenesis of VTV

In the first phase of the project, we hypothesized that different proteins facilitate the sorting and packaging of nascent VLDL into VTVs and nascent proteins into protein transport vesicles (PTVs). The first objective was to determine presence of CideB in VTV and other ER derived vesicles and establishing CideB interaction with apoB100. The second objective was to understand the functional role of CideB in VTV budding from ER, hence VLDL secretion.

Specific aim of the first project involves:

1. Determine the presence of CideB in VTV, PTV and PCTV.
2. Determine the interaction of CideB with apoB100.
3. Examine interaction of CideB with COPII complex components.

4. Determine how blockade of CideB may affect VTV budding.
5. Examine the effect of silencing of CideB on VTV budding using siRNA approach.

Myristic acid dependent recruitment of SVIP protein on hepatic ER membrane regulates VTV formation

In the second phase of the project we hypothesized that SVIP forms an intricate COP II coat to make larger vesicle i.e. the VTV. Since our overall hypothesis was that different proteins are involved in the sorting of VLDL into the VTV and biogenesis of the VTV, our first objective was to determine interaction of SVIP with Sar1. As MA is known to increase apoB100 secretion and SVIP is known to have sites for myristoylation we will also study the effect of MA on SVIP recruitment on ER, if any. In the second objective we will determine the functional role of SVIP by silencing SVIP and study the effect on VTV budding. We will also study effect of MA recruitment of SVIP on ER in context of VTV budding.

Specific aim of the second project involves:

1. Determine presence of SVIP in VTV.
2. Study SVIP recruitment on ER followed by MA treatment and its effect on Sar1 recruitment on ER.
3. Examine interaction of SVIP with Sar1 and other COPII components.
4. Determine the effect of silencing of SVIP on VTV generation.
5. Determine the effect of MA on the VTV generation.

**Figures**

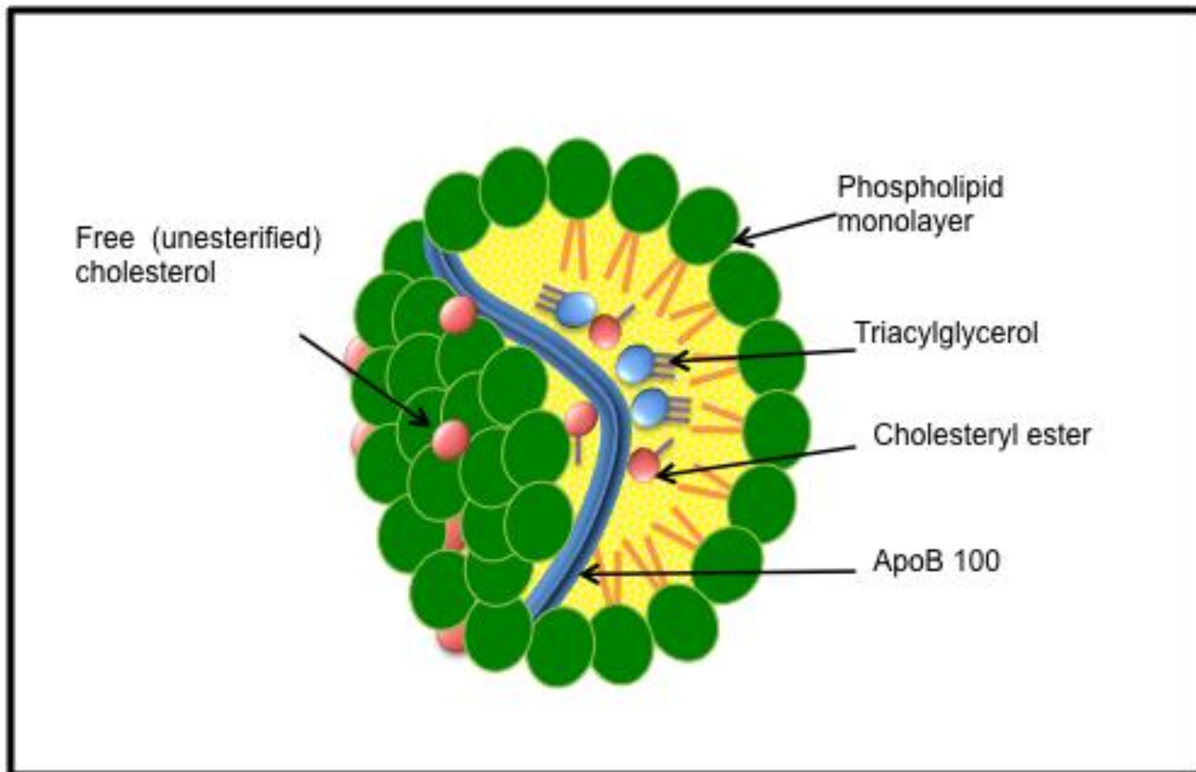


**Figure 1: Structure of VLDL**

The core of VLDL is made up of nonpolar lipids like TAG and cholesteryl ester. The outer surface of VLDL is made up of free cholesterol and phospholipids monolayer that interacts with plasma and the neutral lipids present in the core. ApoB100 is a large protein, which binds to the lipid core by its hydrophobic domain while the hydrophilic part of apoB100 remains towards the plasma.

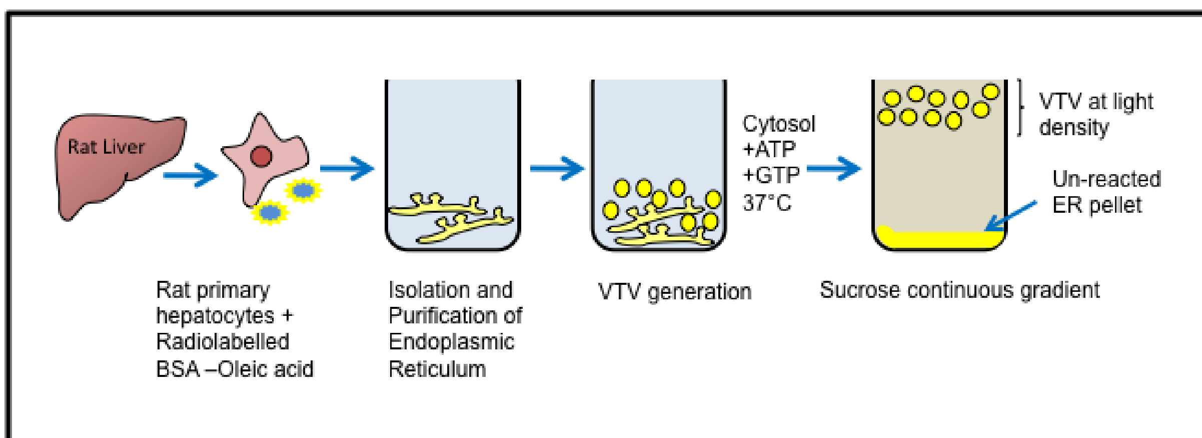


**Figure 2: Cell free *in vitro* VTV budding assay**

It is a detailed assay that involves isolation of rat primary hepatocytes followed by its incubation with radiolabelled BSA-oleic acid complex. This is followed by isolation of radiolabelled ER by sucrose step gradient methodology. Next, radiolabelled ER is utilized for the generation of VTV at 37$^{o}$C temperature, which require cytosol, ATP regenerating system, and GTP. VTV can be isolated from the reaction mixture by centrifugation. Due to their light density of VLDL, VTV floats on the reaction mixture while the unreacted ER gets pelleted. The most TAG counts remain in the light density of the sucrose gradient, which is an expected place for VTV.

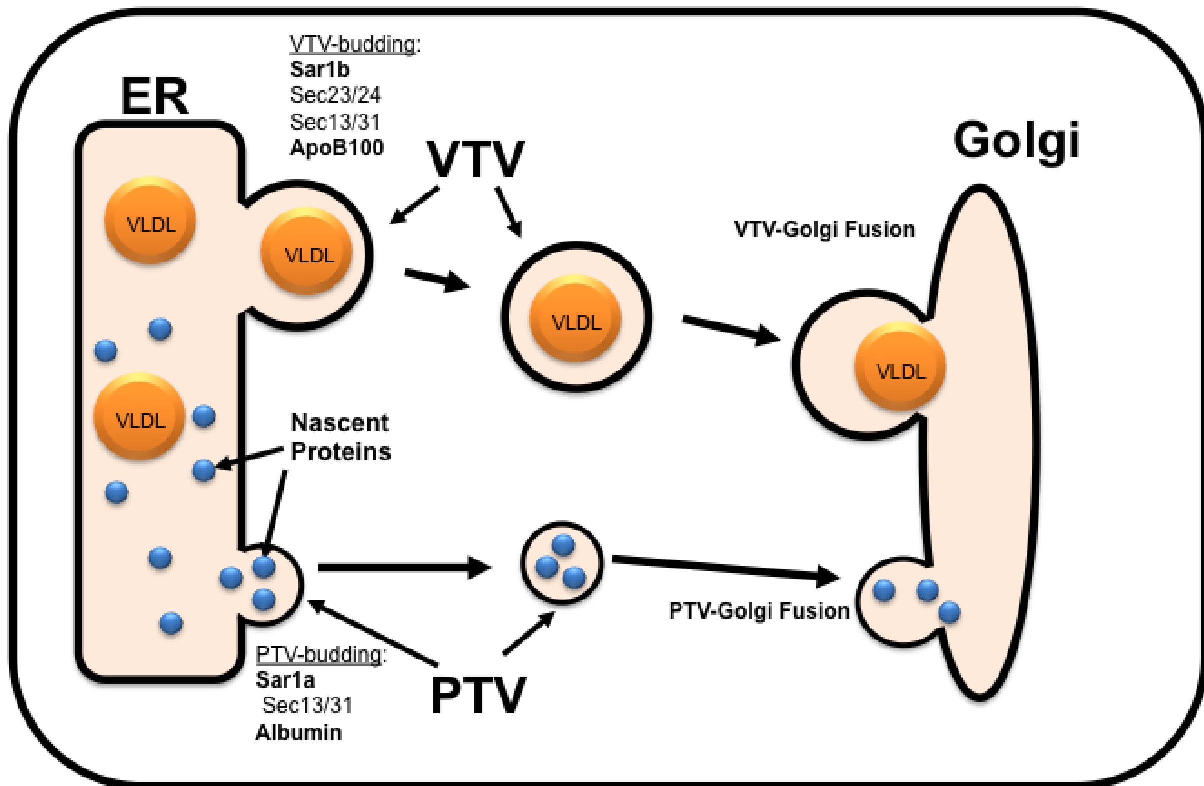


**Figure 3: Transport of VLDL from ER to the Golgi apparatus in hepatocytes**

VLDL assembly takes place in the ER lumen. Next, VLDL is packed into specialized vesicle, the VTV for its subsequent transport to Golgi apparatus. VTVs exit the ER membrane and reach Golgi lumen and fuse with Golgi lumen in order to transport VLDL to Golgi apparatus. Proteins involved in VLDL-selection into VTV and VTV-Golgi fusion are yet to be characterized. On the other hand protein transport vesicles (PTVs) transport nascent proteins from the ER to the Golgi lumen. Both VTVs and PTVs require COPII proteins however they differ in the Sar1 homologs as shown in the figure. VTVs and PTVs differ in their size and cargo proteins and they also utilize different sets of SNARE proteins for their fusion with Golgi lumen

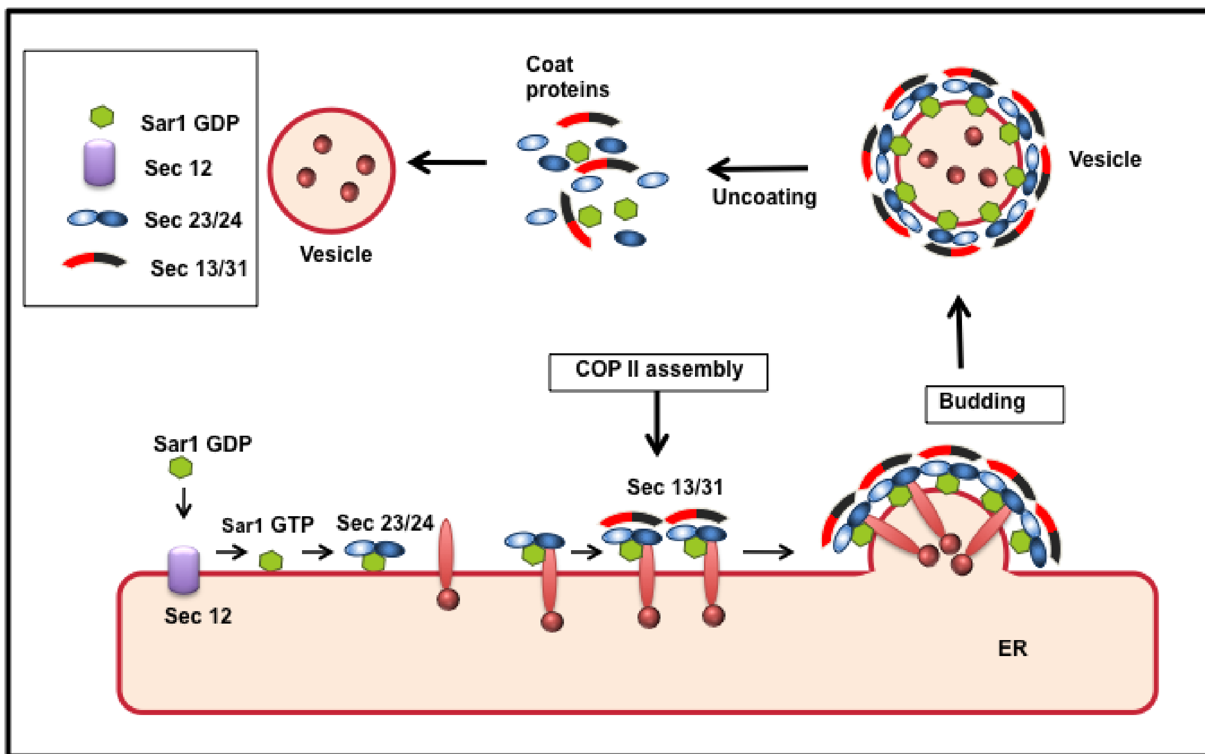


**Figure 4: Schematic diagram summarizing COPII vesicle formation**

The first of step of VLDL budding from ER lumen is activation of inactive GDP bound Sar1 into active GTP bound Sar1 by a guanine nucleotide exchange factor Sec12. After activation Sar1 gets attached to specific sites on ER surface known as ER exit sites. The next step is attachment of Sec23 and Sec24 complex to Sar1 resulting into a pre-budding complex. The final step is recruitment of Sec 13/31 on the pre-budding complex that results in the budding of VTV from ER lumen.

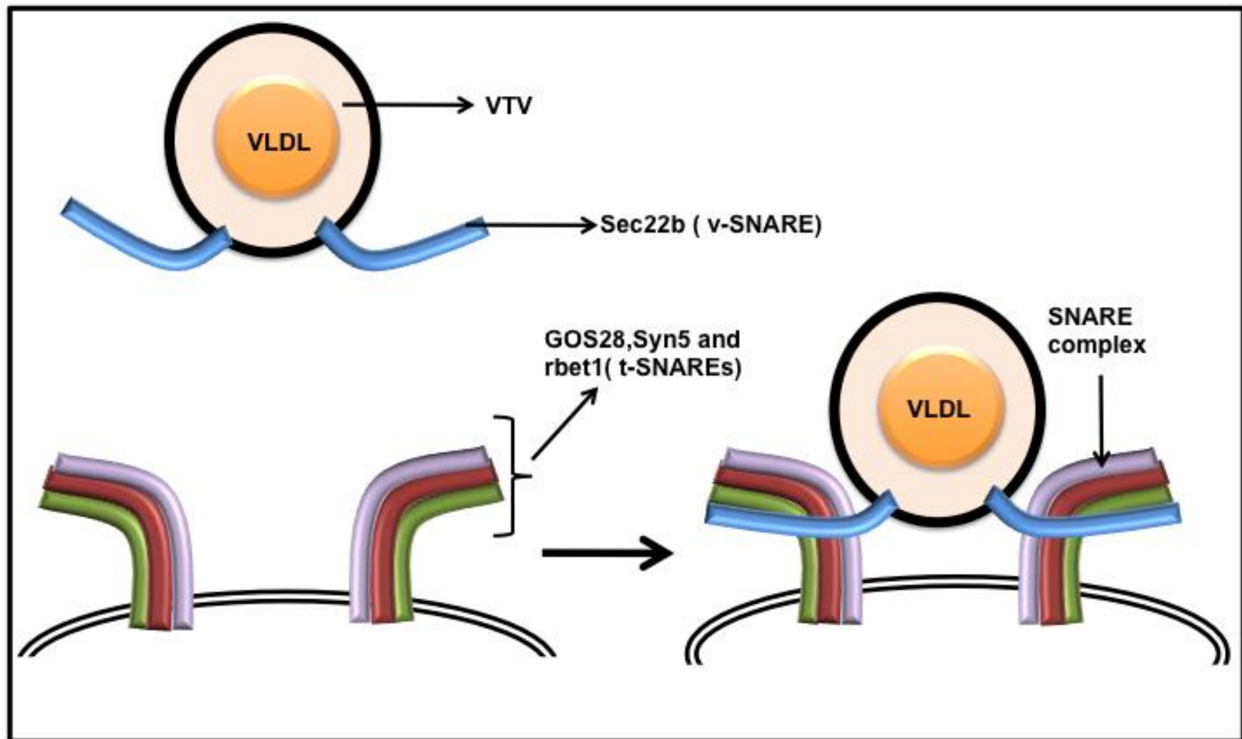


**Figure 5: SNARE Complex Formation**

SNARE proteins are involved in the facilitation of targeting, docking and fusion of transport vesicles with their respective target membrane. SNARE proteins are present on transport vesicles as v-SNARE and on target membranes as t-SNARES. As discussed in the text, SNARE have been re-classified as R-SNAREs (transport vesicles) and Q-SNARES (target membrane) based on the presence of arginine (R) and glutamine (Q) amino acids in the center of the fusion complex. Sec22b is identified as v-SNARE while GOS28, syntaxin 5 and rBet1 were identified as t-SNARE proteins involved in the VTV fusion with Golgi apparatus.

**Table 1: Differences between VLDL Transport Vesicles and Pre-Chylomicron Transport Vesicles**

| VTV | PCTV | References |
|---|---|---|
| Transports VLDL from ER to Golgi in Liver | Transports chylomicron from ER to Golgi in small intestine | (95,98,191) |
| VTV-budding requires Sar1 | PCTV-budding requires L-FABP | (95,98,191,192) |
| GTP is required for VTV-budding | GTP is not required for PCTV-budding | (95,98,191) |
| ≈100 nm -120 nm in size | ≈142 nm -500 nm in size | (95,191) |
| VAMP7 is absent on VTV | VAMP7 is present on VTV | (95,191) |
| Sec22b is present on VTV | Sec22b is absent on VTV | (95,191) |
| VTVs are denser than PCTV consistent with their proportionally smaller amount of TAG | PCTVs are of lighter density than VTVs consistent with their proportionally higher amount of TAG | (95,191) |

# CHAPTER TWO: CIDE B PROTEIN IS REQUIRED FOR THE BIOGENESIS OF VTV

## Introduction

Intracellular transport of newly synthesized lipoproteins from the ER to the Golgi is of utmost importance because abnormalities associated with this transport step lead to the pathogenesis of various metabolic diseases (8,193). The liver and the small intestine are two organs that primarily produce lipoproteins VLDL and chylomicrons, respectively. In liver, the biogenesis of VLDLs occurs in the ER and this process is facilitated by microsomal triglyceride transfer protein (MTP) (30,38,43,46,47,82,87,118). Once synthesized in the ER lumen, nascent VLDLs are exported to the Golgi where several essential modifications occur to VLDL particles (16,123,125,131,194). Their structural protein, apolipoprotein B100 (apoB100) gets further glycosylated and phosphorylated (125,131,194). Moreover, it has been proposed that additional triglycerides are added to the nascent VLDL in the Golgi lumen (98,122-124). Based on a number of biochemical and histological data, it has been suggested that Golgi is the site of VLDL maturation, however, this supposition is still a subject of debate (98,116,120-125,131,194). Regardless of their maturation site, the transport of nascent VLDL particles from the ER to the Golgi is imperative and determines the rate of VLDL-secretion from the liver (16).

