Structure and function of mitochondria rough-er contact sites in mouse liver

Mémoire

Rana Ghandehari Alavijeh

Maîtrise en neurosciences - avec mémoire
Maître ès sciences (M. Sc.)

Québec, Canada

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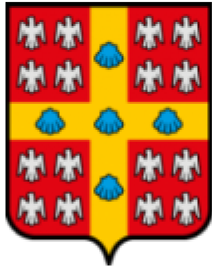

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Résumé

La biologie des sites de contact entre organelles est une nouvelle branche de la biologie cellulaire qui étudie comment les organelles coopèrent ensemble pour coordonner des activités cellulaires complexes. Le premier contact de ce type à être décrit de manière fonctionnelle est celui qui est formé par le réticulum endoplasmique lisse (RE) et la mitochondrie, la MAM (Mitochondria-Associated ER Membrane). La MAM coordonne en effet les échanges de phospholipides et de calcium entre ces deux organelles. Cependant, on ignore si les mitochondries peuvent également former des contacts fonctionnels avec d'autres types de RE. Pour combler ce manque de connaissances, j'ai contribué, dans ma thèse, à étudier pour la première fois l'organisation morphologique et la fonction du contact entre l'ER brut et les mitochondries.

Grâce à l'utilisation de la microscopie électronique (EM) et de la tomographie 3D, j'ai contribué à découvrir l'existence d'un nouveau type d'ER brut, que nous avons appelé wrappER, qui enveloppe étroitement les mitochondries dans les hépatocytes hépatiques de la souris. Sa caractérisation biochimique a révélé la présence de protéines et de transcrits impliqués dans la biogenèse des lipoprotéines de très basse densité (VLDL). De plus, mes études ont permis d'établir que la protéine Rrbp1 sert à lier les deux organelles et de découvrir que sa dérégulation dans le foie de la souris est caractérisée par une augmentation de la distance entre la mitochondrie et le wrappER ainsi qu'une diminution du taux de sécrétion des VLDL. Sur la base de ces résultats, nous avons proposé que les contacts wrappER-mitochondrie participent à l'homéostasie des lipides en régulant le taux de sécrétion des VLDL.

Abstract

The biology of inter-organelle contact sites is a new branch of cell biology that studies how organelles cooperate together to coordinate complex cellular activities. The first contact of this type to be functionally described was that that is formed by the smooth endoplasmic reticulum (ER) and the mitochondrion, the MAM (Mitochondria-Associated ER Membrane). The MAM indeed coordinates phospholipid and calcium exchanges between these two organelles. However, whether mitochondria can also form functionally competent contacts with other types of ER remains unknown. To fill this gap of knowledge, in my thesis I contributed to investigate for the first time the morphological organization and the function of the contact between the rough-ER and the mitochondria.

Through the use of electron microscopy (EM) and 3D tomography, I have contributed to discover the existence a new type of rough-ER, which we called wrappER, that closely wraps the mitochondria in the liver hepatocytes of the mouse. Its biochemical characterization revealed the presence of proteins and transcripts involved in the biogenesis of the very low-density lipoproteins (VLDL). Furthermore, my studies have helped establish that the Rrbp1 protein serves to tether between the two organelles and discovered that its down-regulation in the mouse liver is characterized by an increase in the distance between mitochondrion and wrappER as well as to a decrease in the secretion rate of VLDL. Based on these results, we have proposed that wrappER-mitochondrion contacts participate in lipid homeostasis by regulating the rate of VLDL secretion.

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Abbreviations list

Ca^{2+}: Calcium
MCSs: membrane contacts sites
PI: phosphatidylinositol
MAM: Mitochondria-Associated Membranes
PTP: Permeability Transition Pore
VDACs: Voltage-Dependent Anion Channels
OMM: Outer Mitochondrial Membrane
IMM: Mitochondrial Membrane
MCU: Mitochondrial Uniporter
IP3R: Inositol Triphosphate Receptor
IP3R-Grp75: Glucose-Regulated Protein 75
ERM: Endoplasmic Reticulum Membrane
SYNJ2BP: Synaptojanin-2 Binding Protein
Rrbp1: Ribosome-Binding Protein 1
AD: Alzheimer's Disease
PD: Parkinson's Disease
EOAD: Early-Onset AD
LOAD: Late-Onset AD
APP: Amyloid Precursor Protein
PS1 and PS2: Presenilin 1 and 2
AICD: APP Intracellular Domain
Aβ: Amyloid Beta
α-syn: α-Synuclein
DJ-1: Deglycase
pink1: PTEN-Induced Kinase 1
MFN2: Mitofusin-2
ApoB: Apolipoprotein B
Mttp: Microsomal Triglyceride Transfer Protein
VLDL: Very Low Density Lipid
LD: Lipid Droplet
TAG: Triglycerides
CE: Cholesterol Esters
TCA: Tricarboxylic Acid Cycle
DGAT: Diglyceride Acyltransferase
ET: Electron Tomography
WAMs: Wrapper-Associated Mitochondria
PAGE: Polyacrylamide Gel Electrophoresis
DTT: Dithiothreitol
BPB: Bromophenol Blue
SDS: Sodium Lauryl Sulfate
PVDF: Polyvinylidene Difluoride
HRP: Horseradish Peroxidase
P-407: Poloxamer-407

LPL: Lipoprotein Lipase
FFA: Free Fatty Acids

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First and foremost, I am extremely grateful to my supervisor, Prof. Luca Pellegrini, and to my colleagues, Irene Anastasia and Nicolò Ilacqua, for their invaluable advice, continuous support and patience during my master studies. Their extensive knowledge and plentiful experience have encouraged me throughout my academic research and daily life. I would also like to thank Prof. Katalin Toth and Mme Marie-Ève Chouinard for their invaluable support during my studies. I would like to thank all the members of Faculty of Medicine, their kind help and support have made my study and life in Québec a wonderful time.

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Introduction

The endoplasmic reticulum (ER) is the largest membrane-bound organelle in eukaryotic cells and it is involved in several important cellular functions, including protein synthesis and processing, lipid synthesis, calcium (Ca^{2+}) storage and release (Phillips and Voeltz, 2016). The most extensive ER domain is located around the cell nucleus to form the double membrane shield known as the nuclear envelope. The outer nuclear membrane is branching out in the peripheral ER, which is organized in two structural domains: the flat membrane cisternae (also known as sheets) and the tubules. ER sheets are covered with ribosomes for the synthesis, translocation, and folding of proteins.

ER tubules have considerably fewer ribosomes and are therefore known as the 'smooth' ER. The tubular ER network is known to form several membrane contacts sites (MCSs) with several organelles and with the plasma membrane (Rolls et al., 2002). The concept of contact sites is a relatively new concept in the field of structural biology. Contact sites are defined as areas of close juxtaposition between the membrane of two organelles. They are divided in homotypic (between similar organelles) and heterotypic (between two different organelles or two different membranes type). Examples of heterotypic interactions are those that the ER establishes with mitochondria, plasma membranes (Pichler et al., 2001; Stefan et al., 2011), Golgi (Mesmin et al., 2013), peroxisomes (Costello et al., 2017) and lipid droplets (Gao and Goodman, 2015) (Figure 1.1).

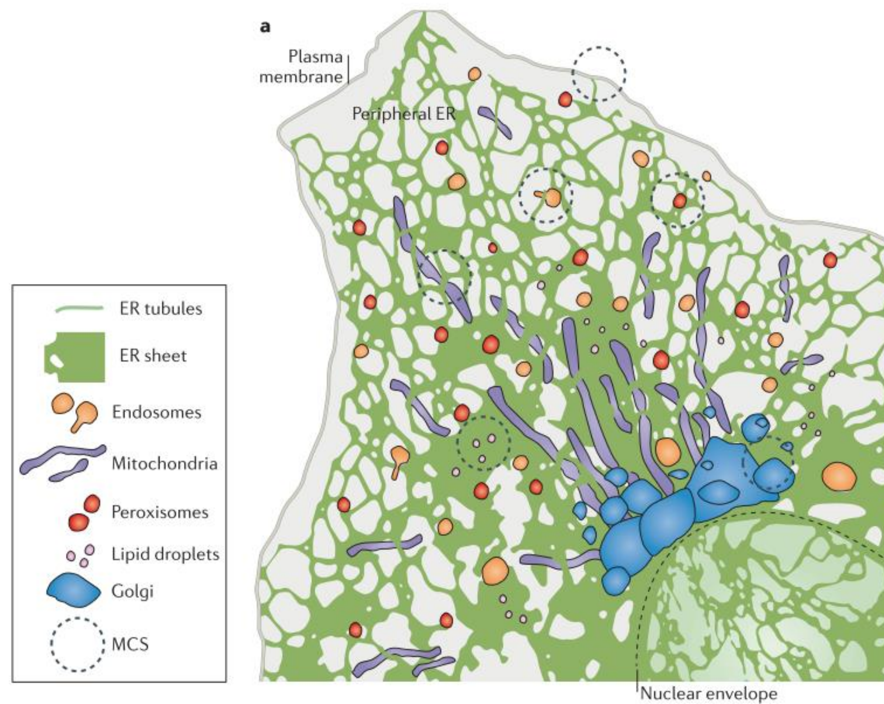


Figure 1.1: Structure of the endoplasmic reticulum (ER) membrane-contact sites (MCSs). The ER consists of the nuclear envelope (outlined with a dashed line) and the peripheral ER, which spreads into the cytosol as a network of sheets and tubules. The peripheral ER forms MCSs with the plasma membrane, mitochondria, endosomes, peroxisomes, lipid droplets and the Golgi (Phillips and Voeltz, 2016).

Studies of electron microscopy have shown for the first time the existence of a contact between two different organelles in 1956. Bernhard and colleagues revealed the presence of close juxtaposition between mitochondria and the ergastoplasm in liver cell (Bernhard and Rouiller, 1956). However, it was only many decades later that new insights were provided about the existence of different ER domains in contact with different organelles.

Chapter 1: The Membrane Contact Sites

1-1 General Principles

In the last two decades, large recognition has been gained in the field of biological communication at membrane contact sites, attracting scientists from several and different fields. The novelty and the constant expansion of the field of organelle contacts has led in 2019 the leading experts to come together to define and unify the determining characteristics of a contact site (Scorrano et al., 2019).

To be called contact site, the juxtaposition of organelles must meet the following characteristics:

- **Must be established by the presence of tethers**: a tether is the the interaction among protein-protein or protein-lipid which is creating a physical bridge between two organelles and leads to be together at a constant distance, which typically spans within 10-80 nm.

- **There must be no fusion between the membranes of the organelles**.

- **Must exist to perform a specific function:** initially the contact sites identified were the ones involving the ER, focusing on the function of the contact sites in the transport of lipids and Ca^{2+}. However, it was noted that more or less all the organelles are able to interact with each other, leading to expand the spectrum of functions performed by these organelles.

To date, three types of functions have been suggested:

(i) The bi-directional transfer of molecules like different ions, Ca^{2+}, metals, amino acids and lipids (Burgoyne et al., 2015; Lahiri et al., 2015).

(ii) Transmission of signaling information or force essential to remodeling activities, including regulation of organelle biogenesis, morphology, succession, placement, fission and autophagy (Wu et al., 2018; Friedman et al., 2011)

(iii) Regulation of enzymes activity, such as the phosphatidylinositol (PI) phosphate phosphatase, Sac1 (Stefan et al., 2011; Dickson et al., 2016).

- **The contact sites must be defined by a specific proteome/lipidome** which is required for all the above (Scorrano et al., 2019).

Initially, contacts were considered to be populated by tethering proteins (tethers) that would establish/maintain the connection between two membranes and by a second collection of proteins that would fulfill specific functions of that special contact site (Levine, 2004). Recently this concept has evolved by formulating the hypothesis that the contact site between the organelles is due to the presence of different molecules that perform both the tethers and enzymatic or transporter functions. This describes why, to date, most interactions have had multiple tethering molecules identified and that removing contact by deleting a single tethering molecule has proven difficult (Eisenberg-Bord et al., 2016).
Nevertheless, at least one role should be played by each contact protein and we define four possible roles that should be identified and enriched at contact sites (Figure 1.2).

