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Role of the 17-beta-hydroxysteroid dehydrogenase type 12 (HSD17B12) in hepatitis C and related flaviviruses replication.

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Résumé

Dans le monde entier, les infections virales causent des problèmes de santé majeurs et récurrents, engendrant de sérieux problèmes socio-économiques. Notamment, les virus de la famille *Flaviviridae* qui représentent un fardeau considérable sur la santé mondiale et font partie des domaines prioritaires de la virologie médicale selon le rapport 2016 du 'Global Virus Network'. Bien que le traitement actuel contre le virus de l'hépatite C (VHC) ait un taux de guérison dépassant 98%, d'autres comme le virus de la dengue (DENV) et le virus zika (ZIKV) n'ont pas encore de traitement spécifique autorisé.

En prenant avantage de la grande expertise de notre laboratoire dans l'étude du VHC, nous avons utilisé des données d'une étude de biologie des systèmes visant à identifier l'interactome des différentes protéines virales. Les techniques utilisées ont combiné l'immunoprécipitation des protéines virales suivie de l'identification des protéines interacteurs humaines par spectrométrie de masse. Des études de génomique fonctionnelle par ARN interférent (ARNi) ont permis d'étudier l'effet de la diminution de l'expression des protéines identifiées sur la réplication du VHC. Cette étude a conduit à la découverte de l'interactant spécifique 17-bêta-hydroxystéroïde déshydrogénase de type 12 (HSD17B12 ou DHB12) de la protéine virale Core comme facteur cellulaire requis à la réplication du VHC. HSD17B12 est une enzyme cellulaire dont l'activité catalytique est requise pour l'élongation des acides gras à très longue chaîne (VLCFA) lors de la deuxième des quatre réactions du cycle d'élongation.

Dans cette étude, nous avons déterminé que les cycles de réplication du VHC, ZIKV et DENV dépendent de l'expression et de l'activité métabolique du facteur cellulaire HSD17B12. Ainsi, nous avons étudié les effets de l'inhibition de l'expression génique par ARNi et de façon pharmacologique sur la réplication de plusieurs flavivirus dans une approche antivirale à large spectre. Nous avons démontré que le silençage de HSD17B12 diminue significativement la réplication virale, l'expression des protéines virales et la production de particules infectieuses de cellules Huh7.5 infectées par la souche JFH1 du VHC. L'analyse de la localisation cellulaire de HSD17B12 dans des

cellules infectées suggère une colocalisation avec l'ARN double brin (ARNdb) aux sites de réplication virale, ainsi qu'avec la protéine Core (et les gouttelettes lipidiques) aux des sites d'assemblage du virus. Nous avons également observé que le silençage de HSD17B12 réduit considérablement le nombre et la taille des gouttelettes lipidiques. En accord avec ces données, la diminution de l'expression de HSD17B12 par ARNi réduit significativement l'acide oléique et les espèces lipidiques telles que triglycérides et phosphatidyl-éthanolamine dans l'extrait cellulaire total. Ces travaux suggèrent une contribution de la capacité métabolique de HSD17B12 lors de la réplication du VHC.

De même, nous avons démontré que le silençage de HSD17B12 réduit significativement les particules infectieuses de cellules infectées par DENV et ZIKV. Ces études supportent le rôle de HSD17B12 dans l'efficacité des processus de la réplication de l'ARN viral et de l'assemblage de particules virales. De plus, l'inhibiteur spécifique de HSD17B12, INH-12, réduit la réplication du VHC à des concentrations pour lesquelles aucune cytotoxicité notable n'est observée. Le traitement avec 20 μM d'INH-12 réduit jusqu'à 1,000 fois les particules infectieuses produite par des cellules Huh-7.5 infectées par DENV et ZIKV lors de plusieurs cycles de réplication, et bloque complètement l'expression des protéines virales.

En conclusion, ces travaux ont conduit à une meilleure compréhension du rôle de HSD17B12 lors de la synthèse de VLCFA et de lipides requise à la réplication du VHC, permettant d'explorer l'inhibition de HSD17B12 et de l'élongation d'acides gras à très longue chaîne comme nouvelle approche thérapeutique pour le traitement à large spectre des infections par les virus de la famille *Flaviviridae*.

Mots clé: Médecine Moléculaire, Découverte de médicament, Cible antivirale, Agents Antiviraux à large spectre, *Flaviviridae,* VHC, DENV, ZIKV, 17-bêta-hydroxystéroïde déshydrogénase de type 12, HSD17B12, DHB12, KAR, acide gras à longue chaîne, 3-oxoacyl-CoA réductase à très longue chaîne, inhibiteur de HSD17B12.

Abstract

Infections with viruses are major recurrent socio-economical and health problems worldwide. These include infections by viruses of the *Flaviviridae* family, which present a substantial global health burden and are among the priority areas of medical virology according to the Global Virus Network 2016 report. While the current treatment regimens for hepatitis C virus (HCV) infection have cure rates of more than 98%, other important members of *Flaviviridae* like dengue virus (DENV) and zika virus (ZIKV) have no specific licensed treatments.

By taking advantage of the most-studied HCV, which our lab has developed a vast expertise in the last 20 years, we used proteomics data of an HCV interactome study, combining viral protein immunoprecipitation (IP) coupled to tandem mass spectrometry identification (IP-MS/MS) and functional genomics RNAi screening. The study uncovered the 17-beta-hydroxysteroid dehydrogenase type 12 (HSD17B12, also named DHB12), as a specific host interactor of core that promotes HCV replication. HSD17B12 catalytic activity is involved in the synthesis of very-long-chain fatty acids (VLCFA) upon the second step of the elongation cycle.

In this study, taking HCV as a virus model, we elucidated the dependency of HCV, dengue virus (DENV) and zika virus (ZIKV) replication on expression and metabolic capacity of the host factor HSD17B12. We investigated the effects of the inhibition of gene expression by RNAi and of its pharmacological enzymatic inhibition on flavivirus replication in a broad-spectrum antiviral approach. We showed that silencing expression of HSD17B12 decreases viral replication, viral proteins and

infectious particle production of the JFH1 strain of HCV in Huh7.5 cells. The cellular localization analysis of HSD17B12 showed a co-staining with double-stranded RNA (dsRNA) at viral replication sites and with core protein (and lipid droplets) at virus assembly sites. Furthermore, HSD17B12 gene silencing drastically reduced the number and size of lipid droplets. In association, the reduced expression of HSD17B12 by RNAi decreases oleic acid levels and lipids such as triglycerides (TG) and phosphatidylethanolamine (PE) in whole-cell extract. The data suggested the requirement of the metabolic capacity of HSD17B12 for HCV replication.

Similarly, we provide evidence that HSD17B12 silencing significantly reduces DENV and ZIKV infectious particles. The studies support a role of HSD17B12 for effective viral RNA replication and particle assembly processes. Moreover, the specific HSD17B12 inhibitor, INH-12, reduces HCV replication at concentrations for which no appreciable cytotoxicity is observed. The treatment of DENV- and ZIKV-infected Huh-7.5 cells with 20 μM of INH-12 dramatically reduces production of infectious particles by up to 3-log10 in infection assays, and completely block viral protein expression.

In conclusion, these studies extends our understanding of the role of HSD17B12 in VLCFA synthesis required for the replication of HCV, allowing to explore the inhibition of HSD17B12 and elongation of VLCFA as a novel therapeutic approach for the treatment of a broad-spectrum of viruses of the *Flaviviridae* family.

Keywords: Molecular Medicine, Drug Discovery, Antiviral host target, Broad-spectrum antiviral, *Flaviviridae*, HCV, DENV, ZIKV, 17-beta-hydroxysteroid

dehydrogenase type 12, HSD17B12, DHB12, KAR, very-long-chain fatty acids, very-long-chain 3-oxoacyl-CoA reductase, HSD17B12 inhibitor.

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List of Abbreviations & Acronyms

AA	Arachidonic Acid
ACC	Acetyl-CoA Carboxylase
ACSL	long chain acyl-CoA synthetase
ACP	Acyl Carrier Protein of fatty acid synthase
AMPK	AMP-activated protein Kinase
AP2M1	clathrin Assembly Protein complex 2 Medium chain
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ARFP	Alternative Reading Frame Protein
CD81	Cluster of Differentiation 81
cDNA	Complementary DNA
CE	Cholesterol Ester
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CK IIα	Casein Kinase IIα
cLD	cytosolic Lipid Droplet
CLDN1	Claudin 1
CoA	Co enzyme A
CPT1	Carnitine Palmitoyl Transferase-1
CypA	Cyclophilins A
DCI	Dodecenoyl Coenzyme A delta isomerase
DH	Dehydratase of fatty acid synthase
DG	Diglycerides
DGAT	Diglyceride acyltransferase
DMV	Double membranous vesicles
DRM	Detergent resistance membrane
ER	Enoyl Reductase of fatty acid synthase
ER	Endoplasmic reticulum
ESCRT	Endosomal Sorting Complex Required for Transport

FASN	Fatty Acid SyNthase
FA (CX, Y)	Fatty acid (X is the number of carbons in its acyl chain, while Y is the number of double bonds)
GAGs	GlycosAminoGlycans
GTP	Guanidine Tri-Phosphate
HACD	3-Hydroxy Acyl Dehydratase
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HCVpp	Hepatitis C Virus pseudo-particles
HIV	Human Immunodeficiency virus
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl Coenzyme A
HSD17B12	17 Beta-Hydroxy Steroid Dehydrogenase type 12
HSPG	Heparan Sulfate Proteo Glycans
HTS	High Throughput Screening
HUH7	Type of human Hepatoma cell line
IP-MS/MS	Imunno-precipitation coupled to mass spectroscopy
IRES	Internal ribosome entry site
IV	Intravenous
JC1	Chimeric genome HCV clone
JFH-1	Japanese Fulminant Hepatitis-1 infectious strain
kDa	kilo Dalton
KR	Keto-Reductse of fatty acid synthase
LDL	Low Density Lipoprotein
LDL-R	Low Density Lipoprotein-receptor
let767	*Caenorhabditis elegans* HSD17B12 analogue
mAbs	monoclonal Antibodies
MAPK	Mitogen Activated Protein Kinase
mir-122	micro RNA 122
MTP	Microsomal triglyceride Transfer Protein

MUFAs	Mono Unsaturated Fatty Acids
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NANBH	Non-A, Non-B Hepatitis
NPC1L1	Niemann-Pick C1-Like 1
OCLN	Occludin
OCT1	Organic Cation Transporter 1
ORF	Open Reading Frame
OSBP	Oxysterol-Binding Protein
PC	Phosphatidyl Cholin
PE	Phosphatidylethanoleamine
PH	Pleckstrin Homology
PHHs	Primary Human Hepatocytes
PI (4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PI4KIII	Phosphatidylinositol-4-kinase III
PI(4)P	Phosphatidylinositol-4-phosohate
PLA2	Phospholipase A2
PPAR	Peroxisome Proliferator-Activated Receptor
PRR	Pathogen Recognition Receptor
PPT	Phosphopantetheine Transferase
RdRp	RNA dependant RNA polymerase
RF	Replication Factory
RNA	Ribo Nucleic Acid
RTK	Receptor Tyrosine Kinase
RXR	Retinoid X Receptor
SCD	Stearoyl-CoA Dehydrogenase
siRNA	Silencing RNA
SR-BI	Scavenger Receptor class B type I
SREBP1c	Sterol Regulatory Element-Binding Protein 1c
SL-	Stem-Loop
TECR	Trans-2,3,-Enoyl-CoA Reductase
TG	Triglycerides

TE	Thioestrase domain
TM	Transmembrane segments
TMDs	Transmembrane Domain
UTRs	Untranslated Regions
VAP-A	Vesicle-Associated membrane Protein-associated protein-A
VLCFA	Very-Long-Chain Fatty Acids
VLDL	Very Low Density Lipoprotein
vRNA	viral RNA
WNV	West Nile Virus
YFV	Yellow Fever Virus

Alhamdulillah

You think that you are a small luminary (in the sky)

While inside you, the whole universe is hidden!

Ali ibn Abi-Taleb

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Chapter 1. Introduction

1.1. *Flaviviridae* family

Viruses of the *Flaviviridae* family are enveloped single-strand positive-sense RNA viruses, with the nucleocapsids surrounded by two or more types of envelope glycoproteins and a lipid layer [1]). It is composed of different genera, including Hepacivirus [e.g., hepatitis C virus (HCV)], Flavivirus [e.g., Zika Virus (ZIKV), Dengue Virus (DENV)], Pegivirus and Pestivirus [2]. Multiple members within the Flavivirus and Hepacivirus genera are significant human pathogens.

HCV is a major human liver-specific pathogen that causes chronic infection, and is the primary cause of liver dysfunction worldwide with more than 70 million people at risk of progression to liver cirrhosis and hepatocellular carcinoma (HCC) [3]. Medically relevant flaviviruses, including ZIKV, DENV, West Nile Virus (WNV), Japanese Encephalitis Virus (JEV) and Yellow Fever Virus (YFV), are usually arboviruses (transmitted by arthropods, mainly mosquitoes and ticks) that are responsible for severe mortality in humans and animals worldwide. DENV and YFV infections can cause severe disease in infected patients, including vascular leakage and haemorrhage [4-6]. DENV causes 60 million symptomatic infections annually, leading to approximately 10,000 deaths per year [3]. On the other hand, JEV and WNV infections tend to cause neurological diseases [7, 8]. ZIKV infection is associated with microcephaly (a serious birth defect) and other neurological disorders [9]. In the last decade, targeting HCV by direct-acting antiviral drugs achieved unprecedented cure rates reaching about 98% of the treated patients; however, there is still an urgent need for medications and vaccines against other *Flaviviridae* viruses such as DENV and ZIKV. These viruses represent a

considerable threat to public health due to the increase in their outbreaks and geographical spread [10].

On the level of molecular virology, viruses within the hepacivirus and flavivirus genera are similar in their general genome organization and the general principle of the replication cycles [2]. The similarities in the ways of host cell manipulation by these viruses may offer the opportunity to learn from the data related to HCV. We can focus on the importance of the virus-host lipid interaction, in the context of antiviral therapy, to discover new avenues to target DENV and ZIKV.

1.2. General aspects of HCV

1.2.1. Discovery of HCV

The problem of parentally transmitted hepatitis appeared following the development of serological tests to detect hepatitis A virus (HAV) and hepatitis B virus (HBV) infections in the 1970s [11]. Most cases of unknown origin surprisingly represented hepatitis. The condition was termed non-a, non-b hepatitis (NANBH). Further, it has been demonstrated that NANBH is progressing gradually and has led to liver cirrhosis in 20% of infected patients [12]. In order to solve this problem, early studies on viral hepatitis used chimpanzees as reliable models for infection transfer from human biological materials [13, 14], further proving the existence of NANBH-causing agents. The chimpanzees' hepatocytes showed tubule-like structures in their cytoplasm [15]. Tubule forming agent (TFA) is described as a lipid-enveloped agent that can be filtered and inactivated by organic solvents [16]. After many efforts to identify the causative agent of NANBH, a team lead by Dr. Michael Houghton at Chiron Corporation identified, for the first time, an agent termed HCV by using bacterial expression cDNA libraries to immune-screen NANBH patient sera [17] **(Fig. 1.1).** Upon discovery, the same group produced the first enzyme specifically for NANBH-specific antibodies. The use of this testing tool demonstrated that the major cause of parentally transmitted NANBH is HCV [18].

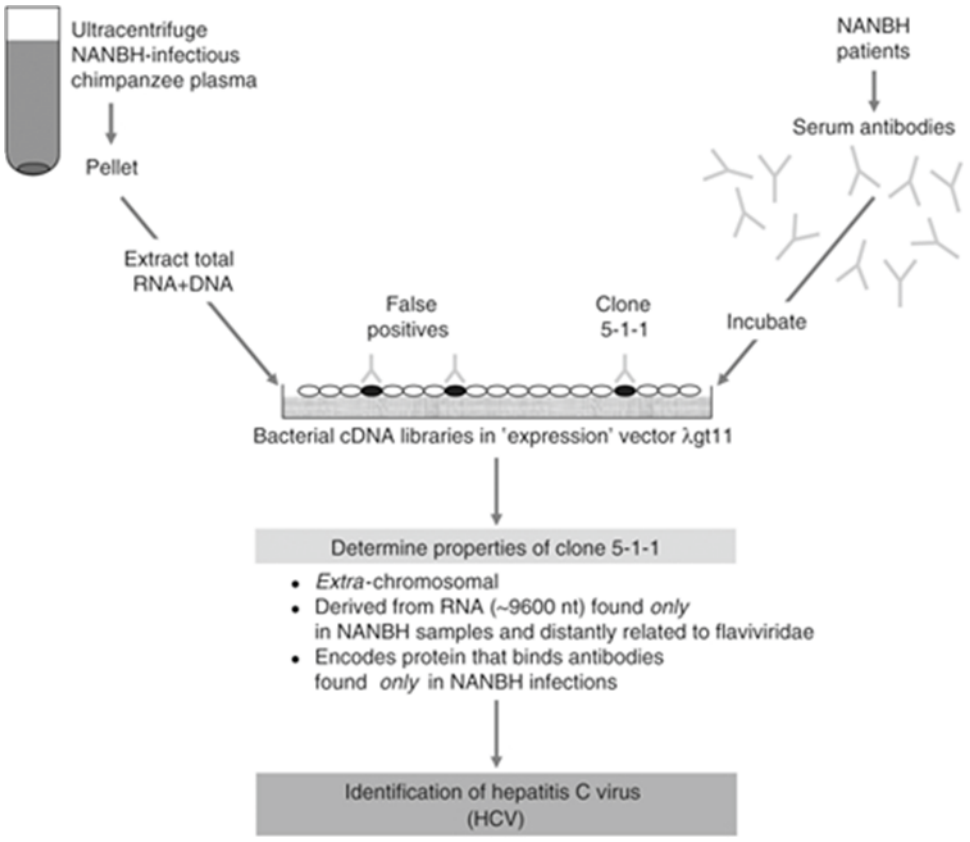

Figure 1.1. Schematic illustration for HCV first identification.

Figure 1.1 shows the use of bacterial expression cDNA libraries to immune-screen NANBH patient sera. This led to discovery of clone 5-1-1 (HCV genome). The figure is adapted with permission from (Houghton, M., Discovery of the hepatitis C virus. Liver Int, 2009. 29 Suppl 1: p. 82-8) [19].

1.2.2. HCV infection: disease and fate

HCV is a significant chronic disease, as 70% of infections progress to persistent infections [20]. During the first week of infection, HCV titers increase exponentially as the virus is able to generate up to 10^{12} virions daily [21, 22]. The patients are mainly asymptomatic; this leads to the spread of the infection. Further, the patients become clinically symptomatic late after the liver disease is established, and its symptoms become apparent [23].

HCV infection is the disease most causative of liver transplantation [24]. The liver damage occurs despite HCV not being a cytopathic virus. Several studies showed that the liver pathology leading to HCC is due to direct cytotoxic T-lymphocyte-mediated killing of hepatocytes [25].

1.2.3. HCV transmission

In developing countries, the primary cause of HCV transmission is iatrogenic, stemming from the use of blood-contaminated medical instruments. For example, Egypt has one of the highest populations of HCV infection. This high prevalence was before the introduction of treatment campaigns with direct-acting anti-HCV drugs. The HCV surge among the Egyptian population was due to anti-schistosomiasis campaigns of mass intravenous (IV) injections using glass syringes in the 1950s [26]. In developed countries, the primary source of transmission is the recreational injection drug use. In Canada, for example, the drug-using population represents near 80% of acquired HCV infection [27].

1.2.4. Virus composition

HCV has an outer envelope containing two proteins: E1 and E2. Underneath the membrane is a layer of the viral core protein, which binds to the viral genome forming the nucleocapsid where the RNA is located [28].

HCV particles purified from sera of humans and chimpanzees exhibit a wide range of low densities between 1.03 g/cm^3 and 1.20 g/cm^3 [29, 30]. Further characterization showed that the particles of lower density have higher infectivity [31]. The virus from patient sera was described for the first time as a lipo-viro-particle when it was found to be associated with a very low-density lipoprotein (VLDL) or low-density lipoprotein (LDL). Virus particles obtained from cell culture confirmed the association with lipoprotein and apolipoproteins ApoE and ApoB. However, they had slightly higher density and a lower specific infectivity [32, 33]. HCV has a unique lipid profile among the *Flaviviridae* family. HCV particles are composed mainly of neutral lipids like cholesterol esters and triglycerides. In contrast, the lipidomic characterization of bovine viral diarrhea virus (BVDV), from the same family, did not reveal the presence of those neutral lipids [34].

1.2.5. HCV genome

The HCV genome is about 9.6 kb. The genome is represented by a non-interrupted open reading frame flanked by two untranslated regions (UTRs). The 5'UTR contains the internal ribosomal entry site (IRES) where the virus genome translation starts. The 3'UTR has *cis*-acting RNA elements that stimulate translation. The viral open reading frame codes for about 3000 amino acids. The HCV polyprotein is processed by a combination of viral and cellular proteases. The viral polyprotein is processed into three

structural proteins [35] - capsid protein and (E1 and E2) envelope proteins – and seven non-structural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [19] **(Fig. 1.2).**

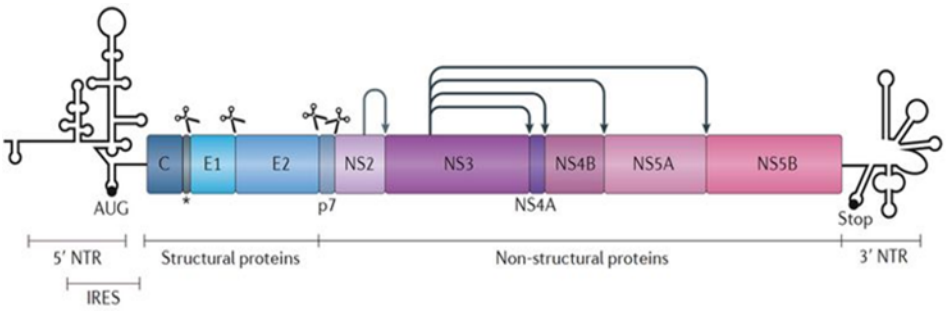

Figure 1.2. Genomic organization of HCV virus.

The figure shows the HCV genome and the protein code. Scissors indicate cleavage by cellular proteases, whereas the arrows depict cleavage by the viral proteins. The figure is adapted with permission from (Neufeldt, C.J., et al., Rewiring cellular networks by members of the Flaviviridae family. Nat Rev Microbiol, 2018. 16(3): p. 125-142.) [2].

1.2.6. Genetic variance, genotypic distribution, and origins

HCV is very diverse genetically and based on phylogenetic and sequence analysis of the viral genome it is classified into seven genotypes (1-7) [19]. Genotyping is based on genome areas which are highly conserved, namely E1, core, NS5B, and 5'UTR [36]. The different genotypes diverge at about 30-35% of the nucleotide sequence. The strains in different genotypes have less than 15% difference in nucleotide sequence [37].

HCV genotype 1, as opposed to 2 and 3, is the most distributed genotype in the developed Western world. The distribution includes North America and Northern and Western Europe. Regarding genotypes 4, 5, and 6, they are more confined to some regions of the world. For example, genotype 4 is found mainly in the Middle East [38]. Genotype 5 is found in South Africa [39], while genotype 6 is predominantly found in South Asia [40]. Genotype 7 is most probably related to Central Africa [41].

1.3. Molecular biology of HCV

1.3.1. HCV proteins

HCV Core

The first structural protein encoded by the HCV open reading frame (ORF) is the core protein **(Fig.1.3)**, which forms the viral nucleocapsid [42]. The premature core (191 amino acids) is constituted by three domains. Recruitment of the premature core to the endoplasmic reticulum (ER) depends on the carboxyl terminal (C-terminal) of domain III [43]. However, the association and targeting lipid droplets (LDs) are dependent on the proteolytic cleavage of the distal C-terminal (domain III) by the signal peptide peptidase enzyme, and generation of the mature core (177 amino acids) [44]. The mature core has two domains. The amino terminal (N-terminal) of domain I is rich in positively charged amino acids and is mostly hydrophilic. This characteristic contributes to RNA-binding ability of the protein [45, 46]. Moreover, the same domain catalyzes the homo-oligomerization of the core and its interaction with glycoprotein E1 [47, 48]. On the other hand, the hydrophobic C-terminal of domain II is responsible for membrane association, for instance, to the membrane surrounding LD [47]. Alternative reading frame protein (ARFP) is a result of a ribosomal frame-shift near codon 11 in core sequence. The protein is composed of 160 amino acids and is expressed in HCV-infected patients. The function of this protein is still to be elucidated [49]. HCV core, particularly of genotype 3, induces the expression of lipid *de novo* synthesis genes and contributes to the development of hepatic steatosis [50].

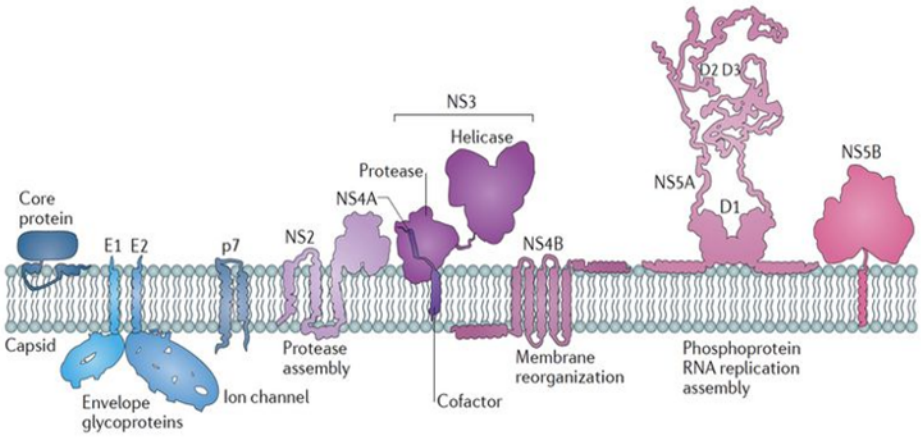

Figure 1.3. HCV proteins attached to ER membranes.

Schematic representation of HCV proteins shows three structural proteins; (core) capsid protein, (E1 and E2) envelope proteins, and seven nonstructural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [35]. The figure is adapted with permission from (Neufeldt, C.J., et al., Rewiring cellular networks by members of the Flaviviridae family. Nat Rev Microbiol, 2018. 16(3): p. 125-142.)[2].

The envelope glycoproteins

HCV has two envelope glycoproteins namely, E1 of 35kDa and E2 of 70kDa. The two proteins are heavily glycosylated. They have a small C-terminal transmembrane domain that contains the retention signal in the ER, and a large N-terminal ectodomain facing the ER lumen [51]. The two envelope proteins are heterodimerized through the transmembrane domain [52]. E1 and E2 are responsible for the early stage of viral entry through the interaction with permissive cell receptors [53].

P7

P7 is a protein with the ability to form pores in artificial membranes. It also has ion channel activity. In virtue of these characteristics, it belongs to the family of viroporins [54]. P7 resides in the ER and has two hydrophobic transmembrane passages linked together through a short hydrophilic segment [55]. P7 has a significant role in HCV particle infectivity through its ion channel activity and interaction with other viral proteins. However, the P7 protein is dispensable in HCV RNA replication and virus entry [56].

NS5A

NS5A plays an essential role in viral replication, particle production, and modulation of cellular environment [57]. No enzymatic activity has been reported for NS5A. NS5A has three domains: one highly structured domain (DI) and two unstructured domains (DII and DIII). DI integrity is essential for RNA replication, while deletion of large parts of DII and DIII could be done without affecting it. A large deletion of DIII domain was used to generate a fluorescent fusion protein of NS5A to produce an RNA

replication-competent replicon [58]. However, the NS5A mutants are impaired in particle production due to the loss of the interaction with core protein [59]. NS5A has two different phosphorylated forms. They are named according to their molecular mass: the basal p56, and the hyper-phosphorylated p58. The p56/p58 ratio was reported to alter HCV RNA replication efficiency [60]. The NS5A N-terminal alpha-helix anchors the cytosolic leaflet of the ER, which is critical for HCV RNA replication [61]. NS5A interacts with ER vesicle-associated membrane protein A (VAP-A), which could play an essential role in the recruitment of effector enzymes such as oxysterol binding protein (OSBP), which is capable of binding VAP proteins [62]. NS5A importantly interacts with phosphatidylinositol-4-kinase III alpha (PI4KIIIA). This interaction stimulates the enzyme. Further, the stimulation of the enzyme leads to the accumulation of phosphatidylinositol-4-phosphate (PI(4)P) which is essential to the recruitment of other effector enzymes that have pleckstrin homology domains (PH domains) [63, 64]. Indeed, NS5A plays a vital role in the preparation of HCV RNA for encapsidation. It is proposed that NS5A and possibly NS2 [65] deliver the virus RNA genome from sites of RNA replication to the LD at which core protein processes the encapsidation [66]. Further, NS5A Domain I was shown to be critical for the induction of double-membrane vesicles associated with hepatitis C virus replication [67].

NS2

NS2 encodes a cysteine protease that catalyzes the cleavage of the HCV polyprotein precursor at the NS2/NS3 junction. NS2's cysteine protease function is enhanced by the N-terminal one third of NS3 [68, 69]. NS2 is not directly required for HCV replication. However, it plays a pivotal role in the regulation of HCV assembly.

The role involves complex interactions with structural and non-structural viral proteins [70]. NS2 is proposed to act as a scaffold bringing the replicase machinery (NS3, NS5A) and the envelope glycoproteins to assembly sites. Mutations in NS2 abrogate the interaction with NS3, NS5A, P7, and E2, consequently impairing HCV particle production [65, 71, 72].

