Dynamic remodeling of membranes and their lipids for cholesterol transport during steroid biosynthesis in Leydig cells

By

Sathvika Venugopal

Division of Experimental Medicine, Faculty of Medicine,

McGill University

Montreal, Quebec, Canada

October 2017

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Notice

Dedication

To my father and my husband, for their support and belief in me.

Table of content

List of figures and tables

Chapter I

Chapter II

Chapter III

List of Abbreviations

17βHSD	17-hydroxysteroid dehydrogenase
22*R*-HC	22*R*-hydroxycholesterol
22*S*-HC	22*S*-hydroxycholesterol
3βHSD	3β-hydroxysteroid dehydrogenase
ACAT	Acyl-coenzyme A: cholesterol acyltransferase
ACBD1	Acyl-CoA Binding Domain-containing 1
ACBD3	Acyl-CoA Binding Domain-containing 3
ACSL4	Acyl-CoA synthetase 4
ACTH	Adrenocorticotropic hormone
AMG	DL-aminoglutethimide
ANT	Adenine nucleotide translocase
APCI	Atmospheric-pressure chemical ionization
apoB	Apolipoproteins B
ATAD3	ATPase family, AAA domain containing 3
ATP	Adenosine triphosphate
BN-PAGE	Blue-native polyacrylamide gel electrophoresis
BODIPY	Boron-dipyrromethene
cAMP	Cyclic AMP
CE	Cholesteryl esters
Cer	Ceramides
CL	Cardiolipins

COX IV	Cytochrome c oxidase subunit IV
CPT	Carnitine palmitoyltransferase
CRAC	Cholesterol recognition amino acid consensus sequence
CREB	cAMP Response Element-Binding protein
CYP11A1	Cytochrome P450 side chain cleavage enzyme
CYP17A1	Cytochrome P450c17
D4	Domain 4 of the Θ toxin
DAG	Diacylglycerol
dbcAMP	Dibutyryl-cAMP
DBI	Diazepam Binding Inhibitor
DMEM	Dulbecco's modified Eagle medium
Drp1	Dynamin-related protein
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
ERC	Endosomal Recycling Compartment
FA	Fatty Acid
FBS	Fetal Bovine Serum
FDX	Ferradoxin
FDXR	Ferradoxin Reductase
FSH	Follicle Stimulating Hormone
GFP	Green Fluorescent Protein
hCG	Human Chorionic Gonadotropin
HDL	High-Density Lipoprotein

HMG-CoA	Hydroxymethylglutaryl-CoA
HMGR	HMG-CoA reductase enzyme
HRP	Horse radish peroxidase
HSC70	Heat shock cognate 70 kDa chaperone protein
HSL	Hormone Sensitive Lipase
HSP90	Heat shock protein 90 kDa
IC 100	Maximal inhibitory concentration
IC 50	Half-maximal inhibitory concentration
IMM	Inner Mitochondrial Membrane
IMS	Inner Mitochondrial Space
IS	Internal standard
LAL	Lysosomal Acid Lipase
LAM	Lipid droplet Associated Membranes
LAMP-2	Lysosome-associated membrane protein 2
LDL	Low-density lipoprotein
LDs	Lipid droplets
LH	Luteinizing Hormone
MA-10	Mouse tumor Leydig cells
MAM	Mitochondrial Associated Membranes
mCherry-D4H	mCherry-D4^{D434S}
MFN2	Mitofusin2
MPTP	Mitochondrial Permeability Transition Pore
MTBE	Methyl Tert-Butyl Ether

MβCD	methyl-β-cyclodextrin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NPC	Niemann-Pick type C
NPC1	Niemann-Pick type C 1
NPC2	Niemann-Pick type C 2
OMM	Outer Mitochondrial Membrane
ORAi1	Calcium release–activated calcium channel protein 1
PA	Phosphatidic acid
PACS2	Phosphofurin acidic cluster sorting protein-2
PAMs	Plasma membrane associated membrane
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PKA	Protein kinase A
PKA-Riα	Protein Kinase A-Regulatory Protein subunit I alpha
PM	Plasma membrane
PMCA1	Plasma membrane $Ca^{(2+)}$ ATPase 1
PS	Phosphatidyl serine
PVDF	Polyvinylidene fluoride
RIA	Radioimmunoassay
RT	Room Temperature
SIGMAR1	Sigma-1 receptor
siRNA	Small interfering RNA

SM	Sphingomyelin
SR-BI	Scavenger receptor class B, type I
SREBP	Sterol regulatory element-binding protein
StAR	Steroidogenic acute regulatory protein
START	StAR-related lipid transfer β-barrel domain
STIM1	Stromal-interacting molecule 1
TM	Transmembrane α-helical domains
TOF	Time of flight
TSPO	Translocator Protein (18 kDa)
VDAC1	Voltage dependent anion channel 1
VLDL	Very low-density lipoproteins
Θ toxin	Perfringolysin O

Abstract

Cholesterol is the only precursor for all steroids produced in mammals. During hormone induced acute steroidogenesis, large quantities of cholesterol is trafficked from its intracellular stores to the cholesterol-poor organelles, mitochondria, to be converted to pregnenolone, the first of all steroids. Although steroidogenesis is extensively studied, the actual source organelle that stores and then mobilizes cholesterol for utilization in steroid production and the pathway that assists this hydrophobic molecule to be trafficked to mitochondria are yet to be determined.

Utilizing the domain 4 (D4) of the Perfringolysin O protein produced by *Clostridium perfringens,* which binds to high concentrations of cholesterol in membranes without cytotoxicity, we first determined the source organelle. Live cell imagining analysis in a Leydig tumor cell line and primary rat Leydig cells utilizing mCherry tagged D4 revealed release of a pool of cholesterol from the plasma membrane within 30 minutes of hormonal stimulation. Thus leading us to conclude that the bulk of steroidogenic cholesterol destined for mitochondria originates from the plasma membrane during acute steroidogenesis. Further we identified a pregnenolone mediated feedback mechanism that stops excessive cholesterol movement from the plasma membrane and thus protects mitochondria from cholesterol-induced toxicity.

We also investigated possible trafficking pathways. A hydrophobic cholesterol molecule from the plasma membrane needs to traverse the aqueous milieu to reach the mitochondria for steroid production. Many pathways have been proposed to assist cholesterol in this endeavor, but a precise mechanism that facilitates rapid movement of large quantities of cholesterol to the mitochondria had yet to be determined. Previous studies showing an increased interaction

between the endoplasmic reticulum and mitochondria during acute steroidogenesis, led us to hypothesize that cholesterol from the plasma membrane enters the endoplasmic reticulum via a membrane association and thus reaches mitochondria by plasma membrane – endoplasmic reticulum – mitochondria associations called as PAMs. These membrane associations and cellular signals are facilitated by a variety of lipid classes. Hence, we addressed this hypothesis by subcellular fractionation of hormonally induced and also hormonally induced but steroidogenesis-inhibited MA-10 cells subjected to lipidomics analysis utilizing mass spectrometry. The results obtained from this study further supported the notion that PAMs are the route for cholesterol movement from plasma membrane to the mitochondria. Further, we also noted a dynamic reorganization of multiple lipid classes that facilitate the membrane associations and cellular signals.

Résumé

Le cholestérol est le seul précurseur pour tous les stéroïdes produits chez les mammifères. Au cours de la stéroïdogenèse aiguë, induite par des hormones, de grandes quantités de cholestérol sont relocalisées des réservoirs intracellulaires vers les organites cellulaires pauvres en cholestérol, les mitochondries. Le cholestérol y sera converti d'abord en prégnénolone, le premier de tous les stéroïdes à être synthétisé. Bien que la stéroïdogenèse soit largement étudiée, l'organite cellulaire source qui emmagasine et mobilise le cholestérol pour la production de stéroïdes ainsi que la voie de transport qui permet au cholestérol, cette molécule hydrophobe, d'être transporté jusqu'aux mitochondries n'ont pas encore été déterminés.

Nous avons pu déterminé l'organite cellulaire source, en utilisant le domaine 4 (D4) de la protéine Perfringolysine O, produite par *Clostridium perfringens*. Le D4 se lie à des concentrations élevées de cholestérol dans les membranes, sans causer de cytotoxicité. L'analyse d'imagerie de cellules vivantes dans une lignée cellulaire tumorale de Leydig et des cellules primaires de Leydig de rat, en utilisant le D4 marqué au mCherry, a révélé la libération d'un pool de cholestérol à partir de la membrane plasmique dans les 30 minutes suivant la stimulation hormonale. Ceci nous mène à conclure que la majeure partie du cholestérol stéroïdien, destinée aux mitochondries, provient de la membrane plasmique pendant la stéroïdogenèse aiguë. En outre, nous avons identifié un mécanisme de rétroaction à la prégnénolone qui arrête le transport excessif du cholestérol à partir de la membrane plasmique et protège ainsi les mitochondries de la toxicité induite par le cholestérol.

Nous avons également étudié les voies possibles du transport du cholestérol. Une molécule hydrophobe de cholestérol, située dans la membrane plasmique, doit traverser le milieu aqueux pour atteindre les mitochondries pour la production de stéroïdes. De nombreuses voies ont été proposées pour aider le cholestérol dans cette entreprise, mais un mécanisme précis qui facilite le mouvement rapide de grandes quantités de cholestérol vers les mitochondries n'a pas encore été déterminé. Des études antérieures montrant une interaction accrue entre le réticulum endoplasmique et les mitochondries, pendant la stéroïdogenèse aiguë, nous ont amené à émettre l'hypothèse suivante; le cholestérol de la membrane plasmique entre dans le réticulum endoplasmique via une association membranaire et atteint ainsi les mitochondries par la membrane plasmique - réticulum endoplasmique - les associations de mitochondries appelées PAM. Ces associations de membranes et signaux cellulaires sont facilités par une variété de classes de lipides. Par conséquent, nous avons vérifié cette hypothèse par l'analyse lipidomique utilisant la spectrométrie de masse. Plus précisément, nous avons comparé les différents lipides retrouvés dans les fractions sous-cellulaires de cellules MA-10 induites hormonalement vs ceux retrouvés dans les fractions sous-cellulaires de MA-10 induites hormonalement mais dont la stéroïdogenèse avait été inhibée. Les résultats obtenus à partir de cette étude ont également soutenu l'idée que les PAM sont la voie du transport du cholestérol de la membrane plasmatique vers les mitochondries. En outre, nous avons également noté une réorganisation dynamique de multiples classes de lipides qui facilitent les associations de membranes et les signaux cellulaires.

Acknowledgments

First and foremost my deepest gratitude goes to my PhD supervisor, Dr. Vassilios Papadopoulos, for his continual guidance, unconditional support, advice, encouragement, patience and passion for science. I am forever grateful for giving me the chance to be you student and be a part of your amazing team.

I would like to give my special thanks to Dr. Martine Culty, for your guidance, always positive attitude and support throughout my time in the lab. I would like to thank all members of the Dr. Papadopoulos and Dr. Culty laboratories for your continuous support and friendship. It has been a great pleasure working with all of you over the years. Special thank you to our lab manager Charles Essagian for your patience, organization skills and ever-helpful nature. To Dr. Jinjiang Fan, for being my second mentor, critic and for sharing your immense knowledge in science. Also to Dr. Daniel B. Martinez-Arguelles for all the help and support you provided with planning and executing multiple challenging experiments.

I would like to thank my thesis advisory committee, Drs Jacques Lapointe, Jean-Guy Lehoux, Julie St-Pierre, and Bertrand Jean Claude, for their time, helpful suggestions and enthusiasm in my work and their continuous encouragement. It is a great honor to have you as my doctorate committee members and gain knowledge from you.

Last, but certainly not least, I would like to express love and gratitude to my amazing husband Ravi Kiran, parents Venugopal and Vasanthi and sister Deekshitha. Special love and thanks to my son Aadhav, who got to accompany me in writing this thesis. Thank you for your unconditional love, support, patience and sacrifices made for me and my goals. My doctorate studies would not have been possible without you.

Preface

Originality of scholarship and distinct contributions to knowledge

In these studies we identified plasma membrane to be the first source organelle for cholesterol trafficked to mitochondria during acute steroidogenesis in Leydig tumor and primary cells. Further we identified a pregnenolone mediated feedback mechanism that stops excess cholesterol movement from the plasma membrane that protects mitochondria from cholesterol-induced toxicity. We show that cholesterol from plasma membrane reaches the mitochondria through membrane associations between plasma membrane – endoplasmic reticulum – mitochondria called as PAMs. Moreover we also noted a dynamic reorganization of multiple lipid classes via lipidomics analysis that facilitate PAM formations and modulate cellular signals during steroidogenesis.

For the two manuscripts, Sathvika Venugopal designed the project, conducted the experiments, analyzed the data and wrote the manuscript. Dr. Vassilios Papadopoulos conceived the study, designed the project, analyzed the data and supervised the projects.

Contributions of authors to manuscripts

Manuscript I: Plasma membrane origin of the steroidogenic pool of cholesterol used in hormone-induced acute steroid formation in Leydig cells. Venugopal, S., Martinez-Arguelles, D. B., Chebbi, S., Hullin-Matsuda, F., Kobayashi, T., and Papadopoulos, V. 2016. Journal of biological chemistry 291, 26109-26125.

- Sathvika Venugopal: research design, performed experiments, analyzed data and wrote manuscript
- Daniel B. Martinez-Arguelles: designed and assisted in primary Leydig cell isolation from rats and lentiviral transfection
- Seimia Chebbi: assisted in live cell imaging using confocal microscopy
- Francoise Hullin-Matsuda: assisted in designing and executing live cell imaging techniques
- Toshihide Kobayashi: research design and host principal investigator at RIKEN institute
- Vassilios Papadopoulos: research design, analyzed data and wrote manuscript

Manuscript II: Dynamic subcellular lipid organization in hormone induced MA-10 mouse tumor Leydig cells. Venugopal, S., Issop, L., Chan, R., Sanyal, E., Taylor, L., Kaur, P., Daly, E., Papadopoulos, V. 2017. Manuscript under revision.

- Sathvika Venugopal: research design, performed experiments, analyzed data and wrote manuscript
- Leeyah Issop: Designed and assisted in electron microscopy analysis
- Rachel Chan: assisted in subcellular organelle isolation and lipid extraction procedures
- Esha Sanyal: assisted in subcellular organelle isolation and lipid extraction procedures
- Lorne Taylor: designed and assisted in Lipidomics analysis using mass spectrometry
- Pushwinder Kaur: designed and assisted in cholesterol analysis using mass spectrometry
- Edward Daly: designed and assisted in cholesterol analysis using mass spectrometry
- Vassilios Papadopoulos: research design, analyzed data and wrote manuscript

CHAPTER I

BACKGROUND

1. Steroid biosynthesis

Steroids are an indispensable class of molecules playing key roles in development, fertility and behavior, and thus, there has been substantial research over the last century (1). A vast majority of circulating steroids are produced by the classic hormone-dependent steroidogenic cells in the adrenals, male and female gonads and hormone-independent placental tissues (1). But other non-classical tissues, such as cells in central nervous system (2), adipose tissue (3), skin (4) and thymus (5), have also been shown to be steroidogenic. Steroidogenic tissues are characterized by the expression of the cytochrome P450 side chain cleavage (CYP11A1) enzyme in mitochondria, which converts the parent molecule cholesterol to the first steroid pregnenolone (6). Further, pregnenolone undergoes a series of enzymatic reactions to form other classes of steroids depending on various physiological needs (1).

The adrenals and gonads make basal levels of steroids constitutively, but stimulation with pituitary trophic hormones, such as adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), and follicle stimulating hormone (FSH), causes an increase in intracellular cyclic AMP (cAMP), a second messenger (7,8). This induction not only stimulates chronic expression of steroidogenic enzymes, but also induces the mobilization of free cholesterol from intracellular stores to the outer mitochondrial membrane (OMM), where a multitude of proteins form a complex called the transduceosome that facilitate the transfer of cholesterol from the OMM, across the inner mitochondrial space (IMS) and into the inner mitochondrial membrane (IMM) (9). The first step in steroidogenesis is considered to happen on the matrix side of the IMM, where the CYP11A1 enzyme is present (7). But the concept of steroidogenesis may very well

begin when the cholesterol molecules destined to become steroids are released from intracellular stores and subsequently trafficked to mitochondria.

Decades of research have been dedicated to steroidogenesis post cholesterol import to mitochondria, but very little is known on how high quantities of free cholesterol are made available to be trafficked to the mitochondria during hormone stimulated acute steroidogenesis (10). Understanding of this pathway is essential due to multiple disorders found in humans. In particular, Wolman Disease, adrenoleukodystrophy, cholesterol ester storage disease, and Niemann-Pick Type C disease have been caused due to disorders in cholesterol internalization and its intracellular processing steps (11). Further, the disorders in the trafficking of cholesterol to steroidogenic mitochondria have been known to cause classic congenital adrenal lipoid hyperplasia and non-classical disorders including glucocorticoid, mineralocorticoid, sexual steroid deficiencies (11,12). The studies discussed in this doctoral thesis are focused on various aspects of how cholesterol is trafficked from the intracellular stores to the mitochondria for steroid biosynthesis.

2. Cholesterol, the precursor of all steroids

Steroidogenic cells are well equipped with a number of enzymes that metabolize cholesterol and precursor steroids, some being unique to the expressing steroidogenic tissue (13). All steroids come from the parent molecule cholesterol, which gets converted to pregnenolone by the CYP11A1 enzyme, a member of a super family of p450 enzymes (14), of which six members are crucial for steroidogenesis (13). The CYP11A1 enzyme converts cholesterol to pregnenolone by utilizing two sequential electron transfer donors ferredoxin reductase (FDXR) and ferredoxin

(FDX). The FDXR accepts two electrons from nicotinamide adenine dinucleotide phosphate (NADPH), and transfers them to the iron-sulfur cluster of FDX which in turn donates the electrons to CYP11A1 (15). The CYP11A1 uses the protons and oxygen molecule to hydroxylate its target cholesterol at the carbon 22 and carbon 20 in the aliphatic chain. Further oxidation cleaves the 20,22 carbon removes the 6-carbon acyl chain yielding pregnenolone (15). The now more hydrophilic pregnenolone moves from the mitochondria to the endoplasmic reticulum (ER) where it is converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3βHSD). In the gonads, P450c17 (CYP17A1) present in the ER converts progesterone to dehydroepiandrosterone by 17α-hydroxylase and 17,20 lyase activities. The androstenedione is then metabolized to testosterone by 17β-hydroxysteroid dehydrogenase (17βHSD). The androgens are further converted to estrogens by the aromatase enzyme CYP19. In the adrenals, CYP17A1 and CYP21A1 catalyze the formation of deoxycorticosterone and 11-deoxycortisol). These steroid intermediate products then re-enter the mitochondria to be converted to cortisol by the CYP11B1 11β-hydroxylase enzyme or CY11B2 (aldosterone synthase) catalyzes the 11β-hydroxylation, 18-methyl oxidation to convert deoxycorticosterone to aldosterone. Thus cholesterol acts as the parent molecule for all steroids.

3. Source of cholesterol

There are two ways by which cholesterol is supplied to a cell, either by *de novo* synthesis or by dietary intake. According to studies published by Grundy and colleagues, 70% of cholesterol required by a cell is contributed by *de novo* synthesis and the rest comes from the diet (16). However, this ratio may be dependent on the genetic constitution of the individual, as well as how much dietary cholesterol is available.

3.1. *De novo* cholesterol synthesis

All mammalian tissues and cells are capable of synthesizing cholesterol *de novo*. Steroidogenic organs such as brain, adrenals, testis and the ovaries are all known to synthesize cholesterol at high rates (17-20). The source molecule of the 27- carbon skeleton of cholesterol is acetate (acetyl-CoA) that undergoes a series of enzymatic conversions through the mevalonate pathway (21). This pathway is initiated by condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. Another molecule of acetyl-CoA mediated by HMG-CoA synthase condenses to form hydroxymethylglutaryl-CoA (HMG-CoA) (22). The rate-limiting step in this second pathway is the conversion of HMG-CoA to squalene by the HMG-CoA reductase enzyme (HMGR) (23). The 30-carbon linear structure is further cyclized to yield lanosterol, which in turn undergoes removal of three carbons to produce cholesterol. HMGR and multiple other enzymes in this pathway are integral membrane proteins in the ER, which also harbors the sterol regulatory element proteins (SREBPs) and SREBP cleavage-activating protein (SCAP) complex that transcriptionally controls their gene expression (24,25). Upon decrease in the levels of cholesterol in the cell, SCAP transports SREBPs from the ER to the Golgi apparatus, where it is cleaved by two proteases (26,27). The resulting N-terminus part of the SREBPs is an active transcription factor that translocates to the nucleus, which results in the increase in transcription of genes involved in making enzymes that synthesize cholesterol, including HMGR (23). It is also known that HMGR is easily degraded under high sterol conditions and can be inhibited by low levels of adenosine triphosphate (ATP) (23). In steroidogenic cells, the enzymes in the cholesterol synthesis pathway have also known to be up regulated upon trophic hormone stimulation (17-19). Thus ER regulates endogenous cholesterol production through the SCAP/SREBP complex. Although this *de novo* synthesis of cholesterol is important for the

maintenance of homeostasis in cells, during steroidogenesis the increase in cholesterol levels via this pathway may not be destined for steroid production, but in turn be utilized as replacement of cholesterol that was utilized for the production of steroids (28).

3.2. Dietary cholesterol intake

Although cellular cholesterol synthesized *de novo* and cholesteryl esters stored in the lipid droplets may be adequate to support steroidogenesis, multiple studies indicate that adrenal glands, the testis and the ovary prefer cholesterol from dietary intake (29,30). Cholesterol from the diet is first transported from the gut to liver (31). Dietary cholesterol is then absorbed by the enterocytes in the small intestine that pack the cholesterol into chylomicrons, which are then taken to the liver by hepatocytes (32). Here they are processed into very low-density lipoproteins (VLDL), which eventually become low-density lipoprotein (LDL) during circulation (33). LDL is the main lipoprotein that transports cholesterol to the peripheral cells (33). When the hepatic tissues have an excess of cholesterol, it is released to the high-density lipoprotein (HDL) (34). The HDL usually circles back to the liver where the cholesterol is used for the production of bile acids that enter the small intestine (34).

Steroid producing cells can obtain circulating cholesterol from the cholesterol carrying plasma LDL, which is recognized by LDL receptors on the plasma membrane (33). Once the receptor has been internalized, it fuses with the endosomal pathways and the budding of clathrin-coated pits into the cytoplasm (35). These vesicles fuse with the early endosome, release the clathrin coats and allow the LDL receptors to cycle back to the membrane after dropping cholesterol in the cytoplasm, thus delivering cholesterol to the cell (36). The other pathway

through which cholesterol is delivered to the cell is by the scavenger receptor class B, type I (SR-BI) receptor, a cell surface receptor for HDL responsible for the selective uptake of lipoprotein derived cholesteryl esters (CE) (37). Though SR-BI receptors are found in many tissues, such as the intestines, macrophages, and the endothelial cells, the highest concentrations are found in the steroidogenic adrenals, ovary and testis (38). SR-BI receptor is localized in the microvillar channels of the plasma membrane and is regulated by the hormones that stimulate steroid production. This pathway involves a two-step process that requires binding with the HDL, followed by the transfer of the CE to the membrane. Once CE reaches the membrane, it is hydrolyzed to free cholesterol by hormone sensitive lipase (HSL) (37). This non-aqueous channel allows a large influx of cholesterol directly into the plasma membrane (29). Studies done in the large intestine show that other proteins, such as the CD36 found in the plasma membrane are involved in this process, suggesting a multiprotein interaction for the transfer of cholesterol (39).

4. Membrane cholesterol

Membranes are versatile parts of mammalian cells, capable of performing several functions simultaneously. They act as a semi-permeable barrier that controls various ion and solute concentrations on either side of the membrane, thus allowing different organelles to carryout their specialized functions and also maintain a transmembrane electrical potential (40). They also provide a rigid scaffold for membrane proteins to burrow into them, yet also be flexible enough to facilitate rapid diffusion when necessary. Flexibility of the membrane is also an important characteristic for fusion with other membranes or budding to form vesicles. Apart from these characteristics, studies in the last two decades have demonstrated intramembranous

lateral heterogeneity, termed lipid rafts, that plays an essential role in cell signaling and trafficking. (41,42). These lipid rafts of highly regulated, varying lipid compositions and lipid phases are maintained in membranes to orchestrate their biological functions.

The principle lipid components of eukaryotic membranes are the glycerophospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipins (CL), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA). Glycerophospholipids have a hydrophobic diacylglycerol (DAG) backbone that carries a phosphate and has attached either saturated or *cis*-unsaturated fatty acyl chains of varying lengths (43). PCs are important for membrane structure, providing fluidity and stability to the membrane. They have one *cis*-unsaturated fatty acyl chain that renders a cylindrical conformation with a polar head group facing the aqueous medium. The PE is another structural lipid class that assumes a conical conformation, but because of a small polar head group, facilitates membrane protein insertion and also provides curvature to the membrane and membrane budding, fusion and fission (44). PEs are almost entirely synthesized in the mitochondria and exported to other membranes by various trafficking methods, discussed later in this thesis. CLs are unique class of phospholipids synthesized and found exclusively in the eukaryotic mitochondria. They have two-phosphatidylglyceride backbones and have four acyl chains of varying lengths, with a small glycerol head group, which again gives a conical conformation to the lipid in membrane organization (45). PSs are negatively charged phospholipids predominantly found in the inner leaflet of the plasma membrane and endosomal recycling compartments. They are a relatively minor constituent of biological membranes, but play essential roles is protein binding to membranes by electrostatic association (46). PI is

another minor constituent of membranes usually present in the leaflet facing the cytosolic side of the cell, playing key roles in carbohydrate and protein binding to the membranes (47). PAs are the simplest glycerophospholipids, yet play a key role in lipid biosynthesis, signaling and membrane dynamics (48). All the above-mentioned glycerophospholipids originate from this key biosynthetic precursor. In eukaryotes, an interplay between ER, mitochondria and peroxisomes is involved in the biosynthesis of PA (49). Once synthesized, PAs are trafficked to other organelles of the cell to perform various functions.

The sphingolipids are another major class of lipids that are essential for the structural maintenance of the membranes. Ceramides (Cer) act as the hydrophobic backbone for this lipid class, with varying lengths of saturated acyl chains. The major sphingolipids include sphingomyelins (SM) and glycosphingolipids present in all mammalian cellular membranes. The saturated acyl chains create a narrower and taller cylindrical conformation for these lipids and hence pack tightly, providing rigidity and stability to the membranes.

Hydrolysis of these glycerophospholipids and sphingolipids produces a series of messenger lipids including lyso-PC, lyso-PA, PA, Cer and DAG (43). Depending on the lipid, they either stay in the membrane and recruit a cytosolic receptor or leave and bind to receptors that trigger signal transduction in cells (50). Ceramides are interesting signaling lipids, since generation of large quantities of these lipids change membrane properties and can drive the movement of cholesterol from the cells by displacing them in the membrane (51-53).

Cholesterol being hydrophobic is predominantly found in membranes, where it is another important determinant for membrane organization (54) and plays a key role in the organization of other lipids in the bilayer (43). Cholesterol is ubiquitous throughout cell membranes, yet is a tightly regulated component in animal cells. Cholesterol has varying affinity for both phospholipids and sphingolipids, thus the lipid composition of the membrane determines the degree of retention or extraction of cholesterol from the membrane (55). Cholesterol binds to other lipids with its hydrophilic 3β-hydroxyl group, oriented towards the external side of the membrane with the more hydrophobic side chain facing the membrane core (56). In membranes containing phospholipids with unsaturated fatty acyl chains, cholesterol can intercalate between the spaces and provide rigidity to the membrane (57). Whereas in membranes containing more di-saturated fatty acyl chains containing phospholipids and sphingolipids, cholesterol intercalation into tight spaces provides more flexibility to the membrane (58). Previous studies, specifically in the plasma membrane, molar concentration of cholesterol between leaflets was shown to be different based on the differences in the phospholipid compositions of the leaflets (59,60). Even though the concentrations of cholesterol vary between leaflets, cholesterol was shown to move freely within the membranes, either transversely or to flip-flop between leaflets (59).

Cellular membranes are also capable of lateral segregation of cholesterol, sphingolipids and other proteins that form fluctuating nano-scale assemblies (<20nm) called lipid rafts (61,62). The rigid ring structure of cholesterol drives the interaction, along with straight and long hydrocarbon chains of sphingolipids and other saturated lipids, into conformations that increase membrane thickness and promote the segregation process by hydrophobic mismatch (63).

Membranes that may seem uniform laterally may still form lipid rafts with short life spans of about ~10ms. These rafts have long known to play an important roles in cellular signaling, trafficking, including exocytosis and endocytosis, and anchoring various molecular ligands (64). Work on lipid heterogeneity and its role in cellular functions has mostly addressed the plasma membrane, with its rich cholesterol content (42,65). But the growing lipidomics field has begun to focus on sphingolipids and sterol concentrated regions in other organellar membranes, including the endoplasmic reticulum, Golgi apparatus and mitochondria.

5. Distribution of cholesterol

Cholesterol represents about 30-40% of total cellular lipids in a cell (58), and in human fibroblast cells, at least, the amount of cholesterol remains constant even after months in culture (55). It is generally agreed that several pathways exist to regulate intracellular cholesterol levels, many of which have yet to be completely understood (24,33,66). Free (i.e., unesterified) cholesterol, predominantly found in membranes, is present at constant levels of ~ 0.3-0.4 mol/mol phospholipids and sphingolipids in all mammalian cells (67). Free cholesterol in excess after complexing with other membrane lipids, can be readily trafficked to other intracellular organelles for other uses. This sub-pool of free cholesterol is called the active cholesterol and is not chemically different from free cholesterol but can be distinguished kinetically (68). This active cholesterol is thought to be the substrate for steroidogenesis. Whereas the esterified form of cholesterol is chemically different and is stored in lipid droplets. It is a product of acyl-coenzyme A: cholesterol acyltransferase (ACAT), present in the endoplasmic reticulum and is activated in the presence of high levels of cholesterol (69). The abundance of esterified cholesterol varies per cell type and its requirements.

Cholesterol is considered an essential constituent for all cellular membranes in mammals, but the overall cholesterol content varies drastically even between organellar membranes in the same cell. Although various studies differ in exact numbers, all show that the highest content of cholesterol is present in the plasma membrane at about 60-80% of the total cellular cholesterol (70). The organelles that intimately associate with the plasma membrane, such as the Golgi apparatus and endosomal recycling compartment (ERC), have intermediate levels of cholesterol. But the endoplasmic reticulum (ER), the site of cholesterol synthesis and the organelle with the largest surface area in a cell, has as low as 1% of total cholesterol content (71-73). It is thought that cholesterol synthesized by the ER is quickly trafficked to other organelles or acetylated by ACAT enzyme and delivered to lipid droplets, which are considered the lipid rich buds of the ER. Thus, the ER is considered the central cholesterol distribution center of the cell (74). Mitochondria, the organelle where steroid production is initiated, among other functions, also has surprisingly low concentrations of cholesterol in its membrane. For this reason, when a signal to trigger steroid production is given by the cell, free cholesterol from intracellular stores is trafficked to the mitochondria to be converted to pregnenolone (69).

6. Intracellular cholesterol trafficking during steroidogenesis

Due to the importance of cholesterol in multiple cellular functions, it is crucial to tightly regulate intracellular cholesterol transport. Cholesterol is transported within a cell by both vesicular and non-vesicular pathways (75). Both these pathways help orchestrate the maintenance of appropriate amounts of lipids in the various organelles.

6.1. Vesicular trafficking during steroidogenesis

Vesicular trafficking refers to the process by which portions of the donor membrane buds to form vesicles and fuses to another membrane, thus delivering lipids, proteins and other cargo throughout the cell (75). The formation of vesicles is facilitated by the enrichment of cholesterol in these membranes that interact with the phospholipid acyl chains, hence stretching hydrocarbon chains into an extended conformation. Thus, a lateral hydrophobic mismatch is formed, along with an increase in membrane thickness that facilitates the budding of vesicles. Vesicular trafficking of cholesterol involves internalization of LDL derived cholesterol through the plasma membrane, formation of vesicles, and cholesterol egress from early and late endosomes to the lysosomes (76). In fact, studies using fluorescently labeled cholesterol, such as dehydroergosterol and BODIPY-cholesterol, when applied to the medium is internalized through the plasma membrane and engulfed by clathrin dependent endocytosis (77).

Once the cholesterol reaches the lysosome, it is either sent to other organelles, destined to be degraded or stored as esters for future use. Lysosome-associated membrane protein 2 (LAMP-2) is an essential glycosylated protein involved in the esterification of cholesterol in the lysosomes (78). In fact, mutations in LAMP-2 has been linked to Danon disease characterized by cardiomyopathy, mental retardation, skeletal muscle myopathy and also accumulation of cholesterol in the late endosomes or lysosomal compartments. This substantiates the importance of intracellular cholesterol movement through vesicular trafficking (79). Although cholesterol synthesized *de novo* in the ER and esters stored in the lipid droplets could potentially be adequate to support hormone induced steroidogenesis, overwhelming evidence suggest that

adrenal, ovary and testicular Leydig cells utilize lipoprotein derived cholesterol for steroid biosynthesis (80).

6.1.1. LDL receptor mediated endocytic trafficking of cholesterol

The endosomes have among the highest concentrations of cholesterol in the cell, just behind the plasma membrane and sometimes even rivaling them (74,81). The endosome cholesterol trafficking pathway has been of great interest since its known involvement in a number of the human diseases, including Niemann-Pick type C (NPC), Wolman disease and cholesteryl ester storage disease (58). Other studies done by Karten and colleagues suggest a direct interaction between endosomes and mitochondria that facilitate cholesterol trafficking between the organelles in steroidogenic Chinese hamster ovary cells (82).

The endosome pathway is initiated when plasma LDL or other apolipoproteins B (apoB) or apoE that carry cholesterol through out the peripheral organs are recognized by the LDL receptor in the plasma membrane (Fig. 1.1) (83). Once recognized, the complex along with clathrin and other accessory proteins congregate at a specific region called the coated pits which get invaginated and pinch off from the plasma membrane (33). These vesicles are then rapidly delivered to the acidic early endosomes, where the vesicles shed their coat and fuse with the early endosome to form large vesicles (38). The acidic nature of the endosome forces the LDL and the LDL receptor complex to dissociate. The LDL receptor is then delivered back to the plasma membrane using the ERC, whereas the cholesteryl esters are hydrolyzed by the lysosomal acid lipase (LAL), enriched in the endosome (84). The free cholesterol thus obtained accumulates in the late endosomes/lysosomes. Mutations in LAL (encoded by the *LIPA* gene)

have been linked to Wolman disease, which is characterized by accumulation of cholesteryl esters and triglycerides and secondary adrenal insufficiency due to limited cholesterol availability for steroidogenesis (85). Mutations may also cause the milder cholesteryl ester storage disease (86).

Endosomal lipid trafficking occurs through interactions with the microtubules that are controlled by the Rab proteins (35,87). The Rabs family of proteins are small GTPases that regulate trafficking at the interaction sites of membranes, allowing cholesterol to be transported through out the ERC (36). The early endosomes bind to recycling endosomes that facilitate LDL receptor transport by Rab5 (88). The early endosomes also interact with the late endosomes with Rab7 that help distribute free cholesterol to the cell (89). From the late endosomes/lysosomes, free cholesterol is thought to be trafficked to various organelles for a variety of functions including back to the plasma membrane or to the lipid droplets where it is stored as the esterified cholesterol or to the mitochondria for steroid biosynthesis (69,90-94).

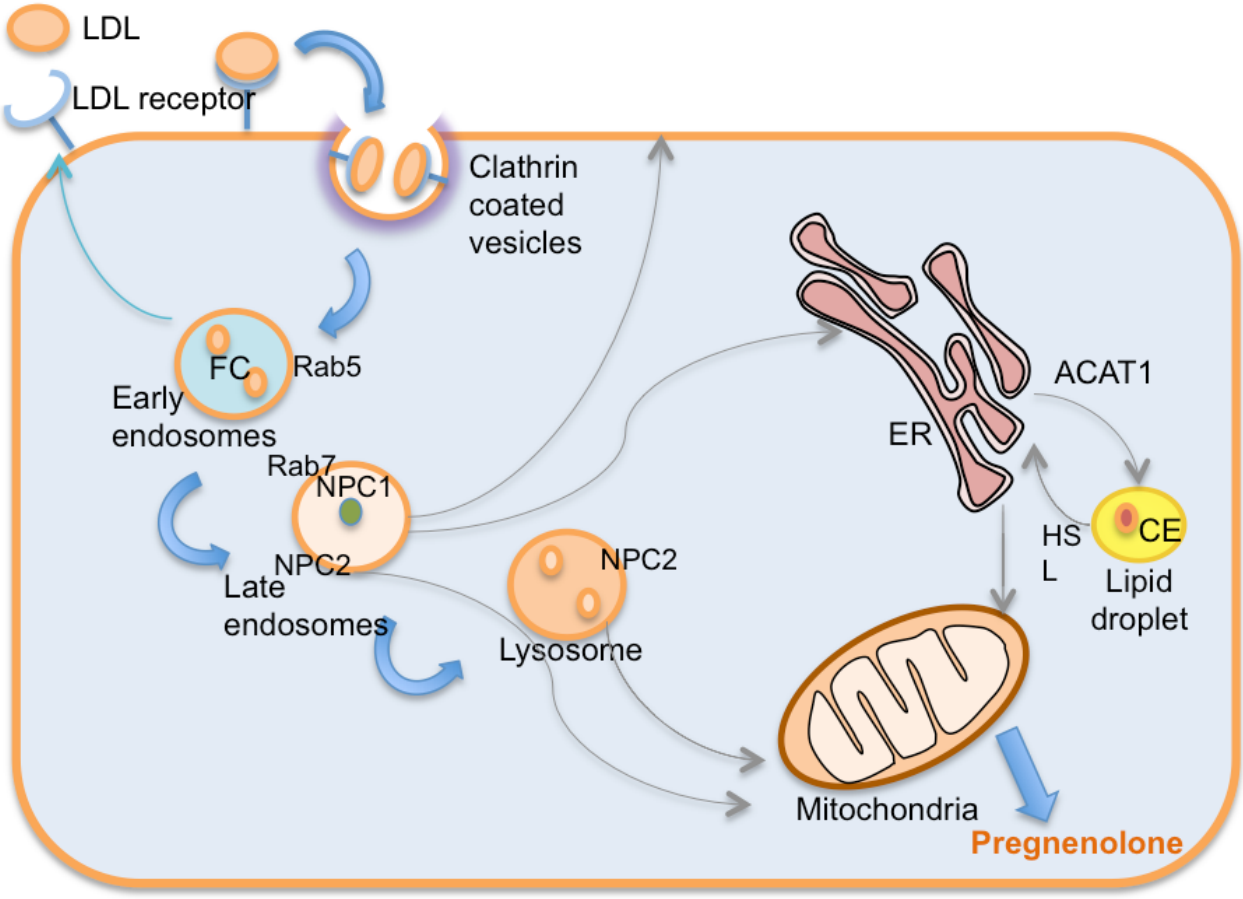


Figure 1.1. Summary of vesicular transport of cholesterol during steroidogenesis. Plasma LDL cholesterol is taken up by the endocytic pathway and further trafficked for steroid biosynthesis in adrenal and gonadal cells. FC-Free cholesterol; CE- cholesteryl ester; ER-Endoplasmic reticulum; NPC1-Niemann-Pick type C1; NPC2- Niemann-Pick type C2.

The Niemann-Pick type C1 and C2 (NPC1 and NPC2) proteins have been previously shown to play an essential role in the trafficking of free cholesterol out of the late endosomes and lysosomes. Mutations in the two genes have been linked to NPC disease, with 95% of cases having mutations in NPC1 and 5% of cases in NPC2 (95). Affected patients are normal during

infancy but soon develop ataxia, dementia, loss of speech at 2-4 years of age. Cholesterol and other lipids accumulate in the brain resulting in neurodegeneration and glial cell infiltration and eventual death in the second decade of life (96).

NPC1 is a 1278-residue integral membrane glycoprotein in the endosomes containing 13 transmembrane domains, whereas NPC2 is a soluble 151-residue glycoprotein found in both endosomal and lysosomal lumens (97). The n-terminal side of the NPC1 protein efficiently binds the 3β-OH side of cholesterol, and NPC2 envelops the aliphatic end of the molecule, thus suggesting that NPC1 and NPC2 act in tandem to transport cholesterol out of the ERC (98). According to this model, the soluble NPC2 delivers free cholesterol to NPC1 from the invaginated lipoproteins and thus facilitate free cholesterol movement from the organelle. Studies utilizing BALB/c mice with a spontaneous mutation in NPC proteins were hypogonadal and had diminished capacity to synthesize testosterone (99). Also NPC proteins have been shown to interact with StARD3, a protein closely related to the Steroidogenic Acute Regulatory protein (StAR) protein and involved in cholesterol trafficking to the mitochondria (100-102). Thus suggesting NPC proteins play an important role in cholesterol trafficking for steroidogenesis.

6.2. Non-Vesicular cholesterol trafficking during steroid biosynthesis

Cholesterol can also be moved between cellular compartments by non-vesicular transport pathways. In contrast to vesicular trafficking, non-vesicular transport of cholesterol has the obvious advantage of time. Non-vesicular trafficking involves rapid movement of lipids between cellular compartments via high affinity cholesterol-binding soluble proteins or by membrane proteins that facilitate membrane interactions between organelles (103). The first evidence of

non-vesicular transport was presented by DeGrella and Simoni in 1982 who showed *de novo* synthesized cholesterol moving from the ER to the plasma membrane (104). Here the cholesterol trafficking took place with a half time of about 10 minutes, much faster than secretory traffic via membrane proteins (104). Although a multitude of studies have shown various protein and membrane interactions during hormone-induced steroidogenesis that may facilitate cholesterol trafficking, tracking sterol movement has been difficult (9,58,105,106). Previous studies using liposomes and membrane isolations from cells suggest cholesterol can spontaneously move between membrane bilayers (107-109), making the study of non-vesicular cholesterol trafficking quite challenging.

According to Prinz and his colleagues, there are four possible mechanisms by which non-vesicular sterol transfer happens (103). The first mechanism is passive diffusion of free cholesterol without any protein assistance. The rate of this passive transfer is inversely related to the lipid composition of the donor membrane, that is, the more phospho- and sphingolipids present in the membrane the less is the chance of diffusion of cholesterol from the membrane. According to Lange, Steck and coworkers, there are two pools of cholesterol that exist in a membrane, one with high chemical activity and the other with low. The pool with high chemical activity readily escapes the membranes, whereas the low chemical activity pool is important for the membrane structure and function (110). We will not discuss passive diffusion further.

The second mechanism is the movement of cholesterol through the aqueous phase with the assistance of soluble proteins. They envelop cholesterol in a hydrophobic pocket with 1:1 stoichiometry and traffic it between membranes (111). A classic example of soluble lipid transfer

protein is StAR, a cytoplasmic protein that carries cholesterol to the mitochondria upon hormonal stimulation during steroid biosynthesis (12). The third mechanism is the transfer of cholesterol by membrane proteins that facilitate the cholesterol movement between soluble proteins or other membranes. A perfect example for this mechanism is the Translocator Protein 18 kDa (TSPO) in mitochondria, shown to be essential for cholesterol trafficking between the outer and the inner mitochondrial membranes (112). These two mechanisms, which work in tandem, are part of the transduceosome-mediated cholesterol import pathway discussed below.

The final mechanism is through membrane-membrane interactions by transient hemifusions. Although the membranes do not fuse in this process, there is a brief interaction between them, facilitated by proteins and microdomains (113), that mediates movement of cholesterol. Example of transient membrane-membrane interactions include lipid droplets interactions (LAMs and LERMITs), mitochondria-ER interactions (MAMs) and plasma membrane-ER-mitochondria interaction (PAMs). All of these are discussed below.

During steroidogenesis, the mitochondria are flooded with cholesterol from various sources for steroid production within minutes, if not seconds, after hormonal stimulation and all four of the mechanism discussed above are operational to one degree or another. Below we discuss in detail the various non-vesicular trafficking of cholesterol from donor organelles to the mitochondria during steroidogenesis.