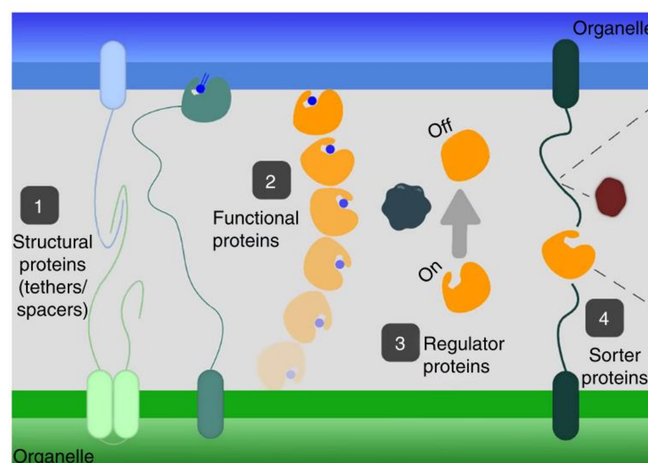


Figure 1.2: Graphical representation of the four types of proteins that should reside in contact sites. Importantly, many proteins can have multiple roles at a contact site (Scorrano et al., 2019).

Class 1: structural protein. These proteins include the tethers, which keep the organelles together, and the spacers, which keep the two membranes at a certain distance, thus forming the contact site and inhibiting the fusion.
The characteristic of tethers is to keep two organelles close together through the realization of a physical bridge. Generally, the tether is on one side anchored to the membrane thanks to a transmembrane domain or lipid modification and on the other side is associated to proteins and/or lipids on the membrane of the other organelle.

Class 2: functional proteins. As mentioned before, a specific function is one the defining characteristic of a contact site. This function is allowed by the of functional proteins that reside in and are enriched by contact. They can transfer ion, protein, lipid, or metabolites, like ion channels and pumps, lipid transfer proteins or metabolite channels/transporters.

Class 3: sorter/recruitment proteins. Sorting and recruiting proteins have been used to characterize the contact-site proteome and lipidome. This can be achieved either by recruiting proteins into the contact site or by repelling contact site non-residents. Sorting can happen through the direct connection to proteins or indirectly by altering lipids or the proteins themselves (such as adding post-translational modifications).
For example, it has been proposed that the mitochondria – ER contact site has a specific lipid structure compared to either mitochondrial and ER membrane (Hayashi and Fujimoto, 2010; Sano et al., 2009) and that palmitoylation acts as a signal to enrich proteins in these particular membrane patches (Blaskovic et al., 2013). Proteins in this category can also identify lipid properties to enrich tethers or functional/regulatory proteins to contacts (Raturi and Simmen, 2013).

Class 4: regulator proteins. These proteins regulate the extent of the contact site itself as well as the function of the active proteins in the contacts. For example, p53 modify the redox level of Ca^{2+} protein handling, thus changing ER-mitochondrial relations. It has recently been shown that any newly defined contact site is characterized by variable combination of those protein classes (Giorgi et al., 2015)

1-2 The Mitochondria-Associated Membranes (MAMs), The First Characterized Interaction Between Endoplasmic Reticulum and Mitochondria

In the last few centuries, different studies have shown that ER and mitochondria form an interaction termed mitochondria-associated membranes (MAMs). These association was described for the first time in 1990 by Jean Vance: she purified this fraction from the rat liver, and she discovered that was enriched in proteins associated with lipid synthesis and trafficking (Vance, 1990).

Since her discoveries, several other studies have shed light on the roles of these appositions, discovering that they are related to lipid metabolism (Vance, 1990), calcium homeostasis (de Vos et al., 2012), cellular respiration (Bravo et al., 2011), mitochondrial DNA replication and mitochondrial division (Lewis et al., 2016), the formation of autophagosomes (Hamasaki et al., 2013) and the onset of apoptosis (Simmen et al., 2005).

These specific interactions and crosstalk between the ER and mitochondria control the crucial functions of the two organelles and thus establish important elements of cell proliferation. In fact, the alternations in the composition of the MAM and the abnormal induction of this interaction between ER and mitochondria lead to different pathological conditions, for instance Alzheimer disease (Marchi and Pinton, 2016).

1-2-1 Synthesis and Exchange of Phospholipids at The Endoplasmic Reticulum-Mitochondria contact sites

The biological membranes are the vital structural elements of all cell types. They preserve the cell from external effects and arrange the interior in separate compartments. In addition to their particular proteome, organelles show unusual lipid structures that affect their structure, physical properties, and function. The major lipid groups present in biological membranes include phospholipids, sterols and sphingolipids which are synthesized in the ER (Flis et al., 2013; van Leer et al., 2008).

Moreover, the primary step of ceramide synthesis is in the ER which provides the precursors for the formation of complex sphingolipids (Futerman, 2006). In addition to the export of ceramides, ER provides a significant portion of lipids to other organelles which are unable to generate their own lipids or have minimal ability to do so, like mitochondria.

Transport of lipids between ER and mitochondria requires a specific membrane contact site. The MAM is a subfraction of the ER which was shown to be in contact or close proximity to the OMM (outer mitochondrial membrane). It is involved in lipid translocation to the mitochondria and harbors a number of lipids synthesizing enzymes (Gaigg et al., 1995; Vance, 1990). The Figure 1.3 represents a scheme of the synthesis and exchange of lipids between ER and mitochondria.

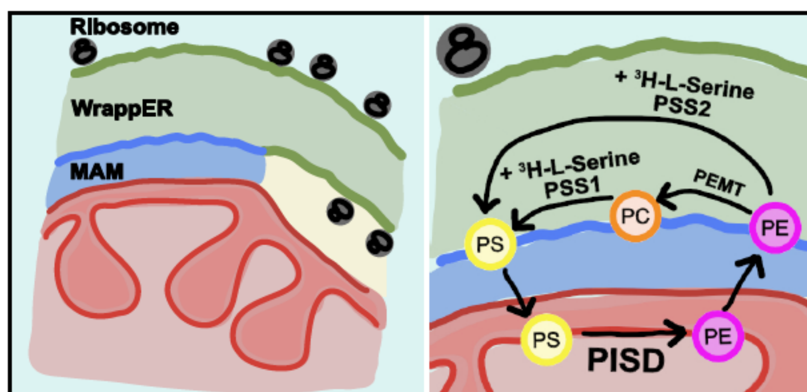


Figure 1.3: Phospholipids are synthesized and transferred between ER and mitochondria at the MAMs.

1-3 Ribosome-Binding Protein 1 (Rrbp1), a rough-ER-mitochondria tether

As previously described, mitochondrial and ER cytosol-facing membranes engage in a wide variety of essential processes (X. Chen et al., 2010; Calvo et al., 2016). At their surface there are proteins that enable signal transduction, regulation of morphology and trafficking, protein import and export, and other specialized processes in the cytosol-facing membranes of cellular organelles.

In 2017 Hung and colleagues performed in living human fibroblasts a screening of endogenous proteins on the OMM and endoplasmic reticulum membrane (ERM) by peroxidase-mediated proximity biotinylation (Hung et al., 2017). The purification of OMM and ERM simply by centrifugations are difficult and available protocols lose a big amount of the residence proteins, hence the need to develop new techniques to acquire more complete data on the proteome of these specialized membranes.
Proximity-based labeling is a biochemical approach that tags biomolecules that are spatially proximal to a protein of interest. These biomolecules can then be selectively identified with biotin for pulldown. Hung et al., in their study, showed a high-quality proteomic map for mitochondrial and ER membrane proteome.
They discovered numerous proteins that were not before known to reside at these membranes. This list is an important reference for better understand OMM and ERM (Hung et al., 2017).

Their study found that Synaptojanin-2 Binding Protein, or SYNJ2BP, was the most heavily enriched among the proteins that appeared in both OMM and ERM proteomes.
SYNJ2BP is a 16 kDa tail-anchored OMM protein with a cytosol-facing PDZ domain. In a mitochondrial transmembrane protein, the presence of a PDZ domain is highly unusual and is suggestive of a binding partner. According to immunofluorescence staining, the localization of endogenous SYNJ2BP protein is in the outer mitochondria membrane and does not appear enriched at sites of ER overlap (Hung et al., 2017).

As a next step, they looked in the list of 56 SYNJ2BP interactors for an ER-localized PDZ-binding motif-containing protein that might serve as a tethering partner for SYNJ2BP. Just one of these proteins has a PDZ-domain, the so-called Ribosome-Binding Protein 1 (Rrbp1), a 166 kDa single-pass transmembrane ER protein, highly enriched in the sheet-like ER (Shibata et al., 2010).

Hung et al. performed immunoprecipitation to confirm the interaction between Rrbp1 and SYNJ2BP. Their results showed that the two proteins are indeed binding across mitochondria-ER junctions through probable PDZ domain-PDZ binding motif interaction (Figure 1.4).

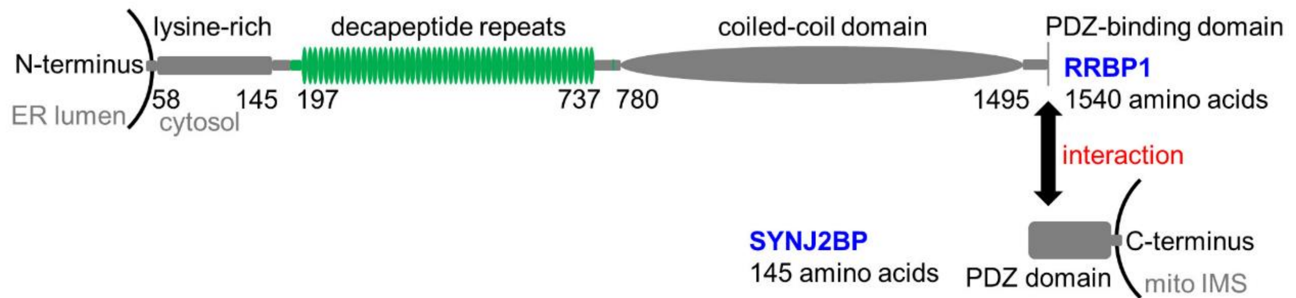


Figure 1.4: Structural domains of SYNJ2BP and Rrbp1. For Rrbp1 (top), regions in green vary among isoforms; isoforms vary in the number of decapeptide repeats (up to 54 repeats) and in the presence or absence of three amino acids after the decapeptide repeat region. The C-terminus of Rrbp1 contains a consensus PDZ-binding domain. SYNJ2BP is a tail anchored OMM protein with a cytosolic PDZ domain (Hung et al., 2017).

Altogether, their results indicate that SYNJ2BP-Rrbp1 could be a novel tether that joins rough ER and mitochondrial membranes and connects these contact sites to protein translation.

1-4 Altered ER-Mitochondria Contact in Neurodegenerative Disease

Communications impairments between ER and mitochondria can lead to neurodegenerative disorders, cancers and metabolic diseases. Various studies indeed have shown that some neurological diseases like Alzheimer's disease (AD) and Parkinson's disease (PD) display structural or functional changes at the MAM (Burns and Iliffe, 2009).

1-4-1 Alzheimer’s Disease, General Principles

Alzheimer's disease (AD) is a common chronic neurodegenerative disease, which typically starts mildly and slowly and eventually worsens over time. It is the cause of more than 50% of dementia cases and it presently affects more than 24 million people worldwide. The

difficulty in remembering recent activities is the most frequent early symptom. As the disease progresses, symptoms can include language difficulties, disorientation (including quickly being confused), mood swings, loss of motivation, not controlling self-care, and behavioral problems.