NS3

NS3 has multiple enzymatic activities, including protease and helicase activities. The protease activity is stimulated when the enzyme binds to its co-factor NS4A and mediates the processing of the polyprotein in the nonstructural protein region [73]. The C-terminus carry the helicase activity. This activity mediates ATP hydrolysis together with unwinding HCV RNA [74, 75]. NS3 is critical for HCV replication and particle assembly [76]. NS3, together with its co-factor NS4A, is localized to the ER membrane and the contact sites between the ER and the mitochondrial membrane. This localization promotes its ability to hinder the induction of innate cellular immunity. Indeed, NS3/4A can cleave mitochondrial antiviral signalling protein MAVS, which is the adaptor of the retinoic acid-inducible gene (RIG-I) [77, 78].

NS4A

The central part of the NS4A protein acts as an NS3 co-factor, while the N-terminus plays a role in tethering NS3 to cellular membranes [79]. However, the function of the C-terminus part is not clear. Mutagenesis studies suggest that through interaction with other nonstructural proteins, it may play a role in HCV RNA replication and particle production [80].

NS4B

NS4B is an integral membrane protein composed of a central core of four transmembrane domains, and C- and N- terminuses facing the cytoplasm [81]. NS4B can induce the rearrangement of ER membranes and generation of vesicle-like structures, which act as HCV replication complex sites [82, 83]. NS4B has shown to bind viral RNA and to interact with HCV nonstructural proteins [84]. Further, NS4B was shown to play a role in viral assembly [85]. Moreover, it was demonstrated to harbour NTPase activity [86], and, similar to the other HCV nonstructural proteins, NS4B has been reported to form oligomers [87]. Interestingly, it was demonstrated that NS4B protein oligomerization is critical for membranous web formation and HCV RNA replication, and hence is required for the assembly of a replication complex, possibly through the induction of membrane curvature and vesicle formation [70].

NS5B

HCV replication proceeds via synthesis of a complementary negative-strand RNA using the genome as a template and the subsequent synthesis of genomic positive-strand RNA from this negative-strand RNA template. The enzyme responsible for this process is NS5B RNA-dependent RNA polymerase (RdRp), which is the core of the replication machinery responsible for the amplification of the HCV genome. The NS5B protein is composed of two domains: a hydrophobic transmembrane domain, and the C-terminal catalytic domain to which the former is linked [58, 88]. The active site of the polymerase includes a GDD motif that is involved, along with the contribution of Mg2+ ions, in the binding of nucleotide substrates and nucleotide polymerization [78].

1.3.2. HCV life cycle

The HCV life cycle includes three major steps: the viral entry, the translation and replication of the virus genome, and the assembly of the viral particles [89]. The HCV life cycle is represented in **(Fig. 1.4)**.

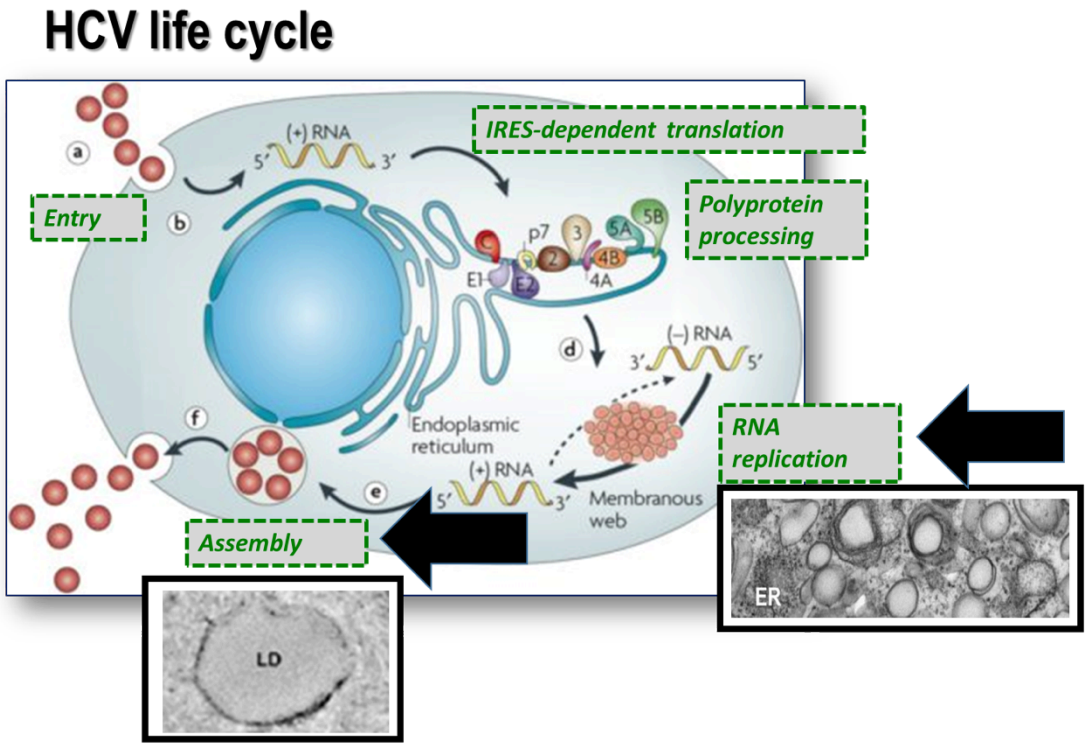

Figure 1.4. Schematic representation of the HCV life cycle.

Virus binding and internalization (a); cytoplasmic release and uncoating (b); IRES-mediated translation and polyprotein processing (c); RNA replication (d); packaging and assembly (e); virion maturation and release (f). The steps are illustrated as separate steps for simplicity. The bold black arrows refer to the replication and assembly compartments (representative electron microscope images are shown). The steps occur in a tightly coupled fashion. The figure is adapted with permission from (Moradpour, D., F. Penin, and C.M. Rice, Replication of hepatitis C virus. Nat Rev Microbiol, 2007. 5(6): p. 453-63.) [89].

1.3.2.1. HCV entry

HCV, during circulation in the blood, is directly in contact with the basolateral membrane of hepatocytes. The contact allows it to bind to the receptors on the surface of these cells and trigger its entry, as reviewed in Lindenbach *et al*. 2013 [72]. LDL receptor (LDL-R), glycosaminoglycans (GAGs), and heparan sulfate proteoglycans (HSPGs) mediate low-affinity attachment of HCV to hepatocytes' surfaces. This attachment occurs by virtue of the HCV particle apolipoprotein E (ApoE) content [72]. In addition to this, there are five hepatocyte surface molecules necessary for HCV particle entry. These are: CD81, scavenger receptor class B type I (SR-BI), claudin 1 (CLDN1), occludin (OCLN) and Niemann-Pick C1-like 1 (NPC1L1). As well, receptor tyrosine kinase (RTK) and tight junction cadherin are regulatory molecules essential for HCV entry [72]. **(Fig. 1.5)**

(i) CD81

Human CD81 is widely expressed in many cell types. The molecule is a tetraspanin adaptor cell surface protein [90]. CD81 promotes a conformational change in the HCV E1/E2 glycoproteins. The conformational change is important for low-pH-dependent fusion and viral endocytosis [91]. CD81, through its large extracellular loop, binds HCV envelope glycoprotein E2 to facilitate HCV entry [53, 92]. The sequences of the extracellular loops of CD81 are conserved between humans and chimpanzees. However, cells from different species with different sequences can support HCV entry in vitro [93].

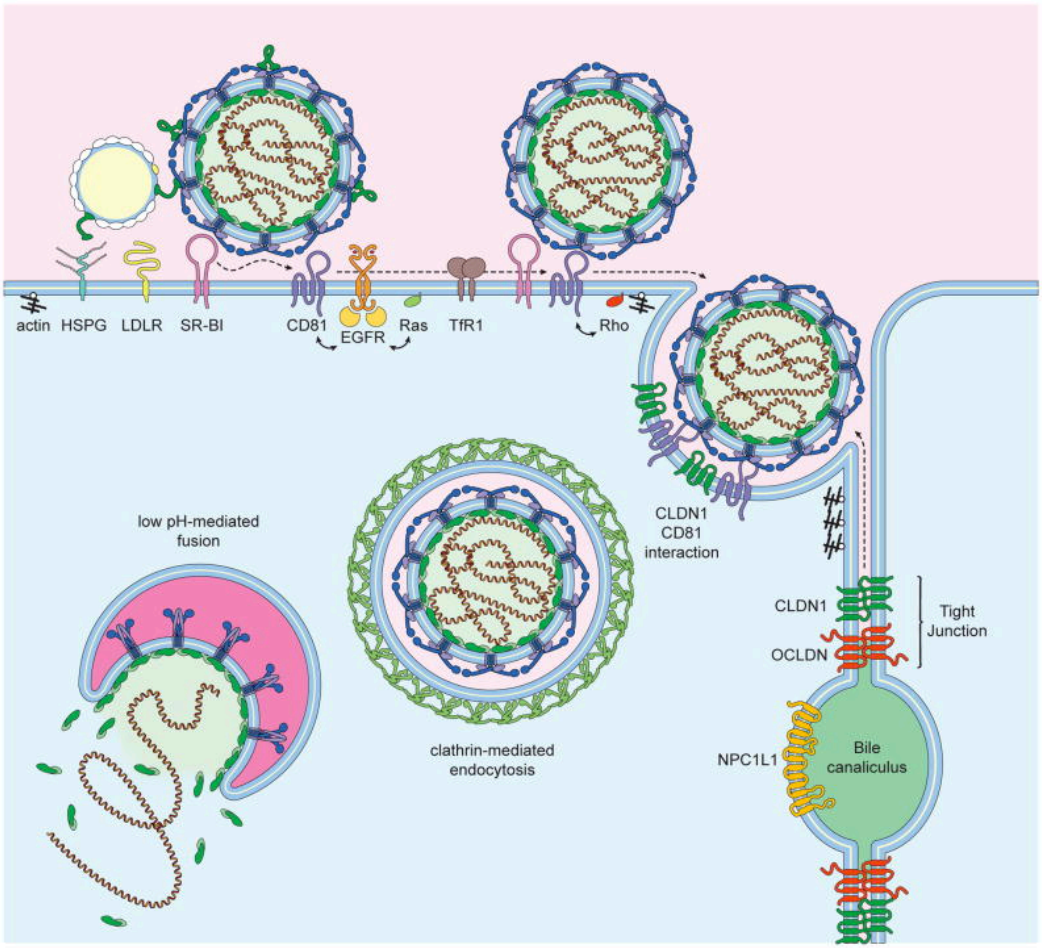

Figure 1.5. Schematic representation of hepatocyte HCV entry factors.

HCV lipo-viro-particle attaches to the cell membrane through interaction with HSPG, LDLR, and SR-BI. SR-BI may produce conformational changes in E2, leading to the exposure of the CD81 binding site. Interaction of E2 with CD81 leads to the activation of the signal transduction through EGFR, Ras, and Rho (Ras homology) GTPases. These signalling events enhance the lateral movement of CD81-HCV complexes to the cell-cell contact sites. Then, CD81 interacts with CLDN1 and promotes HCV internalization via clathrin-mediated endocytosis. The low pH of the endosomes leads to HCV fusion. The figure is adapted with permission from(Lindenbach, B.D. and C.M. Rice, The ins and outs of hepatitis C virus entry and assembly. Nat Rev Microbiol, 2013. **11**(10): p. 688-700.) [72].

(ii) Scavenger receptor class B type I (SR-BI)

SR-BI is a 509-amino acid polypeptide, and it regulates lipid metabolism through its function as a primary receptor for high-density lipoproteins (HDLs), supporting its selective uptake into hepatocytes [94, 95]. SR-B1 was initially identified as a putative receptor for HCV because it binds soluble E2 (sE2). This binding was suggested to occur through the interaction with E2 hypervariable region 1 (HVR1) [96]. SR-BI mediates primary attachment of HCV particles of intermediate density to cells. These initial interactions involve apolipoproteins, such as ApoE, which is present on the surface of HCV particles [97]. This suggests that the lipoprotein components in the virion act as host-derived ligands for important entry factors such as SR-BI. It has been shown that HCV entry relies on the lipid transfer activity of SR-BI [97]. Indeed, SR-BI antagonist was demonstrated to have additive and synergistic potency when used in combination with other antiviral agents [98].

(iii), (iv) Claudin 1 and Occludin

CLDN1 and OCLN are two tight junction proteins. The two proteins were identified as factors able to render non-human cells supportive of HCV entry [99, 100]. It was suggested that CLDN1 and OCLN support the later phase of HCV entry, after SRB1 and CD81. Interestingly, CLDN1 and OCLN do not directly interact with the HCV envelope proteins. However, CLDN1 and CD81 interact to form a part of the HCV receptor complex [101, 102]. It was also shown that HCV envelope glycoproteins stimulate endocytosis of both CD81 and CLDN1 and support their fusion with the early endosome, supporting a model wherein HCV stimulates receptor trafficking to promote particle internalization [103].

(v) Niemann-Pick C1-like 1

NPC1L1, a 13 transmembrane cell surface cholesterol-sensing receptor, is expressed in the gastrointestinal tract on the apical surface of intestinal enterocytes, in addition to the human hepatocytes like Huh7 cells. Further, NPLC1L1 is responsible for the regulation of cellular cholesterol absorption and controls body cholesterol homeostasis [104].

Silencing or antibody-mediated blocking of NPC1L1 impairs cell culture-derived HCV (HCVcc), demonstrating that NPC1L1 expression is required for HCV infection initiation [105]. The requirement was shown to be dependent on virion cholesterol content, and to occur before the virion-cell membrane fusion step [105]. Further, ezetimibe, the clinically available FDA-approved NPC1L1 antagonist, potently blocks HCV uptake *in vitro*. [105].

Regulatory molecules

(i) Receptor tyrosine kinases

Using kinase RNAi screens, two receptor tyrosine kinases RTKs, including the epidermal growth factor receptor (EGFR) and ephrin type-A receptor 2 (EphA2), were identified as HCV entry cofactors [106]. The two receptors regulate CD81-claudin-1 co-receptor associations and viral glycoprotein-dependent membrane fusion [106]. GTPase HRas, (Harvey Rat Sarcoma oncogene homolog) activation downstream of EGFR signalling was shown as a critical host signal transducer for EGFR-mediated HCV entry. Moreover, HRas signalling drives the lateral membrane diffusion of CD81 [107].

(ii) E-cadherin

E-cadherin is a transmembrane glycoprotein, which acts as a significant adherent junction protein. It plays an essential role in maintaining cell-cell adhesion. Further, it plays a vital role in maintaining the epithelial architecture and cell polarity and differentiation [108, 109]. Li and co-workers showed that E-cadherin regulates HCV entry [110]. Their functional studies demonstrated that E-cadherin is closely associated with CLDN1 and OCLN on the cell membrane. It was also demonstrated that the depletion of E-cadherin severely diminished the cell-surface distribution of these two tight junction proteins. The phenotype indicates that E-cadherin plays a vital role in the regulation of CLDN1/OCLN localization on the cell surface [110].

1.3.2.2. HCV translation and replication

HCV has highly structured 5'- and 3'-untranslated regions (UTRs) flanking a single open reading frame instead of a 5'-terminal cap and a 3'-terminal poly (A) tract structure characterizing the host cell mRNA [111]. HCV translation initiation occurs by a cap-independent mechanism mediated by the type III internal ribosomal entry site (IRES) in the 5' UTR [112]. The IRES encompasses 5' UTR stem-loop II-IV [111]. The IRES includes two stem-loop structures located in the core-coding region. Beside the IRES, several *cis*-acting RNA elements in the 3' UTR stimulate the translation of HCV RNA. Recent reports suggest the circularization of the HCV genome occurs as a result of the interaction between stem-loop structures residing in the NS5B coding region and motifs in the IRES [113].

Further, it has been shown that possible circularization of the HCV genome is likely facilitated by cellular and viral proteins, for instance, PCBP2 (poly(rc)-binding protein 2), ILF3 (interleukin enhancer-binding factor 3) and IGF2BP1 (insulin-like growth factor II mRNA-binding protein 1) [114, 115]. The circularization of the HCV genome facilitates the translation (5' to 3' direction) and the viral replicase-mediated RNA synthesis (3' to 5' direction). Furthermore, liver-specific microRNA-122 (miR-122) [116] binds to two sites in the 5' UTR of the HCV RNA genome, leading to stimulation of IRES-mediated translation, and protecting viral RNA from degradation via recruitment of Argonaute 2 [111, 117].

HCV RNA translation leads to a single polyprotein, which is subsequently processed by viral and host encoded proteases into 10 mature proteins (discussed in section 1.3.1). Host signal peptidases and signal peptide peptidases process the structural proteins of HCV by mediating the cleavage at the core/E1, E1/E2, E2/p7 and p7/NS2 junctions. Further, the NS2 cysteine protease, whose activity is enhanced by the N-terminus of NS3, mediates the cleavage of the NS2/NS3 junction releasing the NS3/4A serine protease. NS3/4A processes most of the non-structural (NS) proteins by mediating the cleavage of the NS4A/4B, NS4B/5A, and NS5A/5B junctions while also cleaving itself and other host factors [70].

On the other hand, viral and cellular proteins orchestrate the process of HCV RNA replication in the multi-step process [118]. The first step of RNA synthesis generates a negative-strand genome, which serves as a template for progeny positive-strand RNA that is produced in 5- to 10-fold excess [118].

It is unclear how RNA replication and translation are co-regulated. As described before, the circularization of the RNA genome might be one mechanism. Another possibility is the formation of alternative RNA structures. For instance, it was shown that the 5' UTR of the positive-strand RNA genome and its complementary sequence – i.e., the 3' UTR of negative-strand RNA – have very different secondary structures [119]. Moreover, RNA sequences in domain II of the IRES are essential for RNA replication [120]. These characteristics could present multiple signals that might be involved in the coordination between RNA translations and replication.

HCV, like the other positive-strand RNA viruses, remodels the intracellular membranes, leading to the formation of organelle-like membranous structures named as replication factories (RFs) or membranous web (MW). The MW plays many essential roles, which include (i) compartmental concentration of factors required for efficient viral RNA replication, (ii) spatial coordination of (RNA translation, replication, assembly) in the viral replication cycle, and (iii) protecting viral proteins and RNA from intracellular innate immunity [120].

Positive-strand RNA virus-induced membranous rearrangements could be included in two morphological types: the double-membrane vesicle (DMV) type and the invaginated vesicle/spherule type [89, 121]. Interestingly, picornavirus and coronavirus replication sites belong to the DMV type, though they have only a very distant evolutionary relationship with HCV, whereas the more closely HCV-related flavivirus such as DENV and West Nile virus (WNV) have replication sites in the form of invaginated vesicles within the ER [120].

Electron microscopy (EM) studies of HCV-infected cells show the formation of DMVs of ~150 nm diameter [122]. The DMVs aggregate to form the structure defined as the membranous web. The DMVs originate as a protrusion from the ER to the cytoplasm, by the action of HCV viral proteins NS4B and NS5A [67, 82]. Importantly, the outer membrane is often linked to the ER membrane. The membranous structures also contain Single Membrane Vesicles (SMVs) and Multi-membrane Vesicles (MMVs) [123]. Biochemical characterizations have demonstrated that HCV RNA and replicase activity reside within nuclease-resistant and protease-resistant environments [121]. This suggests that the HCV replication occurs inside the lumen of DMVs. Only 10% of DMVs have pores like a connection to the cytoplasm [124]. Hence, it may be only a minor fraction of the DMVs that support active replication at a given time. Otherwise, DMVs have nuclear pore complex-like structures, which might enable the traffic in and out of a closed membrane compartment [125].

Host factors, besides HCV proteins, critically contribute to RF formation. For instance, cyclophilin (CypA), by acting on NS5A, is suggested to contribute to the formation of HCV RFs [126]. Another example is PSTPIP2 (proline-serine-threonine phosphatase interacting protein 2), which belongs to the BAR (Bin-Amphiphysin-Rvs) domain-containing protein family that acts as a sensor or inducer of positive membrane curvature [127]. A study by Chao *et al.* [127] showed that PSTPIP2 is required for HCV-induced membrane alterations and, thus, RNA replication. Both NS4B and NS5A interact with PSTPIP2, and recruit it to HCV-remodeled membranes. Further, depletion of this host factor abrogates DMV formation [127].

HCV uses a sterol regulatory element-binding protein-1 (SREBP1) pathway to induce *de novo* lipid and membrane biosynthesis [128]. The induction has been shown to cause distinct changes in the lipidomic profile of HCV-infected cells [129]. The SREBP1 pathway was also activated in the core- and NS4B-overexpressing cells [128, 130]. Further, the overexpression stimulates the transcription of lipogenic genes such as fatty acid synthase (FASN) and 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA), the rate-limiting enzyme of the cholesterol biosynthesis of the mevalonate pathway. Further, viral replication requires the metabolic intermediate geranylgeranyl phosphate in protein prenylation [131]. NS5B has been shown to also interact with FASN to stimulate its RdRp activity [132].

HCV is strongly dependent on the lipid kinase PI4KIIIα and its product, phosphatidylinositol-4-phosphate (PI(4)P) [120]. PI(4)P is predominantly found in Golgi membranes and the inner leaflet of the plasma membrane of non-infected cells. However, in HCV infected cells, the PI(4)P is highly enriched in DMVs and RF [63, 133]. PI4KIIIα knockdown impairs HCV replication and causes the aggregation of DMVs of significantly reduced diameter [63]. The same phenotype was shown when a PI4KIIIα pharmacological inhibitor was used [62]. The phenotype shows that PI(4)P is critically required for DMV functionality. Notably, DMV induction by the distantly related picornaviruses also requires PI(4)P, suggesting an evolutionarily conserved mechanism [134].

One pathway linked to PI(4)P which involves non-vesicular cholesterol transport is the oxysterol binding protein (OSBP)-mediated cholesterol transport [62 352]. OSBP catalyzes the cholesterol transport to PI(4)P-containing HCV-remodeled membranes [63].

Importantly, rhinoviruses manipulate this cellular lipid transport system in a strikingly similar way [135], demonstrating an evolutionarily conserved mechanism of DMV biogenesis. In HCV-infected cells, it has been shown that DMV diameters were reduced both by blocking OSBP-mediated cholesterol transport [130] and by depletion of cholesterol from purified DMVs [121], showing that cholesterol is an essential structural component of HCV-remodeled membranes. The knockdown of PI4KIIIα or OSBP results in very similar DMV morphologies. However, PI4KIIIα knockdown blocks HCV RNA replication potently [120]. In the same context, HCV infection induces the synthesis of specific sphingolipids that enhance NS5B-mediated RNA replication [136]. Sphingolipids, in addition to cholesterol, play a critical role in detergent-resistant membranes (DRM) [structural part of RF composed of condensation of phospholipids, sphingolipids, and cholesterol [120]].

Autophagy is another cellular pathway eventually used by HCV and other positive-strand RNA viruses to establish RFs. One obvious link is the similarity of the autophagosomes and DMV-type RFs morphologies. Moreover, HCV infection stimulates a key event in autophagosome formation, LC3 lipidation [137]. It was demonstrated that lipidated LC3 associates HCV proteins on viral replication-specialized cell membranes [138]. However, it is still not well defined which steps of the HCV replication cycle are modulated by autophagy. For instance, autophagy was reported to stimulate the translation of incoming HCV RNA without affecting RNA replication [137]. Others suggest that autophagosomes serve as platforms for HCV RNA synthesis [139]. Further, autophagy might affect virus production [140]. Hence, autophagy may have a

multifaceted impact on HCV replication. However, to what extent the autophagy machinery plays a role in viral DMV biogenesis remains to be investigated.

1.3.2.3. HCV assembly

The core protein is the maestro protein of viral assembly. (The HCV core protein interaction with other viral proteins in the assembly process is represented in **(Fig. 1.6)**) The mature core with a positively charged N-terminal binds viral genome RNA, whereas the C-terminal domain facilitates membrane binding via palmitoylated cysteine residues [47, 141]. The core protein is synthesized on ER ribosomes and then homodimerizes and traffics to cytosolic LDs. Mutations that prevent core protein from trafficking and binding to LD strongly inhibit virus assembly and lead to core protein degradation [142, 143]. Cellular factors were demonstrated for core-cLD (cytosolic LD) association. For instance, diacylglycerol-O- acyltransferase 1 (DGAT1), an enzyme required for the synthesis of triglycerides that are stored in cytosolic LDs [144], and the activity of cytosolic phospholipase which is regulated by mitogen-activated protein kinase (MAPK), were reported to be involved in core trafficking to LDs [145]. Further, core protein processes a conserved motif YXX* in which X is any amino acid and * corresponds to a hydrophobic amino acid. This motif interacts with clathrin assembly protein complex 2 medium-chain (AP2M1) and is involved in transporting core from cLD to ER [146]. Inhibition of RTKs that regulate AP2M1 activation is reported to strongly inhibit HCV assembly (i.e., anti-cancer compounds erlotinib and sunitinib) [147].

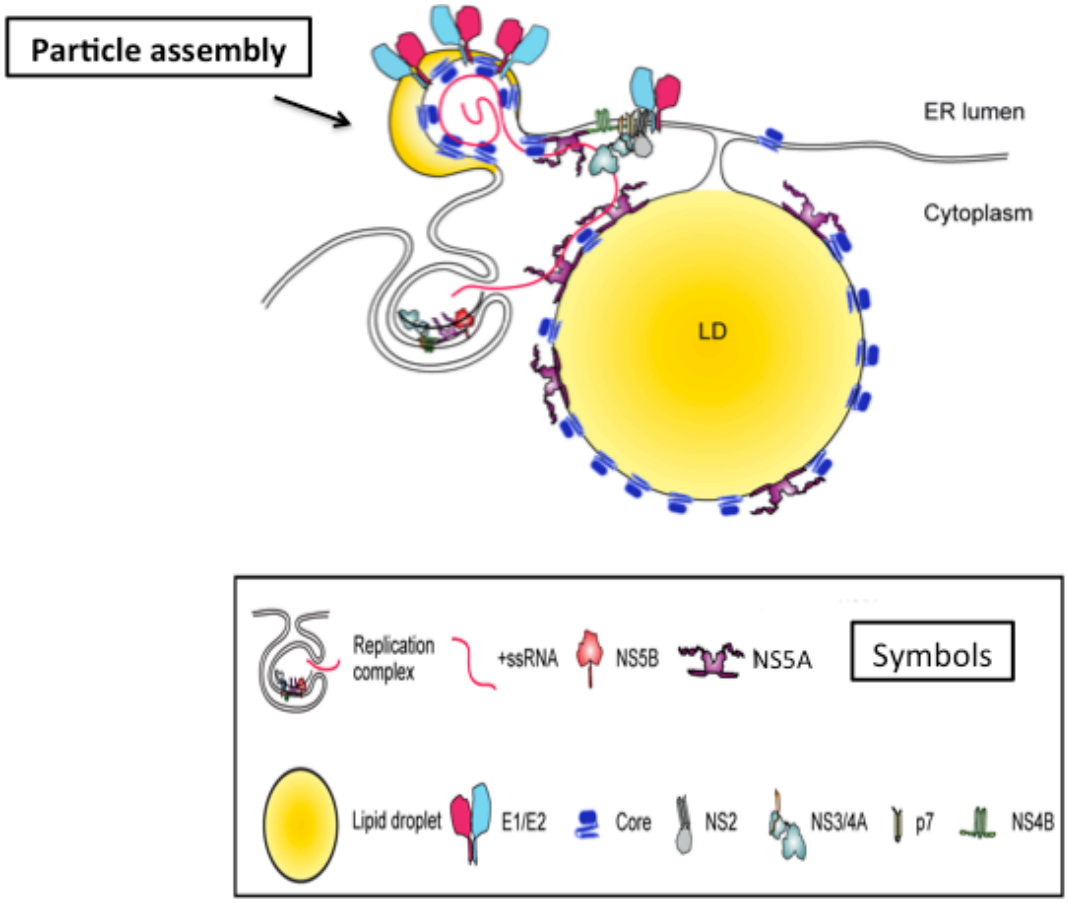

Figure 1.6. Schematic representation of HCV protein interactions necessary for virus particle assembly.

NS2, NS3, P7, and phosphorylated NS5A interact with core and envelope proteins to promote particle assembly. The figure is adapted with permission from (Zayas, M., et al., Coordination of Hepatitis C Virus Assembly by Distinct Regulatory Regions in Nonstructural Protein 5A. PLoS Pathog, 2016. **12**(1): p. e1005376.) [66]

Recently, imaging studies were conducted on virus-producing cells to obtain further insight into the trafficking of core protein during virus assembly and release [148]. It has been demonstrated that the core protein is rapidly trafficked to the surface of cLDs, and then recruited from the surface of cLD into motile puncta that move in correspondence with cell microtubules. The movement with microtubule architecture represents the virus particle within the secretory pathways [148].

While the trafficking of a core protein to LD is critical for the assembly, the core interaction with other viral factors at the assembly sites is crucial. For example, NS5A interaction with the LDs bound core protein is a crucial step in HCV assembly [59]. The C-terminal unfolded domain of NS5A has an essential role in HCV assembly. In particular, a key event for the virus assembly is the phosphorylation of specific serine in this domain by casein kinase IIα (CK IIα) [149]. Also, NS5A interactions with ApoE and annexin A2 were shown to enhance the assembly process [150, 151]. Further, NS2 and P7 play a pivotal role in organizing the formation of the virus assembly complex. NS2 processes the three transmembrane domains (TMDs). Moreover, the C-terminal cysteine protease domain of NS2 induces the protein homodimerization. The two parts of the protein – TMDs and the cysteine protease – are required for the production of the virus particle [152, 153]. Additionally, NS2 interacts with membrane-bound P7, and the interaction is essential to localize the proteins to the LD bound core [154].

Moreover, significant defects in HCV assembly were reported when a mutation in the NS3 helicase domain was created. The demonstration of this suggests the possibility that NS3 helicase activity serves a purpose in packaging viral RNA during nucleocapsid assembly [155].

E1 and E2 are located on the luminal side of the ER and associate the newly composed virion envelope. They fold and form non-covalent heterodimers [156]. The folding of each HCV glycoprotein is dependent on the other one. However, the E2 ectodomain can independently fold on its own into a structure that can be recognized by conformation-specific antibodies [157].