6.2.1. Transduceosome

Mitochondria play an indispensable and highly specialized role in the production of steroid hormones (75). The first and the rate-limiting step in steroid biosynthesis is the conversion of cholesterol to pregnenolone in the mitochondria by the CYP11A1 enzyme. Steroidogenic cells have specialized machinery to limit the access of cholesterol to CYP11A1 and control the amount of steroids they produce (114). They are capable of continually synthesizing steroids at basal rates, but during hormonal stimulation, the rate of steroid synthesis can increase up to a 100 fold, if not more in certain steroidogenic cells (69). Circulating pituitary hormones, such as ACTH, LH or hCG, bind their cognate cell surface receptors on the steroidogenic cells, and initiate a downstream signaling cascade, which involves the activation of cAMP through protein kinase A (PKA). This signaling cascade also induces expression of steroidogenic enzymes, rapid import of cholesterol to the mitochondrial OMM and eventually to the IMM, where synthesis of pregnenolone production takes place (115).

Once the hydrophobic cholesterol reaches the OMM, it encounters another obstacle, the aqueous IMS between the OMM and the IMM. Traversing this aqueous microenvironment has been shown to require a complex of proteins that facilitate the transduction. Previous studies in our laboratory, utilizing blue-native polyacrylamide gel electrophoresis (BN-PAGE) coupled with mass spectrometry, identified a novel mitochondrial protein complex of 800kDa mass, comprised of cytosolic, OMM and IMM proteins (9). This multimeric complex of proteins that come into contact with each other at the OMM is called the transduceosome (116). The term transduceosome was first coined in 2006 as a complex of proteins that propagates cAMP signaling and initiates cholesterol transfer to the mitochondria (116). As shown in Figure 1.2, the

transduceosome proteins include both cytoplasmic (PKA-RI, StAR, ACBD3 and ACBD1) and mitochondrial proteins (TPSO, VDAC and ATAD3) that respond to hormonal signals and form large non-covalent complexes.

Multiple cytosolic proteins come into contact with each other at the OMM and play important roles in the transportation of cholesterol, regulation of flow of cholesterol into the mitochondria and also regulation of the transduceosome assembly. These proteins include Protein Kinase A-Regulatory Protein subunit I alpha (PKA-RIα), hormone induced mitochondrion targeted StAR, Acyl-CoA Binding Domain-containing 3 (ACBD3), and another ACBD protein family member, ACBD1. PKA has been previously shown to play a crucial role in the assembly of the transduceosome, where suppression of the protein using PKA inhibitor H-89 caused inhibition of TSPO polymerization (117). ACBD3 is a Golgi protein that migrates to the mitochondria upon hormonal stimulation and forms a scaffold for binding PKA-RI subunits to the transduceosome (118). ACBD1, previously known as diazepam binding inhibitor (DBI) was first identified through its ability to displace benzodiazepine bound to GABA receptor sites in the neurons (119). Later studies confirmed ACBD1 has the ability to bind TSPO and also stimulate steroidogenesis (120,121).

Of the cytosolic proteins in the transduceosome complex, StAR has garnered a lot of research interest, since it was originally identified as the protein factor that mediated acute mitochondrial transport of cholesterol to facilitate steroidogenesis in hormone sensitive cells (12). It was first identified by Orme-Johnson and colleagues, using two dimensional polyacrylamide gel electrophoresis, where a 30 kDa mitochondrial phosphoprotein was rapidly

up-regulated by hormonal stimulation in steroidogenic cells (122). Once isolated and characterized, StAR was revealed to be a 285 amino acid protein, with the first 65 amino acids being the mitochondrial targeting sequence attached to the 220 amino acid START domain (123). Once hormonally stimulated the 37 kDa cytoplasmic protein loses the mitochondrial targeting sequence tag and takes an active 30 kDa form and enters the mitochondrial matrix (124). But once in the matrix, it assumes an inactive form, making the role of StAR import and processing unclear. A more detailed discussion on StAR's role in mitochondrial site of action is discussed below.

The OMM transmembrane proteins involved in transduceosome were identified as the TSPO, voltage dependent anion channel 1 (VDAC1) (9). These particular proteins have long been of considerable interest in the field of steroid biosynthesis, owing to their ability to bind cholesterol (112,125). Hormone stimulation promotes clustering of TSPO, which has been shown to control the amount of cholesterol trafficked to the IMM (126,127). Also, knocking down TSPO ablates steroidogenesis in multiple steroidogenic cells (126,128). TSPO has also procured a lot pharmacological interest for its ability to bind benzodiazepine and other classes of drug ligands with the capacity to stimulate steroidogenesis (129,130). The other OMM protein identified in the complex is the 30 kDa VDAC1, known to interact with TSPO to enhance drug-binding ability of TSPO and also facilitate cytosolic cholesterol to enter mitochondria (131,132). An important IMM protein identified in the transduceosome complex is ATAD3 (105). ATAD3 is the only transduceosome protein that extends through both the membranes of the mitochondria and into the matrix. Because of this, it was hypothesized that ATAD3 may play a role ER-mitochondria organellar interaction and also cholesterol trafficking (133). In fact, in ATAD3

depleted cells it was noted that steroid production was replenished when treated with 22R-hydroxy cholesterol, a less hydrophobic precursor of pregnenolone, suggesting the importance of ATAD3 in cholesterol trafficking during steroidogenesis (105). Important transduceosome proteins StAR, TSPO, VDAC and ATAD3A directly involved in cholesterol trafficking will be discussed in more detail below.

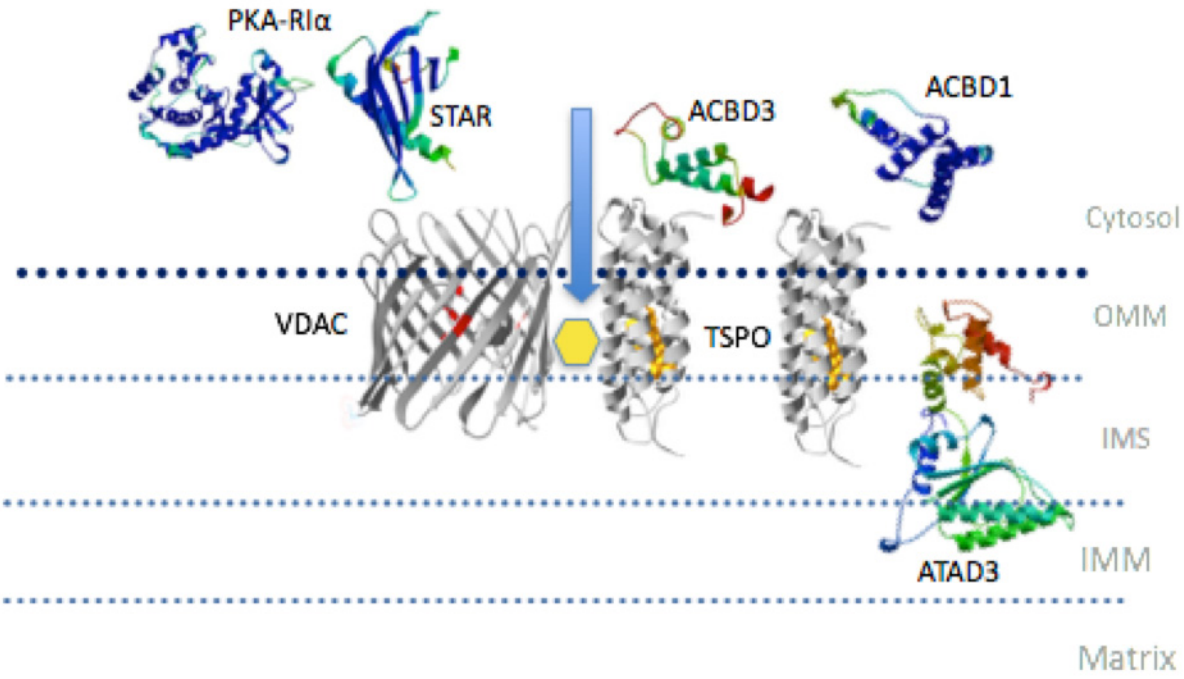


Figure 1.2. Transduceosome protein complex. Hormonal stimulation triggers the formation of a transduceosome at the OMM. The transduceosome complex contains cytoplasmic StAR, ACBD3, ACBD1, and PKA-RI and transmembrane OMM proteins TSPO and VDAC. This complex formation facilitates cholesterol import into the mitochondria during steroid biosynthesis.

6.2.1.1. StAR

StAR was first identified as a hormone sensitive protein that was rapidly induced upon hormonal stimulation in steroidogenic cells (134). Upon hormonal stimulation StAR levels rise in a manner parallel to the level of induction of steroidogenesis, and phosphorylation enhances its function (135,136). In mouse Leydig cells and cell lines, Star mRNA is induced within 120 minutes following cAMP stimulation by the transcription responsive, cAMP Response Element-Binding protein 1 (CREB) (137). However, StAR protein levels in the same cells increase within 30 minutes and reach significant levels within 60 minutes of hormonal stimulation (137). Cells treated with compounds that inhibit protein synthesis, such as cycloheximide or actinomycin, inhibit steroidogenesis as well, suggesting rapid protein synthesis is an essential driver for steroid biosynthesis (138). The role of StAR in steroidogenesis first came into prominence from work done in humans, showing multiple mutations in StAR contributed to congenital adrenal lipoid hyperplasia, a condition characterized by reduced steroid synthesis, impaired sexual development, adrenal dysfunction and eventual death in infants, if not supplemented with glucocorticoids (139). Furthermore, in mouse StAR knockout models, high levels of lipid deposition were visualized in the adrenal cortex, and when corticosteroid supplements were given for the survival of the animals, detectable levels of testosterone were produced and

therefore spermatogenesis was observed in these animals (140). However, lower levels of both testosterone and spermatogenesis were observed when compared to wild-type mice.

StAR is expressed as a 37kDa protein that contains an N-terminal mitochondrial targeting leader sequence, which restricts the function of StAR to the mitochondria (141). Once the protein reaches the mitochondria, the presequence is cleaved by the Lon protease in the matrix to leave a 30 kDa mature protein. The C-terminus of the protein contains the StAR-related lipid transfer (START) β-barrel domain that has been shown to bind cholesterol with low micromolar affinity, thus bringing cholesterol to the mitochondria (142,143). This low affinity to cholesterol was thought to help the release of cholesterol once the protein reached the OMM. But other studies also suggest StAR can bind cholesterol with an insufficient stoichiometry of 1:1, suggesting that cholesterol brought by StAR to the mitochondria may not meet the requirements for acute steroidogenesis (111). Recent findings that StAR physically interacts with VDAC1 and can be found in the transduceosome complex after stimulation of steroidogenesis suggests that StAR acts as a key to start the cholesterol transport machinery at the OMM (144).

6.2.1.2 TSPO

TSPO, previously known as Peripheral Benzodiazepine Receptor (PBR), was first described in 1977 by Braestrup and Squires, when radiolabeled diazepam was found binding with high density in the kidney instead of the expected central nervous tissue (145). It was later found to be present in most tissues of the body; hence the nomenclature was change to TSPO in 2006 (146). TSPO role in steroidogenesis was proposed when various chemically distinct ligand-binding studies revealed that it could stimulate steroidogenesis in model and physiological

systems, as well as playing a central role in the OMM transduceosome that facilitates cholesterol transport (147,148).

TSPO, a 169-residue protein, spans the OMM through its five transmembrane α-helical domains (TM1 to TM5) (149). These domains are tightly packed together in a clockwise manner in the order of TM1-TM2-TM5-TM4-TM3, when visualized from the cytosol (150). Like most mitochondrial proteins, TSPO does not have a mitochondrial targeting sequence, but instead depends on cytosolic chaperons, specifically Heat shock cognate 70 kDa chaperone protein (Hsc70) and Heat shock protein 90 kDa (HSP90) (151). The cytosolic C-terminus is highly positively charged and harbors the cholesterol recognition amino acid consensus domain (CRAC) between residues 147-159, which has been shown to bind cholesterol with nano-molar affinity (152,153). The positive charge patches may play a key role for interaction with proteins carrying cholesterol. Mutations in the CRAC domain resulted in the loss of mutant recombinant TSPO the ability to bind cholesterol (154). The N-terminus part of the protein was previously considered to contain the drug binding site of the protein, but recent studies have shown that there are in fact sixty-one contact sites of the drug ligand PK 11195 to TSPO creating a binding pocket by all 5 transmembrane helices (130). Several of these contact sites in the binding pocket are highly conserved residues, and one such residue A^{147} is of particular interest since a natural polymorphism to threonine can strongly affect several ligands binding to TSPO in humans (154).

For the past 3 decades, a multitude of studies have emphasized the role of TSPO in steroidogenesis. Snyder and colleagues first showed that hypophysectomy in rats reduced the expression of TSPO in both the testis and adrenals, suggested that TSPO expression was

hormonally regulated, and also showed its possible relationship to testicular and adrenal steroidogenesis (155). The presence of the endogenous ligand ACBD1 (previously known as Diazepam Binding Inhibitor, DBI) for TSPO was able to stimulate cholesterol transport in isolated mitochondria (156,157). When DBI was knocked down, steroidogenesis was blocked, demonstrating the importance of TSPO in steroidogenesis. Further studies showed that known TSPO-binding drug compounds were able to stimulate steroid formation in primary cells, multiple cell lines, and also isolated mitochondria (158). Thus, multiple TSPO drug ligands have pharmacological properties to regulate steroid synthesis in the brain and have been used to alleviate multiple neurological and psychiatric disease symptoms in humans (158,159). Further, functional antagonists of TSPO were able to block steroid production, and partial agonists of TSPO were able to stimulate steroid formation in primary cells and cell lines (129,148). Recent *Tspo* conditional knockout mouse studies suggest an essential role for TSPO in pre-implantation embryo development and ACTH stimulated steroidogenesis (149).

6.2.1.3. VDAC

VDAC is 32-kDA β-barrel channel forming protein that regulates the passage of ions and other small molecules through the OMM (160). VDAC also plays an essential role in regulation of apoptosis and cellular metabolism (161). VDAC is the most abundant protein in the OMM, and three isoforms are expressed in the human genome. VDAC1 is the predominant form, followed by VDAC2, and VDAC3 is expressed the lowest in comparison (162). Although the three isoforms were thought to have functional redundancy, new studies unraveled unique functionalities for these proteins. Studies on *Vdac1* and *Vdac3* null mice displayed viability with minimal phenotypical changes; however, another study with *Vdac2*$^{-/-}$ mice displayed embryonic

lethality. *Vdac1* and *Vdac3* null mice and cell lines null for all three isoforms did not have any effect on mitochondrial function or apoptosis, questioning the role of VDAC or indicating the presence of another mechanism that could bypass VDAC's function (163).

The first study to show VDAC's association with TSPO was when it was shown to interact with TSPO in digitonin-solubilized mitochondria separated by gel filtration column chromatography (125). The interaction between TSPO and VDAC has also been shown to play a key role in the ligand binding affinity with TSPO. Later VDAC was also shown to be associated with the IMM protein adenine nucleotide translocase (ANT); together they have been known to form the mitochondrial permeability transition pore (MPTP), a structure that is important for small molecular trafficking and apoptosis (164). VDAC also complexes with other proteins, such as hexokinase, creatine kinase and the apoptotic Bcl-2 family of proteins (165). Although it is unlikely that VDAC acts as a cholesterol channel during steroidogenesis, its key role in cholesterol trafficking is discussed in greater detail below.

While the transduceosome moves cholesterol from the cytosol to the IMM, additional proteins come into play to move it across the IMM into the mitochondrial Matrix. As shown in Figure 3, the IMS and IMM have their own sets of proteins involved in the continued transport of cholesterol. Members of the transduceosome, TSPO, VDAC and ATAD3, belong to another multi-protein complex termed a Metabolon that brings cholesterol to the matrix side of IMM, where CYP11A1 is located and metabolizes to pregnenolone to complete the journey.

6.2.1.4. ATPase family, AAA domain containing 3 (ATAD3)

The ATAD3 protein belongs to a large AAA^+ family of ATPases implicated in very diverse cellular functions. ATAD3 was first identified by Martinou and colleagues in 2003 and was shown to be enriched in the mitochondria, especially at mitochondrial contact sites (166) (133). Although the exact structure is not yet known, trypsin digestion suggests that ATAD3 has two N-terminal coiled coil domains that anchor the protein to the OMM, a transmembrane segment in the IMM and a C-terminal ATPase domain localized in the mitochondrial matrix (80) (167). The C-terminus region of the protein contains two domains: one that binds ATP called *Walker A* and an ATPase domain called *Walker B*. This *Walker B* in the mitochondrial matrix facilitates the homo-oligomerization of the protein and also is thought to play a role in the organellar interaction between mitochondria and ER (133).

The first implication that ATAD3 plays a role in steroidogenesis and mitochondrial organization came from a study done on H295R adrenal cortical cell lines. ATAD3 was identified in an 800-kDa bioactive complex transduceosome by BN- PAGE and subsequent mass spectrometry analysis (9). RNA silencing studies revealed retardation in steroid formation and critical modification in mitochondrial ultrastructure in MA-10 mouse tumor Leydig cells (105). Based on the above studies and its localization through both the membranes of the mitochondria, it was hypothesized that ATAD3 may play a role ER-mitochondria organellar interaction and also cholesterol trafficking. In fact, in ATAD3 depleted cells it was noted that steroid production was replenished when treated with 22R-hydroxy cholesterol, a less hydrophobic precursor of pregnenolone, suggesting the importance of ATAD3 in cholesterol trafficking during steroidogenesis. ATAD3 depleted cells have also been shown to disrupt interactions between

mitochondria and the ER, and a decrease in cholesterol trafficking was noted (105,168). Putting together these data, ATAD3 is considered a key player for the transduceosome in cholesterol trafficking.

6.2.1.5. Metabolon

The concept of metabolon was first proposed by Welch in 1977 to define the role of multiprotein systems in cellular metabolism (169). These protein associations have long since been known to play crucial roles in multiple metabolic pathways. Eventually in 1990's Lieberman and Prasad used the term for a group of proteins that associate together for steroidogenic metabolism (170). However, there have been many difficulties in experimentally demonstrating the concept of a metabolon, since they tend to be very transient. Recent techniques, such as BN-PAGE and high-resolution fluorescent microscopic analysis, have facilitated the understanding of these transient protein interactions (9).

The mitochondrial metabolon overlaps with the transduceosome complex as TSPO, VDAC and ATAD3 are considered members of both the complexes(133). During steroid biosynthesis, the transduceosome OMM proteins TSPO and VDAC drive the transfer of cholesterol from the OMM whereas IMM-OMM protein, ATAD3 facilitates cholesterol movement from the OMM to the IMM. Once at the IMM, CYP11A1 enzyme along with Fdx and FdxR at the inner side of IMM are involved in the metabolism of cholesterol to pregnenolone in a rate limiting way (171-173). Using BN-PAGE coupled with mass spectrometry, we noted that CYP11A1 and FdxR physically associate during steroidogenesis in tumor Leydig cells, suggesting the formation of a metabolon in the IMM for sterol metabolism (9). Previous studies

in our laboratory suggest an increased interaction between the transduceosome and metabolon complexes, confirmed by FRET analysis, where the distance between TSPO, a transduceosome OMM transmembrane protein, and CYP11A1, a metabolon IMM protein, is greatly reduced upon hormonal stimulation (9). Thus it is thought the CYP11A1 metabolon interacts with the transduceosome, which then transducts cholesterol from the OMM to the IMM and also sends signals for sterol metabolism (116).

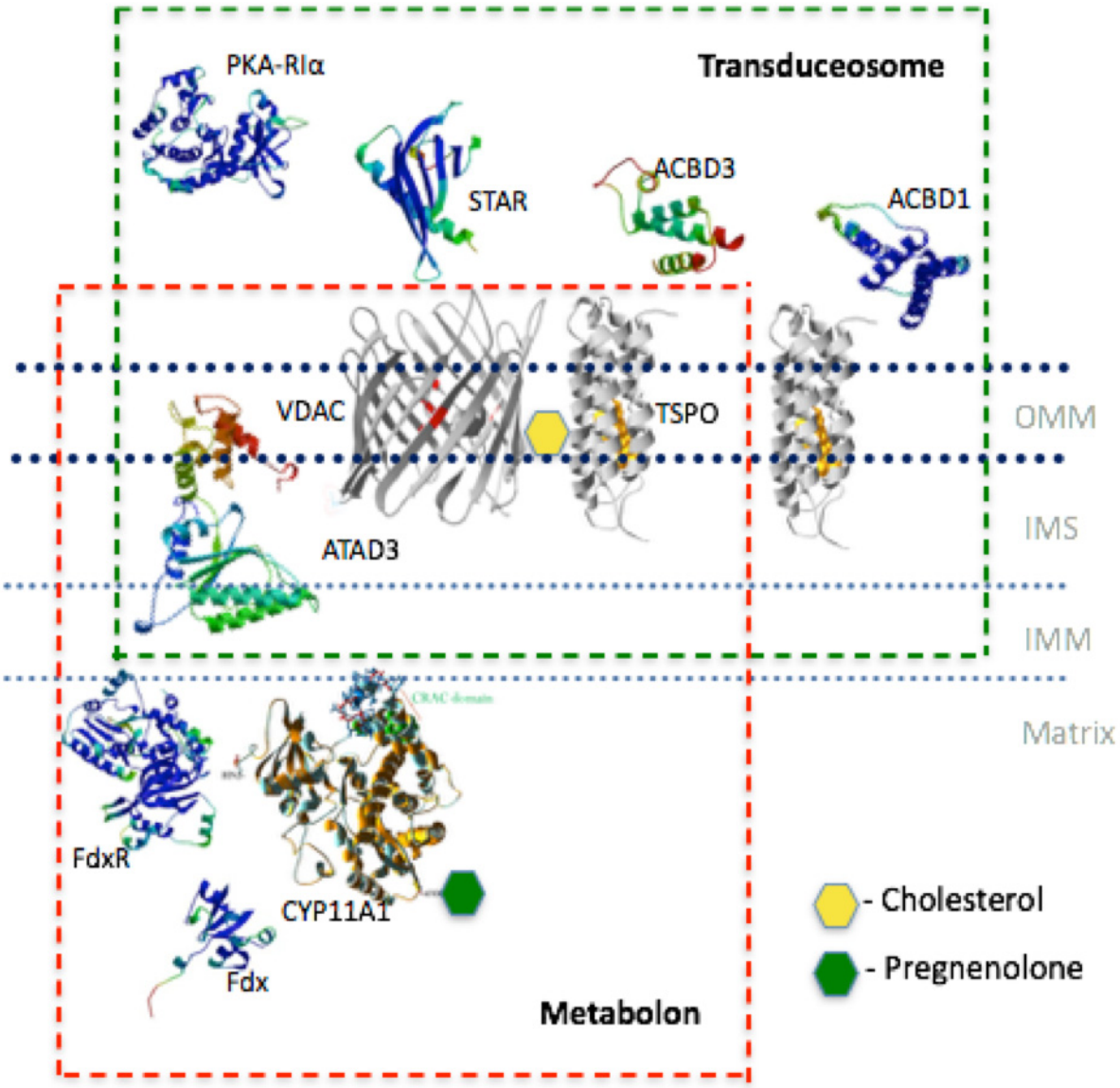


Figure 1.3. Formation of transduceosome and metabolon complexes. The mitochondrial cholesterol import machinery, the transduceosome, and metabolism machinery, metabolon, are shown, demarcated in green and red respectively. The transduceosome contains cytoplasmic proteins including and PKA-RIα, StAR, ACBD3, ACBD1, OMM proteins VDAC and TSPO; and IMM ATAD3. VDAC, TSPO and ATAD3 also belong to the metabolon complex, that

directs the cholesterol molecule to CYP11A1, which metabolizes cholesterol to pregnenolone, the precursor to all other steroids.

6.2.1.6. CYP11A1

Human CYP11A1 is a 521 amino acid residue protein with 39 amino acid residue of mitochondrial signaling sequence (13). Once imported into the mitochondria, the target sequence is cleaved, and the active CYP11A1 enzyme anchors to the IMM on the matrix side. Point mutation studies in the presequence suggested that three positively charged amino acids on the N-terminus are absolutely essential for the transport of the protein to the mitochondrial matrix (174). The N-terminus part of the protein facilitates the anchoring of the protein to the mitochondrial membrane through the F-G loop and residues surrounding the α-helix (175-178). During steroidogenesis, the proximity between the transduceosome protein TSPO and CYP11A1 is increased, confirmed by FRET analysis, suggesting an interaction between the OMM and the IMM (9). It is thought that this tight association with the IMM is important for CYP11A1 to access the cholesterol delivered from the transduceosome complex (9).

The presence of the CYP11A1 enzyme characterizes a cell as steroidogenic. The catalytic reaction model of CYP11A1 was first proposed by Shikita and Hall in 1974 (179). This enzyme catalyzes the conversion of cholesterol to pregnenolone by cleaving the aliphatic side chain of cholesterol. The cleavage happens via three sequential modifications of cholesterol. In the first step, CYP11A1 catalyzes hydroxylation at carbon 22 in the aliphatic chain; in the second step, carbon 20 is hydroxylated; and in the third step, the C20-22 bond is cut by oxidation yielding pregnenolone and isocaproaldehyde (13,179) (Fig. 1.4).

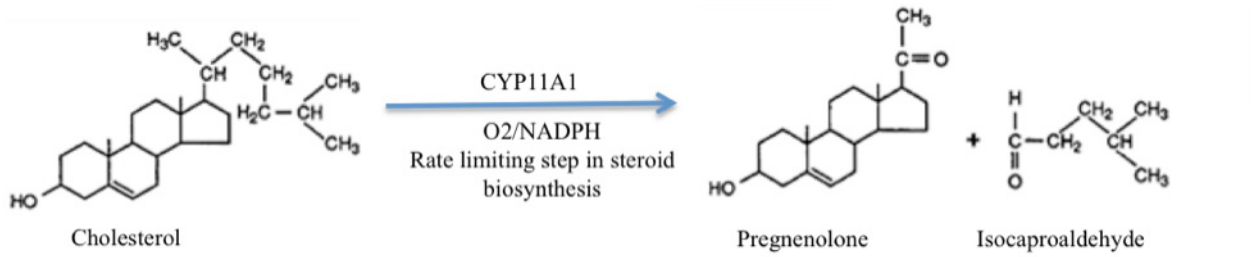


Figure 1.4. Cholesterol metabolism to pregnenolone. CYP11A1 enzyme catalyzes the conversion of cholesterol to pregnenolone by hydroxylation of 20α and 22 carbon and further scission of 20, 22 carbon-carbon bond, forming pregnenolone and isocaproaldehyde.

The activity of CYP11A1 is regulated by the rate of electron transfer from the Fdx and FdxR proteins (180). These electron transfer donors are evolutionarily conserved proteins and found in all domains of life (181). FdxR is expressed in a number of tissues, but found abundance in steroidogenic ones (172). FdxR is a flavoprotein found attached to the IMM like CYP11A1 and has two functional domains, one of which binds to NADPH and the other binds to flavin adenine dinucleotide (171). This 54.5 kDa protein also has a cleft that contains basic residues that facilitate the binding to its partner Fdx by interacting with its acidic residues (171,180). Both proteins function together to reduce NADPH by electron transfer to flavin adenine dinucleotide and then transfer electrons to the CYPs for their catalytic activities. Hence, CYP11A1 together with Fdx, FdxR are considered the IMM metabolon, where cholesterol delivered from the transduceosome is converted to pregnenolone, a precursor steroid for all other steroids in the body (80).

6.2.2. Cholesterol transport between lipid droplets, ER and mitochondria during steroidogenesis

Lipid droplets are dynamic organelles constantly growing or shrinking and known to play an important role in multiple cellular functions, including cellular homeostasis, signaling, membrane trafficking, lipid storage, and lipid metabolism, among other functions. (182,183). Common pathologies, including diabetes, obesity, atherosclerosis and metabolic syndromes, are caused due to lipid storage anomalies in lipid droplets (184). Additional genetic disorders, including various lipodystrophies and congenital lipoid adrenal hyperplasia, are also linked to disorders in lipid storage in droplets (12,184). They are ubiquitous in all eukaryotic organisms, but vary drastically in number and volume based on the cell type. For example, adipocytes can harbor about 100μm sized lipid droplets, whereas fibroblasts cells contain lipid droplets that rarely exceed 1μm (185). Excess lipids can prove detrimental to a cell, hence cells esterify these lipids to achieve a neutral form and store them in the form of lipid droplets for later use (186). Thus, lipids droplets are made up mainly of a neutral lipid core, such as triacylglycerols and cholesterol esters, and a surfactant phospholipid monolayer that efficiently exclude them from the soluble cytoplasmic environment (187). The composition of the lipid droplets differs vastly based on the cell type and the neutral lipid they store (188). In steroidogenic cells, lipid droplets primarily store cholesterol esters, but again the size and amount of lipid droplets vary (189,190). For example, normal adult rat, hamster, and bovine Leydig cells contain very low levels of lipid droplets, whereas normal adult mouse, dog, and monkey Leydig cells have significantly higher levels of lipid droplets (190). However, aging rat (191) and human Leydig cells (192) were shown to accumulate more droplets in Leydig cells than their younger counterparts. Also multiple studies show that testicular feminization causes decreased steroidogenesis and increased

lipid accumulation in lipid droplets and further substantiates the role of lipid droplets in steroidogenesis (193).

A series of *in vivo* and *in vitro* studies performed in the 1970s led to the conclusion that lipid droplets are the source of the steroidogenic pool of cholesterol. Early evidence from an ultrastructural study showed a decrease in the volume of LDs in adrenocortical cells after exposure to stimulatory hormones (194). Subsequently, the testes of adult male mice treated *in vivo* with human chorionic gonadotropin (hCG) were shown to have fewer LDs one day after treatment than untreated mice (195). Other evidence for the importance of LDs came from knockout studies of the vimentin gene in mice, which codes for an LD-associated intermediate filament. Gene knockout disrupted steroidogenesis in adrenal but not testicular tissue (196), consistent with the known slower response of testes to steroid-synthesis-inducing hormones compared to adrenal cortex. It was also demonstrated that hormones regulate the enzyme cholesterol ester hydrolase in LDs, effecting de-esterification of esterified cholesterol and increasing the pool of free cholesterol for steroid formation (197). Although LDs could provide a constant supply of cholesterol to sustain steroidogenesis, the kinetics of this process does not suggest that it is important in the acute response of these tissues to hormones.

6.2.2.1. Lipid droplet- ER interaction

Lipid droplets are generally accepted to be formed from the ER since the 1970's, although direct evidence showing the budding is still missing (198). Once formed, ER proteins such as FATP1 and the lipid droplet protein DGAT2 are involved lipid synthesis and expansion of the lipid droplets (199). The enzymes that make triacylglycerols (200) and cholesterol esters

(69) are present in the ER. Furthermore, many other proteins, including the Rab protein family, are localized in both organelles (201). During steroid synthesis, apposition between the ER and the lipid droplets has been visualized, suggesting that this interaction may facilitate the synthesis of cholesteryl esters and its metabolism as well (202-204). In the mouse tumor Leydig cell line MLTC-1, lipid droplet associated protein PLIN1 was seen in perinuclear lipid droplets, but upon hormonal stimulation, the same protein was found in small lipid droplets overlapping with calnexin, an ER protein in the cytoplasmic side, suggesting a close association of lipid droplets with the ER (204). Electron microscopy analysis shows an association between the two organelles, and a new study in yeast shows direct apposition of lipid droplets and the ER (205), thus strongly suggesting a dynamic association between the two organelles. Thus, an association between ER and lipid droplets is termed Lipid droplet associated membranes (LAM) (133).

6.2.2.2. Lipid droplet- mitochondria interaction (LAM)

The first association between lipid droplets and mitochondria was noted by electron microscopy and proteomic studies. Lipid droplets have also been shown to interact with mitochondria for fatty acid (FA) metabolism and steroid biosynthesis (206). FA hydrolysis goes hand in hand with steroid biosynthesis, since FA is the source of ATP essential for the rate-limiting step in the conversion of cholesterol to pregnenolone (206). In the liver, the FAs from lipid droplets are transported to the mitochondria by carnitine palmitoyltransferase (CPT) and carnitine. For the transport of FA into the mitochondria, CPT interacts with VDAC at the OMM (207). Once the FA-carnitine complex reaches the mitochondrial matrix, FA is repeatedly metabolized to generate acetyl-CoA, a reaction catalyzed by coenzyme A. Furthermore, the

acetyl-CoA produced enters the TCA cycle to form NADH and FADH2, used for the production of ATP in the electron transport chain.

HSL, a major cholesterol ester hydrolase in steroidogenic tissues, was previously shown to interact with both vimentin, increasing the contact sites between ER and lipid droplets, and also with StAR protein in the cytoplasm, which translocates to the mitochondria during steroidogenesis (208). In another study, active transport of LDs along microtubules in Y-1 mouse adrenocortical tumor cells was observed with non-perturbational imaging. The results also suggested an interaction between mitochondria and LDs, consistent with cholesterol delivery from LDs to mitochondria during steroidogenesis (209). Perilipin 3 and 5, belonging to the perilipin family of scaffolding proteins, play an important role in the tethering between lipid droplets and mitochondria under stress conditions (210). It is speculated that this phenomenon also happens during steroidogenesis (211).

LD, an important source organelle for cholesterol; ER, the organelle that regulates cholesterol trafficking; and mitochondria, the site for steroidogenesis, undergo physical interactions in MA-10 Leydig cells during steroidogenesis, noted by electron microscopy studies (133). Thus, this interaction involving LD-ER-mitochondria is termed LERMIT (133). In addition, the OMM protein VDAC can also bridge LD-mitochondria and ER-mitochondria interactions (133). Other proteins, including diglyceride acyl transferase (212) and ACAT1 (9), both from the ER, have also been shown to be actively involved in the LERMIT interaction.

.

6.2.2.3. Mitochondria-ER interaction (MAMs)

As discussed earlier, the ER is a complex organelle, distributed through out the cell and plays a key role in lipid synthesis and trafficking, among other functions (213). ER is the site of *de novo* synthesis of cholesterol, but cholesterol brought to the cell by lipoproteins comes to the ER eventually, as it is the site for esterification of cholesterol. ER also harbors the sterol sensing machinery, the SREBP protein, that regulates transcription of genes for cholesterol synthesis (1,214). Furthermore, ER is the site of multiple enzymatic reaction during steroidogenesis. Recently, ER has gained even more emphasis in the field of steroid research, as it is now considered a source organelle for cholesterol (105,215).

The first study to note an interaction of the ER with mitochondria came four decades ago in 1971 from the Franke and group (216). Later in depth studies by Vance and colleagues noted that crude mitochondria isolated from rat liver had multiple enzymes involved in phospholipid synthesis, but purified mitochondria from the same cells lacked them (217). He also noted the increased presence of multiple ER markers in the crude fraction when compared to other organelles, suggesting that ER and mitochondria interaction to play an important role in phospholipid synthesis. Later multiple electron microscopy and biochemical studies have revealed specific association sites between mitochondria and ER representing about 5-20% of the mitochondrial surface (218-220). This association was noted for multiple processes, including lipid synthesis, lipid trafficking, protein trafficking and in maintenance of calcium homeostasis as well, so much so that this interaction was termed Mitochondrial Associated Membranes (MAMs) (221). Later studies by Hayashi and colleagues found that MAM associations occurred

at specific lipid raft-like microdomains, rich in cholesterol and other lipids including ceramides (222).

Apart from having a unique lipid makeup at the association site, recent studies have identified a unique protein makeup at MAM sites (221). Mitofusin2 (MFN2), is one such protein that is harbored in the OMM and ER and tethers the ER to the mitochondria at MAM sites (223). Phosphofurin acidic cluster sorting protein-2 (PACS2) mediates the localization of the ER protein calnexin to the MAM site and has been shown to be important for MAM integrity (224). Dynamin-related protein (Drp1) is intimately involved in mitochondrial fusion/fission, so it may be involved in other membrane-membrane interactions, perhaps stabilizing them. So it may not have a direct effect on steroidogenesis, but facilitates it via membrane interactions (225,226). Therefore, it is hypothesized to be involved in steroidogenesis, but as of now no studies exist on this particular protein to support this hypothesis.

Recent studies have demonstrated that a number of proteins involved in steroidogenesis are also involved in MAM formation. VDAC, the channel forming transmembrane protein, was found in these MAM sites (227). In fact, VDAC2 appears to interact with StAR protein at MAM sites in steroidogenic cells, an interaction already deemed important for cholesterol import to mitochondria (113). Another interesting MAM protein is the sigma-1 receptor (SIGMAR1) previously known to play a role in cholesterol export and compartmentalization in the ER and in another study was shown to co-immunoprecipitate with VDAC2 in steroidogenic cells (228,229). Acyl-CoA synthetase 4 (ACSL4), another important component recognized at MAM sites, has been previously shown to participate in arachidonic acid metabolism and transport through

mitochondria (230). The role of ACSL4 in steroidogenesis came into prominence in 2005, when silencing the protein using siRNA reduced the amount of steroids produced in Leydig cells and overexpression caused an increase in steroid production in the same cells (231). Finally, ATAD3A a transduceosome complex protein, known to link the IMM to the OMM for cholesterol trafficking during steroid production, is also thought to physically link the ER to mitochondria at MAM sites (105). Thus, we could confidently say that the transduceosome complex is intrinsically linked with MAM formation during steroidogenesis.

6.2.2.4. Plasma membrane-ER-mitochondria interaction (PAMs)

The plasma membrane has the highest content of cholesterol in the cell and has been shown to be the prime source of active cholesterol for many intracellular processes (67). Recent studies have shown cholesterol comprises 40 % of plasma membrane bilayer on a molar basis and associates with phospholipids in 1:1 ratio (56,106). It is generally accepted that the plasma membrane has asymmetric bimolecular leaflets. Compared to the inner leaflet, the outer leaflet is more rigid, with high concentrations of sphingolipids, increasing the association with cholesterol by strong van der Waal's forces. In contrast, the inner leaflet has fewer sphingolipids and more glycerolipids, and cholesterol binds less efficiently due to weak Van der Waal's forces. This may make it easier for cholesterol to be mobilized from the inner leaflet. For steroidogenesis, the plasma membrane has long since been considered a source organelle for cholesterol. Specifically, our study has determined that the inner leaflet of the plasma membrane looses significant levels of cholesterol within 15-30 minutes of hormone stimulation in mouse tumor Leydig cells (106). Further, the same phenomenon was noted in primary rat Leydig cells as well.

The first study to suggest the plasma membrane to be the source organelle for cholesterol came from Freeman and colleagues, where cholesterol movement was noted from the plasma membrane through endosomes after hormone stimulation in Leydig tumor cell model (232). A series of metabolic labeling studies in Leydig and adrenal cell lines, using radiolabelled acetate and cholesterol, also led them to suggest the importance of the plasma membrane in steroidogenesis (233). Further subcellular fractionation studies indicated that the plasma membrane was the major source of cholesterol from changes in the cholesterol content in hormone stimulated MA-10 cells, with no corresponding change in phospholipid composition (234).

The plasma membrane interacts with other organelles for both vesicular and non-vesicular trafficking of cholesterol. Freeman and colleagues suggested that the plasma membrane interacted with endosomes and lipid droplets to transported cholesterol to the mitochondria (235). Maxfield and coworkers observed dehydroergosterol, an analog of cholesterol, trafficking to endosomes with a half time of about 2.5 minutes in a modified CHO cell line (236). The transfer was even faster from the plasma membrane to lipid droplets with a half time of 1.5 minutes.

The plasma membrane and the ER also forms dynamic contact sites, with the ER covering about 20%-45% of the surface of the plasma membrane (237). The distance between the two organelles at such association sites is usually between 10 and 25 nm (238). According to Wu and colleagues a distance between 30nm or less between two organelles can be considered an association with purpose (238). The structural interaction between the PM and ER was first

described by Pichler and colleagues, and they were successfully able to isolate these interaction sites by differential centrifugation based, subcellular fractionation (239). This interaction plays essential role in cell signaling, calcium homeostasis, lipid trafficking and metabolism, organelle morphology and ER function (240).

The term PAMs came into prominence when MAM fractions isolated from HeLa cells or rat liver contained markers for the plasma membrane (241). Similar direct contact sites between the plasma membrane, ER and mitochondria were noted during the activation of Ca^{2+} channels (242-244). Compared to MAMs, the molecular composition of PAMs is not well characterized. But several proteins including stromal-interacting molecule 1 (STIM1), an ER protein; calcium release–activated calcium channel protein 1 (ORAi1), a plasma membrane protein: steroidogenic OMM protein VDAC and ACSL4 have all been shown to be integral components of PAM association in eukaryotic cells (245).

The fact that ER-mitochondria contact sites increase within 15 minutes of hormonal stimulation and plasma membrane cholesterol depletion occurs around the same time led us to hypothesize that PAM association could be a good route for steroidogenic free cholesterol to rapidly move from the plasma membrane to the mitochondria (105,106). The studies discussed in this doctoral thesis are focused on finding the major source organelle for cholesterol during steroidogenesis, hypothesized to be the plasma membrane, and the dynamic interplay between organelles for the transport of cholesterol to the mitochondria upon hormonal stimulation in testicular Leydig cells.

Chapter II

Manuscript I

Plasma membrane origin of the steroidogenic pool of cholesterol used in hormone-induced acute steroid formation in Leydig cells*

Sathvika Venugopal[§], Daniel Benjamin Martinez-Arguelles[§], Seimia Chebbi[§], Françoise Hullin-Matsuda[¶⌘], Toshihide Kobayashi[¶⌘#], and Vassilios Papadopoulos[§||]**

[§]*Research Institute of the McGill University Health Centre and the Department of Medicine, McGill University, Montreal, H4A 3J1, Canada*

[¶]*Lipid Biology Laboratory, RIKEN Advanced Science Institute, Wako, Saitama, Japan*

[⌘]*INSERM U1060, Université Lyon 1, INSA Lyon, Villeurbanne, France*

[#] *UMR 7213 CNRS, University of Strasbourg, 67401 Illkirch, France*

[||]*Department of Pharmacology and Therapeutics, McGill University, Montreal, H3G 1Y6, Canada*

***Department of Biochemistry, McGill University, Montreal, H3G 1Y6, Canada*

Running title: *Plasma membrane cholesterol in steroidogenesis*

To whom correspondence should be addressed: V. Papadopoulos, Research Institute of the McGill University Health Centre, 1001 Decarie Blvd, Bloc E, Montreal, Quebec, H4A 3J1, Canada; phone: 514-934-1934 ext. 44580; fax: 514-934-8439; e-mail: vassilios.papadopoulos@mcgill.ca; Present address: Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, USA; e-mail: vpapadop@usc.edu

Keywords: cholesterol, lipid transport, plasma membrane, mitochondria, steroidogenesis, testis

Abstract

Hormone-sensitive acute steroid biosynthesis requires trafficking of cholesterol from intracellular sources to the inner mitochondrial membrane. The precise location of the intracellular cholesterol and its transport mechanism are uncertain. Perfringolysin O, produced by *Clostridium perfringens,* binds cholesterol. Its fourth domain, D4, retains cholesterol-binding properties but not cytotoxicity. We transfected steroidogenic MA-10 cells of mouse Leydig-cell tumors with the mCherry-D4 plasmid. Tagged D4 with fluorescent proteins enabled us to track cholesterol. The staining was primarily localized to the inner leaflet of the plasma membrane and was partially released upon treatment with dibutyryl-cAMP (dbcAMP), a cAMP analog. Inhibitors of cholesterol import into mitochondria blocked steroidogenesis and prevented release of D4 (and presumably cholesterol) from the plasma membrane. We conclude that the bulk of the steroidogenic pool of cholesterol, mobilized by dbcAMP for acute steroidogenesis, originates from the plasma membrane. Treatment of the cells with steroid metabolites, 22*R*-hydroxycholesterol and pregnenolone, also reduced D4 release from the plasma membrane, perhaps evidence for a feedback effect of elevated steroid formation on cholesterol release. Interestingly, D4 staining was localized to endosomes during dbcAMP stimulation suggesting that these organelles are on the route of cholesterol trafficking from the plasma membrane to mitochondria. Finally, D4 was expressed in primary rat Leydig cells with a lentivirus and was released from the plasma membrane following dbcAMP treatment. We conclude that the plasma membrane is the source of cholesterol for steroidogenesis in these cells as well as in MA-10 cells.

Introduction

Cholesterol is an important component of cell membranes, with unique structural and physical properties (43). The relative amount of cholesterol varies drastically between membranes in a cell and this apparently critical balance is maintained by vesicular and non-vesicular trafficking; the latter is currently an intense area of research (40,246). Cholesterol trafficking has been implicated in a number of diseases, including cardiovascular and brain diseases, cancer, and several rare monogenic diseases (58,66).