Transport of nascent VLDL from the ER is mediated by a specialized vesicle, the VTV, which buds off the hepatic ER membranes (95). Even though, these vesicles have been shown to be morphologically and biochemically different from classical ER-derived

PTVs, their biogenesis requires COPII proteins (92,101,195-199). COPII proteins consist of five cytosolic proteins Sar1, Sec23-Sec24 and Sec13-Sec31 (102,200). These five proteins have been shown to be sufficient to select cargo proteins and facilitate vesicle formation from the ER membrane. Sar1, a COPII component that initiates vesicle biogenesis, has two mammalian homologs – Sar1a and Sar1b (111). Several studies have shown that Sar1b is involved in lipoprotein trafficking and secretion because mutations in Sar1b lead to chylomicron retention disease (201-205). The definitive role of Sar1 in VLDL-exit from the hepatic ER has been evident from our and others data. Using H89, an inhibitor of Sar1-recruitment to the ER membrane, we found a significant reduction in VTV formation (95). Over-expression of dominant negative Sar1 (Sar1T39N) in rat hepatoma cells significantly blocks ER-to-Golgi transport of apoB100, a core component of VLDL (98).

That both nascent VLDL and secretory proteins exit the same ER in two different kind of vesicles which require the same initiator for their genesis, Sar1, and other COPII components, has been shown in many reports (95,98). These observations, however, raise the possibility of potential involvement of an additional factor(s) that is required for either cargo-selection or vesicle biogenesis. It has been demonstrated that several cargoes require additional cytosolic proteins for their exit from the ER (21,95,144,191,192,206,207). For example, the formation of pre-chylomicron transport vesicle (PCTV) from the intestinal ER requires four cytosolic proteins in addition to five COPII proteins (207). These nine proteins form a pre-budding complex that leads to the

formation of the PCTVs, which contain pre-chylomicrons and are able to fuse with and deliver their cargo to the Golgi lumen (192,207). It is possible that the generation of PCTV requires extra proteins because of its very large size and these additional proteins facilitate the formation of a larger cage. However, the requirement of protein(s) other than COPII is not limited to larger cargoes or their carrier vesicles, smaller cargoes such as amyloid precursor protein needs additional cytosolic factor(s) for their exit from the ER (208). These findings suggest that proteins other than COPII are required either for sorting and packaging of specific cargoes into vesicles or for the genesis of specialized vesicles that transport specific cargoes to the Golgi.

Because the size of the VTV (100 -120 nm) is larger than the size of a normal COPII vesicle (~ 55 – 70 nm), it is likely that generation of the VTV requires supplementary protein(s). We recently carried out a detailed proteomic analysis of the VTV to find out proteins present exclusively in VTV (99). Of several important proteins, which are not present in other ER-derived vesicles such as PCTV and PTV, one protein was identified as cideB (cell death-inducing DFF45-like effector b) (99). Interestingly, cideB has been shown to be involved in secretion of VLDL (186,209). CideB belongs to CIDE family, which includes cideA, cideB and cideC also called as Fsp27 in mice and are reported to play important role in lipoprotein metabolism (184). CideB is expressed in liver and kidney and is associated with maturation and secretion of VLDL and apoptosis (176,184). It has been reported that cideB$^{-/-}$ mice have reduced secretion of VLDL

particles, which are also small in size (209). However, the presence of cideB or its functional role in VTV biogenesis has not been investigated so far.

In the current study, we sought to identify the role of cideB in VTV budding. We found that cideB interacts with apoB100 in the ER whereas it does not interact with albumin, which is excluded from the VTV. Our data suggest that cideB interacts with Sar1 at the ER level. The interaction of cideB with VLDL-cargo protein, apoB100, and COPII-component, Sar1, indicates its role in VTV biogenesis and thus VLDL-exit from the ER. We further examined the effect of blocking the function of cideB or cideB knockdown on VTV generation from the ER. We report that cideB forms a specialized complex with COPII proteins that leads to the biogenesis of the VTV.

## Materials and methods

### Reagents

[$^3$H] oleic acid (45.5 Ci/mM) was obtained from PerkinElmer Life Sciences (Boston, MA). Reagents used for immunoblotting were purchased from Bio-Rad, Corp (Hercules, CA). ECL (enhanced chemiluminescence) reagents were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Protease inhibitor mixture tablets were obtained from Roche Applied Science (Indianapolis, IN). Albumin was purchased from Sigma Chemical Co. (St. Louis, MO). Other biochemicals used were of analytical grade and were purchased from local companies. Sprague–Dawley rats, 150–200 g, were obtained from Harlan (Indianapolis, IN). All procedures involving animals were conducted according to the

guidelines of the University of Central Florida's Institutional Animal Care and Use Committee (IACUC) and strictly following the IACUC approved protocol.

Antibodies

Goat polyclonal antibodies to cideB, calnexin, Ykt6, GOS28; rabbit polyclonal antibody to syntaxin 5, Sec13, Sec23, Sec31 and Sec24, goat anti-rabbit IgG Texas Red and bovine anti-goat IgG-fluorescein isothiocyanate conjugated antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-Sar1 antibodies were generated commercially and have been described previously (95). Rabbit polyclonal antibodies against rat VAMP7 (vesicle-associated membrane protein 7; amino acids 105–123) were described earlier (143). Mouse monoclonal antibodies to rBet1, and membrin were procured from StressGen (Vancouver, Canada). Rabbit anti-rat albumin antibody was purchased from Bethyl Laboratories, Inc. (Montgomery, TX) and rabbit polyclonal anti-apoB antibodies were a gift from Dr. Larry Swift (Department of Pathology, Vanderbilt University, Nashville, TN, USA). Rabbit anti-goat IgG and goat anti-rabbit IgG conjugated with agarose beads were purchased from Sigma Chemical Co. (St. Louis, MO).

Primary hepatocyte culture

Primary hepatocytes were isolated from adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN). Hepatocytes were isolated using collagen perfusion as described previously (210,211). Briefly, liver was perfused with Kreb's buffer (121 mM

NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 2 mM $MgSO_4$, and 1.2 mM $KH_2PO_4$;pH7.2). This was followed by perfusion with type II collagenase (Worthington Biochemical Corp. Lakewood, NJ). Cell suspension was obtained in RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 1µM dexamethasone (Sigma Chemical Co. St. Louis, MO), 100 nM insulin (Sigma Chemical Co. St. Louis, MO), 5%FBS (Life Technologies, Grand Island, NY), 1% penicillin streptomycin (Sigma Chemical Co. St. Louis, MO). Cells were seeded on a 60 mm collagen coated dish (BD Biosciences, San Jose, CA) with the density of $3x10^6$ cells per plate. After 4 hours, unattached cells were removed and fresh media was added followed by overnight incubation.

Preparation of cell extract

Rat hepatocytes were lysed using RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with protease inhibitor. Lysed cell extract was centrifuged at 13,000x g for 15 minutes. Supernatant obtained was used to determine protein concentration.

SDS-PAGE and Immunoblot analysis

Concentration of protein in ER and whole cell lysate was determined by Bradford method (95). Protein samples were separated by SDS-PAGE followed by transblotting onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Detection of protein was done by ECL western blot detection reagent (GE Healthcare Life Sciences, Pittsburgh, PA) and autoradiography film (MIDSCI, St. Louis, MO).

Preparation of radiolabeled hepatic ER and cis- and trans- Golgi

ER labeled with [$^{3}$H]-triacylglycerol (TAG) was prepared from rat liver using the same method as we have described previously (95). Briefly, primary hepatocytes in buffer B (136 mM NaCl, 11.6 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 7.5 mM KCl, 0.5 mM dithiothreitol; pH 7.2) were incubated with BSA-bound [$^{3}$H]-oleate (100 µCi) for 35 minutes at 37 °C and washed twice with 2% BSA in PBS to wash the excess of [$^{3}$H]-oleate. Cells were then homogenized in 0.25 M sucrose in 10 mM Hepes, 50 mM EDTA and protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) in a Parr bomb at 1,100 psi for 40 minutes followed by isolation of ER, *cis*- and *trans*-Golgi in a sucrose step gradient (95,100,191).

Preparation of hepatic cytosol

Hepatic cytosol was prepared by following the same method as described previously (95). After washing with Kreb's buffer cells were washed in cytosol buffer (25 mM Hepes, 125 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM DTT and protease inhibitors; pH 7.2) and homogenized using Parr bomb at 1,100 psi for 40 minutes. This was followed by centrifugation at 40,000 rpm for 95 minutes (Beckman rotor 70.1 Ti). Supernatant was dialyzed overnight against ice-cold fresh cytosol buffer and concentrated using centricon filter (Amicon, Beverly, MA) and ultra-filtration membrane (Millipore, Billerica, MA) with a cut-off of 10 kDa to a final concentration of protein to 17 mg/ml.

In vitro VTV formation

The *in vitro* VTV formation was carried out as established previously in our laboratory (95,99,100). In brief, ER having [$^3$H]-TAG (500 μg) was incubated at 37 °C for 30 minutes with hepatic cytosol (1 mg prot.), an ATP-regenerating system, 5 mM $Mg^{2+}$, 5 mM $Ca^{2+}$, 5 mM DTT, 1 mM GTP, 1 mM E600. Reaction mixture volume was adjusted to 500 μl by addition of transport buffer (30 mM Hepes, 250 mM sucrose, 2.5 mM MgOAc, 30 mM KCl; pH 7.2). Next, reaction mixture was placed on a sucrose continuous gradient made from 0.2 and 2.1 M sucrose respectively and centrifuged using Beckman rotor SW41 at 25,900 rpm for 2 hours at 4 °C resulting in resolution of VTV in lighter fractions. Fractions (500 μl) having VTV were separated from sucrose continuous gradient.

Measurement of radioactivity

Radioactivity associated with [$^3$H]-TAG was measured in terms of dpm by using Tri-Carb 2910 TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Shelton, CT) (95,191).

Co-immunoprecipitation

ER membranes (250 μg) were solubilized in ice cold PBS containing 2% (v/v) Triton X-100 (Fisher Scientific, Pittsburgh, PA) at 4 °C for 15 minutes. Next, rabbit anti-apoB100 antibodies were added and incubated for 4 hours at 4 °C. Similarly, parallel experiments

were performed with goat anti-cideB and rabbit anti-albumin. After 4 hours, either anti-goat or anti-rabbit IgGs bound to agarose beads were added and incubated overnight at $4^{o}$C. Beads bound to immune complexes were washed 12 times with ice cold PBS (95,207).

Effect of antibody treatment on VTV budding

ER containing [$^{3}$H]-TAG (450 µg protein) was incubated with same amount of indicated (figure legends) antibody or pre-immune IgG for 1 hour at 4 $^{o}$C as previously described (207). The ER was washed with cold 0.1 M sucrose in Hepes buffer to remove unbound antibody. The ER-pellet was re-suspended in transport buffer (30 mM Hepes, 250 mM sucrose, 2.5 Mm MgOAc, 30 mM KCl; pH7.2) and used in *in vitro* VTV-budding assay.

Transfection with siRNA

Rat primary hepatocytes were transfected with cideB siRNA (Silencer select Pre-designed SiRNA, Life Technologies, Grand Island, NY). The sequence of siRNA was 5'CAUGAGCUGCGAUUUUCAATT3'. Transfection was carried out by lipofectamine by following the method according to manufacturer's protocol (Life Technologies, Grand Island, NY.

Immunochemistry

Primary rat hepatocytes were plated on 22 mm round cover slips coated with collagen type I (BD Biosciences, San Jose, CA). Cells were washed three times with PBS, fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 minutes and permeabilized with 0.2% Triton-X100 (Fisher Scientific, Pittsburgh, PA) for 10 minutes at room temperature. After washing three times with PBS, cells were blocked with 5% BSA solution in PBS for 30 minutes at room temperature. Cells were then double labeled with goat anti-cideB (1:100) and either rabbit anti-apoB100 (1:100) or rabbit anti-calnexin antibodies (1:100) for 1 hour at room temperature and washed three times with PBS. Next, cells were incubated in dark with species-specific secondary antibody, bovine anti-goat IgG-FITC (1:500) and goat anti-rabbit IgG–Texas Red (1:500) for 45 minutes at room temperature. After washing with PBS, cells were mounted on glass slide using mounting media (Electron Microscopy Sciences, Hatfield, PA) and visualized using Zeiss Spinning Disk Confocal microscope and velocity image analyzer software.

Immunoelectron microscopy

In an attempt to examine the localization of cideB on VTVs by immuno-electron microscopy, we adopted the negative-staining approach and followed the same methodology for immuno-gold labeling of the VTVs as described previously (99,100,144). In brief, VTVs adsorbed on formvar-carbon-coated nickel grids were

incubated with 10% (w/v) BSA containing either anti-rabbit pre-immune IgG (1:100) or rabbit polyclonal anti-cideB antibodies (1:100) for 4 hours. Samples were washed with PBS and incubated with anti-rabbit IgG (1:50) conjugated with 15 nm colloidal gold. After washing with PBS, samples were subsequently fixed in 1% (w/v) glutaraldehyde in PBS for 10 minutes, stained with 0.5% aqueous uranyl acetate for 1 minute, and examined at 12,000x magnification.

Statistical analysis

Data were compared using a one-way analysis of variance (ANOVA) using GraphPad software (GraphPad Prism 5 Software for Mac OS X version).

## Results

CideB is present in hepatic ER, Golgi, cytosol and VTV

Our first goal was to determine the distribution of cideB in various subcellular organelles in primary hepatocytes. To achieve this, we separated the ER, Golgi and cytosol from primary hepatocytes. Since the purity of sub-cellular fractions isolated from primary hepatocytes is considered crucial for the success of our *in vitro* assays, we carried out western blotting utilizing organelle specific protein markers for ER and Golgi to establish their purity as reported previously (95,100). Our results show that ER did not contain recognizable GOS28, a Golgi marker protein whereas Golgi was free from calnexin, an ER marker protein as shown in Figure 6. Both ER and Golgi membranes were found to

be devoid of Rab11, an endosomal/lysosomal marker protein (data not shown). After confirming that our sub-cellular organelles are of adequate purity, we performed a pre-established *in vitro* VTV-budding assay to prepare VTVs, which were purified and characterized as we reported earlier (95). To make sure that our vesicular fraction contains *bona fide* VTVs, we probed for VTV markers, Sar1 and apoB100. As shown in Figure 6, both proteins were concentrated in VTV fractions, suggesting that we have adequate VTV fractions to perform further analysis. To examine that cideB is present in the VTV, ER, Golgi, and cytosol, we performed western blotting using specific cideB antibodies. The data presented in Figure 6 show that hepatic ER, Golgi and cytosol contain cideB, which is consistent with previous reports (95). However, cideB was found to be the least in cytosolic fraction (Figure 6). Interestingly, we observed that cideB is concentrated in VTV fractions as compared with their parent membrane, hepatic ER (Figure 6).

To illustrate the localization of cideB to the VTVs morphologically, we carried out immunogold labeling of cideB on the VTVs and probed the results by electron microscopy using the negative-staining technique. As shown in Figure 7 (panel II), cideB is localized on the surface of the VTVs as established by immunogold labeling. By contrast, control experiments using pre-immune IgG displayed no immunogold labeling (Figure 7, panel I). Together, these biochemical and morphological data strongly suggest that cideB is not only present on the surface of the VTV but is concentrated in the VTVs.

CideB is not present in ER derived PTV and PCTV

Our next aim was to find out if cideB is present in other ER-derived vesicles such as PTVs that contain nascent secretory proteins and PCTVs, which transport nascent lipoproteins of intestinal origin, the pre-chylomicrons (21,191). We isolated and purified these vesicles as discussed previously (143). Our data reveal that cideB is not present in either PTV or PCTV (Figure 8); however, we observed a strong band of cideB in the VTV fractions (Figure 8) indicating that cideB is present in VTVs only but not in other ER-derived vesicles. When we probed for PTV marker protein, albumin (a hepatic secretory protein), we found a strong band of albumin in PTV fraction. In addition, we immunoblotted for VAMP7, which is an endosomal or post-Golgi protein but uniquely present in ER-derived PCTVs (144). As expected, VAMP7 is enriched in PCTV fraction (Figure 8); however, both VTV and PTV do not have identifiable VAMP7 (Figure 8), which is consistent with previous studies (95). Taken together, these data suggest that cideB is specifically present in the VTVs.

CideB interacts with apoB100 but not with albumin

Since both PTV and VTV are budding off the same ER at the same time and cideB is not present in the PTV but is enriched in VTV, we questioned whether it interacts with albumin (PTV cargo protein) and apoB100 (a VTV cargo protein) at the ER level. To address this issue, we carried out co-immunoprecipitation experiments utilizing ER membranes solubilized in 2% triton-X100 and specific cideB antibodies. Our results

demonstrate that cideB co-immunoprecipitates apoB100 but not albumin, however, albumin is present in the ER (Figure 9). These findings suggest that cideB specifically interacts with VTV cargo protein but not with PTV-cargo indicating its potential role in VLDL transport from the ER to the Golgi.

To substantiate our observations pertaining to cideB-apoB100 interaction at the ER level, we sought to provide additional morphological evidence using confocal microscopy. We used double-labeled immunofluorescence technique. As can be seen in Figure 10, cideB co-localizes with apoB100. Middle panel of Figure 10 clearly shows the co-localization of apoB100 with cideB in the characteristic reticular structure of the ER. Interestingly, we observed punctate co-staining most likely in VTVs because this is characteristic staining of the ER-derived vesicles (middle panel, Figure 10; arrowheads). To show that this interaction occurs at the ER level, we used an ER marker, calnexin. The data presented in the lower panels of Figure 10 display that cideB co-localizes with calnexin. Together, these data confirm that the interaction between cideB and apoB100 is specific and occurs at the level of ER.

CideB interacts with COPII components

Since the VTV originates from hepatic ER membrane in a COPII-dependent fashion and cideB is specifically present in the VTV, it is likely that cideB may interact with VTV-coat proteins i.e. COPII proteins. Another reason for this assumption is that VTVs are larger in size than the typical COPII-coated PTVs, suggesting that the assembly of VTV-coat

may require additional protein(s) to form a larger/modified COPII-cage. To find out whether cideB interacts with COPII proteins, we performed co-immunoprecipitation experiments. Since all five COPII proteins (i.e. Sar1, Sec23, Sec24, Sec13 and Sec31) are cytosolic, we used hepatic cytosol anti-cideB antibodies in our co-immunoprecipitation assays.

In order to have high confidence in our results, we re-probed the same membrane with different antibodies against each of COPII proteins. The immunoblotting data presented in figure 4 suggest that cideB strongly interacts with Sar1 and Sec24, however, its binding with Sec23 was minimal. Also, we did not observe a strong signal for cideB interaction with Sec13 and Sec31 (Figure 11). These data suggest that cideB interacts with select COPII components (Sar1 and Sec24). Interaction between cideB and Sec24 indicates that these two proteins form a heterodimer, which in turn facilitates the assembly of an intricate COPII-coat necessary for VTV-formation.

Blockade of CideB reduces VTV formation

That cideB is specifically present in ER-derived VTVs and its interaction with VTV-cargo and VTV-coat proteins raise the possibility of its active involvement in the process of VTV biogenesis and thus, ER-exit of nascent VLDL particles. To determine whether cideB has some role in VTV-budding from hepatic ER, we first decided to examine the effect of blocking cideB using specific antibodies on VTV-budding. To accomplish this, we performed an *in vitro* VTV-budding assay that we have standardized previously (95).

To block cideB on the ER, [$^{3}$H]-TAG containing hepatic ER membranes were pre-incubated at 4$^{o}$C with equal amount of either control IgG or anti-cideB antibodies. Post-antibody treatment, excess antibodies were removed by washing. Since cytosol also contains little amount of cideB, we treated cytosol with anti-cideB antibodies or the pre-immune IgGs (control) bound to agarose beads prior to budding assay and the excess antibodies were removed. Anti-cideB antibody treated ER membranes and cytosol were used in VTV-budding assay (see Experimental Procedures section). As presented in Figure 12, the treatment of hepatic ER with anti-cideB antibodies significantly inhibited VTV generation. However, IgG treatment did not have any effect on VTV-budding activity (Figure 12). These results show that cideB blockade results in reduced VTV formation suggesting an active role of cideB in VTV biogenesis.

It is, however, possible that antibody treatment results in non-specific inhibition of VTV-budding process due to a number of factors. To rule out the possibility of non-specific inhibition, we treated the ER and cytosol with a variety of antibodies against proteins that are known to be present on hepatic ER and involved in ER-to-Golgi transport of proteins and lipoproteins. We used antibodies against syntaxin5, rbet1, ykt6 and membrin, which have been well characterized to be present on hepatic ER (95). We followed exactly the same protocol of antibody treatment as we used for cideB antibody treatment. Our data reveal that the treatment with each of these antibodies did not have any effect on VTV formation (Figure 13) suggesting that the blocking effect of cideB on VTV generation was specific. Moreover, we treated hepatic ER with VAMP7, which is

required for the generation of PCTVs from small intestinal ER (144). As shown in Figure 13, there was no decrease in VTV budding as compared to control when VAMP7 antibodies were used.