HCV particle production is tightly linked to very-low-density lipoprotein (VLDL) [158]. The link was shown in cell cultures and in humans. For instance, the cell culture-produced HCV particles have incorporated apolipoproteins [159] as in human serum-derived virus particles [160]. Apolipoprotein E (ApoE) has a particular importance in the HCV assembly. Many observations indicate, for example, that antibodies targeting ApoE efficiently neutralize HCV particles, highlighting its essential role in the particles' determinants of infectivity [161]. Further, the genetic knockdown of ApoE reduces HCV particle production, and this can be restored upon ectopic expression of ApoE, pointing to its essential role in particle production [161-163]. In the same context, ApoE was shown to interact with NS5A and viral envelope glycoproteins [159, 164, 165]. Recent functional data are in consensus with earlier reported imaging studies in pointing toward the role of ApoE in a post-envelopment step of HCV particle production. The data suggested that HCV secretion utilizes the same pathway as VLDL secretion [159, 166, 167].

The endosomal sorting complex required for transport (ESCRT) pathway is involved in the scission of budding membrane compartments that curve away from the original cellular membranes. Several reports demonstrated that ESCRT could be required for HCV in post-assembly steps during virus egress [168].

1.4. *In vitro* models to study HCV

1.4.1. Huh7 cells

The discovery of the Huh7 cell line that was isolated from HCC of a Japanese patient was a cornerstone in the advancement of HCV research [169]. The cell line was used to create HCV replicon that could express the virus proteins and replicate the viral RNA continuously [83]. This accomplishment led to the development of *in vitro* cell assays for screening of small molecules antivirals. It was found that some mutations in the virus genome increase the replication *in vitro*. However, these mutations render the HCV unable to be infectious to chimpanzees [170].

1.4.1.1. Subgenomic replicon system

The HCV subgenomic replicon model encodes for the viral proteins NS3 to NS5B of HCV genome **(Fig. 1.7)**. Two IRES sequences govern the translation of the replicon. The first IRES is derived from HCV and mediates the translation of a neomycin selection marker that is resistant against the cytotoxic compound G418. Additionally, it mediates expression of firefly luciferase (FLuc) gene as a quantifiable readout. The second IRES is derived from Encephalomyocarditis virus (EMCV) and mediates the translation of the five HCV viral proteins necessary for replication [171]. There are variations to the replicon system; for example, an additional GFP within the NS3 to NS5B is added for immunofluorescence microscopy investigations. The replicon system is a model of choice for high-throughput RNA interference (RNAi) and chemical screens to identify potential replication stimulators/inhibitors and co-factors/restriction factors [171].

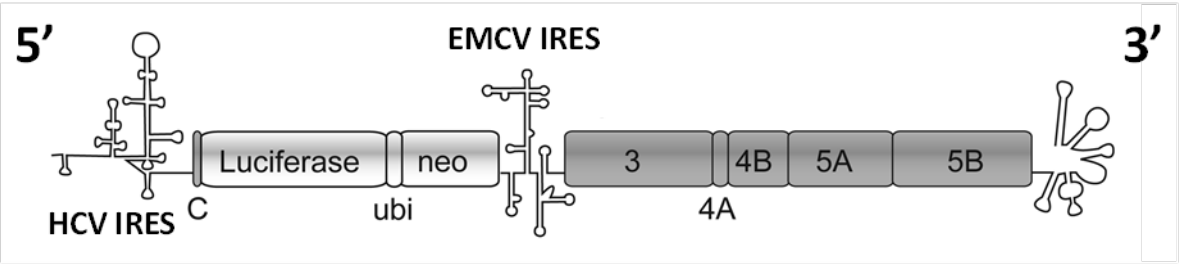

Figure 1.7. An example of an HCV subgenomic replicon.

Luciferase and antibiotic resistance are expressed under HCV IRES, while the nonstructural proteins are expressed under EMCV IRES. The figure is adapted with permission from (Lohmann, V. and R. Bartenschlager, On the history of hepatitis C virus cell culture systems. J Med Chem, 2014. **57**(5): p. 1627-42.) [171].

1.4.1.2. JFH-1 System

In 2005, the attempts to isolate *in vitro* infectious strains of HCV were successful in isolating Japanese fulminant hepatitis 1(JFH-1) genotype 2a clone [172]. This strain was isolated from a Japanese patient who developed fulminant hepatitis [173]. JFH-1 is able to replicate in cell culture systems [172]. Various chimeras were created to increase infectivity, one of which is JC1. JC1 is a chimeric HCV genome composed of a hybrid of two isolates genomes. This includes J6 (encoding core to NS2 from another genotype 2a isolate), and JFH-1 (encoding NS3 to NS5B). This chimeric HCV genome yields a viral titer 1000 times more efficient than the original JFH-1 genome [171]. JC1 has also been modified to contain a luciferase gene, renilla luciferase (RLuc), to measure the modulatory effects on the entire HCV genome **(Fig. 1.8)**. Like the replicon, JC1 can be used in high-throughput RNAi and chemical compound screens. Further, other constructs containing fluorescent proteins are used for visualization in microscopic investigations [171].

Many mutations were introduced to the JFH-1 system in order to boost viral titers. However, permissive cell lines such as Huh7.5 can also increase viral replication due to a point mutation in RIG-I, a cytoplasmic RNA sensor, which disrupts the activation of IRF3 for the production of ISGs to induce an antiviral state in the cell [174, 175]. After the discovery of JFH-1, several infectious isolates were developed for genotypes 1a, 1b, 3a, 4a [176, 177].

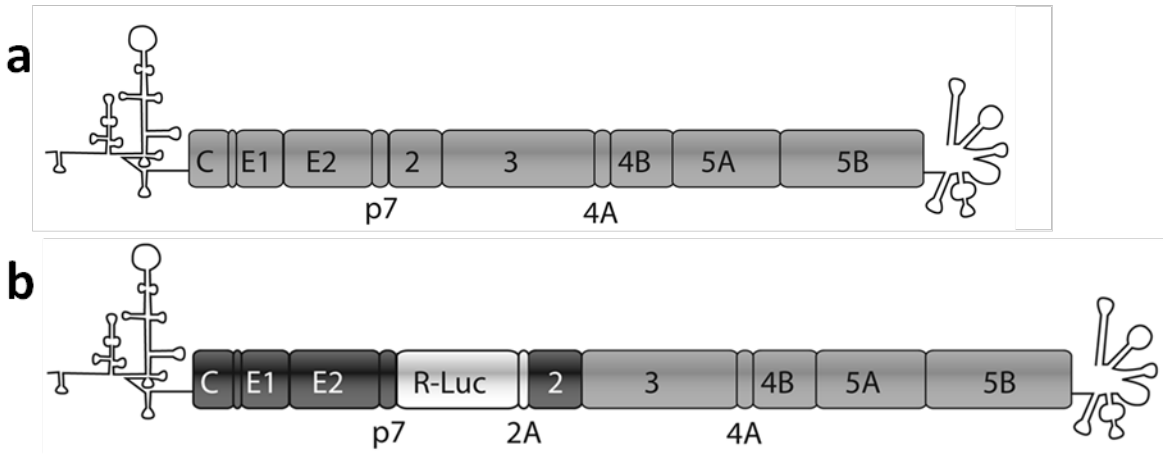

Figure 1.8. Schematic representation of a) JFH-1 genome and b) hybrid JC1 genome.

Viral proteins are indicated from J6 and JFH-1 in dark grey and grey, respectively. The figure is adapted with permission from (Lohmann, V. and R. Bartenschlager, On the history of hepatitis C virus cell culture systems. J Med Chem, 2014. **57**(5): p. 1627-42.) [171].

1.4.1.3. Primary Human Hepatocytes (PPHs)

PHHs are the best *in vitro* model to study hepatocyte metabolism and function. They are usually isolated from healthy tissues adjacent to tumors in patients undergoing liver resection [178]. Fetal livers of aborted embryos can also be a source of PHHs [179]. PHHs have a short lifespan that ranges from days to two weeks, as they do not divide [180]. Several reports have demonstrated HCV-productive infections in PHH after culture [181]. The productive HCV infection was also shown in PHHs isolated from HCV-infected patients [182, 183]. Importantly, PHH could be transplanted in mice livers to establish an *in vivo* humanized model [184].

1.4.1.4. HCV pseudo-particles

Human immunodeficiency virus (HIV) core particles were used to express HCV glycoprotein on their envelope, creating HCV pseudo-particles (HCVpp). These pseudo-particles are infectious to hepatocytes and hepatoma cells [185]. HCV pseudo-particles contributed to studies of the virus entry and assessments of neutralizing antibody (nAb) responses [186].

1.5. Fatty acid metabolism is an underexplored area during HCV and flaviviruses infection

A fatty acid is a carboxylic acid with a long aliphatic chain. They are categorized according to the number of the carbon atoms in their aliphatic tail and whether their aliphatic tail contains double bonds between its carbons [187]. Short fatty acids have less than 6 carbons, medium fatty acids have from 6 to 12 carbons, long fatty acids have from 12 to 16 carbons, and very long fatty acids have more than 16 carbons. As an energy source for the cell, their synthesis and the oxidation of their β-carbon (β-oxidation) are highly regulated [188]. Fatty acids are mainly secured via metabolic by-products, directly through dietary intake, as well as through the catabolism of glycerolipids [188].

Indeed, the synthesis of fatty acids has long been identified as a requirement for the replication of a large number of viruses including the *Flaviviridae* family (HCV, DENV, YFV, WNV, JEV) [189], and antiviral effects have been reported with inhibitors of acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN) [187, 189, 190].

The very-long-chain fatty acids (VLCFA) are the a major component of cell lipidome, and are obtained from dietary intake and *de novo* synthesis [191]. Importantly, it was shown that the most enriched lipids in HCV- and related *Flaviviridae* virus-infected cells have acyl chains longer than 16 carbons (VLCFA). These lipid species are integrated in the replication factory membrane (membranous web) and LDs [129, 144, 192-194]. To our knowledge, no studies investigated the mechanisms involving *de novo* VLCFA synthesis that may underlie these specific enrichments.

Fatty acids up to 16 carbon atoms in length (C16) are synthesized in the cytosol by the multifunctional fatty acid synthase (FASN) to elongate fatty acids in two-carbon

increments [195]. Palmitic acid (16:0) is the primary product of FASN, and it is the major precursor for VLCFA synthesis [196, 197]. The synthesis of VLCFA species (≥ C18) takes place at the ER membrane [191].

VLCFA are very abundant and represent the necessary components of cellular lipid families and species [191]. For example, phospholipids, sphingolipids, and triglycerides have VLCFA incorporated in their molecules and controlling their physical and chemical properties [191]. The intracellular pool of VLCFA is determined by several molecular mechanisms, including the regulation of their *de novo* synthesis, cellular uptake, and oxidation to produce energy. Further, their availability involves the balance between their use in complex lipid species building (anabolism) and their release from lipid breakdown (catabolism) [198].

Lipid droplets (LDs) are the major neutral lipid storage compartments inside the cell, which play an essential role in HCV and flavivirus replication [199]. The main components of LDs are triglycerides (TGs) and cholesterol ester (CEs). Interestingly, oleic acid, and stearic acid VLCFA are the primary fatty acid tails for TGs and CEs in LDs[200, 201]

Interestingly, several VLCFA-elongating enzymes are human hubs interacting with proteins of different RNA viruses. The interacting RNA viruses include HIV-1 from the *Retroviridae* family, and human respiratory syncytial virus (RSV) from the *Paramyxoviridae* family, in addition to influenza A virus (FLU) from the *Orthomyxoviridae* family. On the other hand, the interacting DNA viruses include the human papillomavirus (HPV) and Epstein-Barr virus (EBV) [202-206] **(Fig. 1.9).**

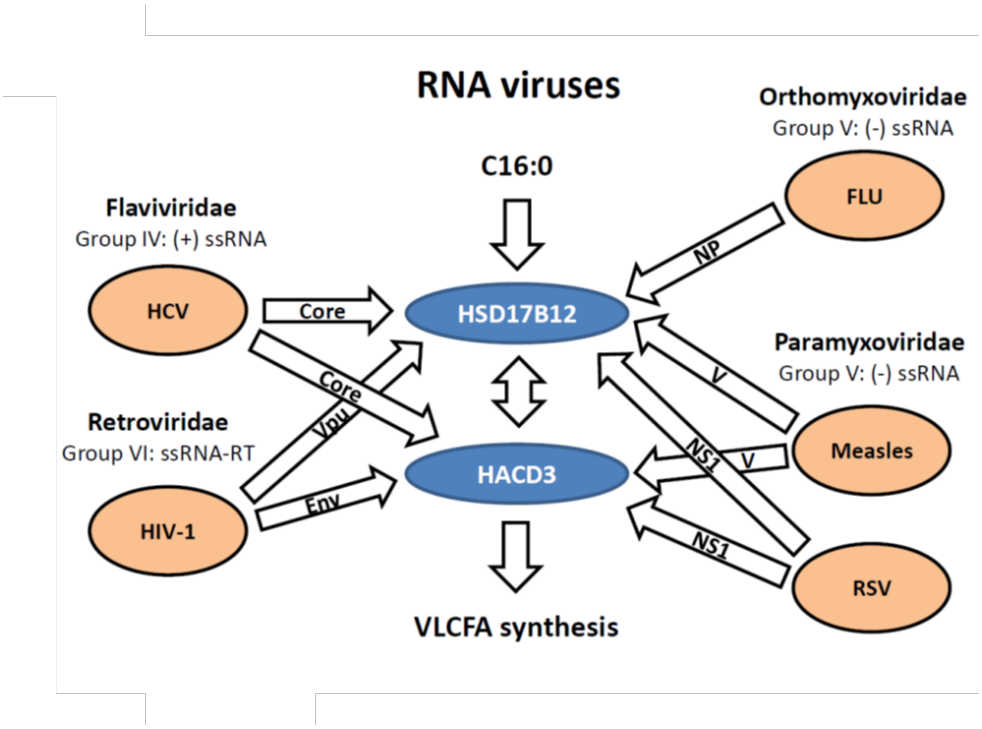

Figure 1.9. Schematic representation of VLCFA *de novo* synthesis enzymes as hub proteins for several families of viruses

The interactions highlight the importance of VLCFA for many viruses.

VLCFAs need more investigations regarding their suggested pro-viral roles. Acquiring more knowledge of the molecular details of VLCFA structure and function in HCV and flavivirus replication, and also the mechanisms whereby their derived lipids are generated and trafficked to the relevant intracellular sites, may enable more targeted antiviral strategies without global effects on the host cell.

In the next sections, we will discuss the requirements of HCV for fatty acid metabolism. The discussion will show how VLCFA *de novo* synthesis, which is not an investigated pathway, may represent an essential requirement for HCV replication and its related flaviviruses.

1.6. HCV and the requirement for fatty acid metabolism

Human liver has distinctive mechanisms for lipid metabolism. Fatty acids handled in the hepatocytes can be summarized into three processes: (i) acquiring fatty acids from extracellular sources, including the uptake, and the *de no*vo fatty acid synthesis (*de novo* lipogenesis), (ii) fatty acid storage, including triglyceride synthesis and the formation of LDs, and (iii) fatty acid consumption and extracellular transportation, including fatty acid degradation (lipolysis), β-oxidation, and the secretion of VLDL [188].

1.6.1. Biogenesis of fatty acids up to 16 carbon (palmitic acid)

Flaviviridae viruses induce cell lipid synthesis and energy production to establish lipid structures that host the viral genome and proteins for proper replication [207]. The *de novo* lipogenesis machinery has been demonstrated to be very essential for virus replication and virion production. The *de novo* lipogenesis includes fatty acid synthesis by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) at the cytosol, and very-long-chain fatty acid (VLCFA) elongation at the ER (> 16 carbons). To synthesize fatty acid acyl chains up to 16 carbons long (palmitic acid), ACC is activated in parallel to FASN to maintain the synthesis process [207]. Different sources of the intracellular pool of fatty acids are represented in **(Fig. 1.10)**. ACC is a highly regulated enzyme in the fatty acid synthesis pathway [208]. It catalyzes the reaction of producing malonyl-CoA by Acetyl-CoA carboxylation. ACC is allosterically up-regulated by citrate and glutamate, and allosterically down regulated by long- and short-chain fatty acyl-CoAs such as palmitoyl-CoA. AMP-activated protein kinase (AMPK) inactivates ACC by phosphorylation [208]. There are two ACCs in the human genome, ACC1 and ACC2.

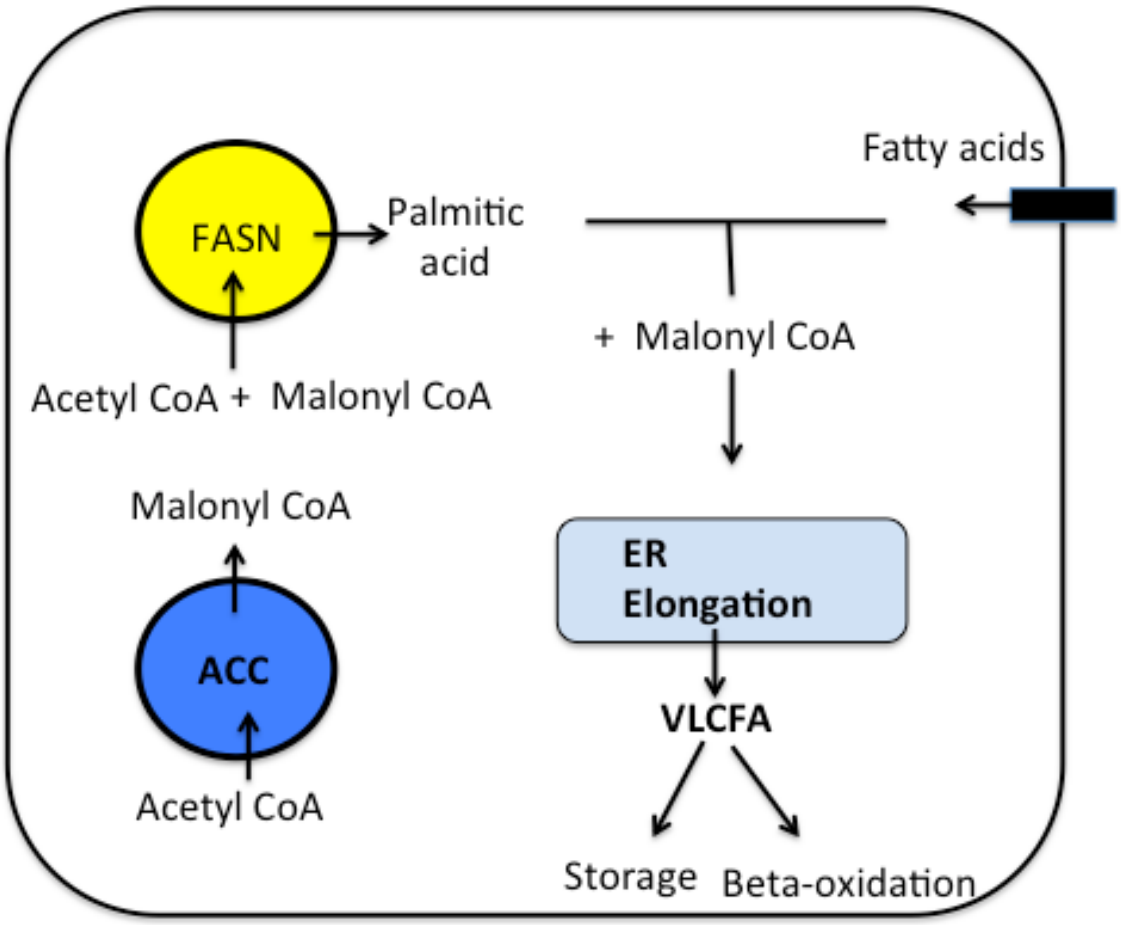

Figure 1.10. Different sources of the intracellular pool of fatty acids.

Fatty acids may come from extracellular sources or *de novo* synthesis. Fatty acid synthase (FASN) produces fatty acids up to 16 carbons (palmitic acid), and fatty acids more than 16 carbons (VLCFA) are synthesized at the ER. Acetyl CoA carboxylase (ACC) provides malonyl CoA for both cytoplasmic FASN and ER-mediated fatty acids chains elongations.

ACC1 is highly enriched in lipogenic tissues, whereas ACC2 occurs in oxidative tissues. Two different metabolic roles are executed by ACC1 and ACC2. ACC1 makes malonyl-CoA serve as a substrate for fatty acid synthesis, whereas ACC2 makes malonyl-CoA

serve as a carnitine palmitoyltransferase 1 (CPT1) inhibitor, thus preventing fatty acid degradation [188, 208, 209].

FASN is a multifunctional enzyme of a 270 kDa. The FASN protein molecule includes seven enzymatic domains that compose complete machinery required to synthesize palmitic acid *de novo* from acetyl and malonyl esters of coenzyme A (CoA) in addition to NADPH [195]. The FASN machinery includes ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), acyl carrier protein (ACP), and thioesterase (TE) [195]. FASN is expressed in two main tissues that produce fatty acids: the liver and adipose tissue. FASN enzyme undergoes posttranslational activation. In this process, the central acyl carrier domain (ACP) of FASN must be modified by an intrinsic phosphopantetheine transferase (PPT) that covalently attaches the phosphopantetheine moiety of coenzyme A (CoA) onto a conserved serine residue of the ACP [210-212].

1.6.1.1. ACC and FASN requirement in HCV replication

Inhibition of ACC, the rate-limiting enzyme in the *de novo* fatty acid synthesis, leads to inhibition of HCV replication. The effect was shown with pharmacological inhibition or siRNA depletion, and also in the case of enzyme phosphorylation [209, 213]. The inhibition of virus replication involves disruption of HCV-induced membranous web [214]. Furthermore, inhibition of ACC is associated with a significant reduction of LDs, and this leads to significant inhibition of virus assembly and release [209]. ACC inhibition also hinders flavivirus replication, specifically WNV [215].

A large body of research evidence supports FASN's importance for HCV replication. Clinically, in HCV-infected livers, FASN protein expression is significantly

increased [216-218]. Experimentally, FASN is up-regulated in HCV-infected Huh7 cells [219]. Further, Huh7.5 infected with HCV was demonstrated to have more free fatty acid than non-infected cells [220]. Moreover, FASN has palmitoylation activity, and it palmitoylated NS4B. NS4B palmitoylation is important for the formation of the replication complex [87]. Also, FASN interacts with NS5B. This physical interaction enhances NS5B RdRp activity [87, 221]. HCV proteins can stimulate FASN activity. This includes HCV core protein [222], NS2 [223] and NS4B [130]. Importantly, FASN induction by HCV promotes the synthesis of phospholipids, which are important components for membranous web [219].

In the same context, FASN enzyme activity participates in the creation of the specialized membranous compartment-specific for the replication of *Flaviviridae* viruses. The viruses include HCV, DENV, YFV, and WNV. Cerulenin, orlistat, and C75 are FASN inhibitors that drastically inhibit the replication of these viruses [224-228]. Interestingly, DENV and WNV infection does not increase FASN expression. However, FASN relocates to the virus membranous replication compartment. The relocation could provide the expanding replication specialized membranes with the fatty acids and lipids required to support virus replication [224, 226, 229].

1.6.2. Desaturation and activation of fatty acid acyl chains and HCV

Fatty acids synthesized from palmitic acid can undergo desaturation to yield mono- or poly-unsaturated fatty acids. Mono-unsaturated fatty acids are of special importance as they control biophysical characteristics of the membrane like fluidity and curvature [230], including those of HCV-infected cells [225]. The stearoyl-CoA desaturase (SCD) is an enzyme involved in the very-long-chain monounsaturated fatty

acid synthesis. SCD is a membrane-bound enzyme at the ER that catalyzes the insertion of the first *cis*-double bond at the delta-9 position of saturated fatty acids. Thus, it converts saturated fatty acids into monounsaturated fatty acids (MUFAs) [231]. The monounsaturated products are the major substrates for the synthesis of more complex lipids such as phospholipids, diacylglycerols, triglycerides, and cholesterol esters. SCD is a highly regulated enzyme. It has four isoforms with distinct tissue distribution and substrate specificity [232-237].

SCD1 catalyzes a rate-limiting step in the synthesis of oleic acid and palmitoleic acid from stearoyl- and palmitoyl-CoA, respectively [238]. SCD1 has been shown to be important in chronic liver diseases and is linked to aggressive hepatocellular carcinoma migration and invasion [239]. Notably, the SCD1 product oleic acid is accumulated in the livers of chronic HCV patients in the form of triglycerides [240].

SCD1 inhibition leads to the inhibition of HCV replication. The effect was shown by genetic depletion of the enzyme, or by pharmacological inhibition, in subgenomic replicon cells and JC1 infected cells [241, 242]. HCV proteins were associated with SCD1 on HCV specialized detergent-resistant membranes. Also, NS4B-mediated membrane rearrangement is supported by SCD1 enzyme activity to provide a suitable microenvironment for HCV replication [241]. SCD1 inhibition disrupts the formation of the HCV membranous web. Hence, SCD1 inhibition disturbs HCV replication complexes and renders HCV RNA susceptible to nuclease-mediated degradation [242].

The fatty acids synthesized by the *de novo* pathway are already active as their acyl chains are covalently linked to CoA group and can be used by several enzymes to be incorporated in HCV membranous structures. For non-esterified fatty acids (coming from

external sources or recycled from the action of lipases on complex cellular lipids) to enter bioactive pools, they must be activated by members of the long-chain acyl-CoA synthase (ACSL) enzyme family, which generate fatty acid-CoA [243]. Mammals have five ACSL isoforms (ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6) [243]. ACSL3 has shown to be important for phosphocholine and phospholipid synthesis [244]. The products of fatty acid activation may serve as substrates for the synthesis of more complex lipids or contribute to cellular signalling, e.g., synthesis of phosphoinositides. It also contributes to protein myristoylation and palmitoylation, which are post-translational modifications that are important in cell physiological processes [245].

Fatty acid activation by ACSL3 is essential for very low-density lipoprotein (VLDL) assembly and secretion in Huh7. It was shown that ACSL3 depletion inhibits HCV secretion in Huh7 cells, without affecting HCV replication. The phenotype goes in the same context as inhibition of VLDL assembly, and secretion inhibits HCV secretion [158]. It is suggested that ACSL3-targeting with small molecules can be useful in both inhibiting VLDL secretion and HCV infection [246].

1.6.3. Fatty acid β-oxidation and HCV

Fatty acid β-oxidation is the process of breaking down fatty acids to release energy [208]. The role of fatty acid β-oxidation in HCV infection is controversial. While data coming from liver samples of patients indicate that HCV induces a decrease in fatty acid β-oxidation in the liver, *in vitro* studies showed that HCV replication is dependent on fatty acid β-oxidation [227, 247, 248].

Three proteins play a pivotal role in regulating β-oxidation in hepatocytes; peroxisome proliferator-activated receptor alpha (PPARα), carnitine palmitoyltransferase (CPT1), and AMP-activated protein kinase (AMPK) [208].

Peroxisome proliferator-activated receptor α (PPARα) is a nuclear receptor that has an important role in positive regulation of fatty acid β-oxidation [249, 250]. PPARα requires heterodimerization with receptor X for retinoids (RXR) in order to exert its function [249, 250]. The PPARα: RXR heterodimer, upon ligand binding, perform conformation changes and bind to DNA at a specific sequence, which is a PPARα response element-promoter, resulting in gene transcription [250, 251]. Activation of PPARα increases the expression of the enzymes involved in lipid catabolism and fatty acid β-oxidation in the mitochondria and the peroxisomes [249]. PPARα knockout mice show steatosis when exposed to high-fat diets [252, 253]. PPARα agonist has been shown to decrease hepatic steatosis in mice receiving a methionine and choline-deficient diet [254].

The expression of PPARα appears to be impaired with HCV [247, 248]. However, the pro-viral role of PPARα stimulation was shown in several studies. Rakic *et al.* demonstrated that HCV replication was inhibited by the inhibition of PPARα [227]. The authors suggested two mechanisms for the inhibition: PPARα inhibition (i) induces local hyperlipidemia that can disrupt replication complexes by altering membranous structures in which replication occurs [83, 255], and (ii) alters the lipidation state of host proteins that are necessary for replication to occur [131, 225]. Recently, Lin *et al.* showed that calcitriol inhibits HCV replication in Huh7.5 cells. This inhibition was reversed by

treatment with the PPARα activator. The authors suggested that calcitriol-mediated HCV inhibition occurs through PPARα inhibition [256].

CPT1 acts as a rate-limiting enzyme in β-oxidation by importing long-chain and very-long-chain fatty acids to the mitochondria [208]. CPT1, similarly to PPARα, was shown to be down-regulated in chronic HCV-infected livers [248]. On the other hand, etomoxir, a pharmacological CPT1 inhibitor, significantly inhibits HCV replication. Further, depletion of mitochondrial fatty acid β-oxidation enzyme dodecenoyl coenzyme A delta isomerase (DCI) inhibits HCV replication [257]. These effects suggest that fatty acid β-oxidation may play a pro-viral role in HCV replication.

AMP-activated protein kinase (AMPK) is a sensory enzyme of cellular energy supply [258]. AMPK activation switches off lipid anabolic pathways and switches on β-oxidation by phosphorylating multiple targets. The AMPK targets include ACC and HMG-CoA [258]. ACC phosphorylation leads to enzyme inhibition and decreases fatty acid synthesis. ACC synthesizes malonyl CoA, which is a potent inhibitor of fatty acid oxidation in the mitochondria. A decrease of intracellular malonyl CoA leads to CPT1 activation and channelling of fatty acids from lipogenesis to mitochondrial β-oxidation [208]. On the other hand, ACC activation by AMPK inhibition inhibits fatty acid β-oxidation via inhibition of CPT1. Failure to activate AMPK leads to hyperlipidemia and hypertriglyceridemia [208]. This phenotype has been demonstrated in AMPKα2-/- mice [259, 260]. Further, AMPK expression has been shown to suppress liver sterol regulatory element-binding protein 1c (SREBP1c) [216], a significant activator of cellular lipogenesis [261, 262].

