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Molecular Regulators Of Post-golgi Vldl Transport Vesicle (pg-vtv) Biogenesis

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MOLECULAR REGULATORS OF POST-GOLGI VLDL TRANSPORT VESICLE (PG-VTV) BIOGENESIS

by

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ABSTRACT

Amongst its numerous functions, the liver is responsible for the synthesis and secretion of very low-density lipoprotein (VLDL). VLDL particles play the important role of facilitating the transport of lipids within the aqueous environment of the plasma; yet high plasma concentrations of these particles result in the pathogenesis of atherosclerosis, while low VLDL secretion from the liver results in hepatic steatosis. VLDL synthesis in the hepatocyte is completed in the Golgi apparatus, which serves as the final site of VLDL maturation prior to its secretion to the bloodstream. The mechanism by which VLDL's targeted transport to the plasma membrane is facilitated has yet to be identified. Our lab has identified this entity. Our findings suggest that upon maturation, VLDL is directed to the plasma membrane through a novel trafficking vesicle, the Post-Golgi VLDL Transport Vesicle (PG-VTV). PG-VTVs containing [3H] radiolabeled VLDL were generated in a cell-free in vitro budding assay for study. First, the fusogenic capabilities of PG-VTVs were established. Vesicles were capable of fusing with the plasma membrane and delivering the VLDL cargo for secretion in a vectorial manner. The next goal of our study is to characterize key regulatory molecular entities necessary for PG-VTV biosynthesis. A detailed analysis was undertaken to determine the PG-VTV proteome via western blot and two-dimensional difference in gel electrophoresis. The identification of key molecular regulators will potentially offer therapeutic targets to control VLDL secretion to the bloodstream.

Dedicated to my Loving Family Ashraf, Riham, & Rana

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LIST OF ABBREVIATIONS

2D-DIGE – Two Dimensional Difference In Gel Electrophoresis

2D-Gel – Two Dimensional Gel Electrophoresis

apoA – apolipoproteinA

apoB – apolipoproteinB

apoE – apolipoproteinE

COPII – Coat-Complex II

dpm – disintegrations per minute

ECL – Enhanced Chemiluminescence

ER – Endoplasmic Reticulum

ERAD – ER Associated Degradation

FA – Fatty Acids

HRP – Horseradish Peroxidase

IDL – Intermediate Density Lipoprotein

IEF – Isoelectric Focusing

kDa – Kilodalton

LDL – Low Density Lipoprotein

MTP – Microsomal Triglyceride Transfer Protein

OA – Oleic Acid

PBS – Phosphate Buffered Saline

PBS-T – Phosphate Buffered Saline with Tween20

PCTV – Pre-Chylomicron Transport Vesicles

PG-VTV – Post-Golgi VLDL Transport Vesicle

PM – Plasma Membrane

SDS – Sodium Dodecyl Sulfate

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SNARE – Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein Receptor

TAG – Triacylglycerol

t-SNARE – target-SNARE

VLDL – Very Low Density Lipoprotein

v-SNARE – vesicular-SNARE

VTV – VLDL Transport Vesicle

CHAPTER ONE: GENERAL INTRODUCTION

For anything to have a significant impact it not only must be made correctly, but must be transported to its destination intact and functional. This is an absolute truth at the cellular level.

Lipoproteins are particles consisting of proteins and lipids that facilitate the movement of lipids through the aqueous environment of the bloodstream. Lipoproteins are formed in order to protect the cell from the cytotoxic effect of high cytosolic concentrations of free Fatty Acids (FA) which lead to the disruption of the cell membrane and would result in cell death (*1, 2*). VLDL is secreted into circulation where it is then digested into Intermediate Density Lipoprotein (IDL) and more importantly the atherogenic particle Low Density Lipoprotein (LDL).

The vesicle-mediated secretory pathway, specifically the lipoprotein secretory pathway is an issue of high significance in the regulation of circulating lipoproteins implicated in coronary heart disease. Increased plasma concentrations of apolipoprotein B (apoB) containing lipoproteins, specifically LDL, have been shown to stimulate the pathogenesis of atherosclerosis (*3, 4*). Atherosclerosis currently poses an egregious predicament in the United States where 1 out of every 6 deaths in 2008 was attributed to coronary heart disease and it is estimated that each minute an American dies due to a coronary event (*5*). The pathogenic atherogenic particles in circulation originate from hepatic secretion VLDL, which upon metabolism generates IDL and then LDL. VLDL biogenesis occurs in the liver where upon maturation is secreted into the circulatory system.

Absorption and metabolism of lipids is necessary for all forms of life, but due to the innate chemical properties of lipids which render them insoluble in the aqueous environment of blood a specialized organ is necessary for proper lipid metabolism. The liver is the organ that specializes in this process in mammalian species. It is here where potentially cytotoxic non-esterified free fatty acids are converted to a more physiologically useful form of triacylglycerol (TAG) in the ER, which are then incorporated into TAG-rich VLDL molecules that are eventually secreted into the bloodstream. This is imperative not only due to the hydrophobic chemical properties of lipids which render them insoluble in the aqueous environment of the blood, but also in order to create a process which can be regulated depending on the fluctuating dietary needs of the organism. Therefore the liver must adapt to the FA influx and bind these hydrophobic lipids to lipoproteins that confer aqueous solubility and indirectly facilitate a process that allows for the regulation of lipid secretion into circulation.

The sources of FA to the liver include the chylomicron remnants, dietary FA from the portal hepatic vein, and FA influx from adipocytes (*1*). The chylomicron is the lipoprotein particle synthesized and secreted from the intestine. Within the intestine, the nascent chylomicron is transported from the ER, its site of synthesis, to the Golgi membranes, its site of maturation, via a unique vesicle termed the Pre-Chylomicron Transport Vesicle (PCTV) (*1, 6*). The PCTV signifies the importance of transport vesicles in intracellular lipoprotein trafficking and alludes to the mechanism that mediates VLDL transport within the hepatocyte.

VLDL Biogeneration

VLDL consists of a core of neutral triacylglycerol (TAG), cholesterol esters, specific apolipoproteins, surrounded by a monolayer of amphipathic phospholipids and unesterified cholesterol (*7, 8*). Newly synthesized VLDL particles range in size between 30-100 nm in diameter (*7, 9*). The main apolipoprotein associated with VLDL is apoB, which serves as the structural backbone for VLDL, IDL, and LDL (*3, 10*). Each VLDL particle contains one and only one apoB molecule, and thus understanding this protein is of great importance.

There exist two major forms of apoB, the fully translated apoB100 and apoB48. ApoB is a large hydrophobic glycoprotein with the fully translated apoB100 sequence consisting of 4536 amino acids and molecular weight of 520 kDa (*11-13*). The apoB48 form corresponds to the peptide of 48% of the full-length protein of approximately 250 kDa. This occurs due to an apobec-1 mediated mRNA editing of the apoB transcript which causes a C-to-U deamination of a cytidine at nucleotide 6666 which normally would encode a glutamine codon (CAA), resulting in a premature translational stop codon (UAA) (*14, 15*). In humans, apoB100 is associated with VLDL produced in the liver, while apoB48 is associated with chylomicrons produced in the intestine (*1*). In rodents however, both apoB100 and apoB48 are synthesized and secreted by the liver (*15*). ApoB100 has a pentapartite structure ($NH_2-\alpha_1-\beta_1-\alpha_2-\beta_2-\alpha_3-COOH$) consisting of three alpha helix domains that bind lipid reversibly and two beta sheet domains that irreversibly bind lipids (*12*). This association allows the peptide to be exposed on the surface while interacting with lipids and thus allows its associated lipids to traverse the aqueous

environment of the bloodstream. The synthesis and regulation of this protein are unique and allude to its significant role in the hepatocyte.

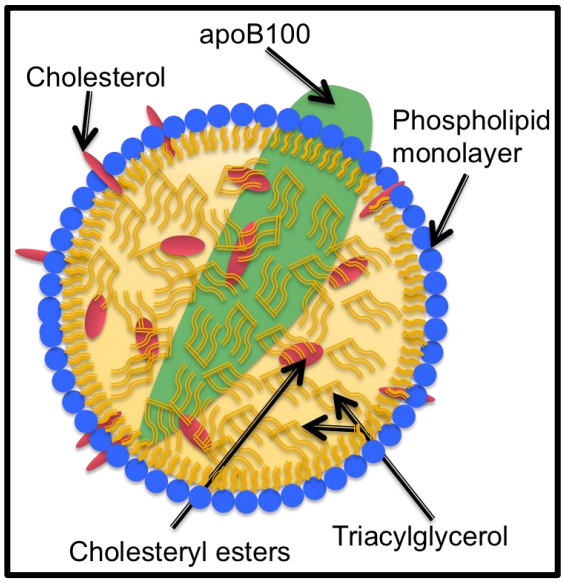


Figure 1 – VLDL Molecule
Cross-section of a Very Low Density Lipoprotein particle.

Amongst the liver's many vital functions, the synthesis and secretion of VLDL is a complex pathway of utmost importance. The first step of VLDL biogeneration begins at the surface of the hepatic Endoplasmic Reticulum (ER) with the synthesis of apoB. Nascent apoB is targeted into the ER by its N-terminal signal-peptide sequence of 27-amino acids. The translocation of nascent apoB occurs through translocons occurs simultaneously with translation, however this translocation is not continuous (*16, 17*).