To rule out the possibility of steric hindrance caused by cideB antibody binding on ER surface, we boiled these antibodies and used in our budding assay. As expected, boiled cideB antibodies did not inhibit VTV formation (Figure 12). Since both Sar1 and Sec24 interact with cideB and apoB100, it is possible that this interaction triggers the process of VTV formation. To test this speculation, we decided to immuno-deplete hepatic cytosol with specific antibodies against Sar1 and Sec24 as we have done successfully previously (212). Also, we washed our ER membranes with urea to remove peripheral Sar1 and Sec24 as we have done in the past (95). As can be seen in Figure 14, Sar1 and Sec24 depleted system did not support the formation of the VTV, which is consistent with published reports (100,212). Taken together, these data strongly suggest that cideB plays an important role in the biogenesis of the VTV.

Silencing of CideB abrogates VTV budding

In an attempt to provide more concrete evidence that cideB is crucial for the genesis of the VTV, we sought to knockdown cideB in primary rat hepatocytes. We decided to use siRNA with lipofectamine because this approach has been used successfully in primary rat hepatocytes (213). Post-transfection, the level of cideB knockdown was ascertained in hepatocytes, ER and cytosol. As shown in Figure 15, 50 nM concentration of cideB

siRNA significantly decreased cideB protein levels as compared with control siRNA in rat primary hepatocytes, whereas there was no effect on the expression of β-actin (Figure 15). Next, we isolated the ER and the cytosol from these hepatocytes and examined the presence of cideB using western blotting. Figure 16 shows that the ER, isolated from cideB siRNA-treated hepatocytes, contains significantly low level of cideB whereas, there is no effect on calnexin, an ER resident protein. Similarly, reduced level of cideB protein was found in cytosol prepared from cideB knocked down hepatocytes, while there was no effect on β-actin level in the same cytosol (Figure 17). Treatment of control siRNA had no effect on the expression of cideB in hepatocytes, ER or cytosol (Figure 15, 16 and 17).

To find out the effect of knocking down cideB on VTV formation process, we carried out *in vitro* VTV-budding assay. As we expected, VTV-budding activity was significantly decreased when cideB was knocked down as shown in Figure 18. In contrast, there was no effect on VTV formation when control siRNA was used (Figure 18). These data strongly suggest a functional role for cideB in VTV budding.

## Discussion

Increased secretion of very low-density lipoproteins from the liver is associated with hyperlipidemia, which is a major risk factor for the development of various cardiovascular diseases. The assembly of VLDL occurs in the ER lumen and this step is mediated by MTP, which possesses binding sites for both apoB100 and triglycerides

(30,82). After their biogenesis in the ER, newly synthesized VLDL particles are exported to the Golgi and this step determines the rate of VLDL secretion from the liver. The transport of nascent VLDL particles from the ER to the Golgi is mediated by specialized vesicles the VTVs (16,95). Our previous reports have shown that the VTVs are different from the classical ER-derived COPII-coated PTVs that carry nascent proteins to the Golgi (95). Despite their distinct cargoes, size and protein compositions, both VTVs and PTVs utilize the COPII system for their biogenesis (95). This indicates that the formation of the VTVs requires other protein(s) in addition to COPII machinery. Taking these observations into account, we reasoned that additional protein(s) that interacts specifically with VLDL core protein (apoB100) forms an intricate COPII-complex, which facilitates the formation of the VTV from the ER membranes. In this study, we tested our hypothesis and provide the evidence that support the involvement of an additional protein, cideB, in VTV-biogenesis. We found that cideB interacts with VLDL-protein (apoB100) and COPII-components.

Our recent study describing the VTV proteome suggested the presence of a small *M*r protein, cideB, in VTVs (99). In this report, using biochemical methods, we confirmed that the VTVs contain cideB. CideB has been shown to be present in ER, Golgi and lipid droplets, however, the presence of cideB in ER-derived VTV is of particular interest because: (i) the VTV exports nascent VLDL to the Golgi lumen; (ii) VLDL gets further lipidated in the Golgi; and (iii) cideB has been recently shown to promote VLDL lipidation and maturation in the Golgi lumen (186). Together, these observations led us

to postulate that cideB might have an un-identified role in VLDL export from the ER to the Golgi.

The role of cideB in intracellular transport processes has not been reported previously; however, a number of findings related to cideB are consistent with the novel role proposed in this report. For example, the liver of cideB knockout mice has been shown to secret reduced amounts of triglycerides (184). Moreover, knockdown or complete ablation of cideB leads to the secretion of triglyceride-poor VLDL particles from hepatocytes (209) – in other words, cideB plays an important role in VLDL-delivery to the Golgi where VLDL undergoes additional lipidation.

Although COPII proteins are sufficient to form vesicles from the ER membranes, they interact with other proteins to make specific COPII-coats for distinct cargoes (21,191,195,206,207,214). We contemplated the feasibility that cideB may interact with COPII components of the VTV to form a specialized larger COPII cage because cideB is present in COPII-coated VTVs, which are lager in their size than standard PTVs. Our results clearly indicate that cideB interacts with COPII components, Sar1 and Sec24. Binding of cideB with Sec24 is especially significant because the resulting cideB-Sec24 heterodimer might be involved in the formation of larger ER-derived vesicles that can accommodate VLDL-sized particles, which is consistent with data reported previously (214). Moreover, the presence of Sec23 in the VTV (95) raises the possibility that Sec24

forms two heterodimers, cideB/Sec24 and Sec23/Sec24 to facilitate the biogenesis of the VTV and this supposition is consistent with other studies (214).

Does cideB interact with other secretory proteins and facilitate their ER-exit? Co-immunoprecipitation analysis suggested that cideB does not associate with liver secretory protein, albumin, which raises another important question whether the classical COPII-coated PTVs contain cideB or not. Since a number of previous studies have revealed that albumin utilizes COPII-coated PTVs for its transport to the Golgi (92,95,98,101,102,195-199); we speculated that cideB might not be present in PTVs.

The data presented in this report demonstrates that PTVs indeed do not contain cideB, suggesting that typical secretory cargo proteins do not require proteins in addition to COPII components, which is consistent with previous unambiguous findings suggesting that COPII proteins are sufficient to recruit nascent proteins and generate PTVs from the ER membranes (92,101,102,111,195-200,215). In contrast, it has been shown that special secretory cargo proteins control the formation and the size of ER-derived PTVs (214). Using temperature sensitive form of vesicular stomatitis viral glycoprotein (VSV-$G^{ts}$), Aridor *et al*, showed that the generation of PTVs from the ER membranes could be controlled by biosynthetic cargoes (216). The ability of secretory cargo proteins to modulate the size of ER-derived vesicles has been demonstrated by many groups (214,217,218). Recently, it has been shown that ubiquitination of Sec31 by ubiquitin ligase CUL3-KLHL12 regulates the size of the COPII coat (217). However, several

studies suggest the requirement of additional protein(s) to form assorted COPII coats that lead to the generation of larger ER-derived vesicles (214). The formation of pre-chylomicron transport vesicle requires four proteins in addition to COPII proteins (207). Moreover, Shimoni *et al*. showed that the transport of PMA from the ER to Golgi requires Lst1 protein, which substitutes for Sec24 and forms the heterodimer with Sec23 leading to PMA-selection into PTVs (214). Interestingly, these vesicles were found to be larger than typical COPII-coated PTVs supporting the thesis that cargo can influence the size of the vesicle. These observations support our findings that VLDL regulates the formation and size of specialized ER-derived VTVs.

The specific nature of the interaction between cideB and apoB100 raises the possibility that cideB plays a crucial role in VLDL sorting and the VTV biogenesis. Since previous published data suggested that VTVs do not carry albumin to the Golgi (95,98), it is likely that cideB functions as VLDL-selecting protein for the VTV. However, the absence of cideB in the PTV and its interaction with COPII components support the thesis that it facilitates the process of VTV formation. Figure 19 summarizes a novel role of cideB in VTV biogenesis. The potential role of cideB in VLDL selection as well as VTV formation is consistent with other reports in which Lst1 has been shown to mediate the selection of specific secretory cargo and the formation of larger ER-derived vesicles (206,214).

Interaction of cideB with apoB100, Sar1, Sec24 and its presence in the VTV but not in other ER-derived vesicles (e.g. PTV or PCTV) indicated that it might play a functional

role specifically in VTV-generation. The data presented in this report clearly demonstrate that both blocking cideB with specific antibodies and cideB silencing using siRNA approach, significantly reduced the generation of the VTV confirming its functional role in VTV-genesis. Unlike Sar1, a COPII component that initiates VTV-budding from hepatic ER membranes, knockdown of cideB is not lethal. Primary hepatocytes lacking cideB (hepatocytes isolated from cideB null mice) continued to secrete VLDL particles, however, these particles were found to be smaller in their size (209). Since cideB has been proposed to be a multi-functional protein, which is involved in various stages of VLDL lipidation in the ER and the Golgi as well (186,209), knockdown of cideB from hepatocytes would lead to incomplete VLDL lipidation both in the ER and Golgi. Under these circumstances, immature VLDLs (smaller than normal VLDL particles) would not require larger ER-derived vesicles; instead, they can be transported to the Golgi in conventional COPII-coated PTVs. However under normal conditions, a larger vesicle (i.e. VTV) is required for exporting VLDL to the Golgi and cideB plays an essential role in VTV biogenesis (Figure 19).

In the present study, we have identified a new physiological role for cideB as we demonstrated that cideB facilitates the formation of ER-derived large vesicles. Using siRNA approach, we were able to demonstrate that the knockdown of cideB significantly reduced the VTV generation. Our co-immunoprecipitation data show that cideB interacts with the COPII components that might be necessary to build a larger COPII cage and thus for the formation of VTV. We suggest that the interaction between cideB and

apoB100 (a VTV-cargo protein) triggers the process of VTV formation. These findings suggest that the size and the type of cargo regulate the assembly of assorted coat complexes leading to the genesis of vesicles of various size.

**Figures**

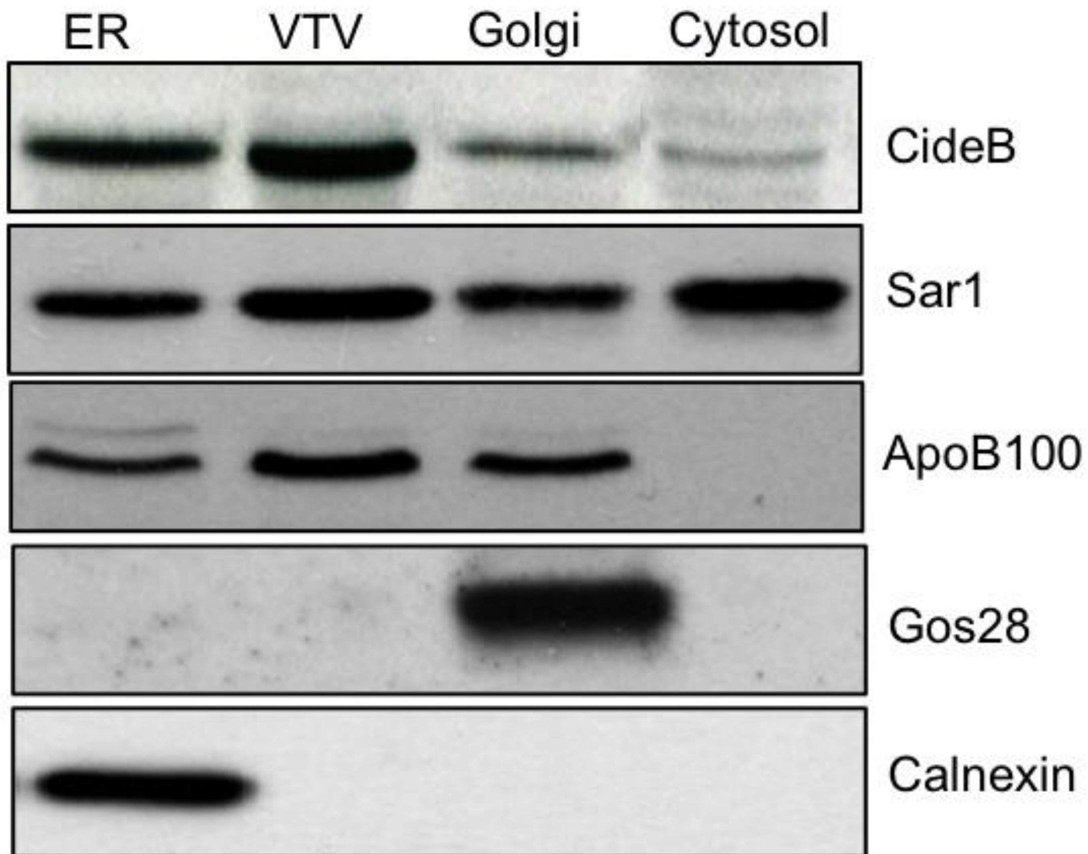


**Figure 6: Cideb Concentrates in the VTV**

Protein samples of purified fractions of ER, VTV, Golgi and cytosol (each sample contains 35 μg of protein) were separated by 12% SDS-PAGE (except for apoB100; a 4-20% gel was utilized), transblotted on to a nitrocellulose membrane and probed with specific antibodies against the indicated proteins. ECL reagents were used to detect proteins. The data are representative of three independent experiments.

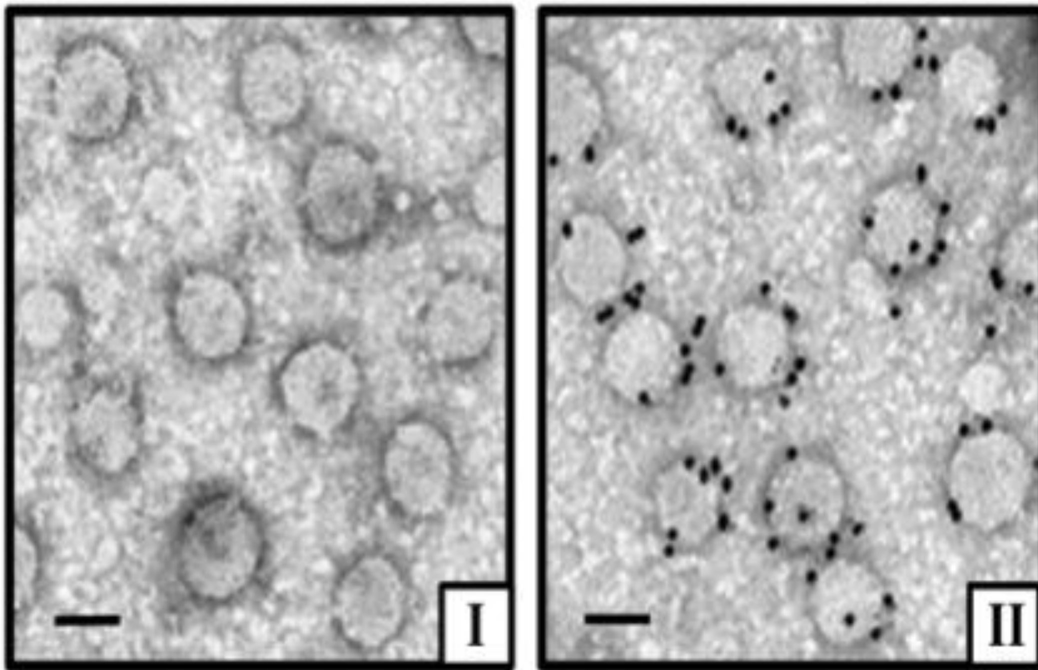


**Figure 7: Visualization of cideB localization on VTVs by immunoelectron microscopy using the negative staining**

Freshly prepared VTVs were adsorbed on formvar-carbon-coated nickel grids, incubated with either (I) anti-rabbit pre-immune IgG; or (II) rabbit polyclonal anti-cideB antibodies and probed with anti-rabbit IgG conjugated with 15 nm gold particles. Bars size = 100 nm.

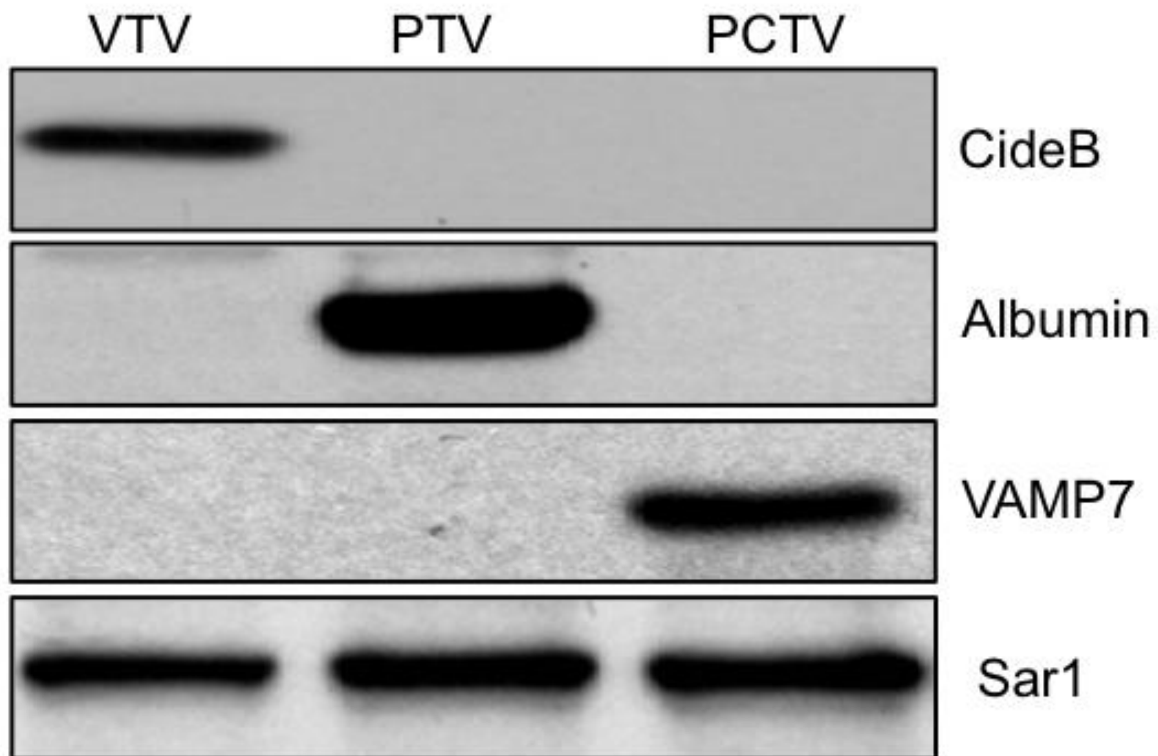


**Figure 8: CideB is present in VTV but not in PTV or PCTV**

Protein samples of purified VTV, PTV and PCTV (each sample containing 40 μg of protein) were separated by 12% SDS-PAGE, transblotted on to a nitrocellulose membrane and probed with specific antibodies against the indicated proteins. Proteins were detected using ECL reagents. The data are representative of four independent experiments.

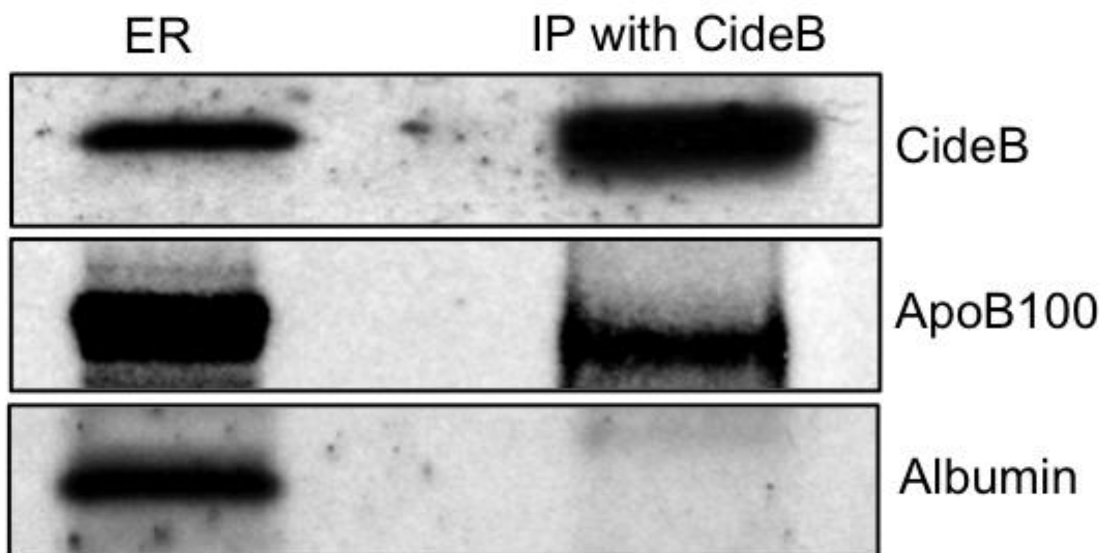


**Figure 9: CideB interacts with apoB100 but not with albumin**

ER (250 µg protein) were solubilized in 2% (v/v) Triton X-100 and incubated with anti-goat cideB antibody (10 µg) for 4h at 4°C. Anti-goat IgGs bound to agarose beads were added and incubated overnight at 4°C. Immune-complexes bound to agarose beads were isolated and washed 10 times with ice cold PBS to remove unbound proteins. Protein sample was separated by SDS-PAGE (8-16% gel) and probed with anti-cideB, anti-apoB100 and anti-albumin antibodies. *IP,* Immunoprecipitation.