Modulation of AMPK activity has been demonstrated to impact HCV replication. For instance, inhibition of AMPK activity increases HCV replication [257, 263]. The same results were shown when using siRNA interference to achieve AMPK knockdown [264]. Pharmacological restoration of AMPK activity efficiently inhibits virus replication [213]. Interestingly, a report by Tsai *et al.* showed that the AMPK activation drug metformin increased phosphorylation of STAT1 and STAT2 in HCV-infected huh7.5 cells. The effect was down regulated when AMPK inhibitor was used, suggesting that one mechanism of metformin-induced HCV inhibition could involve activation of IFN-β signalling [265].

Altogether, the discussed *in vitro* studies show that fatty acid β-oxidation exerts a pro-viral role in HCV replication.

1.7. Lipid droplet as an important organelle for HCV and flaviviruses

Viruses of the *Flaviviridae* family show substantial differences in transmission, tissue tropism, and pathogenesis. However, they use similar intracellular replication strategies and utilize LDs to support their replication and assembly [1].

LDs are essentially the storage compartment of insoluble oil dispersed in aqueous cytoplasm. LDs have a unique structure, containing a hydrophobic core and a single layer of amphipathic phospholipids. The neutral lipid core contains predominantly triacylglycerols (TGs) and cholesterol esters (CEs) [266].

The protein composition of the phospholipid layer of LD determines its function [267]. Perilipin, adipose differentiation-related protein (ADRP), and tail anchoring protein 47 (TIP47) are of particular interest. These proteins can control neutral lipid

storage. Perilipin, when phosphorylated, switches its role from neutral lipid storage to lipid mobilization by recruiting hormone-sensitive lipase (HSL)[268]. ADRP has a similar function through the interaction with adipose triglyceride lipase (ATGL) [269]. TIP47 plays potential role in LD biogenesis [270]. Importantly, the COPI system regulates LD lipolysis. The knockdown of COPI components increases neutral lipid storage. Also, it increases ADRP and TIP47 on LD surfaces and decreases ATGL [271].

HCV and flaviviruses exploit LDs to promote their replication. For HCV infection, LD surface protein ADRP is replaced by viral capsid protein. The activity of DGAT1 catalyzes this procedure [144]. The loss of LD ADRP leads to loss of LD mobility balance, which forces LDs to move towards the nucleus [272]. The gathering of LDs in the proximity of the nucleus leads to contact with HCV replication complexes [272]. NS5A mediates the traffic of HCV RNA from the replication complex to the LD. This process is followed by nucleocapsid formation. The nucleocapsid buds into the ER, and fuses with VLDL to form viral lipo-viro-particles in the ER lumen reviewed in [199, 273]. **(Fig. 1.11)**

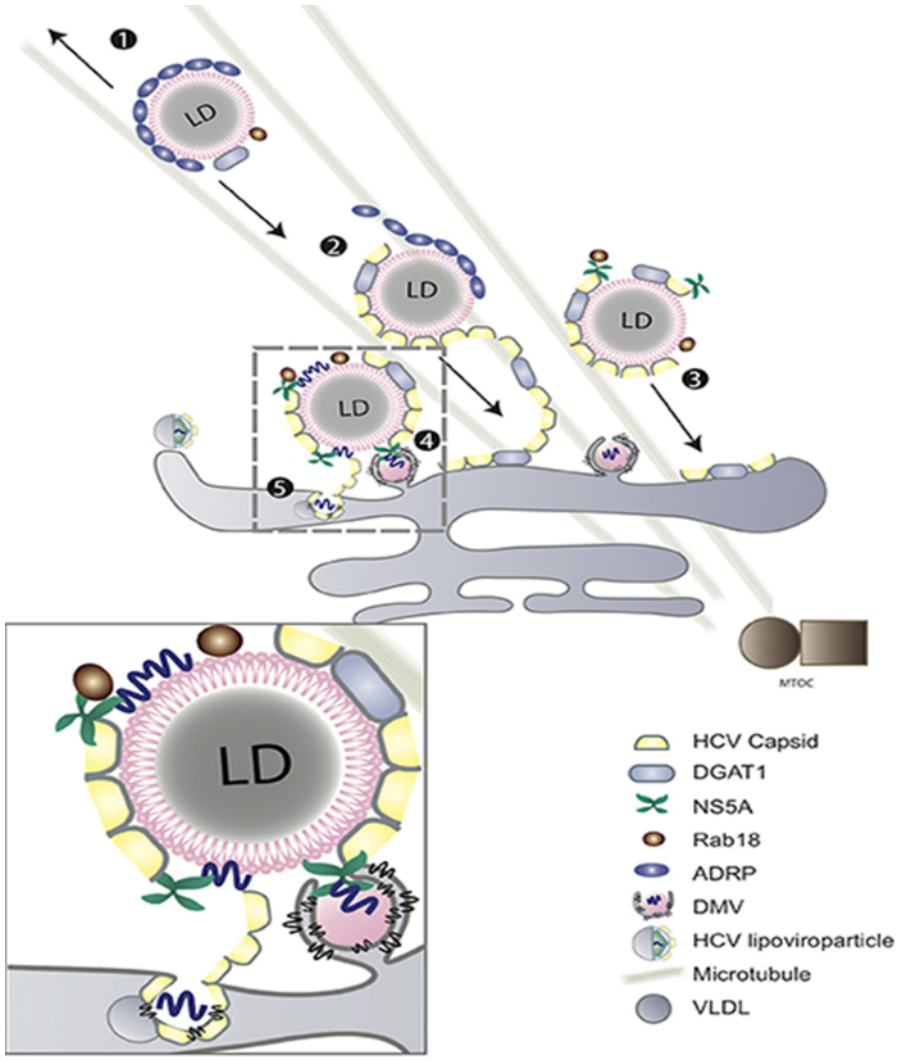

Figure 1.11. LDs are the platforms for HCV assembly.

LD loses its mobility balance due to the replacement of ADRP by core protein. LD moves towards the HCV replication complexes close to the nucleus (1, 2, 3) forming nucleocapsid (4) and fuses with VLDL (5) forming viral lipo-viro-particles in the ER lumen. The figure is adapted with permission from (Zhang, J., Y. Lan, and S. Sanyal, Modulation of Lipid Droplet Metabolism-A Potential Target for Therapeutic Intervention in *Flaviviridae* Infections. Front Microbiol, 2017. **8**: p. 2286.)[199].

On the other hand, DENV capsid protein utilizes host Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1)-ARF-COPI pathway to localize to the surface of LDs [274]. The accumulation of DENV capsid protein on LDs is associated with cellular perilipin 3 and intracellular K+ concentration. Exposure of the capsid protein cationic surface to the aqueous environment through binding to LDs is critical for DENV assembly [275]. Replicated DENV genomes are transferred through the vesicle pores, and then contained by the nucleocapsids that bud through the ER membrane nearby. Packed virions accumulate within the lumen of the vesicle packet-containing ER network before being transported to the Golgi [199, 272, 273]. **(Fig. 1.12)**

LDs are made of triglycerides (TGs) and cholesterol esters (CEs) that mainly incorporate VLCFA, especially stearic and oleic acids [200]. The importance of LDs for HCV and flavivirus infection emphasizes the importance of VLCFA *de novo* synthesis for such viruses.

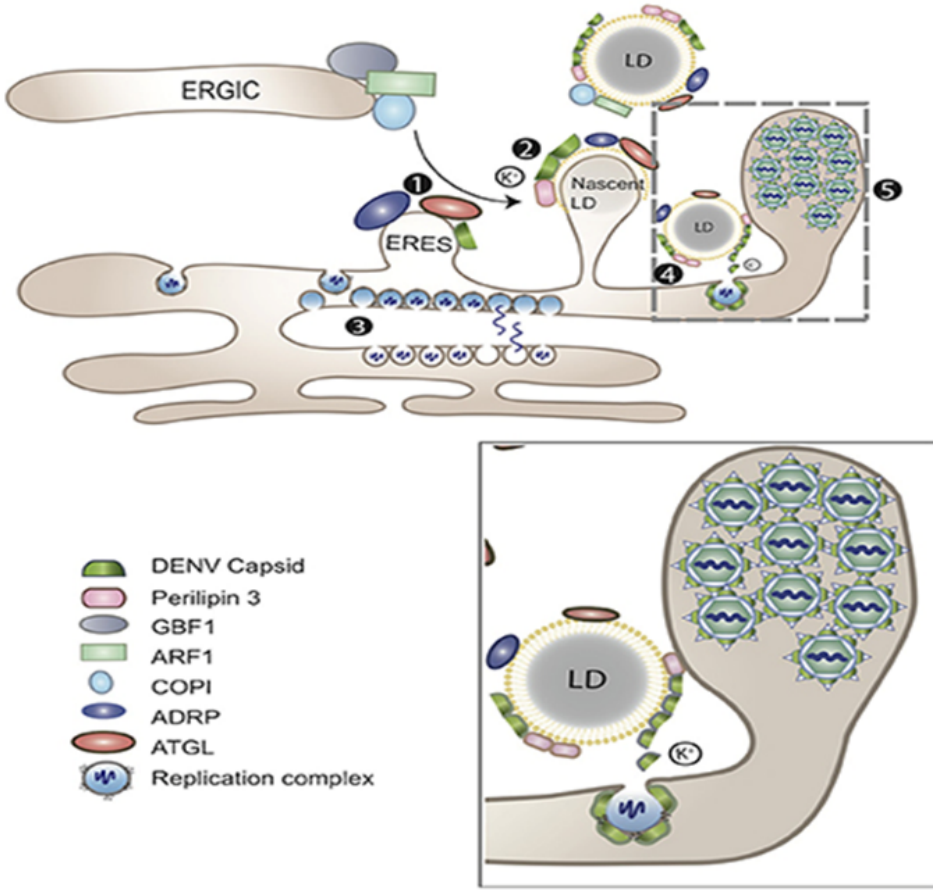

Figure 1.12. Importance of LDs for DENV assembly.

The (GBF1)-ARF-COPI pathway delivers DENV capsid to the surface of LDs (1). DENV capsid protein on LDs is associated with cellular perilipin 3 and intracellular K+ concentration (2). Replicated DENV genomes are released through the vesicle pores, then engaged into nucleocapsids [276]. Capsid protein can be released from LDs to the cytosol or other cellular compartments for subsequent viral assembly (4). Packed virions accumulate within the lumen of the vesicle packet-containing ER network before transportation to the Golgi (5). (ERGIC): ER–Golgi intermediate compartment. (ERES): ER export sites. The figure is adapted with permission from (Zhang, J., Y. Lan, and S. Sanyal, Modulation of Lipid Droplet Metabolism-A Potential Target for Therapeutic Intervention in *Flaviviridae* Infections. Front Microbiol, 2017. 8: p. 2286.)[199].

1.8. *De novo* synthesis of very-long-chain fatty acids (VLCFAs)

Fatty acids (≥16 carbons) go through elongation by ER-resident enzymes following the conversion of their acyl chains to acyl-CoAs. Fatty acid elongation takes part by cycling through a four-step process (condensation, reduction, dehydration, and reduction). In the first rate-limiting reaction step, malonyl-CoA is condensed with acyl-CoA to produce 3-ketoacyl-CoA. This reaction is catalyzed by fatty acid elongase. There are seven elongases in mammals (ELOVLs 1-7) that exhibit characteristic substrate specificity [277]. In the second step, 3-ketoacyl-CoA is reduced to 3-hydroxy acyl-CoA by 17-Beta hydroxysteroid dehydrogenase type 12 (HSD17B12, KAR, DHB12, 3-ketoacyl-CoA reductase) [278]. Nicotinamide adenine dinucleotide phosphate (NADPH) is used as a reducing agent in this reaction [278]. Third, 3-hydroxy acyl-CoA dehydratase (HACD1–4) dehydrates 3-Hydroxyacyl-CoA, generating 2,3-trans-enoyl-CoA [279]. Finally, 3-hydroxy acyl-CoA is reduced to an acyl-CoA having two more carbon chain units than the original acyl-CoA. A 2,3-trans-enoyl-CoA reductase (TER) uses NADPH as a reductant to catalyze this reaction [191, 278]. **(Fig. 1.13)**

1.8.1. ELOVLs

ELOVLs are the rate-limiting enzymes in VLCFA synthesis. They catalyze the condensation of the acyl-CoA with malonyl-CoA, producing 3-ketoacyl-CoA which undergoes the following reactions in the VLCFA elongation cycle. ELOVLs have different protein expression profiles in different cells and organs, which highlight the different requirements for their VLCFA products and the containing lipid species. The members of ELOVLs family are as follows:

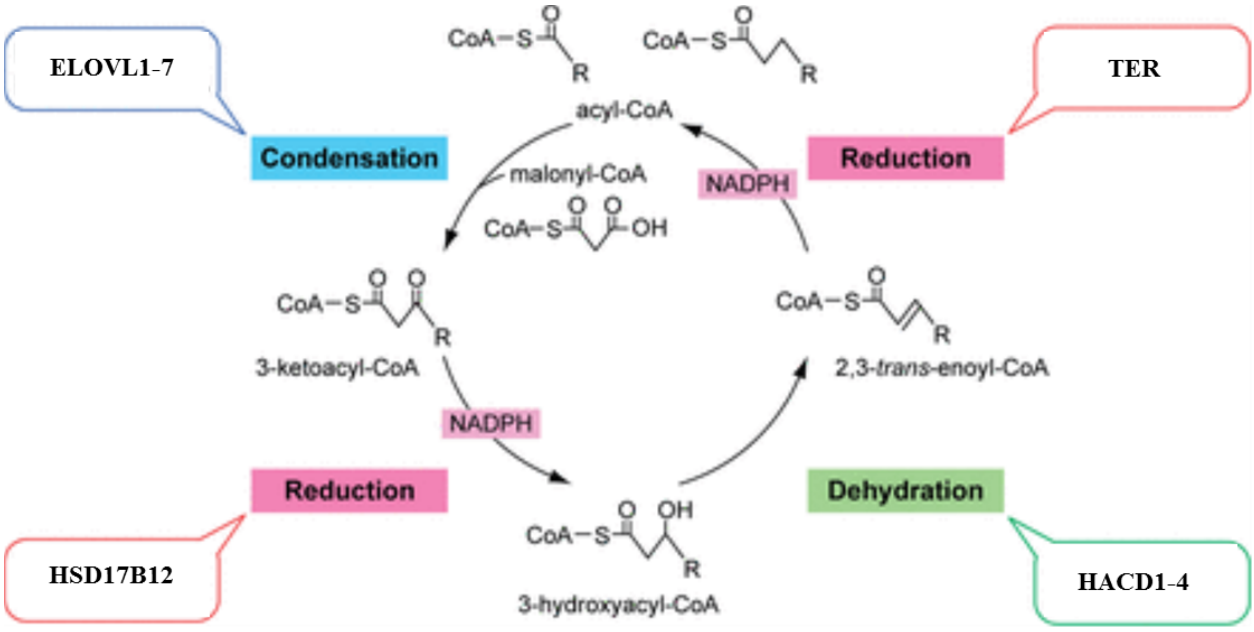

Figure 1.13. Very-long-chain fatty acid (VLCFA) elongation cycle at ER.

Proceeding from ELOVLs1-7, HSD17B12, HACD1-4 and ending with TER [191]. ELOVLs carry out the condensation step by adding two carbons to the acyl chain, HSD17B12 catalyzes the reduction of the resulting keto-acyl, HACDs carry out the dehydration step, and TER catalyzes the final reduction step. The figure is adapted with permission from (Kihara, A., Very long-chain fatty acids: elongation, physiology and related disorders. J Biochem, 2012. 152(5): p. 387-95.)[191].

ELOVL1 plays a very critical role in the synthesis of saturated VLCFA beyond palmitic acid up to a saturated chain of 26 carbons. This activity makes the enzyme very important in the building of membrane lipids, especially sphingolipids. Further, the enzyme was shown to be involved in synthesizing nerve sphingomyelin [280].

ELOVL2 was also discovered by the sequence similarity to ELOVL1 and ELOVL3. ELOVL2 is involved in the catalysis of the elongation of polyunsaturated 20-carbon VLCFA [281].

ELOVL3 was the first ELOVL discovered. The enzyme was identified in the brown adipose tissue and liver of mice. It showed very high expression in the brown adipose tissue upon exposure to cold stimuli and sympathetic activation. Due to this characteristic expression, the enzyme was called cold-induced glycoprotein of 30 kDa (Cig30). The enzyme has a role in the elongation of saturated and monounsaturated fatty acid beyond palmitic acid up to 24-carbon VLCFA [280, 282].

ELOVL4 is involved in the synthesis of VLCFA beyond carbon 26 with saturated and unsaturated chains. Defect in the enzyme is linked to the Stargardt-like macular dystrophy, which is a retinal disease that leads to loss of vision [283, 284].

ELOVL5 catalytic activity is involved in the elongation of polyunsaturated fatty acids with 18 and 20 carbon chain. It has been demonstrated that ELOVL2 and ELOVL6 compensate ELOVL5 deficiency in ELOVL5 ablated mouse [285, 286].

ELOVL6 plays a central role in bridging palmitic acid that is synthesized by FASN to fatty acid chains of 18 carbons and more. The enzyme catalyzes the elongation of palmitic acid and palmitoleic acid to stearic and oleic acids [287].

ELOVL7 is preferentially involved in fatty acid elongation of saturated VLCFA [288].

In summary, synthesis of VLCFA requires first ELOVL6, which elongates C16:0 to C18.0, and then ELOVL1, which elongates these VLCFA further to C24:0 and ≥C26:0. C18.1-C24.1 requires ELOVL6 and ELOVL3, while polyunsaturated VLCFA synthesis requires ELOVL2/4/5 [289, 290]. **(Fig. 1.14)**

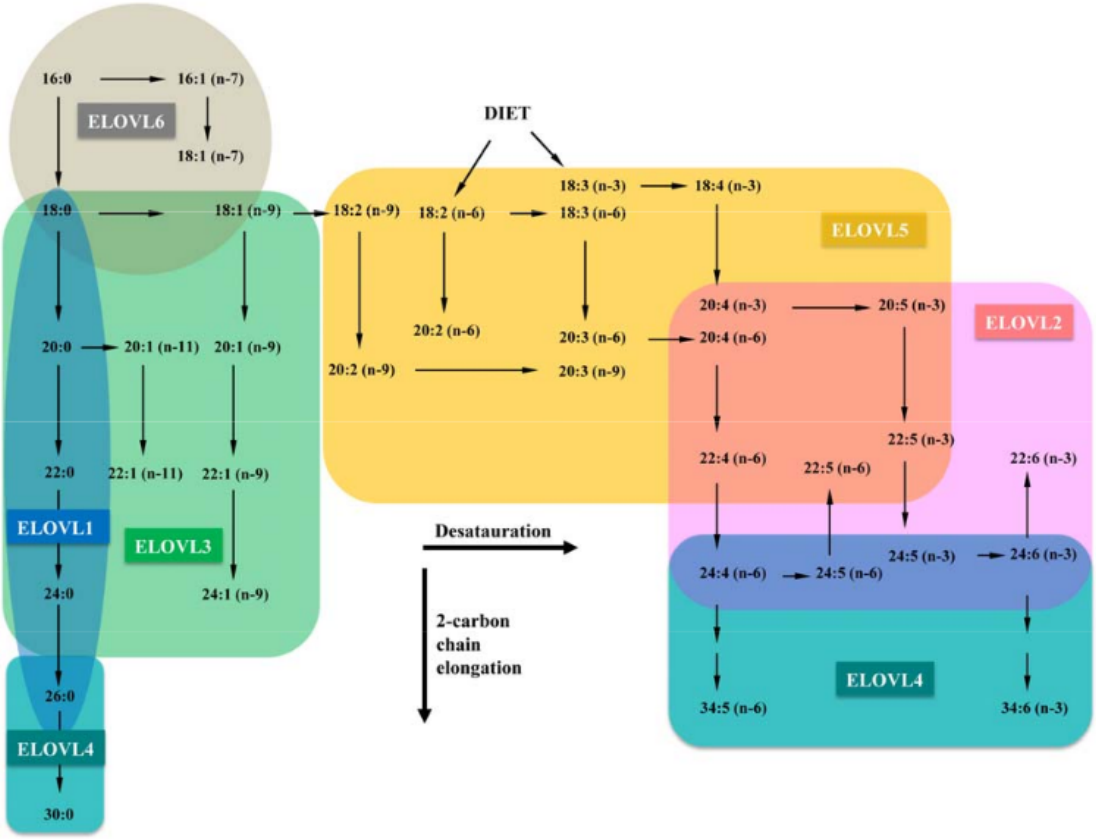

Figure 1.14. ELOVL family substrates and products.

First, ELOVL6 elongates C16:0 to C18.0, and then ELOVL1 elongates these VLCFA further to C24:0 and ≥C26:0. C18.1-C24.1 requires ELOVL6 and ELOVL3, while polyunsaturated VLCFA synthesis requires ELOVL2/4/5. The figure is adapted with permission from (Brolinson, A., Regulation of ELOVL and fatty acids metabolism. 2009, University of Stockholm.)[290].

1.8.2. HSD17B12

HSD17B12 (GenBank accession nos. AF078850 and NM_016142) is a member of 14 different mammalian steroid dehydrogenases. HSD17Bs are the key enzymes responsible for the synthesis of sex steroids and their inactivation [291-295]. They synthesize the active androgens and estrogens by reductive HSD17Bs, which catalyze the molecular hydrogenation at position 17β of the steroid backbone. Also, they inactivate the steroids through the removal of hydrogen by oxidative HSD17Bs [292]. A particular property of this family of enzymes is the wide difference of their primary structures (an average of only ~20% amino acid identity). However, they have high specificity for substrates that have closely related structures [292]. Human HSD17Bs need two types of nucleotide cofactor for their activity: NAD and NADH. On the other hand, they differ in subcellular localization and tissue-specific expression patterns [292]. The best-characterized HSD17B enzyme is HSD17B1 [296, 297], which catalyzes the conversion of estrone (E1) into estradiol (E2) [298].

Employing structure-function relationship analysis using sequence alignment, enzyme modeling, and site-directed mutagenesis showed that HSD17B12 is homologous to HSD17B3, the enzyme responsible for testosterone (T) synthesis [299]. *HSD17B12* gene is located at p11.2 on human chromosome 11. Genomic structures of *HSD17B3* and *HSD17B12* genes are very similar. The two genes consist of 11 exons. However, HSD17B12 shows longer 5′- and 3′-untranslated regions. Further, the first and last exons of *HSD17B12* possess additional 0.2- and 1.2-kb sequences, respectively **(Fig. 1.15)**. Nevertheless, the rest of the 11 exons have the same numbers of nucleotides, except for exons 5 and 6. HSD17B3 and HSD17B12 proteins share 41% identity in the amino acid

sequence alignment. Interestingly, the two enzymes have a conserved active site (YXXXK) and a slightly modified cofactor-binding (NADPH) motif (GXXXGXL) instead of GXXXGXG as described for HSD17Bs [298]. **(Fig. 1.16)**

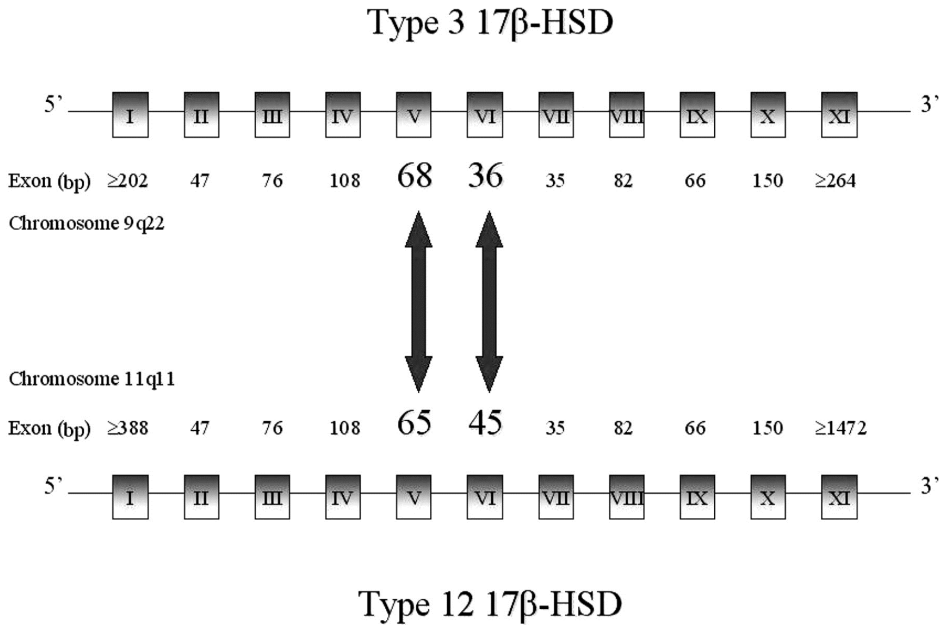

Figure 1.15. Schematic representation of the Genomic Structure of *HSD17B3* and *HSD17B12*.

The exons are numbered I–XI inside boxes. The exon sizes for *HSD17B3* and *HSD17B12* are indicated below and above the boxes, respectively. Chromosomal locations are also indicated. The figure is adapted with permission from (Luu-The, V., P. Tremblay, and F. Labrie, Characterization of type 12 17beta-hydroxysteroid dehydrogenase, an isoform of type 3 17beta-hydroxysteroid dehydrogenase responsible for estradiol formation in women. Mol Endocrinol, 2006. 20(2): p. 437-43.) [300].

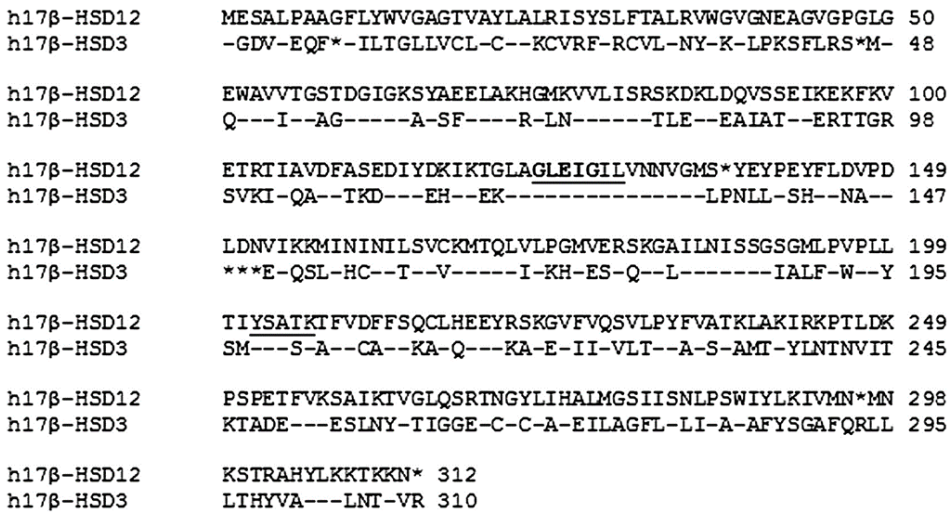

Figure 1.16. Comparison between amino acid sequences of HSD17B3 and HSD17B12.

Dashes and stars represent identical and missing amino acids, respectively. The consensus sequences for active sites and cofactor binding are underlined. The figure is adapted with permission from (Luu-The, V., P. Tremblay, and F. Labrie, Characterization of type 12 17beta-hydroxysteroid dehydrogenase, an isoform of type 3 17beta-hydroxysteroid dehydrogenase responsible for estradiol formation in women. Mol Endocrinol, 2006. 20(2): p. 437-43.) [300].

In 2003, Moon and Horton characterized HSD17B12 as an ER-bound keto-acyl reductase [278] **(Fig. 1.17)**. They showed that the primary function of the enzyme is suggested to be fatty acid chain extension to form VLCFA. Studies on HSD17B12 orthologous in Caenorhabditis elegans (let767) and yeast (YBR159w) have also supported the role of HSD17B12 in VLCFA synthesis [301]. Interestingly, HSD17B12 has activity similar to the FASN keto-acyl reductase subunit, which is among the complexes responsible for *de novo* fatty acid synthesis [195, 208] **(Fig. 1.18)**. Further, HSD17B12 is partially regulated by SREBP1, similarly to FASN [302]. FASN has been widely studied as a candidate target for inhibiting virus replication [224, 303] and cancer cell growth and division [304], and for the treatment of obesity-related disorders [305], suggesting that HSD17B12 may also be a candidate for those therapeutic targets.

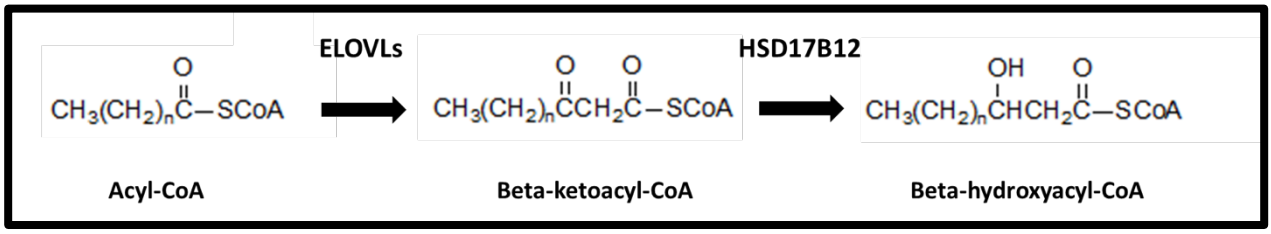

Figure 1.17. ELOVLs and HSD17B12-catalyzed reactions in the elongation of VLCFA.

ELOVLs carry out the condensation step by adding two carbons to the acyl chain; HSD17B12 catalyzes the reduction of the resulting keto-acyl (http://www.lipidhome.co.uk).