There is a poor understanding of the causes of Alzheimer's disease (Burns and lliffe, 2009; Bekris et al. 2010). It is known that approximately 70% is inherited by the parents, with several genes typically involved. Other risk factors include a history of head trauma, depression, and hypertension. Plaques and neurofibrillary tangles in the brain are associated with the disease phase (Ballard et al., 2011).

Depending on the age of onset, AD is classified into 2 subtypes: early-onset AD (EOAD) and late-onset AD (LOAD). Early-onset AD reports for around 1% - 6% of all cases and ranges from around 30 years to 60 years. The most common form of AD is LOAD which is classified as AD an age at onset later than 60 years. In people with family history of AD, both LOAD and EOAD can occur. About 60% of EOAD cases have multiple cases of AD within their families, and of these familial EOAD cases, 13% are inherited in an autosomal dominant manner with at least 3 generations affected (Campion et al., 1999; Brickell et al., 2006).

Most autosomal dominant FAD can be linked to mutations in one of these three genes: amyloid precursor protein (APP) encoding and Presenilin 1 and 2 (PS1 and PS2) (Waring and Rosenberg, 2008).

At this date, there are several controversies about the pathophysiology of AD. It is suggested that AD develops when amyloids and probably tau proteins accumulate in the brain, forming plaques that lead to a remarkable lack of neurotransmitters which causes loss of brain function interrupting its normal function and chemistry (Selkoe, 2011; Hardy, 1992).

The current theory is called "amyloid cascade", it explains the progressing of the disease by placing the β-amyloid accumulation at the center of the process.

APP is a type 1 transmembrane protein that is expressed in many tissues and is localized in neuronal synapses. The main function of APP is not well known; however, it has been implicated as a regulator of synapse development (Priller et al., 2006), neuronal plasticity (Turner et al., 2003) and iron export (Duce et al., 2010). APP can undergo series of

proteolytic cleavage by secretase enzymes. APP can chevage via two different pathways: normal cleavage of APP (not amyloidogenic) and abnormal cleavage of APP leading to excess amyloid accumulation (Figure 1.5).

In the first, APP is cleaved by α-secretase and produce a sAPPα and a membrane-bound C-terminal 83 amino acid fragments called C83. This fragment (C83) is then cleaved by the γ-secretase forming a secreted fragment called P3 peptides (P3), which are have a role in neuronal death and in the increase inflammatory response in AD and leaves the membrane-bound APP intracellular domain (AICD) (Soria Lopez et al., 2019).

In the second pathway, called amyloidogenic pathway, APP is cleaved by β-secretase to create the secreted extracellular product called sAPPβ. sAPPβ includes the domains needed to promote the outgrowth of neurites, and reduces cell adhesion and enhances axonal outgrowth like sAPPα (Chasseigneaux and Allinquant, 2012) and the membrane-bound 99 amino acid C-terminal fragment, C99. C99 is then cleaved by γ-secretase generating ~ 50-aa APP intracellular domain (AICD) and a range of Amyloid beta (Aβ) fragments that have ~ 40-aa in normal individuals ~ but 42-aa in AD patients, with a concomitant increase in the Aβ42: Aβ40 ratio. When compared to Aβ40, Aβ42 is fibrillogenic and settles in plaques. This amyloid is poisonous for the cells, and the resulting tension promotes tau to hyperphosphorylate. In healthy neurons, tau normally binds to and stabilizes microtubules, axonal transport, and modulation of signaling pathways, while in Alzheimer's disease tau detaches from microtubules and sticks to other tau molecules, forming threads that eventually join to form tangles inside neuron (Harris et al., 1995; Holtzman et al., 2016; Soria Lopez et al., 2019), with both extra-neural plaques and intraneuronal tangles conspiring to develop the disease by inducing cell death (Francis et al., 1999).

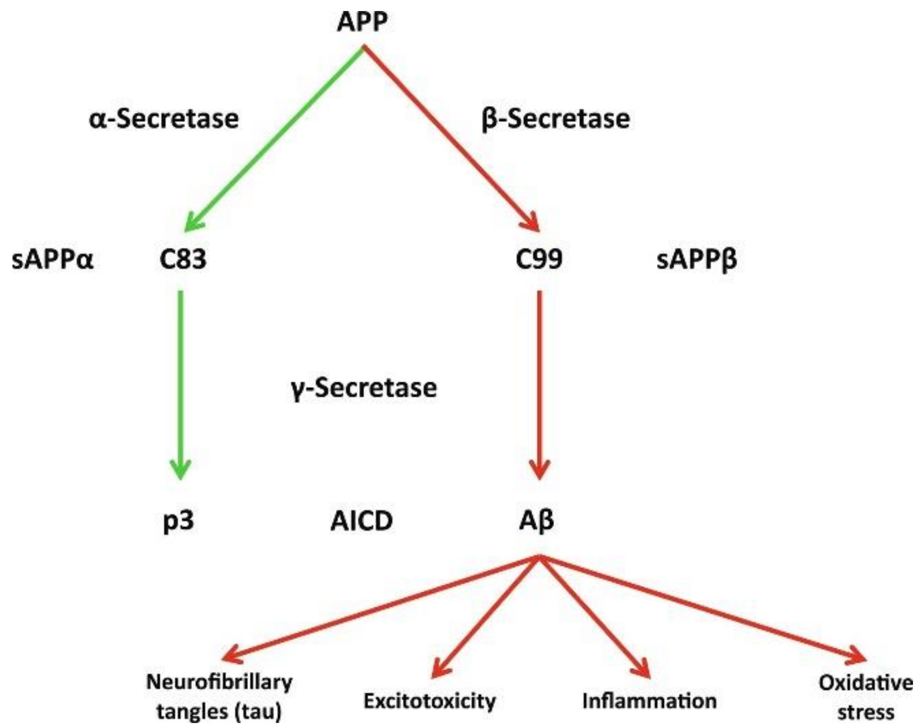


Figure 1.5: **Scheme of APP cleavage by β-secretase and α-secretase** (Soria Lopez et al., 2019)

However, there are other mechanisms that are related to AD and that have received less attention in the field. These include altered cholesterol (Stefani and Liguri, 2009), fatty acid (Fraser et al., 2010), glucose (Hoyer et al. 1988), phospholipid metabolism (Pettegrew et al., 2001), improper calcium homeostasis (Bezprozvanny et al., 2008) and mitochondrial dysfunction (Wang et al., 2009). All these are MAM- related functions, and these are the very ones that are disturbed in AD. As already discussed, proteins that enriched or are localized at the MAMs are associated with fatty acids metabolism and calcium homeostasis. Based on the hypothesis that AD pathogenesis may be affected by changes in the MAMs, Area-Gomez et al. have observed that PS1 and PS2, as well as γ-secretase, are primarily located at MAM (Area-Gomez et al., 2016).

1-4-2 The MAM Hypothesis: A New Way of Thinking Alzheimer's Disease

According to the central function of MAM in Ca^{2+} homeostasis, phospholipid, and cholesterol metabolism, different studies showed that there is an association in the levels of lipids synthesized and regulation of protein complexes present in MAM of AD patients (Area-Gomez et al., 2016).

Gomez and colleagues have proposed that AD pathogenesis is regulated by enhanced ER–mitochondrial contacts, which consequentially alters the role of proteins residing at the interface of these two organelles. The primary site of activity for γ-secretase and APP localization along with the PS1 and PS2 proteins is in the MAM (Pettegrew et al., 2001) (Figure 1.6). The increase in ER-mitochondria apposition is consistent with the increased traffic in calcium between the two organelles (Liang et al., 2015; Mattson, 2010), the aberrant phospholipid profiles (Pettegrew et al., 2001), the disrupted cholesterol homeostasis (Stefani and Liguri, 2009), the shifts in mitochondrial structure, morphology and distribution (Wang et al., 2009) and the increased Aβ42: Aβ40 ratio exhibited in the AD. The altered topology of the ER membrane at the MAM in AD could clarify the shift from Aβ40 to Aβ42 at the position of the γ-secretase cleavage site on APP-C99 (Winkler et al., 2012, Area-Gomez et al., 2016, Schon and Area-Gomez, 2013).

In the end, the functional cause of AD can be enhanced by ER–mitochondrial contact and upregulated MAM function.

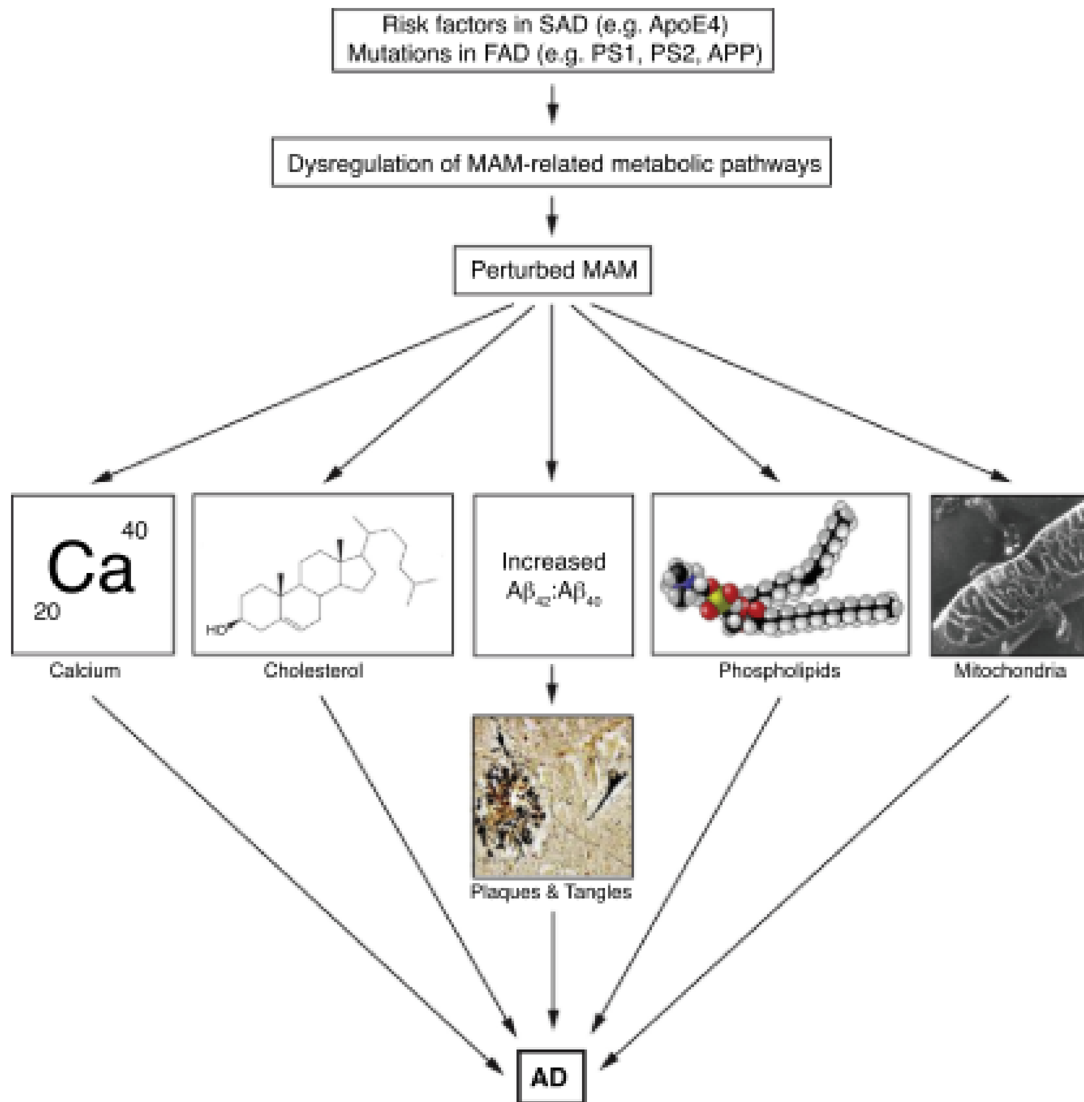


Figure 1.6: The MAM hypothesis. The theory suggests that increased ER – mitochondria contact is the physiological cause of AD. This results in alterations in the specified functions as well as a rise in the Aβ42: Aβ40 ratio; plaques and tangles occur as a result of the downstream disruption. Increased ER-mitochondrial connectivity is the outcome of a disruption in specific biochemical pathways triggered by mutations in PS and APP (in the case of FAD) or by other factors (in the case of SAD) (Area-Gomez et al., 2016).