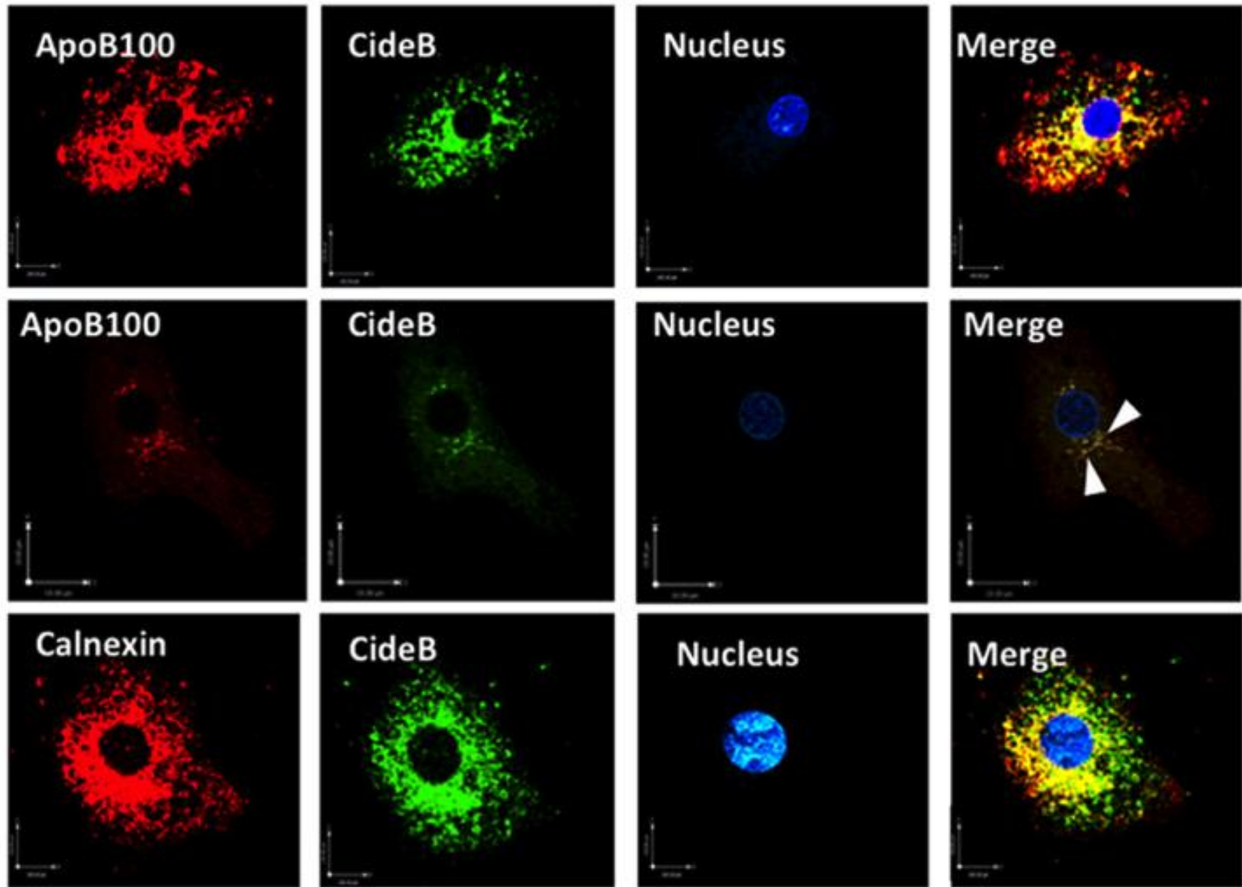


**Figure 10: CideB co-localizes with apoB100 in hepatic ER**

Primary hepatocytes were double-labeled with either CideB (FITC, green) and apoB100 (Texas Red, red) (upper and middle panels) or CideB (FITC, green) and calnexin, an ER marker (Texas Red, red) (lower panel). In the middle panel, we used higher magnification and less saturation of both channels. Arrowheads in the middle panel show punctate vesicular staining in the VTVs. The nucleus is stained with DAPI (blue). Merged figures show co-localization of CideB with apoB100 and calnexin.

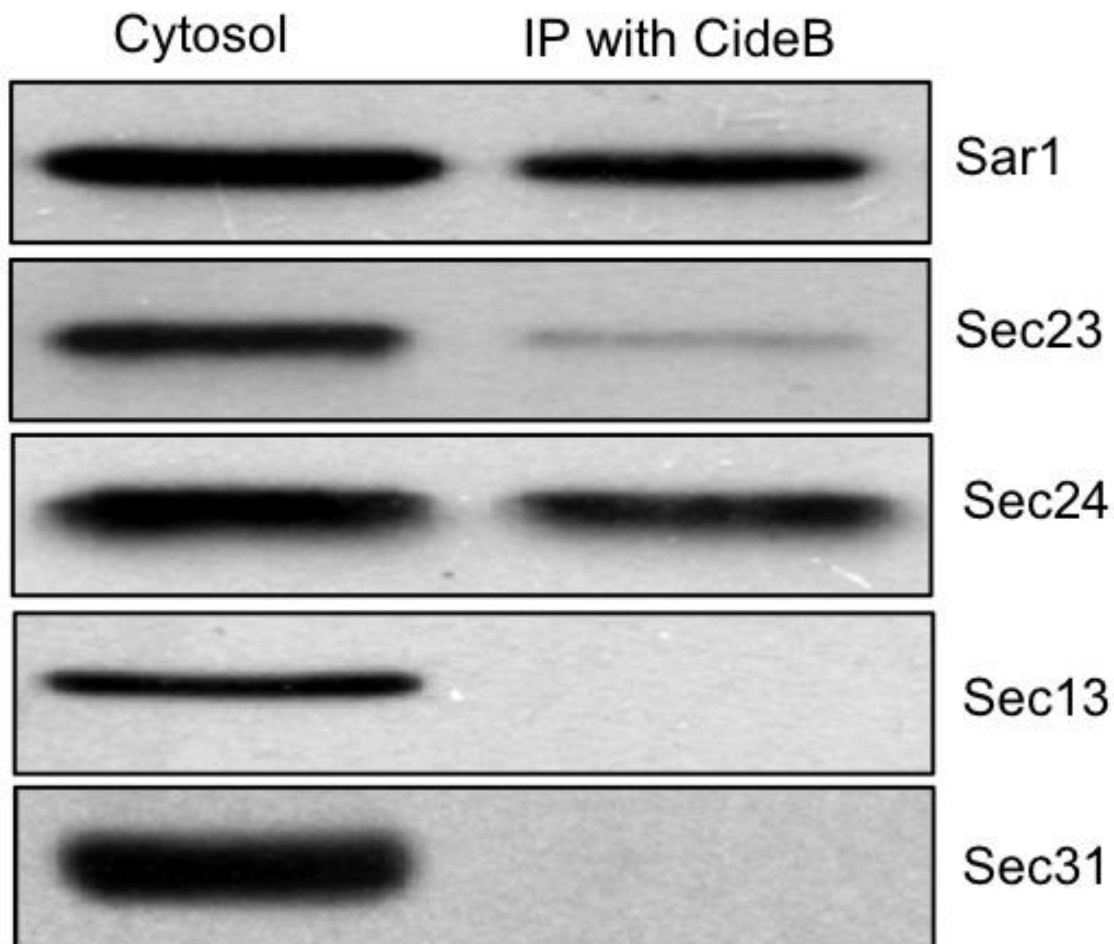


**Figure 11: CideB interacts with COPII components**

Rat hepatic cytosol (150 µg protein) was incubated with anti-goat cideB antibody bound to agarose beads at 4°C overnight. Immune-complexes bound to agarose beads were isolated and washed thoroughly. The proteins were separated by SDS-PAGE (5-15%) and probed with antibodies against the indicated proteins. A single membrane was used, which was sequentially probed with the indicated antibodies after washing. Protein detection was carried out using ECL reagents. The results are representative of four experiments. *IP,* Immunoprecipitation

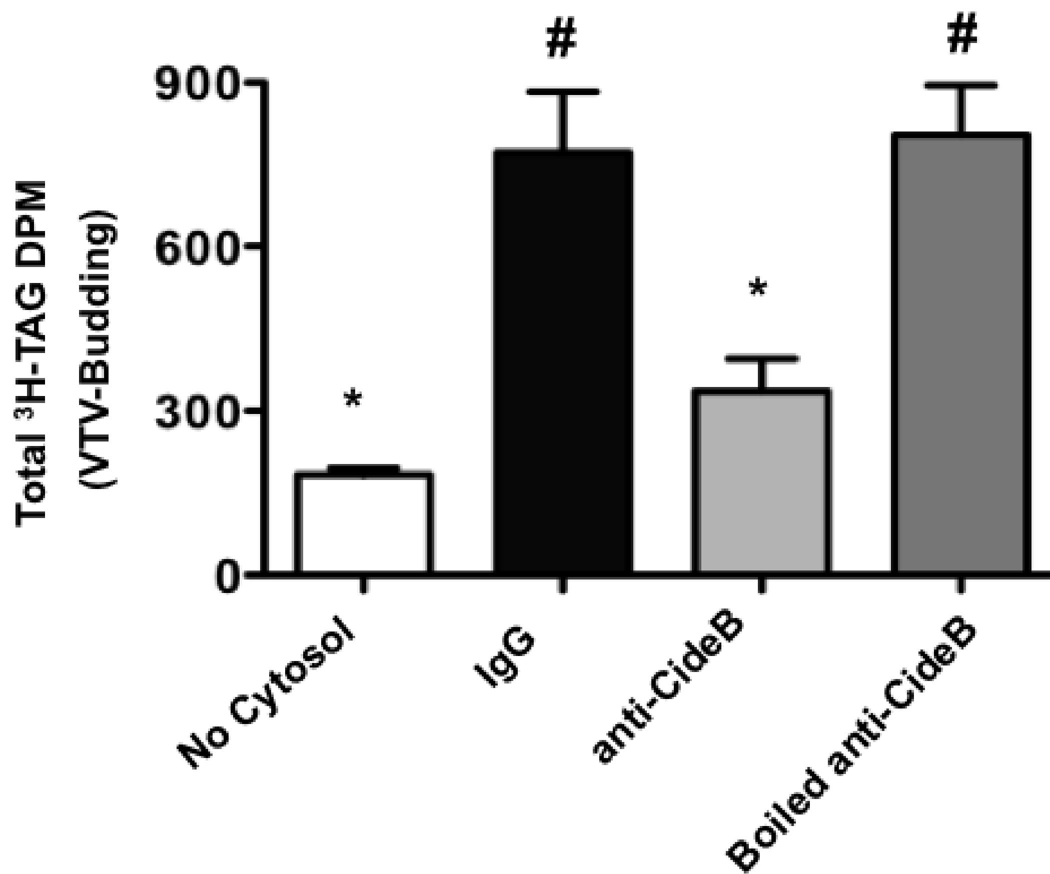


**Figure 12: The effects of cideB blockade on VTV formation**

VTV budding assays were performed in which hepatic ER containing [$^3$H]-TAG was incubated with or without rat hepatic cytosol in the presence of an ATP-regenerating system. Post-incubation, reaction mixture was resolved on a continuous sucrose density gradient and fractions (0.5 ml) were collected. The [$^3$H]-TAG dpm in all fractions (500 µl) were measured. A. Prior to budding assay, the ER containing [$^3$H]-TAG was incubated at 4°C for 2 hours with pre-immune IgG, anti-cideB or boiled anti-cideB antibodies and antibodies in each case were removed by washing. Cytosol was pre-treated at 4°C for 2 hours with pre-immune IgG, anti-cideB or boiled anti-cideB antibodies bound to agarose

beads and the antibodies were removed by centrifugation. Results are mean ±SD (n =4). Bars labeled with different symbols show $P < 0.01$ using one-way ANOVA

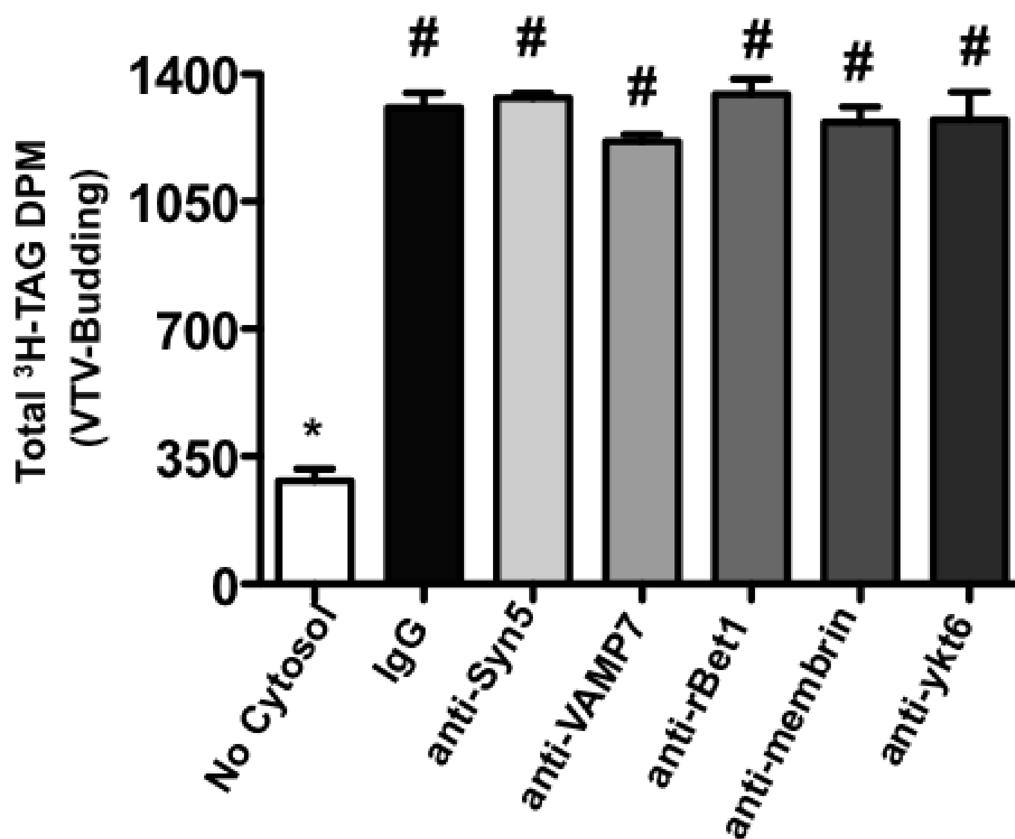


**Figure 13: The effect of blocking of proteins involved in ER to Golgi transpot of proteins and lipoproteins**

The ER containing [$^{3}$H]-TAG and hepatic cytosol similar to 5A were treated prior to performing budding assays, with indicated antibodies. Data are mean ±SD (n =4). Bars labeled with different symbols are *P* < 0.005 using one-way ANOVA.

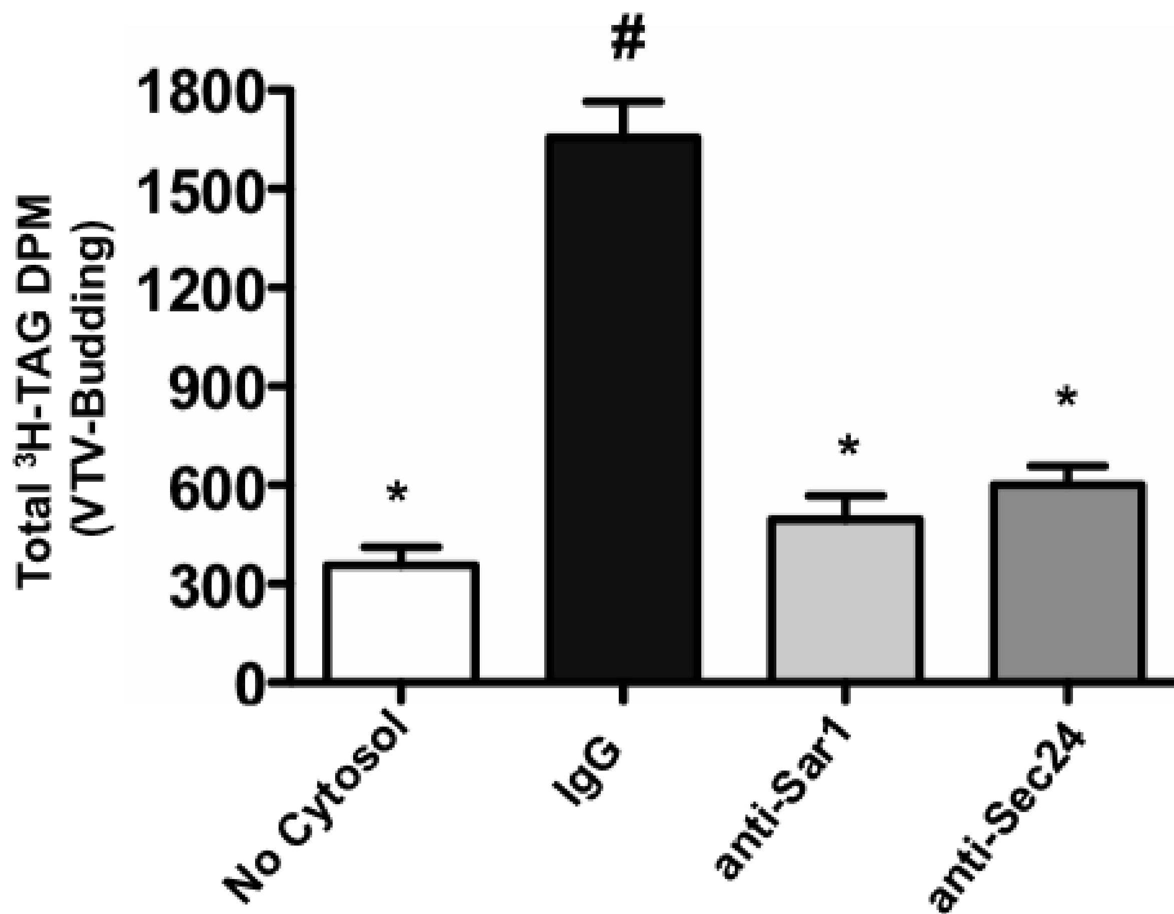


**Figure 14: The effects of blocking of Sar1 and Sec24 on VTV budding**

ER containing [$^3$H]-TAG and hepatic cytosol were pre-treated with indicated antibodies and use in VTV budding assays as described above. For details, see the Experimental section. Values are mean ±SD (n =4). Bars labeled with different symbols have *P* < 0.001 using one-way ANOVA.

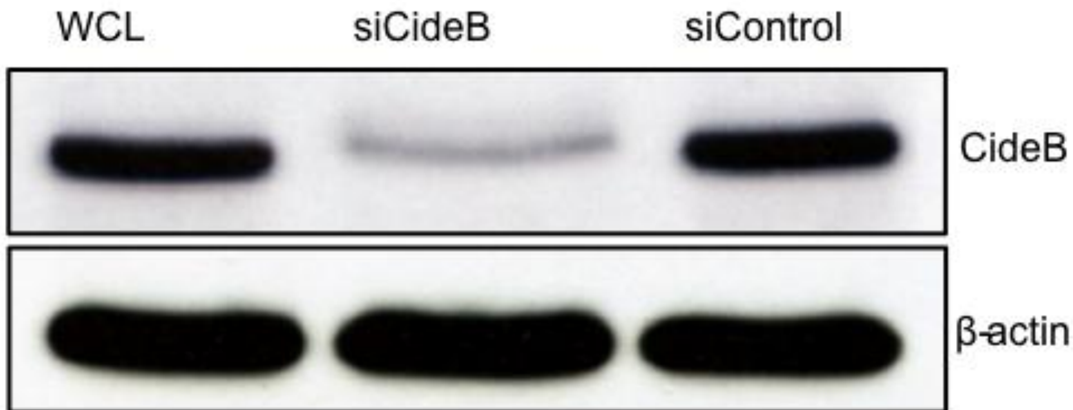


**Figure 15: Protein samples of whole cell lysate (WCL) following Knockdown of cideB in primary hepatocytes**

Primary hepatocytes were transfected with either cideB siRNA (5'CAUGAGCUGCGAUUUUCAATT3') or control siRNA (silencer select siRNA, Ambion). ER containing [$^3$H]-TAG and cytosol were prepared and purified from both sets of hepatocytes as described in the methods.