The level of apoB100 in the liver is regulated not by its synthesis, but by the level of degradation (*18*). Nascent apoB100 that fails to be lipidated in the lumen of the ER is directed to the ubiquitin-proteasome pathway for degradation in a process called ER Associated Degradation (ERAD). While most protein that are directed to the ERAD are degraded due to improper translation ultimately leading to misfolding, apoB100 is unique in that when it is poorly lipidated, it is sent for degradation, thus regulating its synthesis based on the availability of core lipids (*18*). This has been shown through the use of MTP inhibitors which reveal that upon inhibition of MTP function, apoB is degraded and if proteasome inhibitors are also introduced there leads to an accumulation of secretion-incompetant apoB100 within the hepatocytes (*19*). The mechanism of ERAD degradation involves the partial translation and translocation of the nascent apoB100, then interaction with the cytosolic chaperone Hsp70, followed by polyubiquitinylation and association with Hsp90 that directs the peptide to the proteasome (*20-22*). This process also involves the association of $P58^{IPK}$, Hsp110, p97, and BiP, which regulate the secretion of apoB through the ERAD (*23-26*).

VLDL assembly occurs via a two-step process (*27*). This newly synthesized apoB100 molecule is first partially lipidated on its N-terminus through the action of microsomal triglyceride transfer protein (MTP). MTP is a lipid transfer enzyme consisting of a dimer of two polypeptides, a 55 kDa 'P' subunit which is an ER resident enzyme protein disulfide isomerase (PDI), and a 97 kDa 'M' subunit which is the subunit responsible for catalyzing the transfer of phospholipids, cholesterol esters, and TAG to apoB100 (*28, 29*). MTP has three functional domains, an apoB binding domain, a lipid transfer domain, and a membrane associating domain

(*28*). The second step of VLDL formation involves the conjugation of this poorly-lipidated VLDL particle with the bulk of its lipid (*30*).

VLDL Trafficking

Upon synthesis, this premature VLDL particle must be transported from the ER to the Golgi apparatus for maturation (*31, 32*). The VLDL Transport Vesicle (VTV) has been identified as the vesicle that mediates this transport step (*33*). The VTV is unique to other ER-to-Golgi transport vesicles characterized presently, such as the Protein Transport Vesicles (PTVs) responsible for transporting protein to the Golgi (*7, 33*). The VTV is unique in both its proteome, morphology, tethering proteins and cargo (*33, 34*).

The formation of the VTV from the ER membranes is dependent upon coat-complex II (COPII) proteins. These are a group of cytosolic proteins that facilitate the formation of vesicles and mediate cargo-selection for these vesicles. The COPII complex consists of the proteins Sar1, Sec23-Sec24, and Sec13-Sec31 (*35-38*). The formation of the COPII coat begins with the guanine nucleotide exchange factor Sec12, which mediates the exchange of the GDP form of Sar1 to Sar1-GTP (*39*). Sar1-GTP then recruits the heterodimer Sec23-Sec24, which is followed by the recruitment of the Sec13-Sec31 heterotetramer. This formation triggers membrane deformation and the budding of the vesicle from the ER membrane (*7, 38*). It is important to note that there are two mammalian isoforms of Sar1. Sar1a is responsible for the ER-to-Golgi transport of nascent proteins, while Sar1b is the isoform that mediates the transport of

lipoproteins (*40-42*). To accommodate the larger cargo, the COPII cage is expanded by complex with the cideB protein (*43*).

The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are the tethering proteins responsible for the targeted delivery of vesicles to their destination and allow for the formation of the vesicular fusion complex (*44, 45*). The SNARE complex required for fusion of VTV with Golgi membranes consists of the vesicular-SNARE (v-SNARE) Sec22b on the VTV, and the target-SNAREs (t-SNARE) syntaxin5, rBet1, and GOS28 on the Golgi membranes (*46*).

Upon arrival within the Golgi lumen, several important modifications occur to VLDL that are prerequisites to its secretion from the hepatocyte including additional phosphorylation and glycosylation of its structural apoB100 protein (*47-50*). It is within the Golgi lumen that VLDL matures further by the incorporation of apolipoproteinAI (apoAI) and apolipoproteinE (apoE) (*49*).

Mature VLDL must then be delivered to the plasma membrane (PM) for secretion from the hepatocyte. Its large size of approximately 80-100nm in diameter suggests that it requires a specific intracellular compartment to facilitate this trafficking step (*33*). The presence of lipases and proteases in the cytosol also strongly suggest the need for a sealed compartment to protect its cargo from digestion. Moreover the mature VLDL molecule must be specifically directed towards the plasma membrane for secretion.

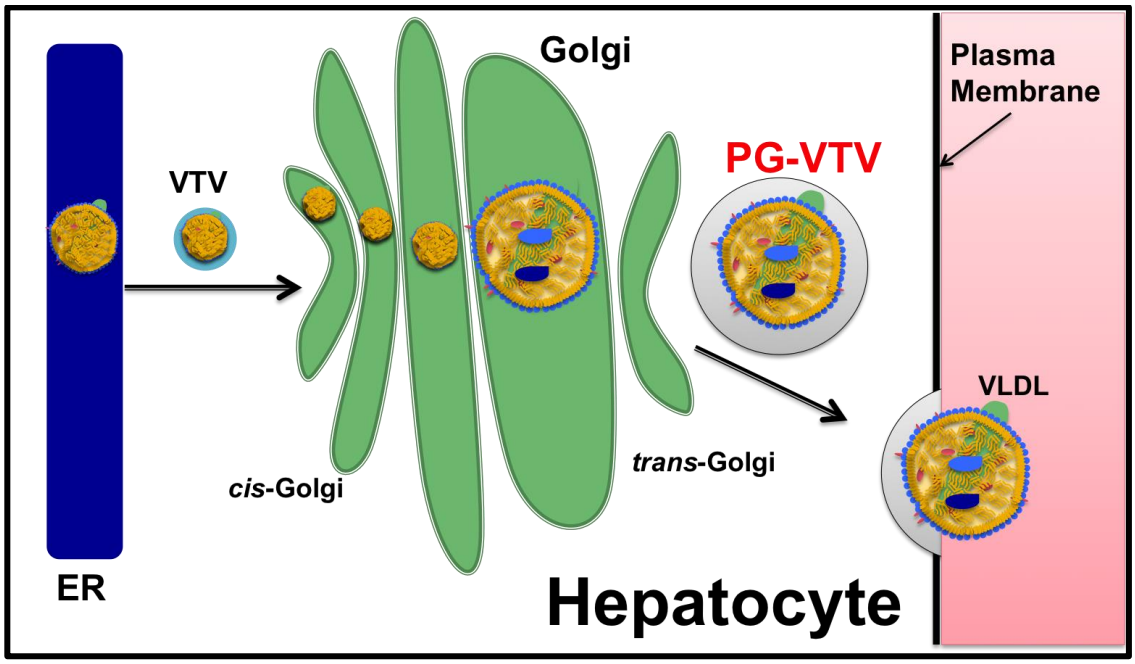


Figure 2 – VLDL Transport Pathway
Overview of the intracellular VLDL trafficking pathway. VLDL is synthesized in the ER lumen and transported via the VTV to the *cis*-Golgi for maturation. Upon maturation it is transported to the plasma membrane for secretion from the hepatocyte via the newly isolated PG-VTV vesicle.

The transport of mature VLDL to the plasma membrane is a prerequisite for its eventual secretion from the hepatocyte. This trafficking step is a directed means and thus alludes to the fact that a vesicle must facilitate this transport. The fact that this cargo is unique in size and biochemical composition to other molecules suggests that a specific vesicle must be responsible for the transport step. Moreover this specific vesicle must have the capability to enclose its mature VLDL cargo, traverse the cytosol and specifically deliver its cargo to the plasma membrane for secretion.

Vesicle budding and fusion is of utmost importance for transport of specific cargo between subcellular organelles and eventual secretion out of cells (*51*). Regulation of vesicular transport at the level of the trans-Golgi network (TGN) is physiologically vital to the generation of basolateral and apical membranes in polarized cells, and for sorting cargo for transport from the TGN (*52-55*). The significance of Golgi regulation has been shown with studies on protein containing vesicles where Golgi lipids such as phosphoinositides have been shown to play a role in the recruitment of trafficking components such as clatherin coats (*56, 57*), while glycosphingolipids and cholesterol levels have been hypothesized to play a role in segregating components into lipid rafts (*58*). Furthermore these studies have indicated the significance of the continuous membrane flow characteristic to the Golgi as being important for directing cargo for secretion (*59*), and have identified that these Golgi derived vesicles are initially formed by a tubule like extension of *trans*-Golgi network membranes and their subsequent membrane fission resulting in vesicles (*60, 61*).

Although much is known on clathrin- (mediate plasma membrane to TGN vesicular transport of endosomes), COPI- (retrograde vesicle transport from Golgi to the ER and more recently retrograde transport from endosomes to TGN), and COPII- vesicles (anterograde ER to Golgi transport) (*62, 63*), the synthesis, protein composition, and tethering SNARE protein complex responsible for directional trafficking and targeting of Golgi-to-Plasma membrane vesicles is not yet fully characterized.