1-5 Lipid Metabolism in The Liver

The liver performs uptake, synthesis, packaging and secretion of lipids and lipoproteins.
In order to improve the solubility of fat particles in the first digestive stage, they must be converted to microscopically small micelles before absorption into the intestine. This is accomplished by bile salts in the small intestine after consumption of fatty food (Figure 1.7). Bile salts are formed by the liver and stored in the gall bladder.
The solubility of these finely dispersed micelles formed from the detergent action of bile salts has now increased. Triacylglycerol, diacylglycerols and monoacylglycerols are first hydrolyzed by lipases in fatty acids and glycerol and then diffused in the epithelial mucosa. They are converted back within the epithelial mucosa to triacylglycerols and aggregated with cholesterol and apolipoproteins to form chylomicrons.

Proteins that bind lipids and transport lipids, such as TAG, cholesterol, cholesterol esters and phospholipids, are called apolipoproteins. Different types of particles, varying in densities, are produced depending on the combination of the lipid and protein. From very low-density lipoproteins to very high-density lipoproteins, the range can vary.
Cell surfaces have unique lipoprotein receptors for their cells. The chylomicrons including apolipoprotein C-II are thus absorbed through attachment to the receptors for passage from the intestinal mucosa to the lymphatic system. These chylomicrons, depending on their characteristics, enter either the adipose tissue or the muscle through the blood.

Apolipoprotein C-II stimulates the enzyme lipoprotein lipase in the capillaries of adipose tissue and muscles, which breaks down chylomicrons and hydrolyses the triacylglycerols into fatty acids and glycerol. The final products can be used for energy by the muscles, while they are re-esterified into triacylglycerols for storage in the adipose tissue.
The remaining fraction of chylomicrons, which still has the cholesterol and apolipoproteins, travels to the liver through the blood and thanks to the specific receptors they are taken up by endocytosis (Mu and Høy, 2004; Høy and Mu, 2000; Mukherjee, 2003).

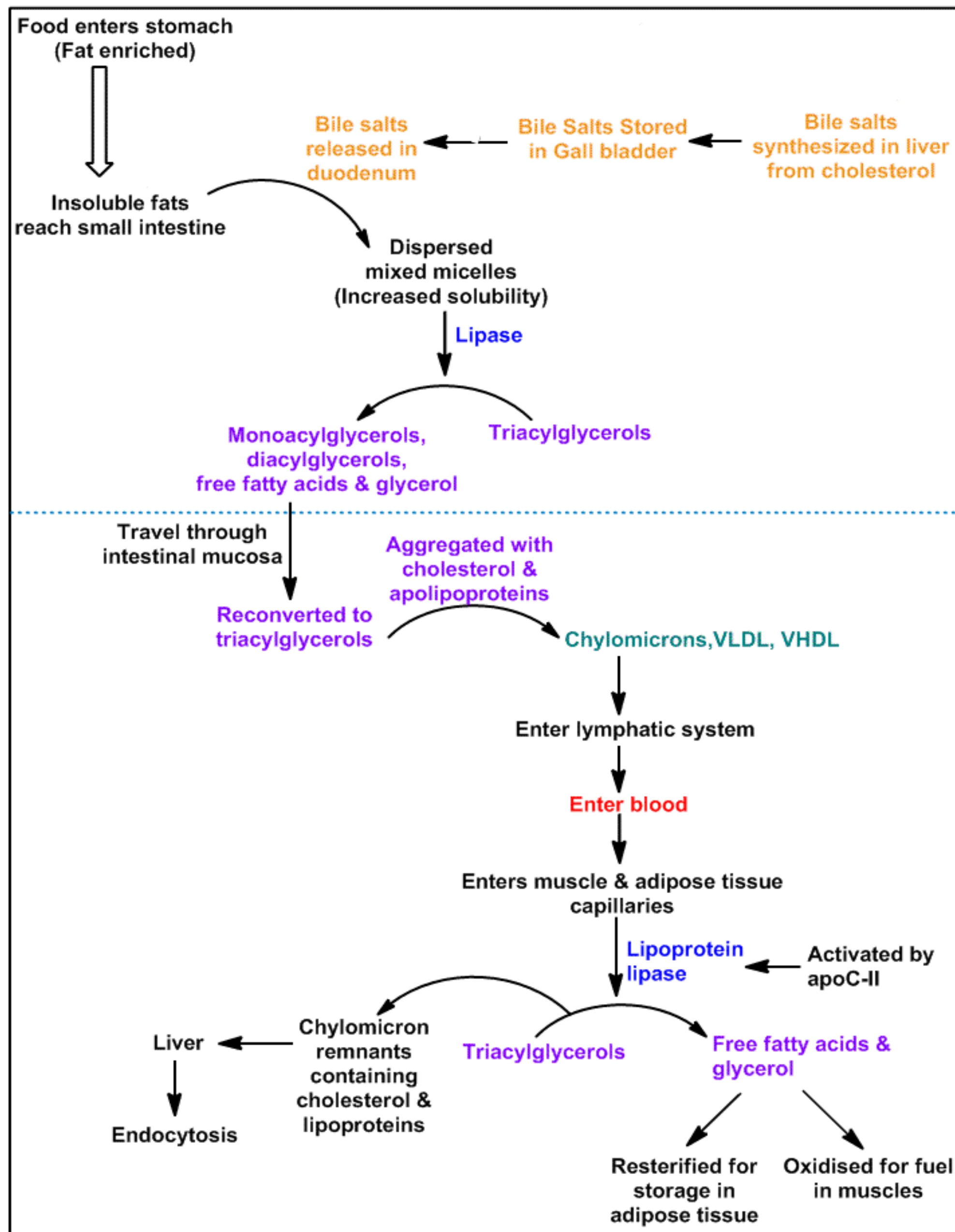


Figure 1.7: Summary of absorption and distribution of lipids in the body (Mehta S., 2013).

The liver can use the fatty acids as an internal source of energy by oxidative pathways, but it can also provide energy by the formation and release of the ketogenic compounds to other organs. For organisms undergoing intense fasting or consuming incredibly low amounts of dietary carbohydrates, this capacity to provide ketones as an energetic substrate is fundamental. The release of ketones from the liver avoids the excess formation of intermediates in the tricarboxylic acid cycle and may thus maintain the oxidative state (Figure

1.8 A). The liver is also essential for the supply of lipid substrates to the body during feeding times. It can indeed assemble fatty acids and glycerol into triglycerides which are packed into very low-density lipoprotein particles (VLDLs) and released into the bloodstream for their secretion from hepatocytes (Figure 1.8 B).

Moreover, the liver plays a critical role in the homeostasis of cholesterol. The cholesterol in the liver can be absorbed from the diet in the intestine or can be *de novo* synthesized. Cholesterol is a molecule necessary for the assembling of cellular membranes and for preserving membrane fluidity. While cholesterol deficiencies can be unhealthy, its excess is also detrimental to health. Excess dietary cholesterol and *de novo* synthesis may lead to unhealthy interactions in the cell membrane, which may promote inflammatory processes that contribute to atherosclerosis or cardiovascular disease (Trefts et al., 2017).

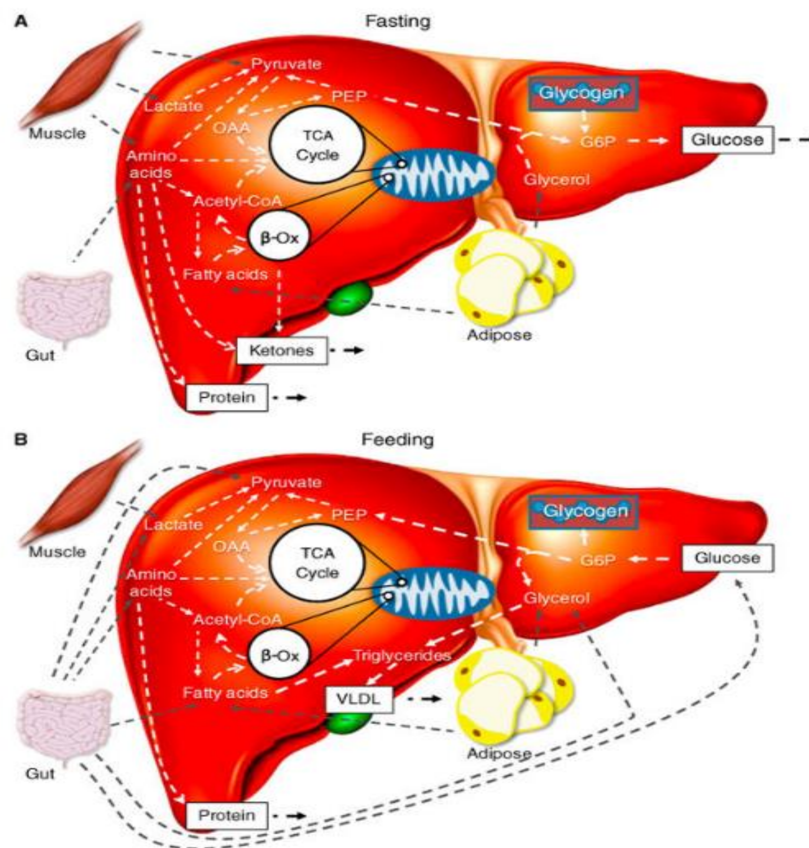


Figure 1.8: Lipid metabolism in the liver. (A) In a fasting state, the liver is in a net hepatic glucose production mode due to a reduced insulin-glucagon level. Glucose is created from both glycogen and gluconeogenesis.

Gluconeogenic substrates are administered in the form of amino acids (gut and muscle), lactate (muscle), pyruvate (muscle) and glycerol (adipose tissue). Fatty acids from adipose tissue lipolysis are also directed to a few pathways, like beta oxidation and the tricarboxylic acid cycle (TCA) cycle. These processes support gluconeogenesis by the processing of ATP and the removal of equivalents. Ketone bodies may also be generated by lipid oxidation and act as an external energy shuttle between the liver and other organs.
Also, amino acids may enter the TCA cycle as anaplerotic substrates and can be used for protein synthesis. Nitrogen released as a result of deamination during amino acid metabolism is discarded during ureagenesis. Urea is expelled from the liver and excreted into the kidneys. (B) During feeding, water-soluble foods join the portal of venous circulation from the intestine. In the liver, the ratio of insulin to glucagon is increased, leading to a net absorption of hepatic glucose. Glucose may undergo glycolysis as a means of production of ATP or may be processed as glycogen. Amino acids can be oxidised for energy production or used as anaplerotic substrates for the TCA cycle. Consumed fats are formed from the fatty acids and glycerol to form triglycerides. Into chylomicrons, these triglycerides are package then enter the lymphatic system. Chylomicrons drain from the lymphatics into the bloodstream, and the remaining fatty acids and glycerol are unloaded upon reaching the liver. Fatty acids should be used to restore energy supply, repletion of TCA cycle intermediates, or re-esterified to triglycerides. Triglycerides may be loaded on to lipoproteins of very low density which transfer lipid to other tissues, including muscle and adipose depots (Trefts E. et al., 2017).