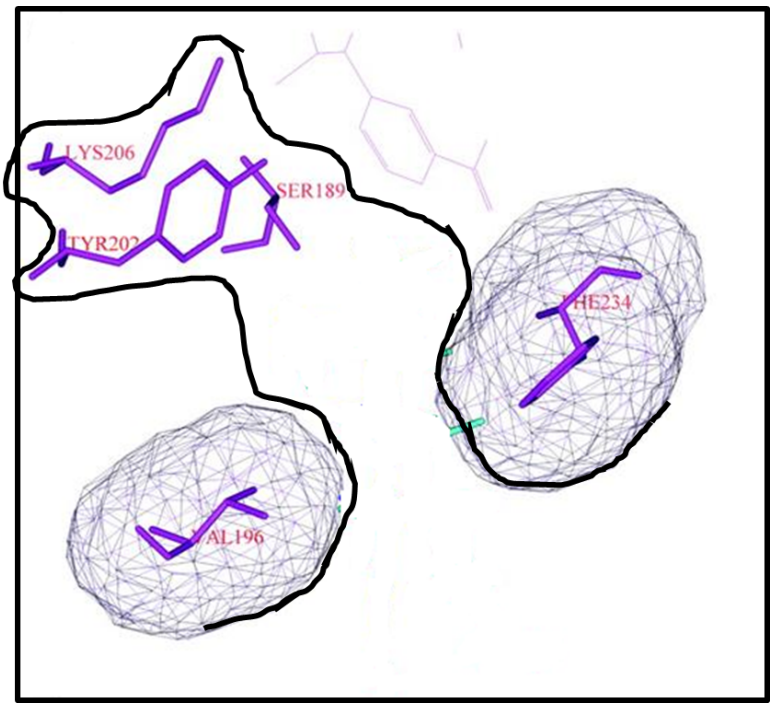

Figure 1.18. Schematic representation of the modeled HSD17B12 active site.

Valine at position 196 (VAL169) and phenylalanine at position 234 (PHE234) are located in the substrate-recognition domain of the enzyme and potentially involved in substrate specificity. Tyrosine at position 202 and lysine at position 206 are the conserved active site residues involved in estrone and VLCFA keto-acyls reduction. The figure is adapted with permission from (Luu-The, V., P. Tremblay, and F. Labrie, Characterization of type 12 17beta-hydroxysteroid dehydrogenase, an isoform of type 3 17beta-hydroxysteroid dehydrogenase responsible for estradiol formation in women. Mol Endocrinol, 2006. 20(2): p. 437-43.) [300].

On the other hand, the HSD17B12 enzyme has been suggested to catalyze the conversion between estrone and estradiol [300]. It was found that HSD17B12 expression is significantly increased in breast cancer tissue samples. Besides, HSD17B12 was detected in both the cytoplasm and nuclei of these cells [306]. Interestingly, recent studies showed no significant correlations detected between HSD17B12 expression and estradiol concentration in breast carcinoma [307]. Moreover, it has been shown that the reduced cell proliferation of breast cancer in the absence of HSD17B12 was rescued by the addition of arachidonic acid (AA), but not estradiol, to the cell culture [302]. Thus, HSD17B12 affects cancer cell growth via the production of VLCFA, especially arachidonic acid. HSD17B12 was shown in a study from our lab to have a pro-viral role in HCV replication [308]. Further, literature mining showed that HSD17B12 is a hub protein that physically interacts with proteins of several viral families. These interactions emphasize the important role of the VLCFA *de novo* synthesis pathway for different viral infections.

1.9. Project rationale

Host-targeting agents based on virus-host interactome studies are expected to increase the therapeutic antiviral arsenal for infectious diseases. Indeed, cellular proteins required for viral replication provide a vast reservoir of targets that can be modulated with small molecules to develop broad-spectrum antiviral therapies. Our research project focused on *Flaviviridae* viruses causing human diseases. By taking advantage of the most studied HCV, we exploited the data of our previous studies that elucidated the HCV interactome, combining viral protein immune-precipitation (IP) coupled to tandem mass spectrometry identification (IP-MS/MS) and functional genomics RNAi screening [308, 309]. This study identified 426 host proteins interacting with HCV protein core, NS2, NS3/4A, NS4B, NS5A, and NS5B. Further, a set of 98 human proteins was statistically enriched as a specific interactor with one of the HCV proteins. The overlap between these 98 proteins with reported HCV-human interactors showed 24 common proteins, demonstrating the reliability and value of the used approach. Furthermore, assessment of the biological significance of these human proteins with an RNA interference (RNAi) gene silencing screen identified 11 novel cofactors that promote viral replication [308].

The cofactors included the 17-beta-hydroxysteroid dehydrogenase type 12 (HSD17B12, also named DHB12) as a specific core interactor that promotes HCV replication [308]. HSD17B12 catalyzes the second of the four reactions of the VLCFA elongation cycle. HSD17B12 participates in the production of VLCFA of different chain lengths that are involved in multiple biological processes as precursors of membrane lipids and lipid mediators, and which also catalyze the transformation of estrone (E1) into estradiol (E2). Interestingly, literature mining showed that HSD17B12 is a human hub

interacting with several viral proteins of RNA viruses and DNA viruses [202-206]. These interactions highlight the significance of studying the possible dependence mechanisms of HSD17B12-mediated VLCFA synthesis for HCV and related flaviviruses replication.

1.10. Hypothesis and objectives

1.10.1. Hypothesis

The premise of my project is based on the dependence of HCV replication on co-opted host factors as common themes employed by different viruses to promote their life cycle. In HCV, we found that core capsid protein physically interacts with HSD17B12 to promote virus replication (Germain *et al*., Mol. Cell. Proteomics 2014). The interaction led us to postulate that this interaction is important for the virus's establishment and promotion of its specialized cellular compartments for replication and assembly/release. Further, HSD17B12 may be important for the replication of related flaviviruses, as they need similar cellular compartments.

1.10.2. Objectives

The objective of my project is to characterize the role of HSD17B12 in HCV replication. The goal is to comprehend the importance of the HCV-HSD17B12 interaction in order to uncover dependence mechanisms for other viral infections and to validate novel antiviral targets for a large spectrum of RNA viruses.

1.10.3. Aims

-To investigate the dependency of HCV on HSD17B12-mediated VLCFA *de novo* synthesis

-To determine the mode-of-action of HSD17B12 catalytic activity and its pharmacological inhibition as an antiviral approach.

- To investigate the dependency of related flaviviruses DENV and ZIKV on HSD17B12 activity and explore its inhibition as a broad-spectrum antiviral target.

Chapter 2

This chapter features the article, currently submitted to Nature Scientific Reports, titled **" Very-long-chain fatty acid metabolic capacity of 17-beta-hydroxysteroid dehydrogenase type 12 (HSD17B12) promotes replication of hepatitis C virus and related flaviviruses."** By **Bassim Mohamed**, Clément Mazeaud, Martin Baril, Donald Poirier, Laurent Chatel-Chaix, Vladimir Titorenko, and Daniel Lamarre.

AUTHOR CONTRIBUTION

Bassim Mohamed, Martin Baril, Laurent Chatel-Chaix, and Daniel Lamarre conceived and designed the experiments.

Bassim Mohamed performed **all experiments** (all figures).

Clément Mazeaud assisted in performing flaviviruses experiments (figures 6, 7)

Bassim Mohamed, Donald Poirier, Laurent Chatel-Chaix, Vladimir Titorenko, and Daniel Lamarre interpreted the results.

Bassim Mohamed and Daniel Lamarre wrote the manuscript.

All authors reviewed the manuscript.

2.0. Very-long-chain fatty acid metabolic capacity of 17-beta-hydroxysteroid dehydrogenase type 12 (HSD17B12) promotes replication of hepatitis C virus and related flaviviruses.

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Running Title: HSD17B12 promotes *Flaviviridae* virus replication.

2.1. ABSTRACT

Flaviviridae infections represent a major global health burden. By deciphering mechanistic aspects of hepatitis C virus (HCV)-host interactions, one could discover common strategy for inhibiting the replication of related flaviviruses. By elucidating the HCV interactome, we identified the 17-beta-hydroxysteroid dehydrogenase type 12 (HSD17B12) as a human hub of the very-long-chain fatty acid (VLCFA) synthesis pathway that promotes HCV replication. Here we show, in Huh7.5 cells, that HSD17B12 knockdown (KD) induces dispersion and dysfunction of HCV replication sites and drastically reduces virion production. Mechanistically, depletion of HSD17B12 induces alteration in VLCFA-containing lipid species and drastic reduction of lipid droplets (LD) that play a critical role in virus assembly. Oleic acid (C18.1) supplementation rescues production of infectious particles in HSD17B12 KD cells, supporting a specific role of VLCFA at the HCV replicative life cycle. Furthermore, the small-molecule HSD17B12 inhibitor, INH-12, significantly reduces replication and infectious particle production of HCV as well as flaviviruses dengue virus and zika virus revealing a conserved requirement across *Flaviviridae* virus family. Overall, the data provide a strong rationale for the full evaluation of HSD17B12 inhibition as a promising broad-spectrum antiviral target for the treatment of *Flaviviridae* viral infections.

2.2. INTRODUCTION

RNA viruses are exploiting basic cellular pathways and cytoplasmic organelles to achieve different stages of their replicative life cycle [310]. This is especially characteristic of *Flaviviridae*, *Coronaviridae* and *Picornaviridae* families of positive-strand RNA viruses that utilize cytoplasmic membranes derived from endoplasmic reticulum (ER) for the formation of viral RNA replication and virion assembly compartments. Hepatitis C virus (HCV), a member of the *Flaviviridae* family, induce extensive endoplasmic reticulum (ER) membrane protrusions that generate double-membrane vesicles (DMVs) organized within a membranous web (MW), while other such as Dengue virus (DENV), Zika virus (ZIKV) and West Nile virus (WNV) use distinct ER invaginated vesicles as replication factories [273]. Mechanistically, the biogenesis of these virus-induced replication factories requires substantial structural changes to the ER membrane that involve membrane deformations, extensions and contractions to generate the appropriate architecture. As such, *de novo* synthesis of host lipids is crucial to the formation and functioning of these replication factories by facilitating membrane curvature and stimulating the activity of viral enzymes in the replication complex (review [311]). Furthermore, the proximity between these replication factories and lipid droplets (LDs), an organelle which is important in lipid storage and metabolism, contributes to the generation of infectious viral particles. Indeed, LDs are essential host components for the assembly of several *Flaviviridae* members [122, 312-314]. As these viruses lack the appropriate enzymatic machinery to conduct their own lipid synthesis, they have evolved multiple mechanisms to hijack host fatty acid and lipid

metabolic pathways for completing their intracellular replication cycles [31, 224, 315, 316].

Fatty acids are constituents of triglycerides, phospholipids and complex lipids, and their syntheses have long been identified as a requirement for the replication of many viruses (FLU, HCMV, EBV) including *Flaviviridae* (HCV, DENV, YFV, WNV, JEV). They contribute to the structural integrity of membranes, energy production and storage, and generation of LDs. Saturated fatty acids up to 16 carbon atoms in length are synthesized in the cytosol of the cell by the multifunctional protein fatty acid synthase (FASN), which utilizes acetyl-CoA (C2:0-CoA), malonyl-CoA and NADPH to elongate fatty acids in two-carbon increments. Upon infection, the production of fatty acids and neutral lipids is often provided by an increase in FASN abundance and activity, which allow HCV propagation without transporting FASN to replication sites [190]. Others, like DENV, directly manipulate *de novo* fatty acid synthesis of palmitic acid (C16:0) by re-localization of FASN to these sites [190, 224, 317]. Moreover, antiviral effects have been reported with inhibitors of acetyl-CoA carboxylase 1 (ACC1) and FASN, which severely impede the replication of *Flaviviridae* family members and enteroviruses [190, 224, 317]. Fatty acid elongation to very-long-chain fatty acids (VLCFA, ≥ C18) and regulation during *Flaviviridae* life cycle, however, are poorly understood. While fatty acids and lipids are used at different stages of viral life cycles, little is known about the modulation and requirement for *de novo* VLCFA synthesis to promote infection. The elongation of fatty acid beyond palmitic acid takes place at the ER membrane and involves multiple enzymes that are acting together as one physiological functional unit for each two-carbon increment elongation cycle. These enzymes include elongation of very-long-chain fatty

acid elongase subtypes 1 to 7 (ELOVL1-7) (1st reaction), 17-beta-hydroxysteroid dehydrogenase type 12 (HSD17B12, also named DHB12) (2nd reaction), very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase subtypes 1 to 4 (HACD 1-4) (3rd reaction) and very-long-chain enoyl-CoA reductase (TECR) (4th reaction).

HSD17B12 was first described as a key enzyme of steroid metabolism pathway [300] and then identified as the human homolog of the yeast 3-ketoacyl-CoA reductase that catalyzes the second reaction in each VLCFA elongation cycle [278]. It interacts with all seven elongase ELOVL1-7 and of the 4 dehydratase HACD1-4 to generate the diversity among saturated, mono and polyunsaturated VLCFA species [318]. HSD17B12 is a metabolic hub of the VLCFA synthesis pathway [319] that was discovered in our HCV interactome screen as an interactor of viral structural protein core [308]. Furthermore, HCV infection of Huh7.5.1 was recently reported to increase intracellular concentrations of VLCFA consistent with the formation of intrahepatic lipid droplets during infection, which are important during viral assembly and the accumulation of hepatic lipids during steatosis [320]. Hence, we investigated the role of HSD17B12 during the replication of HCV and related flaviviruses. We showed that HSD17B12 knockdown (KD) disrupts the functional integrity of ER-associated replication complexes upon HCV infection and severely reduces the number of cytoplasmic lipid droplets (LD). The KD of HSD17B12 result in significant inhibition of HCV, DENV and ZIKV infectious particle production and is associated with the reduced relative abundance of phosphatidylethanolamine (PE), triglycerides (TGs) and oleic acids (C18.1) of whole cell extracts as revealed by shotgun lipidomics analysis. We further show that INH-12, a small-molecule steroid-based HSD17B12 inhibitor, reduces the replication of HCV, DENV and ZIKV. The data

support a contribution of *de novo* VLCFA synthesis for *Flaviviridae* virus infections and provide a strong rationale to explore the broad-spectrum antiviral potential of targeting HSD17B12 to treat and/or prevent RNA virus infections.

2.3. RESULTS

2.3.1. HSD17B12 redistributes to replication and assembly sites during HCV infection.

HSD17B12 was previously shown to interact with HCV core [308] suggesting that HCV co-opts HSD17B12 functions during the infection. To further assess the cellular localization of HSD17B12, naïve and HCV-replicating Huh7.5 human hepatoma cells were imaged using confocal fluorescence microscopy (**Fig. 2.1. and 2.S1**). HSD17B12 contains a C-terminal di-lysine motif that confers ER localization for type I membrane proteins, and previous study showed that the enzyme co-localizes with the ER resident protein calnexin [278]. We confirmed that HSD17B12 is an ER resident protein in Huh7.5 cells by the co-staining with protein disulfide isomerase (PDI) used as an ER marker (**Fig. 2.1A**). Upon HCV infection, we showed that a fraction of endogenous HSD17B12 overlaps viral replication and assembly sites as revealed by co-staining with anti-double-strand RNA (dsRNA) and anti-core antibodies, respectively (**Fig. 2.1B-C**). Similar results are obtained with ectopic expression of a FLAG-tagged HSD17B12 fusion protein and detection with specific anti-FLAG antibodies in HCV-infected Huh7.5 cells (**Fig. 2.S1**). The data support the relocation of HSD17B12 at virus-induced RNA replication sites and core-associated lipid droplets suggesting that HCV promotes *de novo* synthesis of VLCFA and derived lipids for the benefit of its replication.

2.3.2. HSD17B12 promotes HCV RNA replication and infectious particle production

To get an insight into the role of HSD17B12 in virus infection, we first evaluated the impact of HSD17B12 knockdown (KD) on cell toxicity by transducing Huh7.5 cells with lentivirus expressing short hairpin RNA (shRNA) specific to HSD17B12 and to non-target sequence (NT). Expression KD was validated at both mRNA and protein levels (**Fig. 2.2A**). We used Huh-7 cells stably expressing a firefly luciferase gene under the control of then EF1α housekeeping promoter to assess on- and off-target cytotoxic effects of shRNA-mediated HSD17B12 depletion (**Fig. 2.S2A**). We showed that the luciferase signal was minimally affected indicating that HSD17B12 KD does not significantly affect general protein synthesis. In parallel, we demonstrated that HSD17B12 KD has no major effect on cell viability and proliferation using an Alamar Blue assay (**Fig. 2.S2B)**. Lentiviruses with best silencing efficiency of HSD17B12 gene were used to assess its role in cells harboring a full-length infectious genome or HCV replicating subgenome (Huh7-Con1-Fluc) for which viral RNA replication is occurring in the absence of virus entry and capsid assembly due to the lack of HCV structural proteins. We showed that KD of HSD17B12 in subgenomic replicon-containing Huh7-Con1-Fluc cells significantly decreased viral replication as monitored by luciferase assays performed at 4 days post-transduction compared to cells expressing shNT (**Fig. 2.2B**). To further investigate the contribution of HSD17B12 to viral replication and virion assembly, we took advantage of the ribozyme-based full-length JFH-1 clone that upon DNA transfection of Huh7.5 generates the authentic ends of HCV RNA genome, and subsequently replicates to produce infectious particles that spread in the cell culture

[321]. We first determined the intracellular HCV RNA levels at 4 days post-transfection by real-time qRT-PCR. Surprisingly, we found that HSD17B12 KD led to a significant intracellular accumulation of HCV RNA compared to cells expressing shNT (**Fig. 2.2C**). Similar results were observed in HSD17B12-depleted HepG2 cells, another liver cell line (**Fig. 2.S3A**). However, we detected a significant decrease of NS3 and core proteins in HSD17B12 KD Huh7.5 cells when assessed by western blot analysis (**Fig. 2.2D**). While the low levels of viral proteins should negatively impact HCV replication kinetics, the increased intracellular HCV RNA in HSD17B12 KD cells possibly reflect an impaired nucleocapsid assembly promoting its retention. The assembly of HCV particles relies on the close proximity between virus-induced MW structures and LDs. As HSD17B12 interacts with core and co-localizes to core-containing LD, and that HSD17B12 KD decreased core protein levels, we postulated that HSD17B12 also contributes to the production of infectious viral particles. To test this, we evaluated the release of extracellular HCV RNA and of supernatant-associated infectivity of HSD17B12 KD cells. We found a significant reduction of the extracellular HCV RNA levels using qRT-PCR analysis (**Fig. 2.2E**). In parallel, we demonstrated a significant decreased production of extracellular infectious particles by up to 9-fold as revealed by infection of naive Huh7.5 with supernatants of HSD17B12 KD cells compared to control cells expressing shNT (**Fig. 2.2F**). Similar results were obtained using the supernatants of HSD17B12 KD HepG2 cells (**Fig. 2.S4**). Finally, the KD of HSD17B12 KD was investigated on translation of HCV proteins by co-expression of an HCV IRES-driven firefly luciferase (Fluc) and CMV-driven cap-dependent renilla luciferase (Rluc). No significant difference of HCV IRES-dependent translation is observed in HSD17B12 KD cells and cells

expressing shNT when both reporter activities were monitored (**Fig. 2.S2C**). Altogether, the data strongly support a role of HSD17B12 during both replication and particle production steps of HCV life cycle.

2.3.3. HSD17B12 contributes to a lipid metabolic environment favoring particle assembly

Lipid droplets (LDs) are essential to HCV assembly and their depletion decreased production of infectious virus particles [322]. LDs are composed mainly of triglycerides (TGs), which extensively incorporate VLCFA, especially stearic (C18:0) and oleic (C18:1) acids. As HSD17B12 is a key player of the VLCFA synthesis pathway, we postulated that its metabolic capacity contributes to the biogenesis and maintenance of LDs through requirement of newly synthesized TGs. To better understand the molecular mechanism by which HSD17B12 contributes to virus assembly and particle production, we analyzed the cytoplasmic LD organelles in HSD17B12-depleted cells by staining LDs with LipidTOX (**Fig. 2.3**). Upon imaging analysis, we showed that HSD17B12 KD drastically decreases the relative number, areas and density of LDs in both mock uninfected and JFH-1-infected Huh7.5 cells. As compared to control cells expressing shNT, the reduced LDs of HSD17B12 KD cells can be explained by a decreased TGs synthesis as a result of the deficiency in VLCFA availability (≥ C18). To further investigate if HSD17B12 KD induces a lipid metabolic remodeling that disfavors HCV infection, we used a shotgun mass spectrometry lipidomics approach to measure fatty acid species and lipid families in whole cell extracts. We showed a significant decrease in the cellular contents of TGs of HSD17B12 KD cells compared to control cells (**Fig.**

2.4A). Furthermore, we found a decrease of the relative abundance of oleic acids (C18.1) with concomitant increase of palmitic acids (C16.0) resulting in significant decreased of C18:C16 ratios that are expected from reduced HSD17B12 enzymatic activity (**Fig. 2.4B-D**). These results support the requirement of HSD17B12 metabolic capacity for LD maintenance by *de novo* synthesis of VLCFA and TGs, as well as during HCV assembly on LDs. We also examined whether the reduced amount of LDs is explained by an increased expression of catabolic lipid enzymes possibly due to the need of stored fatty acids for metabolic reactions. Interestingly, we detected striking increased mRNA levels of hormone sensitive lipase (LIPE) and phospholipase A2 (PLA2G1B) in HSD17B12 KD cells (**Fig. 2.S5).** Given their role in lipolysis, these results suggest that reduced expression of HSD17B12 induces a feedback response that hydrolyses TGs of LDs. Such rescued response, however, is unable to maintain cellular contents of oleic acids (C18:1) and TGs that are required for MW structures and viral assembly sites. Interestingly, we also showed a significant decrease in the cellular contents of the phospholipid phosphatidylethanolamine (PE) (**Fig. 2.4E**), which is reported to be important for viral replication compartments of positive-strand RNA virus [323, 324], while no changes were observed for phosphatidylcholine (PC) species (**Fig. 2.4F**). The decreased VLCFA and lipid species of HSD17B12 KD cells contrast with the opposite metabolic needs of HCV-infected cells to synthesize ER-derived specialized MW structures and to expand LDs for virion assembly. Overall, our data strongly support the metabolic capacity of HSD17B12 in tailoring a lipid metabolic environment that favors HCV infection, and that its inhibition interferes with the replication and particle assembly processes by reduction of *de novo* VLCFA synthesis and lipid species.

2.3.4. Depletion of HSD17B12 disrupts HCV-induced MW structures

HSD17B12 contributes to the synthesis of oleic acids that produce important concentration-dependent alterations of the lipid membrane structure [325]. In light of the altered metabolic capacity of HSD17B12 KD cells and reduced abundance of LDs (**Fig. 2.3**), one might expect some biochemical and functional changes of HCV-induced MW structures. To test this, we first assessed the cellular distribution of dsRNA as a marker of viral replication sites in HSD17B12 KD cells by confocal microscopy (**Fig. 2.5A**). No anti-dsRNA antibody staining was observed in parental cells transduced with lentivirus-expressing shNT validating the staining of HCV RNA replication compartments. In JFH-1-replicating control cells expressing shNT, we showed a dot-like cytoplasmic staining with a well-defined distribution of replication sites into punctuated foci as previously reported [326]. In HSD17B12 KD cells, however, we observed a more diffuse staining with a wide-spread pattern of distribution. The HCV-induced MW structures are known to be partially resistant to mild detergents and to protect viral components from exogenous proteases and nucleases *in vitro* [172, 327]. To assess the membrane integrity of the diffuse replication sites of HSD17B12 KD cells, we performed an RNase protection assay using membrane permeabilizing detergent (digitonin) and exogenous nucleases as previously reported [242, 312] (**Fig. 2.5B**). The treatment of shNT-expressing control cells with nuclease and digitonin showed less than 10% degradation of HCV RNA and 90% degradation of cytoplasmic actin mRNA used as control. However, more than 50% of HCV RNA is degraded in HSD17B12 KD cells, demonstrating that the depletion of HSD17B12 lead to increased digitonin sensitivity of the MW most probably because of a modified membrane VLCFA composition. As control, cells permeabilized

with NP40 lead to the almost complete degradation of HCV RNA. Overall, the data demonstrate that HSD17B12 KD disrupts the functional integrity of HCV MW.

2.3.5. Oleate supplementation rescues HCV replication in HSD17B12 KD cells

The requirement of oleic acids was previously reported for HCV replication [242]. Furthermore, HCV-infected cells were shown to contain a higher relative abundance of TGs and a strikingly increased utilization of C18 fatty acids, most prominently oleic acids [192]. As HSD17B12 KD decreases the cellular contents of oleic acids (**Fig. 2.4C**) and is associated with increased intracellular HCV RNA levels and a decrease of infectious particle production (**Fig. 2.2**), we hypothesized that supplementing HSD17B12 KD cells with oleic acid-BSA would rescue HCV replication (**Fig. 2.S6**). We showed that addition of oleic acid (20 μM) to cell culture medium of KD cells partially rescues particle production, supporting the requirement of oleic acids and other VLCFA species for viral replication, progeny assembly and MW integrity.

2.3.6. HSD17B12 is required for ZIKV and DENV replication

Many flaviviruses manipulate *de novo* fatty acid synthesis of palmitic acid [190, 224, 317], which serves as the main substrate for the synthesis of VLCFA. As very little is known about the requirement of VLCFA for flavivirus infection, we assessed the replication of DENV and ZIKV in HSD17B12-depleted Huh7.5 cells (**Fig. 2.6**). We showed that HSD17B12 KD significantly decreased DENV (strain 16681) and ZIKV (FSS13025 strain) replication as monitored by Rluc activities for both reporter viruses.

HSD17B12 KD also significantly reduced infectious titers of supernatants using plaque forming unit (PFU) assays. We then evaluated the effects of INH-12, a small-molecule inhibitor of HSD17B12 on flavivirus replication (**Fig. 2.7**). INH-12 is a steroid-based selective HSD17B12 inhibitor (**Fig. 2.7A**) synthesized at greater than 99% pure, which has great solubility, high cell permeability and from a series of compounds that are rarely cytotoxic at concentrations up to 50 μM [328]. We first confirmed that INH-12 reduces viral replication of HCV replicon containing cells (**Fig. 7B)** and core protein expression of JFH-1 infected cells (**Fig. 2.7C**) at concentrations for which no appreciable cytotoxicity is observed (**Fig. 2.S7**). Outstandingly, the treatment of DENV- and ZIKV-infected Huh7.5 cells with INH-12 dose-dependently reduces the production of infectious particles in infection assays (**Fig. 2.7D, F),** and up to 3-$\log_{10}$ for DENV, and completely block viral protein expression as monitored by Western blotting (**Fig. 2.7E, G)**. The data demonstrate the conserved requirement of HSD17B12 metabolic capacity for the replication of HCV and related flaviviruses DENV and ZIKV.

2.4. DISCUSSION

The elongation of fatty acids to VLCFA and modulation of the pathway for virus-induced specialized membranous structures are poorly understood aspects of the biology of *Flaviviridae*. In this study, we characterized the requirement of HSD17B12 for HCV infection and its mode-of-action. This is a continuation of our HCV interactome study that uncovered HSD17B12 as a specific viral core interactor promoting HCV replication [308]. Interestingly, literature mining showed that HSD17B12 is a hub protein that interacts with proteins of different RNA and DNA viruses [202-206], which underline a potential broad-spectrum role of HSD17B12 against viral infections. HSD17B12 is an

ER-bound keto-acyl reductase that catalyzes conversion between E1 and E2 [300], and later reported to catalyze the second reaction of each VLCFA elongation cycle [278]. Our lipidomics analysis also support a function of HSD17B12 in VLCFA synthesis as revealed by the significant decreased ratio of oleic acid (C18.1) to palmitic acid (C16.0) in HSD17B12 KD cells. In addition, a recent study described that VLCFA amounts are increased in HCV-infected cells [320]. Accordingly, patients with fatty liver display elevated levels of VLCFA in this organ [329]. This suggests an important role of the VLCFA synthesis pathway for HCV infection and pathogenesis. In this study, we provide strong and compelling evidence that the VLCFA metabolic capacity of HSD17B12 promotes the replication of HCV and flaviviruses DENV and ZIKV. First, HSD17B12 KD significantly decreases viral replication and infectious particle production in Huh7.5 cells (**Fig. 2.2**). Second, the inhibitor of HSD17B12, INH-12 [328], reduces virus replication at concentrations for which no appreciable cytotoxicity is observed and dramatically reduces infectious particle production (**Fig. 2.7**). To our knowledge, this is the first report describing a pro-viral role of the metabolic capacity of HSD17B12-mediated VLCFA synthesis to promote viral replication and particle assembly processes of several *Flaviviridae* members. Our data further highlight an additional antiviral mechanism-of-action other than FASN inhibitors [190, 224, 317] through modulation of VLCFA metabolism with HSD17B12 inhibitors.

To mechanistically explore the contribution of HSD17B12 in promoting replication of pathogenic *Flaviviridae* viruses, we focused our study on the replicative life cycle of HCV. We first provide evidence for the re-localization of a pool of ER resident HSD17B12 at virus-induced MW and LD sites (**Fig. 2.1**). The biogenesis of the special

architecture of MW (such as DMVs) and assembly compartments likely requires specific lipid species and stoichiometry that provide the membrane biophysical characteristics needed for optimal viral genome replication and particle assembly processes [241, 242, 330]. FASN has the capacity to synthesize fatty acids up to palmitic acid and was thought to be solely bringing the flux of fatty acids in proximity to replication and assembly compartments of flaviviruses [190, 219, 224, 226]. However, FASN capacity to synthesize fatty acids is not sufficient to explain the enrichment of VLCFA at virus-induced specialized membrane sites [64, 229], since it requires the elongation enzymatic machineries. We provide strong evidence of the requirement for *de novo* VLCFA synthesis with carbon chains ≥ C18 upon virus infection. Indeed, HSD17B12 KD blocks VLCFA elongation flux resulting in the expected increase of its substrate palmitic acid (C16.0) (**Fig. 2.4**). However, such increased palmitic acid levels are not enough to promote virus replication and particle assembly (**Fig. 2.2 and Fig. 2.6**). Whether other elongation enzymes are hijacked to trigger *de novo* VLCFA synthesis will need further investigation of virus-induced specialized membrane compartments using proteomics and cell fractionation studies.