It is for these reasons that the hypothesis is that mature VLDL exits the Golgi in a unique specialized vesicle which is unique in morphology, size, proteomic composition, and that this

vesicle is functional in its ability to deliver its VLDL cargo to the plasma membrane for secretion from the hepatocyte. The mechanism by which this investigation will be carried out involves first, generating Golgi-derived lipoprotein vesicles, followed by conducting morphological and biochemical studies on these vesicles. Upon confirmation that these vesicles are indeed unique and are capable of enclosing mature VLDL cargo, a proteomic analysis will be conducted to analyze these vesicles and compare them to previously identified hepatic lipoprotein vesicles, specifically the VTV. The intent of this study is to elucidate the requirements for Golgi-derived VLDL transport vesicle biogeneration and to identify its proteome. This study indicates that the PG-VTV is a novel Golgi to plasma membrane vesicle unique in size, buoyant density, cargo, and proteome and is responsible for the secretion of fully matured VLDL from hepatocytes. The significance of these studies are vast, as this will provide a basic tool for cardiovascular, and more specifically, lipoprotein research.

CHAPTER TWO: EXPERIMENTAL PROCEDURES

Materials

[^{3}H]OA (oleic acid; 45.5 Ci/mM) was acquired from Perkin Elmer Life Sciences (Boston, MA). Protease inhibitor cocktail tablets were acquired from Roche Applied Science (Indianapolis, IN). Gel electrophoresis and immunoblotting reagents were acquired from Bio-Rad (Hercules, CA). Enhanced chemiluminescence (ECL) reagents were acquired from GE Healthcare Life Sciences (Pittsburgh, PA). Other reagents used were of analytical grade and purchased from local companies. Sprague-Dawley rats (150-200 g) were acquired 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 anti-apolipoproteinB (apoB) antibodies were generated commercially (Protein Tech Group, Chicago, IL) using a synthetic peptide corresponding to amino acids 2055-2067 of rat apoB. Goat polyclonal anti-apoAIV, anti-apoE, anti-calnexin, anti-GOS28, anti-syntaxin antibodies; rabbit polyclonal anti-L-FABP antibody; and mouse monoclonal anti-Sec22b, anti-TGN38 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal anti-Sar1 antibodies were generated commercially and have been described previously. Rabbit polyclonal antibodies against rat VAMP7 (vesicle-associated membrane

protein 7; amino acids 105-123) have been described previously. Rabbit polyclonal antibodies to apoAI; and Mouse monoclonal antibodies to alpha 1 Sodium Potassium ATPase were purchased from Abcam (Cambridge, MA). Goat anti-Mouse IgG, Goat anti-Rabbit IgG, and Rabbit anti-Goat IgG conjugated with horseradish peroxidase (HRP) were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation and Purification of Subcellular Organelle Samples

Preparation of Radiolabelled Hepatic ER, cis-, and trans-Golgi

Perfusion of rat liver with calcium-free Kreb's Buffer [119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$; pH 7.4] followed by collagenase perfusion [11,000 units per liver (51 mg Type II collagenase per 150ml Kreb's buffer)] was performed. The liver was immediately harvested and primary hepatocytes were immediately isolated by chopping the liver and pushing the sample through a 100-micron sieve. Cells were washed in Kreb's buffer twice, then incubated in Buffer B [136 mM NaCl, 11.6 mM KH_2PO_4, 7.5 mM KCl, 0.5 mM dithiothreitol, 8mM Na_2HPO_4; pH 7.2] with BSA complexed to [^{3}H]OA (150 μCi) for 35 minutes at 37°C. Cells were then washed twice with 2% BSA in PBS to remove excess [^{3}H]OA. Cells were then homogenized in [0.25 M Sucrose in 10 mM HEPES pH 7.2, 50mM EDTA, and protease inhibitor] using a Parr Bomb at 1,000 psi for 40 minutes. A post-nuclear supernatant (PNS) was prepared, then pelleted and the resulting pellet was adjusted to 1.2 M sucrose in 10 mM

HEPES. ER, *cis*- and *trans*-Golgi fractions were separated using a sucrose step gradient (1.15 M, 0.86 M, 0.25 M).

Preparation of Hepatic Cytosol

Rat primary hepatocytes were washed in Kreb's buffer following isolation from freshly harvested rat liver perfused with collagenase. Cells were washed with 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 with a Parr bomb at 1,000 psi for 40 minutes, followed by ultracentrifugation at 165,000 × g for 95 minutes (Beckman Rotor Type 70 Ti). To remove endogenous ATP and GTP, supernatant was dialyzed against ice-cold cytosol buffer overnight at 4°C. Cytosol was concentrated using a centricon filter (Amicon, Beverly, MA) and ultra filtration membrane (Millipore, Billerica, MA) with a cut- off of 10 kDa.

Preparation of Hepatic Plasma Membrane

Rat liver was harvested following perfusion with Kreb's buffer, chopped, washed with 0.25 M Sucrose with protease inhibitor and EDTA, then centrifuged at 600 × g for 10 minutes. The Post Nuclear Supernatant (PNS) was collected, homogenized with 0.25 M sucrose and centrifuged at 38,400 × g for 10 minutes (Fiberlite F21S-8x50y rotor). The pellet was homogenized with 57% (w/v) sucrose until 47% sucrose (w/v) based on reading in refractometer and laid under a discontinuous sucrose step gradient of 37.2% (w/v) sucrose and 0.25 M sucrose, then ultracentrifuged at 106,600 × g (Beckman SW 32 Ti) for 16 hours at 4°C. The fraction was collected at the interface of the 0.25 M sucrose and 37.2% (w/v) fractions. Sample was then

adjusted to 14.6% sucrose and the PM was purified from Golgi membranes by being overlaid on a discontinuous sucrose gradient (23.7%, 30.8%, 38.1%), ultracentrifuged at 124,800 × g (Beckman Rotor SW 41 Ti) for 2 hours at 4°C. PM was collected at the 38.1% (w/v) and 30.8% (w/v) interface by aspiration.

Determination of Protein Concentration and Assessment of Sample Purity

The protein concentration of the samples was determined using the Bradford method. Samples were assessed for purity by testing for the presence of marker protein via immunoblotting.

In vitro Golgi-derived Vesicle Budding Assay

PG-VTVs were generated in a cell-free *in vitro* vesicular budding assay. Rat hepatic Golgi membranes (200 µg of protein) were incubated with hepatic cytosol (500 µg of protein), an ATP-generating system [1 mM ATP, 5 mM phospho-creatine and 5 units of creatine phosphokinase], 1 mM GTP, 1mM E600, 5 mM Mg^{2+}, 5mM DTT and 5mM Ca^{2+}, and the 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] for 30min at 37°C in the absence of Plasma Membrane acceptor. The reaction was terminated by placing the tubes on ice and diluted to 0.1 M with cold 10 mM HEPES. The reaction mixture was then overlaid on top of a continuous sucrose gradient (0.1 M to 0.86 M sucrose in 10 mM HEPES), ultracentrifuged at 115,000 × g (Beckman Rotor SW 41 Ti) for 2 hours at 4°C. Fractions of 500 µl were collected by aspiration and dpm of fractions was determined using a liquid scintillation counter.

In vitro PG-VTV – Plasma Membrane Fusion Assay

To demonstrate fusion, PG-VTV (150 µg of protein) was incubated with rat hepatic PM (150 µg of protein), cytosol (500 µg of protein), an ATP regenerating system, Mg^{2+}, Ca^{2+}, DTT, E600, Transport Buffer and incubated at 37°C for 35 minutes. Reaction was stopped by placing tubes on ice and reaction mixture was adjusted to 14.6% (w/v) with 23.7% (w/v) cold sucrose in 10 mM HEPES and overlaid upon a discontinuous sucrose gradient (23.7%, 30.8%, 38.1% sucrose in 10 mM HEPES) and ultracentrifuged at 124,800 × g (Beckman Rotor SW 41 Ti) for 2 hours at 4°C. 500 µl Fractions were collected at intermediate of 38.1% and 30.8% (w/v) interface by aspiration and dpm was determined using a liquid scintillation counter.

Measurement of Radioactivity

Radioactivity associated with [^{3}H]TAG was quantitated in terms of disintegrations per minute (dpm) using the single-isotope mode on a liquid scintillation analyzer (TriCarb, Model 2910, Perkin Elmer Life and Analytical Sciences, Shelton, CT).

Electron Microscopy

The negative staining technique of electron microscopy was utilized to examine the morphology of PG-VTVs. A formvar-carbon coated nickel grid was placed on a drop of concentrated PG-VTV fraction for 2–3 minutes, rinsed with PBS and water. The grid was then

stained with 0.5% aqueous uranyl acetate, air-dried, and examined at 10,000x magnification using an FEI Morgagni 268(D) transmission electron microscope (FEI, Hillsboro, Oregon).

Gel Electrophoresis and Immunoblot Analysis

Samples (40 µg of protein) were solubilized in Laemmli's Buffer (1X final concentration) and resolved via SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (12% gel for single percentage gels, 4-20% gel for gradient gels) at 20 mAmp until tracking dye reached the end of the gel. Proteins were then transblotted onto nitrocellulose membranes (Bio-Rad) at 50 mAmp overnight at 4°C. The membrane was then blocked with 10% (w/v) non-fat dried skimmed milk in PBS-T, incubated with specific primary and then the appropriate secondary antibodies conjugated with HRP. Protein was detected using ECL reagents and exposing to Film (MIDSCI, St. Louis, MO).

Two-Dimensional Gel Electrophoresis (2D-Gel)

Sample (150 µg of protein) was incubated in Rehydration Sample Buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) carrier ampholyte (pharmalyte pH 3-10), 0.002% Bromophenol blue, 20 mM DTT] and incubated at 37°C for 1 hour.