1-5-1 Composition and Function of The Very Low-Density Lipoproteins (VLDLs)

The liver is essential for the regulation of lipids and lipoproteins. Indeed, one of the liver's crucial functions is to synthesize VLDLs, particles that allow the transport of insoluble molecules around the body through the bloodstream. The size of the newly synthesized VLDL particles ranges in diameter from 35 to 100 nm (Alexander et al., 1976). They are composed of a central core of triglycerides (TAG) and a shell of cholesterol, phospholipids and different apolipoproteins (Pan et al., 2002). TAG consist of three fatty acid chains linked to an alcohol called glycerol and represent a very efficient way of storing energy that the body can use during the fasting periods.

Among all the apolipoproteins, which are the proteins that bind lipids to form lipoproteins, apolipoprotein B (ApoB) is the most important and provides structural stability to the nascent VLDL particle. ApoB is a large amphipathic glycoprotein and it has two forms: ApoB100 and ApoB48. ApoB100 is a fully translated protein that contains 4536 amino acids (Lusis et

al., 1985), while ApoB48 represents a truncated form (48%) of the full-length ApoB and contains 2152 amino acid residues (Tiwari et al., 2012).

In humans, ApoB100 is expressed in the liver and it is the main structural protein of VLDL, intermediate-lipoprotein and low-density lipoprotein (low-densities lipoproteins, deriving from the VLDL once that the TAG have been hydrolyzed, thus containing a higher proportion of cholesterol). ApoB48 instead is mainly synthesized by the human intestine and it is a chylomicron structural protein (Kane et al., 1980; Lo CM et al., 2008; Mansbach et al., 2010). However, both ApoB100 and ApoB48 are synthesized by the rodent liver (Tennyson et al., 1989). Like other secretory proteins, ApoB is synthesized at the ER surface and then co-translationally translocated in the ER where the VLDL is thought to be assembled (Figure 1.9) (Chuck et al., 1990; Chuck, 1992).

Indeed, the specific location and mechanism of assembly of the VLDL are still under discussion. The accepted model considers that VLDL biogenesis begins at the ER with the translocation of newly translated ApoB across the rough ER membrane (Swift, 1995; Rustaeus et al., 1999). In the first step, nascent ApoB is partially lipidated to create a primordial VLDL molecule. This step is enabled by the microsomal triglyceride transfer protein (Mttp) (Shelness et al., 1999), a lipid transport protein with three domains: (1) an ApoB-binding domain, (2) a lipid transport domain, and (3) a membrane association domain (Hussain et al., 2003; Hussain et al., 1998). Mttp can transfer neutral and polar lipids to the forming VLDL particle, but recent results from Hussain's studies clearly suggest that the phospholipid transfer domain of Mttp can alone produce VLDL particles (Khatun et al., 2011). Lipidation of ApoB depends on the availability of triglycerides; a significant amount of nascent ApoB is destroyed without lipidation (Yao et al., 1997).

After this initial lipidation, the process that leads to the complete formation of the VLDL still needs to be clarified, but one of the hypotheses is that TAGs are first stored in between the double leaflets of the ER membrane, forming an enlargement similar to a lens. This lens is then embedded in the ApoB-nascent VLDL (Figure 1.9). These precursor particles mature with additional lipidation during their flux through ER and Golgi until they reach a diameter

of 30-80 nm and are eventually secreted from the hepatocytes into the bloodstream to deliver lipids to peripheral tissue (Tiwari et al., 2012).

Surprisingly, despite the fact that these pathways have been studied for nearly half a century, the trafficking of VLDL particles from the ER and the eventual secretion from the hepatocyte into the bloodstream remain largely undefined. The decoding of VLDL biogenesis can shed light on the mechanisms underlying metabolic syndromes and cardiovascular diseases directly related to altered lipoproteins and lipid homeostasis.

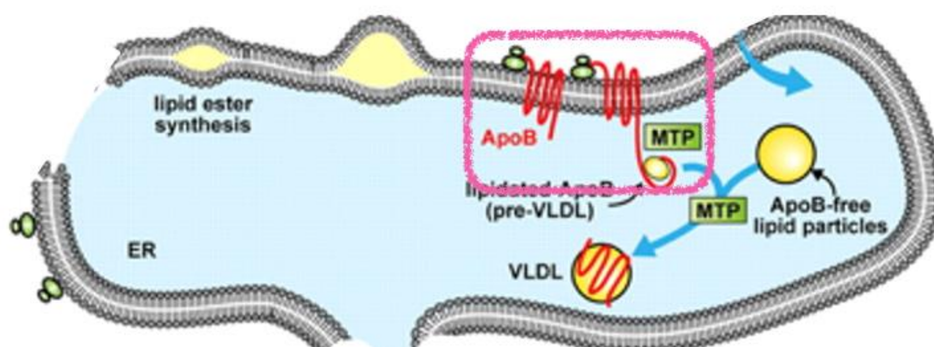


Figure 1.9: VLDL biogenesis in the ER. ApoB is co-translationally lipidated and acquires additional lipids to become mature VLDL (Ohsaki et al., 2008).

1-5-2 Lipid Droplets, The Cellular Storage of Triglycerides and Cholesterol Esters

The lipid droplet (LD) is a phylogenetically conserved organelle, and it is the only organelle which has a micellar structure, surrounded by a monolayer of phospholipids, and that is born from the ER in the eukaryotes.

LDs are cytosolic organelles, which can be as wide as 100 mm in diameter. Compared to other cellular organelles, LDs have a special structure. Approximately 75–90 mol percent of LD consists of a hydrophobic centre consisting of neutral lipids, mainly cholesterol esters (CE) and TAG, which have a highly assorted composition of mixed fatty acyls (Gross and Silver, 2014). It contains more cholesterol than ER membrane and less than plasma

membrane (Tauchi-Sato et al., 2002). The hydrophobic centre of the LD is covered by 2–2.5 nm wide phospholipid monolayer containing numerous proteins, for instance the perilipin family, the most abundant structural protein found on LDs, lipid-synthesis enzymes lipases and membrane-trafficking proteins (Brasaemle, 2007).

The liver enters a state of physiological steatosis during fasting, increasing lipid accumulation in LDs as a way of reserving and providing essential cellular functions energy. Non-esterified fatty acids obtained from hydrolysis of TAG stored in adipose tissue, dietary fatty acids from intestinal chylomicron residues and fatty acids newly synthesised by de novo lipogenesis are the source of fatty acids for hepatic TAG synthesis. In the liver, TAG can be stored in two forms, either in LDs or into TAG-rich ApoB-containing lipoproteins. After re-feeding, the number and the size of LDs decrease in the hepatocytes (Hamada et al., 2020); however, the physiology of fasting and re-feeding process is not well understood yet.

1-5-3 The Biogenesis of The Lipid Droplets

The cycle of LDs begins when the albumin and lipoproteins that carry extracellularly fatty acids enter in cell. Lipoprotein lipases release fatty acids from the lipoproteins' TAG in order to allow their translocation in the cells by passive diffusion facilitated by fatty-acid transport proteins or fatty-acid translocase. The other source of fatty acids is their *de novo* synthesis in many cell types (Ehehalt et al., 2006).

In the hepatocytes, the conjugation of fatty acids with CoA produces fatty acyl-CoA. In the ER, acyl-CoA generates diacylglycerol by glycerolipid- synthesis enzymes. Diacylglycerols are the precursor of TAG in the ER, then they are converted to neutral lipids (triacylglycerols) by Diglyceride acyltransferase (DGAT) enzymes or enter phospholipid- synthesis pathways. Most of the cells can produce sterols; in contrast to fatty acids, sterols are taken up within cells via endocytosis and lysosomal degradation of lipoproteins. In the ER, excess sterols are converted to sterol esters by conjugation with fatty acyl-CoA (Guo et al., 2009).

The cores of LDs consist of the neutral lipids which are synthesized in the ER.

The lipid formation and accumulation are still debated. At this moment, there are three hypothesis that describe the lipid-droplet formation:

1. In the ER-budding model, the neutral lipids form a “lipid lens” in the ER bilayer that subsequently “buds” from the membrane, keeping with itself the phospholipids from the cytosolic leaflet (Figure 1.10 A).
2. In the ER-domain model, LDs remain connected to the ER and consist in lipid-containing protrusions of the ER membrane, forming a specialized ER domain (Figure 1.10 B).
3. In the micelle model, neutral lipids accumulate between the leaflets of the ER membrane but, instead of budding, nascent LDs are excised from the membrane, taking with them phospholipids from both the cytosolic and luminal leaflets (Figure 1.10 C) (Hamada et al., 2020).

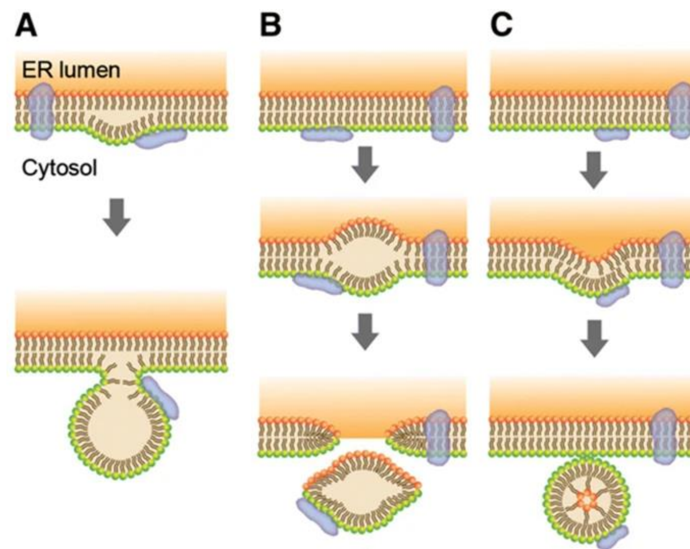


Figure 1.10: Models of lipid droplet formation. (A) Triacylglycerols are deposited between the leaflets of the ER membrane. After reaching a critical size to form a lipid droplet, the TAG core buds off from the ER membrane. Lipid droplets are covered with a monolayer from the leaflet of the cytosol side of the ER membrane. (B) Lipid droplet formation is similar to model in (A). However, lipid droplets are excised from the ER membrane, and both ER membrane leaflets contribute to the lipid droplet surface monolayer. (C) Inclusion of the TAG core in the membrane vesicle requires rearrangement of the leaflet of lumen side of the ER membrane (Hamada et al., 2020).

1-5-4 The Lipid Droplets Forms Contacts with Other Cellular Organelles

There are increasing evidence that LDs are associated dynamically with other cellular organelles. LDs are commonly located in close proximity to ER (Fei et al., 2009), mitochondria (Wang et al., 2011), endosomes (Liu et al., 2007), peroxisomes (Blanchette, 1966) and the plasma membrane (Gao and Goodman, 2015). These organelle associations might facilitate lipid exchange, either for anabolic growth of LDs or for catabolic breakdown. Conversely, LDs may provide a means to transport lipids inside the cell between organelles, much as lipoproteins transport lipids through the blood between tissues (Guo et al., 2009).

1-6 Working Hypothesis

In light of what has been presented in the indroduction to this work, many questions regarding the structure and function of contact sites between mitochondria and the rough endoplasmic reticulum remain unanswered. In this regard, my two major working hypotheses are the following. First, that in the mouse liver the contacts between rough-ER and mitochondria are highly organized and, therefore, necessary for a specific hepatic function. Second, since a major role of the liver is to regulate VLDL biogenesis and secretion, that the integrity of the contact between these two organelles is required for ensuring VLDL-dependent hepatic liver homeostasis.

1-7 Objectives

The overall objective of this study is to characterize for the first time the structure and function of the contact between the rough-ER and the mitochondrion in the liver. To this goal, I have pursued the following specific objects:

1) Analyzed EM and ET images to perform a qualitative and quantitative ultrastructural analysis of the contact between rough-ER and mitochondria in the mouse liver.

2) Classified the mRNAs and proteins localized at this new contact site and measured their relative enrichment level.