HSD17B12 KD decreased replication of subgenomic HCV RNA in absence of virus assembly but unexpectedly increased intracellular viral RNA levels of HCV-infected cells, which correlate with functional alteration of MW distribution and biochemical properties. Since the reduction in membrane VLCFA such as oleic acids may impact the compartment integrity, whether the segregation of viral RNA synthesis at less defined virus-replicating sites contributes to the increased HCV RNA levels needs further investigation. An alternative explanation is the accumulation of intracellular HCV RNA

as a consequence of the significant reduction of LDs that disrupt encapsidation of newly synthesized viral RNA and decrease particle production of HSD17B12 KD cells.

HSD17B12 KD reduces the number and size of LDs and decreases oleic acid and TGs levels, which are the main components of LDs. The induction of lipolytic enzymes is observed and may reflect a cellular feedback requirement to increase cytoplasmic VLCFA involved in various vital metabolic processes. Interestingly, inhibition of lipolytic enzymes by core is rather observed upon HCV infection to maintain LD organelles [331, 332], impeding the activity of the hormone-sensitive lipase enzyme (LIPE) [333] and the adipose triglyceride lipase (ATGL) [334]. The LDs are crucial to several virus assembly processes [122, 224, 312-314] and as such the metabolic capacity of HSD17B12 to maintain LDs most likely contributes to the production of HCV, DENV and ZIKV infectious particles. In addition, the decreased LDs in HSD17B12 KD cells is expected to lead to a rapid degradation of core protein as disrupting its localization to LDs leads to a destabilization of the protein [335-338]. Along with the requirement of *de novo* VLCFA synthesis to maintain LDs, the inhibition of HSD17B12-mediated LD homeostasis as an essential platform for virus assembly provides an antiviral mechanism-of-action for the significant reduction of HCV, DENV and ZIKV infectious particles. Finally, INH-12 is a selective inhibitor of HSD17B12 reductase activity, which supports a competitive mode-of-inhibition by occupation of the active site of the enzyme for its antiviral activity [300, 339, 340]. In summary, our study identifies HSD17B12 as a host co-factor involved in the replication of HCV and related flaviviruses DENV and ZIKV, and as a promising validated antiviral target. This study supports a conserved key role of *de novo* VLCFA

synthesis in *Flaviviridae* infections and reveals the therapeutic potential of targeting HSD17B12 as a broad-spectrum antiviral approach.

2.5. METHODS

2.5.1. Cell lines, antibodies, reagents: 293T, HeLa, VeroE6, Huh-7, Huh7.5 and HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, and 1% nonessential amino acids (all from Wisent). Cells were transfected with linear (25-kDa) polyethylenimine (PEI; Polysciences) or jetPRIME (Polyplus-transfection, Invitrogen) as described by the manufacturer. Huh7 cells stably expressing a reporter Con1 subgenomic replicon (Huh7-Con1-Fluc) were maintained in complete DMEM with 500 µg/ml G418 (Multicell).

2.5.2. Lentivirus production: 293T cells were transfected with PEI by using plasmids pRSV-REV, pMD2-VSVG, and pMDLg/pRRE and shRNA-encoding plasmid pLKO.1-puro (non-target and HSD17B12 TRCN0000027145, TRC 1 generation; Sigma-Aldrich). Lentiviruses were titrated using Hela cells as previously reported [338]. For gene silencing, cells were transduced shRNA-expressing lentiviruses at a multiplicity of infection (MOI) ≥ 2. Cells were collected for analysis according the design of different experiments.

2.5.3. HCV infection assay: First, we knockdown the gene of interest in Huh7.5 cells using shRNA expressing lentivirus, then three days from transduction, we transfect the cells with plasmid pEF/JFH-1-Rz/Neo by using JetPRIME (Polyplus-transfection, Invitrogen) (2ug plasmid DNA/5*10^5 cells) as in [309], and cell media is replaced after 4 h. At 4 days post-transfection, cells and culture media are collected. Virus-containing

culture medium is cleared through a 0.45-µm filter and used to infect naive cells. Infected naive cells are collected after 4 days. Extracellular viral particles from cell culture supernatants are concentrated with 50 kDa Amicon- EMD Millipore) for RNA extraction and RT-PCR determination.

2.5.4. The Con1b Replicon: Huh7 cells stably express the genotype 1b Con1 subgenomic replicon, were used. This bicistronic replicon expresses the neomycin phosphotransferase and the Firefly luciferase through the HCV IRES, while NS3-NS5B polyprotein production is under the control of the encephalomyocarditis virus (EMCV) IRES [309]. Luciferase assays were performed as described.

2.5.5. Cytotoxicity assays: Cells were cultured in transparent 96-well plates. Twenty microliters of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) stock solution (5 mg/ml in PBS) was added to the cells and incubated for 1 h at 37°C in the dark. Following the removal of the medium, cells were incubated at room temperature for 10 min with 150 µl of dimethyl sulfoxide (DMSO) containing 2 mM glycine, pH 11. Absorbance at 570 nm was read with a reference at 650 nm. For the Alamar Blue assay, cells were cultured in black 96-well plates. Ten microliters of Alamar Blue reagent (Invitrogen; diluted 1:4 in PBS) was added to the cells and following a 3-h incubation at 37°C, fluorescence at 595 nm (excitation wavelength, 531 nm) was measured with an EnVision plate reader (PerkinElmer). A control plate with medium only (no cells) or Alamar Blue only was used to determine the background that was subtracted from the fluorescence value.

2.5.6. qRT-PCR: Cells RNA was extracted using RNeasy Plus Kit. DNase treatment, reverse transcription and real-time PCRs were performed at the IRIC Genomic Core Facility with TaqMan-based assays. For HCV RNA detection, we used primers CATGGCGTTAGTATGAGTGTCG and GGTTCCGCAGACCACTATG and TaqMan-labeled probe CAGCCTCC (probe 75; no. 04688988001 from the Roche Universal Probe Library); for ZIKV PF strain, we used with primers GCCCTTCTGTTCACACCATT and CCACATTTGGGCGTAAGACT and for DENV2, we used the primers AGATGAA-CTGATTGGCCGGGC and AGGTCTCTTCTGTGGAAATA. Relative viral RNA and genes mRNA levels were calculated using the 2 -$^{\Delta\Delta}$CT method using ACTIN as an internal control, and plotted as fold change by normalizing to control samples

2.5.7. Luciferase assays: Cells were washed once PBS. For firefly luciferase (Fluc) assays, 1 volume PBS and 1 volume of 2× luciferase buffer (100 mM Trizma acetate, 20 mM magnesium acetate, 2 mM EGTA, 1% Brij 58, 0.7% β-mercaptoethanol, 3.6 mM ATP, 45 μg/ml d-luciferin, pH 7.9) were added to the cells. Cells were incubated for 15 min at room temperature in the dark. For Renilla luciferase (Rluc) assays, 1 volume of PBS and 1 volume of 2 mM EDTA (pH 8)–5 μM coelenterazin (Nanolight) were added to the washed cells. Fluc and Rluc activities were measured with a luminescence counter (PerkinElmer).

2.5.8. Antibodies: The following antibodies were used in this study: mouse anti-actin (EMD Milipore), mouse anti-HCV core (Affinity BioReagents), mouse anti-HCV NS3 (Abcam), mouse anti-Flag tag (Sigma), mouse anti-dsRNA (English Scientific Consulting), mouse anti-PDI (Stressgen), rabbit anti-HSD17B12 (Novus Biologicals), mouse anti-DENV NS3 (GeneTex; cross-reactive with ZIKV NS3) and rabbit DENV-

NS4B (GeneTex; cross-reactive with ZIKV NS4B). Secondary antibodies coupled with horseradish peroxidase and Alexa Fluor were purchased from Bio-Rad and Invitrogen.

2.5.9. Immunofluorescence analysis: Cells were grown on a coverslip in 6-well or 24-well plates, fixed, permeabilized and blocked as previously reported [338]. Following three rapid washes, cells were labeled for 2 h at room temperature with primary antibodies diluted in 5% BSA–0.02% sodium azide–PBS. Slides were washed three times in PBS and then probed with Alexa Fluor 488-, 594-conjugated secondary antibodies (Invitrogen) diluted 1:1000 in 5% BSA–0.02% sodium azide–PBS for 1 h in the dark. Cells were extensively washed and incubated with Hoechst dye (Invitrogen) at a final concentration of 1 μg/ml in PBS for 10 min. Following three rapid washes, the slides were mounted with 1,4-diazabicyclo [2.2.2] octane (Sigma-Aldrich) as an anti-fading agent. Labeled cells were then examined by confocal laser scanning microscopy (Leica TCS-SP5 MP) at the CRCHUM-Imaging Core Facility. For lipid droplets staining, after fixation, permeabilization and blocking, the nuclei were stained with Hoechst. Following three rapid washes, cells were incubated for 1 h with HCS LipidTOX Deep Red (Invitrogen) diluted 1:1000 in PBS, immediately afterward cells were mounted with anti-fading agent. Labeled cells were then examined by Zeiss Observer microscopy. Data were analyzed by CRCHUM-Imaging Core Facility.

2.5.10. RNase protection assay: The RNase protection assay was adapted from Lyn *et al* [242]. Transduced cells with shNT and shHSD17B12 were transfected with JFH-1 plasmid for 4 days. Cells were washed once with cold buffer B (20 mM HEPES-KOH (pH 7.7), 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, and 2 mM dithiothreitol). For samples undergoing digitonin (Sigma-Aldrich, USA) treatment, buffer

B containing 50 µg/ml of digitonin was added to cells for 5 min at 37°C. The reaction was stopped by washing twice with cold buffer B. For samples treated with micrococcal nuclease (Sigma-Aldrich, USA) and/or NP-40 substitute (octyl-I-phenoxypoly-ethoxyethanol; Bioshop Canada Inc.), the cells were washed twice with buffer D (20 mM HEPES-KOH (pH 7.7), 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, and 1 mM $CaCl_2$) and treated with buffer D containing 0.1 unit/mL micrococcal nuclease, with or without, 0.45% NP-40 substitute for 15 min at 37°C. Samples treated with 0.45% NP-40 substitute only were incubated for 10 min at 37°C. Total RNA was extracted using the RNeasy Mini Kits (Qiagen) and treated with DNase. 250 ng of total RNA was reverse transcribed into cDNA, and equal amounts of cDNA processed for qRT-PCR at IRIC. The absolute amount of HCV RNA and actin were calculated.

2.5.11. Lipidomics analysis: Tested cells were collected and pelleted. Cell pellets were kept on ice and re-suspend in 1 ml of nanopure water then were transferred to glass tubes. 20 µl of internal standard mix was added using the 25 µl glass syringe. Next, ~800 µl of glass beads were added using scoop device. Using a glass pipet, 3 ml of 17:1 $CHCl_3$:MeOH was added to each tube. The tubes were vortexed at 4°C (in the cold room) for 2 h and phases were separated by centrifugation at 3000g for 5 min. The lower organic phase was transferred to a clean 15 ml glass tube. The solvent of the organic phase tube was evaporated under nitrogen flow. Using a glass pipet 1.5 ml of 2:1 $CHCl_3$:MeOH was added to the remaining aqueous phase. This phase was vortexed again at 4°C (in the cold room) for 1 h. Phases were separated by centrifugation at 3000g and combined with the organic phase from the 17:1 $CHCl_3$:MeOH extract. The solvent was

evaporated under nitrogen flow. Lipid film was dissolved in 150 µl of 2:1 $CHCl_3$:MeOH and transferred to 2 ml glass vial with either Teflon or aluminum lined caps. Samples were stored at -20°C until ready to be analyzed by mass spectrometry. Prior to infusing the samples in the mass spectrometer, samples were diluted in a 1:1 ratio using 2:1 $CHCl_3$:MeOH + 0.1% NH_4OH. Then samples are run through quantitative shotgun mass spectrometry using a high resolution Thermo Orbitrap Velos instrument. Data were analyzed as previously reported [341]. For free fatty acids (FFA), the relative abundance of specific fatty acid was calculated to total detected FFA. For TG, PC, PE, the relative abundance was calculated to total detected lipid species.

2.5.12. HSD17B12 inhibitor INH-12 and antiviral assays: INH-12 (compound **97**; Table 1 in [339] was synthesized as reported in [340]. The antiviral effects of INH-12 were determined on Huh7.5 cells transfected with JFH-1 plasmid and incubated with various concentrations (0 - 20 µM) of INH-12 for 4 days. Cells were analyzed by western blot. HCV replicon containing cells were treated with INH-12 and analyzed by luminescence assay. For DENV and ZIKV replication, Huh7.5 cells were infected at a MOI of 3 pfu per cell for western blot analysis, and at a MOI of 0.002 pfu per cell for the luciferase replication assay for 2 h at 37°C. Virus inoculum was removed, cells were washed with PBS and incubated with various concentrations (0 - 20 µM) of INH-12. DMSO was used as control. Virus replication was analyzed by western blot after 2 days, by luminescence assay after 3 days, and by plaque assay for virus titration at 3 days post-infection.

2.5.13. Oleic acid supplementation: For rescue experiments, Huh7.5 cells were treated with oleic acids, which was prepared as previously described by Chatel-Chaix *et al* [338].

Briefly, 0.5 g of fatty-acid-free BSA (catalog number A6003; Sigma-Aldrich) was dissolved in 3.6 ml of 0.1 M Tris-Cl, pH 8. A 12.6-mg sample of oleic acid (catalog number O1008; Sigma-Aldrich) was transferred into clean, fatty-acid free BSA (catalog number A6003; Sigma-Aldrich) and then diluted in 3.6 ml of Tris-BSA buffer by gently shaking the solution until it was clear. The oleic acid stock was at 12.5 mM.

2.5.14. DENV/ZIKV titration by plaque assay and Rluc assays: Confluent monolayers of VeroE6 cells were infected with serial 10-fold dilutions of virus supernatants for 2 h at 37°C. Two hours later, inoculum was removed and replaced with serum-free MEM (Gibco, Life Technologies) containing 1.5% carboxymethylcellulose (Sigma-Aldrich). Four days (ZIKV) or seven days (DENV) post-infection, cells were fixed for 2 h at room temperature with formaldehyde directly added to the medium to a final concentration of 5%. Fixed cells were washed extensively with water before being stained with a solution containing 1% crystal violet and 10% ethanol for 30 min. After rinsing with water, the number of plaques was counted at the appropriate dilution and virus titers were calculated. RNA replication of DENV and ZIKV reporter virus [342] was determined by measuring the activity of virus-encoded renilla luciferase (Rluc) as previously described [343]. After lysis of the cells, coelenterazine (1.43 μM final concentration) was added and luminescence was measured.

2.5.15. Statistical analysis: The statistical analysis was performed with the GraphPad Prism 7 software. All Student *t* tests were non-parametric two tailed, and P values of <0.05 were considered significant.

2.6. ACKNOWLEDGEMENTS

We thank Jake Liang for pEF/JFH1-Rz/Neo plasmid; Takaji Wakita (National Institute of Infectious Diseases, Tokyo), Charles Rice (Rockefeller University) and Apath LLC for the use of JFH-1 and Huh7.5 cells; Ralf Bartenschlager (University of Heidelberg) for luciferase-encoding Con1b subgenomic replicon and DENV2 16681 molecular clones; the European Virus Archive goes Global (EVAg) and Dr. Xavier de Lamballerie (Emergence des Pathologies Virales, Aix-Marseille University) for providing ZIKV H/PF/2013 original stock; Dr. Pei-Yong Shi (University of Texas Medical Branch) for ZIKV FSS13025 Rluc-expressing molecular clone; Dr. Tom Hobman and Dr. Anil Kumar (University of Alberta) for VeroE6 cells; Aurelie Cleret-Buhot (CRCHUM Bio-Imagery Core Facility) for confocal microscopy and Guillaume Chouinard (CRCHUM Bio-Imagery Core Facility) for lipid droplet quantifications; Jessy Tremblay (INRS-Armand Frappier Institute imagery facility) for confocal microscopy; Younes Medkour (Concordia University) for lipid quantification; IRIC's Genomics Core Facility for real-time PCR. This work was supported by an operating grant from the Canadian Institutes of Health Research to DL and by A PhD scholarship to BM from National Research Centre of Egypt, Egyptian Government of Higher Education and Scientific Research.

2.7. ADDITIONAL INFORMATION

Competing financial interest: The authors declare no competing financial interests.

2.8. SUPPLEMENTAL INFORMATION

Supplemental information includes seven figures and can be found with this article.

2.9. FIGURES

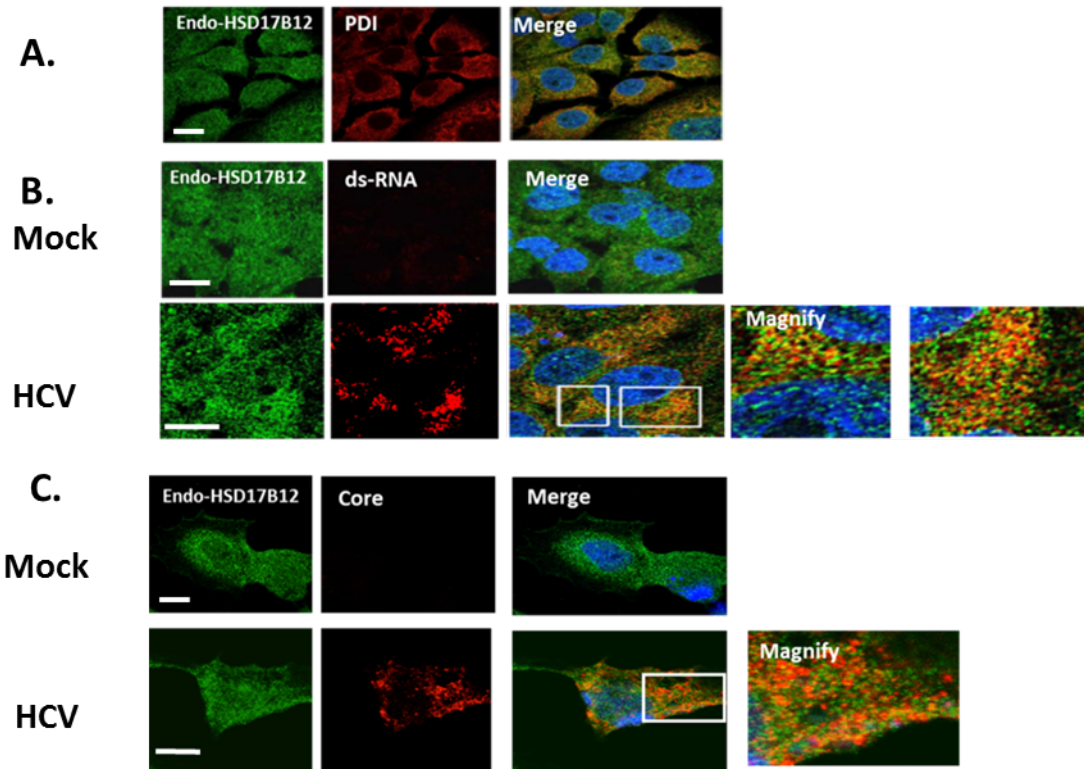

Figure 2.1. HSD17B12 overlaps replication and core-associated assembly sites of HCV infection. (A) Parental Huh7.5 cells were fixed with formaldehyde, permeabilized with 0.1% Triton X-100 and co-stained with anti-HSD17B12 and anti-PDI antibodies. (B) Huh7.5 cells (Mock) and transfected with JFH-1 expressing DNA plasmid (HCV) for 4 days were co-stained with anti-HSD17B12 and anti-dsRNA antibodies and (C) with anti-HSD17B12 and anti-core antibodies. Nuclei were stained with Hoechst. Fluorescent images were obtained using a confocal laser scanning microscope. Merged images are presented on the right column. Scale bars represent 20 μm.

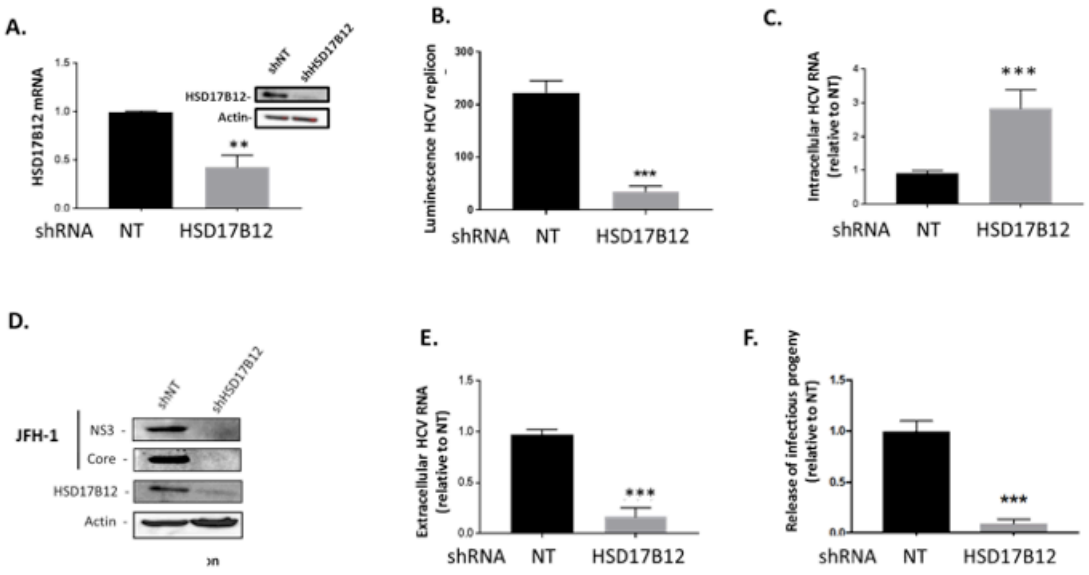

Figure 2.2. HSD17B12 KD decreases HCV replication and infectious viral particle production. (A) Huh7.5 cells transduced with lentivirus-expressing shNT or shHSD17B12 for 4 days are analyzed for HSD17B12 mRNA and protein levels. The effects of shNT (NT) and shHSD17B12 (HSD17B12) were determined on (B) reporter activity of Huh7 cells harboring an HCV subgenomic replicon (Huh7-Con1-Fluc), (C) intracellular HCV RNA levels of JFH-1-infected Huh7.5 cells by qRT-PCR and (D) NS3 and core protein levels by western blot. Supernatants of cells transduced with lentivirus expressing shNT or shHSD17B12 and infected with JFH-1 for four days were analyzed for (E) extracellular HCV RNA levels and (F) extracellular infectivity by re-infecting naive Huh7.5 cells for 4 days and analysis of HCV RNA levels by qRT-PCR. HCV RNA levels are normalized with actin RNA content and arbitrarily set to 1 for cells expressing shNT and infected with JFH-1. Values represent mean ± SD from the analysis of more than three experiments. P values, < 0.05 (*), < 0.01 (**) or < 0.001 (***) are indicated in comparison with shRNA NT cells.

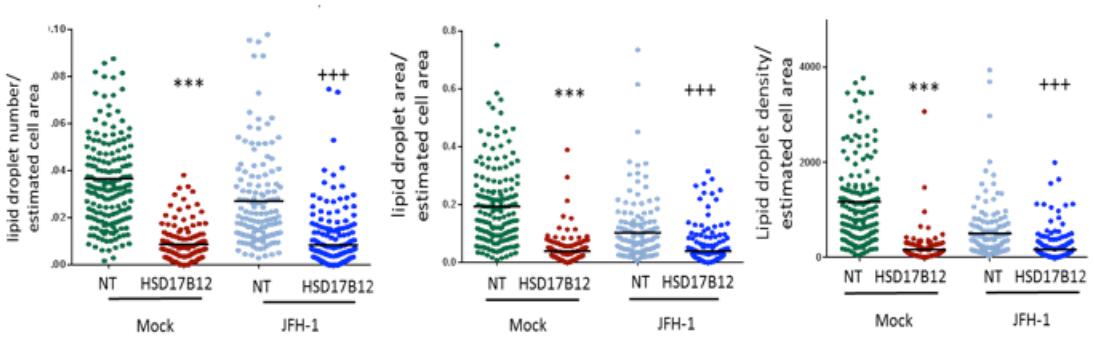

Figure 2.3. HSD17B12 KD reduces the number of cytoplasmic LDs. Huh7.5 cells transduced with lentivirus-expressing shNT (NT) or shHSD17B12 (HSD17B12) are either uninfected (Mock) or infected with HCV (JFH-1) for four days and analyzed for the contents of LDs. Cells were fixed with formaldehyde, permeabilized with 0.1% Triton X-100 and stained with Lipidtox and Hochest. Cells were imaged with 7 field views for each condition to quantify number, area and density of LDs per cell. P values < 0.001 (***) and (+++) are indicated in comparison with cells expressing shNT for non-infected and JFH-1-infected cells, respectively.

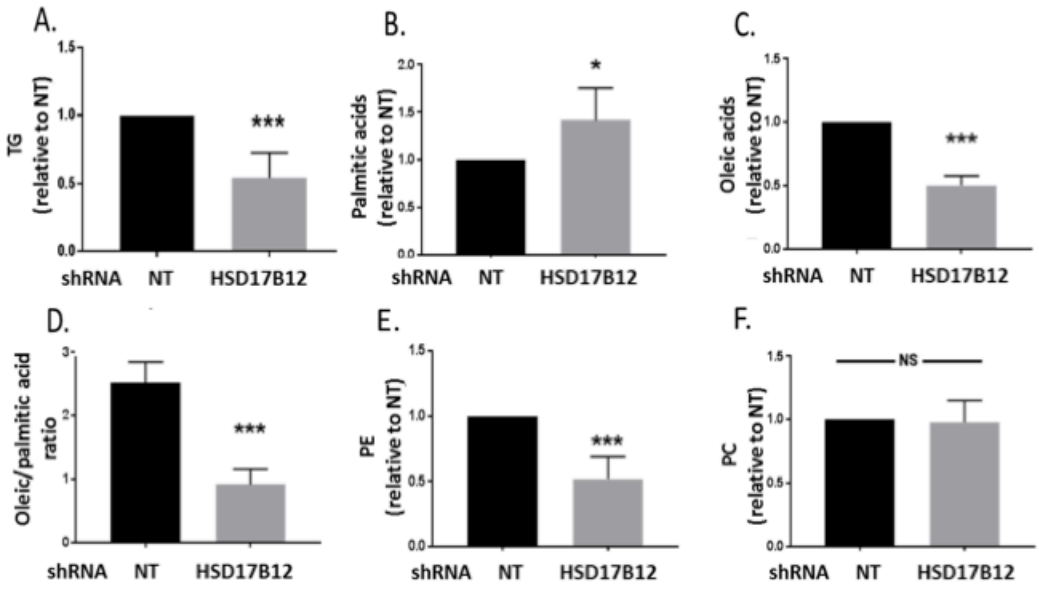

Figure 2.4. HSD17B12 KD induces alteration in cellular lipid metabolism. Huh7.5 parental cells transduced with lentivirus-expressing shNT (NT) or shHSD17B12 (HSD17B12) were used to determine the relative cellular abundance of (A) triglycerides (TGs), (B) palmitic acids (C16.0), (C) oleic acids (C18.1), (D) oleic/palmitic (C18/C16) ratios (E) phosphatidylethanolamine (PE) and (F) phosphatidylcholine (PC) among all detected lipid species by shotgun lipidomics analysis of whole cell extracts. The data of three experiments were pooled for the analysis. P values, < 0.05 (*), < 0.001 (***), or non-significant (NS) are indicated in comparison with cells expressing shNT as control. NS: non-significant. (Data were normalized to control arbitrary value of 1 "except D", non-parametric two tailed student t test was used to compare each test condition with the control).

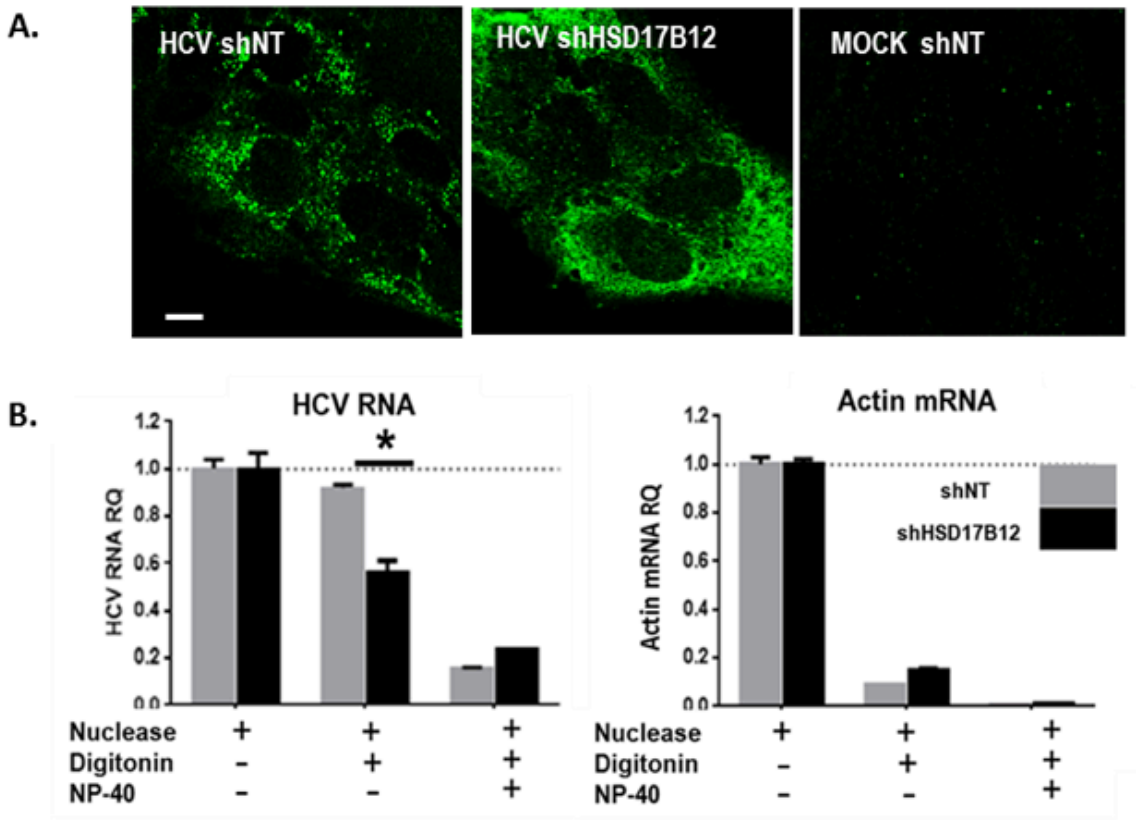

Figure 2.5. HSD17B12 KD induces dispersion and disruption of HCV replication sites. (A) Huh7.5 parental cells (MOCK shNT), infected with JFH-1 and transduced with lentivirus-expressing shNT (HCV shNT) or shHSD17B12 (HCV shHSD17B12) were stained with anti-dsRNA antibodies as makers of viral replication sites by confocal microscopy. Results are representative of multiple field views. (B) Huh7.5 cells transduced with lentivirus-expressing shNT or shHSD17B12 and transfected with JFH-1 DNA plasmids for four days are analyzed for RNA susceptibility to exogenous nucleases. Cells were treated with micrococcal nuclease, digitonin and NP40 as indicated by a +, and the relative abundance of HCV RNA (left panel) and actin mRNA (right panel) levels were determined by qRT-PCR analysis. RNA levels of cells treated with nuclease only are normalized to one. Values represent mean ± SD from the analysis of two experiments. P values < 0.05 (*) are indicated in comparison with shNT treatment.