Sample was immediately loaded via anodic cup loading onto 11 cm IPG strip (pH 3-10 linear gradient) (GE Healthcare) that had been rehydrated at room temperature overnight in Rehydration Buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) carrier ampholyte

(pharmalyte pH 3-10), 0.002% bromophenol blue, 1.2% DeStreak Reagent]. The IPGphor III system was used to subject samples to isoelectric focusing for 52,000 Volt hours at 20°C at 50 µAmp/strip.

The IPG strip was then equilibrated for SDS-PAGE immediately following IEF by incubating for 15 minutes in SDS Equilibration Buffer I (Reducing Buffer) [6 M urea, 75 mM Tris-HCl (pH 8.8), 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 1% DTT] at room temperature, then subsequently incubated for 15 minutes in SDS Equilibration Buffer II (Alkylation Buffer) [6 M urea, 75 mM Tris-HCl (pH 8.8), 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 2.5% iodoacetamide] at room temperature. The IPG strip was then loaded onto a 10% SDS-PAGE gel, sealed with 0.75% agarose, and resolved at 15 mAmp for 1 hour, then at 20 mAmp until the dye reached the end of the gel.

The gel was immediately silver stained and scanned.

Two-Dimensional Difference In Gel Electrophoresis (2D-DIGE)

Sample Preparation and Minimal Fluorescent Labeling

Samples (50 µg of protein) were incubated with Lysis Buffer [7 M urea, 2 M thiourea, 30 mM Tris, 4% (w/v) CHAPS; pH 8.5] for 15 minutes on ice. No primary amines, DTT, or carrier ampholytes were in the lysis buffer as these could react with the N-hydroxysuccinimide ester group of the CyDye resulting in reduced concentration of fluorophore available for protein labeling. Samples were separately labeled with 400 pmol Cy3 or Cy5 fluorescent dye, vortexed,

briefly centrifuged, and incubated on ice for 30 minutes in the dark. The labeling reaction was stopped and unreacted dye was quenched by the addition of 10 mM L-lysine to each sample and incubation on ice for 10 minutes in the dark. Both of the labeled samples were then combined and incubated with 2D DIGE Sample Buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) carrier ampholyte (pharmalyte pH 3-10), 0.002% Bromophenol blue, 15 mM DTT] and incubated on ice for 15 minutes in the dark.

Isoelectric Focusing (IEF) and SDS-PAGE

Samples were immediately loaded via anodic cup loading onto 11 cm IPG strip (pH 3-10 linear gradient) (GE Healthcare) that had been rehydrated at room temperature overnight in Rehydration Buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) carrier ampholyte (pharmalyte pH 3-10), 0.002% bromophenol blue, 1.2% DeStreak Reagent]. The IPGphor III system was used to subject samples to isoelectric focusing for 52,000 Volt hours at 20°C at 50 µAmp/strip.

The IPG strip was then equilibrated for SDS-PAGE immediately following IEF by incubating for 15 minutes in SDS Equilibration Buffer I (Reducing Buffer) [6 M urea, 75 mM Tris-HCl (pH 8.8), 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 1% DTT] at room temperature, then subsequently incubated for 15 minutes in SDS Equilibration Buffer II (Alkylation Buffer) [6 M urea, 75 mM Tris-HCl (pH 8.8), 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 2.5% iodoacetamide] at room temperature. The IPG strip was then loaded onto a 10% SDS-PAGE gel, sealed with 0.75% agarose, and resolved at 15 mAmp for 1 hour, then at 20 mAmp until the dye reached the end of the gel.

Image Scan and Data Analysis

The gel was immediately scanned using a Typhoon TRIO scanner (GE Healthcare) at the manufacturer specified excitation and emission wavelengths at 100 µm resolution. Scanned images were generated and analyzed using ImageQuant software (version 5.2, GE Healthcare).

CHAPTER THREE: PG-VTV BIOGENERATION

In order to study the downstream steps of VLDL trafficking within the hepatocyte, a cell free *in vitro* budding assay was utilized to study the formation of the VLDL containing vesicles from Golgi membranes. Golgi membranes, cytosol, ER, and PM subcellular organelles were isolated from hepatocytes for use in this cell free assay. The aim was to establish that VLDL exits the Golgi apparatus in a process mediated by a specialized vesicle; to establish this vesicle's functionality and specificity in targeting the plasma membrane for delivery of its VLDL cargo; and to identify the requirements for its biogenesis.

Assessment of Hepatic Subcellular Organelle Purity

As our means of studying VLDL vesicles is under cell free in vitro assays, organelle purity is of great importance. In order to proceed with confidence, the purity of isolated organelle samples were initially assessed by the presence of their specific marker proteins. Marker proteins were utilized in order to verify isolation of organelles was indeed successful and that no contamination was present. Specific marker proteins, or proteins unique to that organelle were tested for by western blot analysis. Table 1 shows the organelle marker proteins used for assessing purity.

Table 1 – Hepatic Subcellular Organelle Marker Proteins

Organelle	Marker Protein	Protein Molecular Weight
Endoplasmic Reticulum	Calnexin	90 kDa
Golgi Apparatus	GOS28	28 kDa
	TGN 38	38 kDa
Plasma Membrane	Na-K ATPase	112 kDa

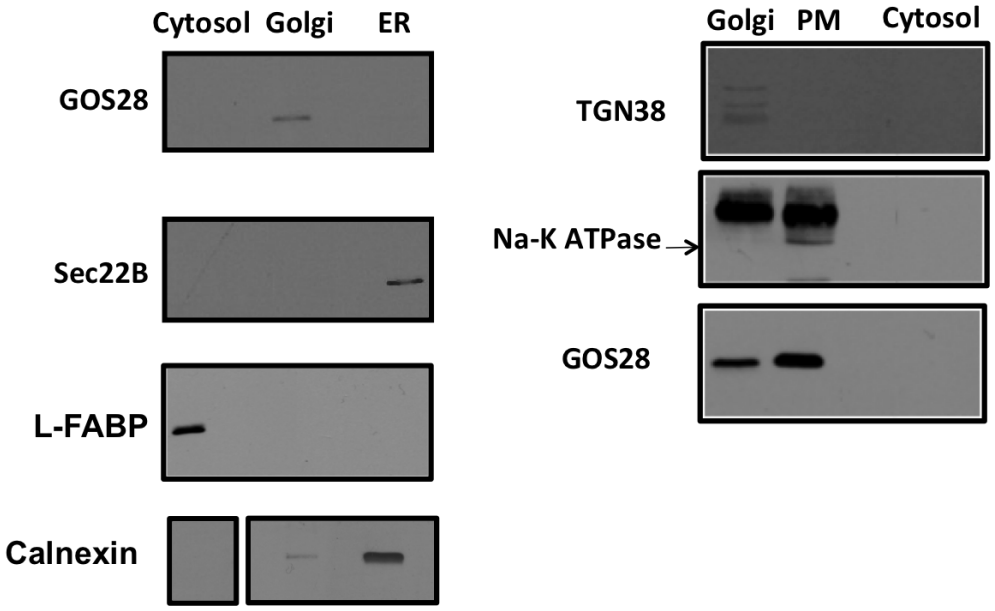


Figure 3 – Organelle Purity

Western blot data for hepatic cytosol, Golgi, and ER samples (left); and Golgi, Plasma Membrane, and Cytosol samples (right). Samples (40 µg of protein) were resolved on a 12% SDS-PAGE gel.

As shown in Figure 3, isolated Golgi membranes contained GOS28 and TGN38, marker proteins for the *cis*-Golgi and *trans*-Golgi, respectively. The Golgi membranes isolated were free of ER contamination as shown by the absence of Calnexin, a resident ER protein. Isolated ER membranes contained Calnexin and sample purity was assessed by the absence of GOS28 and TGN38. As expected, PM fractions contained the Na-K ATPase protein. Purity of the cytosol sample was assessed by the absence of organelle specific marker proteins Calnexin, GOS28, TGN38, and Na-K ATPase while containing the cytosolic protein L-FABP.

To further demonstrate that each organelle sample was unique in proteome, two dimensional electrophoresis gel (2D-Gel) analysis was conducted. The 2D-gel spot pattern for each organelle was unique as shown in Figure 4.

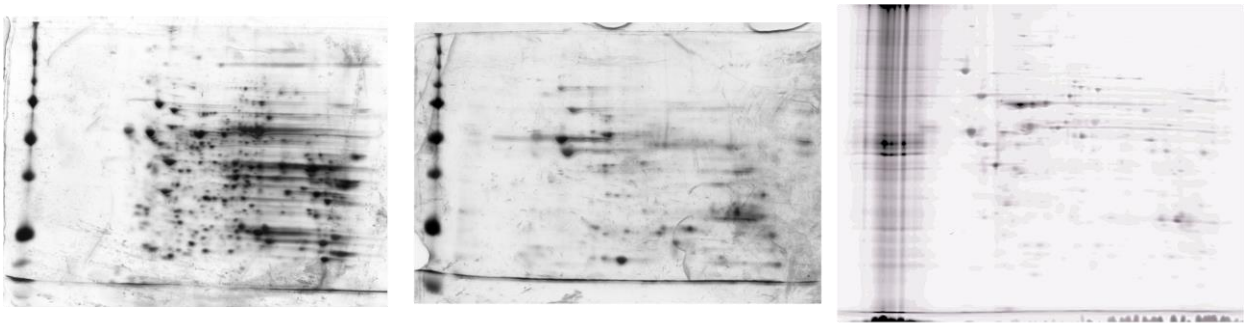


Figure 4 – Organelle 2D-Gels
Hepatic cytosol, ER, and Golgi membranes were resolved on 2D-Gel (pH 3-10 first dimension, 10% SDS-PAGE second dimension). Cytosol and ER samples (150 µg protein) were silver stained immediately after the second dimension. Golgi sample (50 µg protein) was labeled with Cy3 and

scanned immediately following the second dimension. Gels were analyzed for general shape and spot pattern.