3) Identified a possible tether protein regulating the juxtaposition between the two organelles.

4) Performed genetic and functional studies aiming to demonstrate *in vivo* the role of this contact site in hepatic lipoproteins (VLDL) biogenesis.

Chapter 2 - Materials and Methods

2-1 Electron Tomography

Electron tomography (ET) is a technique for obtaining detailed 3D structures of sub-cellular macro-molecular objects. Electron tomography is an extension of traditional transmission electron microscopy and uses a transmission electron microscope to collect the data. This technique exploits the rotation of the sample with respect to an axis, during which sequential images are taken. The images thus generated are then arranged and merged using computational techniques to recreate a 3D image or tomogram. ET has been successfully operated to study contact sites such as the ER–mitochondria or ER–plasma membrane.
Using the software Amira, I then generated the 3D reconstruction by manually drawing mitochondria, ER and ribosomes in every slide of each tomogram.

2-2 WrappER-Associated Mitochondria Fractionation Protocol

To study the composition of the contacts between wrappER and mitochondria, I took advantage of the fractionation protocol previously developed in our laboratory. Briefly, the fractionation technique utilises high viscosity and gravitational acceleration to isolate organelles dependent on density. Since mitochondria wrapped in ER are the heaviest components in the cell, once the nucleus and plasma membranes have been eliminated, it is possible to sediment and enrich them by low-speed centrifugation. For each experiment showed in Figure 3-2, the mice were sacrificed at 3 hours postprandial, the liver was extracted, homogenised and separated from cell debris and nuclei. The homogenate obtained was placed on a viscous solution and centrifuged, obtaining a pellet containing the wrappER-associated mitochondria (WAMs). The WAM fractions were then analysed by western blot or send to the Proteomic Platform of the CHU Research Center Universitè Laval. For the

mRNA sequencing, the transcripts contained in the WAM fractions were isolated by Dynabeads mRNA Direct Micropurification Kit and sequenced by the Genomics Center of the CHU de Québec. The Proteomics and Transcriptomics data were analyzed using the Microsoft Excel Software.

2-3 Immunoblot Analysis

Western blot or immunoblot consists of the use of polyacrylamide gel electrophoresis (PAGE) to isolate proteins according to their size, their subsequent transfer to a membrane and their selective detection using an antibody targeted against the protein of interest.
Protein samples were analyzed by SDS-PAGE (Sodium lauryl sulfate-polyacrylamide gel electrophoresis) using the following types of precast gels according to manufacturer's instructions: Bolt 8%, 12% or 4-12% Bis-Tris Plus gels. The concentration of acrylamide defines the resolution of the gel - the higher the acrylamide concentration, the better resolution of lower molecular weight and the lower the acrylamide concentration, the better resolution of higher molecular weight proteins. Before loading the protein on the SDS-PAGE, samples are incubated for 5 minutes at 95℃ in sample buffer which consists of:

- 2% SDS
- 50 mM Dithiothreitol (DTT)
- 10% Glycerol
- 0.02% Bromophenol blue (BPB)
- 10 mM Tris HCl
- pH 6.8

When sodium lauryl sulfate (SDS) is mixed to the protein samples, it charges the polypeptides negatively according to their length, with equal charge density per unit length. Migration is therefore defined by molecular weight, rather than by the polypeptide's intrinsic charge. Dithiothreitol is used to eliminate disulfide bridges (disulfide bonds [S-S] to sulfhydryl groups [SH and SH]), which are important for size-by-size separation.

To increase the density of the sample to be loaded, Glycerol (10% w/v) is added to the buffer. This molecule is denser than the running buffer, allowing samples to “sink” down to the bottom of the wells rather than diffuse into the buffer.
BPB is a small anionic dye molecule that helps the visualization of the sample during its loading into the SDS-PAGE and allows to track progress of the electrophoresis. BPB, indeed, has a slightly negative charge that allows its migration in the same direction of proteins during electrophoresis.

After the electrophoretic run, the proteins were transferred to a 0.45 micrometres PVDF membrane for 60 minutes at 100V.

Transfer buffer:

- 20% methanol
- 320 mM glycine
- 20 mM Tris-base pH 8.4

Polyvinylidene fluoride or polyvinylidene difluoride (PVDF) is used as an artificial membrane to which proteins are transferred using electricity. PVDF needs to be quickly activated in methanol before contact with the PAGE-gel because of its strong hydrophobicity, which could hinder aqueous buffer movement and protein binding to the membrane.

The electroblotting technique uses an electrical flow to pull the negatively charged proteins from the gel towards the positively charged anode, and into the PVDF membrane. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein.

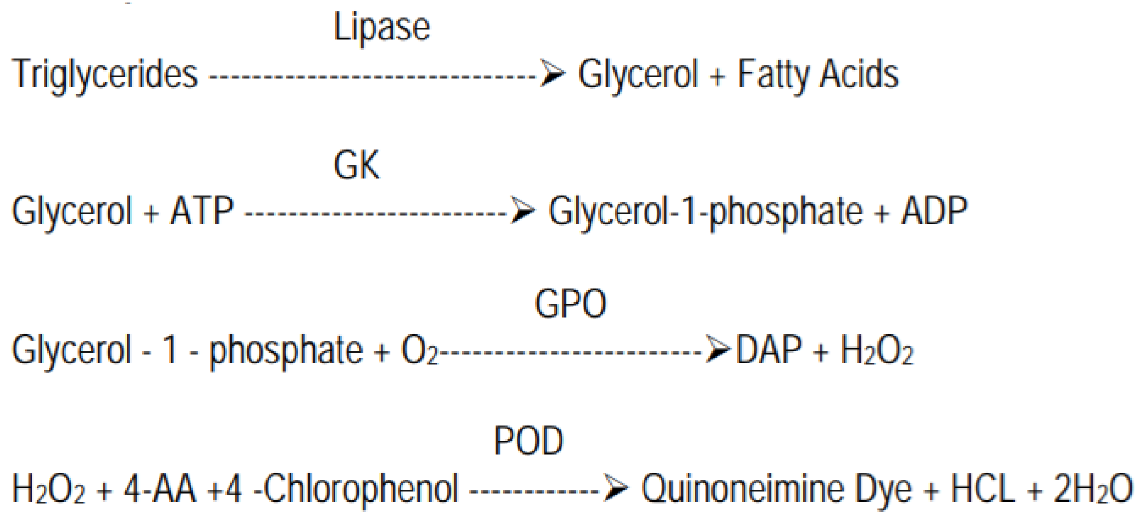
Since the membrane is chosen to bind protein and since both antibodies and the target protein are proteins, steps must be taken to avoid the associations between membrane and antibody used for detection of the target protein. Blocking of non-specific binding is accomplished by placing the membrane in a dilute protein solution with 7.5% non-fat milk in Tris-buffered saline, 0.1% Tween-20. The milk proteins will bind to the areas of the membrane where the

target proteins have not been transferred. Thus, when the antibody is added, the only binding site available are the target proteins. After blocking, the membrane is incubated with primary antibodies overnight at 4 °C in 5% non-fat milk or 5% bovine serum albumin in Tris-buffered saline, 0.1% Tween-20 (TBS-T). The primary antibodies used in this study were the following: β-actin (1:10,000), ADFP/Perilipin-2 (1:1000), ApoB (1:2,000), BiP/Grp78 (1:50,000), Calnexin (1:30,000), Calreticulin (1:50,000), GFP (1:1,000), MnSOD (1:7,000), OPA1 (1:10,000), PMP70 (1:15,000), Rrbp1 (1:2,000), phosphorylated-S6 (Ser204/244, 1:1000). After the overnight incubation, membranes are washed tree times with TBS-T and incubated for 1 hour with the secondary antibody. Its role is to bind the primary antibody and increase its signal, causing a much lower concentration of proteins to be identified than can be expected by SDS-PAGE alone. The Horseradish peroxidase (HRP)-conjugated secondary antibodies used in this study were the following: anti-Mouse IgG (1:5,000) and anti-rabbit IgG (1:5,000). HRP is an enzyme conjugated to the antibody that, after exposure to a chemiluminescent substrate (SuperSignal ELISA Femto substrate), permits the production of luminescence allowing the identification of the target protein. Therefore, luminescence production is proportional to the quantity of HRP-conjugated secondary antibodies and thus indirectly measures the existence of the target protein, which is then visualized through the use of the VersaDoc 3000 CCD imaging system.

2-4 *In vivo* VLDLs Secretion Analysis

Mice were fasted for 6 h and injected intraperitoneally with the lipase inhibitor Poloxamer-407 (P-407; 1g/kg;), which leads to TAG accumulation in the blood by blocking their release from VLDL to the tissue. Blood was then collected at 0 ,1 hr, 2 hrs and 3 hrs after inhibitor Poloxamer-407 (P-407) injection. Around 60 μl of blood was obtained from the caudal vein with the heparinized capillary tubes and instantly centrifuged at 2,100 g for 12 min to prepare plasma fractions. An aliquot of each samples was conserved to measure the Albumin expression level through Western Blot and the total proteins amount through Coomassie gel stain. The amount of TAG in the blood was than assessed with the GPO-Colorimetric kit. The triglycerides glycerol method (GPO) is based on the enzymatic determination of glycerol

using the enzyme phosphate oxidase GPO after hydrolysis by lipoprotein lipase (LP). The peroxide generated during this reaction is used to produce a red colored quinonimine dye which absorbs at 500 nm. The intensity of the colored complex formed is directly proportional to the triglycerides concentration of the sample and can be detected using a spectrophotometer. Here below a schematic representation of the reactions exploit during the assay:



Chapter 3 - Research Project

3-1 Results

3-1-1 A Wrapping Type of Rough-ER (Wrapper) Forms Contact Sites With Mitochondria *In Vivo*

The ultrastructure of the mitochondrion and of the rough-ER in the mouse liver has been studied by electron microscopy analysis and 3D-electron tomography reconstruction studies. For these experiments, mice were fasted overnight and sacrificed the day after, three hours after refeeding with chow diet and sugary water (30% sucrose), and tissue samples from the liver were collected.

Electron tomography with 3D reconstruction analysis of 5 complete mitochondria showed that most of the mitochondrion surface in the hepatocytes was largely wrapped by rough-ER (Figure 3-1 A, upper panel). It also revealed that this contact was widely abundant in the cell, with nearly every mitochondrion associated with the rough-ER (120 mitochondria out of 121 analyzed, Figure 3-1 A, lower panel). Morphometric analysis on EM images showed that 48 ±1.2% (SEM; n=250 from 5 mice; Figure 3-1 B) of the mitochondrial perimeter was wrapped by rough-ER, at an average distance of 45.1 ±0.4 nm (SEM; n=2100 from 5 mice; Figure 3-1 B). Since this type of rough-ER is highly curved and able to wrap itself around the mitochondrion, we called it wrappER. Moreover, EM analysis revealed that this type of rough-ER contained ribosomes-free areas of close juxtaposition with the mitochondrion (MAM), suggesting that the MAM is a sub-compartment of the wrappER. Consistently, in 3D reconstructions of ET at least one MAM-like domain was observed in 89% of the mitochondria covered by the wrappER (Figure 3-1C, D), characterized by lack of ribosomes and a closer ER juxtaposition to the mitochondrion (10-30 nm).