All vertebrate steroid hormones are derived from the enzymatic metabolism of cholesterol (1,13). In steroidogenic cells, such as those in the gonads and adrenal cortex, steroid biosynthesis is initiated by circulating pituitary peptide hormones. These hormones bind to their cognate receptors on the plasma membrane (247) and enhance the synthesis of cAMP (152). cAMP acts to promote cholesterol transport from intra-cellular stores to the mitochondria, where steroidogenesis is initiated (248). When the pool of free cholesterol dedicated to steroid biosynthesis reaches the mitochondria, it is inserted into the outer mitochondrial membrane (OMM)[1], where it is segregated from the structural cholesterol until it is imported to the inner mitochondrial membrane (IMM) via an 800-kDa protein complex known as the steroidogenic metabolon. This complex includes the mitochondrial proteins: translocator protein (TSPO; 18kDa), the voltage-dependent anion channel (VDAC), the ATPase family AAA Domain-containing protein 3 (ATAD3), and the cytochrome P450 side chain cleavage enzyme (CYP11A1) (9). This latter enzyme cleaves cholesterol to pregnenolone, which is the precursor of all cellular steroids (69). Steroidogenic acute regulatory protein (StAR) acts on this complex to accelerate cholesterol transfer from the OMM to the IMM (9,69,152).

Despite this detailed understanding of cholesterol transport and metabolism in steroid biosynthesis, it is not yet certain what the source of cholesterol is, particularly during acute steroidogenesis. A series of in vivo and in vitro studies performed in the 1970s led to the conclusion that lipid droplets (LDs), which contain esterified cholesterol, are the source of the steroidogenic pool of cholesterol. Early evidence came from an ultrastructural study that showed a decrease in the volume of LDs in adrenocortical cells after exposure to stimulatory hormones (194). Subsequently, the testes of adult male mice treated in vivo with human chorionic gonadotropin (hCG) were shown to have fewer LDs one day after treatment than untreated mice (195). In another study, active transport of LD along microtubules in Y-1 mouse adrenocortical tumor cells was observed with non-perturbational imaging. The results also suggested an interaction between mitochondria and LD, consistent with cholesterol delivery from LDs to mitochondria (209). Other evidence for the importance of LD came from knockout studies of the vimentin gene in mice, which codes for a LD-associated intermediate filament. Gene knockout disrupted steroidogenesis in adrenal but not testicular tissue (196), consistent with the known slower response of testes to steroid-synthesis-inducing hormones compared to adrenal cortex. It was also demonstrated that hormones regulate the enzyme cholesterol ester hydrolase in LDs, effecting de-esterification of esterified cholesterol and increasing the pool of free cholesterol for steroid formation (197). Although LDs could provide a constant supply of cholesterol to sustain steroidogenesis, the kinetics of this process does not suggest that it is important in the acute response of these tissues to hormones.

The first evidence that the plasma membrane may provide the free cholesterol for steroidogenesis in the mitochondria came from studies by Freeman and colleagues. A series of

metabolic labeling studies of Leydig and adrenal cell lines with radiolabeled acetate and cholesterol led them to suggest the importance of the plasma membrane (232-235,249). Among the cell membranes, the plasma membrane has the highest concentrations of cholesterol, with the next highest concentrations observed in endosomal recycling compartments and the Golgi apparatus (250). It is surprising that mitochondria, the site where steroid synthesis is initiated, and the endoplasmic reticulum (ER), where cholesterol is synthesized de novo, have lower concentrations of cholesterol than other cell membranes (250,251). Because the cholesterol concentrations in cell membranes vary so widely, most researchers believe that vesicular trafficking using the secretory machinery is essential in cholesterol homeostasis (103,252). However, recently there has been increased interest in non-vesicular cholesterol trafficking, especially in dynamic inter-organellar interactions (253). During these interactions, organelles come into close contact but do not fuse. Instead, they form microdomains rich in cholesterol that allow rapid communication between the organelles (254).

This is relevant here as several studies implicate organelle plasticity and inter-organelle associations in steroidogenesis (133). These include electron microscope and biochemical studies that reveal increased incidence of ER-mitochondria interactions under hormone exposure (217,220). We recently found that, in MA-10 mouse tumor Leydig cells, hormonal stimulation was followed by increased apposition of the ER and the OMM, known as mitochondria-associated membranes (105). In addition to the MAMs, plasma membrane-associated microdomains involving the ER and mitochondria are also of current interest, as they are involved in signaling pathways, calcium influx, lipid synthesis, and, most importantly, nonvesicular cholesterol trafficking (255-259). In a study using ultra-thin electron microscopy

images, workers noted that plasma membrane-ER associations were ten times more prevalent than ER-mitochondria interactions (239,260).

The plasma membrane also interacts with other organelles in non-vesicular cholesterol trafficking. Maxfield and colleagues observed that dehydroergosterol, an analogue of cholesterol, trafficked from the plasma membrane to the endosome recycling compartments with the very short half-time of 2.5 minutes in TRVb-1 cells, a modified CHO cell line (236). It was even more rapidly transferred from the plasma membrane to LDs in macrophage foam cells, in which the half-time was 1.5 minutes (261).

Despite the improved understanding on how cells handle cholesterol trafficking and the knowledge available on cholesterol metabolism to steroid products, there is still a gap in our knowledge on the origin of free cholesterol used for acute steroid synthesis. To identify and visualize cholesterol used for steroid formation, we employed Perfringolysin O (Θ toxin), a cytolysin produced by *Clostridium perfringens* that has the ability to bind cholesterol with high affinity (262,263). Domain 4 of the Θ toxin (D4) is the C-terminal fragment and displays the same cholesterol binding affinity of the full protein, but does not exert cytotoxicity (263). Tagging D4 with fluorescent proteins enabled us to track cholesterol movement in living cells. Here, we report on a study in which we used the D4 probe in Leydig cells treated with hormones or cAMP and examined the ability of the cells to form steroids. We also used a variety of steroidogenesis and cholesterol trafficking inhibitors to examine the specificity of the process. We conclude that cholesterol from the plasma membrane supplies hormone-induced acute steroidogenesis.

Results

Tracking free cholesterol movement in MA-10 mouse tumor Leydig cells transfected with mCherry-D4 – Free cholesterol was tracked during steroidogenesis in MA-10 cells by transfecting them with the mCherry-D4 plasmid and visualizing the fluorescent D4 protein with scanning confocal microscopy. The results are shown in Figure 1A. In untreated cells, the fluorescent protein was predominantly bound to the plasma membrane and there were few aggregates inside the cell. When the MA-10 cells were treated with dbcAMP, fluorescent intensity at the plasma membrane began to decline within 30 min, continued to decline over the next 30 min, and remained constant and low after that, while there was minimal change in untreated control cells (Fig. 1A,B). Also, more fluorescent aggregates were seen in the treated than in the untreated cells. Acute stimulation of the cells by dbcAMP was confirmed by the measurement of progesterone, which increased during the 2-h treatment time. The accumulation of progesterone was unaffected by expression of mCherry-D4 (Fig. 1C).

We assessed the binding of mCherry-D4 to cholesterol in MA-10 cells by depleting the plasma membranes of cholesterol, which can be accomplished by treating cells with methyl-β-cyclodextrin, MβCD. The data in Figure 1D show that, following MβCD treatment, mCherry-D4 was no longer bound to the plasma membrane but formed aggregates in the cell as already observed (264). Upon depletion of cholesterol in the plasma membrane mCherry-D4 loses its affinity to bind to it, confirming its specific binding to cholesterol-rich membranes.

We next probed the effects of arresting cholesterol trafficking on the release of mCherry-D4 from the plasma membrane and on steroidogenesis. MA-10 cells were treated with U18666A,

an inhibitor of cholesterol transfer from the plasma membrane to intracellular membranes (265). When cells were pre-treated with U18666A and dbcAMP was added 4 h later, mCherry-D4 was retained in the plasma membrane, as evidenced by persistent high fluorescence 2 h later (Fig. 1E). We conclude that in the presence of the inhibitor, the cAMP analog was prevented from its expected action: the release of cholesterol from the plasma membrane. Furthermore, this cholesterol source appears to be important in steroidogenesis, as treatment with U18666A inhibited progesterone production in the presence of dbcAMP (Fig. 1F), but did not affect basal steroid production in control cells.

Intracellular cholesterol distribution in mCherry-D4 expressing MA-10 cells - Considering the ability of D4 to bind cholesterol rich membranes in live cells raised the question if the protein had an influence on cholesterol distribution in these cells. In order to examine this, MA-10 cells transfected with mCherry-D4 were stained with filipin, which binds to non-esterified cholesterol throughout the cell and compared to cells not expressing mCherry-D4 (Fig. 2A, B). No change in cholesterol distribution was noticed throughout the cells and the fluorescence intensity at the plasma membrane, where mCherry-D4 predominantly bound, remained unchanged (Fig 2E). To study the effect of D4 in cholesterol distribution during hormone-induced steroidogenesis, MA-10 cells transfected with mCherry-D4 were treated with dbcAMP and then fixed and stained with filipin (Fig. 2 C, D). Cholesterol distribution remained unchanged between control and mCherry-D4 expressing cells (Fig. 2C, D). However, a significant drop in the filipin fluorescence intensity was noticed in plasma membrane of dbcAMP-treated cells when compared to control cells (Fig. 2 C, D, E).

To further examine D4's effect on cholesterol distribution, we measured cholesterol levels in subcellular organelles involved in steroidogenesis. For this we first isolated mitochondria, plasma membrane and endosomes from MA-10 control and mCherry-D4 transfected cells. The purity of the organelle fractions were confirmed by immunoblot analysis (Fig. 2F). Rab5, an endosome marker, VDAC1, a mitochondrial marker, plasma membrane Ca(2+)-ATPase 1 (PMCA1), a plasma membrane marker, and β-actin, a cytoplasmic marker were all found to be enriched in their respective organelle fractions indicating the purity and efficiency of the isolation procedure used (Fig. 2F). Isolated fractions were processed for cholesterol content measurement by mass spectrometry. The results obtained revealed an overall similarity between control and mCherry-D4 expressing MA-10 cell organelle fraction cholesterol content (Fig. 2G). The plasma membrane, known to be rich in cholesterol, had the highest content of cholesterol, followed by endosomes and then the mitochondria and the cytoplasm which had the least amount of cholesterol as expected (Fig. 2G). No change in cholesterol levels were noticed between control and mCherry-D4 treated fractions, suggesting that mCherry-D4 expression in the MA-10 cells does not alter cholesterol distribution in plasma membrane, mitochondria and endosomes.

Cholesterol trafficking in MA-10 cells upon treatment with steroidogenic inhibitors – The next question examined was if the cholesterol originating from plasma membrane was targeted to mitochondria. The mCherry-D4-transfected MA-10 cells were treated with a variety of steroidogenic inhibitors, specifically with a cholesterol recognition/interaction amino acid consensus CRAC domain ligand (266), erastin (9), aminoglutethimide (9) and a START domain of the steroidogenic acute regulatory StAR protein ligand (267). They are inhibitors of TSPO,

VDAC, CYP11A1, and StAR protein, respectively and play important roles in cholesterol transport into mitochondria and steroid biosynthesis.

When aminoglutethimide was added to the MA-10 cells in the presence of 1 mM dbcAMP to inhibit CYP11A1 (Fig. 3A, E), the rapid decline in mCherry-D4 fluorescence that occurred at the plasma membrane with dbcAMP alone was partially prevented indicating that cholesterol was retained in the plasma membrane, with a maximal significant effect at 60 min. In addition, Figure 3E shows that retention of cholesterol in the plasma membrane in the presence of the inhibitor effectively blocked dbcAMP-induced steroid formation. Similar results were observed with erastin, an inhibitor of the OMM protein VDAC, which is involved in cholesterol translocation to the IMM for steroidogenesis (Fig. 3B).

The drug ligand (N-[2-(4-ethyl-5-[2-oxo-2-(4-toluidino)ethyl] sulfanyl-4H-1,2,4-triazol-3-yl)ethyl]-4-methylbenzamide) binding to the cholesterol recognition amino acid consensus sequence (CRAC) domain of TSPO, an inhibitor of steroidogenesis (266), reduced the dbcAMP-induced movement of mCherry-D4 from the plasma membrane to inside the cell in a dose-dependent manner (Fig. 1D). The half-maximal inhibitory concentration (IC 50) and maximal inhibitory concentration (IC 100) of the CRAC domain ligand have been shown to be 1 μM and 10 μM, respectively (266) (Fig 3D, E), and these concentrations were used here. We used the CRAC domain ligand in combination with dbcAMP to block steroidogenesis while inducing the movement of cholesterol to the mitochondria. A similar experiment was conducted with the START domain ligand (16-[4-(difluoromethoxy)benzylidene]androst-5-ene-3,17-diol) of the StAR protein (Fig. 3C, E). The mCherry-D4 fluorescence intensity at the plasma membrane was

unaffected by the steroidogenic inhibitors alone, but the inhibitors did partially prevent the release of cholesterol from the plasma membrane, consistent with the proposal that cholesterol in the plasma membrane is trafficked to the mitochondria and used in steroid biosynthesis upon acute stimulation of MA-10 cells by dbcAMP.

Cholesterol trafficking in the presence of steroids in MA-10 cells **-** To determine cholesterol trafficking in the presence of endogenous steroids, MA-10 cells expressing mCherry-D4 protein were incubated with 22*R*-HC, a membrane permeable metabolic intermediate. When *22R*-HC enters the mitochondria, MA-10 cells readily made progesterone, up to 1000 ng/mg of protein (Fig. 4C), levels that are similar to MA-10 cells stimulated with 1mM dbcAMP (Fig. 4A, C, D). The steroid intermediate added to MA-10 cells did not affect mCherry-D4 labeling at the plasma membrane, indicating that intracellular progesterone production does not induce cholesterol movement from the plasma membrane (Fig 4A). However, when 22*R*-HC and dbcAMP were applied in combination to induce both steroid formation and cholesterol movement from the plasma membrane, mCherry-D4 labeling of the plasma membrane remained unaffected, even after 2 h of combined 22*R*-HC and dbcAMP treatment (Fig 4A). These results could be explained by the existence of a feedback signal generated by the synthesized progesterone that blocks cholesterol mobilization from the plasma membrane. When MA-10 cells were treated with 22*S*-HC, an inactive isomer of 22*R*-HC, we observed, as expected, no stimulation of progesterone production (Fig 4C). However, when the inactive isomer was combined with dbcAMP, progesterone accumulated to about 700 ng/mg protein (Fig. 4D). Thus, in contrast to the active isomer 22*R*-HC, MA-10 cells treated with 22*S*-HC and dbcAMP readily trafficked cholesterol from the plasma membrane, as evidenced by the decreased fluorescence, which was

close to the background signal within 30 minutes of treatment (Fig. 4B). We interpret these results as strongly indicative that, in the absence of endogenous steroid synthesis, the feedback signal is not activated in response to dbcAMP and cholesterol is trafficked to the mitochondria for steroid formation.

Two candidates for feedback regulation of cholesterol trafficking are progesterone and pregnenolone. When MA-10 cells expressing mCherry-D4 were treated with these steroids in the presence and absence of dbcAMP (Fig. 4E, F), pregnenolone and to some extent progesterone blocked cholesterol trafficking. This effect was apparent even 2 h after treatment. It may be, then, that the steroids trigger a feedback signal that inhibits the movement of cholesterol from the plasma membrane.

Identification of other organelles involved in cholesterol trafficking – In addition to its binding on the plasma membrane, mCherry-D4 was also detected within cells. When steroidogenesis was enhanced with dbcAMP, there was an increase in this accumulation. We wished to determine if mCherry-D4 formed aggregates in the cell or localized to another organelle; the latter should be detectable by assessing increased free cholesterol in these organelles. The mitochondria, LD, lysosomes and ER in live MA-10 cells were identified using their respective fluorescent dyes. Whereas, early endosomes, late endosomes and Golgi apparatus were labeled using specific plasmids transduced into MA-10 cells using insect baculovirus (Bacmam 2.0 technology, Thermo Fischer Scientific). Thus organelles in MA-10 cells were screened for co-localization with mCherry-D4 upon dbcAMP stimulation. An increase in co-localization with early and late endosome markers Rab5a-GFP and Rab7a-GFP (CellLight® Late Endosomes-GFP, BacMam

2.0), respectively, were detected 60 min after dbcAMP treatment, suggesting that these endosomes might be part of the route by which cholesterol is trafficked from the plasma membrane to the mitochondria (Fig.5A, B).

To study the role of endosomes in steroid biosynthesis, NPC-2, previously shown to be involved in cholesterol trafficking between endosomes and mitochondria, was knocked down by transfecting MA-10 cells with specific siRNA at 50 and 75 nM concentrations (Fig. 5*C*) (48). The reduction of NPC-2 levels was accompanied by an increase in progesterone production at basal productions (Fig. 5*D*), but no change in Bt_2cAMP stimulated MA-10 cells (Fig. 5*E*). These data suggest that NPC-2 may play a negative role in cholesterol trafficking during basal steroidogenesis but does not play a role during acute steroidogenesis.

To further analyze the role of endosomes in steroidogenesis, endosomes from MA-10 control and Bt_2cAMP-treated cells were isolated, and their enrichment was verified by immunoblot analysis using anti-Rab5 antibody. Cholesterol extracted from the samples was analyzed by LC-MS/MS. There was no change in cholesterol distribution in the whole cell homogenate samples, and in endosome fractions slight but not significant increases in cholesterol levels were noticed in control and treated groups (data not shown).

Identification of the plasma membrane leaflet from which cholesterol is mobilized for steroidogenesis **-** The plasma membrane comprises two leaflets, an inner and outer. We wanted to know which leaflet the free cholesterol made available for steroidogenesis originates from. Untreated and dbcAMP-treated cells were fixed with paraformaldehyde and incubated with

recombinant EGFP-D4 protein. In intact cells, the exogenous recombinant EGFP-D4 was bound to the outer leaflet even 2 h after treatment with dbcAMP (Fig. 6A), and despite convincing movement of the endogenously expressed mCherry-D4 (Fig. 6A, B). It would seem that cholesterol for steroidogenesis comes from the inner leaflet of the plasma membrane.

Cholesterol movement in other mammalian tumor Leydig cells transfected with mCherry-D4 - Another Leydig-cell tumor cell line, rat R2C cells were transfected with mCherry-D4 plasmid. These cells produce high levels of steroids in a constitutive, hormone- and cAMP-independent manner. Cholesterol trafficking was measured in these cells and the results are presented in Figure 7. It was noted that the overall fluorescence intensity of mCherry-D4 at the plasma membrane in control R2C cell line was lower uniformly than the control MA-10 cells (Fig. 7A). In contrast to MA-10 cells, these cells do not respond to dbcAMP (Fig. 7A, B). There were no major changes in the fluorescence intensity at the plasma membrane, despite some movement of mCherry-D4 aggregates following dbcAMP treatment (Fig 7B). This may be due to high levels of constitutive pregnenolone formation in these cells. Progesterone production measured in R2C cells revealed mCherry-D4 transfection did not have an effect in steroid biosynthesis in both control and 2 hour dbcAMP treated cells (Fig. 7C).

Tracking free cholesterol movement in primary rat Leydig cells transfected with mCherry-D4 - To study cholesterol trafficking in primary adult rat Leydig cells, mCherry-D4 was packed into lentiviruses and used to infect the purified cells. The mCherry-D4 readily bound to the plasma membrane and 60-90 min after dbcAMP treatment, decreased fluorescence associated with the plasma membrane was observed, presumably due to movement of mCherry-D4 from the plasma

membrane (Fig 8A, B). We conclude that the plasma membrane is the cholesterol source for steroidogenesis in these cells. We measured testosterone in these cells, 1 h after they were isolated, in control (uninfected) cells 18 h after incubation, and 18 h post infection. After 18 h in culture, the control cells had less testosterone than just after isolation, probably due to loss of steroidogenic capacity in culture (Fig. 8C). However, there was no change in testosterone levels at 18 h in the control and infected cells, suggesting that the lentivirus containing mCherry-D4 did not affect steroid biosynthesis (Fig. 8C).

Discussion

All vertebrate steroid biosynthesis is initiated at the mitochondria (7). The substrate, cholesterol, must move from its intracellular location to the cholesterol-poor IMM, where it will be converted to pregnenolone by the CYP11A1 enzyme. Cholesterol transport is incompletely understood, partly due to its hydrophobicity, but is believed to entail a network of cellular signals, lipid transfer systems, and protein interactions that operate between and within organelle membranes (66). In-depth studies of hormone-induced acute steroidogenesis have shown that a multi-protein, 800-kDa complex is formed at the mitochondria that transports cholesterol from the OMM to the IMM (9). However, the mechanisms of cholesterol transport from its intracellular sources to the mitochondria are not clear.

In planning this study, we recognized the utility of a probe that would permit us to track intracellular cholesterol movement within cells. The θ-toxin protein from *Clostridium perfringens* seemed a likely candidate, as it can bind up to 30 mol% or more of cholesterol-containing membranes (268) and domain 4 of the protein retains the binding affinity without

toxicity. The major advantages of using D4 over other fluorescent probes available to track cholesterol movement are that it does not damage the membranes it binds to (Fig. 2E) and also does not disturb the intracellular distribution of cholesterol (Fig. 2G) (268-270). In addition, live-cell imaging is possible, which is not the case with filipin, another candidate probe. The binding affinity of D4 to membranes is low, so small changes in the cholesterol concentrations could be detected. Finally, it binds specifically to membrane cholesterol, avoiding the background signal that can be present with probes that bind indiscriminately to cellular cholesterol (263). In HeLa cells, D4 tagged with fluorescent proteins bound to both the outer and inner leaflet of the plasma membrane (262,264). In another study with CHO cells, recombinant GFP-D4 successfully bound the outer leaflet of the plasma membrane, but the expressed mCherry-D4 in the cell could not bind the inner leaflet owing to the low levels of cholesterol present in the membrane. To circumvent this, the workers employed a mutant mCherry-D4^{D434S} (mCherry-D4H) that binds to the plasma membrane with higher affinity than the wild-type protein (269,271). Hence both D4 and D4H could be excellent tools to study cholesterol trafficking. For this study, we transfected MA-10 cells with mCherry-D4, permitting us to track cholesterol.

Given that the plasma membrane has a greater concentration of cholesterol than any other organelle (Fig. 2G), we expected D4 to readily bind it, as well as any other organelle with a membrane that contained greater than 30 mol% of cholesterol. Indeed, confocal images of mCherry-D4 transfected cells revealed fluorescence predominantly at the plasma membrane with a few aggregates in the cell (Fig. 1A). After initiating steroidogenesis with dbcAMP, there was a dramatic and significant decline in fluorescence intensity associated with the plasma membrane (Fig. 1A,B). This could have been due either to release of cholesterol below 30 mol% or to a

negative effect of dbcAMP on D4 binding. To distinguish between these possibilities, we used MβCD to expunge cholesterol from the membranes of the cells (272), which it did without binding to the membrane. Release of mCherry-D4 from the plasma membrane with MβCD alone led us to conclude that the removal of cholesterol prevented mCherry-D4 from binding (Fig. 1D).

Expression of mCherry-D4 did not alter dbcAMP-stimulated acute progesterone synthesis (Fig. 1C). Nevertheless the ability of D4 to efficiently bind cholesterol-rich membranes raised the question whether D4 can influence the intracellular cholesterol distribution. Filipin staining analysis revealed no change in intracellular cholesterol distribution between control and mCherry-D4 expressing cells (Fig. 2A-D). However, a significant drop in filipin fluorescence intensity at plasma membrane in dbcAMP-treated cells was seen, substantiating our finding that cholesterol for steroidogenesis is mobilized from the plasma membrane (Fig. 2E). In agreement with the filipin staining analysis, the subcellular distribution of cholesterol analyzed by mass spectrometry also did not reveal any change in cholesterol distribution in mitochondria, plasma membrane and endosomes (Fig. 2G), suggesting that D4 does not affect intracellular cholesterol distribution during steroid biosynthesis.

To ensure that dbcAMP did not negatively affect D4 binding, we employed the inhibitor U18666A, which efficiently blocks cholesterol trafficking and interferes in cholesterol synthesis in the cell (273). In presence of U18666A, cholesterol was trapped at the plasma membrane even after treatment with dbcAMP and steroid formation was inhibited. This is evidence that the presence of dbcAMP did not have a negative influence on mCherry-D4 bound to the plasma membrane and strengthens our conclusion that cholesterol release from the plasma membrane

can be attributed to reduced cholesterol in the membrane during steroidogenesis. Thus, we believe that, during dbcAMP-induced acute steroidogenesis in MA-10 cells, a pool of free cholesterol is trafficked from the plasma membrane.

In addition to inducing cholesterol transport, hormone stimulation affects a multi-protein complex called the transduceosome at the OMM (69) where the mitochondrial proteins are part of the steroidogenic metabolon, described earlier. The transduceosome includes the hormonally induced cytoplasmic protein StAR, the integral outer mitochondrial membrane proteins TSPO (146) and VDAC (274), and the IMM-associated protein CYP11A1 (13). Several studies show that interrupting the functionality of these proteins is detrimental to cholesterol transport to the IMM and, in turn, steroidogenesis. We asked if the pool of cholesterol mobilized from the plasma membrane was, in fact, trafficked to the mitochondria after hormone stimulation. mCherry-D4 transfected MA-10 cells were treated with aminoglutethimide, a CRAC domain of the TSPO ligand, erastin, and a START domain of the StAR protein ligand. These are inhibitors of CYP11A1, TSPO, VDAC, and StAR proteins, respectively (Fig. 3A-D). These inhibitors drastically increased the time required for the dbcAMP-induced movement of mCherry-D4 from the plasma membrane (Fig. 3A-D), indicating that when there is a congestion of cholesterol at the OMM, it acts as a signal that prevents the mobilization of free cholesterol from the plasma membrane. By increasing the concentration of the StAR and TSPO ligands, the time of mCherry-D4 release was also delayed, further substantiating our conclusion (Fig. 3B, D).

We also examined the kinetics of mCherry-D4 labeling of the plasma membrane of MA-10 cells exogenously supplied with the endogenous steroid substrate 22*R*-HC. This treatment

stimulated steroid synthesis and maintained the mCherry-D4 labeling of the plasma membrane. Even when dbcAMP was supplied with 22*R*-HC, there was minimal movement of cholesterol from the plasma membrane. This may be evidence of a feedback signal that blocks the transfer of cholesterol from the plasma membrane. 22*R*-HC actively binds CYP11A1 and is rapidly converted to pregnenolone, so the congestion of cholesterol at the OMM might trigger a feedback signal similar to that observed with aminoglutethimide, which is an inhibitor of CYP11A1. To further probe the possible feedback mechanism, MA-10 cells were treated with 22*S*-HC, an inactive stereoisomer of 22*R*-HC (275). This isomer has the ability to bind CYP11A1 but does not undergo side chain cleavage to be converted to steroid products. In MA-10 cells treated with 22*S*-HC in the presence of dbcAMP, there was rapid displacement of cholesterol from the plasma membrane, even though there was some inhibition of steroid formation. We interpret these data to mean that it is the production of steroids and not the presence of hydroxycholesterols that induced the feedback mechanism. Indeed, presence of progesterone has been previously shown to block cholesterol mobilization from late endosomal/lysosomal compartments in human fibroblast and monocyte cells (276,277). So we next evaluated the release of mCherry-D4 from the plasma membrane in the presence of both steroids and dbcAMP. The release of mCherry-D4 was slightly retarded in the presence of progesterone, but D4 was clearly retained at the plasma membrane when pregnenolone was supplied (Fig 4F). Therefore, It may be that pregnenolone acts in the feedback mechanism.

It is generally accepted that cellular membranes are asymmetric bimolecular leaflets composed predominantly of phospholipids, varying concentrations of cholesterol, other minor species of lipids, and a plethora of proteins (43,278,279). Due to its fast flip-flop rate, the

cholesterol distribution across the two leaflets of the plasma membrane has been quite challenging to determine, experiments suggesting either an outer or inner leaflet enrichment (250,280). A recent study, based on a large-scale molecular dynamics simulation, showed an asymmetrical distribution of cholesterol, in which the outer leaflet of the plasma membrane comprised 54% of the total cholesterol with a slightly higher cholesterol content than the inner leaflet (comprising 46%) (279).

Compared to the inner leaflet, the outer leaflet is more rigid, with a higher concentration of sphingolipids that tightly interact with cholesterol through strong van der Waal's forces, forming rafts. In contrast, the inner leaflet has few sphingolipids and more glycerolipids, and cholesterol binds less efficiently due to weak Van der Waal's forces. This may make it easier for the cholesterol to be mobilized from the inner leaflet. In our study, when the outer plasma membrane leaflet was labeled with recombinant EGFP-D4 and cells were subjected to dbcAMP treatment, no change in the labeling occurred (Fig. 6A, B). This can be explained if the cholesterol for steroidogenesis comes from the inner leaflet.

The first suggestion that the plasma membrane was the source for the steroidogenic pool of cholesterol was made by Freeman and colleagues (233). Based on these results, it was also suggested that cholesterol moves from the plasma membrane passing through endosome compartments during steroidogenesis in MA-10 cells (235). Our screening of various organelles with the appropriate respective fluorescent markers is in agreement with this suggestion: mCherry-D4 colocalized with a late endosome marker and the co-localization increased upon dbcAMP stimulation (Fig. 5B), suggesting that the late endosome might be part of the route for

cholesterol trafficking from the plasma membrane to mitochondria. But in dbcAMP-treated endosome-rich isolates only a slight increase in cholesterol levels was noted when compared to control (Fig. 5D). Although this finding may not add further evidence to the data generated by confocal microscopy, it is likely that the sensitivity of the mCherry-D4 is such that when cholesterol concentrations cross the threshold of 35 mol%, the probe would be able to bind the membrane (262) and small increases in the concentrations of cholesterol may not be observed as significant when studied by mass spectrometry. Furthermore, knocking down NPC-2, a protein previously shown to be involved in the exit of cholesterol from the endosome/lysosome compartment to mitochondria (281), did not have an effect on dbcAMP-induced steroidogenesis although it had a negative effect in basal conditions, suggesting that NPC-2 may not play a role in cholesterol transport between endosome compartment and mitochondria (Supplementary Fig. 2A-C).

In contrast to the hormone-inducible MA-10 cells, the rat tumor R2C Leydig cells constitutively synthesize high levels of pregnenolone and progesterone (282). Interestingly, mCherry-D4 localized at the plasma membrane in R2C cells even though they were constitutively making steroids. Since these cells do not respond to hormone or cAMP stimulation, no release of mCherry-D4 from the plasma membrane was observed, suggesting that a different source organelle supplies cholesterol for steroid biosynthesis in R2C cells.

Even though MA-10 and R2C cell lines appear to be excellent models for studying steroid biosynthesis, the differences in cholesterol trafficking between these tumor Leydig cell lines led to concerns that tumorigenic transition of these cell lines altered the source of

cholesterol utilized in steroidogenesis. To investigate this possibility, primary rat Leydig cells were isolated and infected with lentiviruses containing mCherry-D4 for efficient gene transfer. Even though the efficiency of the infection was approximately 95%, only a few cells had labeling at the plasma membrane. This trend was similar to that in MA-10 cells, in which cholesterol movement at the plasma membrane was apparent 60-90 min after stimulation of steroid biosynthesis by dbcAMP. Thus, we conclude that the plasma membrane is the source of cholesterol used in steroid biosynthesis in primary rat Leydig cells.

Free cholesterol is considered to be the main source for steroid hormone production in rodent testes (58). This is in contrast to rodent adrenal gland and ovary, where an important reservoir of cholesterol for steroidogenesis is present in cholesterol esters stored in the LDs. The results presented herein show that the plasma membrane is one of the richest sources of free cholesterol available for binding to the mCherry-D4 probe under basal conditions (Fig. 9A). During acute stimulation of steroid biosynthesis by cAMP, MA-10 cells and primary Leydig cells mobilize this free cholesterol that is transported to the mitochondria, where the transduceosome complex translocates the cholesterol to the IMM for conversion to pregnenolone by CYP11A1, and then, subject to further metabolism to progesterone and other steroids in the ER (Fig. 9B). Inhibiting one of the transduceosome proteins may create a feedback signal instructing the trafficking system to stop sending cholesterol to the already congested mitochondria, slowing down steroidogenesis (Fig. 9C). Moreover, when a large amount of pregnenolone is added to the system, it may initiate another feedback signal that inhibits the movement of cholesterol from the plasma membrane (Fig. 9D). It seems that these feedback mechanisms serve as protective mechanisms to avoid cholesterol-induced toxicity at the OMM

and excessive steroid production, which could be detrimental to the body. Although pregnenolone was proposed to be the messenger carrying the feedback, its sensor at the plasma membrane remains to be identified. Insights into cholesterol trafficking pathways will increase the understanding of steroid-related diseases and help the development of drugs affecting cholesterol trafficking and metabolism.

Experimental procedures

Cell culture **-** MA-10 cells from mouse Leydig cell tumor (kindly provided by Dr. M. Ascoli, University of Iowa, Iowa City, IA, USA), R2C cells from rat Leydig cell tumor cells (American Type Culture Collection, Manassas, VA, USA) were cultured with Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 (Invitrogen) supplemented with 1% penicillin and 1% streptomycin and maintained at 37°C. The media for MA-10 and R2C cells were supplemented with 5% fetal bovine serum (FBS) and 2.5% heat-inactivated horse serum and the cultures were grown in 3.5% or 5% CO_2, respectively. For time courses, the cells were incubated with DMEM/Ham's F-12 without any supplementation in the presence of appropriate drug treatments.

Live cell imaging **-** Cells were plated onto a 35 mm diameter fluorodish culture dish (World Precision Instruments) that had been pretreated with 0.1% gelatin and incubated at 37°C for 24 h. At 75% confluence, the cells were transiently transfected with 2 μg of mCherry-D4 plasmid using 4 μL jetPRIME reagent (Polyplus transfection) and 200 μL of jetPRIME buffer. Cells were visualized at room temperature using a scanning laser confocal microscope (FluoView™ FV1000; Olympus Corp.) at 100x with an oil immersion objective (UPLSAP). FluoView software (version 3.1) was used to capture the images; quantification and image processing were done with Image-Pro Plus (version 6.3) and ImageJ version 1.47 (http://rsbweb.nih.gov/ij).

Statistical analysis of the quantitative data was performed using Prism version 5.0 (GraphPad Software).

Cholesterol detection assay **-** MA-10 cells (1×10^5) were plated on 35mm diameter Fluorodish and kept on growth medium overnight. The next day, cells were transfected with mCherry-D4 plasmid and incubated for another 24 hours. Following the treatment period, medium was removed, cells were washed 3 times with 1× PBS, and fixed with 4% paraformaldehyde for 30 minutes. The cells were then stained for cholesterol using 50 µg/ml filipin III (Sigma) for 1 hour, washed and visualized under scanning laser confocal microscope.

Isolation of organelles **-** MA-10 cells were grown in 875 cm^2 Falcon® rectangular straight neck cell culture multi-flasks to 80% confluence, washed with PBS and trypsinized. Isolation of cytoplasm, mitochondria and plasma membranes was carried out following a well-established methodology with minor modifications (245). In brief, cells were homogenized with a teflon pestle and an overhead stirrer by using 6-7 strokes at 3000 rpm. Cell integrity was assessed using trypan blue staining and visualized under microscope until 90% cell damage has been attained. Homogenates were centrifuged at 800*g* for 5 minutes 4^{o}C. The collected supernatants were centrifuged twice at 10,000*g* for 10 minutes. The pellets obtained contained crude mitochondria isolates, which were further centrifuged at 10,000*g* for 10 minutes to obtain the mitochondrial fraction. To confirm the enrichment of mitochondria, immunoblot analysis was performed using an anti-VDAC1 antibody. The supernatant was centrifuged at 25,000*g* for 20 minutes at 4^{o}C. The obtained supernatant contained the microsomes and the cytoplasm fractions, which were further subjected to ultracentrifugation at 95,000*g* for 2 h and 30 minutes at 4^{o}C to obtain the isolated

cytoplasm. The purity of the fraction was confirmed by immunoblot analysis using anti-β-actin. The pellet from the 25,000*g* centrifugation step contained primarily plasma membrane and further mitochondrial contaminants. The pellet was resuspended and layered on to 38%, 43% and 53% sucrose gradient and ultracentrifuged at 95,000*g* for 2 h and 30 minutes at 4^{o}C from which the plasma membrane fraction was separated from the mitochondrial contamination. The purity of the plasma membrane fraction was confirmed by immunoblot analysis using an anti-PMCA1 antibody.

To isolate endosomes, MA-10 cell homogenates were subjected to discontinuous density gradient centrifugation using 8%, 35% and 42% sucrose density gradients and ultracentrifuged at 210,000*g* for 1.5h at 4^{o}C as described (283). The purity of the endosome fraction was confirmed with immunoblot analysis with an anti-Rab5 antibody.

Immunoblot analysis - Proteins from subcellular organelle fractions were extracted using M-PER mammalian protein extraction reagent (ThermoFisher Scientific). Proteins were quantified using Bradford assay (Bio-Rad laboratories). 15µg of protein each from whole cell homogenates (WH), cytoplasm (C), endosomes (Endo), mitochondria (Mito), plasma membrane (PM) were separated in a Novex NuPage Bis-Tris 4–12% (wt/vol) precast gel (Invitrogen), transferred to polyvinylidene fluoride (PVDF) membranes for standard western blotting and blocked for 90 minutes at room temperature in blocking buffer (20nM Trizma base, 100mM NaCl, 1% Tween 20, 5% skim milk). Membranes were incubated overnight at 4°C with anti-rab5 (1:2000, Abcam), anti-VDAC1 (1:5000, Abcam), anti-PMCA1 (1:1000, Abcam) and anti-β-actin (1:1000, Abcam), followed by appropriate secondary horseradish peroxidase (HRP)-conjugated

antibodies (1:1000, Cell Signalling Technology). Proteins of interest were visualized using the Amersham chemiluminescence kit and a FUJI image reader LAS4000 (FUJIFILM) for capturing images.

Cholesterol analysis using AB Sciex triple -TOF Mass spectrometer (TOF) - For the purpose of quantification of Cholesterol from sub-cellular organelle fractions - these fractions were spiked with d6-Cholesterol internal standard (IS). Samples were extracted using 2 X 1 ml methyl tert-butyl ether (MTBE). Aliquots were combined in 10 x 13 mm test tubes dried in a Thermo Speed Vac™ for 20 minutes, re-dissolved in 500-µl aliquot of MTBE, vortexed and dried again ~ 10 min. Samples were re-suspended in 200 µl of 50% (aq.) Methanol and vortexed. The contents were transferred in autosampler vials and were stored at -20oC until ready for LC-MS analysis. All measurements were made by AB Sciex 5600+ triple-TOF Mass Spectrometer coupled with Shimadzu Nexera XR UHPLC system. A binary mobile phase consisting of (A) water w/ 0.1 % formic acid and (B) acetonitrile w/ 0.1 % formic acid and acid was utilized. A 2µl aliquot of sample was injected into LC. Analytes were chromatographically resolved by isocratic elution (90% (B) at 350 µL/min) with an Agilent Eclipse Plus C-8 analytical column (50mm X 2.1mm ID x 1.8 µm particle). The total run time was 10 minutes. The mass spectrometer was operated in a positive HESI mode with a vaporization temperature of 550 °C and a spray voltage of 5.5 kV. For quantification purposes, a calibration curve was run along with the samples. After data acquisition, the intensity of each ion was integrated MultiQuant™ software.

Co-localization analysis - To assess the localization of mCherry-D4 protein to the Golgi apparatus, early endosomes, or late endosomes, mCherry-D4-transfected MA-10 cells were

transduced with the appropriate green fluorescent protein (GFP), using BacMam 2.0 (Thermo Fischer Scientific) at 100 virus particles per cell for 24 hours at 37°C. The GFPs were: CellLight Golgi-GFP, CellLight early Endosomes-GFP, or CellLight Late Endosomes-GFP (Thermo Fischer Scientific). To study the localization of the mCherry-D4 probe to mitochondria, mCherry-D4-transfected cells were treated with 100 nM MitoTracker green fluorescent mitochondrial stain (Green FM; Cell Signaling Technology) for 30 min at 37°C. The cells were washed three times with PBS and visualized. To detect localization of the mCherry-D4 probe to LDs, mCherry-D4-transfected cells were treated with 2 μg/mL of boron-dipyrromethene (BODIPY 493/503; Thermo Fischer Scientific), incubated for 30 min at 37°C, washed three times with PBS and then visualized. mCherry-D4 probe in lysosomes was detected by labeling lysosomes with 70 nM LysoSensor Green DND-189 (Thermo Fischer Scientific) and incubated for 30 min at 37°C, washed three times with PBS before visualization. Localization of the mCherry-D4 probe to the ER was assessed by incubating cells with 250nM ER-Tracker™ Blue-White DPX dye for 45 minutes at 37°C, washed three times with PBS and visualized under confocal microscope.

Small Interfering RNA Transfection Analysis- MA-10 cells were plated onto 6-well plates at a concentration of 3 × 105 cells/well and immediately transfected using ON-TARGETplus mouse NPC2 siRNA 50 and 75 nM(Dharmacon) using 4 μl of jetPRIME® reagent (Polyplus-transfection) and 200 μl of jetPRIME® buffer based on the protocol specified by the manufacturers. A scrambled siRNA (ON-TARGETplus non-targeting siRNA; 40 nM) from Dharmacon was used as a transfection control. 72 h post-transfection, medium was collected to analyze progesterone production using RIA before and after Bt2cAMP

treatments. Cells were subjected to protein extraction using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific). Proteins were quantified using Bradford assay. 20 µg of protein each from control, mock, scrambled, and siRNA-transfected samples were subjected to immunoblot analysis.

Cell treatments **-** The cell treatments were: 1mM dibutyryl cAMP (dbcAMP; Sigma-Aldrich); 20 µM 22*R*-hydroxycholesterol (22*R*-HC; Sigma-Aldrich); 20 µM 22*S*-hydroxycholesterol (22*S*-HC; Sigma-Aldrich); 20 µM progesterone; 20 µM pregnenolone; 1 µM or 10 µM TSPO cholesterol recognition amino acid consensus sequence (CRAC) domain ligand (N-[2-(4-ethyl-5-[2-oxo-2-(4-toluidino)ethyl]sulfanyl-4H-1,2,4-triazol-3-yl)ethyl]-4-methylbenzamide) (266); 100 µM START domain of StAR protein ligand (16-[4-(difluoromethoxy)benzylidene]androst-5-ene-3,17-diol) (267); 0.67 mM DL-aminoglutethimide (AMG; Sigma-Aldrich), a CYP11A1 inhibitor, or 100 µM Erastin (Sigma-Aldrich), an inhibitor of VDAC. Chemicals were supplied in serum-free media with or without dbcAMP for 2 hours.

Cholesterol was removed from internal sources by treating MA-10 cells with 10 mM methyl-β-cyclodextrin (MβCD; Sigma-Aldrich), for 30 min at 37°C. Cholesterol trafficking was arrested by treating MA-10 cells with 7 µM 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A; Sigma-Aldrich), for 4 h and re-induced by treating them with dbcAMP.

Steroid measurement **-** MA-10 cells and R2C cells were plated onto 96-well plates (2×10^4 cells/well). Twenty-four hours after incubation, the media were replaced for control cells, while the cells for treatments were transfected with mCherry-D4 plasmid and incubated for another 24

h, after which the media were replaced with serum-free media. At the end of the treatments, the culture media were collected and progesterone production was measured by radioimmunoassay (RIA) with progesterone antisera (MP Biomedicals) and [1,2,6,7-^{3}H]progesterone (specific activity, 94.1 Ci/mmol; PerkinElmer Life Sciences) using the manufacturer's recommended conditions. Progesterone production was normalized to the amount of protein in each well. The RIA data were analyzed with Prism 4.02 from GraphPad.

To assess steroidogenesis in the primary rat Leydig cells, cells were plated into wells of a 96-well plate (1×10^{5} cells/well). Testosterone was measured 2 h and 20 h after plating or, for the cells with lentiviral infection, 20 h after plating. Steroidogenesis was induced with 1mM dbcAMP for 2 h, when the culture medium was collected and testosterone was measured by RIA with testosterone antisera (MP Biomedicals) and radiolabeled testosterone [1,2,6,7-^{3}H(N); specific activity, 83.4 Ci/mmol; PerkinElmer Life Sciences],
according to the manufacturer's instructions.

Recombinant EGFP-D4 protein labeling - MA-10 cells were seeded in medium containing serum. Twenty-four hours after incubation, cells were washed with PBS and then incubated for 2 h in fresh media without serum, either with or without 1 mM dbcAMP. Cells were then washed three times with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS, and incubated in 5 μg/mL enhanced green fluorescent protein (EGFP)-D4 in binding buffer (0.1% BSA/PBS) for 30 min at room temperature. Recombinant EGFP-D4 was prepared as previously described (262). The cells were washed three times again with PBS and visualized using a scanning confocal microscope.