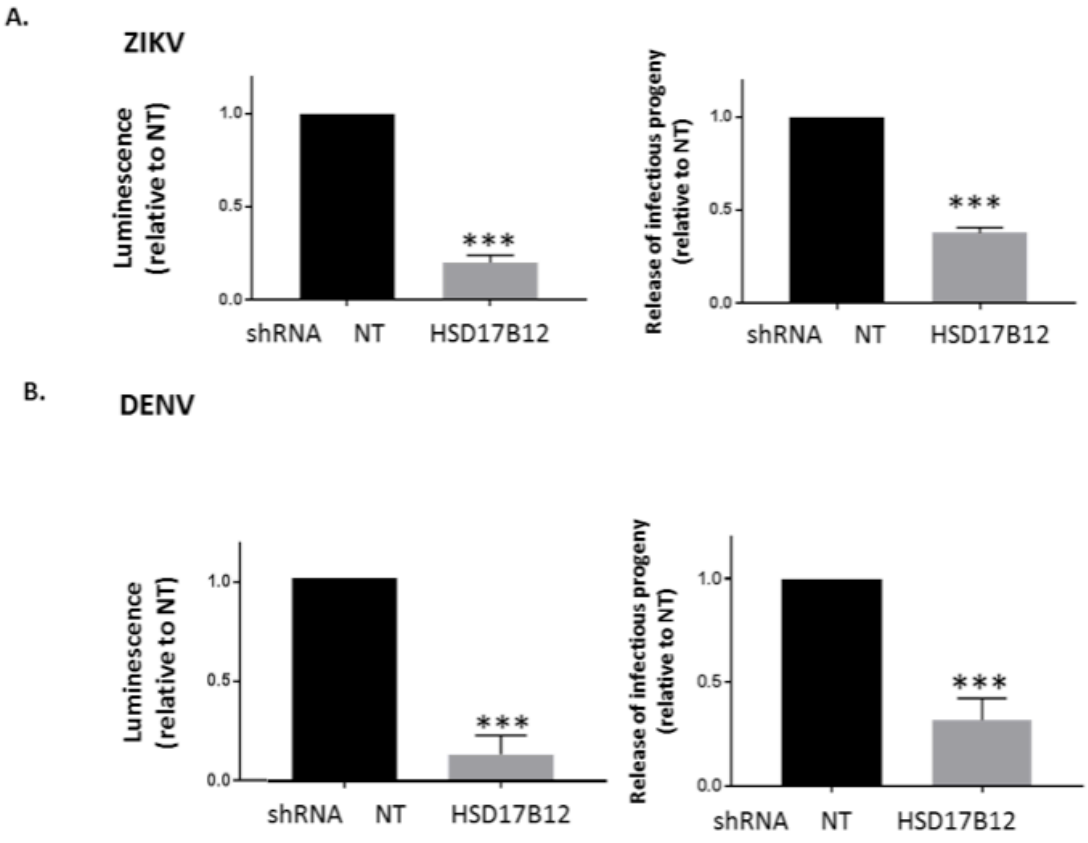

Figure 2.6. HSD17B12 KD decreases DENV and ZIKV replication and infectious viral particle production. Huh7.5 cells transduced with lentivirus-expressing shNT (NT) or shHSD17B12 (HSD17B12) are infected with (A) ZIKV FSS13025 strain and (B) DENV2 16681 strain both expressing a renilla luciferase (Rluc) gene. At 3 days post-infection, cells were analyzed for luminescence as readout of viral replication (left panels). Supernatants were analyzed for the content of infectious particle production by plaque assays (right panels). The luminescence signals and plaque forming units (PPU) of cells transduced with lentivirus-expressing shNT were arbitrarily set to 1. Values represent mean ± SD from the analysis of three experiments. P values < 0.001 (***) are indicated in comparison with shNT treatment. (Data were normalized to control arbitrary value of 1, non-parametric two tailed student t test was used to compare each test condition with the control).

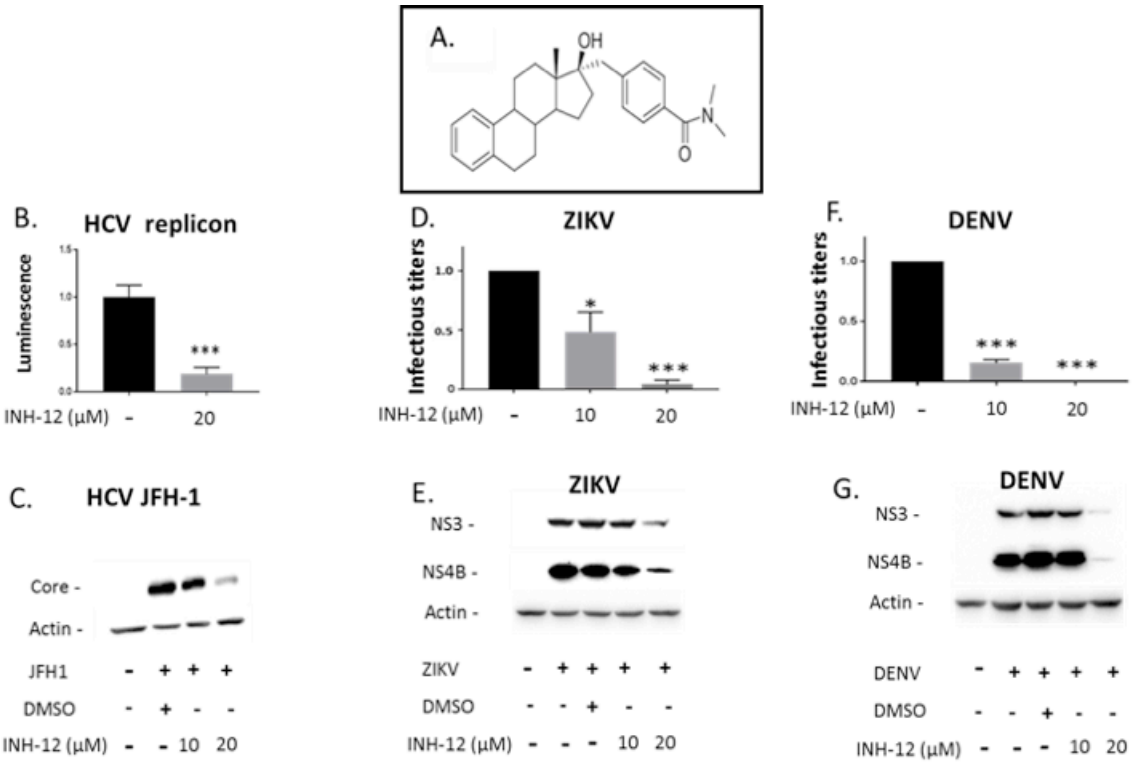

Figure 2.7. HSD17B12 inhibitor INH-12 blocks HCV, ZIKV and DENV replication. (A) Chemical structure of INH-12. (B) Huh-7 cells expressing an HCV subgenomic replicon Con1b luciferase were treated with 0 and 20 μM of INH-12. (C) Huh7.5 cells infected with JFH-1, (D-E) with ZIKV H/PF/2013 strain and (F-G) with DENV2 16681 strain were treated with 0 to 20 μM of INH-12. Infectious virus titers (D and F) were measured at a MOI = 0.002 for 3 days by plaque assays. The expression of viral proteins (E and G) was determined at a MOI = 3 at 2 days post-infection by western blot. P values, < 0.05 (*) or < 0.001 (***) in comparison with DMSO control are indicated from analysis of 3 experiments (Data were normalized to control arbitrary value of 1 (D-F), non-parametric two tailed student t test was used to compare each test condition with the control).

2.S SUPPLEMENTARY FIGURES

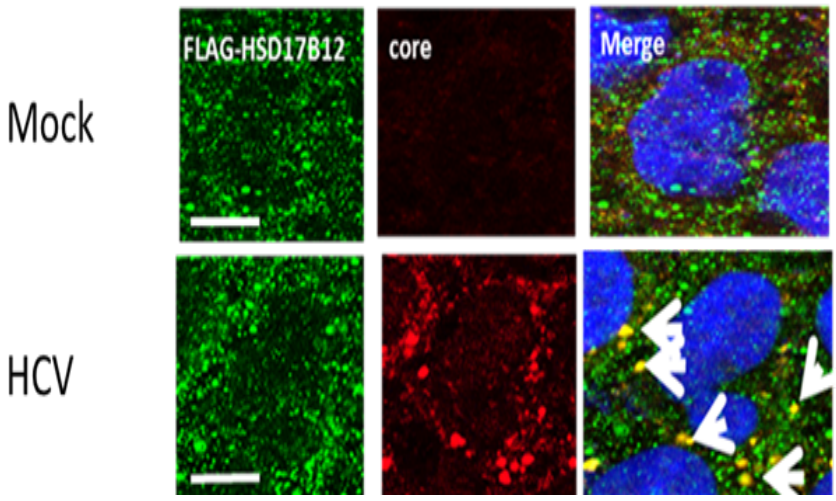

Figure 2.S1.

HSD17B12 overlaps with HCV assembly sites. Huh7.5 parental (Mock) and JFH-1-infected cells (HCV) were transfected with an N-terminal FLAG-tagged HSD17B12 fusion protein expressing vector. Cells were fixed with formaldehyde, permeabilized with 0.1% Triton X-100 and stained with anti-FLAG, anti-core antibodies. Nuclei were stained with Hoechst. Images were obtained using a confocal laser-scanning microscope. Co-localization is indicated by white arrows on merged images from right column. Scale bars represent 20 μm.

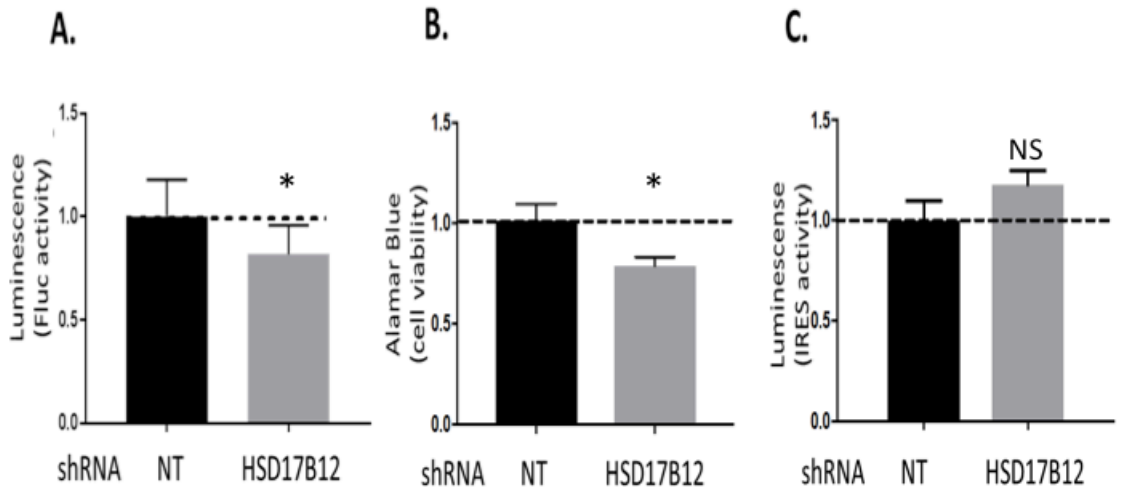

Figure 2.S2.

HSD17B12 KD has minor effects on protein translation, cell survival and no effect on HCV IRES-mediated translation. Huh7 cells transduced with shNT (NT) and shHSD17B12 (HSD17B12) are (A) stably expressing a luciferase gene under an EF1 alpha promoter to assess protein translation (B) used to monitor cell survival using an Alamar Blue assay and (C) HCV IRES-driven Firefly luciferase activities normalized to expression of CMV-driven Renilla luciferase as a cap-dependent translation control. Values represent mean ± SD from the analysis of at least two experiments. P values < 0.05 (*) or non-significant (NS) are indicated in comparison with shRNA NT treatment.

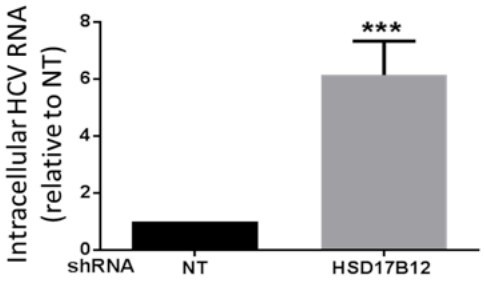

Figure 2.S3.

HSD17B12 KD increases intracellular HCV RNA levels of HepG2-infected cells. HepG2 cells transduced with shNT (NT) and shHSD17B12 (HSD17B12) are transfected with the JFH-1-expressing DNA plasmid for four days. Cell extracts are analyzed for the intracellular HCV RNA levels using qRT-PCR, normalized with actin RNA content and arbitrarily set to 1 for cells transduced with shNT. Values represent mean ± SD from analysis of three experiments. P values < 0.001 (***) are indicated in comparison with shRNA NT treatment.

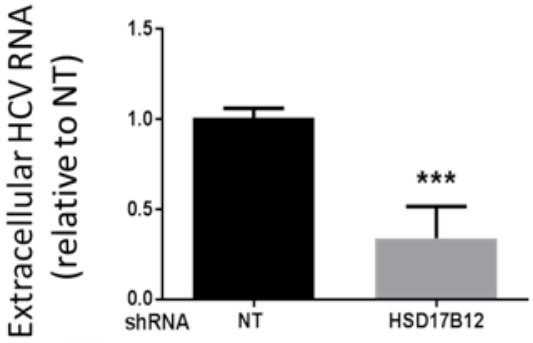

Figure 2.S4.

HSD17B12 KD decreases extracellular HCV RNA levels of HepG2 infected cells. HepG2 cells transduced with lentivirus-expressing shNT (NT) or shHSD17B12 (HSD17B12) are transfected with the JFH-1-expressing DNA plasmid for 4 days. Cell supernatants are analyzed for the extracellular HCV RNA levels using qRT-PCR and arbitrarily set to 1 for cells transduced with lentivirus expressing shNT. Values represent mean ± SD from the analysis of three experiments. P values < 0.001 (***) are indicated in comparison with shRNA NT treatment.

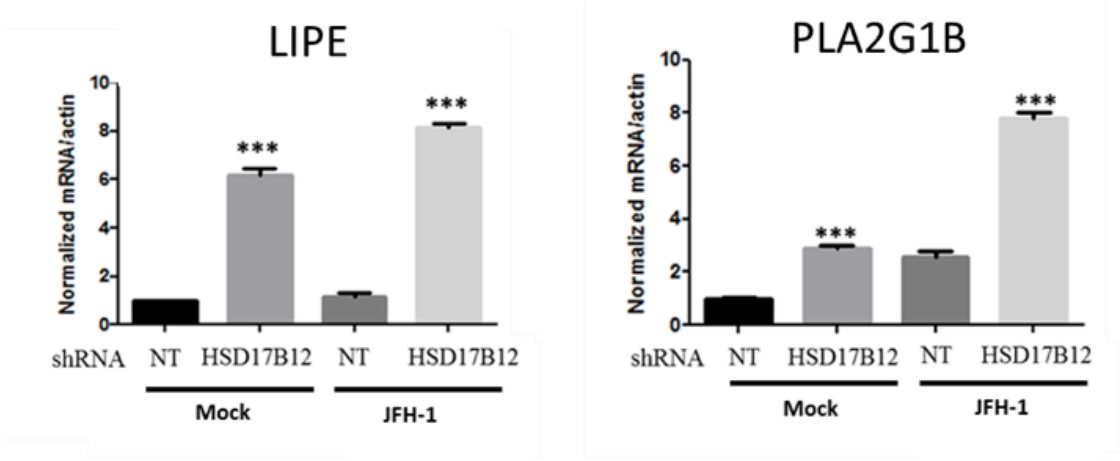

Figure 2.S5.

HSD17B12 KD increases expression of lipolysis genes. Huh7.5 parental cells transduced with lentivirus-expressing shNT (NT) and shHSD17B12 (HSD17B12), either uninfected (Mock) or infected with JFH-1 for four days are analyzed for mRNA levels of hormone sensitive lipase (LIPE) and phospholipase A2 (PLA2G1B) by RT-qPCR. Levels are normalized with actin RNA content and arbitrarily set to 1 for Mock NT cells. Values represent mean ± SD from the analysis of three experiments. P values < 0.001 (***) are indicated in comparison with shRNA NT or NT JFH-1 treatment.

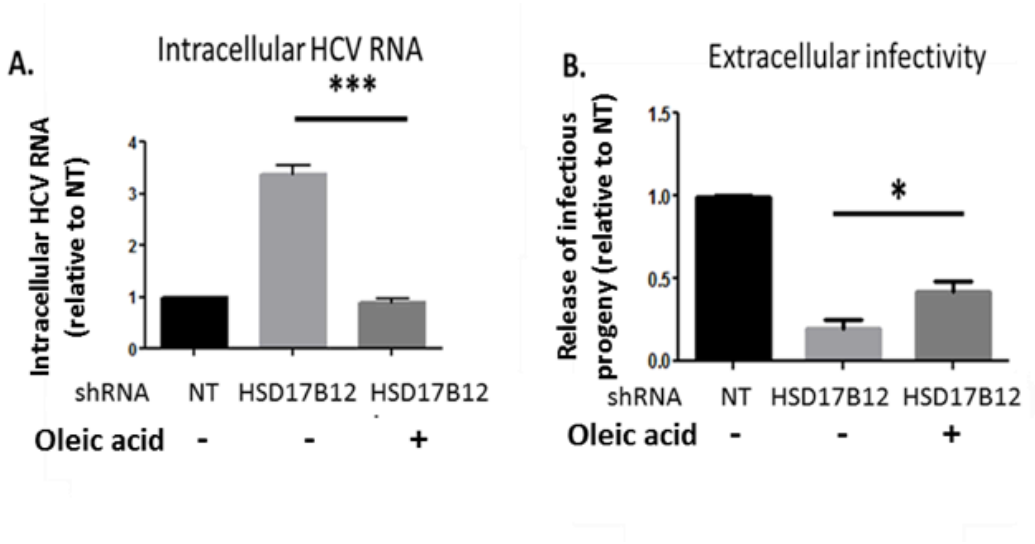

Figure 2.S6.

Oleic acid supplementation restores HCV RNA replication sites and rescues infectious particle production of HSD17B12 KD cells. Huh7.5 cells transduced with lentivirus-expressing shNT (NT) or shHSD17B12 (HSD17B12) and infected with JFH-1 are used to evaluate the rescued effects of BSA-oleic acid (20 μM) to the cell culture media on (A) intracellular HCV RNA levels and (B) extracellular infectivity of supernatants upon re-infection of naive Huh-7.5 cells. HCV RNA levels are normalized with actin RNA contents and arbitrarily set to 1 for cells transduced with shNT and infected with JFH-1. Values represent mean ± SD from the analysis of three experiments. P values, < 0.05 (*) or < 0.001 (***) are indicated in comparison with untreated shHSD17B12 transduced cells.

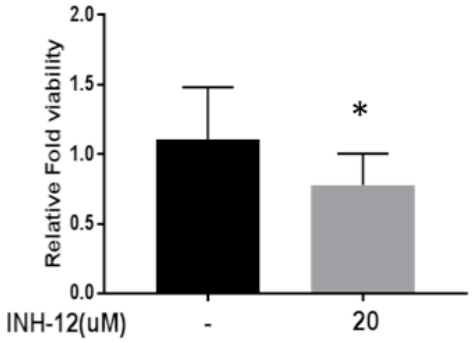

Figure 2.S7

HSD17B12 inhibitor INH-12 has minor effect on cell viability. The effects of small-molecule HSD17B12 inhibitor INH-12 on the cellular viability were determined in HCV Con1b replicon-containing Huh7 cells at 20 μM using an Alamar Blue assay. Minor effect is observed in comparison with DMSO control. Values represent mean ± SD from the analysis of at least two experiments. P value < 0.05 (*) is indicated in comparison with DMSO treatment.

Chapter 3

This chapter features the manuscript, currently under preparation, titled **"Requirement of VLCFA metabolic pathway for HCV replication."** By Bassim Mohamed and Daniel Lamarre.

The first author (Bassim Mohamed) performed all experiments, as well as the analysis of results and the making of all figures for this manuscript. The designing of experiments and the writing of the article were done by the first author, with valuable input, insight and revisions by Dr. Daniel Lamarre.

3.0. Requirement of VLCFA metabolic pathway for HCV replication

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3.1. ABSTRACT

Hepatitis C virus (HCV) as a member of the *Flaviviridae* family depends on lipids in every step of its replication cycle. However, some pathways of lipid metabolism have not being explored regarding a possible pro-viral role for HCV replication. The very-long-chain fatty acids (VLCFA) *de novo* synthesis pathway is an example. VLCFAs are significant constituents of cell lipids, which are used in the synthesis of complex lipids such as phospholipids, sphingolipids and triglycerides, as well as for energy production through beta-oxidation. HSD17B12 is a pivotal enzyme of this synthesis and reacts with several elongation enzymes (ELOVLs) to produce a diversity of VLCFA. In the present study, we showed that HSD17B12 knockdown (KD) inhibits HCV replication using the HCV Con1B subgenomic replicon model to similar levels than the inhibition of fatty acid synthase (FAS) enzyme. Oleic acid supplementation is able to partially restore HCV replication induced by HSD17B12 KD in this *in vitro* cell model. Furthermore, the metabolism of HSD17B12-mediated products, VLCFA, through sphingolipids synthesis and beta-oxidation processes was shown to be essential for HCV replication. The present work supports the requirement of HSD17B12 and VLCFA synthesis pathway in HCV replication and highlights potential areas for antiviral development.

3.2. INTRODUCTION

Host cell lipid metabolic pathways are commonly co-opted by multiple viruses of the *Flaviviridae* family and represented an attractive target for antiviral development. Several studies have highlighted that *Flaviviridae* infections are highly dependent on cellular fatty acid and lipid metabolism for their replication. Fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC) are two key enzymes in fatty acids synthesis that have been pursued as therapeutic targets against some member of *Flaviviridae* viruses [215, 224, 226]. However, it was shown that the most enriched lipids in *Flaviviridae* infected cells have very-long-chain fatty acids longer than 16 carbons (so-called VLCFA) [129, 144, 192, 229]. There are contradicting observations regarding VLCFA metabolism and its role in HCV replication. For example, fatty acid beta-oxidation was shown to be pro-viral in a report by Rasmussen *et al*. [257] but to have an antiviral activity in a report by Levy *et al*. [344]. Besides, the requirement of the oleic acid-synthesizing stearoyl-CoA desaturase enzyme 1 (SCD1) for HCV was also controversial. A report by Lyn *et al.,* [242] showed that inhibition of SCD1 reduces HCV replication while studies by Hofmann *et al*., [192] demonstrated that SCD1 knockdown does not have any effects on HCV replication. This inconsistency may be due to different in vitro culture models for HCV and pathway probing. Fatty acid synthesis up to 16 carbons occurs in the cytoplasm and involves key enzymes like FASN and ACC. However, fatty acids more than 16 carbons are elongated at the endoplasmic reticulum (ER) organelle [278]. HSD17B12 is a pivotal and hub enzyme in VLCFA elongation that in complex with other synthesizing enzymes of the pathway will lead to diverse elongation products such as stearic acid (18:0) (the precursor of oleic acid (18:1)), polyunsaturated γ-linolenic (18:3 n-6),

arachidonic (20:4 n-6) and eicosapentaenoic acid (20:5 n-3) [278]. VLCFA are essential in different aspects of cell biology. In the present study, commercially available small-molecule inhibitory compounds and RNA interference (RNAi) gene silencing approach are used to evaluate the requirement of HSD17B12 and *de novo* VLCFA synthesis for viral replication in HCV genotype 1B replicon (Con1B) containing cells. The study provides a broad picture of HCV replication dependency on the VLCFA elongation pathway.

3.3. RESULTS

3.3.1. Role of *de novo* VLCFA synthesis in HCV replication

FASN enzyme is a crucial enzyme for the synthesis of palmitic acid (C16), which is the substrate for the synthesis of VLCFA species (un-, mono- and poly-saturated) produced by the elongation machinery (ELOVLs, HSD17B12, HACDs, TER). Hopperton *et al.,* showed that 70-80% of labeled acetate, and the same for labeled palmitate, were incorporated in phospholipids synthesis in the tested cell lines [197], demonstrating that *de novo* fatty acid synthesis by FASN is required but not sufficient for the synthesis of cell phospholipids [197].

We first explored the effect of C75, a potent synthetic inhibitor of FASN, on HCV Con1B replicon **(Fig. 3.1)**. We showed that C75 inhibits HCV replication at treatment concentration of 30 μM for four days by monitoring luciferase activities and has minimal effect on cell viability. The inhibition demonstrates the requirement of fatty acid synthesis of 16 carbon chain (palmitic acid) but also supports the need of greater than 16 carbon (VLCFA) for HCV replication. Indeed, we showed that HSD17B12 KD has a comparable

level of HCV replication inhibition than C75 at 30 μM. Finally, the combination demonstrated an additive inhibitory effect (statistically significant by t test) supporting the requirement of *de novo* synthesis of palmitic acid and its elongation to VLCFA for HCV replication. **(Fig. 3.1)**

3.3.2. Role of ELOVLs in HCV replication

Fatty acid elongases (ELOVLs) are physically interacting with HSD17B12 [345], and elongate fatty acids chains by adding two carbon atoms in each VLCFA elongation cycle. The roles of the various elongases, however, have not been investigated in HCV infection. We showed that the KD of ELOVL 1, 3, 4, and 5 significantly inhibits replication of HCV subgenomic replicon and has no effects on cell viability suggesting pro-viral role for each individual ELOVL in HCV infection **(Fig. 3.2 A-B).** We further investigated the KD of ELOVL 1, 3, 4, and 5 in JFH-1-infected cells **(Fig. 3.2 C-D)**. We showed that the KD of ELOVL1 and ELOVL3 decrease HCV replication. ELOVL1 and 3 are involved in the VLCFA elongation of C18:0 to C24:0 and of C18:1 to C24:1, respectively. In contrast, the KD of ELOVL4 and ELOVL5, increases HCV replication. ELOVL4 and 5 are involved in the elongation of polyunsaturated of greater length (C18:2 to C34.6). The data suggest that different HCV models have different responses to ELOVL KD-mediated deprivation of distinctive VLCFA species.

3.3.3. Role of the synthesis of neutral lipids for HCV replication

We next investigated the synthesis of neutral lipids containing VLCFA for HCV replication. The various long-chain acyl-CoA synthase isoforms (ACSL) are a family of enzymes that catalyze the conversion of long and very-long-chain fatty acids to long and

very-long-chain acyl-CoA in the presence of ATP, CoA, and Magnesium. Long and very-long-chain acyl-CoA molecules serve as substrate for each step in the synthesis of neutral lipids including diglycerides, TGs and cholesterol ester [346]. The impact of competitive inhibitors of ACSL 1, 3, and 4 was first assessed by testing the pharmacological inhibitor Triacsin C [347]. ACSL3 is of particular importance as it was shown to be critical in the synthesis of neutral lipids of LDs [201]. This was further supported by the observation that addition of oleic acid to cells leads to an increase in LDs, which is manifested by a significant increase of LDs-ACSL3 association [348]. Inhibition of ACSL by Triacsin C is well documented to block the synthesis diglycerides, TGs, and cholesterol esters and to decrease LDs availability [322, 349]. We used a concentration of 4.5 μM Triacsin C. This concentration is in the same range of the concentrations reported to deplete LDs in Huh7 cells with no obvious effect on cell viability [348]. Our experiments demonstrated that the treatment with 2.5 to 4.5 μM of Triacsin C has a weak (26.7%) but significant inhibitory effect on HCV replication using the replicon model. As a control for cytotoxicity, we evaluated the effect of Triacsin C on Huh7 cells stably expressing the luciferase gene under EF1α promoter and showed no significant effects of the treatment concentrations that have been used **(Fig. 3.3).**

3.3.4. Role of *de novo* synthesis of sphingolipids for HCV replication

ELOVL6 and ELOVL1 are key enzymes of the VLCFA elongation cycle and essential for the synthesis of sphingolipids [350, 351]. We showed that ELOVL1 KD leads to inhibition of HCV replication (section 3.3.2). However, to determine the role of sphingolipids in HCV replication, we used the commercially available compound L-cycloserine as an irreversible inhibitor of 3-ketodihydrosphingosine synthase, which is

the first enzyme of the regulated sphingolipids synthesis pathway. L-cycloserine mainly affects sphingomyelin and ceramide synthesis [352]. HCV subgenomic replicon containing cells were treated with 250 μM and 500 μM L-cycloserine as used by Nieto *et al.,* [352]. After four days, HCV RNA replication was determined using luciferase activity. Alongside, cell viability was determined by an Alamar Blue assay. We showed that L-cycloserine inhibits HCV replicon reporter activity in a dose-dependent manner and did not affect cells survival **(Fig. 3.4)**. The data suggest that *de novo* synthesis of sphingomyelin and ceramides that incorporate VLCFA are essential for HCV genome replication.