Together, western blotting data and 2D-gel data indicate that the isolated hepatic subcellular organelle samples were of sufficient purity to use in subsequent experiments.

Cell-Free *in vitro* Golgi Budding Assay and Assessment of VLDL

Prior to isolating the organelle samples, VLDL within hepatocytes were radiolabeled by incubating the hepatocytes with [^{3}H]Oleic Acid, which would then be incorporated into [^{3}H]TAG and conjugated with VLDL. This allowed the detection of VLDL in downstream assays by assessment of radioactivity. Golgi membranes containing radiolabeled mature VLDL were incubated at 37°C in the presence of cytosol and an ATP regenerating system under conditions that promoted vesicular generation, or budding, from the Golgi membranes. The reaction was terminated and immediately resolved on a continuous sucrose gradient (0.1 M to 0.86 M). Under these conditions it is expected that vesicles containing VLDL would float to the top due to their light buoyant density as a result of their high TAG content, while the remaining Golgi membranes would pellet. This technique is illustrated in Figure 5.

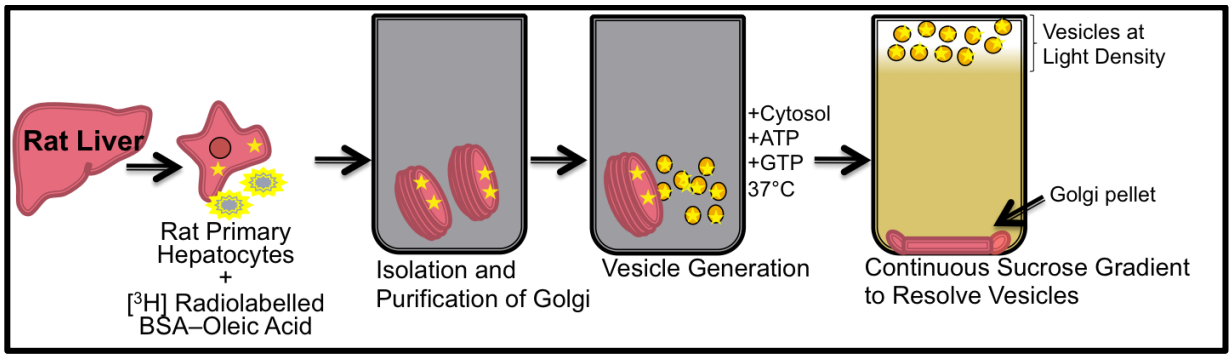


Figure 5 – Budding Assay for PG-VTV Biogeneration

Overview of the cell-free *in vitro* budding assay utilized to generate PG-VTVs. Rat primary hepatocytes were isolated from freshly harvested rat liver and incubated with radiolabelled [^{3}H]OA. Organelles containing radiolabeled VLDL were isolated and incubated under conditions to promote vesicle formation. Vesicles were isolated on a continuous sucrose gradient.

This gradient was then separated into sequential fractions and assessment of radioactivity by dpm count of each fraction was conducted. The presence of VLDL was indicated by a high dpm count indicating the presence of [^{3}H]TAG. As shown in figure 6, the lightest density fractions contained a peak in radioactivity indicating the presence of VLDL containing vesicles of Golgi origin.

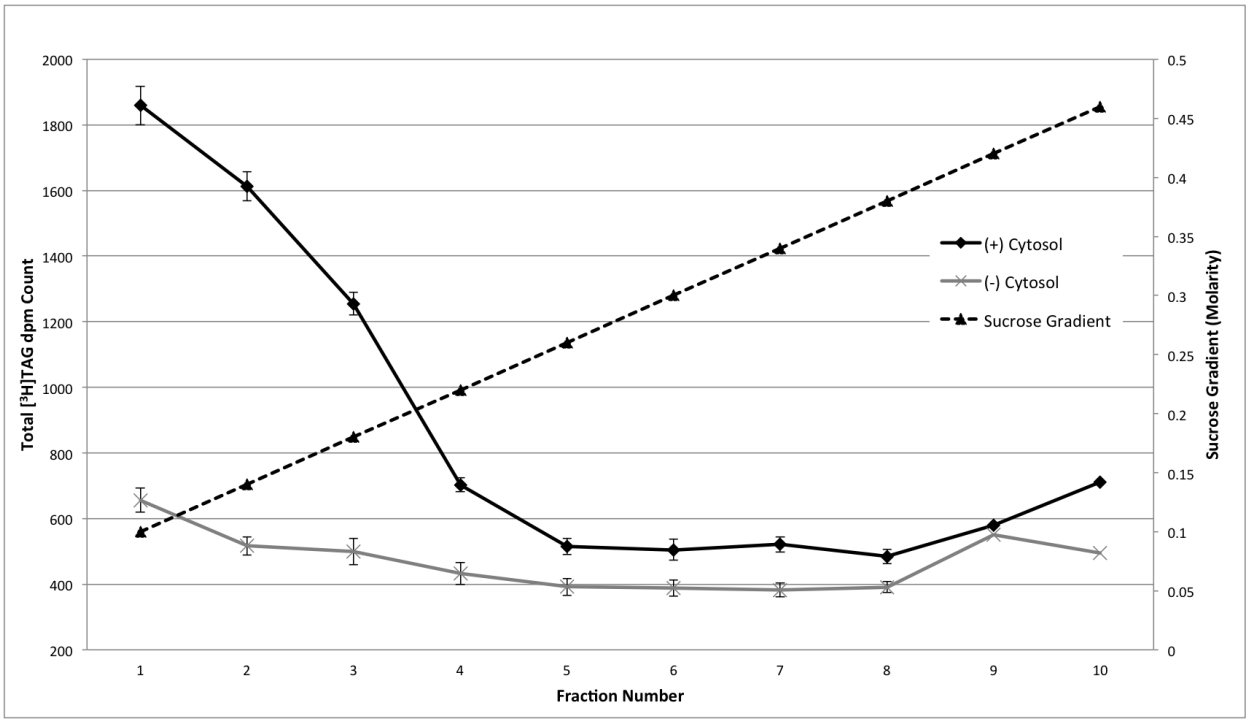


Figure 6 – [^{3}H]TAG Radiolabelled VLDL Along Continuous Sucrose Gradient
Total dpm count along the continuous sucrose gradient. Green line corresponds to the increasing sucrose gradient. Orange line corresponds to the negative control (no cytosol), while the blue line is the actual experiment. Data reveals the high concentration of dpm in the first three fractions.

Vesicle Purity from Organelle Contamination

Isolated PG-VTVs did not contain organelle marker proteins, suggesting they were specifically formed VLDL transport compartments and not merely fragments of broken organelle membranes. As shown in Figure 7 PG-VTVs did not contain the Golgi marker protein

GOS28, nor the SNARE protein Sec22B, which is present on VTVs and localizes to ER membranes.

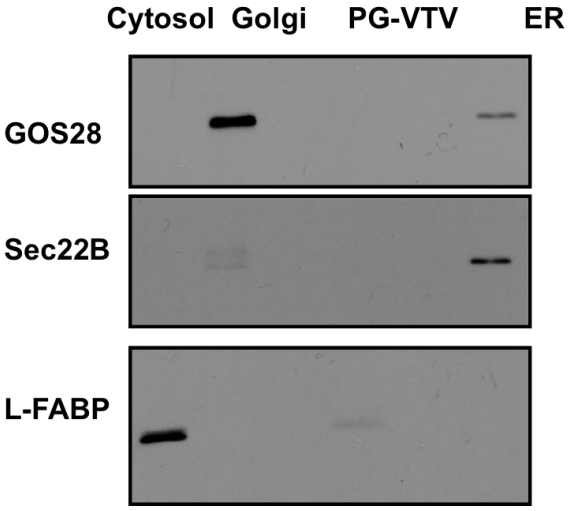
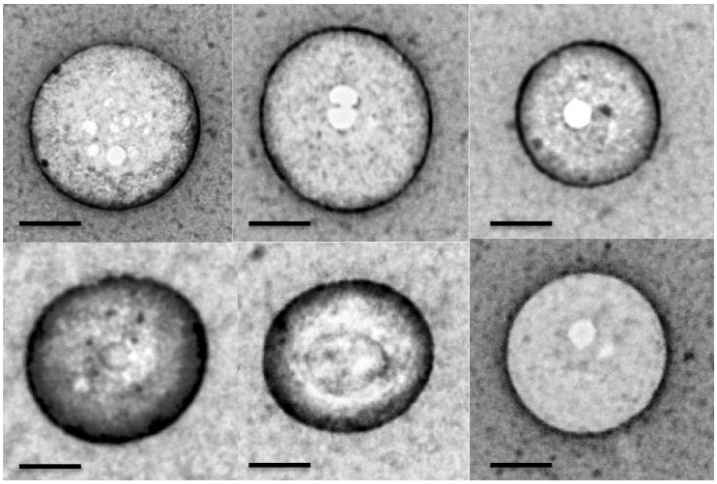


Figure 7 – PG-VTV Purity From Organelle Contamination
Western blot analysis of Cytosol, Golgi, PG-VTV, and ER. Samples (40 µg protein) were resolved on a 4-20% gradient SDS-PAGE gel.