Lentiviral vector packaging using 293FT cells – The D4 cDNA coding sequence was subcloned into the HIV-based lentiviral expression vector, pLVX-mCherry-C1 (Clontech) at the EcoRI and BamHI sites using the primers: 5'-gaattcgtacaagAAGCTTaaggg-3' and 5'-ggatccGCGGGTTTAAACCTCGAG-3'. Three micrograms of the pLVX-mCherry-D4 vector was then co-transfected with 9 μg ViraPower Lentiviral packaging mix in the presence of Lipofectamine 2000 using Opti-MEM I reduced serum media (all from Thermo Fischer Scientific) into a 10 cm diameter culture dish (Corning) containing 6×10^6 293FT cells. Seventy-two hours after transfection, the presence of lentiviral particles was verified with Lenti-X GoStix (Clontech). The media containing the virus particles was removed from the cells and concentrated using the Lenti-X concentrator (Clontech). The viruses were stored at –80°C.

Primary rat Leydig cell isolation and lentiviral infection **-** Leydig cells were isolated from 60-64 day-old Sprague-Dawley rats (Charles River Laboratories) as previously described (284). In brief, the testis were decapsulated, digested in M-199 medium containing 0.05% collagenase-dispase, 0.005% soy trypsin inhibitor, and 0.001% deoxyribonuclease I (all from Sigma-Aldrich) and shaken at 90 cycles/min for 45 min at 34°C. Once dissociated, the seminiferous tubules were removed by filtering the tissue through a 40μm cell strainer (Corning) and media containing enzyme was removed by centrifuging at 900 rpm for 10 min. The pellet was resuspended in fresh M-199 medium and the cells were applied to a Percoll gradient, which yielded 90% pure Leydig cells. The purified cells were plated onto a cyclic olefin co-polymer 96-well plate (Corning) and supplied with 20 μL of 10^7 IFU/ml concentrated lentiviral particles, sufficient to infect 50,000 cells in 200 μL of medium. Eighteen hours after infection, the cells were visualized under a

scanning confocal microscope (FluoView FV1000; Olympus Corp.) at 100x with an oil immersion objective (UPLSAP).

Acknowledgments

We thank Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA) for supplying the MA-10 cell line; Mr. Edward Daly and Ms. Pushwinder Kaur for their assistance with the mass spectrometry studies; and Ms. Rachel Chan and Ms. Esha Sanyal for their assistance in subcellular organelle isolations procedure.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

SV designed, performed, and analyzed experiments; and wrote the paper. DBM designed and constructed lentiviral vectors and assisted with the experiments shown in Figure 8. SC provided assistance with the experiments shown in Figures 1-3. FHM and TK provided the mCherry-D4 vectors, assisted with the design of experiments, and assisted with data analysis. VP conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Footnotes

* This work was supported by a grant from the Canadian Institutes of Health Research (MOP102647 and MOP125983) and a Canada Research Chair in Biochemical Pharmacology to V.P. and S.V. was supported in part by pre-doctoral fellowships from the Experimental Medicine Graduate Program and the Center for the Study of Reproduction. The Research Institute of MUHC was supported by a Center grant from Le Fonds de la recherche du Québec-Santé.

The abbreviations used are: 22*R*-HC, 22*R*-hydroxycholesterol; 22*S*-HC, 22*S*-hydroxycholesterol; ATAD3, ATPase family AAA Domain-containing protein 3; CRAC, cholesterol recognition amino acid consensus sequence; CYP11A1, cytochrome P450 side chain cleavage enzyme; D4, Domain 4 of the Θ toxin; dbcAMP, dibutyryl cAMP; DMEM, Dulbecco's modified Eagle medium; hCG, human choriogonadotropin; ER, endoplasmic reticulum; ERC, endosomal recycling compartments; FBS, fetal bovine serum; HRP, horseradish peroxidase; IMM, inner mitochondrial membrane; LD, lipid droplets; MAMs, mitochondria-associated membranes; MβCD, methyl- β- cyclodextrin; OMM, outer mitochondrial membrane; PMCA1, plasma membrane Ca(2+)-ATPase 1; PVDF, polyvinylidene fluoride; RIA, radioimmunoassay; StAR, steroidogenic acute regulatory protein; TSPO, translocator protein; U18666A, 3-beta-[2-(diethylamino)ethoxy]androst-5-en-17-one; VDAC, voltage-dependent anion channel.

Figures and legends

Figure 1

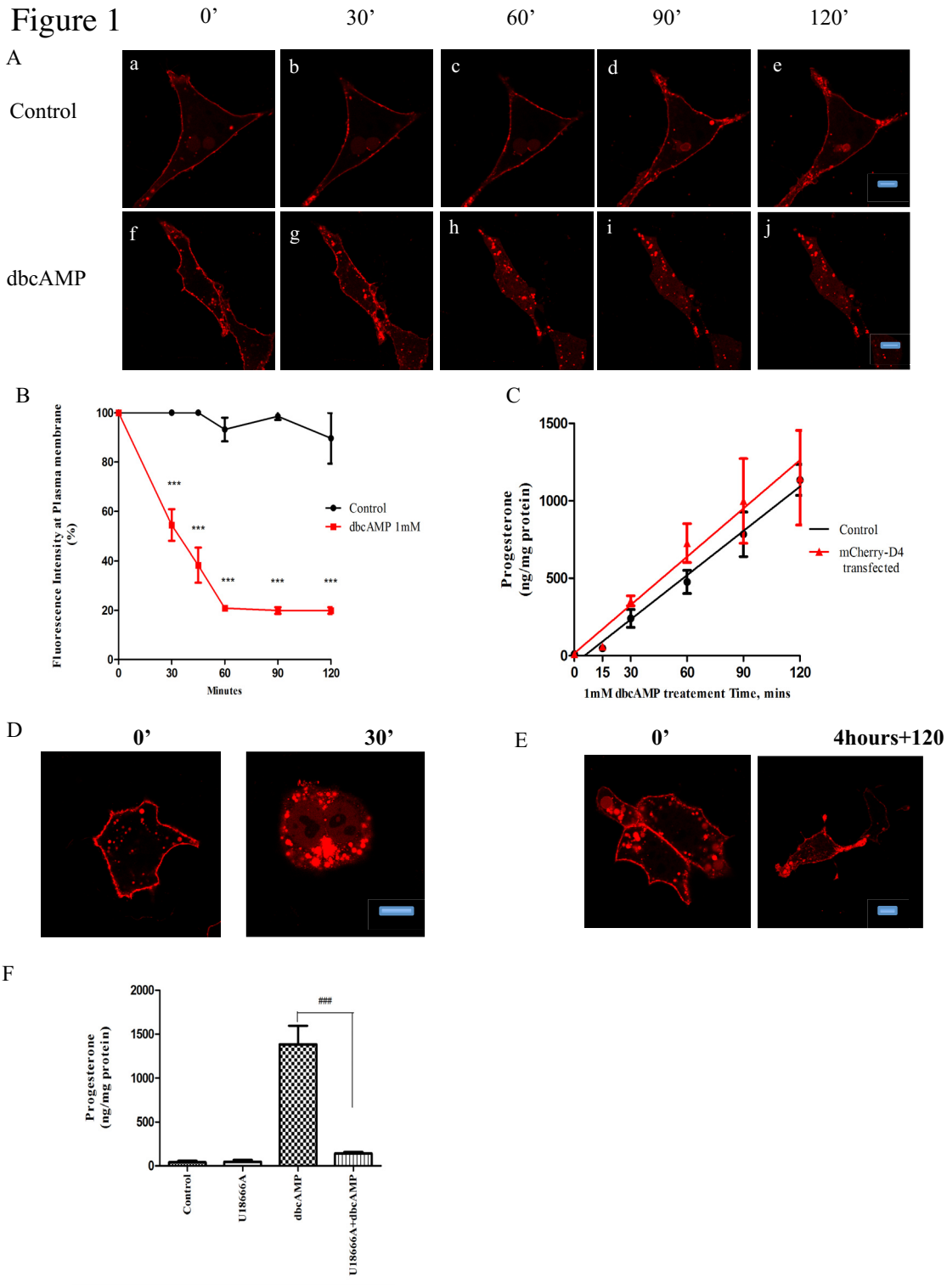
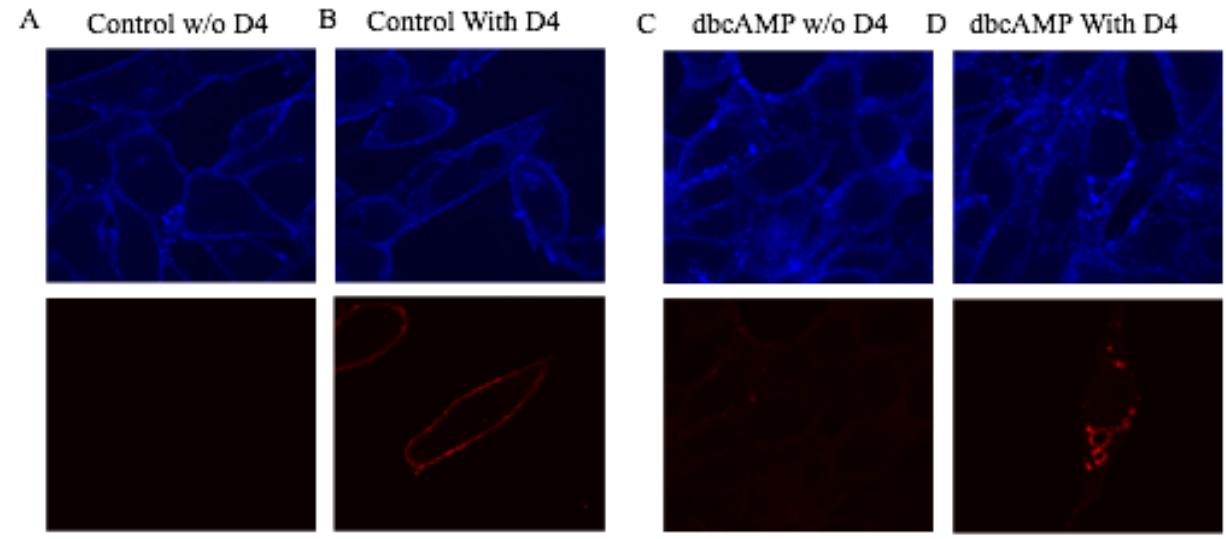
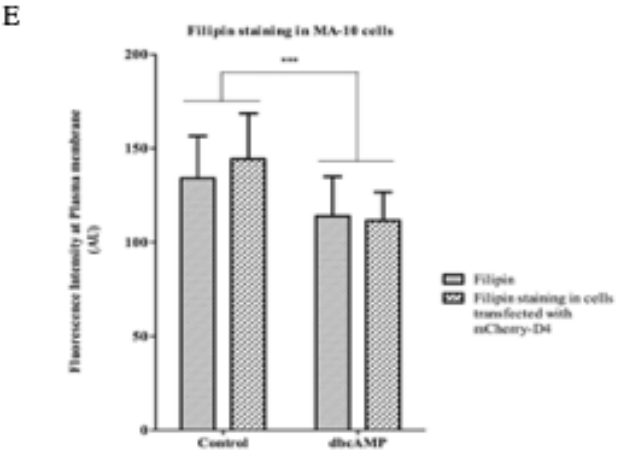
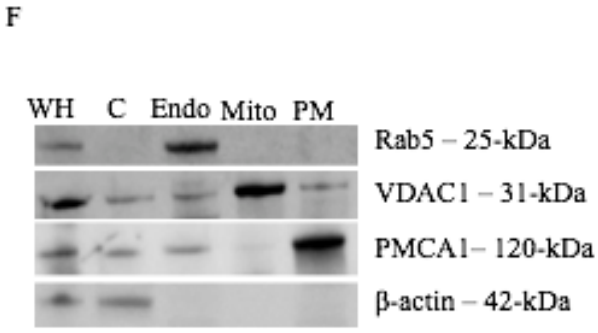


Figure 1. Movement of free cholesterol in MA-10 cells. *A*, Confocal microscope images of cells transfected with mCherry-D4. Images a-e: time course of one control (untreated) cell; images f-j: time course of one cell treated with dbcAMP. Scale bar = 10 μm; *B*, Time course of fluorescence intensity at the plasma membrane of control and dbcAMP-treated cells transfected with mCherry-D4. *C*, Time course of progesterone levels in untransfected cell and cells transfected with mCherry-D4 after treatment with dbcAMP. *D*, Confocal microscope images of cells transfected with mCherry-D4 before treatment and 30 min after treatment with methyl-β-cyclodextrin. Scale bar = 10μm. *E*, Confocal microscope images of cells before treatment and cells incubated with U18666A for 4 h, then treated with dbcAMP for 2 h, Scale bar = 10μm. *F,* Progesterone production in mCherry-D4 transfected cells treated with U18666A, U18666A and dbcAMP, dbcAMP, or untreated. (Data represent means ± SD of at least three independent experiments performed in triplicate; two-way ANOVA followed by Bonferroni's post-hoc test (*) or *t*-test (#) were used to calculate statistical significance; $^{***,\#\#\#}$ $p < 0.001$; scale bars: 10 μm; dbcAMP, dibutyryl cAMP.)


A
Control w/o D4
B
Control With D4
C
dbcAMP w/o D4
D
dbcAMP With D4


E
Filipin staining in MA-10 cells
***
Fluorescence Intensity at Plasma membrane (AU)
200
150
100
50
0
Control
dbcAMP
Filipin
Filipin staining in cells transfected with mCherry-D4


F
WH
C
Endo
Mito
PM
Rab5 – 25-kDa
VDAC1 – 31-kDa
PMCA1– 120-kDa
β-actin – 42-kDa

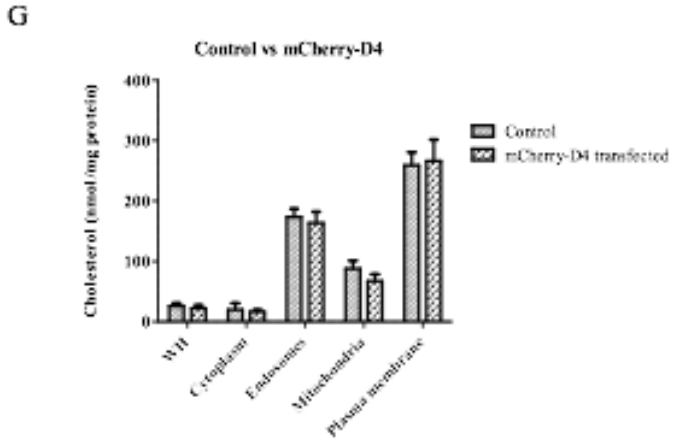

G
Control vs mCherry-D4
Cholesterol (nmol/mg protein)
400
300
200
100
0
WH
Cytoplasm
Endosomes
Mitochondria
Plasma membrane
Control
mCherry-D4 transfected

Figure 2. Intracellular cholesterol distribution in MA-10 cells expressing mCherry-D4. *A and B,* Confocal images of control and mCherry–D4 expressing MA-10 cells fixed with 4% paraformaldehyde and stained with 50 μg/ml filipin, scale bars: 10 μm. *C and D*, 1mM dbcAMP-treated MA-10 cells with and without mCherry-D4 expression, fixed and stained with 50 μg/ml filipin, scale bars: 10 μm. *E*, Fluorescence intensity of filipin staining at the plasma membrane in control and dbcAMP-treated MA-10 cells with or without mCherry-D4 expression. Data represent means ± SD of at least 12 different cells performed in triplicate; two-way ANOVA followed by Bonferroni's post-hoc test (*) were used to calculate statistical significance; *** $p <$ 0.001. *F,* Protein content in whole cell homogenates (WH), cytoplasm (C), endosomes (Endo), mitochondria (Mito), plasma membrane (PM) isolates were quantified using Bradford assay (Bio-Rad laboratories), and 15μg of protein were separated in a Novex NuPage Bis-Tris 4–12% (wt/vol) precast gel (Invitrogen) and transferred to PVDF membranes for standard western blotting. Proteins were detected with anti-rab5 (1:2000, Abcam), anti-VDAC1 (1:5000, Abcam), anti-PMCA1 (1:1000, Abcam) and anti-β-actin (1:1000, Abcam), followed by appropriate secondary HRP-conjugated antibodies (1:1000, Cell Signalling Technology). *G,* Organelle cholesterol distribution was measured by LC-MS/MS in WH, C, Endo, Mito and PM isolates from control and mCherry-D4 expressing MA-10 cells.

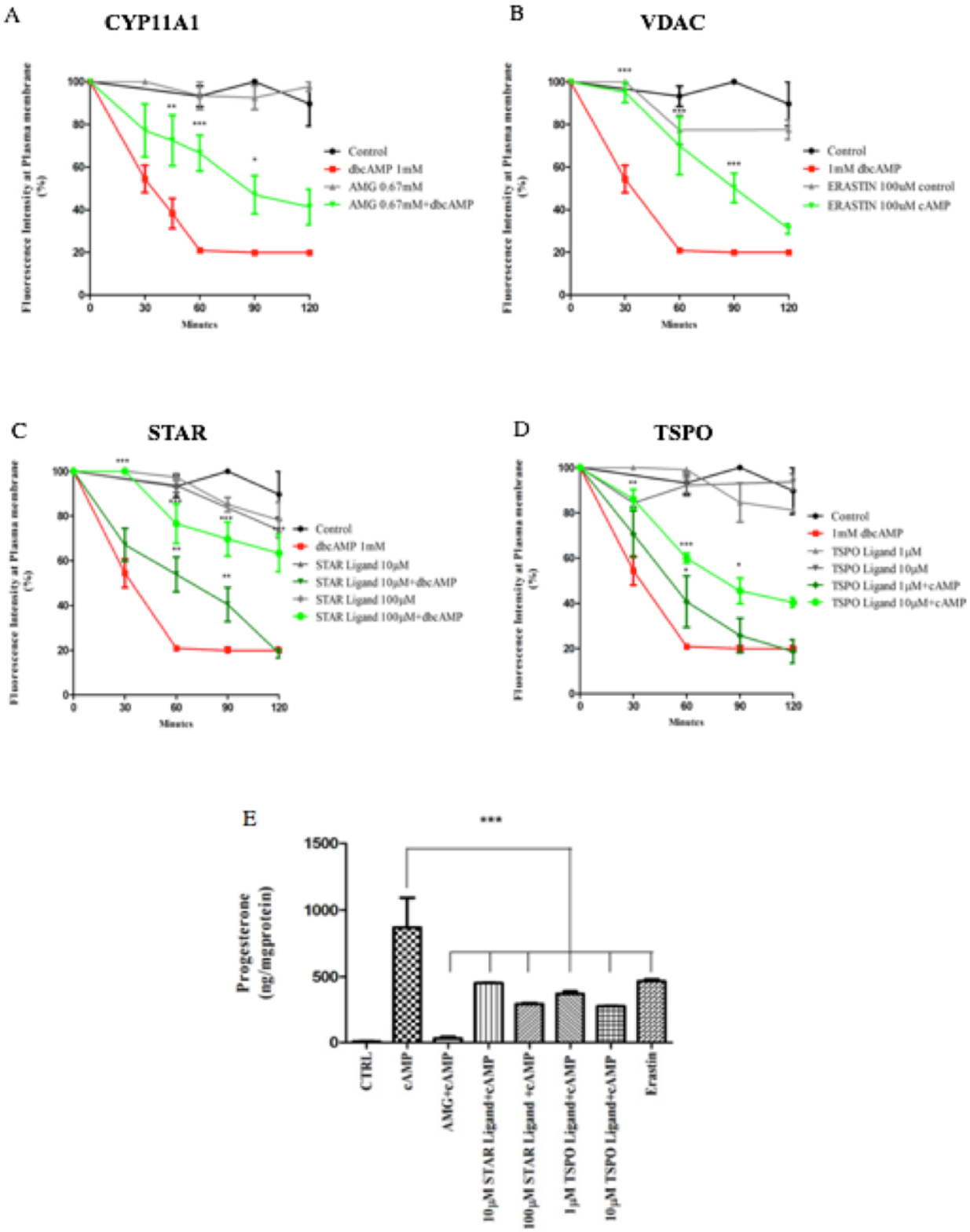
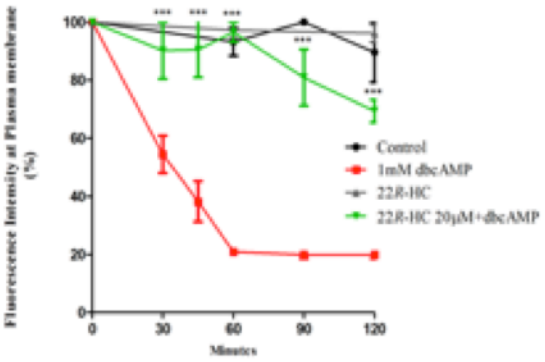

A
CYP11A1
Fluorescence Intensity at Plasma membrane (%)
Minutes
Control
dbcAMP 1mM
AMG 0.67mM
AMG 0.67mM+dbcAMP
B
VDAC
Fluorescence Intensity at Plasma membrane (%)
Minutes
Control
1mM dbcAMP
ERASTIN 100uM control
ERASTIN 100uM cAMP
C
STAR
Fluorescence Intensity at Plasma membrane (%)
Minutes
Control
dbcAMP 1mM
STAR Ligand 10μM
STAR Ligand 10μM+dbcAMP
STAR Ligand 100μM
STAR Ligand 100μM+dbcAMP
D
TSPO
Fluorescence Intensity at Plasma membrane (%)
Minutes
Control
1mM dbcAMP
TSPO Ligand 1μM
TSPO Ligand 10μM
TSPO Ligand 1μM+cAMP
TSPO Ligand 10μM+cAMP
E
Progesterone (ng/mgprotein)
CTRL
cAMP
AMG+cAMP
10μM STAR Ligand+cAMP
100μM STAR Ligand +cAMP
1μM TSPO Ligand+cAMP
10μM TSPO Ligand+cAMP
Erastin

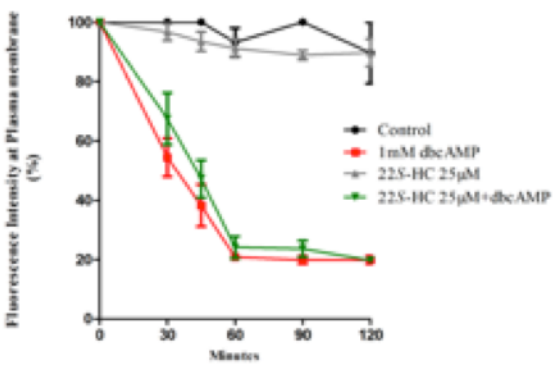
Figure 3. Effect of inhibitors of the proteins associated with the steroidogenic metabolon on cholesterol trafficking from the plasma membrane in MA-10 cells transfected with mCherry-D4. *A-D.* Time course of mCherry-D4 fluorescence associated with the plasma membrane in the presence of various inhibitors in the presence or absence of dbcAMP. *A*, Aminoglutethimide (AMG), an inhibitor of CYP11A1. *B*, Erastin, an inhibitor of VDAC. *C,* Two concentrations of the START domain ligand, an inhibitor of the StAR protein. *D,* Two concentrations of the CRAC domain ligand, an inhibitor of TSPO. *E,* Progesterone production 2 h after exposure of the cells to various inhibitors. (Data represent means ± SD of at least three independent experiments performed in triplicate; two-way ANOVA followed by Bonferroni's post-hoc test (*) or *t*-test (#) were used to calculate statistical significance; $*,^{\#}p < 0.05$, $**,^{\#\#}p < 0.01$, $***,^{\#\#\#}p < 0.001$; CRAC, cholesterol recognition amino acid consensus sequence; CYP11A1, cytochrome P450 side chain cleavage enzyme; StAR, steroidogenic acute regulatory protein; TSPO, translocator protein; VDAC, voltage-dependent anion channel.)

Figure 4

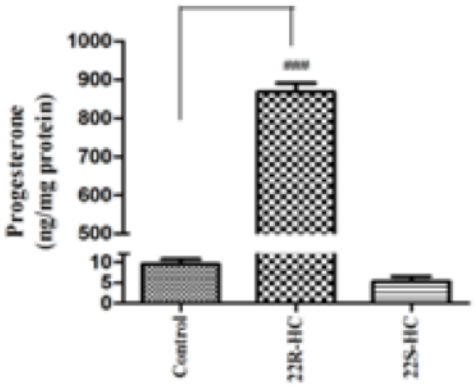
A



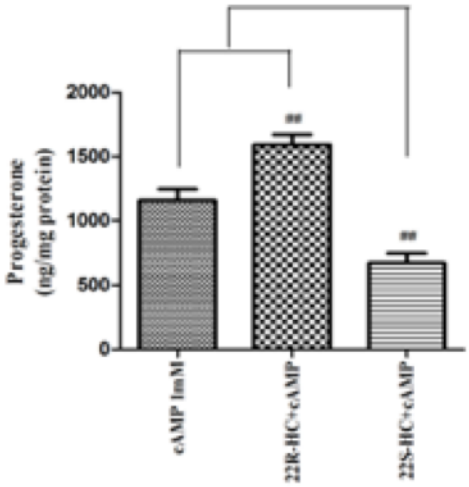
B



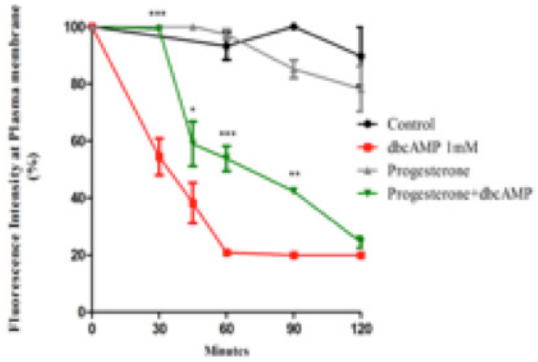
C



D



E



F

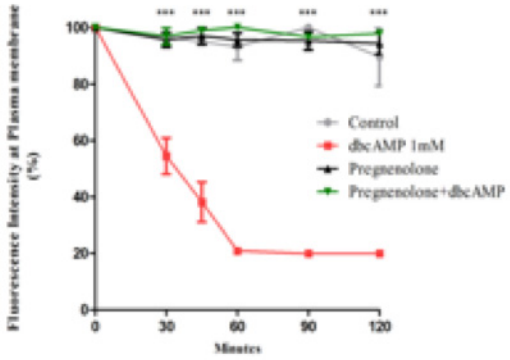


Figure 4. Effect of steroids on trafficking of free cholesterol in mCherry-D4 expressing MA-10 cells. *A and B,* mCherry-D4 fluorescence intensity associated with the plasma membrane in the presence or absence of dbcAMP: *A*, Cells treated with the steroid intermediate 22*R*-HC; *B,* Cells treated with the stereoisomer 22*S*-HC. *C and D,* Progesterone levels in cells 2 h after treatment with 22*S*-HC or 22*R*-HC, either: *C,* without dbcAMP or *D,* with dbcAMP. *E and F,* mCherry-D4 fluorescence intensity associated with the plasma membrane in cells treated with or without dbcAMP and either: *E,* progesterone or *F,* pregnenolone. (Data represent means ± SD of at least three independent experiments performed in triplicate; two-way ANOVA followed by Bonferroni's post-hoc test (*) or *t*-test (#) were used to calculate statistical significance; $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***,\#\#\#}p < 0.001$; 22*R*-HC, 22*R*-hydroxycholesterol; 22*S*-HC, 22*S*-hydroxycholesterol; dbcAMP, dibutyryl cAMP.)

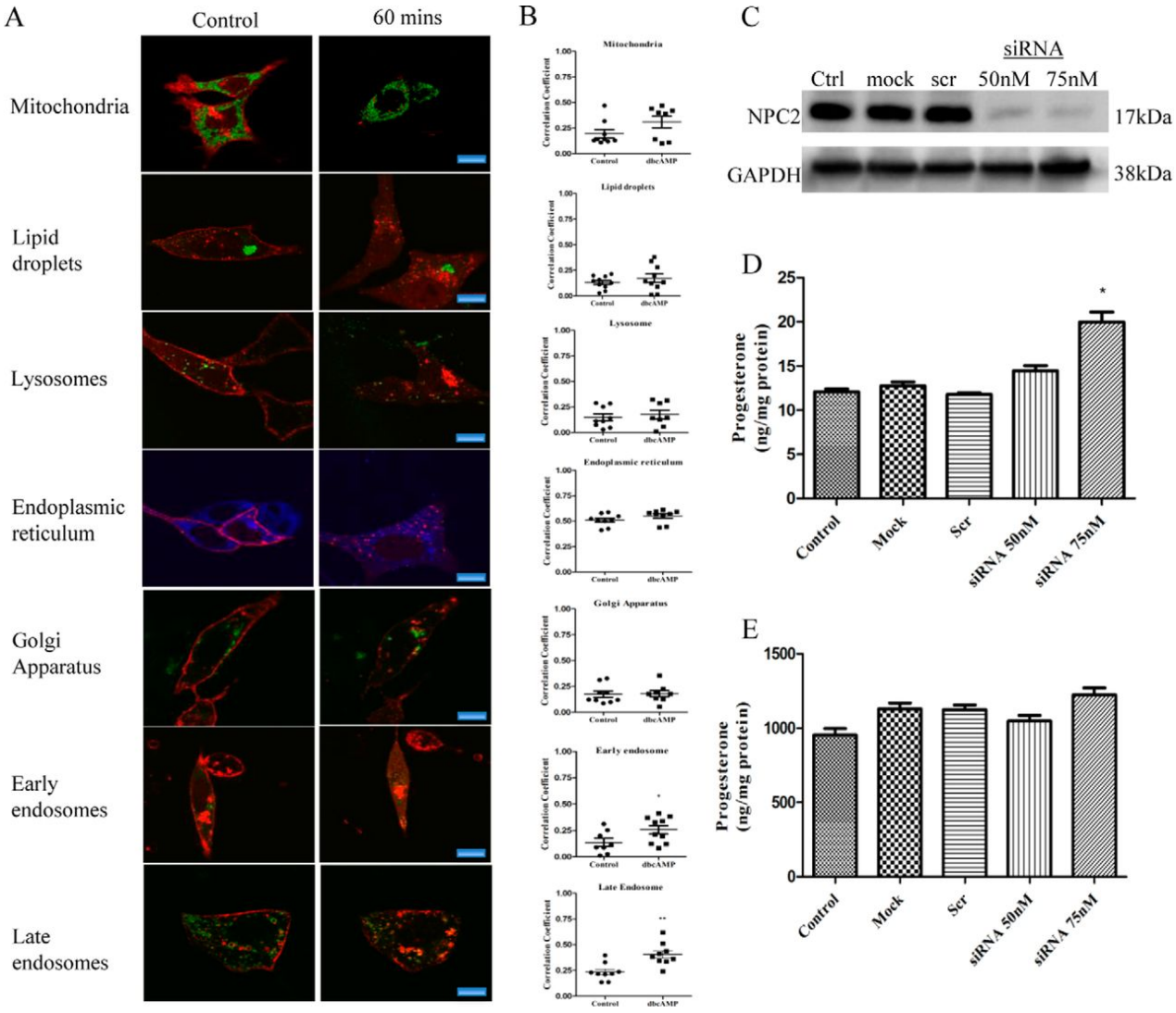

A
Control
60 mins
Mitochondria
Lipid droplets
Lysosomes
Endoplasmic reticulum
Golgi Apparatus
Early endosomes
Late endosomes
B
Mitochondria
Lipid droplets
Lysosome
Endoplasmic reticulum
Golgi Apparatus
Early endosome
Late Endosome
Correlation Coefficient
Control
dbcAMP
C
siRNA
Ctrl
mock
scr
50nM
75nM
NPC2
17kDa
GAPDH
38kDa
D
Progesterone (ng/mg protein)
Control
Mock
Scr
siRNA 50nM
siRNA 75nM
E
Progesterone (ng/mg protein)
Control
Mock
Scr
siRNA 50nM
siRNA 75nM

Figure 5. Identification of organelles involved in cholesterol trafficking during steroid biosynthesis in mCherry-D4-expressing MA-10 cells. Co-localization of mCherry-D4 with organelles was assessed with appropriate fluorescent markers in living cells. *A,* images of the fluorescence staining results. *1st column,* untreated cells; *2nd column,* Bt_2cAMP (*dbcAMP*)-treated cells. *Scale bar,* 10 μm. *B,* correlation coefficients between images and organelles based on quantification of co-localization of images with mCherry-D4, using ImageJ with the plug-in co-localization using Pearson's correlation coefficient. Data represent means ± S.D. (n= 9 cells per group). *, $p <$ 0.05. *dbcAMP*, dibutyryl cAMP. *C,* 72-h post-transfection with siRNA targeting *NPC-2* resulted in reduced levels of NPC-2 levels, confirmed by immunoblotting with anti-NPC-2 (1:1000, Santa Cruz Biotechnology), and GAPDH was used as control using anti-GAPDH (1:1000, Trevigen). *D* and *E,* to assess the role NPC-2 in acute steroidogenesis, NPC-2 knocked down MA-10 cells were treated with 1 mM Bt_2cAMP for 2 h and progesterone production was measured by RIA before and after stimulation. Statistical analysis was performed using Student's *t* test. *, $p < 0.05$.

Figure 6

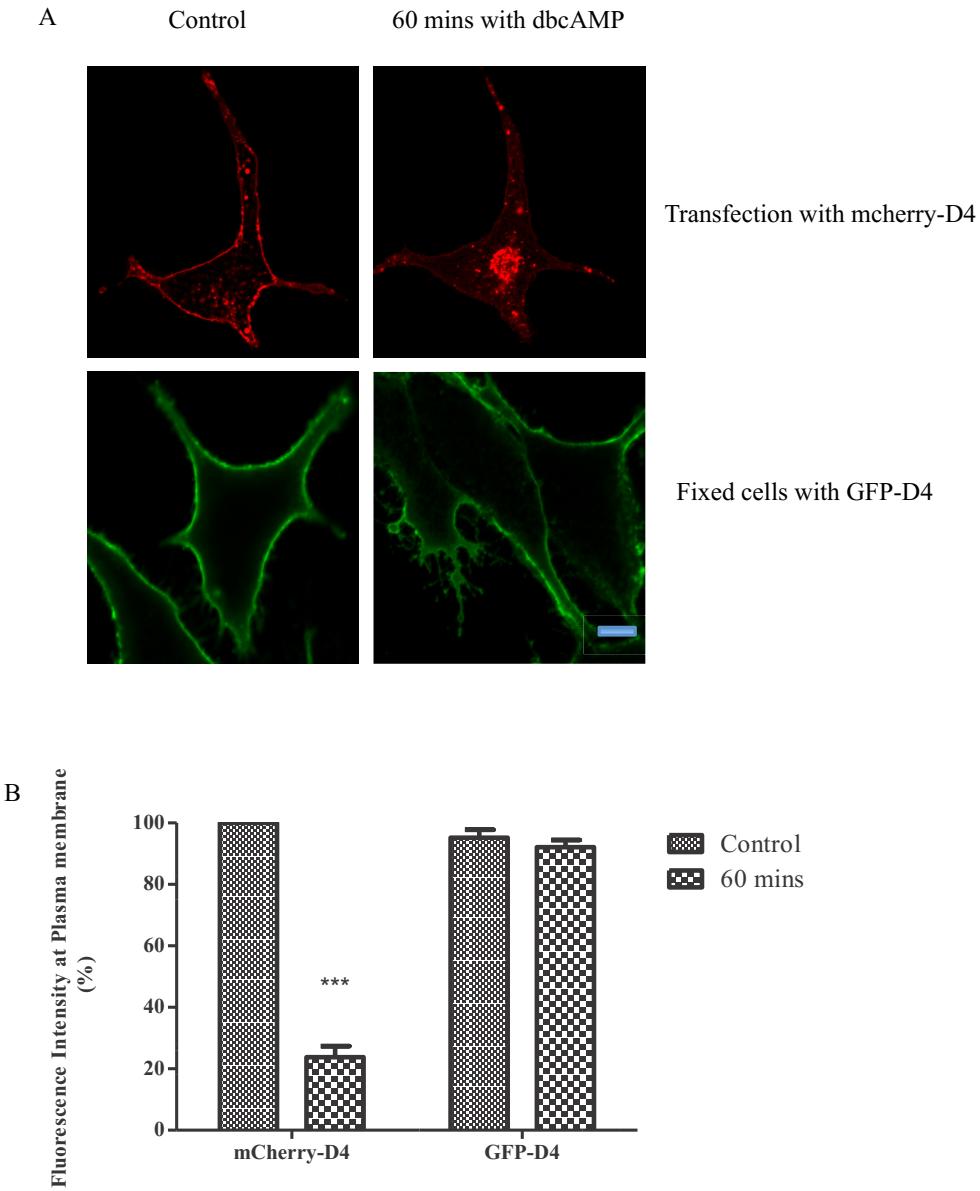

A
Control
60 mins with dbcAMP
Transfection with mcherry-D4
Fixed cells with GFP-D4
B
Fluorescence Intensity at Plasma membrane (%)
100
80
60
40
20
0
***
mCherry-D4
GFP-D4
Control
60 mins

Figure 6. No change in the plasma membrane outer leaflet cholesterol of mCherry-D4 expressing MA-10 cells with and without dbcAMP treatment *A and B,* Cells were fixed with paraformaldehyde and treated with recombinant EGFP-D4 to assess binding to the outer leaflet of the plasma membrane. Where a decrease in the inner leaflet binding mCherry-D4 was noticed, no change in the binding of recombinant EGFP-D4 was observed, Scale bar = 10μm.

Figure 7

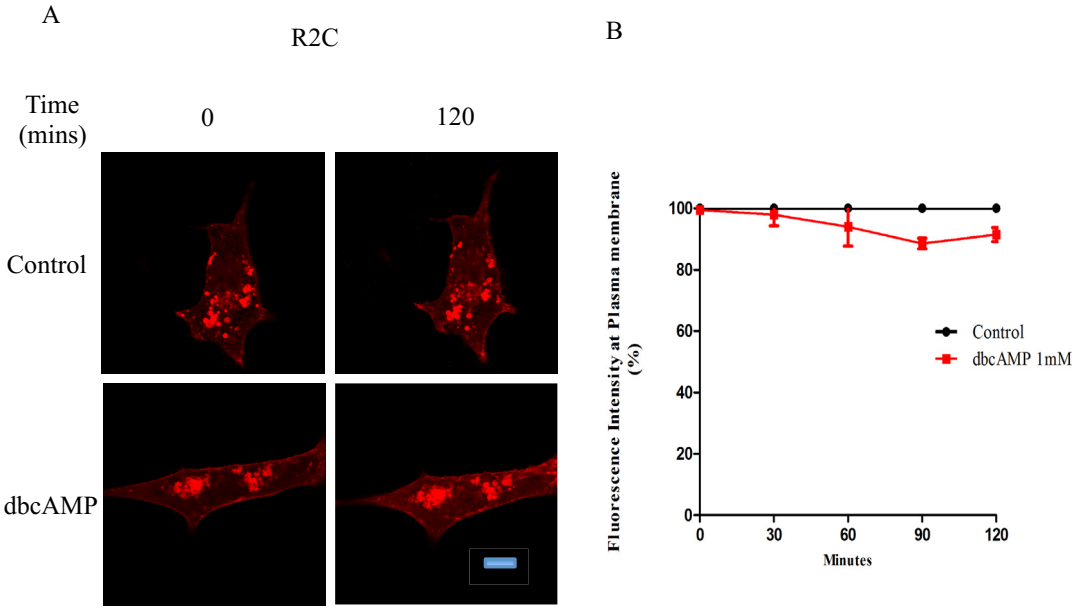

A
R2C
Time (mins)
0
120
Control
dbcAMP
B
Fluorescence Intensity at Plasma membrane (%)
100
80
60
40
20
0
0
30
60
90
120
Minutes
Control
dbcAMP 1mM

Figure 7. Movement of free cholesterol in mCherry-D4 transfected R2C cells, which constitutively synthesize steroids. *A,* Confocal microscope images of cells with and without treatment with dbcAMP, Scale bar = 10μm. *B,* Time course of mCherry-D4 fluorescence intensity associated with the plasma membrane. *C,* Progesterone production measured in control and mCherry-D4 transfected cells, before and after 2 hour dbcAMP treatment. (Data represent means ± SD of at least three independent experiments performed in triplicate; two-way ANOVA followed by Bonferroni's post-hoc test (*) were used to calculate statistical significance; *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$; dbcAMP, dibutyryl cAMP).

Figure 8

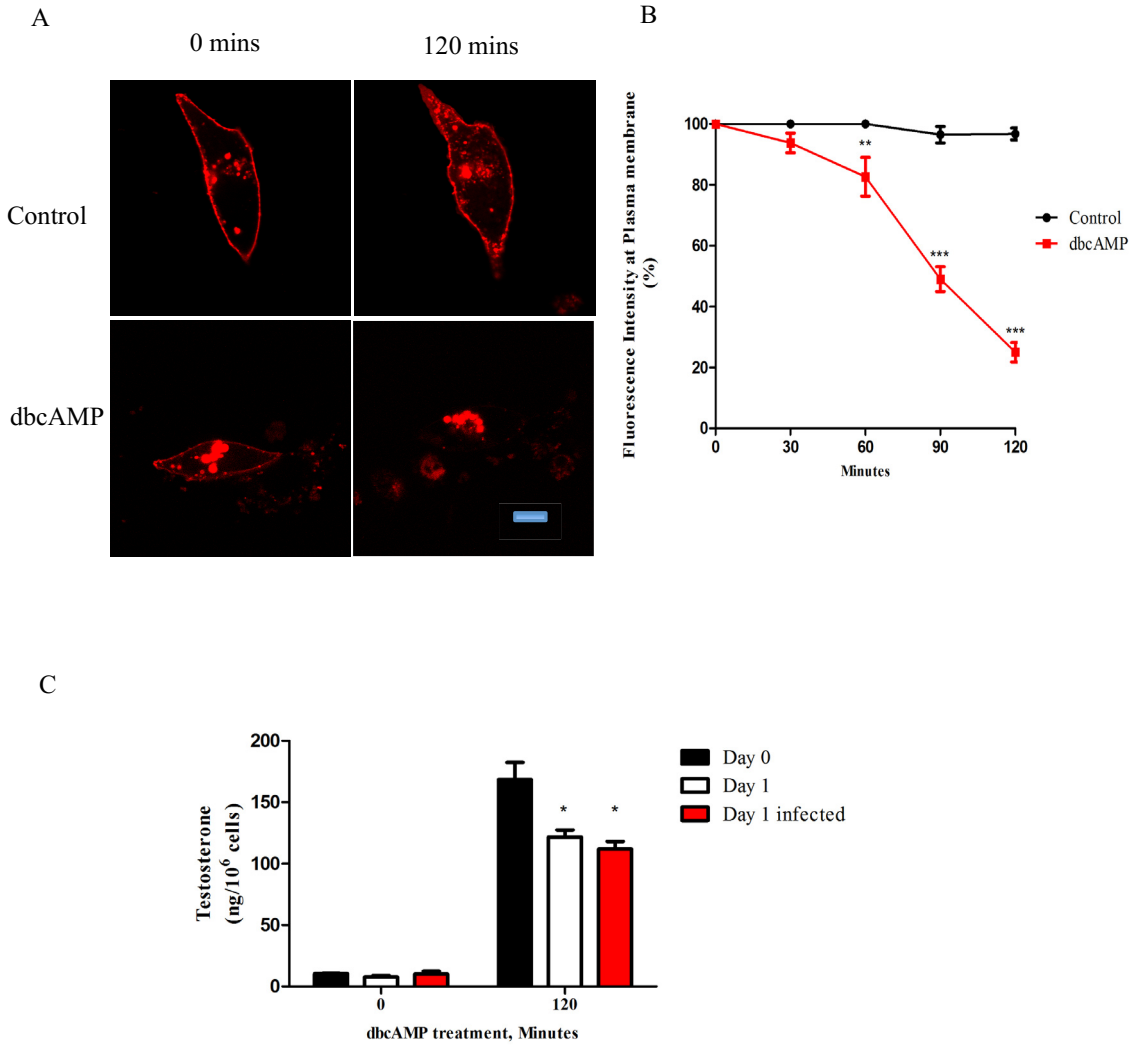

A
0 mins
120 mins
Control
dbcAMP
B
Fluorescence Intensity at Plasma membrane (%)
100
80
60
40
20
0
0
30
60
90
120
Minutes
**
***
***
Control
dbcAMP
C
Testosterone (ng/10^6 cells)
200
150
100
50
0
0
120
dbcAMP treatment, Minutes
*
*
Day 0
Day 1
Day 1 infected

Figure 8. Cholesterol trafficking analysis in primary rat Leydig cells infected with lentivirus containing mCherry-D4. *A,* Confocal microscope images of cells with and without addition of dbcAMP, Scale bar = 10 μm. *B,* Time course of mCherry-D4 fluorescence intensity at the plasma membrane of control and dbcAMP-treated cells. *C*, Testosterone content of control cells and cells infected with lentivirus before and after 2-h treatment with dbcAMP. Testosterone was measured just after the cells were isolated (0 h), in control (uninfected) cells 18 h after incubation and 18 h after infection. (Data represent means ± SD of at least three independent experiments performed in triplicate; two-way ANOVA followed by Bonferroni's post-hoc test (*) or *t*-test (#) were used to calculate statistical significance;*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$; dbcAMP, dibutyryl cAMP).