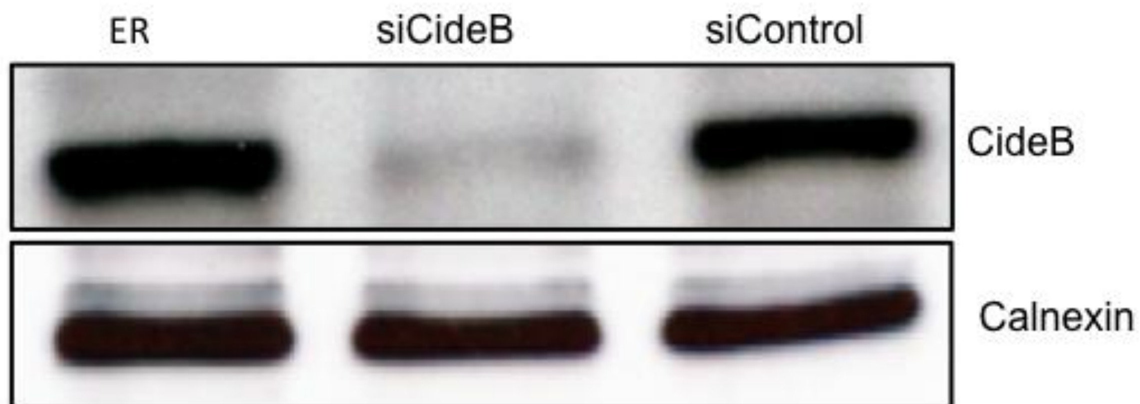


**Figure 16: Protein samples of Hepatic ER membranes following Knockdown of cideB in primary hepatocytes**

Primary hepatocytes were transfected with either cideB siRNA (5'CAUGAGCUGCGAUUUUCAATT3') or control siRNA (silencer select siRNA, Ambion). ER containing [$^{3}$H]-TAG and cytosol were prepared and purified from both sets of hepatocytes as described in the Methods.

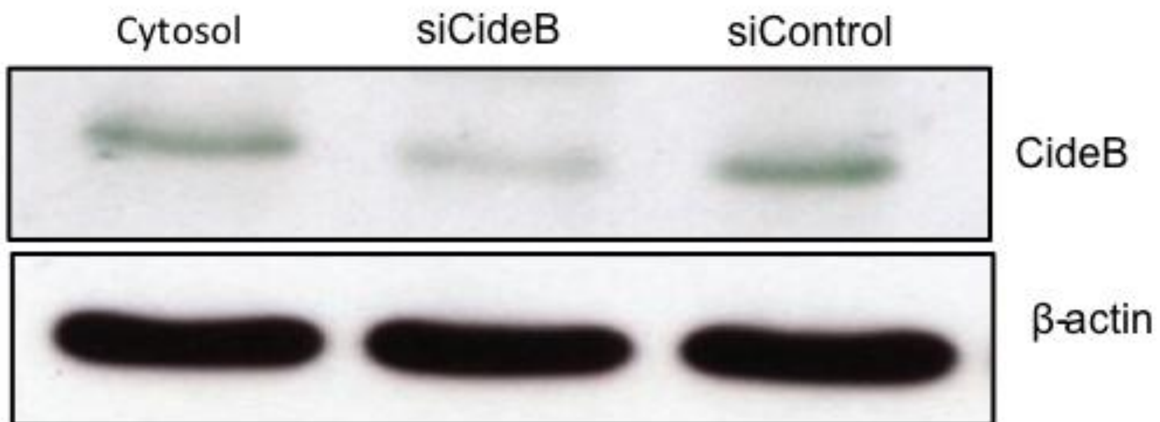


**Figure 17: Effect of Knockdown of cideB**

Cytosol prepared from untreated (Untreated) primary hepatocytes or treated with either cideB siRNA (siCideB) or control siRNA (siControl) were probed with specific anti-cideB, anti-actin or anti-calnexin antibodies.

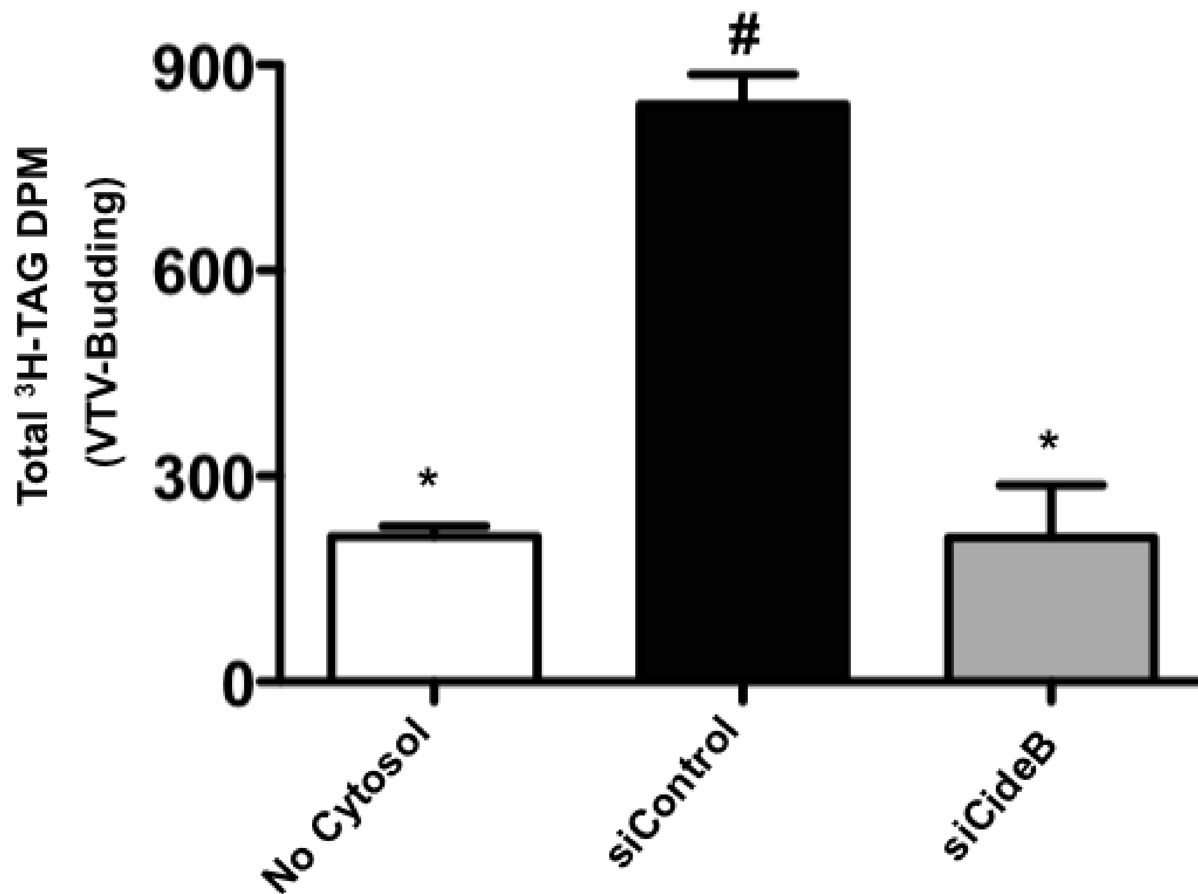


**Figure 18: Effect of Knockdown of Cideb on VTV Budding Assay**

VTV budding assays were performed using ER containing [$^{3}$H]-TAG and cytosol were isolated from primary hepatocytes treated with either cideB siRNA (siCideB) or control siRNA (siControl). As a negative control, ER containing [$^{3}$H]-TAG isolated from untreated hepatocytes was incubated in the absence of cytosol (No cyto). Results are mean ±SD (n =4). Bars labeled with different symbols depict $P < 0.001$ using one-way ANOVA.

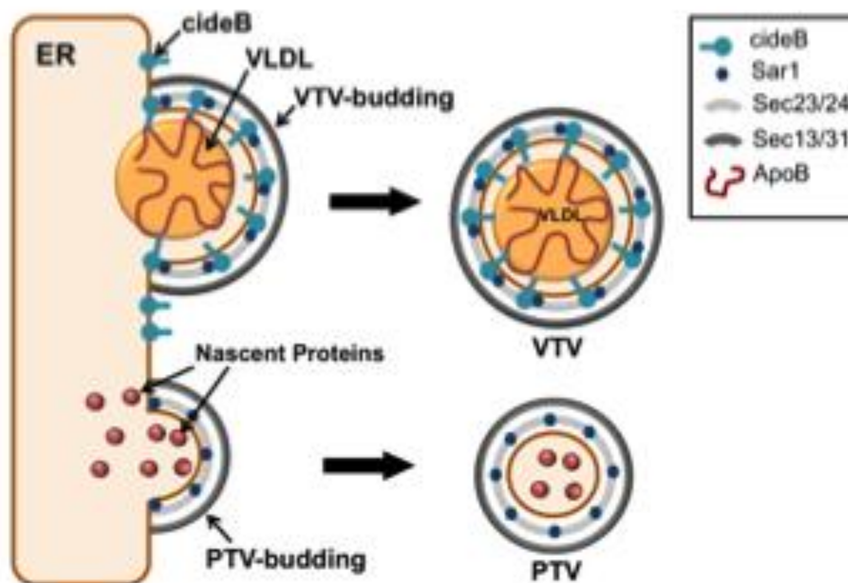


**Figure 19: Schematic diagram summarizing the proposed novel role of CideB in the formation of the VTVs**

CideB is localized to the ER membrane and VTVs. CideB binds to VLDL-apoB and may facilitate the VTV formation. CideB interacts with Sar1 and Sec24 to assemble an intricate COPII coat that may be necessary for the formation of large sized vesicles, the VTV, from hepatic ER membranes. CideB does not interact with nascent secretory proteins such as albumin and is not present in PTVs.

# CHAPTER THREE: MYRISTIC ACID DEPENDENT RECRUITMENT OF SVIP PROTEIN ON HEPATIC ER MEMBRANE REGULATES VTV FORMATION

## Introduction

Liver plays a pivotal role in lipid metabolism as it converts free fatty acids (FFA) into energy rich TAG (11). Liver thus not only protects cells from adverse effect of FFA but also harvest FFA energy into a more useful and transportable form called as lipoprotein (95). Lipoproteins have both protein and lipid moiety. Protein facilitates the transport of lipid in aqueous milieu of plasma to other parts of the cells for its metabolism and other functions (7). VLDL is the TAG rich lipoprotein synthesized in liver (1). The site of VLDL synthesis in hepatocytes is ER (47,117,119,120). Upon its synthesis, VLDL is transported to Golgi body for further modifications (16,123,125,131). The transport of VLDL from ER to Golgi is the rate-limiting step of VLDL secretion (16).

VLDL secretion is of utmost importance as VLDL is a precursor of LDL. High levels of VLDL secretion can relate to pathogenesis of atherosclerosis that results due to accumulation of atherogenic particles like LDL (8,193). Nascent VLDL gets transported from ER to Golgi in a specialized vesicle, the VLDL transport vesicle (VTV) (95). VTVs are larger in size as compared to vesicles that transport protein called as protein transport vesicle (PTV) (95). The coat of both the vesicle is made up of soluble protein complex called as coat protein complex II (COPII) (16,95). COPII complex formation is a sequential and controlled process. The first step of vesicle budding from ER is activation

of inactive GDP bound Sar1 into active GTP bound Sar1 by guanine nucleotide exchange factor (GEF) (111,112,195,196). Upon activation Sar1 recruits itself on ER membrane. Next step is binding of cytosolic factors Sec23/24 to Sar1 resulting in a pre-budding complex (108,113). Finally, Sec13/31 complex recruits on the pre budding complex resulting in budding of VTV from ER (219,220). In spite of having different size and morphology both vesicles utilize COPII protein component and the initiator of budding Sar1. The larger size of VTVs as compared to PTVs suggests involvement of some additional proteins in its biosynthesis. Our group has carried out a detailed proteomic analysis of VTV to characterize proteins associated with VTV. Recent data from the proteomic profiling of VTVs identified SVIP as a putative protein for VTV biogenesis (99).

SVIP is a small protein made of 78 amino acids having N-terminal site for myristoylation (188). It is an adaptor protein of VCP-97 and binds to VCP-97 on its N-terminal. It has been shown that SVIP anchors to ER membrane by myristoylation of its gly2 residue (188). Upon attachment to ER membrane SVIP locates itself on the membrane towards the cytosolic side of ER as shown by its susceptibility to degradation upon proteinase K treatment (190). Interestingly, SVIP also plays a role in inhibiting endoplasmic reticulum associated degradation (ERAD) (190). It is a process of removing misfolded proteins from ER. The misfolded proteins then become available for proteosomal degradation in cytosol. Though ERAD protects the cell from harmful effects of misfolded proteins a controlled ERAD is required for normal transport of proteins from ER to Golgi. SVIP

inhibits the formation of trimeric complex of VCP/97, derlin and gp78, which is required for ERAD by uncoupling binding of VCP/97 and derlin to gp78 (189,190). This inhibitory role of SVIP suggests that it promotes transport of proteins from ER to Golgi. Moreover, gp78 overexpression leads to degradation and decreased secretion of apoB100; the structural protein for VLDL assembly (221). ApoB100 provides structural integrity to VLDL as well as facilitates its transport in plasma. The decreased secretion of apoB100 due to gp78 overexpression can extrapolate to cause decreased secretion of VLDL, as apoB100 only exist in VLDL in plasma. SVIP inhibits gp78 association with other protein of ERAD and in this way may protect apoB100 from degradation and thus facilitate VLDL secretion (189,190).

N-myristoylation is a process in which myristoyl group is attached to the N terminal glycine of a peptide by forming an amide bond (222). It is an irreversible process that can take place both post-transitionally and co-transitionally. The enzyme that catalyzes the reaction is N-myristoyl transferase (NMT) (223,224). Upon myristoylation, proteins may bind to membrane due to hydrophobic forces followed by protein-protein interactions in the membrane once the hydrophobic protein inserts itself into the membrane (225,226). Myristic acid affects the secretion of lipoproteins (227). ApoB100 is required for the synthesis; secretion and transport of VLDL. The amount of VLDL secretion by hepatocytes depends on the number of VLDL loaded into the VTV. As each VLDL contains one apoB100 the amount of VLDL depends on the availability of apoB100. It has been shown that McA-RH7777 cells treated with myristic acid results in

increase in apolipoprotein B100 secretion (227). It also protects apoB100 from proteolytic degradation and thus makes more apoB100 available for lipidation and secretion (227). SVIP plays a role in protecting proteins from ER associated degradation and also undergoes myristoylation, which leads to its attachment on to the ER membrane as shown in Figure 20 (189,190). Here we have studied the effect of myristic acid on the recruitment of SVIP on the ER membrane and how this affects the secretion of VLDL.

In this study we have shown that SVIP recruits Sar1 on ER membrane. We have shown that upon myristoylation SVIP recruitment on ER membrane increases in a concentration dependent manner and it in turn recruits Sar1 on ER from cytosol. Sar1 does not possess any site for myristoylation and in spite of this gets attached to ER membrane after myristic acid treatment suggests a role of SVIP in Sar1 localization. This idea is further strengthened by the fact that SVIP and Sar1 form a complex in cytosol. We have shown that SVIP makes complex with Sar1 in VTV and hence increase the size of vesicle coat. We have also shown that myristic acid treatment to cells increase TAG secretion. This increase in TAG secretion can be associated with increased recruitment of SVIP on ER membrane. In order to sought out the involvement of SVIP in VTV budding we followed an approach to knock down SVIP and studied its effect on VTV budding. We found that SVIP knockdown reduced budding of VTV. We have shown that SVIP recruits on ER membrane and in turn recruits Sar1 to initiate

formation of bigger size COPII cage that can accommodate larger size VLDL. Thus SVIP is important for the budding of VTVs.

**Materials and methods**

Reagents

[$^3$H] oleic acid (45.5 Ci/mM) and [$^3$H] myristic acid (43.5 Ci/mM) were obtained from PerkinElmer Life Sciences (Boston, MA). Reagents used for immunoblotting were purchased from Bio-Rad, Corp (Hercules, CA). ECL (enhanced chemiluminescence) reagents were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Protease inhibitor mixture tablets were obtained from Roche Applied Science (Indianapolis, IN). Albumin and sodium myristate were purchased from Sigma Chemical Co. (St. Louis, MO). Other biochemicals used were of analytical grade and were purchased from local companies. Sprague–Dawley rats, 150–200 g, were obtained from Harlan (Indianapolis, IN). All procedures involving animals were conducted according to the guidelines of the University of Central Florida's Institutional Animal Care and Use Committee (IACUC) and strictly following the IACUC approved protocol.

Antibodies

Rabbit polyclonal antibody against SVIP has been characterized previously (228). Rabbit polyclonal antibody to Sec13, Sec23, Sec31 and Sec24 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-Sar1 antibodies were generated commercially and have been described previously (95).

Mammalian cell culture

McARH7777 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies, Grand Island, NY) and 1% penicillin streptomycin Sigma Chemical Co. St. Louis, MO) in 5%$CO_2$ and fed with fresh media every second day.

SDS-PAGE and Immunoblot analysis

Concentration of protein in ER and whole cell lysate was determined by Bradford method (95). Protein samples were separated by SDS-PAGE followed by transblotting on to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Detection of protein was done by ECL western blot detection reagent (GE Healthcare Life Sciences, Pittsburgh, PA) and autoradiography film (MIDSCI, St. Louis, MO).

In vitro VTV formation

The *in vitro* VTV formation was carried out as established previously in our laboratory (95,99,100). In brief, ER having [$^3$H]-TAG (500 µg) was incubated at 37 $^{\circ}$C for 30 minutes with hepatic cytosol (1 mg protein), an ATP-regenerating system, 5 mM $Mg^{2+}$, 5 mM $Ca^{2+}$, 5 mM DTT, 1 mM GTP, 1 mM E600. Reaction mixture volume was adjusted to 500 µl by addition of transport buffer (30 mM Hepes, 250 mM sucrose, 2.5 mM MgOAc, 30 mM KCl; pH 7.2). Next, reaction mixture was placed on a sucrose continuous gradient made from 0.2 and 1.15 M sucrose respectively and centrifuged

using Beckman rotor SW41 at 25,900 rpm for 2 hours at 4 $^{o}$C resulting in resolution of VTV in lighter fractions. Fractions (500 µl) having VTV were separated from sucrose continuous gradient.

Measurement of radioactivity

Radioactivity associated with [$^{3}$H]-TAG was measured in terms of dpm by using Tri-Carb 2910 TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Shelton, CT) (95,191).

Co-immunoprecipitation

Rabbit anti-SVIP antibody was added in cytosol (1000µg) and incubated for 4 hours at 4 $^{o}$C. Similarly, parallel experiments were performed with anti–sec13 and anti-sec23 antibodies. After 4 hours, either anti-goat or anti-rabbit IgGs bound to agarose beads were added and incubated overnight at 4$^{o}$C. Beads bound to immune complexes were washed 12 times with ice cold PBS (95,207).

Preparation of radio-labeled hepatic ER, cis- and trans- Golgi

ER labeled with [$^{3}$H]-triacylglycerol (TAG) was prepared from rat liver and McARH7777 cells using the same method as we have described previously (95). Briefly, cells in buffer B (136 mM NaCl, 11.6 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 7.5 mM KCl, 0.5 mM dithiothreitol; pH 7.2) were incubated with BSA-bound [$^{3}$H]-oleate (100 µCi) for 35

minutes at 37 °C and washed twice with 2% BSA in PBS to wash the excess of [$^3$H]-oleate. Cells were then homogenized in 0.25 M sucrose in 10 mM Hepes, 50 mM EDTA and protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) in a Parr bomb at 1,100 psi for 40 minutes followed by isolation of ER, *cis*- and *trans*-Golgi in a sucrose step gradient (95,100,191).