Morphology of PG-VTV

The negative staining technique of electron microscopy was utilized to examine the morphology of the PG-VTVs. Fractions 1-3, which contained peak dpm counts, were concentrated and observed. As shown in the figure 8, these light-density fractions contained several vesicles. Moreover, these vesicles were approximately 300nm in diameter, an adequate size to harbor a VLDL sized particle (80-100nm diameter).



Bar = ~100 nm

Figure 8 – Morphology of PG-VTV
PG-VTV vesicles were concentrated from fractions 1-3 of continuous sucrose gradient and analyzed utilizing negative staining technique of electron microscopy.

Requirements for PG-VTV Budding

The requirements for PG-VTV biogenesis from Golgi membranes were identified. The post-Golgi budding assay was repeated under several conditions. To assess the requirement of physiological temperature, the assay was repeated at 4°C. PG-VTV budding requires energy in the form of ATP as vesicular formation was significantly abrogated when the ATP regenerating system was replaced with apyrase, an enzyme which digests ATP. Cytosolic proteins are necessary for proper PG-VTV formation, as no budding occurs when cytosol was replaced with cytosolic buffer. As figure 9 shows, the formation of VLDL containing vesicles requires ATP,

cytosolic protein, and incubation at 37°C. When these conditions are not met, budding was significantly abrogated.

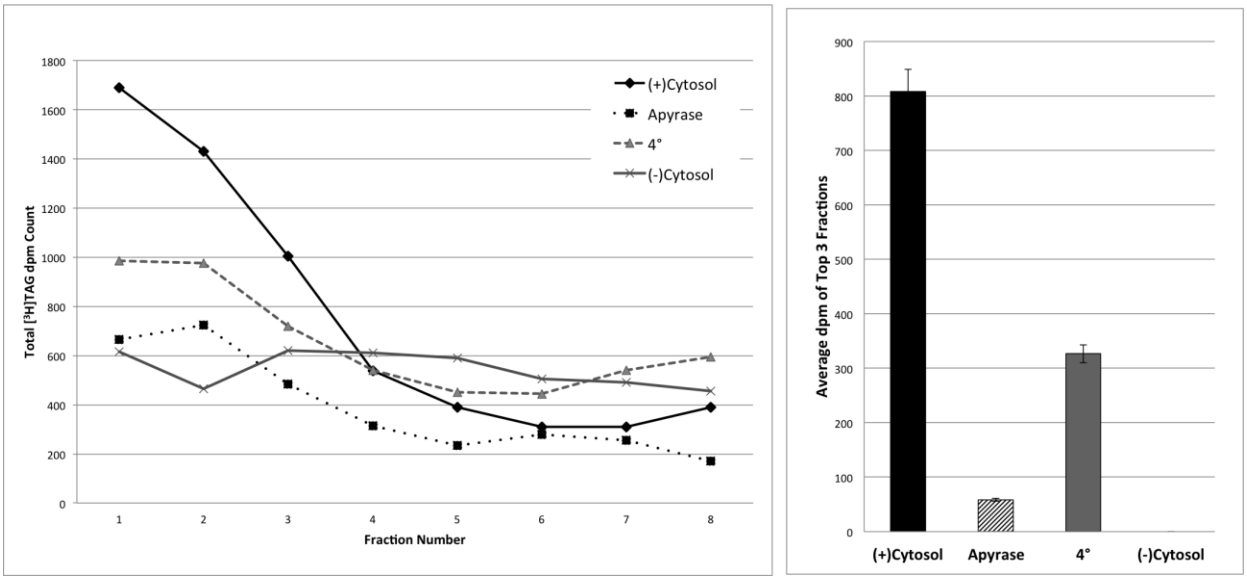


Figure 9 – PG-VTV Budding Requirements
Cell-free *in vitro* budding assay was performed under conditions of apyrase replacing ATP, 4°C, and no hepatic cytosol. Each condition significantly abrogated dpm signal.

Fusion with Plasma Membrane

The functionality of PG-VTVs was assessed by their fusogenic properties, specifically fusion with the plasma membrane and delivery of their VLDL cargo to the plasma membrane. This was characterized through a cell free *in vitro* fusion assay with isolated hepatic plasma membrane sample. A characteristic of vesicle-mediated trafficking is the ability of the specific

vesicle to deliver its cargo to its destination through a directed mechanism. This fusion assay is illustrated in figure 10.

PG-VTVs incubated with hepatic plasma membrane were successful in transferring their VLDL cargo to plasma membrane destination. Importantly this trafficking step required access to the cytosolic side of the plasma membrane. Isolation of plasma membrane results in vesicle like compartments oriented identical to the intact hepatocytes. A series of freeze/thaw cycles in liquid nitrogen are required to flip the plasma membrane vesicles and expose their cytosolic side. Figure 11 illustrates the steps required to expose cytosolic side of plasma membrane to the vesicles for fusion. Upon exposure of the cytosolic side, the PG-VTVs are able to bind with the plasma membrane and deliver their VLDL cargo to these PM compartments.

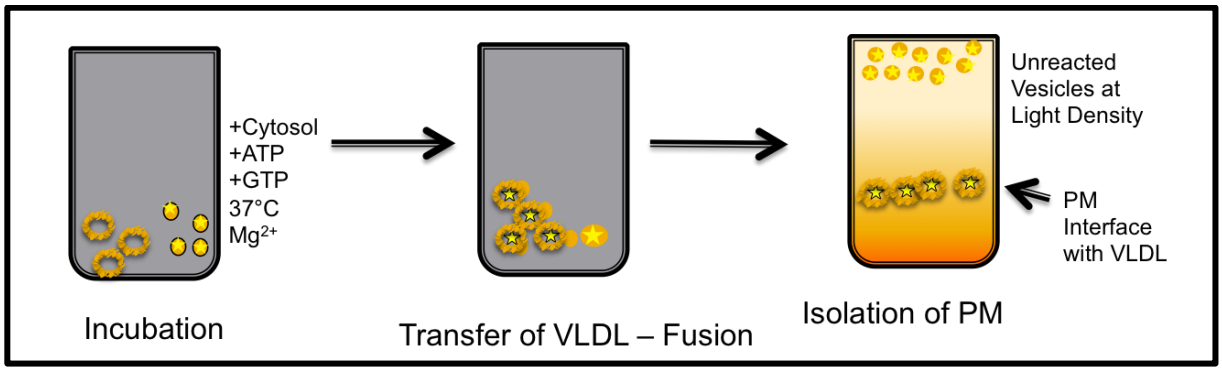


Figure 10 – Fusion Assay
PG-VTVs were incubated with hepatic cytosol and plasma membrane. Reaction was then resolved on a discontinuous sucrose gradient and PM interface was isolated at its expected density. Fusion of vesicles with plasma membrane was signified by VLDL becoming isodense with the plasma membrane signifying a transfer of dpm to the plasma membrane interface.

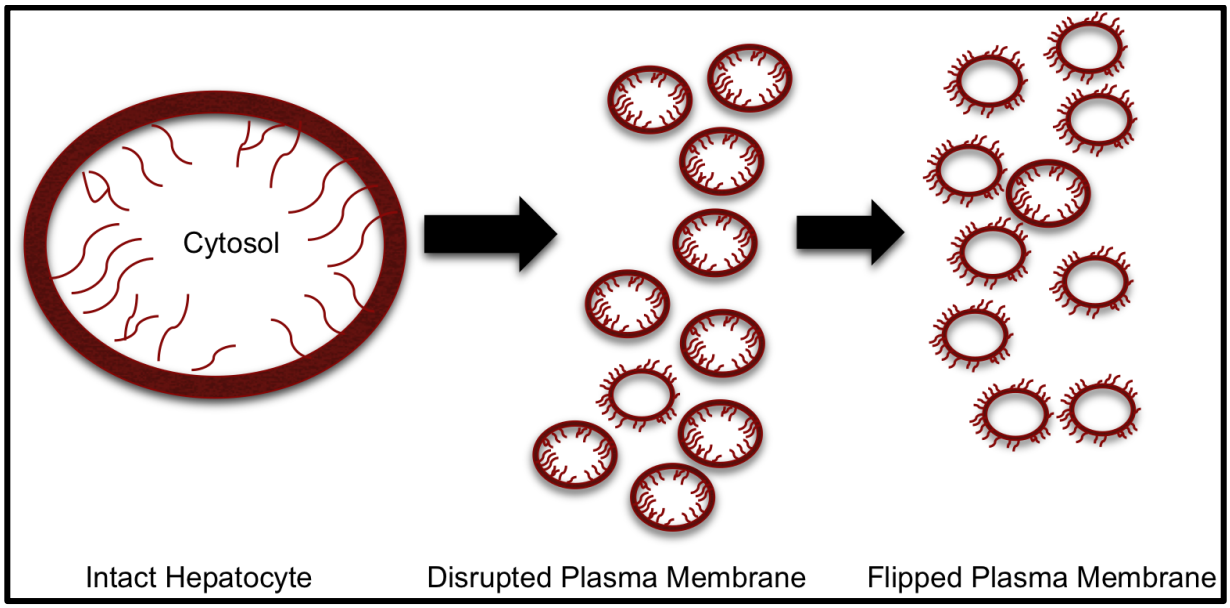


Figure 11 – Plasma Membrane Orientation
Shown is a model illustrating the necessity to flip plasma membrane in order to expose its cytosolic side to vesicles. Upon isolation of plasma membrane from intact hepatocytes, vesicle-like structures form with the same orientation as intact hepatocyte. To expose cytosolic side, plasma membrane sample was freeze/thawed in liquid nitrogen and 20°C water bath for 5 cycles.