Figure 9

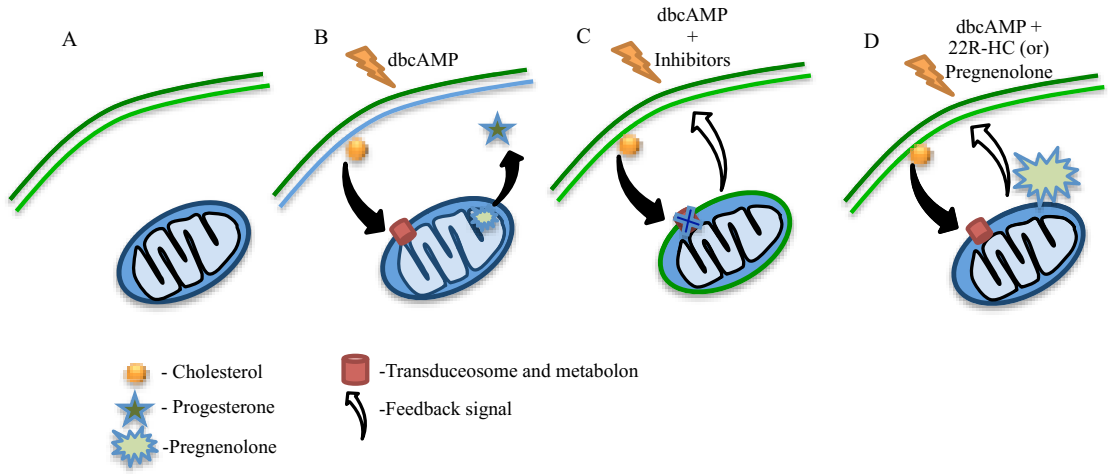

A
B
dbcAMP
C
dbcAMP
+
Inhibitors
D
dbcAMP +
22R-HC (or)
Pregnenolone
- Cholesterol
- Progesterone
-Pregnenolone
-Transduceosome and metabolon
-Feedback signal

Figure 9. Proposed model for cholesterol movement from PM to the mitochondria in Leydig cells during acute steroid formation. *A,* under basal conditions, cholesterol is enriched in both leaflets of the plasma membrane, while the mitochondrial membranes have little cholesterol. *B,* After 30-45 min of acute stimulation with dbcAMP, cholesterol is trafficked from the plasma membrane to the mitochondria, where a transduceosome and metabolon complex translocates cholesterol from the OMM into the IMM and enzymatically converts free cholesterol to pregnenolone. Pregnenolone further undergoes multiple enzymatic reactions and is converted to progesterone. *C,* Inhibition of the protein components in the transduceosome and the metabolon, initiates a negative feedback signal that obstructs the cholesterol release from the inner leaflet of the plasma membrane. *D,* the presence of pregnenolone, progesterone, and the steroid intermediate 22*R*-HC in the system initiates a similar negative feedback signal, inhibiting the trafficking of cholesterol from the plasma membrane to the mitochondria. [22*R*-HC, 22*R*-hydroxycholesterol; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.]

Preface to Chapter III

During hormone induced acute steroid biosynthesis, large quantities of cholesterol from intracellular stores are trafficked to the mitochondria. The precise location from which this cholesterol is mobilized within seconds if not minutes of hormone stimulation remained uncertain. Results from chapter II, utilizing mCherry-D4 to track cholesterol movement in the cell, led us to believe that the bulk of the steroidogenic pool of cholesterol comes from the plasma membrane. This phenomenon was seen in both MA-10 mouse tumor Leydig cells and also primary rat Leydig cells.

From plasma membrane the hydrophobic cholesterol would need a medium to traverse through the aqueous milieu to reach the mitochondria. Previous studies in our lab indicate an increased juxtaposition between ER and mitochondria (MAMs) during steroidogenesis (105), leading us to hypothesize membrane interactions between plasma membrane-ER-mitochondria (PAMs) to be the route for cholesterol trafficking during steroidogenesis. In chapter III we isolated subcellular fractions in an attempt to map this route of cholesterol trafficking between organelles during steroidogenesis and also study other lipid involvements in this process.

Chapter III

Manuscript II

Dynamic subcellular lipid organization in hormone induced MA-10 mouse tumor Leydig cells.

Sathvika Venugopal[1,2], Issop Leeyah[1,2], Chan Rachel[1], Sanyal Esha[1], Taylor Lorne[1], Kaur Pushwinder[1], Daly Edward[1], Vassilios Papadopoulos[1,2,3]

[1]Research Institute of the McGill University Health Centre

[2]Department of Medicine, McGill University, Montreal, Quebec, H4A 3J1, Canada; [3]Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, USA

To whom correspondence should be addressed: Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, USA; e-mail: vpapadop@usc.edu

Abstract

Lipids play an essential role in numerous cellular processes, including membrane remodeling, signal transduction, mediating hormone action, and steroidogenesis. Detailed insight into the function of lipids in steroidogenesis, however, requires an understanding of the subcellular organelle localization of individual lipids. We chose steroidogenic MA-10 mouse tumor Leydig cells to study the relationship of subcellular lipid localization and steroidogenesis because of their ability to rapidly produce progesterone in a hormone-inducible manner. Mitochondria, endoplasmic reticulum (ER), cytoplasm, plasma membrane (PM), PM-associated membranes (PAMs) and mitochondria associated membranes (MAMs) were isolated from MA-10 cells in basal, hormone stimulated, and hormone stimulated with steroidogenesis inhibited states. Lipidomic analyses identified 569 individual/isobaric species, including glycerophospholipids, sphingolipids, and cholesterol and its esters. Each isolated subcellular organelle membrane had a unique lipid composition, and hormone induction of steroidogenesis caused a significant remodeling of the subcellular lipidome. A variety of dbcAMP-induced changes in lipid composition were found, including a substantial increase in phosphatidylinositol and ceramides levels in PAM and PM compartments, a drastic decrease in cholesterol ester levels in the cytoplasm, ER and whole cell lipid extracts, and a dramatic reorganization of phosphatidyl serine and ethanolamine, coupled with increased phosphatidic acid and cardiolipins synthesis. Abundant lipids such as phosphatidylcholine and sphingolipids were not affected by the various treatments.

The observed cAMP-induced dynamic changes in MA-10 cell subcellular membrane lipidome suggest that hormone-induced acute steroid hormone production is a process that

involves extensive organelle remodeling. This study is one of the first to analyze lipid re-organization during steroidogenesis.

Introduction

The fundamental structures of eukaryotic cells are the organelles compartmentalized within membranes. These membranes create a biochemical microenvironment specific to the organelle. A number of essential biological processes take place at or in these membrane structures. Much of the research in the past few decades has attributed the biological processes specific to each organelle to the distinct composition of proteins in each organelle membrane. For instance, the plasma membrane is rich in receptor proteins enabling signal transduction or nutrient uptake (285), while the mitochondrial membrane is rich in proteins involved in carrying out electron transport chain reactions or cholesterol trafficking for steroid biosynthesis (152). But increasing evidence points out that, in addition to having a distinct protein composition, membranes also have their own distinct lipid compositions (286). These compositions determine membrane properties and influence membrane protein localization and their function, in turn influencing the role the organelles play in the cell (43,287,288). Hence, only a comprehensive understanding of the dynamic interplay between the membrane lipidome and membrane associated proteins, can give reasonable information about the biological process being investigated in any organelle.

Traditionally, lipid analysis was carried out by extraction of lipids from whole cells or tissues, followed by lipid separation and measuring the lipid classes and their subspecies. However, this eliminated all information about the subcellular location of these lipids and how lipids assemblies were organized. To truly get complete information on biochemical reactions

happening at the subcellular level, it is essential to determine the location and assemblies of lipids by combining subcellular fractionation and advanced mass spectrometry analysis of lipid species. In this study we focused on the subcellular lipidomics of cells involved in steroidogenesis and illuminated the dynamic interplay of lipids and proteins in multiple organelles for the purpose of trafficking cholesterol to the mitochondria for the synthesis of steroids.

Steroids are vital for development, reproduction and behavior. Synthesis of steroids involves a well-regulated multistep process that effectively meets physiological needs in a tissue specific manner (1). Mitochondria are considered the first sites for steroid synthesis, where free cholesterol from intracellular stores destined to be converted to steroids is trafficked to the outer mitochondrial membrane (OMM), whereupon a multitude of proteins come into contact with each other and form a complex called the steroidogenic metabolon to transfer cholesterol from the OMM to the inner mitochondrial membrane (IMM) (75) where the cytochrome P450 side chain cleavage enzyme CYP11A1, with its cofactors ferredoxin reductase (FdxR) and ferredoxin (Fdx), convert cholesterol to the first of the steroids, pregnenolone (80).

During acute steroid biosynthesis large quantities of cholesterol must be trafficked to the cholesterol-poor mitochondria. Although lipid droplets rich in cholesterol have been considered the classic source for mitochondrial steroid production, recent studies have focused interest on the Endoplasmic Reticulum (ER) as a possible source of cholesterol (105). The ER is a complex subcellular organelle running throughout the cell and has many roles to play, including a broad array of lipid and protein biosynthesis (213). Although ER is poor in cholesterol like the

mitochondria, it is the site for *de novo* cholesterol synthesis, which together with cholesterol obtained from circulating lipoproteins, if not used, eventually will be esterified and incorporated into the lipid droplets (1). The ER is also considered the sterol regulatory machinery of the cell, as it houses the sterol regulatory element-binding protein (SREBP) that senses the cholesterol levels in the ER and regulates the transcription of multiple genes involved in the synthesis of cholesterol and other fatty acids (214). Recent electron microscopic studies have suggested a direct organelle-organelle interaction between the mitochondria and ER upon hormonal stimulation in steroidogenic cells (105). The site of association between the two organelles is thought to be formed by microdomains rich in cholesterol and has been termed Mitochondrial Associated Membranes (MAM) (289). During acute steroid biosynthesis this site represents about 5-20% of the mitochondrial surface and has been shown to possess unique lipid and protein compositions (80). Interestingly, multiple components of the transduceosome complex are located in MAMs, suggesting that MAMs play a key role in cholesterol trafficking.

The Plasma Membrane (PM) is another organelle considered a potential source of cholesterol for acute steroid biosynthesis. The first evidence that the PM could be a potential source of cholesterol for steroid production came from multiple studies done by Freeman and co-workers (232-235,249). This group had previously shown the movement of cholesterol from the PM in multiple steroidogenic cell lines, including Leydig and adrenal cells. Another recent study done using fluorescent-tagged domain 4 of the Θ toxin (D4) from *Clostridium perfringens,* which has the ability to bind cholesterol in membranes with high affinity, showed an increased movement of cholesterol from the PM during hormonally stimulated steroidogenesis in mouse tumor Leydig (MA-10) cells (106), thus further validating the role of PM in steroid biosynthesis

and leading us to hypothesize that cholesterol from the PM may respond to hormonal stimulation and traffic cholesterol by interacting with the ER, which in turn interacts the mitochondria through MAM formation, delivering cholesterol via the steroidogenic metabolon to the IMM for steroid formation. This association between PM–ER-mitochondria via microdomains in these organelles has been termed Plasma membrane Associated Membrane (PAM).

In this study we analyze three major lipid classes and their reorganization in isolated subcellular organelles and PAM and MAM microdomains from MA-10 cells in basal, hormonally stimulated and hormonally stimulated while inhibiting steroidogenesis with cycloheximide. These lipid classes known to play essential roles in membrane structure and function were analyzed by mass spectrometry analysis. We found a dynamic reorganization of lipids in the membranes at the subcellular level upon activation of steroidogenesis.

Experimental procedure

Cell culture **-** Cells from mouse Leydig cell tumor (MA-10, kindly provided by Dr. M. Ascoli, University of Iowa, Iowa City, IA, USA), were cultured with Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 (Invitrogen) with 1% penicillin and 1% streptomycin and maintained at 37°C. The medium for MA-10 was supplemented with 5% fetal bovine serum (FBS) and 2.5% heat-inactivated horse serum. For time courses, the cells were incubated with DMEM/Ham's F-12 without any serum supplementation in the presence of appropriate drug treatments.

Small interfering RNA transfection and live cell imaging- Cells were plated onto 6-well plates at an initial concentration of 3×10^5 cells per well, and immediately transfected using a TriFECTa kit DsiRNA duplex (Integrated DNA Technologies) and Lipofectamine RNAiMAX (Life Technologies). The following small interfering RNA (siRNA) duplexes (150nM) were used for *Atad3* (NM_179203): duplex 1, 5′-CCAUCGCAACAAGAAAUACCAAGAA-3′; duplex 2, 5′-CCAGUUUGACUAUGGAAAGAAAUGC-3′; and duplex 3, 5′-AGGACAAAUGGAGCAACUUCGACCC-3′. Gene expression and target gene knockdown were evaluated by qRT-PCR. After 48 hours, transfected cells were plated onto a 35 mm diameter fluorodish culture dish (World Precision Instruments) that had been pretreated with 0.1% gelatin and incubated at 37°C for 24 h. At 75% confluence, the cells were transiently transfected with 2 μg of mCherry-D4 plasmid using 4-μL jetPRIME reagent (Polyplus transfection) and 200 μL of jetPRIME buffer. Cells were visualized at room temperature using a scanning laser confocal microscope (FluoView^{TM} FV1000; Olympus Corp.) at 100x with an oil immersion objective (UPLSAP). FluoView software (version 3.1) was used to capture the images; quantification and image processing were done with Image-Pro Plus (version 6.3) and ImageJ version 1.47 (http://rsbweb.nih.gov/ij). Statistical analysis of the quantitative data was performed using Prism version 5.0 (GraphPad Software).

Electron microscopy- MA-10 cells cultured in in 6-well plates were grown for 48h, followed by treatment with 1mM dbcAMP at different time points (0, 15,30 60 and 120 min). The cells were washed once in a sodium cacodylate buffer (0.1M, pH7.4) (Mecalab, Montreal, Quebec, Canada) and gently scraped. After centrifugation (1500 g, 5 min), the pellet was fixed with 2.5% glutaraldehyde (Sigma-Aldrich, St-Louis, MO, USA) overnight at 4^{o}C. After 3 washes with

dH_2O, cells were stained with OsO_4 1% Osmium (Mecalab), for 2 hours at 4oC. The stained cells were then dehydrated by incubation in increasing acetone concentrations from 30 to 100%. After an infiltration step with different mixes of acetone-epon (1:1, 1:2 1:1:3 vol/vol), the samples were embedded in pure Epon (Mecalab, Montreal, Quebec, Canada). Polymerization was performed by incubation at 60°C for 48h. Blocks were cut in slices of 100nm, collected on 200 mesh copper grids (Electron Microscopy Sciences, Fort Washington, PA, USA) and post-stained with 4% uranyl acetate for 5 min, then with Reynold's lead for 5 min. Cells on grids were observed with a transmission electron microscope FEI TECNAI 12 operated at 120KV. Images were collected on a CCD Camera (AMT XR 80 C) at the McGill University electron microscopy facility.

Organelles, PAM and MAM isolation- PAMs along with PM and mitochondrial membranes were isolated from MA-10 cells following the protocol published by Suski et al with modifications (290). MA-10 cells were grown in 150 mm dishes to 80% confluence. Cells were washed with PBS, then incubated with serum free media with or without 1mM dbcAMP or 1mM dbcAMP with 0.2mM cycloheximide for 2 hours; after which, cells were washed again with PBS and harvested using cell scrapers. The cells were then centrifuged twice at 600*g* for 5 minutes at 4°C. The pellets were resuspended in 10 ml ice-cold isolation buffer 1 (225mM mannitol, 75mM sucrose, 0.1mM EDTA, and 30mM Tris-HCl pH 7.4). The cells were homogenized using a Teflon pestle attached to a motorized overhead stirrer run at 4,500 rpm, and the integrity of the cells were checked every 25 strokes. Once 90% cell damage was observed, the homogenates were centrifuged twice at 600*g* for 5 minutes at 4°C. The supernatant was taken and centrifuged at 7000*g* for 10 minutes at 4°C. The pellet consisted of crude mitochondrial fractions, which was

resuspended in MRB buffer (250mM mannitol, 5mM HEPES (pH 7.4), 0.5mM EGTA) and layered on Percoll solution (225mM mannitol, 25mM HEPES (pH 7.4), 1mM EGTA and 30% Percoll (vol/vol)) followed by ultracentrifugation at 95,000xg for 30 minutes, yielding crude MAM and mitochondria fractions. The samples were further purified by centrifugation at 630xg for 10 minutes and further ultracentrifugation at 95,000Ig for 60 minutes, yielding pure MAM and mitochondrial fractions. The purity of the sample was verified using immunoblotting with anti-acyl-coenzyme synthetase long-chain family member 4 (ACSL4) (Abcam), anti-cytochrome c oxidase subunit IV (COX IV) (Santa Cruz biotechnology) and anti-voltage-dependent anion channel (VDAC) (Abcam).

The supernatant contained PM, PAM, microsomes, ER, and cytosolic proteins. To isolate ER membranes, the supernatant was centrifuged at 20 000*g* for 30 minutes at 4°C. Further centrifugation of the supernatant (100,000*x*g for 1 hour) resulted in the isolation of the ER (pellet) and cytosolic fraction (supernatant). ER membranes were resuspended in isolation buffer 1, and its purity was checked by immunoblotting with calreticulin antibody (Abcam). The PAM and PM fractions were isolated by laying the samples on the 4ml 38%, 4ml 43%, 3ml 53% sucrose gradient and centrifuged at 95,000xg for 2hours 30minutes at 4ºC. Once collected the samples are further centrifuged at 95,000*x*g for 1 hour at 4ºC to obtain pure PAM and PM pellets that were resuspended in 50µl isolation buffer 2 (225mM mannitol, 75mM sucrose and 30mM Tris-HCl (pH 7.4)). The enrichment of the samples was verified by immunoblots using anti-TRPC3/6/7 (Santa Cruz biotechnology) for the PM fraction and anti-ACSL4 (Abcam) and anti-ORAI1 (Santa Cruz biotechnology) for the PAM fraction.

Lipid extraction and thin layer chromatography- Lipids from isolated membranes were extracted using the Bligh-Dyer method, where samples were diluted to 1ml with NaCl (154mM) and dissolved in chloroform-methanol 1:2 (v/v), vortexed for 1 minute and then kept at room temperature (RT) for 10 minutes. Chloroform-NaCl (154mM) 1:1 (v/v) was then added to the samples, vortexed and centrifuged at 1800 rpm for 5 minutes at RT. The bottom phase containing the lipids was extracted using a Pasteur pipette and dried by subjecting to nitrogen gas. The lipids were then re-suspended in chloroform-methanol 1:1(v/v). The samples were then loaded as a 0.8 cm band onto a chloroform-methanol 1:1 (v/v) prewashed and heat activated (100^{o}C for 1 hour) HPTLC plate (silica gel 60 10 x 10 cm, EMD Millipore Corporation, MA, USA). The lipid standards, 10 µg cholesteryl ester 17:0 and 10µg cholesterol (Avanti Polar Lipids, AL, USA), were added in separate lanes. The plate was developed in a Hexane: diethyl ether: acetic acid (70:30:1) mobile phase. The plate was then sprayed with primuline stain (Sigma-Aldrich, USA). The spots were visualized under UV light and quantified with a BAS 5000 image analyzer (Fuji Film Inc., Tokyo, Japan).

Cholesterol analysis using AB Sciex triple -TOF Mass spectrometer (TOF) - To quantify cholesterol from sub-cellular organelle fractions, the fractions were spiked with d6-cholesterol internal standard (IS). Samples were extracted using 2 x 1 ml methyl tert-butyl ether (MTBE). Aliquots were combined in 10 x 13 mm test tubes, dried in a Thermo Speed Vac™ for 20 minutes, re-dissolved in 500-µl aliquot of MTBE, vortexed and dried again ~ 10 min. Samples were re-suspended in 200 µL of 50% (aq.) methanol and vortexed. The contents were transferred in autosampler vials (Thermo Fisher Scientific) and were stored at -20^{o}C until ready for liquid chromatography-mass spectroscopy (LC-MS) analysis. All measurements were made by AB

Sciex 5600+ triple-TOF Mass Spectrometer coupled with a Shimadzu Nexera XR UHPLC system. A binary mobile phase consisting of (A) water w/ 0.1 % formic acid and (B) acetonitrile w/ 0.1 % formic acid and acid was utilized. A 2µl aliquot of sample was injected into the LC. Analytes were chromatographically resolved by isocratic elution (90% (B) at 350 µL/min) with an Agilent Eclipse Plus C-8 analytical column (50mm X 2.1mm ID x 1.8 µm particle). The total run time was 10 minutes. The mass spectrometer was operated in a positive HESI mode with a vaporization temperature of 550 °C and a spray voltage of 5.5 kV. For quantification purposes, a calibration curve was run along with the samples. After data acquisition, the intensity of each ion was integrated using MultiQuant™ software.

Lipidomics analysis using Hybrid quadruple Time-of-flight (TOF)- Lipid extracts from the subcellular fractions were analyzed by flow injection analysis. Approximately 100 µL of a 10-fold diluted lipid extract in 5 mM ammonium acetate in chloroform: methanol (1:2, v/v) was delivered to the source by isocratic flow at 20 µL/min of methanol: isopropanol (3:1, v/v) with 5 mM ammonium acetate using a Shimadzu Prominence XR UFPLC autosampler and isocratic pump (Shimadzu Corporation). A second isocratic pump was used to deliver a solution of 98% isopropanol and 2% methanol containing 5 mM ammonium acetate as a make-up flow to the source through a T-junction at a rate of 30 µL/min.

Total flow was approximately 50 µL/min at point of entry into the DuoSpray® source through the ESI probe. Positive mode ion spray voltage was set at 5000 and negative mode ion spray voltage at −4000, declustering potential at 40 V, and ESI source operating temperature was set at 300 °C. An atmospheric-pressure chemical ionization (APCI) probe and inlet were connected to

a calibrant pump that delivered mass calibration solution. Each sample was injected twice to complete a positive mode and a negative mode experiment back-to-back upon polarity switching, based on the instrument manufacturer instructions. Positive and negative ion MS and sequential precursor ion fragmentation acquisitions were carried out on a TripleTOF™ 5600 System (AB SCIEX, Concord, ON) controlled with Analyst® TF 1.5.1 software with MS/MSALL mode activated to carry out a series of product ion scans. MS experiments were carried out from m/z 200–1,200 at an accumulation time of 300 ms, followed by 1,000 product ion experiments with 1,000 precursors evenly spaced from m/z 200.051 to m/z 1,200.051, measuring across m/z 100–1,500, accumulated for 300 ms each, and collected in order from low to high m/z. The total time to carry out one MS/MSALL acquisition was 5.48 minutes. The acquired TOF MS and MS/MS_{ALL} data were processed with LipidView™ 1.1 software.

Results

PM-ER-mitochondria communication increases upon hormone treatment in MA-10 cells.

To examine the association between PM-ER and ER-mitochondria during steroidogenesis, we performed EM studies using MA-10 cells treated with or without dbcAMP for 15, 30, 60, 90 and 120 minutes (Fig. 1A, B, C). The blue circles in the EM image (Fig. 1A.) represent the association between PM-ER and ER-mitochondria, previously termed PAMs (245). We observed a significant increase in the interaction between the PM and ER within 15 minutes of treatment with 1mM dbcAMP and a stable increase up to 120 minutes after stimulation when compared to control cells (Fig. 1B). This increase correlated positively with increased and progressive association between mitochondria and ER in a time dependent manner (Fig. 1C), thus suggesting

that not only MAMs but also PAMs may play a role in trafficking cholesterol to the mitochondria during steroidogenesis.

ATAD3 is a key protein involved in the formation of MAMs and cholesterol trafficking into the mitochondria during steroid biosynthesis (105). Free cholesterol movement was tracked after siRNA-induced knockdown of ATAD3 during steroidogenesis in MA-10 cells by transfecting them with the mCherry-D4 plasmid and visualizing the fluorescent D4 protein with scanning confocal microscopy, following the methodology of previously published data (106). In untreated cells, the fluorescent protein was predominantly bound to the PM. When the MA-10 cells were treated with dbcAMP, fluorescent intensity at the PM began to decline within 30 min, continued to decline over the next 30 min, and remained constant and low after that, while there was minimal change in untreated control cells (Fig. 1D). In ATAD3 knocked down MA-10 cells, in the presence of 1 mM dbcAMP (Fig. 1D), the rapid decline in mCherry-D4 fluorescence that occurred at the PM with dbcAMP alone was partially prevented, indicating that cholesterol was retained in the PM, with a maximal significant effect at 60 min. Thus, by decreasing the availability of ATAD3 for MAM formation, the cholesterol at the plasma membrane was stymied, suggesting that the PAM association could be the route for free cholesterol trafficking from the PM to the mitochondria for steroidogenesis (Fig. 1E).

Isolation and analysis of subcellular fractions in MA-10 cells

To investigate the distribution of cholesterol and other lipids in steroidogenic MA-10 cells, we first purified subcellular membrane fractions from 1 billion MA-10 cells each for control, treated with 1mM dbcAMP for 2 hours, and 1mM dbcAMP with 0.2mM cycloheximide for 2 hours, using a modified protocol for cultured cells previously published by Suski and colleagues

and combining it with a protocol published by Wieckowski and colleagues (245,291). A schematic representation of the various differential centrifugation and gradient centrifugation steps involved in PAM and MAM isolation, along with PM, ER, mitochondria and cytoplasm, is shown in Fig 2A, B. Cytoplasm and whole cell homogenates were used as negative and positive controls, respectively, for this analysis. To achieve MAM and PAM quantities sufficient for protein estimation studies, immunoblot analysis and lipidomics analysis, we used as raw material for this fractionation about 1 billion cells, which were subjected to multiple differential centrifugation processes as explained in Fig. 2A to achieve crude PM, PAM, mitochondria, MAM, ER, and cytosol fractions. Further PM and PAM samples were subjected to sucrose gradient (38%, 43%, 53%) ultracentrifugation that separates PAM from the PM (Fig. 2B). Percoll gradient ultracentrifugation was used to isolate MAMs from mitochondria. Using the supernatant from the first centrifugation series (Fig. 2A), an additional ultracentrifugation step was performed to separate the ER from the cytoplasm (Fig. 2B).

Once isolated, the protein levels in each of the samples were measured by Bradford analysis. The enrichment of each membrane fraction isolated was studied using immunoblot analysis. Calnexin was used as an ER marker, COX IV as an IMM marker, VDAC1 as a mitochondrial marker, PM $Ca^{(2+)}$-ATPase 1 (PMCA1) as a PM marker, ACSL4 as a PAM marker, ORAI1 as a MAM marker and β-actin as a cytoplasmic marker. These were all found to be enriched in their respective organelle fractions, indicating the purity and efficiency of the isolation procedure used, except for MAM fraction, where the presence of ORAI1 was not detected in the immunoblot analysis, due to limited amount of sample generated during isolation (Fig. 2C).

Cholesterol composition in isolated subcellular membrane fractions

To study a possible communication between the PM and mitochondria via PAMs for cholesterol trafficking, PM, ER, mitochondria, cytoplasm, PAMs and MAMs from control, 2 hours dbcAMP treated and dbcAMP with cycloheximide treated MA-10 cells were isolated. Protein-normalized samples were subjected to lipid extraction and run on an HPTLC silica plate to study differences in cholesterol concentrations between control and both treatments (Fig. 3A). The thin layer chromatographic analysis showed that the cholesterol extraction was successful and that the PM and PAM samples had the highest levels of cholesterol content when compared to other organelles. However, no significant changes in cholesterol levels were observed in organellar membranes isolated from dbcAMP treated cells when compared to control cells, probably because of the low sensitivity of the method used. Therefore, isolated fractions were processed for cholesterol content measurement by mass spectrometry.

In agreement with the thin layer chromatography analysis (Fig.3A), the mass spectrometry analysis showed PM and PAM samples to have the highest levels of cholesterol (Fig. 3B). ER had the next highest cholesterol content followed by mitochondria and then cytoplasm. Cholesterol concentrations (~20nmol/mg protein) in the whole cell homogenates of treated cells remained unchanged, suggesting that during hormonal stimulation that induces steroid biosynthesis, MA-10 cells strive to maintain stable cholesterol levels, even when steroid biosynthesis is increased by 100-fold during the 2 hour stimulation with dbcAMP (Fig. 3B). Cholesterol content in the ER remained unchanged in control and dbcAMP treated samples, but a significant increase was observed in samples treated with dbcAMP and cycloheximide,

suggesting that by inducing cholesterol trafficking by dbcAMP, but blocking transduceosome complex function at the OMM with cycloheximide, accumulation of cholesterol was found at the ER, indicating that ER could be the route for cholesterol trafficking to mitochondria for steroidogenesis (Fig. 3B). Furthermore, in both PM and PAM cholesterol concentrations were decreased upon hormone stimulation, though not significantly. This decrease may suggest a role for the PM and PAM in cholesterol trafficking. A similar decrease was also noticed between treatments in the cytoplasm samples (Fig. 3B). In mitochondria, a slight increase in the cholesterol levels after cycloheximide treatment again indicates that by inhibiting the transduceosome complex function a pool of unprocessed cholesterol builds up at the mitochondria (Fig. 3B).

Lipidomic analysis of major structural lipids during steroidogenesis

This lipidomics analysis was focused on three major categories of mammalian lipids that are important for membrane associations or play a role during steroidogenesis, i.e., glycerophospholipids, sphingolipids, and cholesterol and its esters. We did not focus this analysis on other categories of lipids, including glycerolipids and fatty acyls because of their extreme hydrophobic nature or relatively hydrophilic nature, respectively. The extreme hydrophobic lipids are usually neutral lipids, do not play an important role in membrane reorganization, and are mostly confined to specialized organelles like lipid droplets; whereas the relatively hydrophilic lipids may release and redistribute from original membranes during the extensive subcellular fractionation. Therefore, glycerophospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA) and cardiolipins (CL), were analyzed in MA-

10 cells before and after dbcAMP and dbcAMP with cycloheximide treatments, followed by sphingolipids, including sphingomyelins (SM) and ceramides (Cer), and neutral lipid cholesteryl ester (CE), to study the role of these lipids in membrane reorganization and steroidogenesis.

A summary of all the lipid species analyzed in 6 subcellular membrane fractions known to play essential roles in cholesterol trafficking and steroid biosynthesis, as well as whole cell homogenates, for control, dbcAMP and dbcAMP with cycloheximide treated MA-10 cells, is presented in Table 1. In total 569 lipid species were analyzed and compared between control and treated samples.

The total lipid abundance data among subcellular distribution were very similar between treatments, with the PM having the highest lipid abundance, closely followed by PAM (Fig 4A). The overall lipid patterns obtained were strikingly different between the PM and PAM, substantiating previously published data that PAM microdomains, even though a part of the PM, contain a different a lipid profile for the unique function carried out by them (255). ER had the next highest abundance, followed by MAM and mitochondria. The PAM, although reminiscent of MAMs, had a very unique lipid profile (239,292). As expected cytoplasm samples had minimal lipid abundance in them (Fig 4A). In general the lipid profiles of each fraction resembled the lipid profile of the whole cell homogenates (Fig 4). PC, the most abundant phospholipid class, was found to remain generally unchanged between treatments in each of the fractions (Fig 4B, supplementary fig. 1), suggesting the quintessential role PC plays in membrane organization and stability. The same goes for the sphingolipids, where no change was noticed between treatments in each fraction (Fig. 4C and supplementary Fig. 2). The abundance

of phosphatidic acid (PA) seemed to be increased with hormonal stimulation at the site of production, which is the ER, and this production was stalled in the presence of cycloheximide (Fig. 4D). Multiple PA species were significantly upregulated upon dbcAMP stimulation as seen in detail in supplementary fig. 4. PE, an important structural membrane lipid class, seems to have a similar lipid abundance profile between fractions, except for PAMs and MAMs where, upon dbcAMP stimulation, a notable increase was seen, whereas in the PM there was a reduction in PE abundance after treatment, indicating an important role for PE in the formation of PAMs and MAMs upon hormonal stimulation (Fig. 4E and supplementary fig. 3). The redistributionn of PE between the PM and PAMs was inhibited by cycloheximide treatment (Fig. 4E and supplementary fig. 3). In mitochondria, the site for PE synthesis, increased lipid abundance that was not inhibited by cycloheximide (Fig. 4E and supplementary fig. 3) was seen. A similar finding was noted in the PS lipid class as well, where there was an increase in PS levels in PAMs, but a decrease was noted in PM samples, suggesting a lateral movement of various PS and PE lipid species from the PM to PAMs upon dbcAMP stimulation (Fig. 4E and supplementary fig. 3). In MAM fractions, an overall increase in PS levels was noted upon dbcAMP stimulation, whereas in the ER a decrease was seen (Fig. 4E and supplementary fig. 3), further suggesting a redistribution phenomenon of lipids by membrane associations during steroidogenesis. PI was the least abundant glycerophospholipid found in MA-10 cells (Fig. 4G), but has been previously shown to be mediate acute responses to stimuli and participate in signal transduction (293). In agreement with previously known data, PI seems to have a general increase in abundance upon dbcAMP stimulation throughout membrane fractions extracted, but this abundance was diminished in the presence of cycloheximide treatment, again throughout

membrane fractions, suggesting inhibitory effects of cycloheximide on the synthesis of PI (294) (Fig. 4G).

dbcAMP induced lipidome remodeling

Cholesterol esters (CE) are predominantly stored in cytosolic lipid droplets, but a small pool is present in the PM (295). Our data reflect previously published work where cytoplasm samples had the highest content of CE, followed by the PM. ER, mitochondria, MAM and PAM samples had negligent amounts in them (Fig. 5A and supplementary fig. 5). The whole cell homogenate samples showed high levels of CE, probably from lipid droplets that were not isolated in the fractionation procedure (Fig. 3A). Most changes in CE levels between treatments were consistant, where the levels drastically decreased upon dbcAMP treatment and further decreased upon dbcAMP with cycloheximide treatment (Fig. 5A and supplementary fig. 5). Out of the eight CE species recorded, most had a significant decrease upon treatment in all fractions, except in PAMs where an increase in cholesteryl esters was noted upon dbcAMP treatment (supplementary fig. 5).

Cardiolipins (CL) are exclusively present in the mitochondria and have been previously shown to play an important role in the electron transport chain and also in steroidogenesis in the mitochondria (74). In the lipidomics analysis a total of 260 species of CL were analyzed. As expected the mitochondria and the MAM fractions had the highest level of cardiolipins, followed by the ER fraction, and a general increase in these fractions was noted upon dbcAMP stimulation, indicating the importance of CL in steroid production (Fig 5B and supplementary fig. 6). But the increase was blocked by cycloheximide treatment. Interestingly, PAM samples

also seem to have some amount of CL in them, but the abundance did not fluctuate between treatments as it did with other fractions. An increase in abundance was also noticed in the whole cell homogenates as well (Fig 5B and supplementary fig. 6).

Ceramides (Cer) are generally synthesized in the cells by the hydrolysis of SM (296). Previous studies have shown Cer can significantly increase progesterone production in MA-10 Leydig cells (297). In our lipidomics study, we noted an overall significant increase in 5 out of the 8 Cer species analyzed in the whole cell homogenates. The ceramides seem to be predominantly present in the PM, ER, MAMs and PAMs, where an increase was noted in dbcAMP treated samples (Fig 5C, D). Specifically in the PM and PAMs a significant increase in six out of the 8 species analyzed was seen, but this increase was vastly inhibited in the cycloheximide treated samples, and this phenomenon was seen throughout all fractions isolated (Fig 5C, D), thus, suggesting cycloheximide involvement in inhibiting protein function in Cer synthesis pathways.

Discussion

Major alterations of lipids in membranes cannot be tolerated by organelles and cells (289). Hence lipid trafficking is a tightly regulated function, closely monitored by a slew of pathways involving proteins and lipids. Lipids synthesized in the cell or obtained from extracellular sources are distributed throughout the cell by mechanisms not yet completely understood (298). Even after decades of research, the basic question of how hydrophobic lipid molecules traverse through the aqueous cytosol has yet to be precisely answered. A number of vesicular and non-vesicular lipid trafficking pathways have been proposed. Especially during acute and chronic

steroid biosynthesis, the pathway by which large quantities of cholesterol are quickly trafficked to the mitochondria across the aqueous milieu is a not a very well delineated. The classic vesicular trafficking methods take longer, and the kinetics do not support the urgent need for cholesterol (58). Soluble protein-mediated non-vesicular lipid transfer pathways have also been suggested, and a few studies have shown the importance of such proteins in steroidogenesis, especially StAR (299). But again, the kinetics do not match the net amounts of cholesterol being trafficked. Increasing evidence showing inter-organellar conduits to be the route for cholesterol traffic, especially ER-mitochondrial tethering called MAM interactions. This mechanism would support the time constraint within which cholesterol needs to be available at mitochondria. However, both these organelles are poor in cholesterol, suggesting that the presence of a third organelle is necessary for this mechanism to be operational. Previous studies from our laboratory have shown the PM to be a potential source organelle for cholesterol (106). Using electron microscopy in this present study, we observed an increased PM- ER interaction within 15 minutes of dbcAMP stimulation, followed by an increased interaction between ER-mitochondria in the same cells (Fig 1A, B and C). Further knockdown of ATAD3A, a MAM interaction protein, seemed to slow down the movement of cholesterol from the PM (Fig 1D). Putting these observations together, we hypothesize PM-ER-mitochondria tethering also known as PAM interactions to be the route for cholesterol trafficking during steroidogenesis (Fig. 1E).

These MAM and PAM tethering has been known to occur in specialized regions of membranes called the microdomains that are quite similar to lipid rafts (300,301). These microdomains are rich in lipids and specialized proteins important for their function (289). Mitochondria-ER (MAM) and PM-ER (PAM) interactions are significantly increased within 15

minutes after starting hormone stimulation in MA-10 cells (Fig 1A, B and C), suggesting rapid reorganization of membranes and their lipids during steroidogenesis. Recent studies have indicated that apart from cholesterol, these membrane-membrane interactions transport a number of other lipids, including sphingolipids such as SM and Cer (302) and major phospholipids including PA, PS, PI and PE (217,293,303-305). It is yet to be understood how organelles tolerate such massive redistribution of lipids during steroidogenesis and still maintain homeostasis.

In this present study we report a comprehensive subcellular lipidomics analysis of steroidogenic MA-10 Leydig cells. The abundance of the major membrane lipids were determined in cellular organelles membrane interactions previously shown to be essential for steroidogenesis. To fully understand the lipidome remodeling during steroidogenesis we compared control, dbcAMP induced acute steroidogenesis, and dbcAMP induced steroidogenesis with steroidogenesis inhibition by the protein synthesis inhibitor cycloheximide (306,307). For this, we first isolated PM, ER, and mitochondria, along with MAM and PAM fractions, from MA-10 cells with the above mentioned treatments, along with cytoplasm and whole cell homogenates for controls following the schematic representation in (Fig. 2A, B).

The observed changes in in the lipid compositions after dbcAMP treatment represent the activated state of MA-10 cells. However, we would like to note that the degree of change induced by dbcAMP treatment in a given lipid class may vary based on the abundance of the lipid itself. Low abundance lipids go through bigger fold changes, whereas as highly abundant lipids may not undergo substantial change in a membrane (308). For example, cholesterol, PC

and SM in Fig. 3B, 4B, and 4C, respectively are highly abundant lipids absolutely essential for membrane integrity. Hence significant changes were not expected between treatments in any fraction. Thus, when a significant increase was noted in cholesterol in the ER fraction with dbcAMP and cycloheximide treatment (induction cholesterol trafficking but inhibition transduceosome complex function), it strongly suggested that the route of cholesterol trafficking to the mitochondria is through the ER (Fig. 3B). Additionally, the slight but consistent decrease in the cholesterol content in the PM and PAM samples after both treatments indicate that the PM could be a source of cholesterol during acute steroidogenesis, and PAM could be the route of transport (Fig. 3B).

CE is an important neutral lipid for steroidogenesis predominantly stored in LDs. For many years it was taken for granted that LDs were the source organelle for cholesterol for steroid production, since one of the first studies showed a decrease in lipid droplet volume upon hormonal stimulation (194). Although LDs could provide a constant supply of cholesterol to sustain steroidogenesis, the kinetics of this process do not support an acute response in cells to hormones. Hence the decrease noted in CE in whole cell homogenate and cytoplasm fractions (Fig. 5A) may suggest utilization of esters for steroid production, but they may not be the first responders to stimulation.

The majority of glycerophospholipids are synthesized in the ER and the mitochondria (309,310). The ER is the site for major production of PA, a lipid class that is a precursor for biosynthesis of many glycerophospholipids including CL (49). Furthermore, previous studies done on preovulatory granulosa cells, showed increased PA formation upon hormonal

stimulation (311). In agreement with this, the significant increase in PA levels at the ER suggested a dbcAMP induced *de novo* synthesis (Fig. 4D). Furthermore, an increase in CL formation that happens exclusively in the mitochondria (Fig. 5B), suggested that dbcAMP also stimulated the newly synthesized PA to be mobilized to the mitochondria and further translocated from the OMM to the IMM for CL production (312). Thus supporting previously claims, dbcAMP stimulates the formation of contact sites between OMM and IMM during steroidogenesis (9).

MAM and PAM formation have been shown to play an substantial role in the transport of multiple lipids to and from the ER (289). PE and PS are two such lipid classes synthesized and transported between the mitochondria and ER through MAM formation (313). In the mitochondria, PE is synthesized by decarboxylation of PS (314). For this synthesis PS is trafficked via MAMs. dbcAMP stimulation seemed to stimulate PE accumulation in mitochondria and MAM fractions, suggesting synthesis of PE at the mitochondria, for which PS would have been translocated from the ER (Fig. 4 E, F). In PAM and PM fractions, both PE and PS seem to have a similar trend, where a reduction was seen in the PM fraction, but an increase was noted in PAM fractions, indicating relocation of these lipids during the formation of PAMs (Fig. 4 E, F). PI is another lipid class primarily synthesized in the ER and delivered to other membranes, especially to the PM, where it is predominantly located, by various pathways yet to be completely understood (Fig. 4G) (293). PI is involved in almost all events that occur at the cell surface, including multiple signal transduction processes. In rat adrenals ACTH treatment induced rapid increases in PI accompanied by several fold increases in corticosterone production (294). In the same study cycloheximide seemed to rapidly inhibit PI, as well as steroidogenesis.

In accordance with this study, an overall increase in PI was noted in the ER, mitochondria, MAM, PM and PAM fractions upon dbcAMP stimulation, suggesting an important role for PI in steroid biosynthesis (Fig. 4G). Also, cycloheximide seems to attenuate the synthesis of PI and its function (Fig. 4G).

Previous studies in the adrenal cortex noted a rapid activation of sphingolipids metabolism upon ACTH or cAMP stimulation (315). This metabolism at the PM has been correlated with cholesterol movement from the PM for steroid hormone secretion in Leydig testicular cells (316). SM hydrolyzed by sphingomyelinases results in the formation of Cer (302). Cer is a known secondary messenger molecule involved in a series of cellular events, including cell proliferation, stress response mediation, senescence, and cell cycle arrest. During dbcAMP mediated steroidogenesis, Cer is significantly increased, especially in PM and PAM fractions, indicating the functional importance of this signaling lipid in steroid production (Fig. 5C, D). However, several previous studies on Cer have suggested a negative role in steroidogenesis (317-319). In contrast, other studies show Cer's importance in lipid raft formation in the PM, and Cer could potentially displace cholesterol from lipid umbrellas (53). The significant increase of Cer in the PM and PAM fractions upon dbcAMP stimulation may suggest a role for Cer in the formation of membrane interactions.

As with all "omics" studies, the dynamic lipidome that was mapped in this study can only be used as an effective hypothesis building system. But this initial analysis allows us to propose several testable hypotheses that in the future could provide deeper understanding of various aspects of lipid metabolism, membrane dynamics and steroidogenic cellular physiology. Results

from this study strongly suggest that the formation of MAM and PAM associations and their essential roles in multiclass lipid trafficking between organelles may be critical the maintenance of homeostasis in the cell during steroidogenesis. This work definitely represents a first step in understanding membrane associations and dynamic subcellular lipid organization in cholesterol trafficking and steroidogenesis.