Preparation of hepatic cytosol

Hepatic cytosol from rat liver and McARH7777 cells was prepared by following the same method as described previously (95). After washing with Kreb's buffer cells were washed in cytosol buffer (25 mM Hepes, 125 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM DTT and protease inhibitors; pH 7.2) and homogenized using Parr bomb at 1,100 psi for 40 minutes. This was followed by centrifugation at 40,000 rpm for 95 minutes (Beckman rotor 70.1 Ti). Supernatant was dialyzed overnight against ice-cold fresh cytosol buffer and concentrated using centricon filter (Amicon, Beverly, MA) and ultra-filtration membrane (Millipore, Billerica, MA) with a cut-off of 10 kDa to a final concentration of protein to 17 mg/ml.

Transfection with siRNA

Rat primary hepatocytes were transfected with SVIP siRNA (Silencer select Pre-designed SiRNA, Life Technologies, Grand Island, NY). The sequence of siRNA was 5'GAGUCAGACUGUAUCAUUAtt3'. Transfection was carried out by lipofectamine by

following the method according to manufacturer's protocol (Life Technologies, Grand Island, NY).

Hepatic ER and cytosol incubation with Myristic acid

Rat hepatic ER (150 µg) and cytosol (300µg) were incubated with increasing concentration of sodium myristate at 4°C for an hour. The mixture was diluted with ice cold Hepes (10 mM) to a final volume of 300µl.Post incubation the reaction mixture was centrifuged at 13g for 20 minutes to yield ER pellet. The ER pellet was dissolved in transport buffer (30 mM Hepes, 250 mM sucrose, 2.5 mM MgOAc, 30 mM KCl; pH 7.2) and recruitment of proteins to ER from cytosol was observed by western blotting.

Rate of TG secretion

McARH7777 cells were washed with warm PBS followed by incubation with experimental media containing complex of myristic acid with BSA. Briefly, sodium myristate was dissolved in sterile distilled water at 50°C for 60 minutes. Next, a complex of BSA and sodium myristate was prepared by incubation at 37°C for 45 minutes. The myristic acid and BSA complex was mixed slowly in DMEM media containing 10% FBS (Life Technologies, Grand Island, NY) and 1% penicillin streptomycin (Sigma Chemical Co. St. Louis, MO) to a final concentration of 0.8mM and 0.2mM respectively and cells were incubated with the experimental media for 24 hours. As a control cells were treated with BSA (0.2mM) alone in the media. Next day cells were washed with PBS and incubated with FBS free media containing 5µCi of BSA bound [$^3$H] myristic acid for

2 hours. Next, cells were washed two times with PBS to remove all the unbound radioactive myristic acid. At each time point 100µl of media was taken from the cells in triplicates and the radioactivity associated with [$^3$H] TAG was measured.

Statistical Analysis

Data were compared using t-test and a one-way analysis of variance (ANOVA) using GraphPad software (GraphPad Prism 5 Software for Mac OS X version).

**Results**

SVIP is present in hepatic ER, Golgi, and Cytosol

In order to determine the presence of SVIP in various subcellular organelles, we isolated ER, Golgi and cytosol from rat primary hepatocytes. We next determined the presence of SVIP in these organelles by immunoblotting the protein samples with antibody specific to SVIP. As shown in Figure 21, SVIP is present in ER, Golgi, and cytosol. Interestingly, we found that most of SVIP in ER is present in multimeric form. However when it transports from ER to Golgi the monomeric form predominates in Golgi. This result is of great interest as SVIP is a very small protein and by virtue of its small size it can make multimer in the VTV, as it is present in the ER in multimeric form.

SVIP is present in VTV

Our next aim was to determine the presence of SVIP in VTV. For this we generated VTV by following a cell free VTV budding assay. After determining the concentration of

protein, equal amount of proteins were loaded for VTV, ER, Golgi and cytosol. As shown in Figure 22, ER, Golgi, cytosol and VTV, all contain SVIP in its multimer form. Interestingly, both SVIP and Sar1 form a multimer in VTV at 75 kDa as shown in Figure 23. The formation of multimer of SVIP can be attributed to its small size, which can accommodate in the geometry of VTV to make a bigger cage of COPII proteins.

SVIP interacts with COPII components

As COPII proteins play a role in forming the coat of VTV and since we found presence of SVIP in VTV, we next determined the interaction of SVIP with COPII proteins in cytosol by co-immunoprecipitation. Figure 24 suggests a strong interaction of SVIP with Sar1, Sec23 and Sec13. We further cross checked our results by performing immunoprecipitation of Sec23 and Sec13 and probed the membrane with SVIP as shown in Figure 25. The interaction of SVIP with COPII proteins can account for the bigger size of VTV.

Silencing of SVIP reduces VTV biosynthesis

As SVIP is present in VTV and interacts with the COPII components; we next investigated the involvement of SVIP in VTV biogenesis. In order to determine the role of SVIP in VTV budding, we silenced SVIP in McARH7777 cells using siRNA approach. As shown in Figure 26 when we used 100 nM of siRNA we found a significant decrease in SVIP protein in McARH7777 cells whereas the control levels of β-actin remained the

same. We next isolated ER and cytosol from the transfected cells and performed VTV budding to determine the effect of loss of function of SVIP on VTV budding. As shown in Figure 27, silencing of SVIP significantly reduced VTV budding from ER. This result established a functional role of SVIP in VTV biogenesis.

SVIP recruits on ER membrane upon myristoylation

Since myristic acid treatment increases apoB100 secretion from McA-RH7777 cells and SVIP is known to have sites for myristoylation that leads to its recruitment on ER surface, our next goal was to determine the effect of myristic acid on the recruitment of cytosolic proteins on ER membrane. We incubated hepatic ER and cytosol with different concentrations of myristic acid. As expected, we found concentration dependent increase in SVIP recruitment on ER membrane, post myristic acid treatment as shown in Figure 28. Interestingly we also observed an increase in recruitment of Sar1 on ER upon myristic acid treatment. This result is interesting, as Sar1 is not known to have any sites for myristoylation. The increase in Sar1 recruitment upon myristoylation suggests that the recruitment of Sar1 is dependent on SVIP recruitment as they both interact in the cytosol. However the increase in protein recruitment, which we observed, may be non-specific. So, in order to rule out this possibility, we probed the same membrane to test recruitment of other proteins, which are not known to have any sites for myristoylation. As shown in Figure 29 there is no change in level of VCP97 recruitment on ER. Similarly, Sec23 a cytosolic COPII protein did not show any increase in its recruitment on ER. Calnexin, an ER marker protein and apoB100 levels remain the

same, which suggest that the recruitment of SVIP on ER membrane upon myristic acid treatment is due to its sites getting myristoylated.

Effect of Myristoylation on total TAG secretion

Myristic acid increases secretion of apoB100 from cells. It also recruits SVIP, which increases VTV budding. We next questioned whether myristic acid increased VLDL-TAG secretion from cells. We incubated McAH7777 cells with 0.8mM concentration of myristic acid. Next we incubated cells with media supplemented with myristic acid overnight. Next day we incubated cells with radiolabelled myristic acid and then washed cells and measure radioactivity in the media at different time points. As shown in Figure 30, myristic acid increased total TAG secretion significantly as compared to control treated with BSA alone. This suggests that myristic acid treatment recruits SVIP and increases TAG secretion.

Effect of Myristoylation on VTV budding

We next determined the effect of myristic acid on VTV budding. As VTV delivers TAG rich VLDL to the Golgi, the rate of VTV budding will determine amount of TAG secretion from cells. As we observed increase in TAG secretion from cells following myristic acid treatment we expected increase in VTV budding after myristic acid treatment to cells. In order to determine this, we incubated McARH7777 cells with myristic acid and isolated ER and cytosol. We next performed VTV budding and measured radioactivity. As shown

in Figure 31 as expected we observed a significant increase in VTV budding upon myristic acid treatment.

## Discussion

Secretion of VLDL from liver is of great importance as the concentration of LDL in blood is dependent on VLDL a precursor of LDL (229). Overproduction of VLDL is a hallmark for serious conditions like atherosclerosis. ER is the site for maturation and secretion of VLDL from hepatocytes (230). Once apoB100, the structural protein of VLDL is transported into the ER, VLDL assembly takes place. Along with apoB100, another protein, MTP is essential for VLDL synthesis (230). After the synthesis of VLDL ER the next step is its transport to the Golgi lumen, which is the site of modifications in apoB100. The step of VLDL transport from ER to Golgi is very critical as this step determines the rate of secretion of VLDL. Previous work done by our group has established the presence of a novel vesicle the VTV that transports VLDL from ER (143). COPII proteins assemble on ER membrane and mediate the formation of VTV (231). These COPII proteins are also involved in the formation of vesicles that transport nascent proteins from ER called as PTV. Irrespective of the involvement of the same building blocks the COPII, both VTV and PTV differ in their size. This suggests involvement of additional proteins in the formation of VTV. In order to study the proteomic profile of VTV our lab has carried out a proteomic profile of VTV using 2D-gel and MALDI-TOF (99). The aim of this study was to identify new proteins involved in VTV

biogenesis that can serve as new targets to control VLDL secretion. One such protein of interest was SVIP.

In the present study we have established the role of SVIP in VTV formation. SVIP is a small 8.6 kD protein that functions as adaptor protein of VCP (188). It is also known to attenuate ERAD by competing with gp78 for the association of VCP97 and derlin 1 (190). It has unique site for myristoylation, a process that localizes SVIP on ER membrane from cytosol. We studied SVIP due to a number of reasons: (1) It is localized in VTV and absent in PTV. (2) It attenuates the function of gp78, which is known to decrease apoB100 secretion from cells (190). (3) It has sites for myristoylation; further, myristic acid, which causes myristoylation, is known to increase apoB100 secretion from cells (188,227). (4) The very small size of SVIP makes it a likely candidate to accommodate in the geometry of COPII cage to form a bigger vesicle. We found that SVIP is present in ER and VTV in a multimer form at 75kD while in Golgi the monomeric form of SVIP prevails. This finding suggests that SVIP makes multimer when it travels from ER to Golgi and later dissociates into the monomeric form.

To understand the formation of multimer of SVIP in more detail we carried out co immunoprecipitation of SVIP to check its association with COPII components. The association of COPII components with SVIP suggests that it helps to form the extrinsic coat of VTV. As the recruitment of COPII proteins on ER for vesicle formation is a sequential process, in order to know in which step of COPII assembly SVIP

incorporates itself, we studied recruitment of SVIP on ER. We found that SVIP recruitment on ER promotes the localization of Sar1 on ER membrane. Also Sar1 and SVIP both form a complex of 75 kD which suggest formation of a hetero-tetramer consisting of two units of SVIP and two units of Sar1b. The next question was does this recruitment of SVIP on ER translate in to increase VTV formation? We determined the answer by incubating cells with myristic acid and performing VTV budding. We found that increased myristoylation results in increase in VTV formation. This finding is interesting as unsaturated fatty acids like myristic acid increase apoB100 secretion from cells (227). ApoB100 level in ER determines the rate of VLDL maturation. The level of apoB100 is regulated by its degradation like via ERAD (232). Role of SVIP as inhibitor of ERAD suggest it protects apoB100 from proteosomal degradation and enhances VLDL formation. It will be interesting to study if SVIP protects apoB100 from ERAD and promote apoB100 secretion from cells. The presence of SVIP in both ER and cytosol suggest that it serves some protective role in ER. We further studied SVIP involvement in VTV budding and formation by silencing SVIP. We found that loss of function of SVIP decreases VTV synthesis significantly. To sum up we have identified a new role of SVIP in VLDL secretion, as it makes complex with Sar1 and increases size of vesicle coat to accommodate large VLDL particle into the VTV. Figure 32 summarizes the proposed role of SVIP in VTV biogenesis.

**Figures**

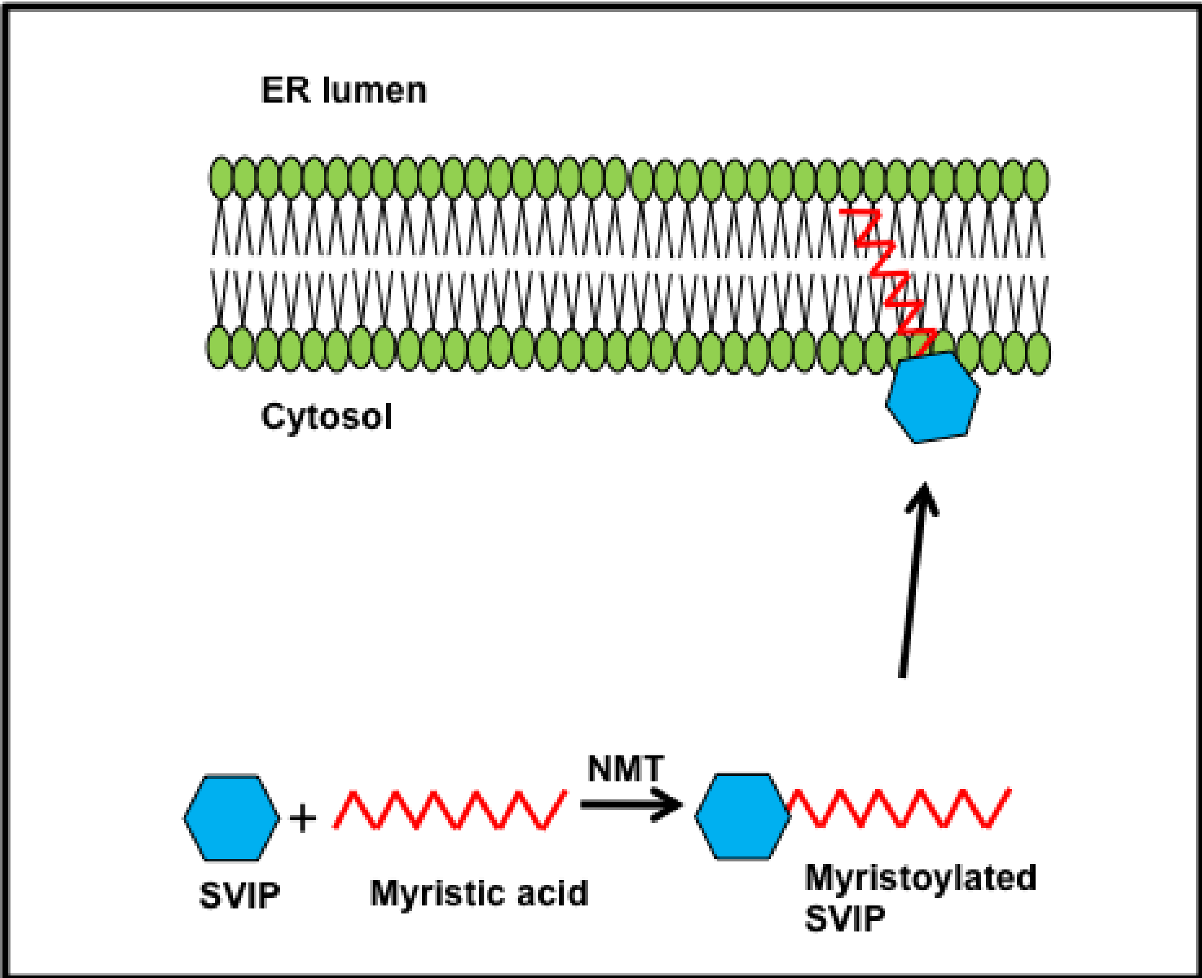


**Figure 20: Schematic diagram showing SVIP and myristoylation**

The N- terminal of SVIP gets myristoylated by the action of myristic acid. This step is catalyzed by enzyme NMT. Upon myristoylation SVIP recruits itself on the ER surface towards the cytosol.

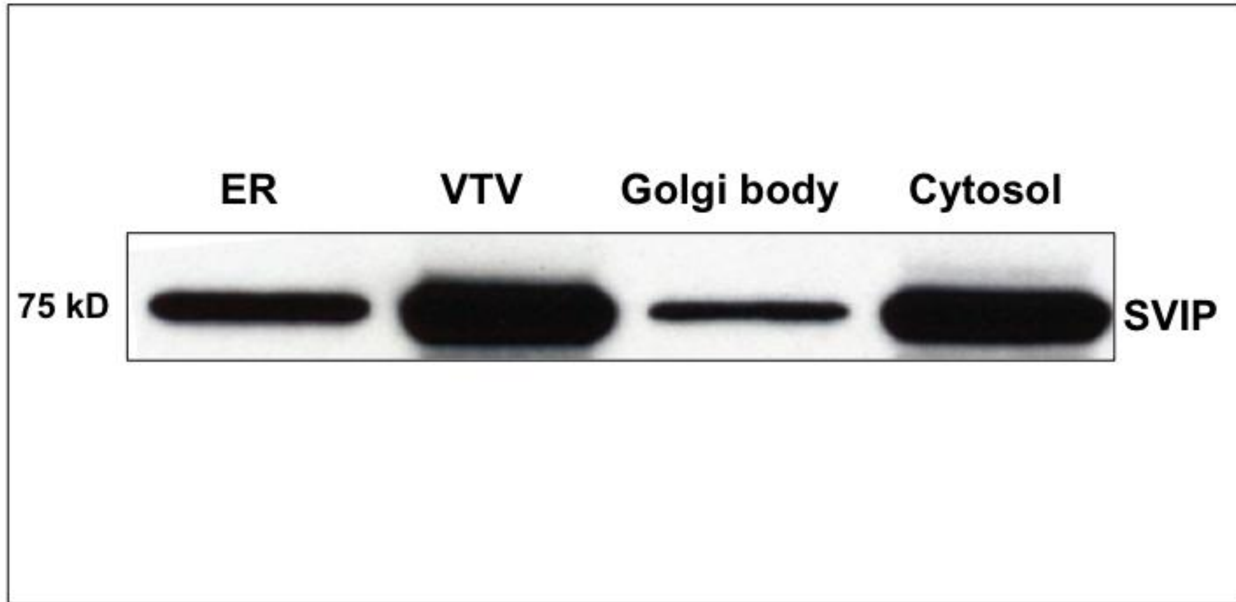


**Figure 21: Immunoblot Analysis showing presence of SVIP in VTV**

Protein samples of purified VTV, ER, Golgi body and cytosol (40μg each) were separated by 8-16% SDS-PAGE and transblotted on a nitrocellulose membrane and probed with antibody against SVIP.

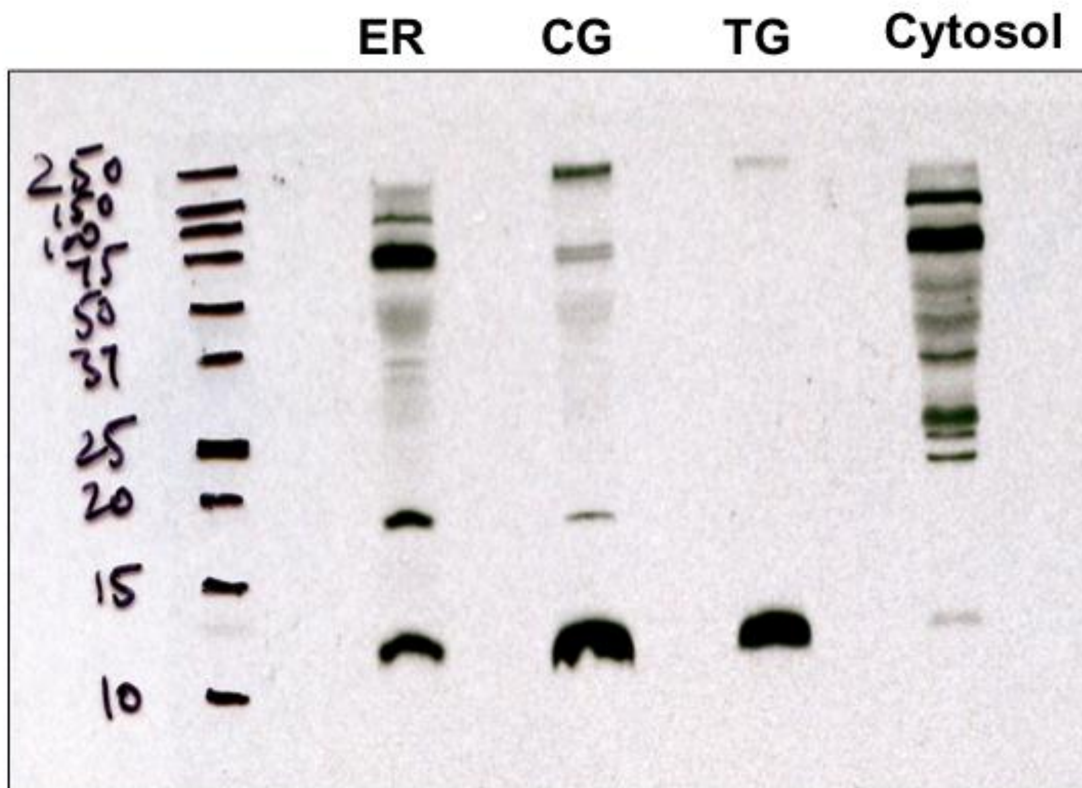


**Figure 22: Immunoblot Analysis showing presence of SVIP in different organelles**
Protein samples of purified VTV, ER, Golgi body and cytosol (40μg each) were separated by 8-16% SDS-PAGE and transblotted on a nitrocellulose membrane and probed with antibody against SVIP. Most of the SVIP in ER is present in multimeric form. However, when it transports from ER to Golgi the monomeric forms predominates in the Golgi apparatus.