Importantly, this transport was unidirectional; vesicles were able to fuse with the plasma membrane but not with Golgi membranes indicating their specificity in transporting VLDL in a vectorial manner along the secretory pathway. As shown in figure 12, PG-VTVs were capable of transferring VLDL, indicated by a transfer of dpm, to the plasma membrane under

conditions of 37°C, access to properly oriented plasma membrane, and presence of cytosolic protein.

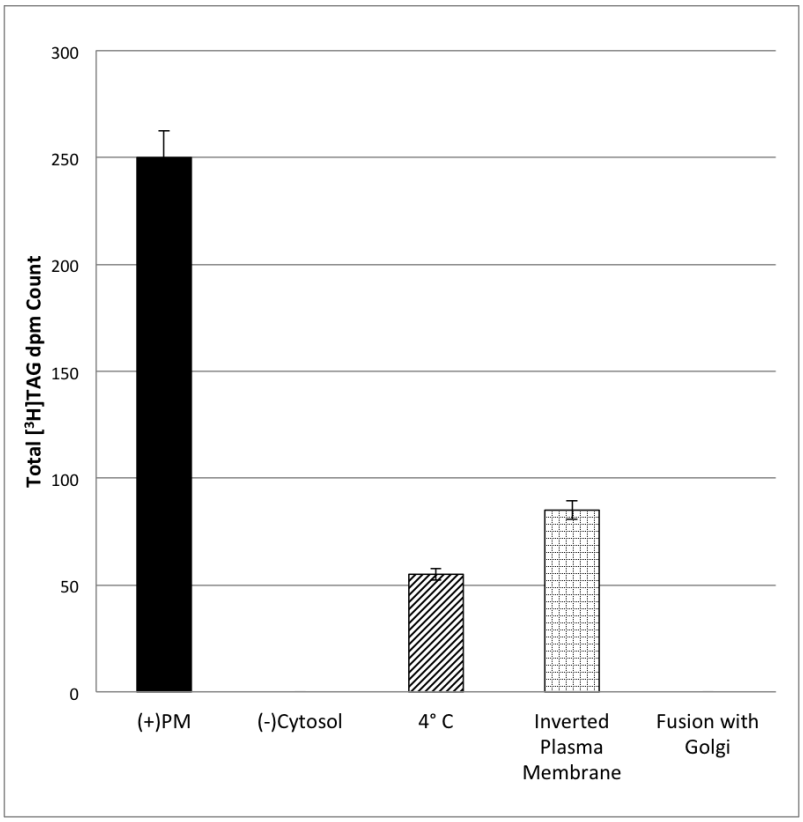


Figure 12 – Transfer of [3H]TAG dpm
Total dpm count of PG-VTV fusion under conditions of no cytosol, 4°C, inverted plasma membrane, and incubation with Golgi membranes.

Taken together, this data strongly suggests the isolation of a specialized VLDL transport vesicle. This vesicle facilitates VLDL exit from the *trans*-Golgi face, directed transport to the plasma membrane, and delivery of cargo for secretion from the hepatocyte. The biogenesis of

the PG-VTV requires the presence of cytosol, energy in the form of ATP, and incubation at 37°C. The fusion and delivery of the cargo requires the presence of cytosol, incubation at 37°C, access to the cytosolic side of the plasma membrane, and importantly is a unidirectional means of transporting mature VLDL from the Golgi to the PM.

This vesicle is unique in its proteome and is fully functional and can not only specifically select its cargo for secretion from the hepatocyte, but transports this cargo in a unidirectional manner further attesting to its specificity and novelty.

CHAPTER FOUR: PROTEOMIC ANALYSIS

The next aim of the study was to conduct a proteomic profile of the newly isolated PG-VTV. Fully describing the PG-VTV proteome will create a new research tool that may prove to be an aid in ultimately elucidating the lipoprotein secretory pathway. This tool may potentially facilitate the identification of key regulatory proteins in the intracellular trafficking of matured VLDL and potentially identify key molecular targets for cardiovascular drug therapy. Moreover, a proteomic analysis will aid in understanding the mechanism PG-VTV function, necessary as its function is a vital downstream step in the secretion of VLDL from the hepatocyte.

Proteomic Analysis via Immunoblot

The first step in the proteomic analysis was to probe for the presence of proteins that are known to be associated with VLDL. ApolipoproteinB100 is the main structural protein of VLDL (*7, 33, 64*). As shown in Figure 13, the PG-VTV concentrated both the 520 kDa apoB100 isoform and the 250 kDa apoB48 forms, indicating the fact that these vesicles do indeed facilitate the transport of VLDL. As expected, the 46 kDa apolipoproteinAIV (apoAIV) was also detected in PG-VTVs, apoAIV serves in interacting with apoB enhancing the ability to expanding VLDL's size thus facilitating the secretion of TAG (*65*). Interestingly as shown in figure 14, apoAI, which is not present on the VTV was present on the PG-VTV, signifying that it is transported to the Golgi apparatus by a mechanism separate from the lipoprotein vesicle. The v-SNARE VAMP7 was detected on the PG-VTV which alludes to its potential role in being the tethering molecule

responsible for directing this vesicle to the plasma membrane. Syntaxin5, a t-SNARE was not detected on the PG-VTV.

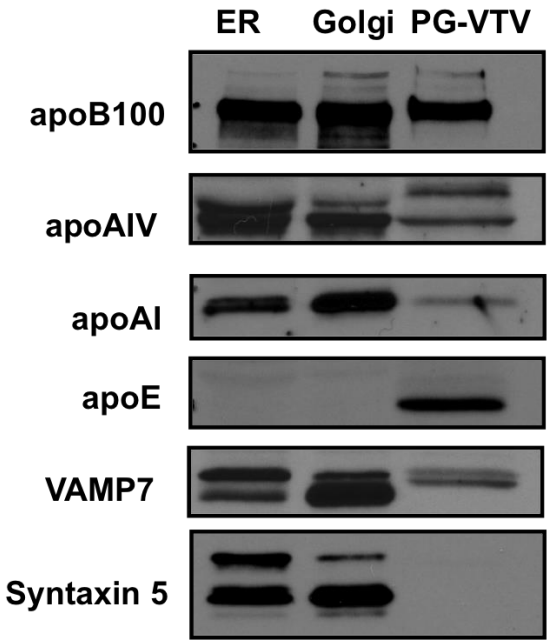


Figure 13 – PG-VTV Western Blot Analysis
Western blot analysis of hepatic ER, Golgi, and PG-VTV. Samples (40 µg of protein) were resolved on a 4-20% gradient SDS-PAGE gel.

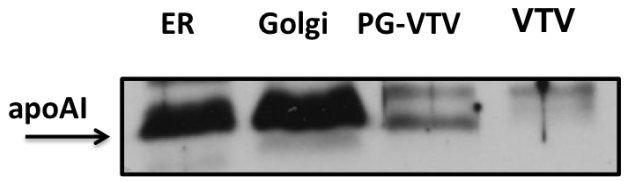


Figure 14 – ApolipoproteinAI added to PG-VTV
Western blot analysis of hepatic ER, Golgi, PG-VTV, and VTV. Samples (40 µg of protein) were resolved on a 4-20% gradient SDS-PAGE gel and probed with anti apoAI antibody and its corresponding secondary antibody and developed on film.

2D Comparative Proteomic Analysis

A comparative proteomic analysis was undertaken to compare the PG-VTV proteome with the currently known VTV proteome. The method utilized was 2D-DIGE as it allows the simultaneous resolution between two samples in the same gel. The scans shown in figure 15 show the VTV protein that was labeled with Cy3 a fluorescent dye that appears in green, and the PG-VTV protein that was labeled with Cy5, a fluorophore that appears in red.

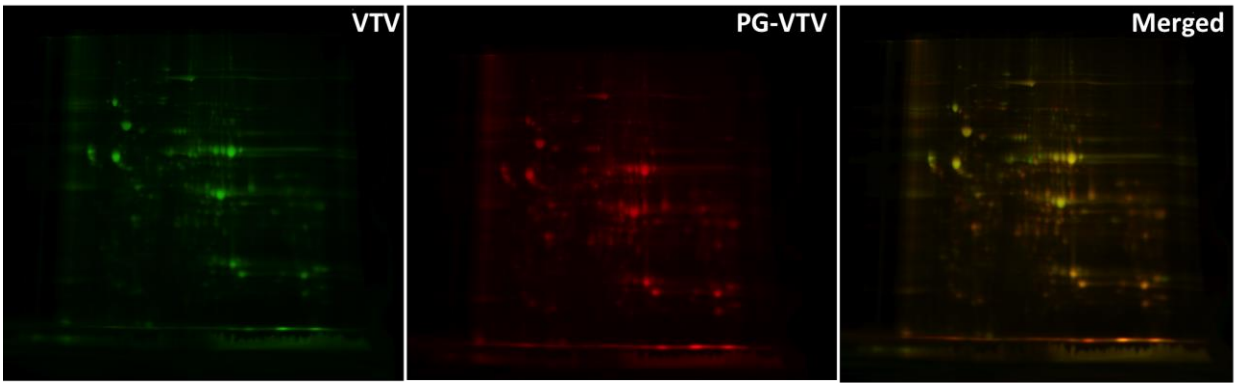


Figure 15 – 2D-DIGE Comparative Analysis
A 2D-DIGE analysis was performed on VTV and PG-VTV samples (50 µg of protein). VTV was labeled with Cy3 fluorescent dye (left), PG-VTV sample was labeled with Cy5 dye (center), and overlay was analyzed (right).