Acknowledgments

We thank Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA) for supplying the MA-10 cell line. Dr. Francoise Hullin-Matsuda and Dr. Toshihide Kobayashi who provided the mCherry-D4 vectors.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

SV designed, performed, and analyzed experiments and wrote the paper. LI performed electron microscopic studies and ATAD3 knockdown analysis in Figure 1. RC and ES provided assistance with the experiments shown in Figures 2 and 3. ED and PK performed mass spectrometry studies in Figure 5. LT performed lipidomics analysis and assisted in data analysis. VP conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

FOOTNOTES

* This work was supported by a grant from the Canadian Institutes of Health Research (MOP102647 and MOP125983) and a Canada Research Chair in Biochemical Pharmacology to V.P. S.V. was supported in part by pre-doctoral fellowships from the Experimental Medicine Graduate Program and the Center for the Study of Reproduction. The Research Institute of MUHC was supported by a Center grant from Le Fonds de la recherche du Québec-Santé.

Figures and legends

Figure 1

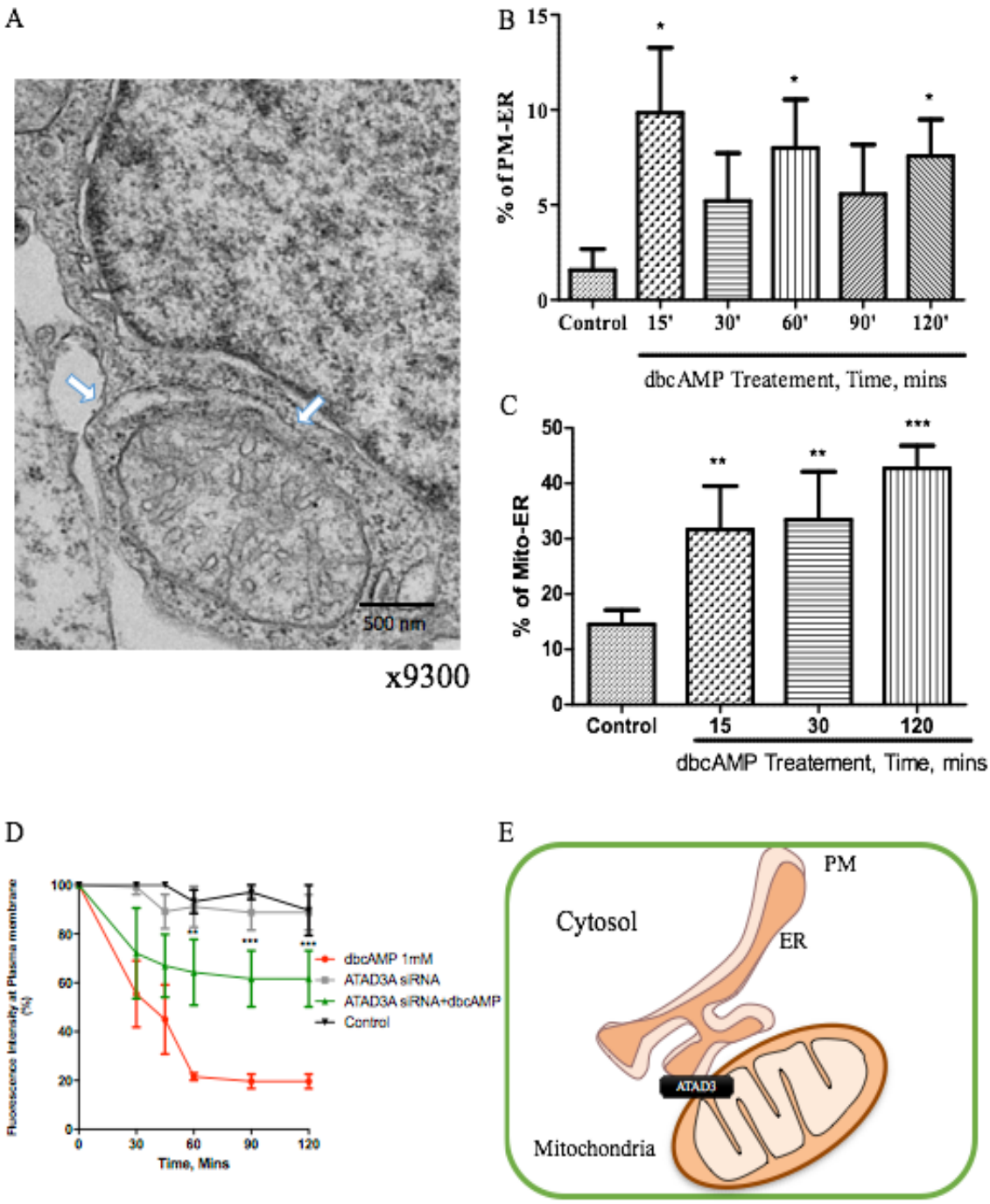


Figure 1. PM-ER-mitochondria interaction in control and hormone stimulated MA-10 cells. *A.* Contact site formation between PM, ER and mitochondria upon 1 mM dbcAMP treatment visualized by electron microscopy (magnification, ×9300; scale bar, 500 nm). The blue circles represent the PM-ER and ER-Mitochondria interactions that form the PAM association. *B.* Quantification of PM-ER interactions at 15, 30, 60, 90 and 120-minute time points based on image analysis of the EM images. *C.* Quantification of ER-mitochondria interactions in the same cells as in B at 15, 30, 60, 90 and 120-minute time points based on image analysis of the EM images. Images from about 30 control MA-10 cells and various indicated time point treated cells each were analyzed for this quantification. *D.* Time course of mCherry-D4 fluorescence associated with the PM in cells treated with siRNA targeting *Atad3,* resulting in reduced ATAD3A expression in control and dbcAMP-treated cells. *E.* Representative model of PAM formation and ATAD3 facilitates ER and mitochondrial membrane associations. (Data represent means ± SD of at least three independent experiments performed in triplicate; two-way ANOVA followed by Bonferroni's post-hoc test (*) was used to calculate statistical significance; ***$p < 0.001$; scale bars: 10 μm; dbcAMP, dibutyryl cAMP.)

Figure 2A

Schematic representation of steps involved in crude PM, Mitochondria, ER and cytosol isolation

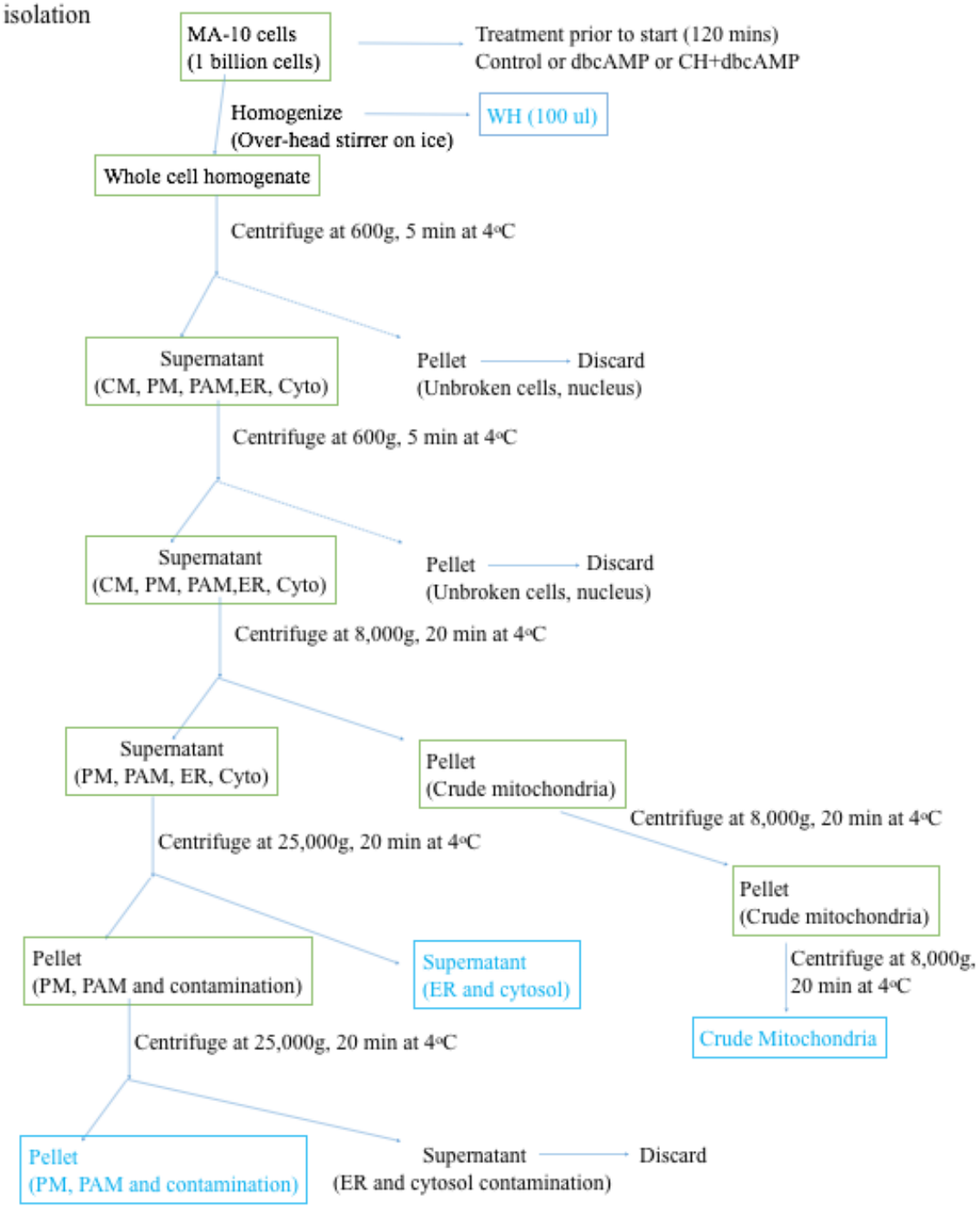


Figure 2B

Schematic representation of steps involved in PM, PAM, ER and cytosol isolation

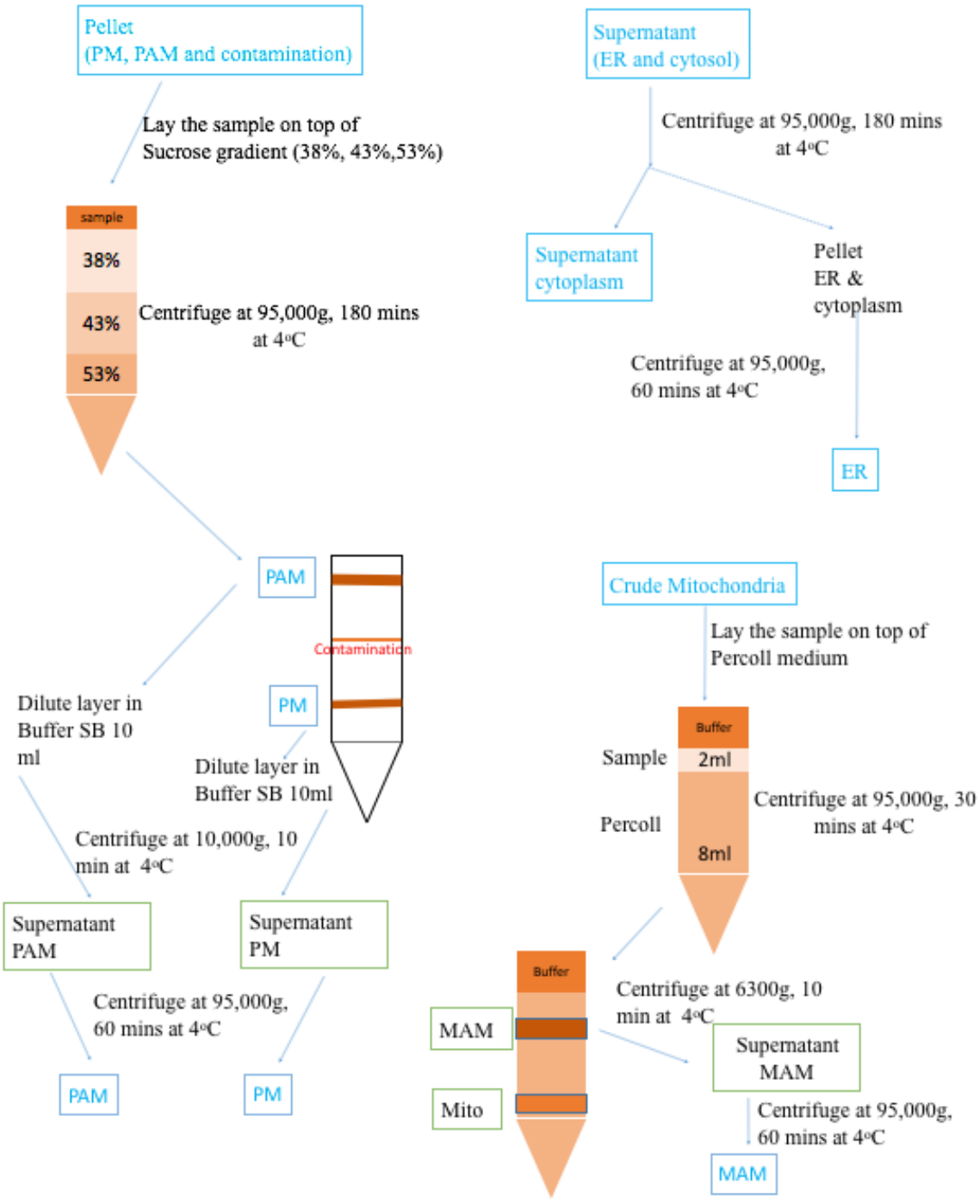


Figure 2C

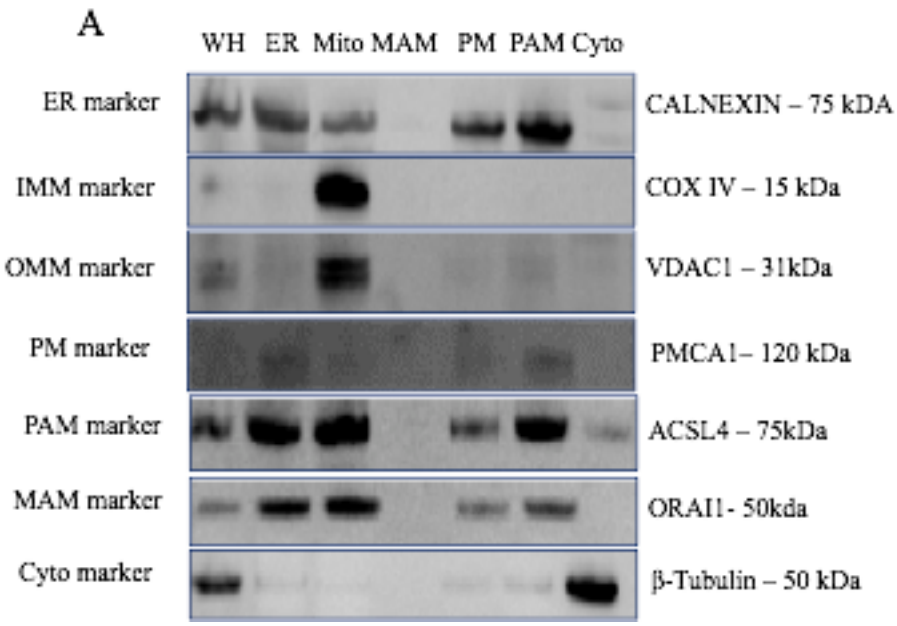
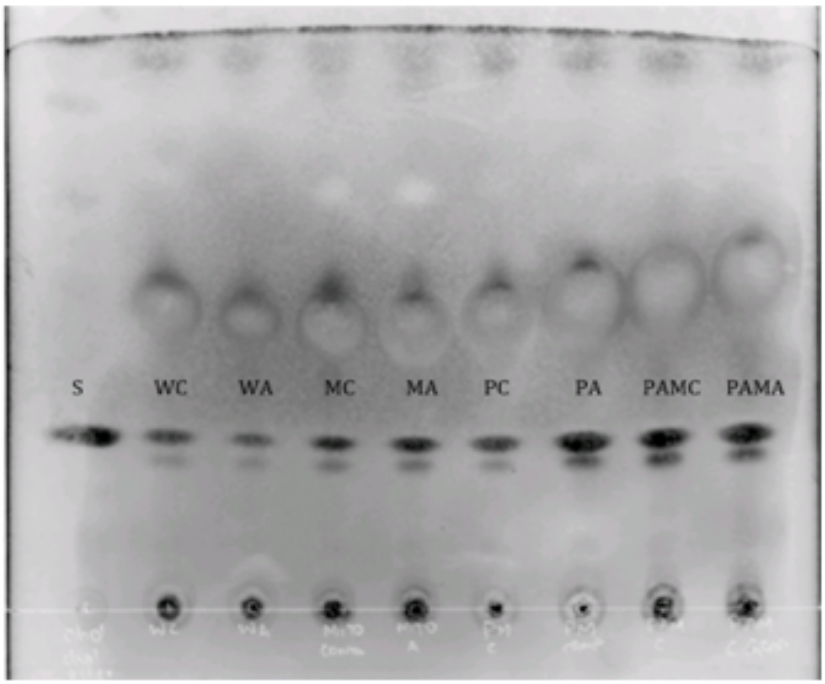


Figure 2. Subcellular fractionation and analysis of enrichment. *A*. Schematic representation of time line and steps involved in isolation of crude PM, ER and mitochondria. One billion MA-10 cells each of control, 1mM dbcAMP or 1mM dbcAMP with 0.2mM cycloheximide treated were used as raw material for the multistep fractionation procedure. Cells were homogenized using an overhead stirrer on ice until 90% cellular disruption confirmed by Trypan blue test. The samples were then subjected to multiple differential centrifugation steps as indicated, leading to crude mitochondria, PM and ER fractions. *B*. The crude PM fraction from 2A was further subjected to a sucrose gradient of 38%, 43% and 53% concentrations and additional multistep centrifugations as shown to achieve pure PM and PAM fractions. The Crude ER fraction was subjected to an additional two-step ultracentrifugation to achieve a pure ER fraction. The crude mitochondria samples from 2A were subjected to a Percoll gradient-based separation that results in pure mitochondria and MAM isolation. *C*. Proteins from isolated organelles were subjected to immunoblot analysis to determine the purity of the samples using the following markers for the

specified organelle: PMCA1 (120kDa) for the PM, VDAC for the mitochondrial OMM, β-Tubulin for the cytoplasm, COX IV for the mitochondrial IMM, Calnexin for the ER, ACSL4 for PAMs and ORALI for MAMs.

Figure 3

A


S
WC
WA
MC
MA
PC
PA
PAMC
PAMA

B

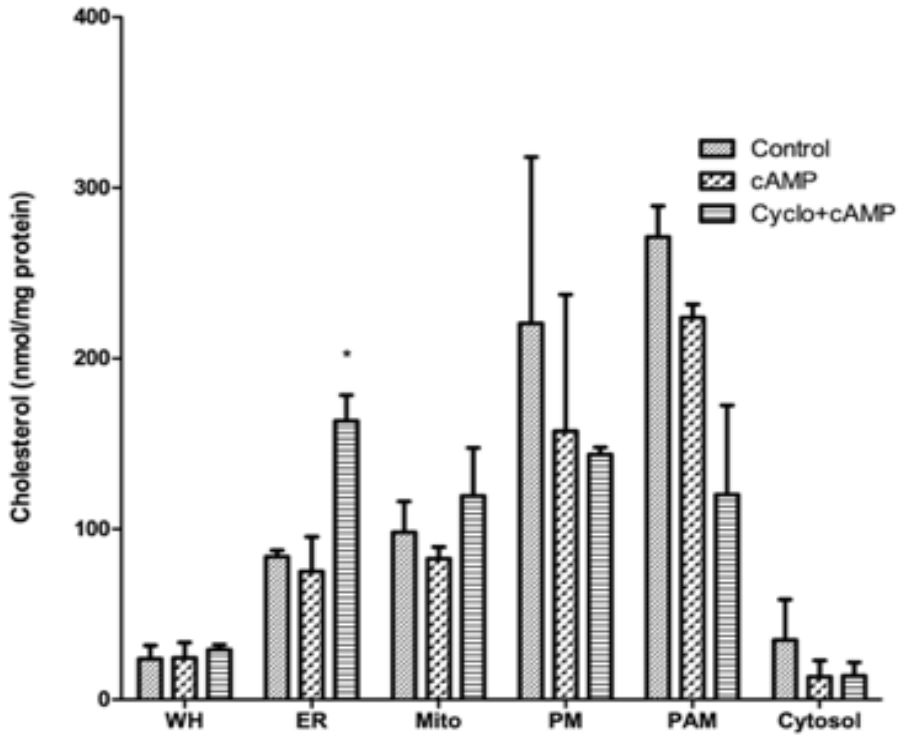

Cholesterol (nmol/mg protein)
400
300
200
100
0
Control
cAMP
Cyclo+cAMP
WH
ER
Mito
PM
PAM
Cytosol

Figure 3: Cholesterol levels in fractions isolated from MA-10 cells. *A.* Organelles isolated were subjected to Bligh-Dyer method to extract lipids and run on an HPTLC silica gel plate. L- Ladder; S- cholesterol Standard; WC-Whole Cell lysate from control cells; WA-Whole cell lysate from dbcAMP treated cells; MC-isolated Mitochondria from Control cells; MA- isolated Mitochondria from dbcAMP treated cells; PC- isolated PM from control cells; PA- isolated PM from dbcAMP treated cells; PAMC – isolated PAM from Control cells; PAMA- isolated PAM from dbcAMP treated cells. *B*. Organelle cholesterol distribution was measured by LC-MS/MS in WH, C, ER, Mito, MAM, PM and PAM isolates from control dbcAMP and dbcAMP with cycloheximide treated MA-10 cells.

	Cytosol			ER			Mito			MAM			PM			PAM			WH		
Lipid Categories	Ctl	cAMP	cAMP + CH	Ctl	cAMP	cAMP + CH	Ctl	cAMP	cAMP + CH	Ctl	cAMP	cAMP + CH	Ctl	cAMP	cAMP + CH	Ctl	cAMP	cAMP + CH	Ctl	cAMP	cAMP + CH
Glycerophospholipids	417	421	411	496	517	519	530	524	531	328	251	219	531	532	522	471	492	496	532	532	532
Sphinglipids	18	17	17	27	29	27	27	25	29	9	8	7	29	29	29	29	26	29	29	29	29
Sterol esters	5	5	5	9	9	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Total	440	443	433	532	555	554	565	557	568	345	267	234	568	569	559	508	526	533	569	569	569

Table 1: Overview of subcellular lipidome – Each box shows the number of lipid species detected and quantified in the specific subcellular fraction in control (Ctl), dbcAMP (cAMP) and dbcAMP with cycloheximide treated (cAMP+CH) states.

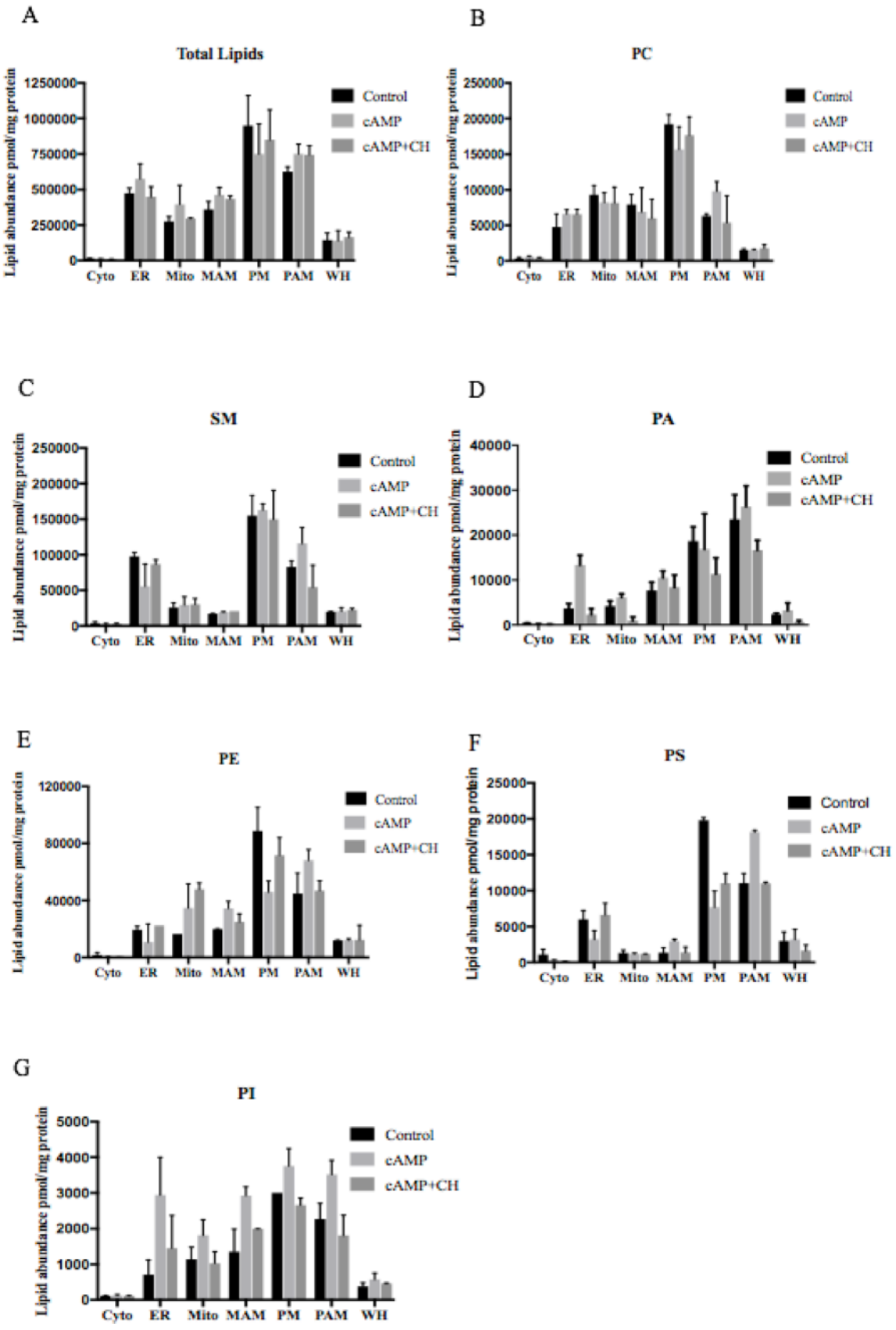

A
Total Lipids
Lipid abundance pmol/mg protein
1250000
1000000
750000
500000
250000
0
Cyto ER Mito MAM PM PAM WH
Control
cAMP
cAMP+CH
B
PC
Lipid abundance pmol/mg protein
250000
200000
150000
100000
50000
0
Cyto ER Mito MAM PM PAM WH
Control
cAMP
cAMP+CH
C
SM
Lipid abundance pmol/mg protein
250000
200000
150000
100000
50000
0
Cyto ER Mito MAM PM PAM WH
Control
cAMP
cAMP+CH
D
PA
Lipid abundance pmol/mg protein
40000
30000
20000
10000
0
Cyto ER Mito MAM PM PAM WH
Control
cAMP
cAMP+CH
E
PE
Lipid abundance pmol/mg protein
120000
80000
40000
0
Cyto ER Mito MAM PM PAM WH
Control
cAMP
cAMP+CH
F
PS
Lipid abundance pmol/mg protein
25000
20000
15000
10000
5000
0
Cyto ER Mito MAM PM PAM WH
Control
cAMP
cAMP+CH
G
PI
Lipid abundance pmol/mg protein
5000
4000
3000
2000
1000
0
Cyto ER Mito MAM PM PAM WH
Control
cAMP
cAMP+CH

Figure 4. Subcellular fraction lipid distribution profile. A: Total lipid. B–F: Total glycerophospholipids, sphingolipids. B-F: Total lipids; phosphatidylcholine, PC; sphingomyelin, SM; phosphatidic acid, PA; phoshatidylethanolamine, PE; phosphatidylserine, PS and phosphatidylinositol, PI respectively. Data shown are sums of data for each molecular species, mean ± SE, n = 3.

Figure 5

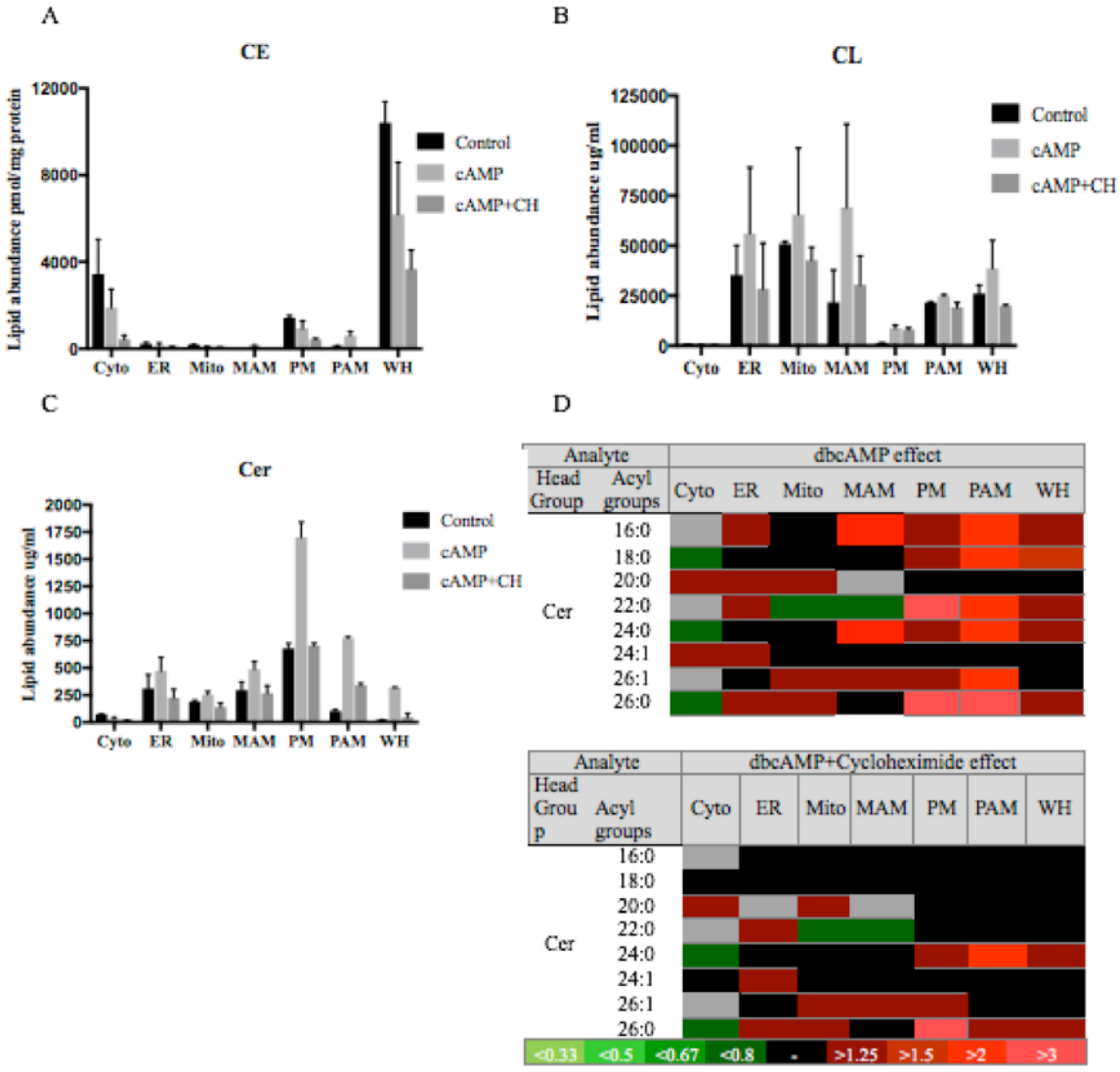
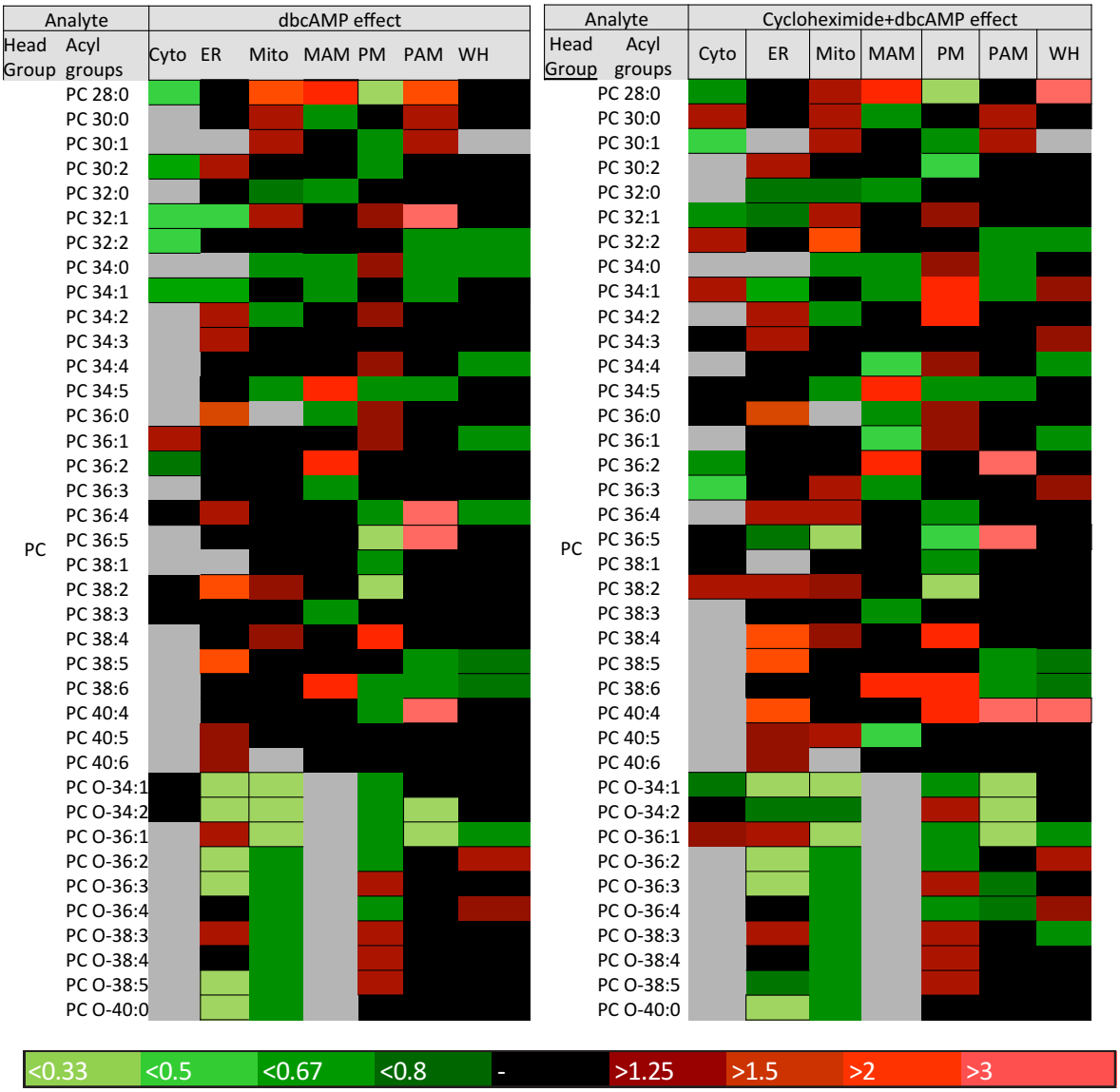


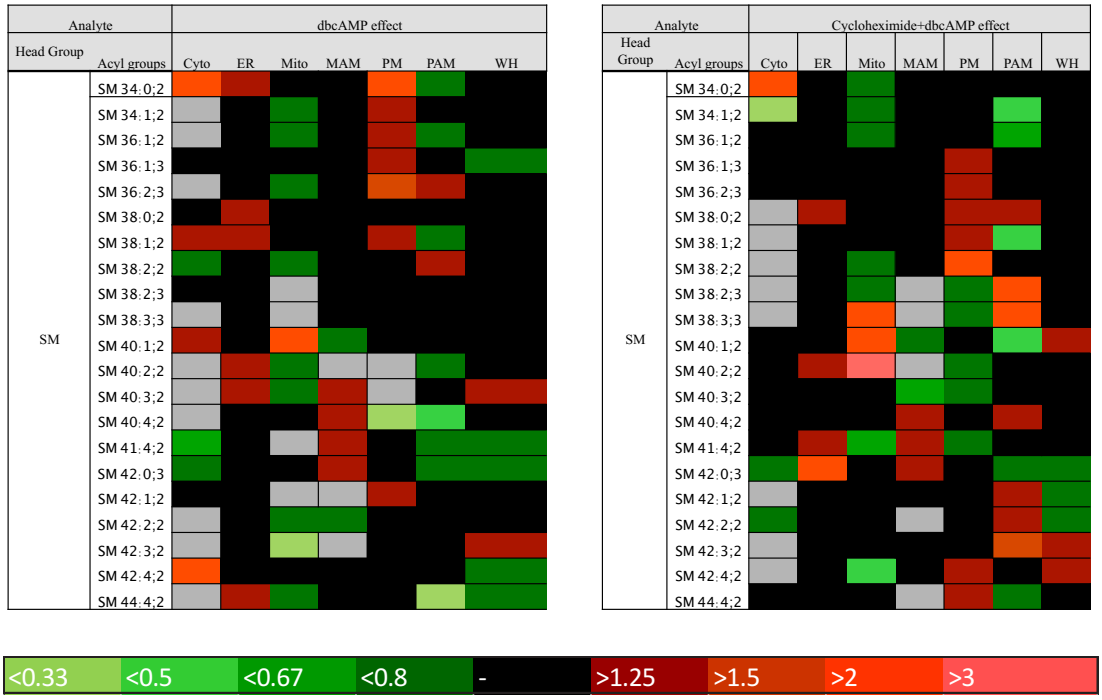
Figure 5. Subcellular fraction lipid distribution profile. A: Cholesteryl esters, CE; B: Cardiolipins, CL; C: Ceramides, Cer; D: Heat map analysis showing fold increase in response to dbcAMP or dbcAMP with cycloheximide treatment. Acyl groups are abbreviated by the number of carbon atoms and double bonds per molecule. Changes are color coded with statistical significance table on the bottom of the figure. Data shown are sums of data for molecular species, mean ± SE, n = 3.

Supplementary fig. 1



Supplementary fig 1: Phosphatidylcholine (PC) heat map analysis. PC heat map analysis showing changes in reponse to dbcAMP and dbcAMP with cycloheximide. Acyl groups are abbreviated by the number of carbon atoms and double bonds per molecule. Changes are color coded with statistical significance table on the bottom of the figure. Data shown are sums of data for molecular species, mean ± SE, n = 3.

Supplementary fig. 2



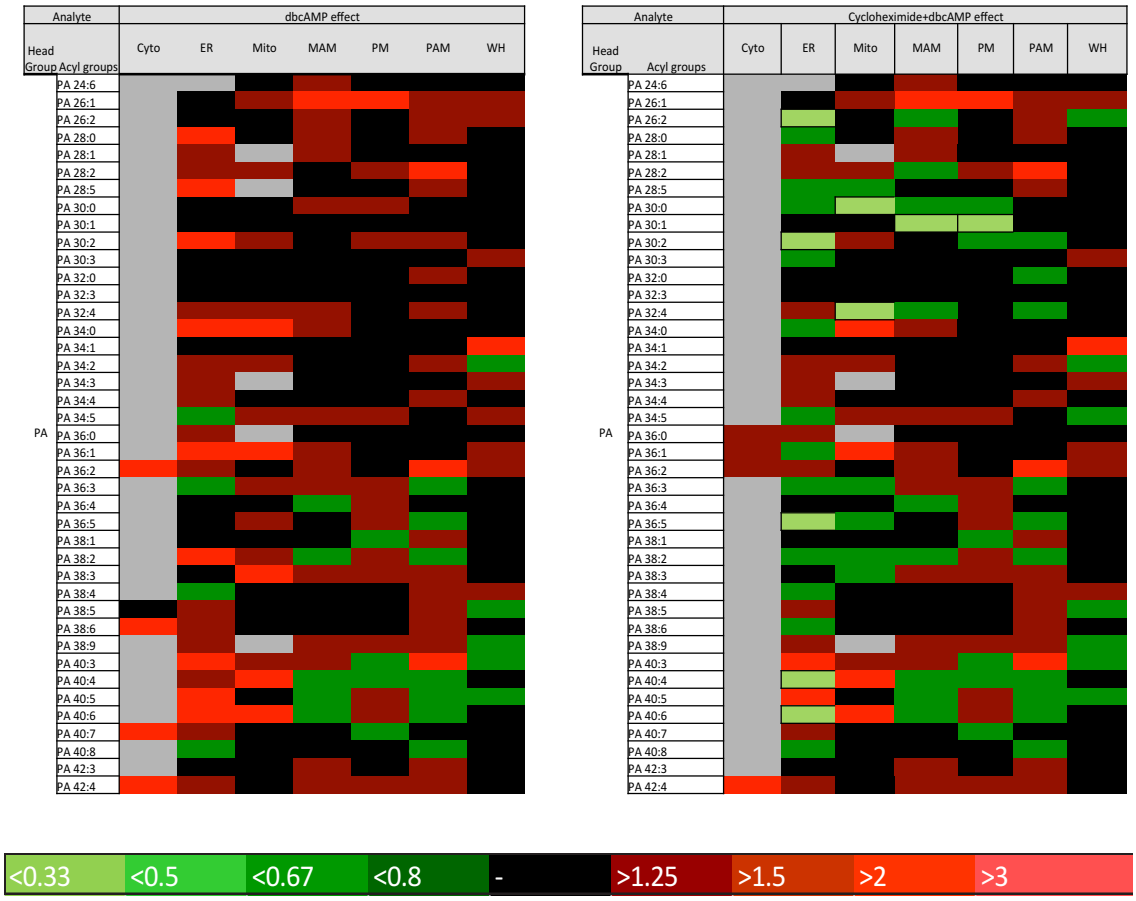
Supplementary fig 2: Sphingomyelin (SM) heat map analysis. SM heat map analysis. showing changes in reponse to dbcAMP and dbcAMP with cycloheximide. Acyl groups are abbreviated by the number of carbon atoms and double bonds per molecule. Changes are color coded with statistical significance table on the bottom of the figure. Data shown are sums of data for molecular species, mean ± SE, n = 3.

Supplementary fig. 3

Supplementary figure 3: Phosphatidylethanolamine (PE) and phosphatidylserine (PS) heat map analysis. PE and PS heat map analysis showing changes in reponse to dbcAMP and

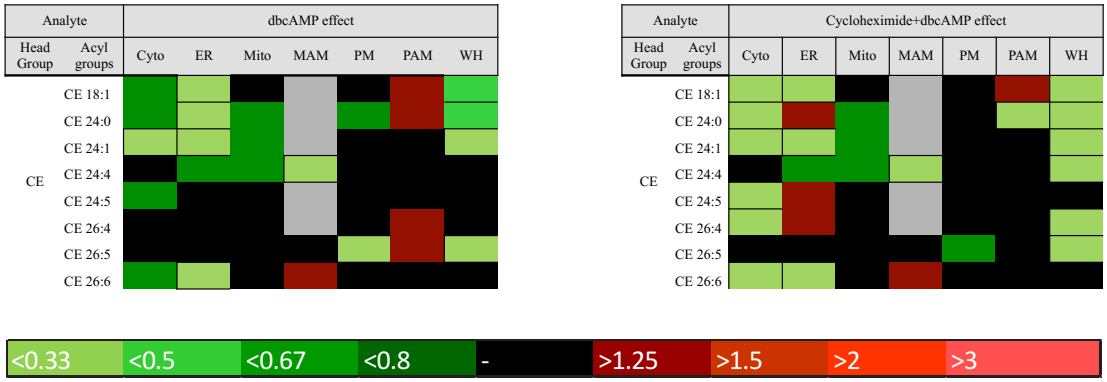
dbcAMP with cycloheximide. Data shown are sums of data for molecular species, mean ± SE, n = 3.