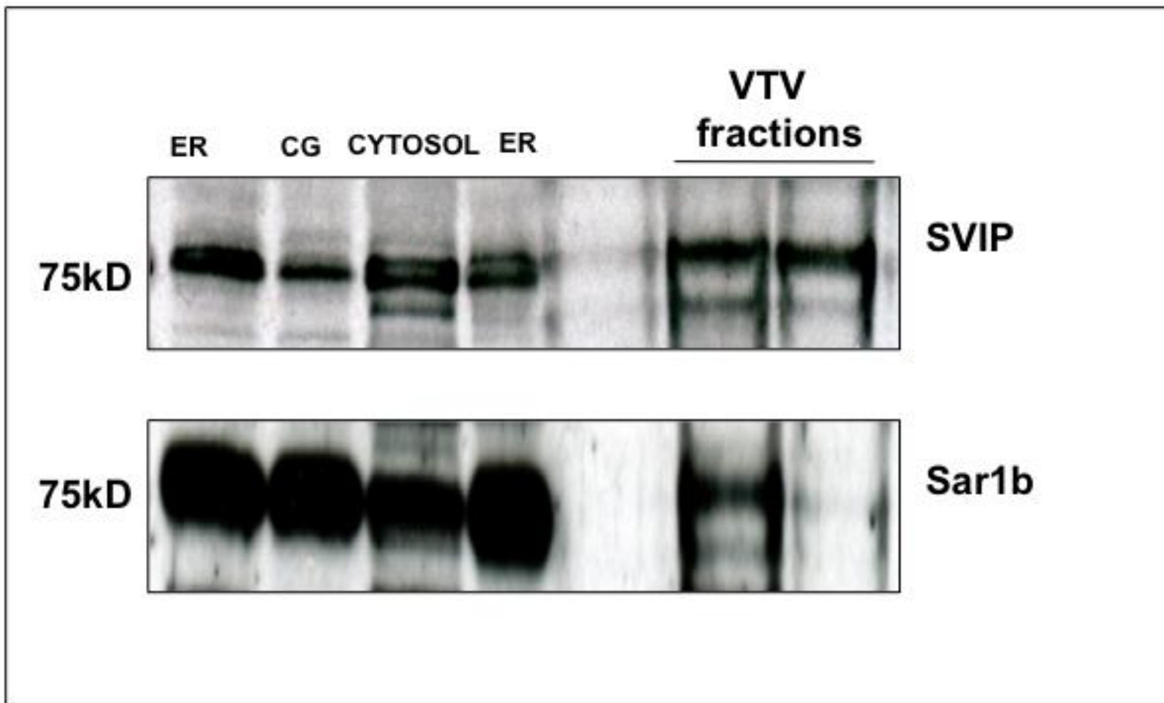


**Figure 23: Multimer Formation of SVIP and Sar1 in VTV fractions**

Protein samples of purified VTVs, ER, Golgi body and cytosol (40µg each) were separated by 8-16% SDS-PAGE and transblotted on a nitrocellulose membrane and probed with antibody against SVIP. The same membrane was re-probed with Sar1.

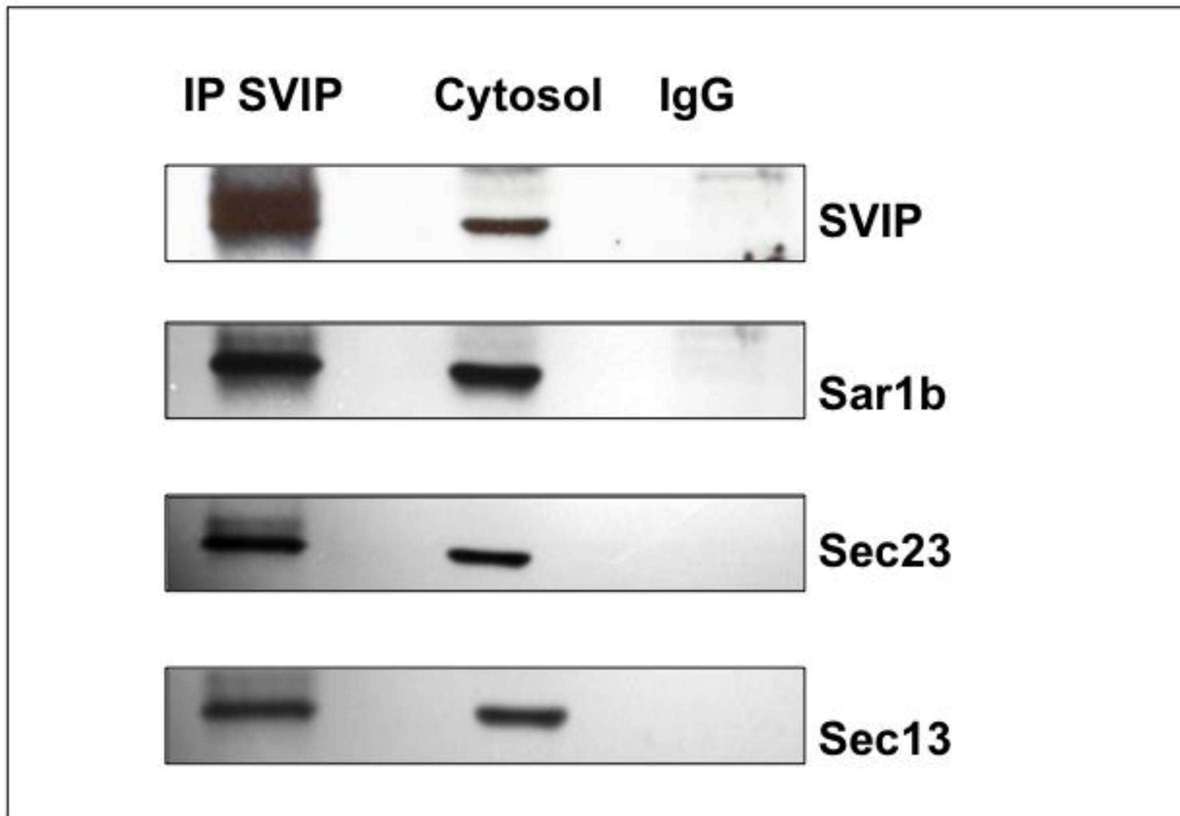


**Figure 24: SVIP Interacts with COPII components**

Rat hepatic cytosol (150 µg protein) was incubated with anti-rabbit SVIP antibody bound to agarose beads at 4$^{o}$C overnight. Immune- complexes bound to agarose beads were isolated and washed thoroughly. Proteins were separated by SDS-PAGE (8-20%) and probed with antibodies against the indicated proteins.

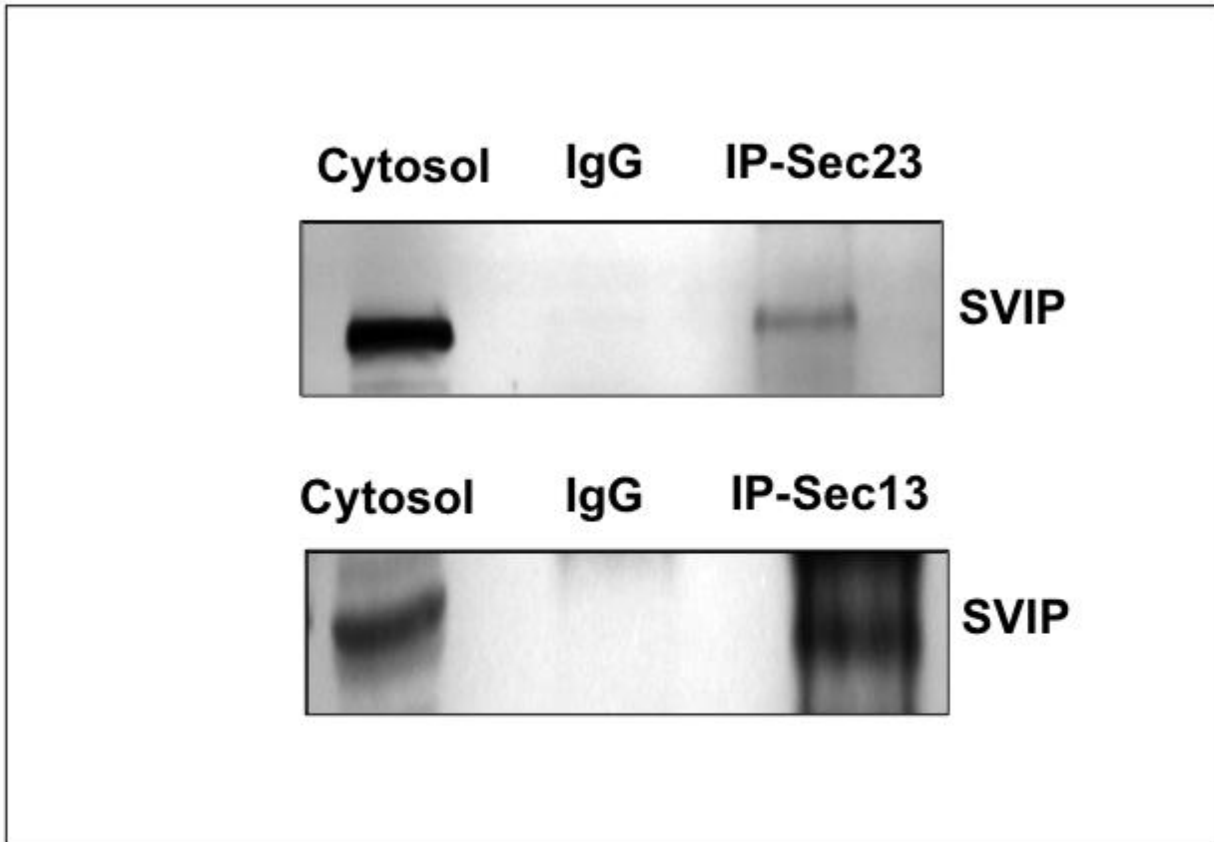


**Figure 25: Co-immunoprecipitation of sec13 and sec23 with SVIP**

Rat hepatic cytosol (150 µg protein) was incubated with anti-rabbit SVIP antibody bound to agarose beads at 4$^{o}$C overnight. Immune- complexes bound to agarose beads were isolated and washed thoroughly. Proteins were separated by SDS-PAGE (8-20%) and probed with antibodies against the indicated proteins.

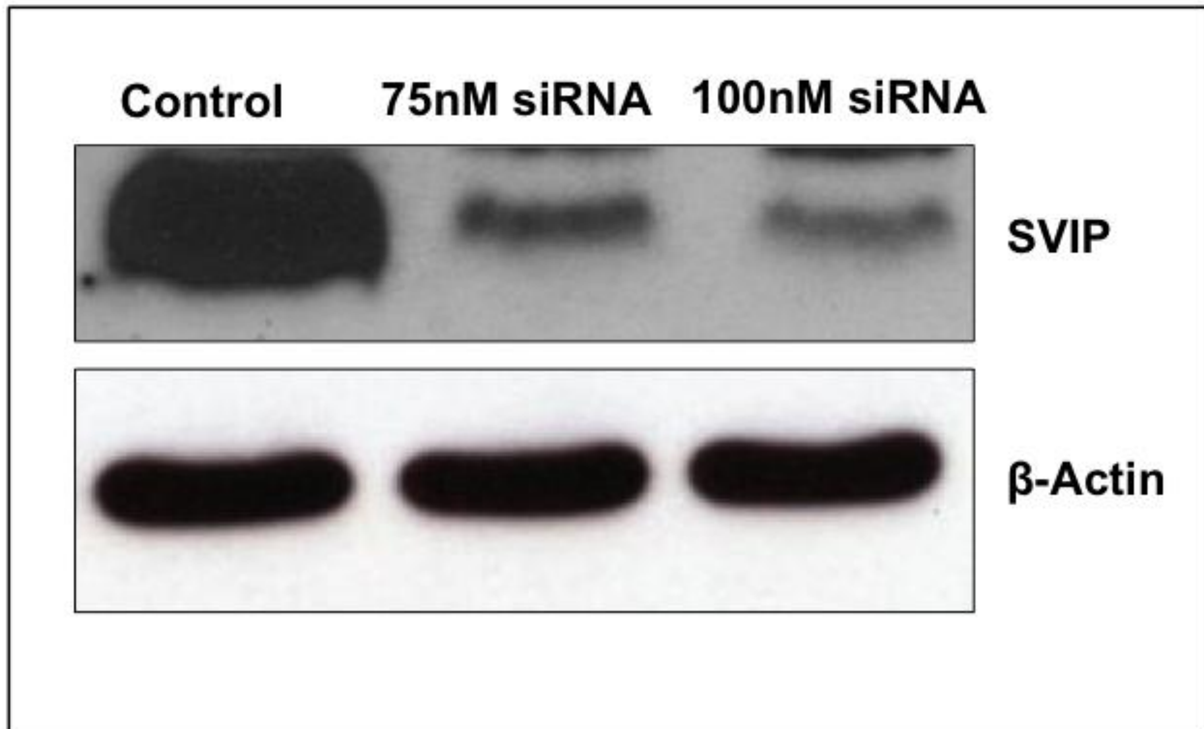


**Figure 26: Effect of SVIP small interfering RNA treatment**

McA-RH7777 cells were transfected with mouse SVIP siRNA. The single-stranded RNAs were dissolved in buffer. Transfection was carried out by lipofectamine following manufacturer’s protocol. The sequence of siRNA used was 5’GAGUCAGACUGUAUCAUUAtt3’

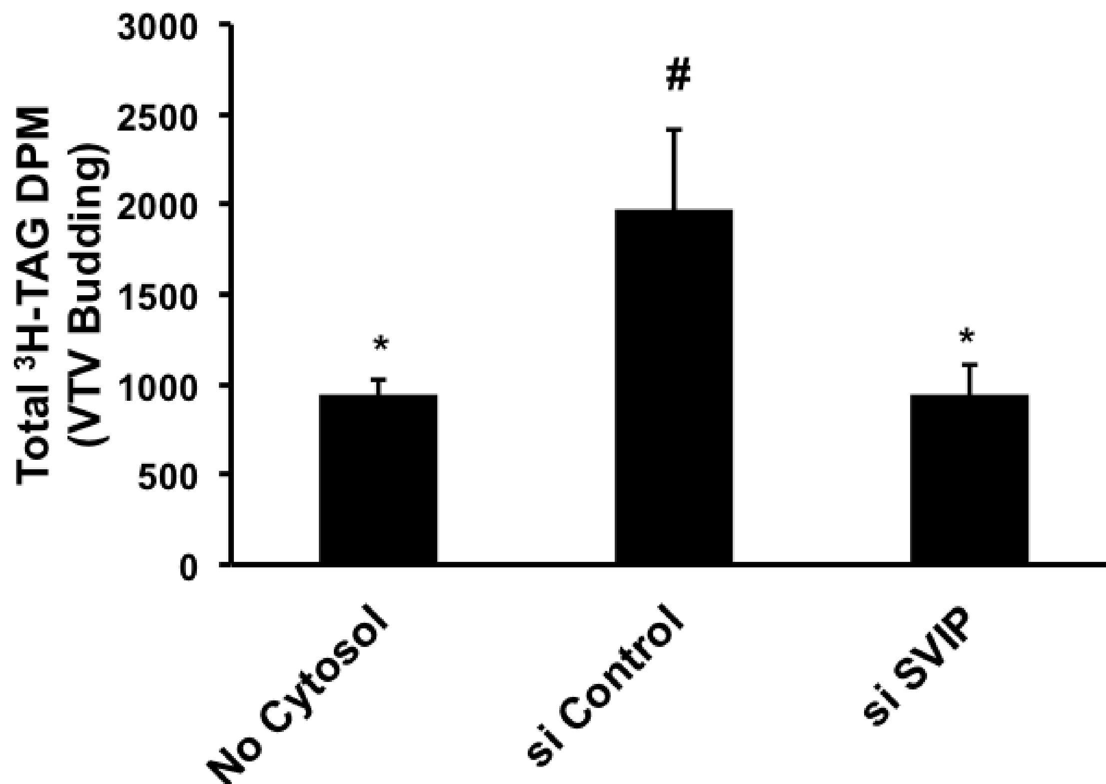


**Figure 27: Effect of Silencing of SVIP on VTV Budding**

McARH7777 cells were transfected with either SVIP siRNA or control siRNA. ER containing $^{3}$H-TAG and cytosol were prepared and purified from both sets of cells. VTV budding assay were performed using ER containing $^{3}$H-TAG and cytosol isolated from cells treated with either SVIP siRNA or control siRNA. Results are mean ±SD (n =4). Bars labeled with different symbols show $P < 0.001$ using one-way ANOVA

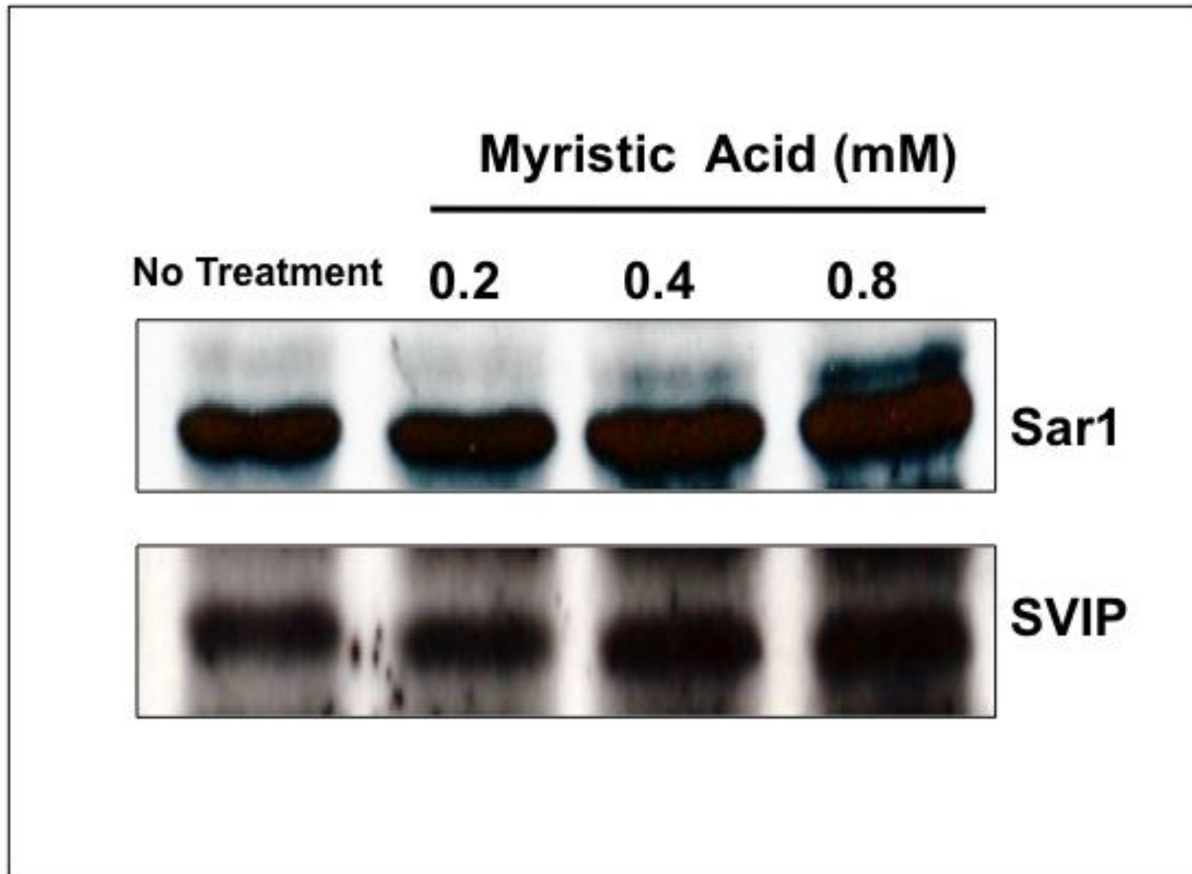


**Figure 28: Effect of Myristic Acid on recruitment of SVIP and Sar1 on ER**

Rat hepatic ER (150 µg) and cytosol (300µg) were incubated with increasing concentration of sodium myristate at 4°C for an hour. The mixture was diluted with ice cold Hepes and centrifuged at 13g for 20 minutes to yield ER pellet. The ER pellet was dissolved in transport buffer (30 mM Hepes, 250 mM sucrose, 2.5 mM MgOAc, 30 mM KCl; pH 7.2) and recruitment of proteins to ER from cytosol was observed by western blotting.