As shown in figure 14, the proteomic profile of the PG-VTV was very similar to the VTV proteome in both shape and composition, however there were several spots that were unique to the post Golgi vesicle. As shown in the merge in figure 16, these spots have been highlighted. These spots are of interest for future study as they may be indicative to regulatory entities that are necessary for PG-VTV biogeneration and direction towards the plasma membrane.

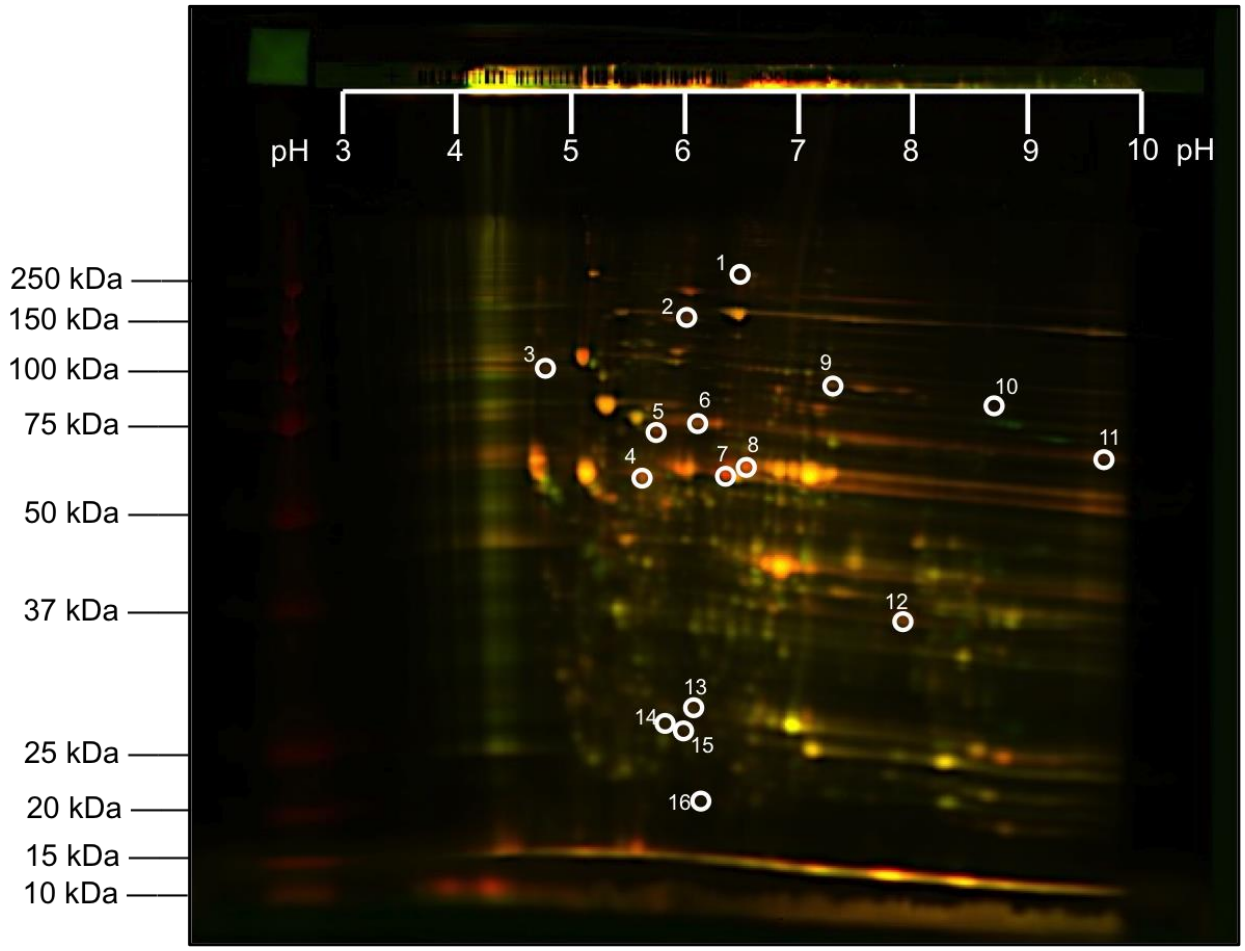


Figure 16 – Analysis of 2D-DIGE Merge

The overlay of the VTV & PG-VTV 2D-DIGE analysis was analyzed for differences. Circled spots are indicative of protein unique to the PG-VTV sample.

CHAPTER FIVE: CONCLUSION

The data resulting from these studies were indicative of the fact that a unique, specialized vesicle is responsible for transporting mature VLDL from its site of maturation, which is the Golgi lumen, to the plasma membrane for its eventual secretion to the bloodstream. This step is required for the secretion of matured VLDL from the hepatocyte and thus studying its required steps are necessary to fully elucidate the hepatic lipoprotein secretory pathway.

The overall secretion pathway, with regards to trafficking, is shown in Figure 2. The VLDL synthesis begins in the lumen of the ER. Upon initial assembly, this premature VLDL molecule is transported to the Golgi lumen for maturation. This first transport step is mediated by the VTV, a unique COPII-dependent vesicle. Upon docking and fusion with the *cis*-Golgi membranes VTV delivers VLDL Golgi lumen. It is here where the VLDL molecule undergoes several modifications as it traverses the cisternae of the Golgi including the incorporation of apoAI and apoE lipoproteins, and the phosphorylation and glycosylation of the apoB100 protein backbone. Upon maturation, this VLDL molecule is ready for secretion from the hepatocyte. In a complex process involving cargo selection, membrane deformation, and curvature, the matured VLDL molecule is incorporated in a vesicle, the PG-VTV.

The PG-VTV facilitates VLDL exit from the *trans*-Golgi face and encloses it in a vesicle of 300-320 nm in diameter, one that is large enough to facilitate a VLDL sized molecule (80-100 nm). This vesicle's morphology is one that confirms the fact that it is a vesicle shaped enclosed

intracellular compartment, which could enclose the VLDL and protect it from the cytosolic lipases and proteases.

The vesicle is free of contamination from organelle marker proteins, and does not contain Sec22b, which is present on the VTV and functions as its v-SNARE. These indicate that the PG-VTV is a newly isolated vesicle unique to the VTV and not fragments of broken organelle membranes.

The formation of this vesicle requires the physiological temperature of 37°C, the presence of energy in the form of ATP, and cytosolic protein. All these conditions that are established conditions for vesicular formation. If any of these conditions are altered, then the presence of [^{3}H]TAG, our marker for VLDL, is severely abrogated in the light density fractions where we normally find the PG-VTV.

The vesicle is functional in its ability to transfer VLDL, indicated by a transfer of dpm, to the plasma membrane. More importantly, this transport step is unidirectional as shown by data indicating the vesicle does not fuse with the Golgi membranes and cannot deliver VLDL to the Golgi lumen. The PG-VTV does indeed deliver VLDL to the plasma membrane successfully. Furthermore, the correct orientation of the plasma membrane is necessary, the PG-VTVs can only deliver their VLDL cargo if the cytosolic side of the plasma membrane is exposed for reaction. The fusion of PG-VTV with correctly oriented plasma membrane also requires the presence of cytosolic protein and the correct physiological temperature of 37°C. These requirements further indicate the function of these vesicles under physiological conditions and correlates with widely established requirements for vesicular fusion.

The vesicle's proteomic profile is unique and contains lipoproteins necessary and known to exist in matured VLDL present in the bloodstream. The comparative 2D gel analysis undergone signifies that this vesicle furthermore is similar to the VTV, stating the fact that it is indeed a vesicle responsible for transporting VLDL, yet this vesicle transports matured VLDL and contains unique protein necessary for the post-Golgi transport step.

Table 2 summarizes key points and differences between the PCTV or the vesicle responsible for transporting nascent chylomicrons in the intestine, the VTV, and the PG-VTV.

Table 2 – Summary of Lipoprotein Transport Vesicles

	PCTV	**VTV**	**PG-VTV**	**Reference**
Size	142-500nm	100-120nm	300-320nm	(*33, 66*)
v-SNARE	VAMP7	Sec22b	VAMP7	(*33, 66-68*)
t-SNAREs	Syntaxin5, rBet1, vti1a	Syntaxin5, rBet1, GOS28	Syntaxin1, SNAP23 (Hypothesized)	(*33, 66-68*)
Coat	COPII independent	COPII dependent		(*33, 68*)
GTP requirement	No	Yes	Yes	(*33, 66*)
Initiator	L-FABP	Sar1		(*33, 67*)
Direction	ER to Golgi - intestine	ER to Golgi - liver	Golgi to PM - liver	(*33, 66*)

In the study herein, the PG-VTV has been shown to be the unique specialized vesicle responsible for transport of mature VLDL from the Golgi apparatus to the plasma membrane for secretion. This vesicle is unique in proteome and morphology from currently known vesicles. Moreover, this vesicle requires physiological conditions for its biogenesis. The vesicle is functional in its ability to fuse with the plasma membrane and deliver its VLDL cargo for secretion. The proteomic profile of PG-VTV that has been conducted will provide a basic tool for studies with focus on lipoprotein and may prove to have vast implications for the future of cardiovascular research.

APPENDIX: IACUC ANIMAL PROTOCOL APPROVAL LETTER

University of Central Florida

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.)

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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