Supplementary fig. 4



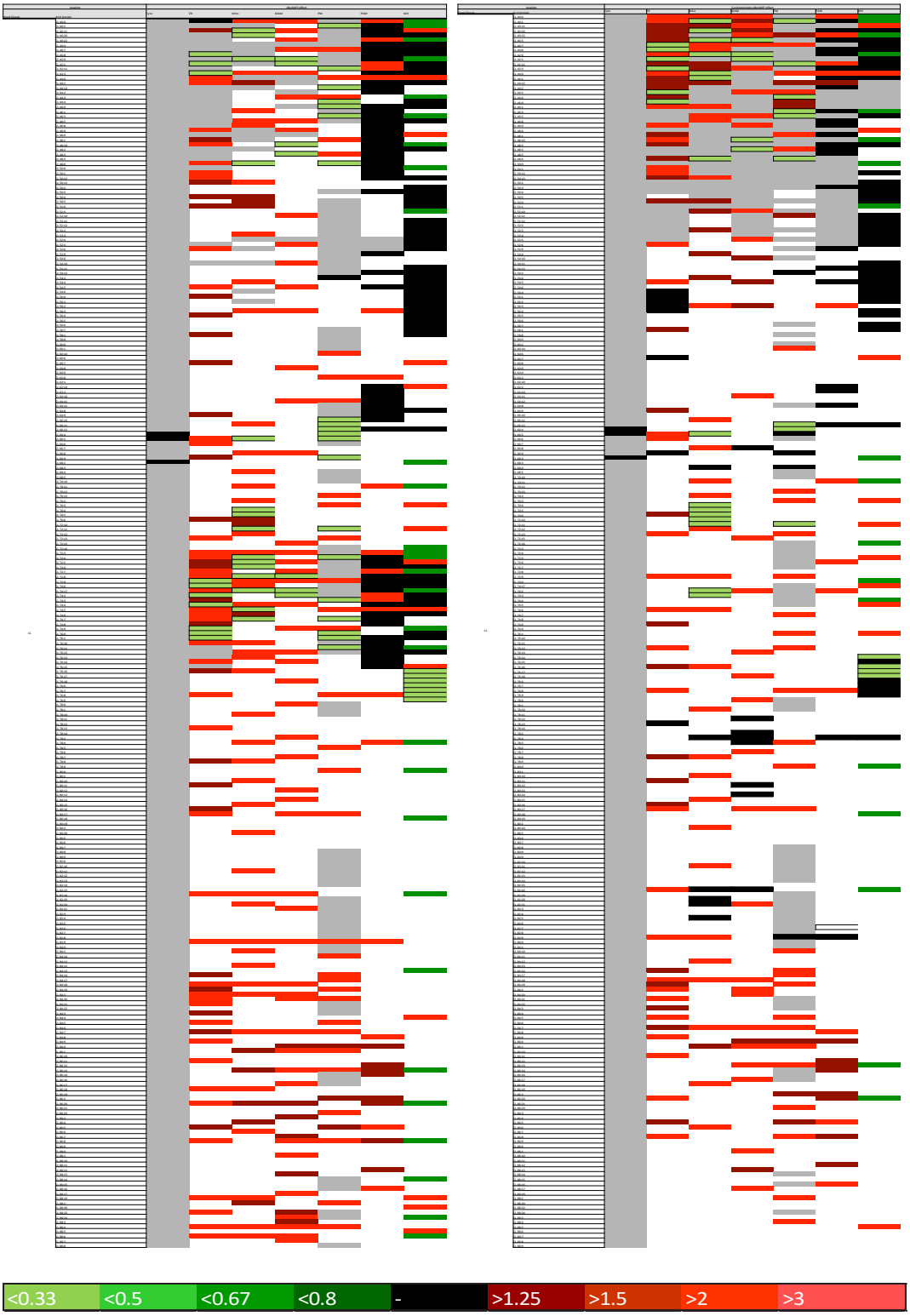
Supplementary fig 4: Phosphatidic acid (PA) heat map analysis. PA heat map showing changes in reponse to dbcAMP and dbcAMP with cycloheximide. Data shown are sums of data for molecular species, mean ± SE, n = 3.

Supplementary fig. 5



Supplementary fig 5: Cholesteryl ester (CE) heat map analysis. CE heat map analysis showing changes in reponse to dbcAMP and dbcAMP with cycloheximide. Data shown are sums of data for molecular species, mean ± SE, n = 3.

Supplementary fig. 6



Supplementary fig 6: Cardiolipin (CL) heat map analysis. CL heat map analysis showing changes in reponse to dbcAMP and dbcAMP with cycloheximide. Data shown are sums of data for molecular species, mean ± SE, n = 3

Chapter IV

Summary and discussion

Steroids are essential for cellular health and well-being. All vertebrate steroid hormone synthesis is initiated at the mitochondria using cholesterol as the parent molecule (10). However, cholesterol needs to traverse from intracellular stores to the cholesterol-poor mitochondrial IMM, where the CYP11A1 enzyme catalyzes the conversion of cholesterol to pregnenolone. During acute steroidogenesis, large quantities of cholesterol must be available for this conversion at the IMM. Cholesterol, being a hydrophobic molecule, is believed to require a number of cellular signals, lipid transfer systems, membrane interactions and protein interactions to reach the IMM (66). Previous in-depth studies in our laboratory have shown that an 800-kDa multi-protein complex forms at the OMM and IMM to transport cholesterol into the IMM where the CYP11A1 enzyme is present (9). However, information on the mechanisms of cholesterol transport from its intracellular stores to the innermost parts of mitochondria still remains elusive.

In mammalian cells, the PM contains the highest concentrations of cholesterol, followed by the endosomes and Golgi apparatus, which contain intermediate levels, and the ER and mitochondria, which contain low levels of cholesterol (74). Lipid droplets (LD) also contain high concentrations of cholesterol, but in their esterified form. In search of the source organelle for steroidogenic cholesterol, since the 1970s multiple studies have suggested that the esters present in the LDs are de-esterified by the cell and thus provide the steroidogenic pool of cholesterol to the mitochondria (194). Although LDs could provide a constant supply of cholesterol to sustain steroidogenesis, the kinetics of this process do not suggest that it is important in the acute response of tissues to hormones. Other studies have suggested that endosomes are involved in bringing cholesterol to the mitochondria (320). Interestingly, endosomal NPC mutations in humans have been identified to cause hypogonadism (but still fertile) with diminished capacity

to synthesize testosterone (97,319). Whether NPC mediated endosomal cholesterol trafficking directly contributes to steroidogenesis or this is caused by a non-specific toxicity remains to be identified. The ER was another organelle proposed to be the source (80), and studies by Freeman and his group indicated that the PM could also provide free cholesterol for steroidogenesis in the mitochondria (233,249). The multiplicity of possible sources led us to look more closely at the problem to resolve this critical issue.

The main focus of my doctoral work was to identify the elusive source organelle for cholesterol during acute steroidogenesis and also to study the mechanisms involved in intracellular cholesterol trafficking. In order to track free cholesterol movement during steroidogenesis, we used fluorescently labeled domain 4 (D4) of the θ-toxin protein from the pathogen *Clostridium perfringens* as a probe that can bind up to 30 % or more on a molar basis of cholesterol-containing membranes without toxicity (263). This probe had advantages over other fluorescent cholesterol tracking probes, as it does not damage the membranes to which it binds and also does not disturb the intracellular distribution of cholesterol. Thus, it could be efficiently used for live cell imaging to track cholesterol movement for up to 2 hours in MA-10 cells upon hormonal stimulation, by which time saturating levels of steroids were produced in these cells.

D4 readily bound to the PM, being the organelle with the highest concentration of cholesterol in its membrane. But within 30 minutes of hormonal stimulations in MA-10 cells, a dramatic and significant decline in fluorescence intensity associated with the PM was seen, suggesting a decline in cholesterol concentrations in these membranes below pre-stimulation

levels. This phenomenon was also noted in primary rat Leydig cells. The decline in fluorescence at the PM could not be due to a negative effect by dbcAMP on the probe, confirmed by analysis using MβCD-based removal of cholesterol from the PM and U18666A-based trapping of cholesterol at the PM. However, D4 might affect the intracellular cholesterol distribution. To address this issue, we co-stained MA-10 cells with mCherry-D4 and filipin. No change in the general distribution of cholesterol was seen, and the subcellular fractionation followed by mass spectrometry analysis did not reveal any change in cholesterol distribution in mitochondria, PM and endosomes.

Hormone stimulation in Leydig cells induces both cholesterol transport and the formation of a multi-protein complex called a transduceosome. The transduceosome protein complex contains OMM proteins TSPO and VDAC; cytosolic proteins such as PKA, STAR and ACBD3, the latter two being targeted to mitochondria; and IMM/OMM protein ATAD3A (133). The transduceosome complex is like a signal amplifier because it targets the PKA-RIα to the mitochondria for local activity. However, the metabolon brings the cholesterol to CYP11A1 without allowing it to mix with the membranes. Inhibiting the function of transduceosome protein STAR and transduceosome/metabolon proteins TSPO, VDAC and ATAD3, also inhibited the movement of cholesterol from the PM, indicating that when there is an accumulation of cholesterol at the OMM, it acts as a signal that prevents the mobilization of free cholesterol from the PM. The same was true when inhibiting the metabolon protein CYP11A1. Studies using 22R-HC and 22S-HC (Chapter II) to bypass the transduceosome-mediated cholesterol transport to the mitochondria revealed that a steroid could also mediate this feedback

signal. In fact, the presence of pregnenolone, rather than progesterone, was found to act as the feedback mechanism.

It is widely known that cellular membranes, like the PM, are comprised of asymmetric bimolecular leaflets composed predominantly of phospholipids with varying concentrations of cholesterol, other minor species of lipids, and a plethora of proteins. The major advantage of fluorescence tagged D4 was that it could not diffuse through the membranes, hence mCherry-D4 transfected into MA-10 cells only bound cholesterol on the cytosolic side of the inner leaflet of the PM, and the cholesterol in the outer leaflet of the PM could be efficiently labeled with the membrane-impermeable recombinant GFP-D4. Thus, the cholesterol movement in both leaflets could be efficiently studied. Live cell-imaging analysis using both probes revealed that cholesterol for steroidogenesis came from the inner leaflet of the PM, since no movement of GFP-D4 from the outer leaflet was noted even after multiple washing steps. This suggests that the inner leaflet of the PM is the source of cholesterol during steroidogenesis in MA-10 cells.

The first suggestion that the PM was the source for the steroidogenic pool of cholesterol was made by Freeman and colleagues (233). It was also suggested that cholesterol moves from the PM, passing through endosome compartments during steroidogenesis (235). Our screening of various organelles in MA-10 cells with the appropriate respective fluorescent markers is in agreement with this suggestion: mCherry-D4 colocalized with a late endosome marker CellLight late endosome-GFP (ThermoFisher Scientific) and the co-localization increased upon dbcAMP stimulation, suggesting that the late endosome might be part of the route for cholesterol trafficking from the PM to mitochondria.

From the data in Chapter II, we showed that the PM is one of the richest sources of free cholesterol available, using the mCherry-D4 probe under basal conditions. During acute stimulation of steroid biosynthesis by dbcAMP, MA-10 cells and primary Leydig cells mobilize free cholesterol from the PM and transport it to the mitochondria, where the transduceosome complex transports the cholesterol to the IMM for conversion to pregnenolone by CYP11A1. Inhibiting one of the transduceosome proteins may create a feedback signal, instructing the trafficking system to stop sending cholesterol to the already congested mitochondria and thereby slowing down steroidogenesis. Moreover, a large amount of pregnenolone added to the system may initiate another feedback signal that inhibits the movement of cholesterol from the PM. It seems that these feedback signals could serve as protective mechanisms to avoid cholesterol-induced toxicity at the OMM and/or excessive steroid production, which could be detrimental to the body. More insights into cholesterol trafficking pathways will increase the understanding of steroid-related diseases and help the development of drugs affecting cholesterol trafficking and metabolism.

Drastic lipid changes in any cellular membrane can be detrimental to the cell. The suggestion that the PM releases large quantities of cholesterol for steroidogenesis within minutes of hormone stimulation would indicate that a feedback mechanism must be available to stabilize the membrane and replace the lost cholesterol with other lipids or replenish it from other sources. Hence lipid trafficking is a tightly regulated function, closely monitored by a number of pathways involving proteins and lipids. For steroidogenesis in particular, *de novo* synthesized cholesterol is the major precursor, rather than cholesterol obtained from dietary sources.

Cholesterol and the majority of other lipids are synthesized at the ER and distributed throughout the cell by mechanisms not yet completely understood. A number of vesicular and non-vesicular trafficking pathways have been proposed for this purpose. Recent studies in our laboratory indicated increased inter-organellar interactions, especially between the ER and mitochondria called MAM interactions, to be the route for cholesterol traffic during steroidogenesis. This mechanism would support the time constraints within which cholesterol needs to be available at the mitochondria. But both these organelles are poor in cholesterol, leading us to look for the presence of a third organelle in this pathway. The results from the Chapter II manuscript led us to believe that the PM could be that third organelle.

The concept of a PM-ER-mitochondria interaction, termed PAM, is not new. Multiple studies in a variety of different cells and tissues have observed this interaction and shown it to be involved in a number of lipid trafficking and signal transduction pathways. In fact, results from Chapter III showed this exact phenomenon, where PM-ER and ER-mitochondrial interactions observed through electron microscopy significantly increased within 15 minutes of hormone stimulation, further supporting the short time constraint required for cholesterol trafficked to the mitochondria and leading us to hypothesize that the route for cholesterol trafficking from the PM to the mitochondria during steroidogenesis is through PAM associations.

In Chapter III we also report a comprehensive subcellular lipidomics analysis of steroidogenic MA-10 Leydig cells to analyze cholesterol trafficking and membrane lipid remodeling that occurs due to membrane associations to facilitate this traffic. The lipid abundance of major membrane lipids were determined in cellular organelle membrane

interactions previously shown to be essential for steroidogenesis. To fully understand lipidome remodeling during steroidogenesis, we compared control, dbcAMP induced acute steroidogenesis and dbcAMP induced cholesterol trafficking but with steroidogenesis inhibition by the protein synthesis inhibitor cycloheximide (306,307). For this, we first isolated the PM, ER, and mitochondria, along with MAM and PAM fractions from MA-10 cells, with above mentioned treatments, as well as cytoplasm and whole cell homogenates as negative and positive controls, respectively. Fractionation was followed by both LC-MS/MS based mass spectrometry to analyze cholesterol and other lipid levels in the fractions and further lipidomic analysis by MS/MS acquisitions on hybrid quadrupole TOF.

We noted that the degree of change induced by dbcAMP treatment in a given lipid class varied based on the abundance of the lipids in the fractions. Low abundance lipids went through bigger fold changes, whereas as highly abundant lipids did not undergo substantial changes (308). Keeping this in mind, a significant increase in cholesterol in the ER fraction from dbcAMP plus cycloheximide treated cells (i.e. induced cholesterol trafficking with inhibition of transduceosome complex function), strongly suggested that the route for cholesterol trafficking to the mitochondria is through the ER. Apart from this observation, a slight but consistent decrease in cholesterol abundance in PM and PAM samples after dbcAMP treatment alone led us to believe that the PM could be a source of cholesterol during acute steroidogenesis and that PAMs could be the route of transport.

The decrease in cholesterol ester in cytoplasmic and whole cell isolates suggested that CE could also be utilized for steroidogenesis, supporting previously published studies suggesting

that LD is a source organelle, since CE is predominantly found in LDs. However, even though LDs could provide a constant supply of cholesterol to sustain steroidogenesis, the kinetics of the de-esterification required for utilizing the cholesterol in LDs do not support the acute response of cells to hormones. Hence, the decreases noted in CE in whole cell homogenates and cytoplasm fractions may suggest utilization of esters for steroid production, but they may not be the first responders to stimulation.

Apart from changes in cholesterol abundance in the fractions isolated. We also observed some very interesting changes in glycerophospholipids and sphingolipids, both of which play key roles in membrane organization and also assist in cholesterol trafficking. PA, the precursor for the biosynthesis of many glycerophospholipids including CL, increased in abundance at the ER during the 2-hour treatment with dbcAMP, suggesting induction of *de novo* synthesis. Furthermore, an increase in CL formation that happens exclusively in the mitochondria suggested that dbcAMP also stimulated the newly synthesized PA to be mobilized to the mitochondria with further translocation of PA from the OMM to the IMM for CL production. This also supports previous findings that dbcAMP stimulates the formation of contact sites between OMM and IMM during steroidogenesis (9).

PE and PS are lipids that have been previously shown to be shuttled between the PM, ER and mitochondria through MAM and PAM interactions (313). PE synthesis seems to be increased in mitochondria. Both PE and PS levels increased in MAM and PAM fractions upon dbcAMP stimulation, suggesting their role in organizing the membrane associations. PI, another important lipid class playing a key role in signaling, seems to increase within 2 hours of

dbcAMP treatment, supporting its important role in signaling in steroidogenesis. Cer, another key lipid subclass in sphingolipids, also seems to be elevated in PM and PAM fractions in particular. Previous studies had indicated a role in displacing cholesterol, facilitating cholesterol movement from the membranes (53). We propose that Cer insertion is the stabilization mechanism that replaces cholesterol that has been removed from the membranes for steroidogenesis in the mitochondria.

The lipidomics study in Chapter III can be used as an effective multi-hypothesis building system and paves the way for several testable hypotheses that in the future could provide deeper understanding of various aspects of lipid metabolism, membrane dynamics and steroidogenic cellular physiology. Results from this study strongly suggest that the formation of MAM and PAM associations and their essential roles in lipid trafficking maintain homeostasis in the cell during steroidogenesis. This report is only the first step in understanding membrane associations and dynamic subcellular lipid organization to support cholesterol trafficking and steroidogenesis.

In conclusion the studies presented here indicate that the PM is the major source organelle for cholesterol during acute steroidogenesis in Leydig cells, and PAMs are the primary route for cholesterol trafficking to the mitochondria.

References

1. Miller, W. L., and Auchus, R. J. (2011) The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev* **32**, 81-151

2. Papadopoulos, V., Lecanu, L., Brown, R. C., Han, Z., and Yao, Z. X. (2006) Peripheral-type benzodiazepine receptor in neurosteroid biosynthesis, neuropathology and neurological disorders. *Neuroscience* **138**, 749-756

3. Li, J., Papadopoulos, V., and Vihma, V. (2015) Steroid biosynthesis in adipose tissue. *Steroids* **103**, 89-104

4. Slominski, A., Zbytek, B., Nikolakis, G., Manna, P. R., Skobowiat, C., Zmijewski, M., Li, W., Janjetovic, Z., Postlethwaite, A., Zouboulis, C. C., and Tuckey, R. C. (2013) Steroidogenesis in the skin: implications for local immune functions. *J Steroid Biochem Mol Biol* **137**, 107-123

5. Vacchio, M. S., Papadopoulos, V., and Ashwell, J. D. (1994) Steroid production in the thymus: implications for thymocyte selection. *J Exp Med* **179**, 1835-1846

6. Shikita, M., and Hall, P. F. (1973) Cytochrome P-450 from bovine adrenocortical mitochondria: an enzyme for the side chain cleavage of cholesterol. II. Subunit structure. *J Biol Chem* **248**, 5605-5609

7. Jefcoate, C. (2002) High-flux mitochondrial cholesterol trafficking, a specialized function of the adrenal cortex. *J Clin Invest* **110**, 881-890

8. Simpson, E. R., and Waterman, M. R. (1988) Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Annu Rev Physiol* **50**, 427-440

9. Rone, M. B., Midzak, A. S., Issop, L., Rammouz, G., Jagannathan, S., Fan, J., Ye, X., Blonder, J., Veenstra, T., and Papadopoulos, V. (2012) Identification of a dynamic mitochondrial protein complex driving cholesterol import, trafficking, and metabolism to steroid hormones. *Molecular endocrinology (Baltimore, Md.)* **26**, 1868-1882

10. Jefcoate, C. R., McNamara, B. C., Artemenko, I., and Yamazaki, T. (1992) Regulation of cholesterol movement to mitochondrial cytochrome P450scc in steroid hormone synthesis. *J Steroid Biochem Mol Biol* **43**, 751-767

11. Miller, W. L. (2017) Disorders in the initial steps of steroid hormone synthesis. *J Steroid Biochem Mol Biol* **165**, 18-37

12. Lin, D., Sugawara, T., Strauss, J. F., 3rd, Clark, B. J., Stocco, D. M., Saenger, P., Rogol, A., and Miller, W. L. (1995) Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267**, 1828-1831

13. Payne, A. H., and Hales, D. B. (2004) Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* **25**, 947-970

14. Hall, P. F. (1985) Role of cytochromes P-450 in the biosynthesis of steroid hormones. *Vitam Horm* **42**, 315-368

15. Nebert, D. W., Wikvall, K., and Miller, W. L. (2013) Human cytochromes P450 in health and disease. *Philos Trans R Soc Lond B Biol Sci* **368**, 20120431

16. Grundy, S. M. (1983) Absorption and metabolism of dietary cholesterol. *Annu Rev Nutr* **3**, 71-96

17. Charreau, E. H., Calvo, J. C., Nozu, K., Pignataro, O., Catt, K. J., and Dufau, M. L. (1981) Hormonal modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in gonadotropin-stimulated and -desensitized testicular Leydig cells. *J Biol Chem* **256**, 12719-12724

18. Azhar, S., Chen, Y. D., and Reaven, G. M. (1984) Gonadotropin modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in desensitized luteinized rat ovary. *Biochemistry* **23**, 4533-4538

19. Golos, T. G., and Strauss, J. F., 3rd. (1988) 8-bromoadenosine cyclic 3',5'-phosphate rapidly increases 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA in human granulosa cells: role of cellular sterol balance in controlling the response to tropic stimulation. *Biochemistry* **27**, 3503-3506

20. Rainey, W. E., Shay, J. W., and Mason, J. I. (1986) ACTH induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase, cholesterol biosynthesis, and steroidogenesis in primary cultures of bovine adrenocortical cells. *J Biol Chem* **261**, 7322-7326

21. Lange, Y., Echevarria, F., and Steck, T. L. (1991) Movement of zymosterol, a precursor of cholesterol, among three membranes in human fibroblasts. *J Biol Chem* **266**, 21439-21443

22. Bloch, K. (1952) Biological synthesis of cholesterol. *Harvey Lect* **48**, 68-88

23. Sato, R., Goldstein, J. L., and Brown, M. S. (1993) Replacement of serine-871 of hamster 3-hydroxy-3-methylglutaryl-CoA reductase prevents phosphorylation by AMP-activated kinase and blocks inhibition of sterol synthesis induced by ATP depletion. *Proc Natl Acad Sci U S A* **90**, 9261-9265

24. Goldstein, J. L., DeBose-Boyd, R. A., and Brown, M. S. (2006) Protein sensors for membrane sterols. *Cell* **124**, 35-46

25. Raghow, R., Yellaturu, C., Deng, X., Park, E. A., and Elam, M. B. (2008) SREBPs: the crossroads of physiological and pathological lipid homeostasis. *Trends Endocrinol Metab* **19**, 65-73

26. Ness, G. C., and Chambers, C. M. (2000) Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase: the concept of cholesterol buffering capacity. *Proc Soc Exp Biol Med* **224**, 8-19

27. Nohturfft, A., Yabe, D., Goldstein, J. L., Brown, M. S., and Espenshade, P. J. (2000) Regulated step in cholesterol feedback localized to budding of SCAP from ER membranes. *Cell* **102**, 315-323

28. Plump, A. S., Erickson, S. K., Weng, W., Partin, J. S., Breslow, J. L., and Williams, D. L. (1996) Apolipoprotein A-I is required for cholesteryl ester accumulation in steroidogenic cells and for normal adrenal steroid production. *J Clin Invest* **97**, 2660-2671

29. Azhar, S., and Reaven, E. (2002) Scavenger receptor class BI and selective cholesteryl ester uptake: partners in the regulation of steroidogenesis. *Mol Cell Endocrinol* **195**, 1-26

30. Azhar, S., Leers-Sucheta, S., and Reaven, E. (2003) Cholesterol uptake in adrenal and gonadal tissues: the SR-BI and 'selective' pathway connection. *Front Biosci* **8**, s998-1029

31. Lestavel, S., and Fruchart, J. C. (1994) Lipoprotein receptors. *Cell Mol Biol (Noisy-le-grand)* **40**, 461-481

32. Cartwright, I. J., and Higgins, J. A. (1998) Molecular and intracellular events in the assembly and secretion of chylomicrons by enterocytes. *Biochem Soc Trans* **26**, 211-216

33. Chang, T. Y., Chang, C. C., Ohgami, N., and Yamauchi, Y. (2006) Cholesterol sensing, trafficking, and esterification. *Annu Rev Cell Dev Biol* **22**, 129-157

34. Rinninger, F., and Pittman, R. C. (1987) Regulation of the selective uptake of high density lipoprotein-associated cholesteryl esters. *J Lipid Res* **28**, 1313-1325

35. Murray, J. W., and Wolkoff, A. W. (2003) Roles of the cytoskeleton and motor proteins in endocytic sorting. *Adv Drug Deliv Rev* **55**, 1385-1403

36. Jordens, I., Marsman, M., Kuijl, C., and Neefjes, J. (2005) Rab proteins, connecting transport and vesicle fusion. *Traffic* **6**, 1070-1077

37. Connelly, M. A., and Williams, D. L. (2003) SR-BI and cholesterol uptake into steroidogenic cells. *Trends Endocrinol Metab* **14**, 467-472

38. Hu, J., Zhang, Z., Shen, W. J., and Azhar, S. (2010) Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr Metab (Lond)* **7**, 47

39. Werder, M., Han, C. H., Wehrli, E., Bimmler, D., Schulthess, G., and Hauser, H. (2001) Role of scavenger receptors SR-BI and CD36 in selective sterol uptake in the small intestine. *Biochemistry* **40**, 11643-11650

40. Maxfield, F. R., and Tabas, I. (2005) Role of cholesterol and lipid organization in disease. *Nature* **438**, 612-621

41. Simons, K., and van Meer, G. (1988) Lipid sorting in epithelial cells. *Biochemistry* **27**, 6197-6202

42. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31-39

43. van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* **9**, 112-124

44. Marsh, D. (2007) Lateral pressure profile, spontaneous curvature frustration, and the incorporation and conformation of proteins in membranes. *Biophys J* **93**, 3884-3899

45. Paradies, G., Paradies, V., De Benedictis, V., Ruggiero, F. M., and Petrosillo, G. (2014) Functional role of cardiolipin in mitochondrial bioenergetics. *Biochim Biophys Acta* **1837**, 408-417

46. Leventis, P. A., and Grinstein, S. (2010) The distribution and function of phosphatidylserine in cellular membranes. *Annu Rev Biophys* **39**, 407-427

47. Clarke, O. B., Tomasek, D., Jorge, C. D., Dufrisne, M. B., Kim, M., Banerjee, S., Rajashankar, K. R., Shapiro, L., Hendrickson, W. A., Santos, H., and Mancia, F. (2015) Structural basis for phosphatidylinositol-phosphate biosynthesis. *Nat Commun* **6**, 8505

48. Kooijman, E. E., and Burger, K. N. (2009) Biophysics and function of phosphatidic acid: a molecular perspective. *Biochim Biophys Acta* **1791**, 881-888

49. Athenstaedt, K., and Daum, G. (1999) Phosphatidic acid, a key intermediate in lipid metabolism. *Eur J Biochem* **266**, 1-16

50. Meyer zu Heringdorf, D., and Jakobs, K. H. (2007) Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. *Biochim Biophys Acta* **1768**, 923-940

51. Kolesnick, R., and Hannun, Y. A. (1999) Ceramide and apoptosis. *Trends Biochem Sci* **24**, 224-225; author reply 227

52. Tepper, A. D., Ruurs, P., Wiedmer, T., Sims, P. J., Borst, J., and van Blitterswijk, W. J. (2000) Sphingomyelin hydrolysis to ceramide during the execution phase of apoptosis results from phospholipid scrambling and alters cell-surface morphology. *J Cell Biol* **150**, 155-164

53. Megha, Sawatzki, P., Kolter, T., Bittman, R., and London, E. (2007) Effect of ceramide N-acyl chain and polar headgroup structure on the properties of ordered lipid domains (lipid rafts). *Biochim Biophys Acta* **1768**, 2205-2212

54. Spector, A. A., and Yorek, M. A. (1985) Membrane lipid composition and cellular function. *J Lipid Res* **26**, 1015-1035

55. Lange, Y., and Steck, T. L. (2008) Cholesterol homeostasis and the escape tendency (activity) of plasma membrane cholesterol. *Prog Lipid Res* **47**, 319-332

56. Lange, Y., Tabei, S. M., Ye, J., and Steck, T. L. (2013) Stability and stoichiometry of bilayer phospholipid-cholesterol complexes: relationship to cellular sterol distribution and homeostasis. *Biochemistry* **52**, 6950-6959

57. Simons, K., and Vaz, W. L. (2004) Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct* **33**, 269-295

58. Miller, W. L., and Bose, H. S. (2011) Early steps in steroidogenesis: intracellular cholesterol trafficking. *J Lipid Res* **52**, 2111-2135

59. Contreras, F. X., Sanchez-Magraner, L., Alonso, A., and Goni, F. M. (2010) Transbilayer (flip-flop) lipid motion and lipid scrambling in membranes. *FEBS Lett* **584**, 1779-1786

60. Drin, G. (2014) Topological regulation of lipid balance in cells. *Annu Rev Biochem* **83**, 51-77

61. Brown, D. A., and Rose, J. K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533-544

62. Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**, 569-572

63. Garcia-Saez, A. J., Chiantia, S., and Schwille, P. (2007) Effect of line tension on the lateral organization of lipid membranes. *J Biol Chem* **282**, 33537-33544

64. Lingwood, D., and Simons, K. (2010) Lipid Rafts As a Membrane-Organizing Principle. *Science* **327**, 46-50

65. Simons, K., and Ehehalt, R. (2002) Cholesterol, lipid rafts, and disease. *J Clin Invest* **110**, 597-603

66. Ikonen, E. (2008) Cellular cholesterol trafficking and compartmentalization. *Nat Rev Mol Cell Biol* **9**, 125-138

67. Lange, Y., and Steck, T. L. (2016) Active membrane cholesterol as a physiological effector. *Chem Phys Lipids* **199**, 74-93

68. Steck, T. L., and Lange, Y. (2010) Cell cholesterol homeostasis: mediation by active cholesterol. *Trends Cell Biol* **20**, 680-687

69. Rone, M. B., Fan, J., and Papadopoulos, V. (2009) Cholesterol transport in steroid biosynthesis: role of protein-protein interactions and implications in disease states. *Biochim Biophys Acta* **1791**, 646-658

70. Liscum, L., and Munn, N. J. (1999) Intracellular cholesterol transport. *Biochim Biophys Acta* **1438**, 19-37

71. Coxey, R. A., Pentchev, P. G., Campbell, G., and Blanchette-Mackie, E. J. (1993) Differential accumulation of cholesterol in Golgi compartments of normal and Niemann-Pick type C fibroblasts incubated with LDL: a cytochemical freeze-fracture study. *J Lipid Res* **34**, 1165-1176

72. Mukherjee, S., Zha, X., Tabas, I., and Maxfield, F. R. (1998) Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog. *Biophys J* **75**, 1915-1925

73. Lange, Y. (1991) Disposition of intracellular cholesterol in human fibroblasts. *J Lipid Res* **32**, 329-339

74. Midzak, A., and Papadopoulos, V. (2014) Binding domain-driven intracellular trafficking of sterols for synthesis of steroid hormones, bile acids and oxysterols. *Traffic* **15**, 895-914

75. Papadopoulos, V., and Miller, W. L. (2012) Role of mitochondria in steroidogenesis. *Best Pract Res Clin Endocrinol Metab* **26**, 771-790

76. Urano, Y., Watanabe, H., Murphy, S. R., Shibuya, Y., Geng, Y., Peden, A. A., Chang, C. C., and Chang, T. Y. (2008) Transport of LDL-derived cholesterol from the NPC1 compartment to the ER involves the trans-Golgi network and the SNARE protein complex. *Proc Natl Acad Sci U S A* **105**, 16513-16518

77. Wustner, D., Solanko, L., Sokol, E., Garvik, O., Li, Z., Bittman, R., Korte, T., and Herrmann, A. (2011) Quantitative assessment of sterol traffic in living cells by dual

labeling with dehydroergosterol and BODIPY-cholesterol. *Chem Phys Lipids* **164**, 221-235

78. Schneede, A., Schmidt, C. K., Holtta-Vuori, M., Heeren, J., Willenborg, M., Blanz, J., Domanskyy, M., Breiden, B., Brodesser, S., Landgrebe, J., Sandhoff, K., Ikonen, E., Saftig, P., and Eskelinen, E. L. (2011) Role for LAMP-2 in endosomal cholesterol transport. *J Cell Mol Med* **15**, 280-295

79. Wang, L., Wang, J., Cai, W., Shi, Y., Zhou, X., Guo, G., Guo, C., Huang, X., Han, Z., Zhang, S., Ma, S., Zhou, X., Fan, D., Eric Gershwin, M., and Han, Y. (2017) A Critical Evaluation of Liver Pathology in Humans with Danon Disease and Experimental Correlates in a Rat Model of LAMP-2 Deficiency. *Clin Rev Allergy Immunol*

80. Midzak, A., and Papadopoulos, V. (2016) Adrenal Mitochondria and Steroidogenesis: From Individual Proteins to Functional Protein Assemblies. *Front Endocrinol (Lausanne)* **7**, 106

81. Kobayashi, T., Beuchat, M. H., Chevallier, J., Makino, A., Mayran, N., Escola, J. M., Lebrand, C., Cosson, P., Kobayashi, T., and Gruenberg, J. (2002) Separation and characterization of late endosomal membrane domains. *J Biol Chem* **277**, 32157-32164

82. Charman, M., Kennedy, B. E., Osborne, N., and Karten, B. (2010) MLN64 mediates egress of cholesterol from endosomes to mitochondria in the absence of functional Niemann-Pick Type C1 protein. *J Lipid Res* **51**, 1023-1034

83. Goldstein, J. L., and Brown, M. S. (2009) The LDL receptor. *Arterioscler Thromb Vasc Biol* **29**, 431-438

84. Goldstein, J. L., Brown, M. S., Anderson, R. G., Russell, D. W., and Schneider, W. J. (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu Rev Cell Biol* **1**, 1-39

85. Aguisanda, F., Thorne, N., and Zheng, W. (2017) Targeting Wolman Disease and Cholesteryl Ester Storage Disease: Disease Pathogenesis and Therapeutic Development. *Curr Chem Genom Transl Med* **11**, 1-18

86. Du, H., Sheriff, S., Bezerra, J., Leonova, T., and Grabowski, G. A. (1998) Molecular and enzymatic analyses of lysosomal acid lipase in cholesteryl ester storage disease. *Mol Genet Metab* **64**, 126-134

87. Sugii, S., Reid, P. C., Ohgami, N., Du, H., and Chang, T. Y. (2003) Distinct endosomal compartments in early trafficking of low density lipoprotein-derived cholesterol. *J Biol Chem* **278**, 27180-27189

88. Grosshans, B. L., Ortiz, D., and Novick, P. (2006) Rabs and their effectors: achieving specificity in membrane traffic. *Proc Natl Acad Sci U S A* **103**, 11821-11827

89. Numrich, J., and Ungermann, C. (2014) Endocytic Rabs in membrane trafficking and signaling. *Biol Chem* **395**, 327-333

90. Buhman, K. K., Chen, H. C., and Farese, R. V., Jr. (2001) The enzymes of neutral lipid synthesis. *J Biol Chem* **276**, 40369-40372

91. Chang, T. Y., Li, B. L., Chang, C. C., and Urano, Y. (2009) Acyl-coenzyme A:cholesterol acyltransferases. *Am J Physiol Endocrinol Metab* **297**, E1-9

92. Goodman, J. M. (2008) The gregarious lipid droplet. *J Biol Chem* **283**, 28005-28009

93. Ohsaki, Y., Cheng, J., Suzuki, M., Shinohara, Y., Fujita, A., and Fujimoto, T. (2009) Biogenesis of cytoplasmic lipid droplets: from the lipid ester globule in the membrane to the visible structure. *Biochim Biophys Acta* **1791**, 399-407

94. Wojtanik, K. M., and Liscum, L. (2003) The transport of low density lipoprotein-derived cholesterol to the plasma membrane is defective in NPC1 cells. *J Biol Chem* **278**, 14850-14856

95. Park, W. D., O'Brien, J. F., Lundquist, P. A., Kraft, D. L., Vockley, C. W., Karnes, P. S., Patterson, M. C., and Snow, K. (2003) Identification of 58 novel mutations in Niemann-Pick disease type C: correlation with biochemical phenotype and importance of PTC1-like domains in NPC1. *Hum Mutat* **22**, 313-325

96. Vanier, M. T., and Millat, G. (2003) Niemann-Pick disease type C. *Clin Genet* **64**, 269-281

97. Infante, R. E., Wang, M. L., Radhakrishnan, A., Kwon, H. J., Brown, M. S., and Goldstein, J. L. (2008) NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes. *Proc Natl Acad Sci U S A* **105**, 15287-15292

98. Wang, M. L., Motamed, M., Infante, R. E., Abi-Mosleh, L., Kwon, H. J., Brown, M. S., and Goldstein, J. L. (2010) Identification of surface residues on Niemann-Pick C2 essential for hydrophobic handoff of cholesterol to NPC1 in lysosomes. *Cell Metab* **12**, 166-173

99. Pentchev, P. G., Comly, M. E., Kruth, H. S., Patel, S., Proestel, M., and Weintroub, H. (1986) The cholesterol storage disorder of the mutant BALB/c mouse. A primary genetic lesion closely linked to defective esterification of exogenously derived cholesterol and its relationship to human type C Niemann-Pick disease. *J Biol Chem* **261**, 2772-2777

100. Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C. B., Tomasetto, C., Gerton, G. L., Rio, M. C., Baker, M. E., and Strauss, J. F., 3rd. (1997) MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. *Proc Natl Acad Sci U S A* **94**, 8462-8467

101. Bose, H. S., Whittal, R. M., Huang, M. C., Baldwin, M. A., and Miller, W. L. (2000) N-218 MLN64, a protein with StAR-like steroidogenic activity, is folded and cleaved similarly to StAR. *Biochemistry* **39**, 11722-11731

102. Olvera-Sanchez, S., Espinosa-Garcia, M. T., Monreal, J., Flores-Herrera, O., and Martinez, F. (2011) Mitochondrial heat shock protein participates in placental steroidogenesis. *Placenta* **32**, 222-229

103. Prinz, W. A. (2007) Non-vesicular sterol transport in cells. *Prog Lipid Res* **46**, 297-314

104. DeGrella, R. F., and Simoni, R. D. (1982) Intracellular transport of cholesterol to the plasma membrane. *J Biol Chem* **257**, 14256-14262

105. Issop, L., Fan, J., Lee, S., Rone, M. B., Basu, K., Mui, J., and Papadopoulos, V. (2015) Mitochondria-associated membrane formation in hormone-stimulated Leydig cell steroidogenesis: role of ATAD3. *Endocrinology* **156**, 334-345

106. Venugopal, S., Martinez-Arguelles, D. B., Chebbi, S., Hullin-Matsuda, F., Kobayashi, T., and Papadopoulos, V. (2016) Plasma membrane origin of the steroidogenic pool of cholesterol used in hormone-induced acute steroid formation in Leydig cells. *J Biol Chem*

107. McLean, L. R., and Phillips, M. C. (1981) Mechanism of cholesterol and phosphatidylcholine exchange or transfer between unilamellar vesicles. *Biochemistry* **20**, 2893-2900

108. Phillips, M. C., Johnson, W. J., and Rothblat, G. H. (1987) Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim Biophys Acta* **906**, 223-276

109. Frolov, A., Woodford, J. K., Murphy, E. J., Billheimer, J. T., and Schroeder, F. (1996) Spontaneous and protein-mediated sterol transfer between intracellular membranes. *J Biol Chem* **271**, 16075-16083

110. Wattenberg, B. W., and Silbert, D. F. (1983) Sterol partitioning among intracellular membranes. Testing a model for cellular sterol distribution. *J Biol Chem* **258**, 2284-2289

111. Tsujishita, Y., and Hurley, J. H. (2000) Structure and lipid transport mechanism of a StAR-related domain. *Nat Struct Biol* **7**, 408-414

112. Krueger, K. E., and Papadopoulos, V. (1990) Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. *J Biol Chem* **265**, 15015-15022

113. Prasad, M., Kaur, J., Pawlak, K. J., Bose, M., Whittal, R. M., and Bose, H. S. (2015) Mitochondria-associated endoplasmic reticulum membrane (MAM) regulates steroidogenic activity via steroidogenic acute regulatory protein (StAR)-voltage-dependent anion channel 2 (VDAC2) interaction. *J Biol Chem* **290**, 2604-2616

114. Miller, W. L. (2013) Steroid hormone synthesis in mitochondria. *Mol Cell Endocrinol* **379**, 62-73

115. Payne, A. H. (1990) Hormonal regulation of cytochrome P450 enzymes, cholesterol side-chain cleavage and 17 alpha-hydroxylase/C17-20 lyase in Leydig cells. *Biol Reprod* **42**, 399-404

116. Liu, J., Rone, M. B., and Papadopoulos, V. (2006) Protein-protein interactions mediate mitochondrial cholesterol transport and steroid biosynthesis. *J Biol Chem* **281**, 38879-38893

117. Boujrad, N., Gaillard, J. L., Garnier, M., and Papadopoulos, V. (1994) Acute action of choriogonadotropin on Leydig tumor cells: induction of a higher affinity benzodiazepine-binding site related to steroid biosynthesis. *Endocrinology* **135**, 1576-1583

118. Li, H., Degenhardt, B., Tobin, D., Yao, Z. X., Tasken, K., and Papadopoulos, V. (2001) Identification, localization, and function in steroidogenesis of PAP7: a peripheral-type benzodiazepine receptor- and PKA (RIalpha)-associated protein. *Molecular endocrinology (Baltimore, Md.)* **15**, 2211-2228

119. Costa, E., and Guidotti, A. (1991) Diazepam binding inhibitor (DBI): a peptide with multiple biological actions. *Life Sci* **49**, 325-344

120. Boujrad, N., Hudson, J. R., Jr., and Papadopoulos, V. (1993) Inhibition of hormone-stimulated steroidogenesis in cultured Leydig tumor cells by a cholesterol-linked phosphorothioate oligodeoxynucleotide antisense to diazepam-binding inhibitor. *Proc Natl Acad Sci U S A* **90**, 5728-5731

121. Fan, J., Liu, J., Culty, M., and Papadopoulos, V. (2010) Acyl-coenzyme A binding domain containing 3 (ACBD3; PAP7; GCP60): an emerging signaling molecule. *Prog Lipid Res* **49**, 218-234

122. Pon, L. A., and Orme-Johnson, N. R. (1986) Acute stimulation of steroidogenesis in corpus luteum and adrenal cortex by peptide hormones. Rapid induction of a similar protein in both tissues. *J Biol Chem* **261**, 6594-6599

123. Clark, B. J., Wells, J., King, S. R., and Stocco, D. M. (1994) The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *J Biol Chem* **269**, 28314-28322

124. King, S. R., Ronen-Fuhrmann, T., Timberg, R., Clark, B. J., Orly, J., and Stocco, D. M. (1995) Steroid production after in vitro transcription, translation, and mitochondrial processing of protein products of complementary deoxyribonucleic acid for steroidogenic acute regulatory protein. *Endocrinology* **136**, 5165-5176

125. McEnery, M. W., Snowman, A. M., Trifiletti, R. R., and Snyder, S. H. (1992) Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier. *Proc Natl Acad Sci U S A* **89**, 3170-3174

126. Boujrad, N., Vidic, B., and Papadopoulos, V. (1996) Acute action of choriogonadotropin on Leydig tumor cells: changes in the topography of the mitochondrial peripheral-type benzodiazepine receptor. *Endocrinology* **137**, 5727-5730

127. Delavoie, F., Li, H., Hardwick, M., Robert, J. C., Giatzakis, C., Peranzi, G., Yao, Z. X., Maccario, J., Lacapere, J. J., and Papadopoulos, V. (2003) In vivo and in vitro peripheral-type benzodiazepine receptor polymerization: functional significance in drug ligand and cholesterol binding. *Biochemistry* **42**, 4506-4519

128. Hauet, T., Yao, Z. X., Bose, H. S., Wall, C. T., Han, Z., Li, W., Hales, D. B., Miller, W. L., Culty, M., and Papadopoulos, V. (2005) Peripheral-type benzodiazepine receptor-mediated action of steroidogenic acute regulatory protein on cholesterol entry into leydig cell mitochondria. *Molecular endocrinology (Baltimore, Md.)* **19**, 540-554

129. Midzak, A., Akula, N., Lecanu, L., and Papadopoulos, V. (2011) Novel androstenetriol interacts with the mitochondrial translocator protein and controls steroidogenesis. *J Biol Chem* **286**, 9875-9887