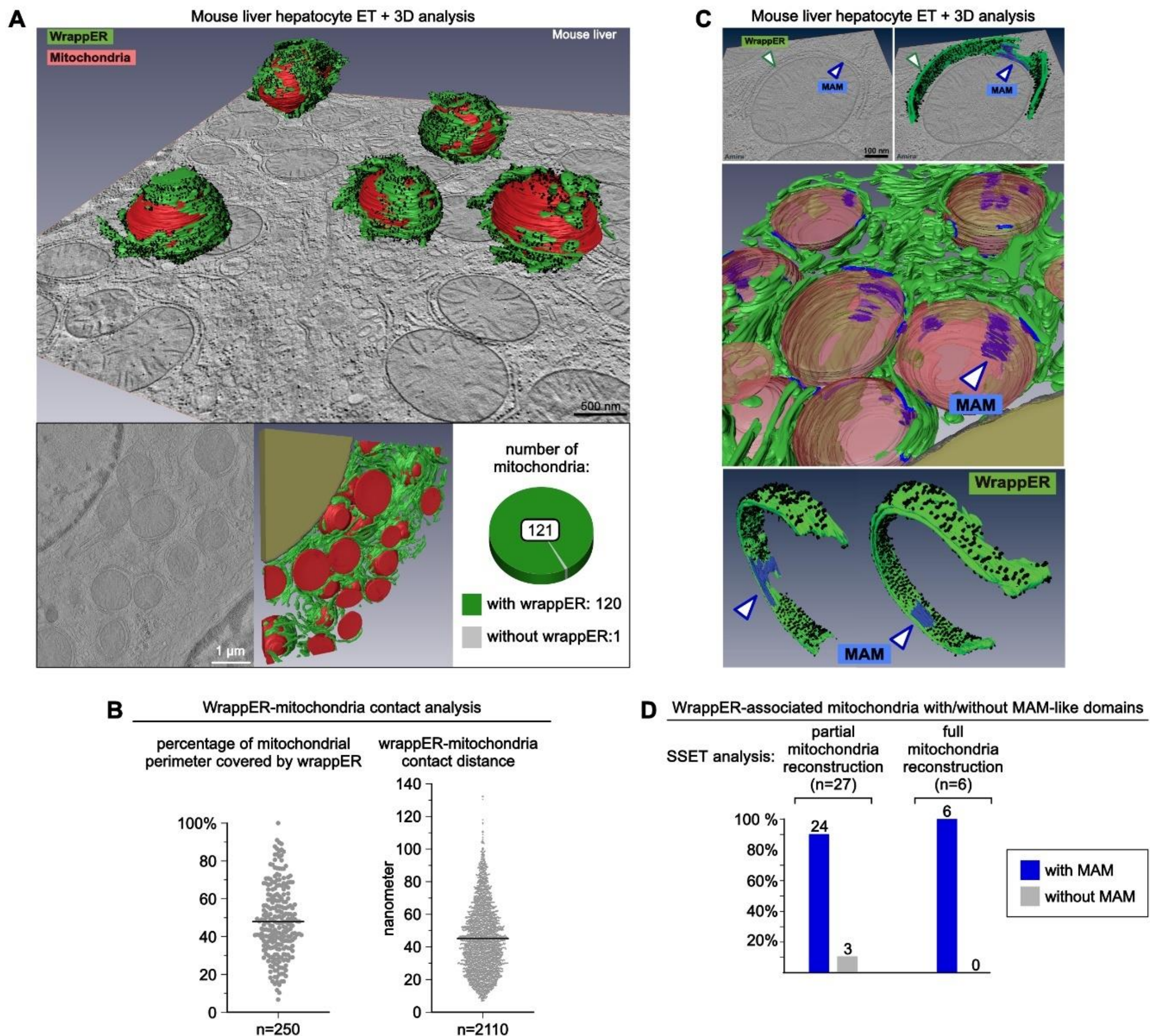
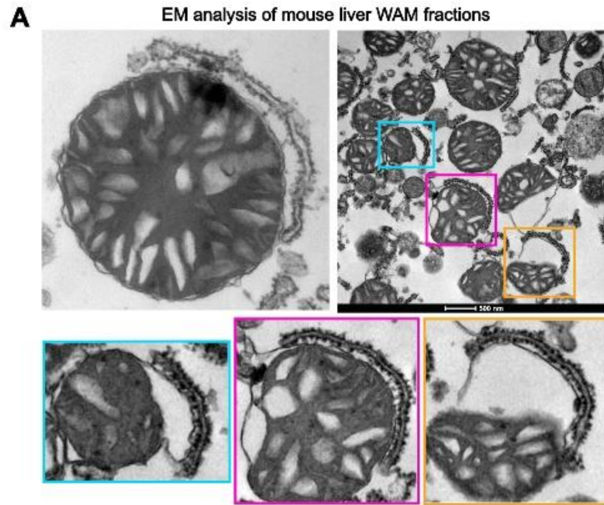


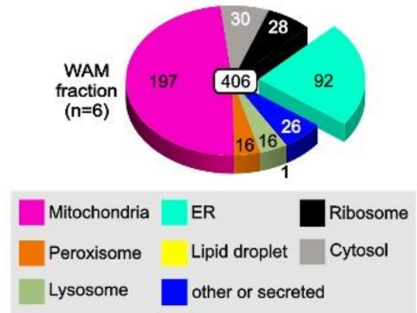
Figure 3-1: A wrapping type of rough-ER (wrappER) forms contact sites with mitochondria *in vivo*. A, ET with quantitative 3D reconstruction analysis of rough-ER (wrappER, green) wrapped around mitochondria (red) in liver hepatocytes. **B**, Scatter dot plot showing the percentage of mitochondrial perimeter covered by wrappER (left panel) and the distance between wrappER and mitochondria (right panel). **C** and **D**, ET with quantitative 3D analysis of mitochondria-associated ER membranes (MAM)-like domains (blue) within the mitochondrial face of the wrappER.

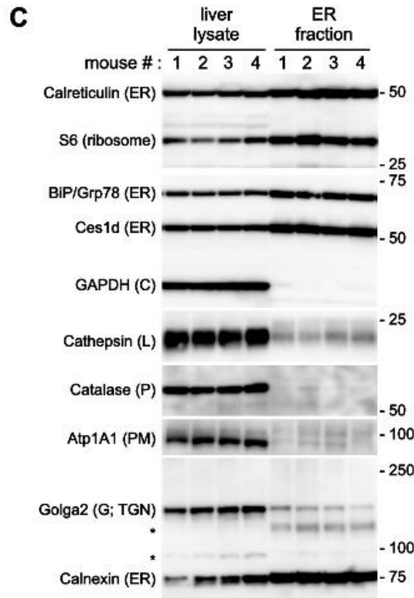
3-1-2 Analysis of The Transcripts And Proteins Present at The Wrapper Mitochondria Contact Sites in The Mouse Liver

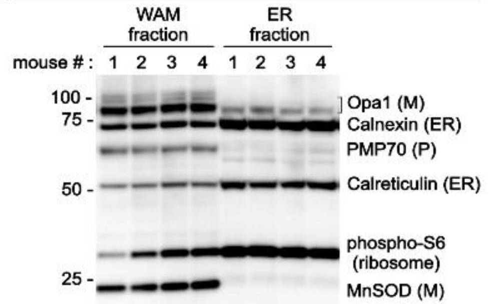
To study the function of the contact sites between wrappER and mitochondria, we developed an *ad hoc* method for the physical purification of wrappER-associated mitochondria (WAM) from mouse liver. Moreover, we isolated an ER fraction from the same samples to be used as control for the subsequent experiments. EM analysis confirmed that the purification yielded wrappER associated with mitochondria in a similar manner to that observed in the EM images of the liver tissue (Figure 3-2 A). The proteomics results confirmed that WAMs were enriched in mitochondrial and ER proteins, with the first pool representing the 50% of the entire population (Figure 3-2 B). The presence of ER and mitochondrial proteins was validated by immunoblot analysis (Figure 3-2 D), in which a fraction enriched in ER proteins was used as comparison, characterized by an abundant expression of ER markers and the absence of other organelles (Figure 3-2 C).

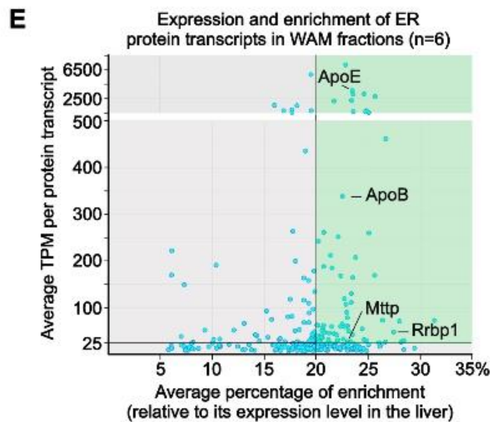
Remarkable, WAM purification was designed to preserve ribosomes and thus permitted not only proteomic analysis, but also transcriptomic. Normalization for relative mitochondrial abundance (measured as reads of the mtDNA-encoded genes) of the WAM deep RNA sequencing results revealed 1,160 recurring transcripts. To determine the relative enrichment of these transcripts, we also evaluated the transcriptome of the corresponding homogenates used to prepare the fractions comprising WAM (that was also normalized for its relative mitochondrial abundance) and determined a percentage enrichment factor (Figure 3-2 E). We obtained a uniform enrichment level across all WAM transcripts varying from 4% to 34%. Transcripts that encoded ER proteins were on average more represented than others (Figure 3-2 E), including transcripts for mitochondrial proteins. We then crossed the transcriptomic and proteomic datasets, which identified a subset of "core" WAM proteins whose mRNAs were enriched in WAMs. A strict filter identified 47 candidates, 33 of them were ER proteins (Figure 3-2 F).


A
EM analysis of mouse liver WAM fractions


B
Proteomic analysis of mouse liver WAM fractions
number of proteins and localization
WAM fraction (n=6)
197
30
28
92
406
26
16
16
1
Mitochondria
ER
Ribosome
Peroxisome
Lipid droplet
Cytosol
Lysosome
other or secreted


C
liver lysate
ER fraction
mouse # : 1 2 3 4 1 2 3 4
Calreticulin (ER)
S6 (ribosome)
BiP/Grp78 (ER)
Ces1d (ER)
GAPDH (C)
Cathepsin (L)
Catalase (P)
Atp1A1 (PM)
Golga2 (G; TGN)
Calnexin (ER)
- 50
- 25
- 75
- 50
- 25
- 50
- 100
- 250
- 100
- 75


D
Biochemical analysis of mouse liver WAM fractions
WAM fraction
ER fraction
mouse # : 1 2 3 4 1 2 3 4
100 -
75 -
50 -
25 -
Opa1 (M)
Calnexin (ER)
PMP70 (P)
Calreticulin (ER)
phospho-S6 (ribosome)
MnSOD (M)


E
Expression and enrichment of ER protein transcripts in WAM fractions (n=6)
Average TPM per protein transcript
6500
2500
500
400
300
200
100
25
ApoE
ApoB
Mttp
Rrbp1
5 10 15 20 25 30 35%
Average percentage of enrichment
(relative to its expression level in the liver)

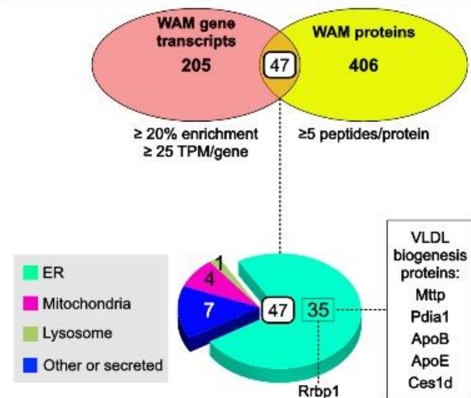

F
Mouse liver WAM fractions
transcriptomic + proteomic analysis (n=6)
WAM gene transcripts
205
47
WAM proteins
406
≥ 20% enrichment
≥ 25 TPM/gene
≥5 peptides/protein
ER
Mitochondria
Lysosome
Other or secreted
4
1
7
47
35
Rrbp1
VLDL biogenesis proteins:
Mttp
Pdia1
ApoB
ApoE
Ces1d

Figure 3-2: Analysis of The Transcripts and Proteins Present at The Wrapper Mitochondria Contact Sites in The Mouse Liver. A, EM analysis of a WAM fraction. **B**, WAM fraction proteomic analysis. **C** and **D,** Immunoblot analysis of the mouse liver WAM fraction and ER fraction (ER: Endoplasmic Reticulum, C: Cytosol, M: Mitochondria, L: Lysosome, P: Peroxisome, PM: Plasma membrane, G: Golgi). **E,** Transcriptomic analysis: each dot represents a gene transcript encoding an ER protein; its average level of expression in the liver and its relative level of enrichment in the WAM fractions are indicated (n=6). **F,** Strategy used to identify proteins and transcripts localized at the WAMs.