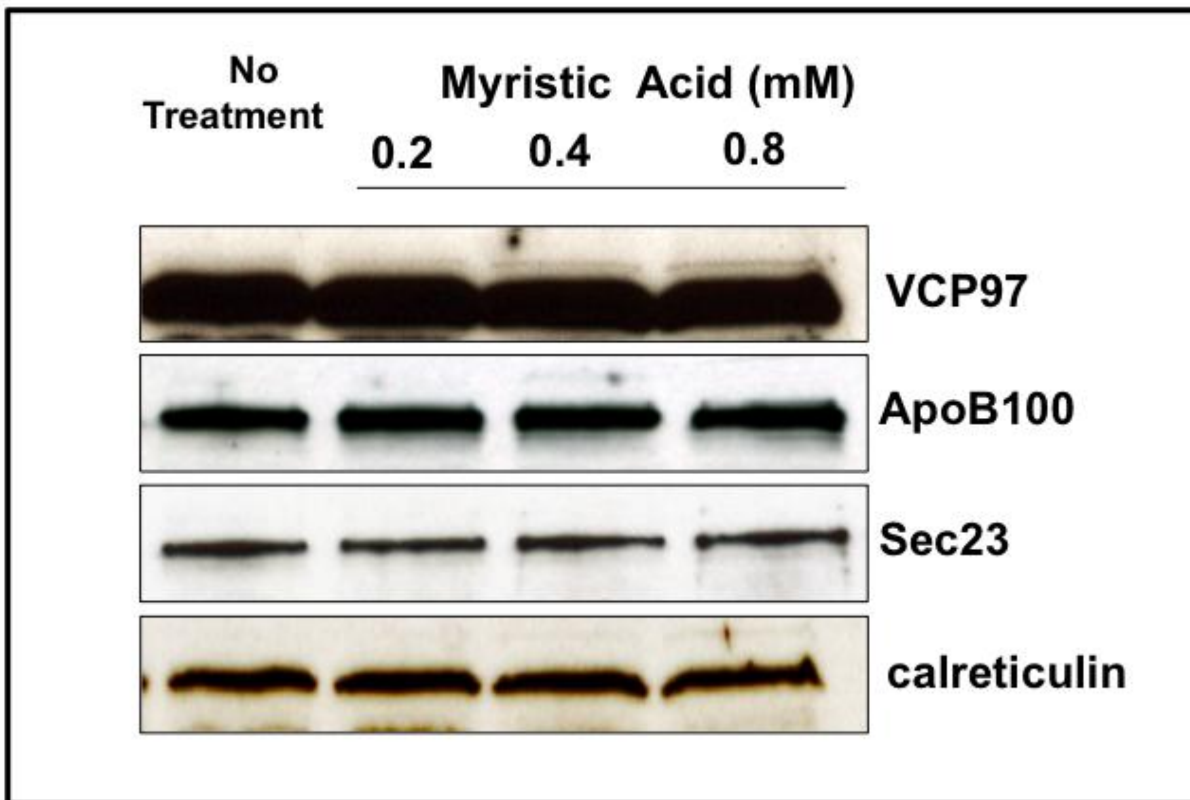


**Figure 29: Effect of Myristic Acid on recruitment of other proteins lacking sites for myristoylation**

Rat hepatic ER (150 µg) and cytosol (300µg) were incubated with increasing concentration of sodium myristate at 4°C for an hour. The mixture was diluted with ice cold Hepes and centrifuged at 13g for 20 minutes to yield ER pellet. The ER pellet was dissolved in transport buffer (30 mM Hepes, 250 mM sucrose, 2.5 mM MgOAc, 30 mM KCl; pH 7.2) and recruitment of proteins to ER from cytosol was observed by western blotting.

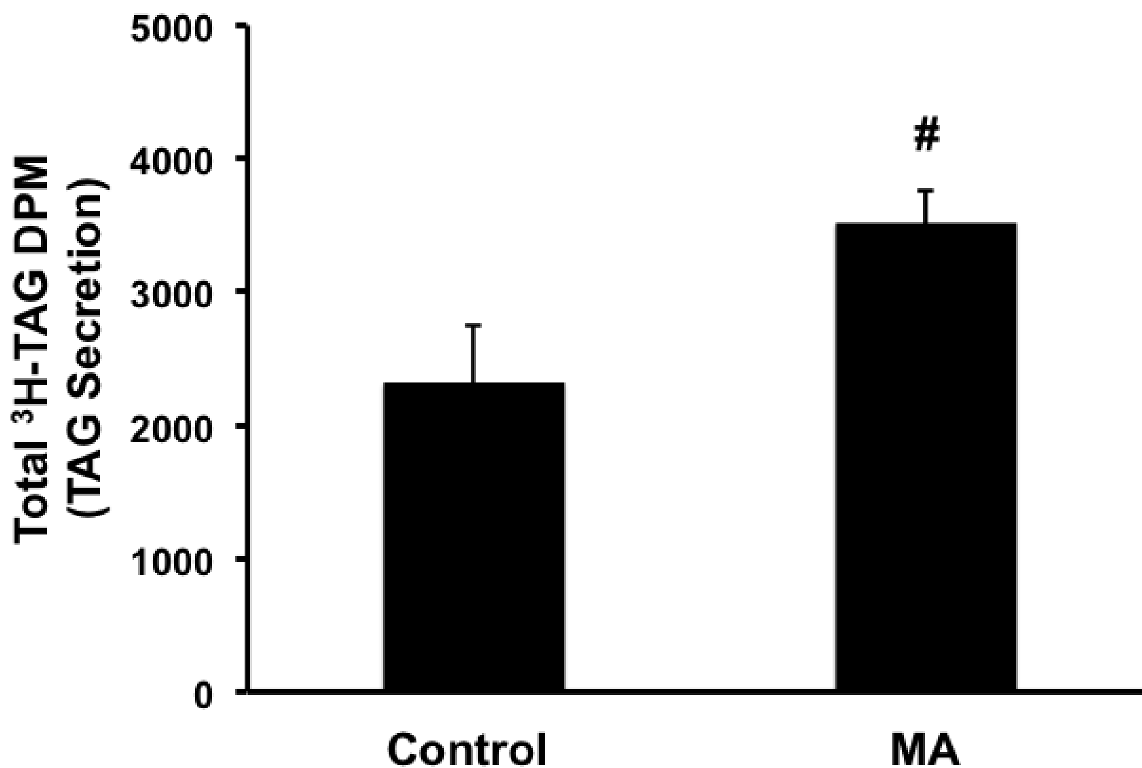


**Figure 30: Effect of Myristic Acid on TAG Secretion**

Sodium myristate was dissolved in sterile distilled water at 50°C for 60 minutes. A complex of BSA and sodium myristate was made by keeping them at 37°C for 45 minutes. Complex was mixed slowly in DMEM media to a final concentration of 0.8mM and 0.2mM respectively. McARH7777 cells were incubation with experimental media containing complex of myristic acid with BSA for 24 hours. As a control cells were treated with BSA (0.2mM) alone in the media. Next day cells incubated with FBS free media containing 5μCi of BSA bound [$^3$H] myristic acid for 2 hours. Cells were washed two times with PBS to remove all the unbound radioactive myristic acid. At every 2h media was taken from the cells and the radioactivity associated with [$^3$H] TAG was

measured. *Control: BSA Treated; MA: Myristic Acid treated.* Results are mean ±SD (n **=**4). Bars labeled with symbols show $P < 0.05$ using t-test.

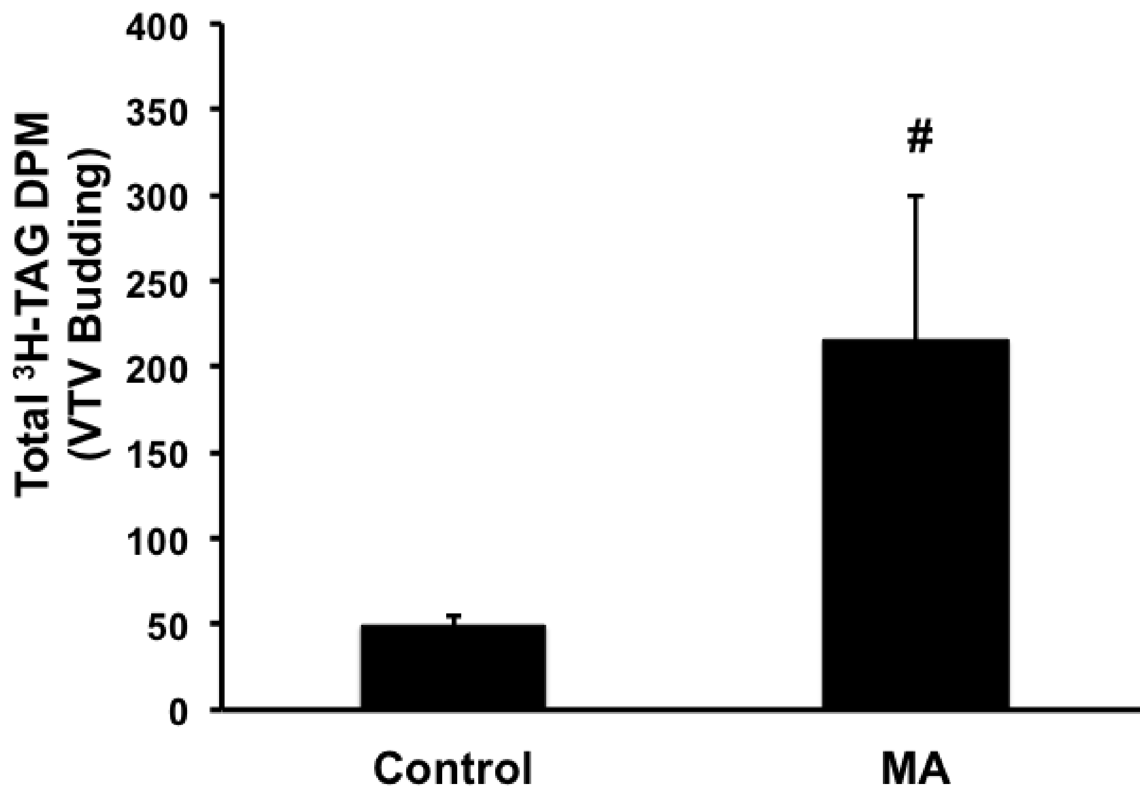


**Figure 31: Effect of Myristic Acid on VTV Budding**

McARH7777 cells were incubation with experimental media containing complex of myristic acid with BSA for 72 hr. ER containing $^3$H-TAG and cytosol were prepared and purified from both sets of cells. VTV budding assay were performed using ER containing $^3$H-TAG and cytosol isolated from cells treated with either sodium myristate or BSA control. *Control: BSA Treated; MA: Myristic Acid treated.* Results are mean ±SD (n =4). Bars labeled with symbols show $P < 0.05$ using t-test.

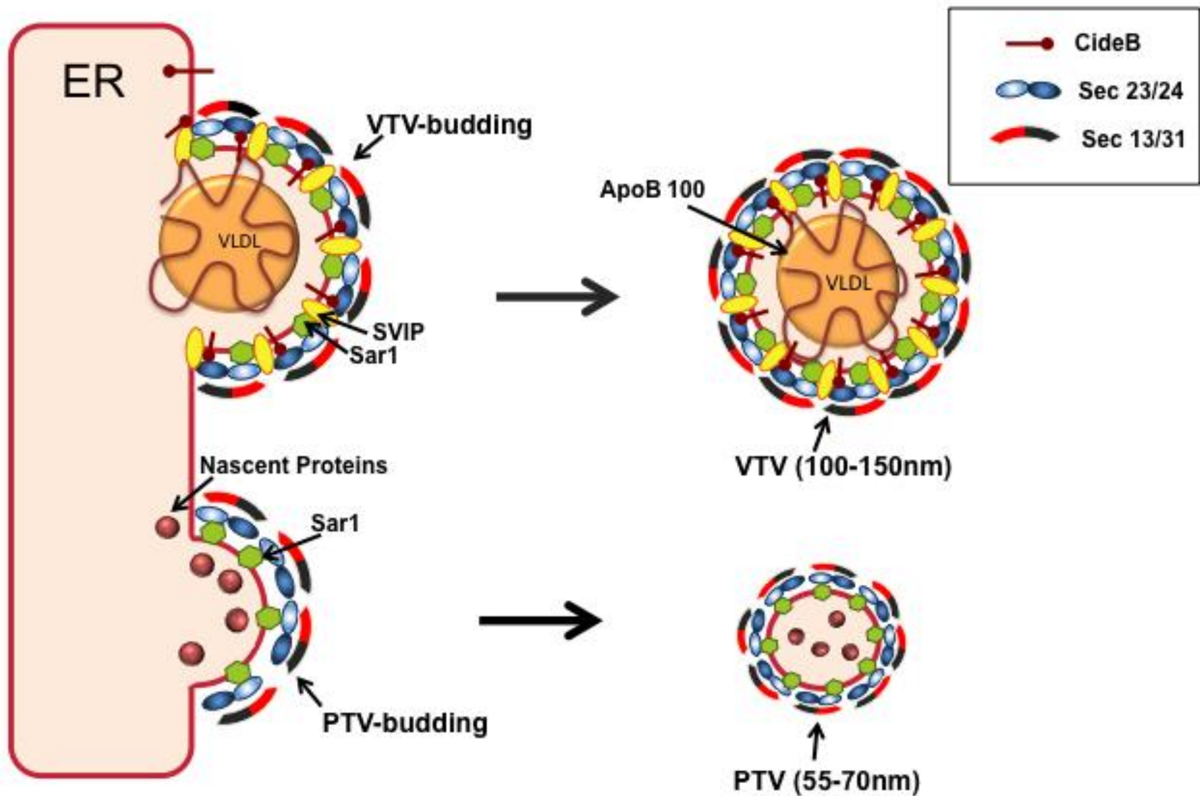


**Figure 32: Schematic diagram summarizing the proposed role of SVIP in the biogenesis of VTV**

SVIP recruits on the ER membranes and facilitates recruitment of Sar1. SVIP interacts with Sar1 and other COPII proteins to form an intricate COPII coat which leads to biogenesis of VTV.

# CHAPTER FOUR: CONCLUSION

The liver is involved in reducing the higher concentration of FFAs by converting them into TAG at the level of ER. Hydrophobic TAG in the ER gets incorporated into lipoproteins which can transport in the aqueous milieu of plasma and thus can be delivered to the sites in the body where they can be utilized to produce energy or can be stored as fats in adipocytes. It is known that higher level of lipoproteins in the blood can lead to atherosclerosis, which is associated with CVDs. VLDL is a lipoprotein synthesized in liver and is known to accommodate majority of TAG in the liver. It is also important as it can be converted into atherogenic lipoprotein LDL that is the key factor leading to atherosclerosis.

The exact site of VLDL synthesis and maturation is not clear and is debatable between ER and Golgi. However in spite of the site of maturation the transport of VLDL from ER to Golgi apparatus is very critical and can be considered as the rate limiting step for overall VLDL secretion. VTV is a unique vesicle that assists in the transport of VLDL from ER to Golgi. Considering the difference in size and function between VTV and other ER derived vesicle PTV; we hypothesized that different proteins are involved in the sorting of VLDL into VTV and in the biogenesis of VTV. Since COPII protein complex is a common denominator in both these vesicles, we assumed that some unidentified proteins interacting with COPII might be involved in VTV biogenesis and thus confer larger size to VTV.

In order to determine the proteins involved in VLDL sorting and biogenesis, we studied the interaction of proteins with apoB100 and Sar1 the two important proteins involved in the formation of VLDL and in its transport. In the first part of the project we found that CideB interacts with ApoB100; the structural protein of VLDL and does not interact with albumin; the protein present in PTV. Moreover, CideB is present and concentrated in ER while it is nonexistent in other ER derived vesicles including but not limited to PTV.

We further studied the involvement of CideB in VTV formation by demonstrating the interaction of CideB with COPII complex proteins. Knocking down of CideB significantly reduced VTV budding thus establishes its role in VLDL secretion. We used rat primary hepatocytes as the cell model to study VLDL secretion, as it is the model that most closely resembles the physiology of liver as compared to the cell lines derived from hepatocytes. Our report concurs with the elegant study by Li *et al*, which demonstrated that CideB null mice exhibit lower VLDL secretion. The involvement of CideB in VTV secretion is a novel observation, as CideB has never been reported previously in context with VTV.

In the second part of the project we investigated the interaction of cytosolic proteins with Sar1 and found that SVIP interact with Sar1. We gained more confidence in studying SVIP and CideB by characterizing VTV and determining its proteome, that indicated the presence of SVIP and CideB exclusively in VTV. The small size of SVIP (≈9 KDa) made it an ideal candidate to study the biogenesis of VTV as it can fit into the geometry of

COPII cage. To reciprocate our finding regarding presence of SVIP in VTV, we studied the interaction of SVIP with COPII components and observed significant association.

Another observation we made was an increase in recruitment of SVIP on ER surface from cytosol upon MA treatment. We selected MA due to two reasons; Firstly SVIP is known to have sites for myristoylation and secondly Fisher and group have reported increase in VLDL secretion after MA treatment. We next assessed the involvement of SVIP in VTV budding after silencing SVIP in McARH7777 cells. We observed reduction in VTV budding upon knock down of SVIP. We further confirmed involvement of MA in VLDL secretion by establishing that addition of MA increases VTV budding. To understand the mechanism behind the involvement of SVIP in VTV budding, we sought to find out if any other proteins are also recruited on ER membrane where the biogenesis of VTV is initiated. Interestingly, we did not observe recruitment of any of the COPII proteins except Sar1. Since Sar1 does not possess any sites for myristoylation we speculate that SVIP recruits Sar1 on ER membrane after itself getting recruited first. Interestingly we observed formation of multimer of SVIP and Sar1 on ER after MA treatment.

In summary, we have shown that CideB plays an important role in the sorting of VLDL and VTV formation. We have also demonstrated that SVIP make an intricate coat complex with COPII proteins and is involved in the biogenesis of VTV. Our results imply that additional proteins are involved in VTV biogenesis and VLDL sorting and may

provide a significant tool to control overproduction and over secretion of VLDL that accounts for development of atherosclerosis.

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# APPENDIX B: IACUC APPROVAL LETTER

Office of Research & Commercialization

3/15/2013

Dr Shadab Siddiqi
Biomolecular Science Center
Lake Nona
10437 Moss Park Road
Orlando, FL 32832

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Dr Shadab Siddiqi:

This letter is to inform you that your following animal protocol was re-approved by the IACUC. The IACUC Animal Use Renewal Form is attached for your records.

| | |
|---|---|
| Animal Project #: | 12-30 |
| Title: | A Cell Biological Approach to Hepatic Lipid Metabolism |
| First Approval Date: | 5/11/2012 |

Please be advised that IACUC approvals are limited to one year maximum. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur. Furthermore, should there be a need to extend this protocol, a renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval. If the protocol is over three years old, it must be rewritten and submitted for IACUC review.

Should you have any questions, please do not hesitate to call me at (407) 882-1164.

Please accept our best wishes for the success of your endeavors.

Best Regards,

Cristina Caamaño

Cristina Caamaño
Assistant Director

Copies: Facility Manager (when applicable.)

12201 Research Parkway • Suite 501 • Orlando, FL 32826-3246 • 407-823-3778 • Fax 407-823-3299

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**THE UNIVERSITY OF CENTRAL FLORIDA**
**INSTITUTIONAL ANIMAL CARE and USE COMMITTEE (IACUC)**
*Re-Approval to Use Animals*

Dear Dr Shadab Siddiqi,

Your application for IACUC Re-Approval has been reviewed and appproved by the UCF IACUC Committee Reviewers.

Approval Date: 3/15/2013

Title: A Cell Biological Approach to Hepatic Lipid Metabolism

Department: Biomolecular Science Center

Animal Project #: 12-30

Expiration: 5/11/2014

You may purchase and use animals according to the provisions outlined in the above referenced animal project. This project will expire as indicated above. You will be notified 2-3 months prior to your expriation date regarding your need to file another renewal.

Christopher Parkinson, Ph.D.
IACUC Chair

Approved ✓ Renewed ✓

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