130. Jaremko, L., Jaremko, M., Giller, K., Becker, S., and Zweckstetter, M. (2014) Structure of the mitochondrial translocator protein in complex with a diagnostic ligand. *Science* **343**, 1363-1366

131. Garnier, M., Dimchev, A. B., Boujrad, N., Price, J. M., Musto, N. A., and Papadopoulos, V. (1994) In vitro reconstitution of a functional peripheral-type benzodiazepine receptor from mouse Leydig tumor cells. *Molecular pharmacology* **45**, 201-211

132. Campbell, A. M., and Chan, S. H. (2007) The voltage dependent anion channel affects mitochondrial cholesterol distribution and function. *Arch Biochem Biophys* **466**, 203-210

133. Issop, L., Rone, M. B., and Papadopoulos, V. (2013) Organelle plasticity and interactions in cholesterol transport and steroid biosynthesis. *Mol Cell Endocrinol* **371**, 34-46

134. Pon, L. A., Epstein, L. F., and Orme-Johnson, N. R. (1986) Acute cAMP stimulation in Leydig cells: rapid accumulation of a protein similar to that detected in adrenal cortex and corpus luteum. *Endocr Res* **12**, 429-446

135. Arakane, F., King, S. R., Du, Y., Kallen, C. B., Walsh, L. P., Watari, H., Stocco, D. M., and Strauss, J. F., 3rd. (1997) Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. *J Biol Chem* **272**, 32656-32662

136. Duarte, A., Castillo, A. F., Podesta, E. J., and Poderoso, C. (2014) Mitochondrial fusion and ERK activity regulate steroidogenic acute regulatory protein localization in mitochondria. *PloS one* **9**, e100387

137. Aghazadeh, Y., Rone, M. B., Blonder, J., Ye, X., Veenstra, T. D., Hales, D. B., Culty, M., and Papadopoulos, V. (2012) Hormone-induced 14-3-3gamma adaptor protein regulates steroidogenic acute regulatory protein activity and steroid biosynthesis in MA-10 Leydig cells. *J Biol Chem* **287**, 15380-15394

138. Crivello, J. F., and Jefcoate, C. R. (1980) Intracellular movement of cholesterol in rat adrenal cells. Kinetics and effects of inhibitors. *J Biol Chem* **255**, 8144-8151

139. Bose, H. S., Sugawara, T., Strauss, J. F., 3rd, Miller, W. L., and International Congenital Lipoid Adrenal Hyperplasia, C. (1996) The pathophysiology and genetics of congenital lipoid adrenal hyperplasia. *N Engl J Med* **335**, 1870-1878

140. Hasegawa, T., Zhao, L., Caron, K. M., Majdic, G., Suzuki, T., Shizawa, S., Sasano, H., and Parker, K. L. (2000) Developmental roles of the steroidogenic acute regulatory protein (StAR) as revealed by StAR knockout mice. *Molecular endocrinology (Baltimore, Md.)* **14**, 1462-1471

141. Arakane, F., Sugawara, T., Nishino, H., Liu, Z., Holt, J. A., Pain, D., Stocco, D. M., Miller, W. L., and Strauss, J. F., 3rd. (1996) Steroidogenic acute regulatory protein (StAR) retains activity in the absence of its mitochondrial import sequence: implications for the mechanism of StAR action. *Proc Natl Acad Sci U S A* **93**, 13731-13736

142. Clark, B. J. (2012) The mammalian START domain protein family in lipid transport in health and disease. *J Endocrinol* **212**, 257-275

143. Fleury, A., Mathieu, A. P., Ducharme, L., Hales, D. B., and LeHoux, J. G. (2004) Phosphorylation and function of the hamster adrenal steroidogenic acute regulatory protein (StAR). *J Steroid Biochem Mol Biol* **91**, 259-271

144. Bose, M., Whittal, R. M., Miller, W. L., and Bose, H. S. (2008) Steroidogenic activity of StAR requires contact with mitochondrial VDAC1 and phosphate carrier protein. *J Biol Chem* **283**, 8837-8845

145. Braestrup, C., and Squires, R. F. (1977) Specific benzodiazepine receptors in rat brain characterized by high-affinity (3H)diazepam binding. *Proc Natl Acad Sci U S A* **74**, 3805-3809

146. Papadopoulos, V., Baraldi, M., Guilarte, T. R., Knudsen, T. B., Lacapere, J. J., Lindemann, P., Norenberg, M. D., Nutt, D., Weizman, A., Zhang, M. R., and Gavish, M. (2006) Translocator protein (18kDa): new nomenclature for the peripheral-type

benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol Sci* **27**, 402-409

147. Papadopoulos, V., Berkovich, A., Krueger, K. E., Costa, E., and Guidotti, A. (1991) Diazepam binding inhibitor and its processing products stimulate mitochondrial steroid biosynthesis via an interaction with mitochondrial benzodiazepine receptors. *Endocrinology* **129**, 1481-1488

148. Papadopoulos, V., Nowzari, F. B., and Krueger, K. E. (1991) Hormone-stimulated steroidogenesis is coupled to mitochondrial benzodiazepine receptors. Tropic hormone action on steroid biosynthesis is inhibited by flunitrazepam. *J Biol Chem* **266**, 3682-3687

149. Fan, J., Campioli, E., Midzak, A., Culty, M., and Papadopoulos, V. (2015) Conditional steroidogenic cell-targeted deletion of TSPO unveils a crucial role in viability and hormone-dependent steroid formation. *Proc Natl Acad Sci U S A* **112**, 7261-7266

150. Jaremko, M., Jaremko, L., Jaipuria, G., Becker, S., and Zweckstetter, M. (2015) Structure of the mammalian TSPO/PBR protein. *Biochem Soc Trans* **43**, 566-571

151. Rone, M. B., Liu, J., Blonder, J., Ye, X., Veenstra, T. D., Young, J. C., and Papadopoulos, V. (2009) Targeting and insertion of the cholesterol-binding translocator protein into the outer mitochondrial membrane. *Biochemistry* **48**, 6909-6920

152. Papadopoulos, V., Liu, J., and Culty, M. (2007) Is there a mitochondrial signaling complex facilitating cholesterol import? *Mol Cell Endocrinol* **265-266**, 59-64

153. Li, H., Yao, Z., Degenhardt, B., Teper, G., and Papadopoulos, V. (2001) Cholesterol binding at the cholesterol recognition/ interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide. *Proc Natl Acad Sci U S A* **98**, 1267-1272

154. Costa, B., Pini, S., Gabelloni, P., Da Pozzo, E., Abelli, M., Lari, L., Preve, M., Lucacchini, A., Cassano, G. B., and Martini, C. (2009) The spontaneous Ala147Thr amino acid substitution within the translocator protein influences pregnenolone production in lymphomonocytes of healthy individuals. *Endocrinology* **150**, 5438-5445

155. Anholt, R. R., De Souza, E. B., Kuhar, M. J., and Snyder, S. H. (1985) Depletion of peripheral-type benzodiazepine receptors after hypophysectomy in rat adrenal gland and testis. *Eur J Pharmacol* **110**, 41-46

156. Besman, M. J., Yanagibashi, K., Lee, T. D., Kawamura, M., Hall, P. F., and Shively, J. E. (1989) Identification of des-(Gly-Ile)-endozepine as an effector of corticotropin-dependent adrenal steroidogenesis: stimulation of cholesterol delivery is mediated by the peripheral benzodiazepine receptor. *Proc Natl Acad Sci U S A* **86**, 4897-4901

157. Papadopoulos, V., Guarneri, P., Kreuger, K. E., Guidotti, A., and Costa, E. (1992) Pregnenolone biosynthesis in C6-2B glioma cell mitochondria: regulation by a mitochondrial diazepam binding inhibitor receptor. *Proc Natl Acad Sci U S A* **89**, 5113-5117

158. Lacapere, J. J., and Papadopoulos, V. (2003) Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. *Steroids* **68**, 569-585

159. Rupprecht, R., Papadopoulos, V., Rammes, G., Baghai, T. C., Fan, J., Akula, N., Groyer, G., Adams, D., and Schumacher, M. (2010) Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. *Nat Rev Drug Discov* **9**, 971-988

160. Colombini, M., and Mannella, C. A. (2012) VDAC, the early days. *Biochim Biophys Acta* **1818**, 1438-1443

161. Mertins, B., Psakis, G., and Essen, L. O. (2014) Voltage-dependent anion channels: the wizard of the mitochondrial outer membrane. *Biol Chem* **395**, 1435-1442

162. Messina, A., Reina, S., Guarino, F., and De Pinto, V. (2012) VDAC isoforms in mammals. *Biochim Biophys Acta* **1818**, 1466-1476

163. Raghavan, A., Sheiko, T., Graham, B. H., and Craigen, W. J. (2012) Voltage-dependant anion channels: novel insights into isoform function through genetic models. *Biochim Biophys Acta* **1818**, 1477-1485

164. Sileikyte, J., Petronilli, V., Zulian, A., Dabbeni-Sala, F., Tognon, G., Nikolov, P., Bernardi, P., and Ricchelli, F. (2011) Regulation of the inner membrane mitochondrial permeability transition by the outer membrane translocator protein (peripheral benzodiazepine receptor). *J Biol Chem* **286**, 1046-1053

165. Brdiczka, D. G., Zorov, D. B., and Sheu, S. S. (2006) Mitochondrial contact sites: their role in energy metabolism and apoptosis. *Biochim Biophys Acta* **1762**, 148-163

166. Da Cruz, S., Xenarios, I., Langridge, J., Vilbois, F., Parone, P. A., and Martinou, J. C. (2003) Proteomic analysis of the mouse liver mitochondrial inner membrane. *J Biol Chem* **278**, 41566-41571

167. Gilquin, B., Taillebourg, E., Cherradi, N., Hubstenberger, A., Gay, O., Merle, N., Assard, N., Fauvarque, M. O., Tomohiro, S., Kuge, O., and Baudier, J. (2010) The AAA+ ATPase ATAD3A controls mitochondrial dynamics at the interface of the inner and outer membranes. *Mol Cell Biol* **30**, 1984-1996

168. Li, S., and Rousseau, D. (2012) ATAD3, a vital membrane bound mitochondrial ATPase involved in tumor progression. *Journal of bioenergetics and biomembranes* **44**, 189-197

169. Welch, G. R. (1977) On the role of organized multienzyme systems in cellular metabolism: a general synthesis. *Prog Biophys Mol Biol* **32**, 103-191

170. Lieberman, S., and Prasad, V. V. (1990) Heterodox notions on pathways of steroidogenesis. *Endocr Rev* **11**, 469-493

171. Heinz, A., Hannemann, F., Muller, J. J., Heinemann, U., and Bernhardt, R. (2005) The interaction domain of the redox protein adrenodoxin is mandatory for binding of the electron acceptor CYP11A1, but is not required for binding of the electron donor adrenodoxin reductase. *Biochem Biophys Res Commun* **338**, 491-498

172. Brentano, S. T., Black, S. M., Lin, D., and Miller, W. L. (1992) cAMP post-transcriptionally diminishes the abundance of adrenodoxin reductase mRNA. *Proc Natl Acad Sci U S A* **89**, 4099-4103

173. Harikrishna, J. A., Black, S. M., Szklarz, G. D., and Miller, W. L. (1993) Construction and function of fusion enzymes of the human cytochrome P450scc system. *DNA Cell Biol* **12**, 371-379

174. DuBois, R. N., Simpson, E. R., Tuckey, J., Lambeth, J. D., and Waterman, M. R. (1981) Evidence for a higher molecular weight precursor of cholesterol side-chain-cleavage cytochrome P-450 and induction of mitochondrial and cytosolic proteins by corticotropin in adult bovine adrenal cells. *Proc Natl Acad Sci U S A* **78**, 1028-1032

175. Ou, W. J., Ito, A., Morohashi, K., Fujii-Kuriyama, Y., and Omura, T. (1986) Processing-independent in vitro translocation of cytochrome P-450(SCC) precursor across mitochondrial membranes. *J Biochem* **100**, 1287-1296

176. Nelson, D. R., and Strobel, H. W. (1988) On the membrane topology of vertebrate cytochrome P-450 proteins. *J Biol Chem* **263**, 6038-6050

177. Headlam, M. J., Wilce, M. C., and Tuckey, R. C. (2003) The F-G loop region of cytochrome P450scc (CYP11A1) interacts with the phospholipid membrane. *Biochim Biophys Acta* **1617**, 96-108

178. Storbeck, K. H., Swart, P., and Swart, A. C. (2007) Cytochrome P450 side-chain cleavage: insights gained from homology modeling. *Mol Cell Endocrinol* **265-266**, 65-70

179. Shikita, M., and Hall, P. F. (1974) The stoichiometry of the conversion of cholesterol and hydroxycholesterols to pregnenolone (3beta-hydroxypregn-5-en-20-one) catalysed by adrenal cytochrome P-450. *Proc Natl Acad Sci U S A* **71**, 1441-1445

180. Muller, A., Muller, J. J., Muller, Y. A., Uhlmann, H., Bernhardt, R., and Heinemann, U. (1998) New aspects of electron transfer revealed by the crystal structure of a truncated bovine adrenodoxin, Adx(4-108). *Structure* **6**, 269-280

181. Ewen, K. M., Hannemann, F., Iametti, S., Morleo, A., and Bernhardt, R. (2011) Functional characterization of Fdx1: evidence for an evolutionary relationship between P450-type and ISC-type ferredoxins. *J Mol Biol* **413**, 940-951

182. Shen, W. J., Azhar, S., and Kraemer, F. B. (2016) Lipid droplets and steroidogenic cells. *Exp Cell Res* **340**, 209-214

183. Thiam, A. R., Farese, R. V., Jr., and Walther, T. C. (2013) The biophysics and cell biology of lipid droplets. *Nat Rev Mol Cell Biol* **14**, 775-786

184. Gross, D. A., and Silver, D. L. (2014) Cytosolic lipid droplets: from mechanisms of fat storage to disease. *Crit Rev Biochem Mol Biol* **49**, 304-326

185. Suzuki, M., Shinohara, Y., Ohsaki, Y., and Fujimoto, T. (2011) Lipid droplets: size matters. *J Electron Microsc (Tokyo)* **60 Suppl 1**, S101-116

186. Krahmer, N., Farese, R. V., Jr., and Walther, T. C. (2013) Balancing the fat: lipid droplets and human disease. *EMBO Mol Med* **5**, 973-983

187. Zanghellini, J., Wodlei, F., and von Grunberg, H. H. (2010) Phospholipid demixing and the birth of a lipid droplet. *J Theor Biol* **264**, 952-961

188. Bartz, R., Li, W. H., Venables, B., Zehmer, J. K., Roth, M. R., Welti, R., Anderson, R. G., Liu, P., and Chapman, K. D. (2007) Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. *J Lipid Res* **48**, 837-847

189. Wrobel, K. H., Sinowatz, F., and Mademann, R. (1981) Intertubular topography in the bovine testis. *Cell and tissue research* **217**, 289-310

190. Zirkin, B. R., Ewing, L. L., Kromann, N., and Cochran, R. C. (1980) Testosterone secretion by rat, rabbit, guinea pig, dog, and hamster testes perfused in vitro: correlation with Leydig cell ultrastructure. *Endocrinology* **107**, 1867-1874

191. Liao, C., Reaven, E., and Azhar, S. (1993) Age-related decline in the steroidogenic capacity of isolated rat Leydig cells: a defect in cholesterol mobilization and processing. *J Steroid Biochem Mol Biol* **46**, 39-47

192. Paniagua, R., Amat, P., Nistal, M., and Martin, A. (1986) Ultrastructure of Leydig cells in human ageing testes. *J Anat* **146**, 173-183

193. Chung, K. W., and Hamilton, J. B. (1975) Testicular lipids in mice with testicular feminization. *Cell and tissue research* **160**, 69-80

194. Zoller, L. C., and Malamed, S. (1975) Acute effects of ACTH on dissociated adrenocortical cells: quantitative changes in mitochondria and lipid droplets. *Anat Rec* **182**, 473-478

195. Aoki, A., and Massa, E. M. (1975) Subcellular compartmentation of free and esterified cholesterol in the interstitial cells of the mouse testis. *Cell and tissue research* **165**, 49-62

196. Shen, W. J., Zaidi, S. K., Patel, S., Cortez, Y., Ueno, M., Azhar, R., Azhar, S., and Kraemer, F. B. (2012) Ablation of vimentin results in defective steroidogenesis. *Endocrinology* **153**, 3249-3257

197. Boyd, G. S., McNamara, B., Suckling, K. E., and Tocher, D. R. (1983) Cholesterol metabolism in the adrenal cortex. *J Steroid Biochem* **19**, 1017-1027

198. Kalantari, F., Bergeron, J. J., and Nilsson, T. (2010) Biogenesis of lipid droplets--how cells get fatter. *Mol Membr Biol* **27**, 462-468

199. Xu, N., Zhang, S. O., Cole, R. A., McKinney, S. A., Guo, F., Haas, J. T., Bobba, S., Farese, R. V., Jr., and Mak, H. Y. (2012) The FATP1-DGAT2 complex facilitates lipid droplet expansion at the ER-lipid droplet interface. *J Cell Biol* **198**, 895-911

200. Wilfling, F., Wang, H., Haas, J. T., Krahmer, N., Gould, T. J., Uchida, A., Cheng, J. X., Graham, M., Christiano, R., Frohlich, F., Liu, X., Buhman, K. K., Coleman, R. A., Bewersdorf, J., Farese, R. V., Jr., and Walther, T. C. (2013) Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocalizing from the ER to lipid droplets. *Dev Cell* **24**, 384-399

201. Ozeki, S., Cheng, J., Tauchi-Sato, K., Hatano, N., Taniguchi, H., and Fujimoto, T. (2005) Rab18 localizes to lipid droplets and induces their close apposition to the endoplasmic reticulum-derived membrane. *Journal of cell science* **118**, 2601-2611

202. Christensen, A. K., and Fawcett, D. W. (1966) The fine structure of testicular interstitial cells in mice. *Am J Anat* **118**, 551-571

203. Rhodin, J. A. (1971) The ultrastructure of the adrenal cortex of the rat under normal and experimental conditions. *J Ultrastruct Res* **34**, 23-71

204. Yamaguchi, T., Fujikawa, N., Nimura, S., Tokuoka, Y., Tsuda, S., Aiuchi, T., Kato, R., Obama, T., and Itabe, H. (2015) Characterization of lipid droplets in steroidogenic MLTC-1 Leydig cells: Protein profiles and the morphological change induced by hormone stimulation. *Biochim Biophys Acta* **1851**, 1285-1295

205. Jacquier, N., Choudhary, V., Mari, M., Toulmay, A., Reggiori, F., and Schneiter, R. (2011) Lipid droplets are functionally connected to the endoplasmic reticulum in Saccharomyces cerevisiae. *Journal of cell science* **124**, 2424-2437

206. Walther, T. C., and Farese, R. V., Jr. (2012) Lipid droplets and cellular lipid metabolism. *Annu Rev Biochem* **81**, 687-714

207. Lee, K., Kerner, J., and Hoppel, C. L. (2011) Mitochondrial carnitine palmitoyltransferase 1a (CPT1a) is part of an outer membrane fatty acid transfer complex. *J Biol Chem* **286**, 25655-25662

208. Shen, W. J., Patel, S., Eriksson, J. E., and Kraemer, F. B. (2010) Vimentin is a functional partner of hormone sensitive lipase and facilitates lipolysis. *J Proteome Res* **9**, 1786-1794

209. Nan, X., Potma, E. O., and Xie, X. S. (2006) Nonperturbative chemical imaging of organelle transport in living cells with coherent anti-stokes Raman scattering microscopy. *Biophys J* **91**, 728-735

210. Fong, T. H., Yang, C. C., Greenberg, A. S., and Wang, S. M. (2002) Immunocytochemical studies on lipid droplet-surface proteins in adrenal cells. *J Cell Biochem* **86**, 432-439

211. Kimmel, A. R., Brasaemle, D. L., McAndrews-Hill, M., Sztalryd, C., and Londos, C. (2010) Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. *J Lipid Res* **51**, 468-471

212. McFie, P. J., Banman, S. L., Kary, S., and Stone, S. J. (2011) Murine diacylglycerol acyltransferase-2 (DGAT2) can catalyze triacylglycerol synthesis and promote lipid droplet formation independent of its localization to the endoplasmic reticulum. *J Biol Chem* **286**, 28235-28246

213. Hu, J., Prinz, W. A., and Rapoport, T. A. (2011) Weaving the web of ER tubules. *Cell* **147**, 1226-1231

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

215. Martin, L. A., Kennedy, B. E., and Karten, B. (2016) Mitochondrial cholesterol: mechanisms of import and effects on mitochondrial function. *Journal of bioenergetics and biomembranes* **48**, 137-151

216. Franke, W. W., and Kartenbeck, J. (1971) Outer mitochondrial membrane continuous with endoplasmic reticulum. *Protoplasma* **73**, 35-41

217. Vance, J. E. (1990) Phospholipid synthesis in a membrane fraction associated with mitochondria. *J Biol Chem* **265**, 7248-7256

218. Mannella, C. A., Buttle, K., Rath, B. K., and Marko, M. (1998) Electron microscopic tomography of rat-liver mitochondria and their interaction with the endoplasmic reticulum. *Biofactors* **8**, 225-228

219. Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A., and Pozzan, T. (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. *Science* **280**, 1763-1766

220. Csordas, G., Varnai, P., Golenar, T., Roy, S., Purkins, G., Schneider, T. G., Balla, T., and Hajnoczky, G. (2010) Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. *Mol Cell* **39**, 121-132

221. Raturi, A., and Simmen, T. (2013) Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). *Biochim Biophys Acta* **1833**, 213-224

222. Hayashi, T., and Su, T. P. (2010) Cholesterol at the endoplasmic reticulum: roles of the sigma-1 receptor chaperone and implications thereof in human diseases. *Subcell Biochem* **51**, 381-398

223. de Brito, O. M., and Scorrano, L. (2008) Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* **456**, 605-610

224. Simmen, T., Lynes, E. M., Gesson, K., and Thomas, G. (2010) Oxidative protein folding in the endoplasmic reticulum: tight links to the mitochondria-associated membrane (MAM). *Biochim Biophys Acta* **1798**, 1465-1473

225. Smirnova, E., Shurland, D. L., Ryazantsev, S. N., and van der Bliek, A. M. (1998) A human dynamin-related protein controls the distribution of mitochondria. *J Cell Biol* **143**, 351-358

226. Palmer, C. S., Osellame, L. D., Stojanovski, D., and Ryan, M. T. (2011) The regulation of mitochondrial morphology: intricate mechanisms and dynamic machinery. *Cell Signal* **23**, 1534-1545

227. Szabadkai, G., Bianchi, K., Varnai, P., De Stefani, D., Wieckowski, M. R., Cavagna, D., Nagy, A. I., Balla, T., and Rizzuto, R. (2006) Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels. *J Cell Biol* **175**, 901-911

228. Marriott, K. S., Prasad, M., Thapliyal, V., and Bose, H. S. (2012) sigma-1 receptor at the mitochondrial-associated endoplasmic reticulum membrane is responsible for mitochondrial metabolic regulation. *J Pharmacol Exp Ther* **343**, 578-586

229. Hayashi, T., and Su, T. P. (2003) Sigma-1 receptors (sigma(1) binding sites) form raft-like microdomains and target lipid droplets on the endoplasmic reticulum: roles in endoplasmic reticulum lipid compartmentalization and export. *J Pharmacol Exp Ther* **306**, 718-725

230. Lewin, T. M., Kim, J. H., Granger, D. A., Vance, J. E., and Coleman, R. A. (2001) Acyl-CoA synthetase isoforms 1, 4, and 5 are present in different subcellular membranes in rat liver and can be inhibited independently. *J Biol Chem* **276**, 24674-24679

231. Maloberti, P., Castilla, R., Castillo, F., Cornejo Maciel, F., Mendez, C. F., Paz, C., and Podesta, E. J. (2005) Silencing the expression of mitochondrial acyl-CoA thioesterase I and acyl-CoA synthetase 4 inhibits hormone-induced steroidogenesis. *FEBS J* **272**, 1804-1814

232. Freeman, D. A. (1987) Cyclic AMP mediated modification of cholesterol traffic in Leydig tumor cells. *J Biol Chem* **262**, 13061-13068

233. Freeman, D. A. (1989) Plasma membrane cholesterol: removal and insertion into the membrane and utilization as substrate for steroidogenesis. *Endocrinology* **124**, 2527-2534

234. Nagy, L., and Freeman, D. A. (1990) Effect of cholesterol transport inhibitors on steroidogenesis and plasma membrane cholesterol transport in cultured MA-10 Leydig tumor cells. *Endocrinology* **126**, 2267-2276

235. Porpaczy, Z., Tomasek, J. J., and Freeman, D. A. (1997) Internalized plasma membrane cholesterol passes through an endosome compartment that is distinct from the acid vesicle-lysosome compartment. *Exp Cell Res* **234**, 217-224

236. Hao, M., Lin, S. X., Karylowski, O. J., Wustner, D., McGraw, T. E., and Maxfield, F. R. (2002) Vesicular and non-vesicular sterol transport in living cells. The endocytic recycling compartment is a major sterol storage organelle. *J Biol Chem* **277**, 609-617

237. West, M., Zurek, N., Hoenger, A., and Voeltz, G. K. (2011) A 3D analysis of yeast ER structure reveals how ER domains are organized by membrane curvature. *J Cell Biol* **193**, 333-346

238. Wu, M. M., Buchanan, J., Luik, R. M., and Lewis, R. S. (2006) Ca2+ store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. *J Cell Biol* **174**, 803-813

239. Pichler, H., Gaigg, B., Hrastnik, C., Achleitner, G., Kohlwein, S. D., Zellnig, G., Perktold, A., and Daum, G. (2001) A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids. *Eur J Biochem* **268**, 2351-2361

240. Manford, A. G., Stefan, C. J., Yuan, H. L., Macgurn, J. A., and Emr, S. D. (2012) ER-to-plasma membrane tethering proteins regulate cell signaling and ER morphology. *Dev Cell* **23**, 1129-1140

241. Wieckowski, M. R., Szabadkai, G., Wasilewski, M., Pinton, P., Duszynski, J., and Rizzuto, R. (2006) Overexpression of adenine nucleotide translocase reduces Ca2+ signal transmission between the ER and mitochondria. *Biochem Biophys Res Commun* **348**, 393-399

242. Zhang, S. L., Yu, Y., Roos, J., Kozak, J. A., Deerinck, T. J., Ellisman, M. H., Stauderman, K. A., and Cahalan, M. D. (2005) STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. *Nature* **437**, 902-905

243. Luik, R. M., Wang, B., Prakriya, M., Wu, M. M., and Lewis, R. S. (2008) Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. *Nature* **454**, 538-542

244. Lebiedzinska, M., Szabadkai, G., Jones, A. W., Duszynski, J., and Wieckowski, M. R. (2009) Interactions between the endoplasmic reticulum, mitochondria, plasma membrane and other subcellular organelles. *Int J Biochem Cell Biol* **41**, 1805-1816

245. Suski, J. M., Lebiedzinska, M., Wojtala, A., Duszynski, J., Giorgi, C., Pinton, P., and Wieckowski, M. R. (2014) Isolation of plasma membrane-associated membranes from rat liver. *Nature protocols* **9**, 312-322

246. Soccio, R. E., and Breslow, J. L. (2004) Intracellular cholesterol transport. *Arterioscler Thromb Vasc Biol* **24**, 1150-1160

247. Ascoli, M., Fanelli, F., and Segaloff, D. L. (2002) The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocr Rev* **23**, 141-174

248. Stevens, V. L., Xu, T., and Lambeth, J. D. (1992) Cholesterol pools in rat adrenal mitochondria: use of cholesterol oxidase to infer a complex pool structure. *Endocrinology* **130**, 1557-1563

249. Gocze, P. M., and Freeman, D. A. (1993) Plasma membrane cholesterol is utilized as steroidogenic substrate in Y-1 mouse adrenal tumor cells and normal sheep adrenal cells. *Exp Cell Res* **209**, 21-25

250. Mesmin, B., and Maxfield, F. R. (2009) Intracellular sterol dynamics. *Biochim Biophys Acta* **1791**, 636-645

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

252. Hughson, F. M., and Reinisch, K. M. (2010) Structure and mechanism in membrane trafficking. *Curr Opin Cell Biol* **22**, 454-460

253. Prinz, W. A. (2010) Lipid trafficking sans vesicles: where, why, how? *Cell* **143**, 870-874

254. Prinz, W. A. (2014) Bridging the gap: membrane contact sites in signaling, metabolism, and organelle dynamics. *J Cell Biol* **205**, 759-769

255. Koziel, K., Lebiedzinska, M., Szabadkai, G., Onopiuk, M., Brutkowski, W., Wierzbicka, K., Wilczynski, G., Pinton, P., Duszynski, J., Zablocki, K., and Wieckowski, M. R. (2009) Plasma membrane associated membranes (PAM) from Jurkat cells contain STIM1 protein is PAM involved in the capacitative calcium entry? *Int J Biochem Cell Biol* **41**, 2440-2449

256. Lebiedzinska, M., Duszynski, J., Rizzuto, R., Pinton, P., and Wieckowski, M. R. (2009) Age-related changes in levels of p66Shc and serine 36-phosphorylated p66Shc in organs and mouse tissues. *Arch Biochem Biophys* **486**, 73-80

257. Perry, R. J., and Ridgway, N. D. (2006) Oxysterol-binding protein and vesicle-associated membrane protein-associated protein are required for sterol-dependent activation of the ceramide transport protein. *Mol Biol Cell* **17**, 2604-2616

258. Vance, J. E., and Tasseva, G. (2013) Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. *Biochim Biophys Acta* **1831**, 543-554

259. Yan, D., and Olkkonen, V. M. (2008) Characteristics of oxysterol binding proteins. *Int Rev Cytol* **265**, 253-285

260. Achleitner, G., Gaigg, B., Krasser, A., Kainersdorfer, E., Kohlwein, S. D., Perktold, A., Zellnig, G., and Daum, G. (1999) Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. *Eur J Biochem* **264**, 545-553

261. Wustner, D., Mondal, M., Tabas, I., and Maxfield, F. R. (2005) Direct observation of rapid internalization and intracellular transport of sterol by macrophage foam cells. *Traffic* **6**, 396-412

262. Ishitsuka, R., Saito, T., Osada, H., Ohno-Iwashita, Y., and Kobayashi, T. (2011) Fluorescence image screening for chemical compounds modifying cholesterol metabolism and distribution. *J Lipid Res* **52**, 2084-2094

263. Shimada, Y., Maruya, M., Iwashita, S., and Ohno-Iwashita, Y. (2002) The C-terminal domain of perfringolysin O is an essential cholesterol-binding unit targeting to cholesterol-rich microdomains. *Eur J Biochem* **269**, 6195-6203

264. Abe, M., Makino, A., Hullin-Matsuda, F., Kamijo, K., Ohno-Iwashita, Y., Hanada, K., Mizuno, H., Miyawaki, A., and Kobayashi, T. (2012) A role for sphingomyelin-rich lipid domains in the accumulation of phosphatidylinositol-4,5-bisphosphate to the cleavage furrow during cytokinesis. *Mol Cell Biol* **32**, 1396-1407

265. Harmala, A. S., Porn, M. I., Mattjus, P., and Slotte, J. P. (1994) Cholesterol transport from plasma membranes to intracellular membranes is inhibited by 3 beta-[2-(diethylamino)ethoxy]androst-5-en-17-one. *Biochim Biophys Acta* **1211**, 317-325

266. Midzak, A. S., Akula, N., Rone, M. B., and Papadopoulos, V. (2015) Computational modeling and biological validation of novel non-steroidal ligands for the cholesterol recognition/interaction amino acid consensus (CRAC) motif of the mitochondrial translocator protein (TSPO). *Pharmacological research* **99**, 393-403

267. Akula, N., Midzak, A., Lecanu, L., and Papadopoulos, V. (2012) Identification of small-molecule inhibitors of the steroidogenic acute regulatory protein (STARD1) by structure-based design. *Bioorganic & medicinal chemistry letters* **22**, 4139-4143

268. Ohno-Iwashita, Y., Shimada, Y., Waheed, A. A., Hayashi, M., Inomata, M., Nakamura, M., Maruya, M., and Iwashita, S. (2004) Perfringolysin O, a cholesterol-binding cytolysin, as a probe for lipid rafts. *Anaerobe* **10**, 125-134

269. Maekawa, M., and Fairn, G. D. (2015) Complementary probes reveal that phosphatidylserine is required for the proper transbilayer distribution of cholesterol. *Journal of cell science* **128**, 1422-1433

270. Maekawa, M., Yang, Y., and Fairn, G. D. (2016) Perfringolysin O Theta Toxin as a Tool to Monitor the Distribution and Inhomogeneity of Cholesterol in Cellular Membranes. *Toxins (Basel)* **8**

271. Johnson, B. B., Moe, P. C., Wang, D., Rossi, K., Trigatti, B. L., and Heuck, A. P. (2012) Modifications in perfringolysin O domain 4 alter the cholesterol concentration threshold required for binding. *Biochemistry* **51**, 3373-3382

272. Christian, A. E., Haynes, M. P., Phillips, M. C., and Rothblat, G. H. (1997) Use of cyclodextrins for manipulating cellular cholesterol content. *J Lipid Res* **38**, 2264-2272

273. Cenedella, R. J. (2009) Cholesterol synthesis inhibitor U18666A and the role of sterol metabolism and trafficking in numerous pathophysiological processes. *Lipids* **44**, 477-487

274. Crompton, M., Barksby, E., Johnson, N., and Capano, M. (2002) Mitochondrial intermembrane junctional complexes and their involvement in cell death. *Biochimie* **84**, 143-152

275. Kase, E. T., Andersen, B., Nebb, H. I., Rustan, A. C., and Thoresen, G. H. (2006) 22-Hydroxycholesterols regulate lipid metabolism differently than T0901317 in human myotubes. *Biochim Biophys Acta* **1761**, 1515-1522

276. Holtta-Vuori, M., Maatta, J., Ullrich, O., Kuismanen, E., and Ikonen, E. (2000) Mobilization of late-endosomal cholesterol is inhibited by Rab guanine nucleotide dissociation inhibitor. *Curr Biol* **10**, 95-98

277. Cox, B. E., Griffin, E. E., Ullery, J. C., and Jerome, W. G. (2007) Effects of cellular cholesterol loading on macrophage foam cell lysosome acidification. *J Lipid Res* **48**, 1012-1021

278. Bretscher, M. S. (1972) Asymmetrical lipid bilayer structure for biological membranes. *Nat New Biol* **236**, 11-12

279. Ingolfsson, H. I., Melo, M. N., van Eerden, F. J., Arnarez, C., Lopez, C. A., Wassenaar, T. A., Periole, X., de Vries, A. H., Tieleman, D. P., and Marrink, S. J. (2014) Lipid organization of the plasma membrane. *J Am Chem Soc* **136**, 14554-14559

280. Holthuis, J. C., and Levine, T. P. (2005) Lipid traffic: floppy drives and a superhighway. *Nat Rev Mol Cell Biol* **6**, 209-220

281. Kennedy, B. E., Charman, M., and Karten, B. (2012) Niemann-Pick Type C2 protein contributes to the transport of endosomal cholesterol to mitochondria without interacting with NPC1. *J Lipid Res* **53**, 2632-2642

282. Rao, R. M., Jo, Y., Leers-Sucheta, S., Bose, H. S., Miller, W. L., Azhar, S., and Stocco, D. M. (2003) Differential regulation of steroid hormone biosynthesis in R2C and MA-10 Leydig tumor cells: role of SR-B1-mediated selective cholesteryl ester transport. *Biol Reprod* **68**, 114-121

283. de Araujo, M. E., Huber, L. A., and Stasyk, T. (2008) Isolation of endocitic organelles by density gradient centrifugation. *Methods Mol Biol* **424**, 317-331

284. Klinefelter, G. R., Hall, P. F., and Ewing, L. L. (1987) Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. *Biol Reprod* **36**, 769-783

285. Klose, C., Surma, M. A., and Simons, K. (2013) Organellar lipidomics--background and perspectives. *Curr Opin Cell Biol* **25**, 406-413

286. Lee, A. G. (2011) Biological membranes: the importance of molecular detail. *Trends Biochem Sci* **36**, 493-500

287. Andersen, O. S., and Koeppe, R. E., 2nd. (2007) Bilayer thickness and membrane protein function: an energetic perspective. *Annu Rev Biophys Biomol Struct* **36**, 107-130

288. Lee, A. G. (2004) How lipids affect the activities of integral membrane proteins. *Biochim Biophys Acta* **1666**, 62-87

289. Vance, J. E. (2014) MAM (mitochondria-associated membranes) in mammalian cells: lipids and beyond. *Biochim Biophys Acta* **1841**, 595-609

290. Suski, J. M., Lebiedzinska, M., Wojtala, A., Duszynski, J., Giorgi, C., Pinton, P., and Wieckowski, M. R. (2014) Isolation of plasma membrane–associated membranes from rat liver. *Nat. Protocols* **9**, 312-322

291. Wieckowski, M. R., Giorgi, C., Lebiedzinska, M., Duszynski, J., and Pinton, P. (2009) Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells. *Nature protocols* **4**, 1582-1590

292. Gaigg, B., Simbeni, R., Hrastnik, C., Paltauf, F., and Daum, G. (1995) Characterization of a microsomal subfraction associated with mitochondria of the yeast, Saccharomyces cerevisiae. Involvement in synthesis and import of phospholipids into mitochondria. *Biochim Biophys Acta* **1234**, 214-220

293. Di Paolo, G., and De Camilli, P. (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**, 651-657

294. Farese, R. V., Sabir, A. M., Vandor, S. L., and Larson, R. E. (1980) Are polyphosphoinositides the cycloheximide-sensitive mediator in the steroidogenic actions of adrenocorticotropin and adenosine-3',5'-monophosphate? *J Biol Chem* **255**, 5728-5734

295. Knecht, T. P., and Pittman, R. C. (1989) A plasma membrane pool of cholesteryl esters that may mediate the selective uptake of cholesteryl esters from high-density lipoproteins. *Biochim Biophys Acta* **1002**, 365-375

296. Cai, K., Lucki, N. C., and Sewer, M. B. (2014) Silencing diacylglycerol kinase-theta expression reduces steroid hormone biosynthesis and cholesterol metabolism in human adrenocortical cells. *Biochim Biophys Acta* **1841**, 552-562

297. Kwun, C., Patel, A., Pletcher, S., Lyons, B., Abdelrahim, M., Nicholson, D., Morris, E., Salata, K., and Francis, G. L. (1999) Ceramide increases steroid hormone production in MA-10 Leydig cells. *Steroids* **64**, 499-509

298. Voelker, D. R. (2009) Genetic and biochemical analysis of non-vesicular lipid traffic. *Annu Rev Biochem* **78**, 827-856

299. Midzak, A., Rone, M., Aghazadeh, Y., Culty, M., and Papadopoulos, V. (2011) Mitochondrial protein import and the genesis of steroidogenic mitochondria. *Mol Cell Endocrinol* **336**, 70-79

300. Hayashi, T., and Fujimoto, M. (2010) Detergent-resistant microdomains determine the localization of sigma-1 receptors to the endoplasmic reticulum-mitochondria junction. *Molecular pharmacology* **77**, 517-528

301. Area-Gomez, E., Del Carmen Lara Castillo, M., Tambini, M. D., Guardia-Laguarta, C., de Groof, A. J., Madra, M., Ikenouchi, J., Umeda, M., Bird, T. D., Sturley, S. L., and Schon, E. A. (2012) Upregulated function of mitochondria-associated ER membranes in Alzheimer disease. *EMBO J* **31**, 4106-4123

302. Lucki, N. C., and Sewer, M. B. (2008) Multiple roles for sphingolipids in steroid hormone biosynthesis. *Subcell Biochem* **49**, 387-412

303. Tamura, Y., Onguka, O., Itoh, K., Endo, T., Iijima, M., Claypool, S. M., and Sesaki, H. (2012) Phosphatidylethanolamine biosynthesis in mitochondria: phosphatidylserine (PS) trafficking is independent of a PS decarboxylase and intermembrane space proteins UPS1P and UPS2P. *J Biol Chem* **287**, 43961-43971

304. Tamura, Y., Onguka, O., Hobbs, A. E., Jensen, R. E., Iijima, M., Claypool, S. M., and Sesaki, H. (2012) Role for two conserved intermembrane space proteins, Ups1p and Ups2p, [corrected] in intra-mitochondrial phospholipid trafficking. *J Biol Chem* **287**, 15205-15218

305. Tatsuta, T., Scharwey, M., and Langer, T. (2014) Mitochondrial lipid trafficking. *Trends Cell Biol* **24**, 44-52

306. Schneider-Poetsch, T., Ju, J., Eyler, D. E., Dang, Y., Bhat, S., Merrick, W. C., Green, R., Shen, B., and Liu, J. O. (2010) Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat Chem Biol* **6**, 209-217

307. Stevens, V. L., Xu, T., and Lambeth, J. D. (1993) Cholesterol trafficking in steroidogenic cells. Reversible cycloheximide-dependent accumulation of cholesterol in a pre-steroidogenic pool. *Eur J Biochem* **216**, 557-563

308. Andreyev, A. Y., Fahy, E., Guan, Z., Kelly, S., Li, X., McDonald, J. G., Milne, S., Myers, D., Park, H., Ryan, A., Thompson, B. M., Wang, E., Zhao, Y., Brown, H. A., Merrill, A. H., Raetz, C. R., Russell, D. W., Subramaniam, S., and Dennis, E. A. (2010) Subcellular organelle lipidomics in TLR-4-activated macrophages. *J Lipid Res* **51**, 2785-2797

309. Henry, S. A., Kohlwein, S. D., and Carman, G. M. (2012) Metabolism and regulation of glycerolipids in the yeast Saccharomyces cerevisiae. *Genetics* **190**, 317-349

310. Osman, C., Voelker, D. R., and Langer, T. (2011) Making heads or tails of phospholipids in mitochondria. *J Cell Biol* **192**, 7-16

311. Liscovitch, M., and Amsterdam, A. (1989) Gonadotropin-releasing hormone activates phospholipase D in ovarian granulosa cells. Possible role in signal transduction. *J Biol Chem* **264**, 11762-11767

312. Potting, C., Tatsuta, T., Konig, T., Haag, M., Wai, T., Aaltonen, M. J., and Langer, T. (2013) TRIAP1/PRELI complexes prevent apoptosis by mediating intramitochondrial transport of phosphatidic acid. *Cell Metab* **18**, 287-295

313. Vance, J. E. (1991) Newly made phosphatidylserine and phosphatidylethanolamine are preferentially translocated between rat liver mitochondria and endoplasmic reticulum. *J Biol Chem* **266**, 89-97

314. Voelker, D. R. (1984) Phosphatidylserine functions as the major precursor of phosphatidylethanolamine in cultured BHK-21 cells. *Proc Natl Acad Sci U S A* **81**, 2669-2673

315. Ozbay, T., Merrill, A. H., Jr., and Sewer, M. B. (2004) ACTH regulates steroidogenic gene expression and cortisol biosynthesis in the human adrenal cortex via sphingolipid metabolism. *Endocr Res* **30**, 787-794

316. Porn, M. I., Tenhunen, J., and Slotte, J. P. (1991) Increased steroid hormone secretion in mouse Leydig tumor cells after induction of cholesterol translocation by sphingomyelin degradation. *Biochim Biophys Acta* **1093**, 7-12

317. Meroni, S. B., Pellizzari, E. H., Canepa, D. F., and Cigorraga, S. B. (2000) Possible involvement of ceramide in the regulation of rat Leydig cell function. *J Steroid Biochem Mol Biol* **75**, 307-313

318. Santana, P., Llanes, L., Hernandez, I., Gonzalez-Robayna, I., Tabraue, C., Gonzalez-Reyes, J., Quintana, J., Estevez, F., Ruiz de Galarreta, C. M., and Fanjul, L. F. (1996) Interleukin-1 beta stimulates sphingomyelin hydrolysis in cultured granulosa cells: evidence for a regulatory role of ceramide on progesterone and prostaglandin biosynthesis. *Endocrinology* **137**, 2480-2489

319. Degnan, B. M., Bourdelat-Parks, B., Daniel, A., Salata, K., and Francis, G. L. (1996) Sphingomyelinase inhibits in vitro Leydig cell function. *Ann Clin Lab Sci* **26**, 234-242

320. Roff, C. F., Strauss, J. F., 3rd, Goldin, E., Jaffe, H., Patterson, M. C., Agritellis, G. C., Hibbs, A. M., Garfield, M., Brady, R. O., and Pentchev, P. G. (1993) The murine Niemann-Pick type C lesion affects testosterone production. *Endocrinology* **133**, 2913-2923