3-1-3 Rrbp1 Downregulation Modifies the Contacts Between WrappER and Mitochondria

Next, we investigated whether mitochondria-wrappER contacts are necessary for WAM function. We focused on the previously proposed tether Rrbp1 (Hung et al., 2017) because our transcriptomics and proteomics results showed it to be highly enriched in the WAM fractions (28% mRNA enrichment; 31 ±1 peptides/protein; Figure 3-2 E and F), making it a strong tether candidate. We confirmed these results by immunoblot analysis, which showed Rrbp1 enrichment in the WAM fraction compared to the ER fraction. (Figure 3-3 A). In particular, WAM fractions contained a specific 100 kDa form of the protein that was barely detectable in the ER fractions and could be responsible for the tethering between the rough ER and mitochondrion.

We then proceeded to Rrbp1 silencing in the liver, by tail injecting mice with AAV8 viruses carrying a short hairpin RNA targeted against Rrbp1 or a scrambled RNA. Immunoblot analysis confirmed that a reduction of more than 60% of the expression level of the protein was achieved (Figure. 3-3 B). As result, EM morphometric analysis revealed that the cleft area (i.e. the area of the space separating the two organelles) was increased in the liver of RRbp1 silenced mice (Figure. 3-3 C), suggesting a contribution of Rrbp1 to the tethering of the two organelles.

3-1-4 The WrappER Is a Site of VLDLs Biogenesis in Mouse Liver

By interrogating the Gene Ontology database, the integrated omics analysis on the WAM reviled that lipoprotein particle assembly was the most represented biological process in this compartment. Indeed, the most upstream regulators of the VLDLs biogenesis, namely Mttp, ApoB and ApoE, were found to be highly abundant in both transcriptomics and proteomics analysis (Figure 3-2 E and F). Given the presence in the WrappER of the machinery involved in the VLDLs synthesis, we hypothesized the involvement of this contact in this pathway.
To test this, we analyzed lipoprotein secretion by injecting mice with Poloxamer-407 (P-407), a lipoprotein lipase (LPL) inhibitor that inhibits the conversion of VLDL to IDL and LDL, which allows the assessment of the hepatic triglyceride secretion rate in fasted mice. Rrbp1 silencing decreased plasma TAG levels after inhibition of the LPL (Figure 3-3 D, left panel). Plasma ApoB-100 content was also reduced (Figure 3-3 E), indicating that the observed reduction in TAG resulted from lower levels of circulating VLDLs. This phenotype was not caused by impaired hepatic secretion because plasma proteins and albumin, markers of liver protein synthesis and secretion, were not affected by Rrbp1 silencing (Figure 3-3 D, right panel). Consistent with these results, we found that the area of lipid droplets, organelles that typically accumulates in the liver after overload with neutral lipids or impairment of the lipoproteins pathway (Raabe et al., 1999), increased in Rrbp1 silenced livers (Figure 3-3 F).

Overall, these results show and characterize for the first time a new *in vivo* contact site between rough-ER and mitochondria in the mouse liver hepatocyte. In particular, they show the wrappER as a site of VLDL biogenesis and of lipid homeostasis regulation whose function is regulated by the presence of the tether Rrbp1.

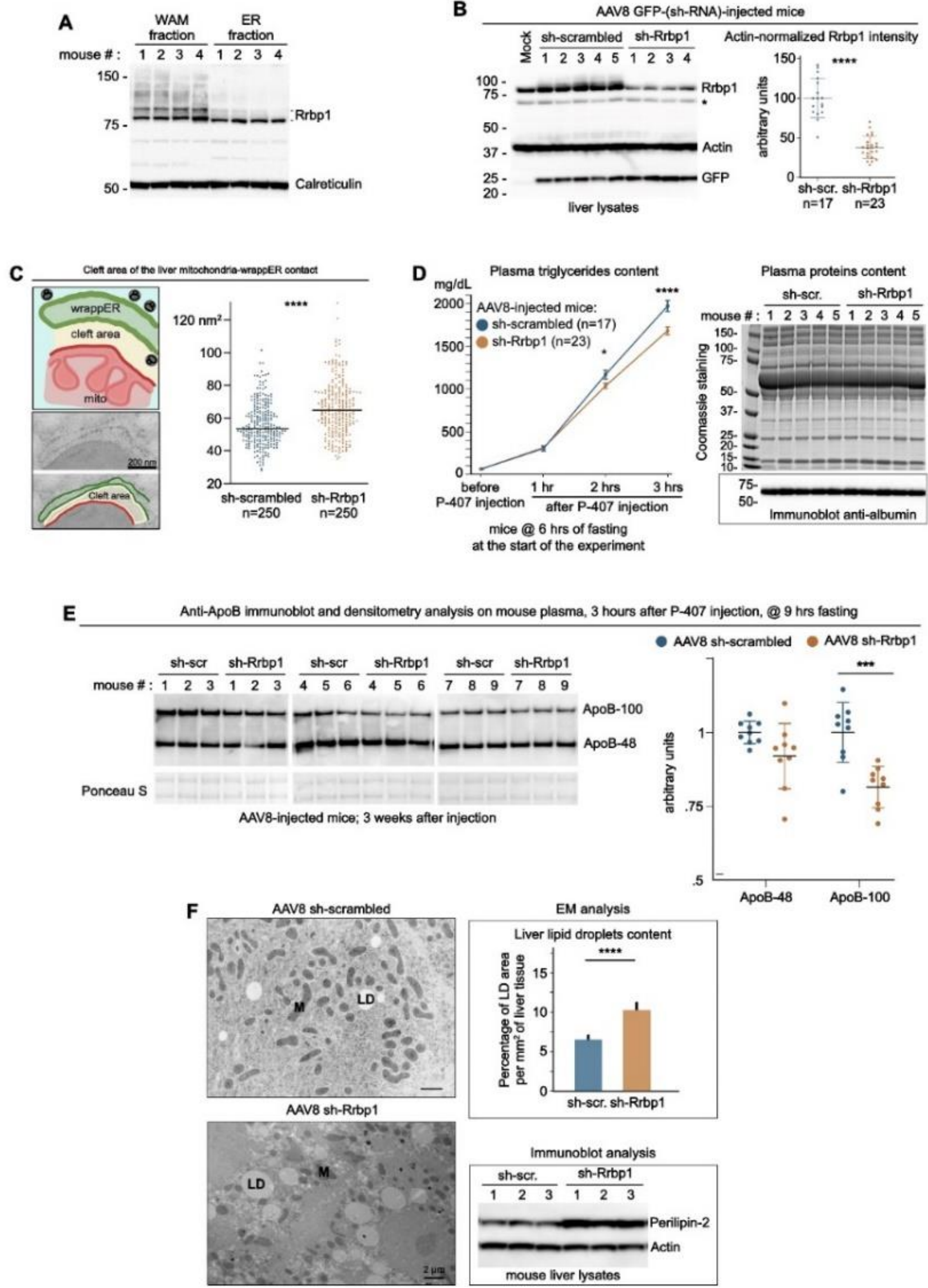

A
WAM fraction
ER fraction
mouse # :
Rrbp1
Calreticulin
B
AAV8 GFP-(sh-RNA)-injected mice
Mock
sh-scrambled
sh-Rrbp1
Actin-normalized Rrbp1 intensity
Rrbp1
Actin
GFP
liver lysates
arbitrary units
sh-scr. n=17
sh-Rrbp1 n=23
C
Cleft area of the liver mitochondria-wrappER contact
wrappER
cleft area
mito
200 nm
Cleft area
120 nm²
sh-scrambled n=250
sh-Rrbp1 n=250
D
Plasma triglycerides content
mg/dL
AAV8-injected mice:
sh-scrambled (n=17)
sh-Rrbp1 (n=23)
before P-407 injection
1 hr
2 hrs
3 hrs
after P-407 injection
mice @ 6 hrs of fasting at the start of the experiment
Plasma proteins content
sh-scr.
sh-Rrbp1
mouse # :
Coomassie staining
Immunoblot anti-albumin
E
Anti-ApoB immunoblot and densitometry analysis on mouse plasma, 3 hours after P-407 injection, @ 9 hrs fasting
sh-scr
sh-Rrbp1
mouse # :
ApoB-100
ApoB-48
Ponceau S
AAV8-injected mice; 3 weeks after injection
AAV8 sh-scrambled
AAV8 sh-Rrbp1
arbitrary units
ApoB-48
ApoB-100
F
AAV8 sh-scrambled
AAV8 sh-Rrbp1
M
LD
2 µm
EM analysis
Liver lipid droplets content
Percentage of LD area per mm² of liver tissue
sh-scr. sh-Rrbp1
Immunoblot analysis
sh-scr.
sh-Rrbp1
Perilipin-2
Actin
mouse liver lysates

Figure 3-3: Rrbp1 Downregulation Modifies the Contacts Between WrappER and Mitochondria and Alters VLDLs Secretion in The Mouse Liver. A, Immunoblot analysis of Rrbp1 enrichment level in mouse liver WAM and ER fractions. **B,** Immunoblot analysis of liver lysates prepared from mice injected with AAV8-GFP-sh-scrambled or AAV8-GFP-sh-Rrbp1. **C,** Distribution of the mitochondria wrappER cleft areas in control and Rrbp1-silenced mouse livers. **D,** Effect of hepatic Rrbp1 silencing on plasma TAG after administration of Poloxamer-407. The left panel shows the means ± SEM of the data collected from AAV8 injected mice. The right panels represent the amount of plasma total proteins (upper panel) and of Albumin (lower panel) of the AAV8 injected mice. **E,** Immunoblot and densitometry analysis of apoB in the plasma of AAV8-sh-scrambled and AAV8-sh-Rrbp1 injected mice. **F,** Quantitative EM analysis of the liver lipid droplets content in AAV8 injected mice. The lower right panel shows the enrichment of the lipid droplets marker Perilipin 2 in the liver homogenate of Rrbp1-silenced mice.

Conclusion

The liver is not the only organ which synthesizes lipoproteins. In the small intestine the enterocytes, epithelial cells responsible for dietary free fatty acids (FFA) adsorption, are able to synthesize and secrete Chylomicrons, a particular type of lipoproteins very similar to VLDL that shuttle dietary lipids to the periphery and the liver. The brain is another organ able to synthesize lipoproteins. It mainly produces ApoE-containing lipoproteins to provide neurons with the lipids necessary to build up their membranes and to repair the damage associated, for example, with aging. For this reason, mutations on the ApoE gene are associated with the development of Alzheimer's disease. Given the ability of both small intestine and brain to produce lipoproteins, we hypothesize the presence of the wrappER in these two organs. To test this possibility, a possible next step requires to analyze the morphology of the ER-mitochondria contact sites by employing the EM and ET technics that we developed for the study of the liver wrappER.

In addition, we found through the analysis of electron microscopy images that the wrappER also appears to be in contact with peroxisomes in the hepatocytes, often forming a tri-organellar contact with both mitochondria and peroxisomes. This interesting observation opens the field to a number of important questions: what is the peroxisome-wrappER contact frequency? What functional role does it play? Do the three organelles work together, or do the two contacts operate independently? What are the peroxisome tethers? These are just few of the issues that need to be answered in the future, not to mention how this relationship could be important for the lipid homeostasis in the liver, considering that the peroxisome is able to oxidize fatty acids and to modify cholesterol to produce bile acids.

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