3.3.5. Role of VLCFA (< C22) beta-oxidation

VLCFA are critical for energy production by providing the very-long acyl chain substrates for beta-oxidation. The effects of fatty acid beta-oxidation on HCV genome replication are controversial. Some reports demonstrated that VLCFA beta-oxidation is vital for virus replication [257], while others showed that it contributes to an antiviral effect [344]. We explored the effect of inhibiting carnitine palmitoyltransferase 1 (CPT1) with etomoxir as a potent inhibitor of beta-oxidation, which blocks the access of long and very-long fatty acids acyl-CoA chains (< C22) to the mitochondria leading to inhibition of mitochondrial beta-oxidation. We used concentrations reported in the study of Fernandes-Siqueira *et al.* [353] to achieve potent inhibition of mitochondrial beta-oxidation. We showed that treatment of HCV subgenomic replicon-containing cells with 400 μM etomoxir significantly decreased HCV replication. Cells viability was not affected as demonstrated by an Alamar Blue assay. The data suggest that the beta-

oxidation of VLCFA (< C22) contributes to the replication of the HCV in a sub-genomic replicon model **(Fig. 3.5)**.

3.3.6. Role of oleic acid synthesis

Oleic acid is the most abundant fatty acid in cell lipids. It contains 18 carbons and has one double bond in its acyl chain (C18.1). It has been shown that oleic acid and derived lipids are incorporated into cell membranes to modulate its biophysical properties [354]. Oleic acid can be *de novo* synthesized from stearic acid (C18.0) by the action of the Δ-9-desaturase (SCD1), which is the rate-limiting step in *the de novo* synthesis of monounsaturated fatty acids (MUFA), specifically from stearoyl- and palmitoyl-CoA to oleic acid and palmitoleic acid, respectively [238].

We tested the effects of the potent and selective SCD1 inhibitor CAY10566 on HCV Con1B replicon-containing cells at concentrations of 5 and 10 μM for 4 days in accordance to previous studies [355, 356]. We observed a significant inhibition of HCV replication **(Fig. 3.6)**, which is similar to the previously reported observations using two different inhibitors of SCD1 [242]. This inhibition was associated with defective membranous web formation [242], due to the requirement of SCD1 for mono-unsaturated fatty acids phospholipid synthesis [356], confirming the requirement of VLCFA in membranous web formation.

3.3.7. Rescue of HCV replication by addition of oleic acid to HSD17B12 KD cells

As the oleic acid precursor "stearic acid" is a significant product of VLCFA elongation [278] and oleic acid has an essential role for HCV replication, we investigated the rescue of HCV replication by addition of oleic acid exogenously to the culture

medium of HSD17B12 KD cells. We showed that treatment of cells harboring Con1b replicon with 10 μM of BSA-oleic acid partially rescued HCV replication as demonstrated by the increased reporter activity **(Fig. 3.7)**. The data suggest that HSD17B12 KD-mediated inhibition of HCV replication is partially through the inhibition of oleic acid synthesis, but also supports a role of HSD17B12 in the elongation of other VLCFA and derived lipid species that are required for HCV replication.

3.4. DISCUSSION

Several human viral pathogens are greatly dependent on cellular lipid metabolism. This suggests that one can be able to exploit these pathways for antiviral intervention. Host-targeted antivirals can increase the barriers to viral drug resistance. However, developing therapeutic strategies for viral infections will need a better understanding of the specific lipid species, their functions in the different viral life cycles and the impact of the reduced levels on host biology. The knowledge will be essential to avoid targeting pathways critical for host cell survival or enhancing the replication of some other viruses.

VLCFA are believed to play an essential role in the replication of HCV and related flaviviruses. In virus-infected cells, lipid species derived from VLCFA are significantly enriched in the membranes of virus replication compartments and of whole-cell extracts [129, 144, 192-194]. Therefore, inhibition of HSD17B12 metabolic capacity provides direct evidence of the requirement of *de novo* VLCFA synthesis in viral infections.

In our study, the inhibition of HCV replication in HSD17B12 depleted cells was comparable to the one achieved by FASN inhibition. Understanding the biology of FASN

that enables the synthesis of fatty acids up to 16 carbons in length and upstream to HSD17B12-mediated VLCFA synthesis, raise the question of whether FASN inhibition can deprive HSD17B12 elongation activity of an important substrate, and subsequently, limit the availability of VLCFA for HCV replication. We previously showed that HSD17B12 KD is associated with an increase in palmitic acid levels. However, the availability of palmitic acid during the absence of HSD17B12 catalytic activity could not promote HCV replication. Furthermore, HSD17B12 KD mediated HCV replication inhibition is partially restored by oleic acid treatment suggesting that the loss of catalytic capacity of HSD17B12 is responsible for the inhibition of HCV replication by limiting the availability of VLCFA such as oleic acid. From these data, we can conclude that FASN-mediated synthesis of palmitic acid is required for its pro-viral activity but not sufficient for HCV replication, which required the additional downstream HSD17B12-mediated VLCFA synthesis.

VLCFA includes diverse species for which the process of synthesis is regulated by different ELOVLs [277]. Our data suggest that HSD17B12 inhibition has a similar effect than the individual knockdown of ELOVL1, 3, 4 and 5 on HCV RNA replication. However, in the presence of a full virus replication cycle using the JFH-1 model, knockdown of ELOVL4 and 5 increase intracellular HCV RNA levels similarly to HSD17B12 KD cells. The different responses could be due to the distinctive genomic structures of the replicon and JFH-1. Specifically, JFH-1 codes for the proteins required for all the viral life cycle steps including viral RNA packaging and assembly, and this is not the case for Con1B replicons. Failure of JFH-1 viral RNA packaging may lead to intracellular viral RNA accumulation, and may this be the case when knocking down

ELOVL4 and 5, however, more experiments are needed to elucidate the specific mechanisms underlining this phenotype.

VLCFA are representing significant components in the synthesis TGs [200]. Our data demonstrated by treating the replicon with Triacsin C are similar to a report by Liefhebber *et al.* using the JFH-1 model [322]. They demonstrated that Triacsin C through inhibiting TGs synthesis and depleting LDs led to slightly reduced viral RNA synthesis, however, the compound significantly impaired assembly of infectious virions in infected cells [322]. The data highlight the significance of TGs synthesis and LDs for infectious virion particles production, but not of the same significance for the virus replication step.

Sphingomyelins (SM) are the most abundant sphingolipids in the cell. In most cells, the acyl moieties of these sphingolipids are of very-long-chain (C16–24) and often saturated [357]. Furthermore, SM play important role for HCV and beside cholesterol organize the solid membrane characterized as the DRM replication sites [358]. We inhibited SM synthesis by inhibiting 3-ketodihydrosphingosine synthase as was reported previously [352]. We observed a potent inhibitory effect on HCV replicon replication. Our data are in the same context with the observations by Umehara *et al.* [359], who tested myrocin, a serine palmitoyltransferase (SPT) inhibitor, against HCV using chimeric mice with humanized liver infected with HCV genotype 1a or 1b. SPT enzyme is another regulatory enzyme in sphingolipids synthesis. Myrocin treatment was able to reduce HCV RNA in the serum and liver. Further, when myrocin was combined with

PEG-IFN the HCV RNA levels were reduced to less than 1/1000 of the control levels [359].

VLCFA are essential substrates for beta-oxidation and represent a vital source of energy for the synthesis of viral proteins. We confirmed that etoxomir, a beta-oxidation inhibitor [353], inhibits HCV replication, supporting the study of Rasmussen *et al.* who demonstrated a similar reduction in HCV replication by inhibition of fatty acid beta-oxidation [257]. The observation supports the pro-viral role of fatty acids beta-oxidation in HCV replication. However, the inhibitor concentration used in our experiments (400 μM) is unusual high concentration although similar concentrations were reported in the literature [353, 360]. The high concentration of etomoxir can cause free radicles generation and leads to off-target biological effects [361]. Further, the resultant metabolic perturbation could not be detected by our cell viability assay. This should be considered as a limitation regarding interpreting the inhibitor effect on the replicon replication. Nevertheless, studies on livers from chronic infected patients showed a downregulation of fatty acids beta-oxidation [247, 248] and suggest that it reflected a defense strategy by the host cells to decrease virus replication. Therefore, these observations are supportive of a pro-viral role of VLCFA beta-oxidation.

Oleic acid is an intracellular VLCFA product of SCD1 enzyme activity, in addition to being imported from the extracellular nutritional sources. Our data with SCD1 inhibition support the study of Lyn *et al.* [242]. They showed that SCD1-mediated oleic acid synthesis is essential to the formation of a functional HCV replication membranous compartment. This is due to the ability of oleic acid to form membranous curvatures and to provide the required fluidity to membranes [241, 242]. Besides, HCV dependence on

the *de novo* synthesis of oleic acid signifies that the intracellular synthesis of oleic acid is more essential for HCV replication than extracellular nutritional sources. However, this preference could be different *in vivo* and the importance of oleic acid *de novo* synthesis pathway for HCV replication should be investigated with *in vivo* models.

Our study shows that intracellular lipid metabolic pathways that handle VLCFA such as oleic acid synthesis, sphingolipids synthesis, and beta-oxidation are essential for HCV replication. Hence, HSD17B12 catalytic activity may be involved in multiple aspects of HCV replication *in vitro*. For instance, HSD17B12 could be vital in supplying the stearic acid for oleic acid synthesis, thus, could be essential for HCV membranous web formation. Furthermore, HSD17B12 could be critical in providing the sphingolipids synthesis with very-long acyl chains, again in forming the detergent-resistant membranes for HCV replication. However, further studies are needed to elucidate the specific mechanisms that may govern HCV dependence on HSD17B12 catalytic activity.

In this study, we used commercially available inhibitors to probe several lipid metabolic pathways and relayed on the literature for the specificity and cytotoxicity of these compounds. This can be a limitation because as we did not characterize the compounds in depleted cell for the target enzyme using shRNA and validating the phenotype achieved by each inhibitor, providing more confidence regarding the results obtained with those inhibitors in our cell models for virus replication.

In conclusion, the present study, along with our group previous study (Mohamed *et al.* Scientific Reports in revision), for the first time provides evidence in support of the importance of HSD17B12-mediated VLCFA synthesis in replication of HCV and related flaviviruses. The viral dependence on VLCFA may open new opportunities for

developing antiviral agents targeting HSD17B12 and other VLCFA synthesis enzymes in a broad spectrum antiviral strategy against HCV, related flaviviruses and possibly others RNA viruses for which there are no approved treatment to date.

3.5. METHODS

3.5.1. The Con1B Replicon: Huh7 cells stably express the genotype 1b Con1 subgenomic replicon (Huh7-Con1B-Fluc) were used. This bicistronic replicon expresses the neomycin phosphotransferase and the Firefly luciferase through the HCV IRES, while NS3-NS5B polyprotein production is under the control of the encephalomyocarditis virus (EMCV) IRES [309]. The cells harboring the replicon were maintained in complete DMEM with 500 μg/ml G418 (Multicell). The medium contained 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 1% nonessential amino acids (all from Wisent).

3.5.2. Chemicals: Enzymes inhibitors (Triacsin, L-cycloserine, CAY10566, etomoxir) were purchased from Cayman chemical Inc., USA. Oleic acid and fat free bovine serum albumin (BSA) were purchased from Sigma-Aldrich Inc., USA.

3.5.3. Luciferase assays: For firefly luciferase (Fluc) assays, cells were washed once with PBS, then, one volume PBS and one volume of 2× luciferase buffer (100 mM Trizma acetate, 20 mM magnesium acetate, 2 mM EGTA, 1% Brij 58, 0.7% β-mercaptoethanol, 3.6 mM ATP, 45 μg/ml d-luciferin, pH 7.9) were added to the cells. After, cells were incubated for 15 min at room temperature in the dark.

3.5.4. Cytotoxicity assay: the Alamar Blue assay, cells were cultured in black 96-well plates. Ten microliters of Alamar Blue reagent (Invitrogen; diluted 1:4 in PBS) was

added to the cells (100µl media) and following a 3-h incubation at 37°C, fluorescence at 595 nm (excitation wavelength, 531 nm) was measured with an EnVision plate reader (PerkinElmer). A control plate with medium only (no cells) or Alamar Blue only was used to determine the background that was subtracted from the fluorescence value.

3.5.5. Lentivirus production: 293T cells were transfected with PEI by using plasmids pRSVREV, pMD2-VSVG, and pMDLg/ pRRE and shRNA-encoding plasmid pLKO.1-puro (non-target and HSD17B12 TRCN0000027145, (sequence: CCGGCCTGCCTTCTTGGATTTATTTCTCGAGAAATAAATCCAAGAAGGCAGGTTTTT), TRC 1 generation; Sigma-Aldrich), or pLKO.1-puro (ELOVL) as shown in **(Table 3.1.)**. Lentiviruses were titrated using Hela cells. For gene silencing, cells were transduced shRNA-expressing lentiviruses at a multiplicity of infection (MOI) ≥ 2.

ELOVL1	Milipore Sigma- Mission shRNA plasmid SHCLND-NM_016031 TRCN0000149553 Sequence: CCGGGATAAACTCTTCCGTGCATGTCTCGAGACATGCACGGAA GAGTTTATCTTTTTTG
ELOVL3	Milipore Sigma- Mission shRNA plasmid SHCLND-NM_152310 TRCN0000149584 Sequence: CCGGGAACTACATGAAGGAACGCAACTCGAGTTGCGTTCCTTC ATGTAGTTCTTTTTTG
ELOVL4	Milipore Sigma- Mission shRNA plasmid SHCLND-NM_022726 TRCN0000011405 Sequence: CCGGGCCTATGCAATCAGCTTCATACTCGAGTATGAAGCTGAT TGCATAGGCTTTTT
ELOVL5	Milipore Sigma- Mission shRNA plasmid SHCLND-NM_021814 TRCN0000151740 Sequence: CCGGCACATTTATCTGCTCTGTCATCTCGAGATGACAGAGCAG ATAAATGTGTTTTTTG

Table 3.1. The table is showing the sequences of ELOVLs shRNA, which were used in the study.

3.5.6. HCV infection assay: Huh7.5 cells (transduced with lentivirus preparation three days before) were transfected with plasmid pEF/JFH-1-Rz/Neo by using JetPRIME (Polyplus-transfection, Invitrogen) (2 μg plasmid DNA/5*10^5 cells), and cell media were replaced after 4 h. At 4 days post-transfection, cells were collected.

3.5.7. qRT-PCR: Cells RNA was extracted using RNeasy Plus Kit. DNase treatment, reverse transcription, and real-time PCRs were performed at the IRIC Genomic Core

Facility with TaqMan-based assays. For HCV RNA detection, we used primers CATGGCGTTAGTATGAGTGTCG and GGTTCCGCAGACCACTATG and TaqMan labeled probe CAGCCTCC (probe 75; no. 04688988001 from the Roche Universal Probe Library). Relative viral RNA and genes mRNA levels were calculated using the 2 -$^{\Delta\Delta}$CT method using ACTIN as an internal control, and plotted as fold change by normalizing to control samples

3.5.8. Statistical analysis: The statistical analysis was performed with the GraphPad Prism 7 Software. One-way ANOVA with Tukey's multiple comparisons test and Student t test (two-tailed and non-parametric), were used. P values of <0.05 were considered significant.

3.6. ACKNOWLEDGEMENTS

We thank Jake Liang for pEF/JFH1-Rz/Neo plasmid; Takaji Wakita (National Institute of Infectious Diseases, Tokyo), Charles Rice (Rockefeller University) and Apath LLC for the use of JFH-1 and Huh7.5 cells; Ralf Bartenschlager (University of Heidelberg) for luciferase-encoding Con1b subgenomic replicon, and IRIC's Genomics Core Facility for real-time PCR. This work was supported by an operating grant from the Canadian Institutes of Health Research to DL and by A PhD scholarship to BM from National Research Centre of Egypt, and Egyptian Government of Higher Education and Scientific Research.

3.7. ADDITIONAL INFORMATION

Competing financial interest: The authors declare no competing financial interests.

3.8. FIGURES

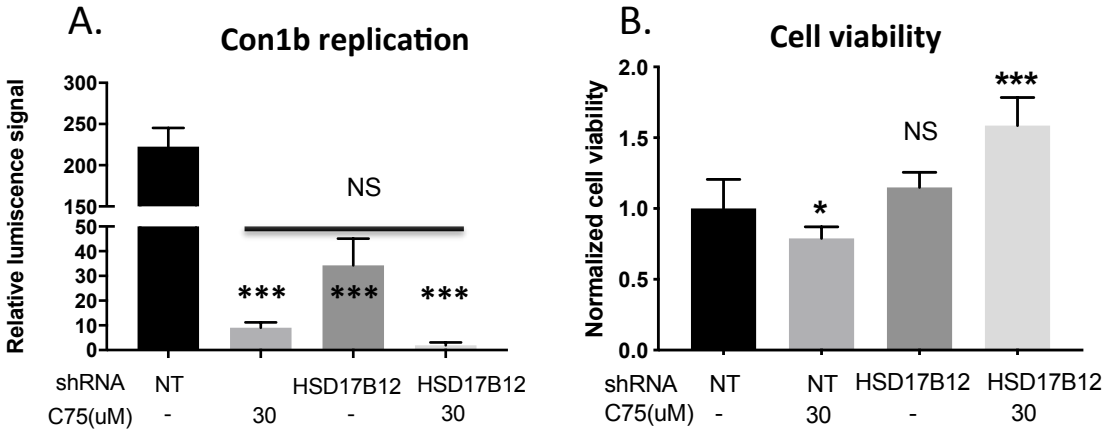

Figure 3.1. Effects of FASN inhibitor and HSD17B12 KD on HCV Con1B replication.

The effects of pharmacological inhibition of fatty acid synthase (FASN) using C75 at 30 μM for 4 days and shHSD17B12 transduction and combination on HCV replication-dependent luciferase activity. As a control, a non-target shRNA (NT) was used. B) Cell viability of different treatments was determined using an Almar Blue assay. Data are representing (mean± SD) of 2 experiments including 8 biological replicates. One-way ANOVA with Tukey's multiple comparisons test were used. P values <0.05 (*), <0.001 (***), and non-significant (NS) are indicated.

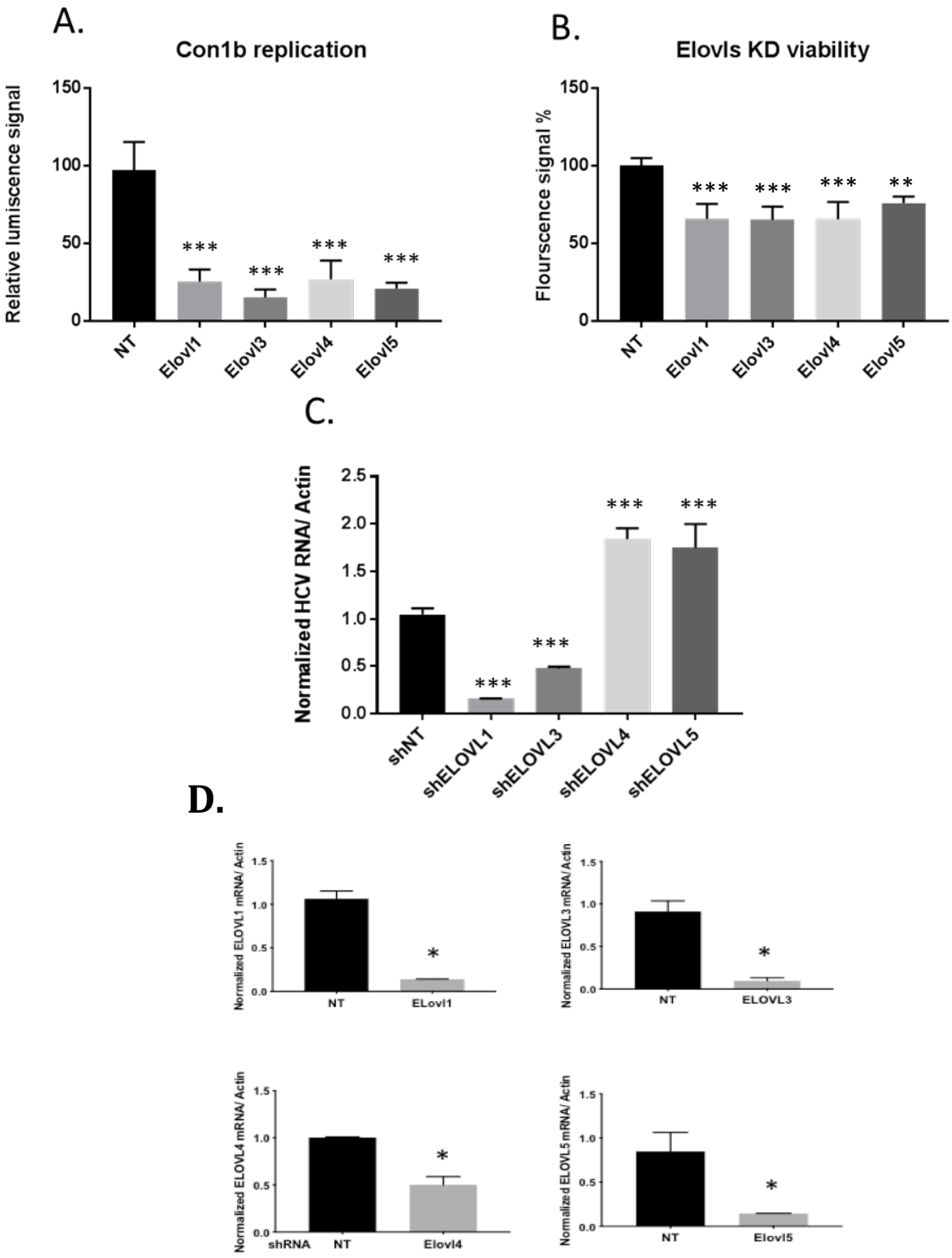

Figure 3.2. Effects of depleting different ELOVLs on HCV RNA replication.

Knockdown effects of shRNA-expressing lentiviruses specific to ELOVl-1, -3, -4, -5 and non-target shRNA (NT) were determined in A) Con1B replicon cells at day 3 post-transduction (MOI of 10) (reporter luminescence as a readout), and C) at day 7 post-

transduction (MOI of 10) 4 days of JFH-1 infection in Huh7.5 cells (qPCR as readout). B) Cell viability of different manipulations was determined using an Almar Blue assay. Data are representing 3 experiments (mean± SD). One-way ANOVA with Tukey's multiple comparisons test were used. P values <0.01(**) <0.001 (***) are indicated in comparison with shNT treatment. D) ELOVLs 1,3,4, and 5 KD efficiency for two biological replicates in Huh7.5. Student t test was used (non-parametric, two tailed). The statistical significance is indicated by (*).

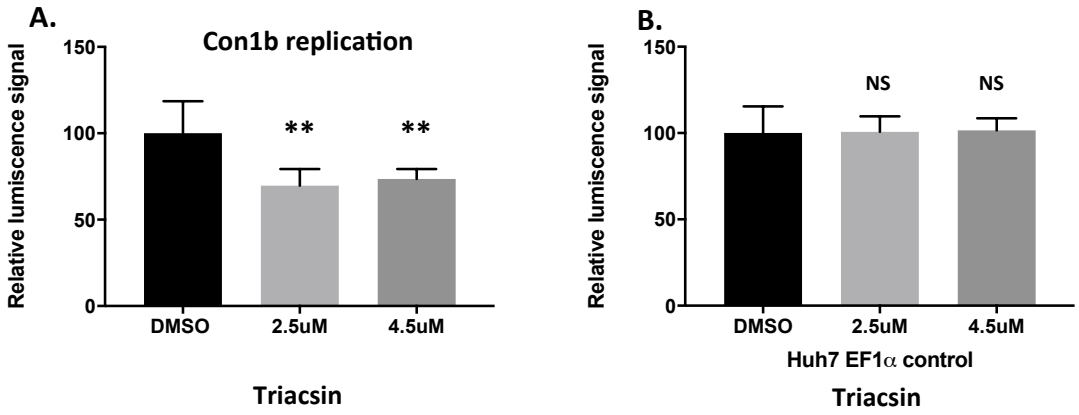

Figure 3.3. Effects of ASCLs inhibition on HCV Con1B replication.

A) The effects of pharmacological inhibition of acyl CoA synthase (ACSL) using Triacsin at 2.5 and 4.5 μM for 4 days on HCV replication-dependent luciferase activity. As a control, DMSO treated cells were used. B) Cells expressing luciferase under control of EF1α promoter were used to determine cells viability. Data are representing 3 experiments (mean± SD). One-way ANOVA with Tukey's multiple comparisons test were used. P values <0.01 (**) and non-significant (NS) are indicated in comparison with DMSO treatment.

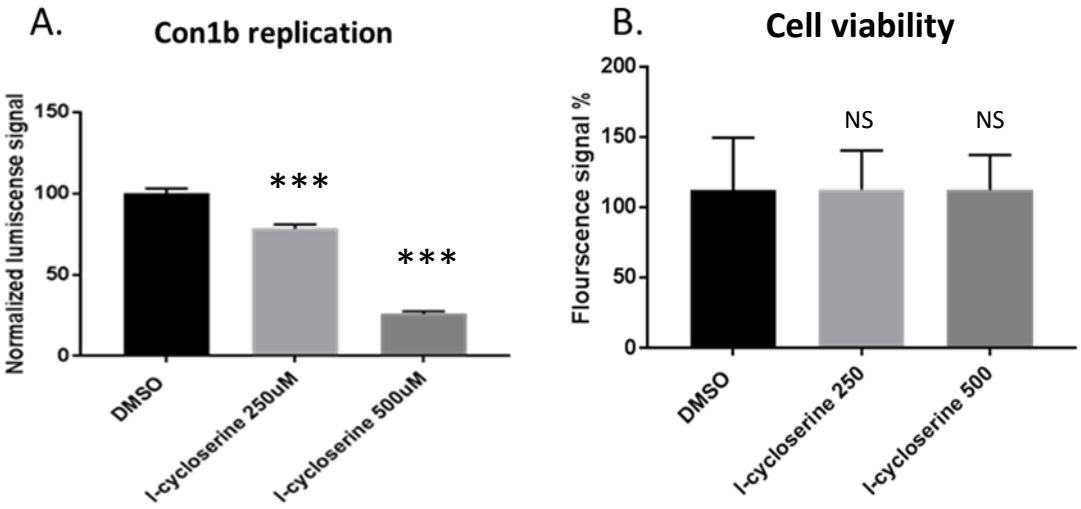

Figure 3.4. Effects of 3-ketodihydrosphingosine inhibition on HCV Con1B replication.

A) The effects of pharmacological inhibition of 3-ketodihydrosphingosine using L-cycloserine at 250 and 500 µM for 4 days on HCV replication-dependent luciferase activity. As a control, DMSO treated cells were used. B) Cell viability of different treatments was determined using Almar Blue. Data are representing 3 experiments (mean± SD). One-way ANOVA with Tukey's multiple comparisons test were used. P values <0.001 (***) and non-significant (NS) are indicated in comparison with DMSO treatment.

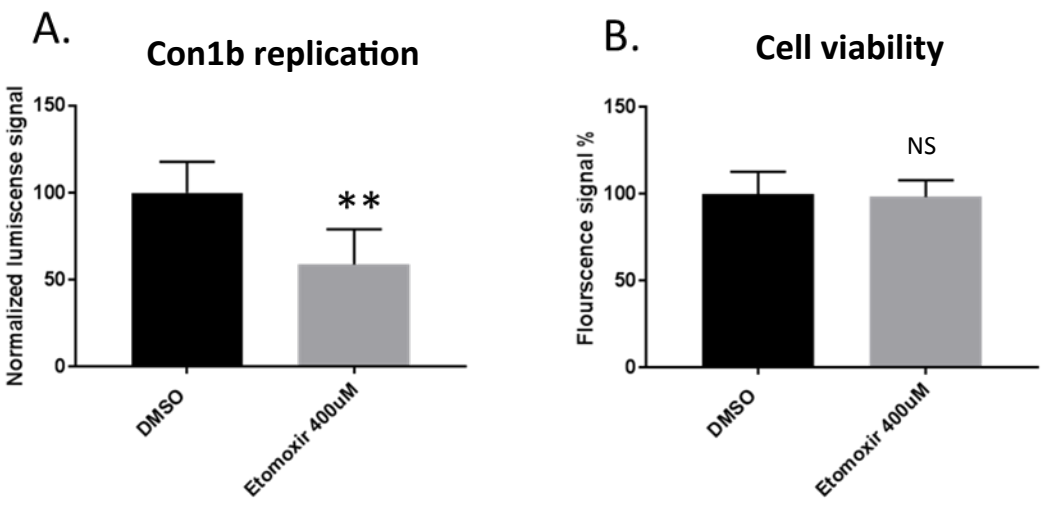

Figure 3.5. Effects of CPT1 inhibition on HCV Con1B replication.

A) The effects of pharmacological inhibition of carnitine palmitoyl acyltransferase-1 (CPT1) using etomoxir at 400 µM for 4 days on HCV replication-dependent luciferase activity. As a control, DMSO-treated cells were used. B) Cell viability of the treatment was determined using an Almar Blue assay. Data are representing (mean± SD) of 2 experiments including 8 biological replicates. Student t test (two-tailed and non-parametric) was used. P values <0.01 (**) and non-significant (NS) are indicated in comparison with DMSO treatment.

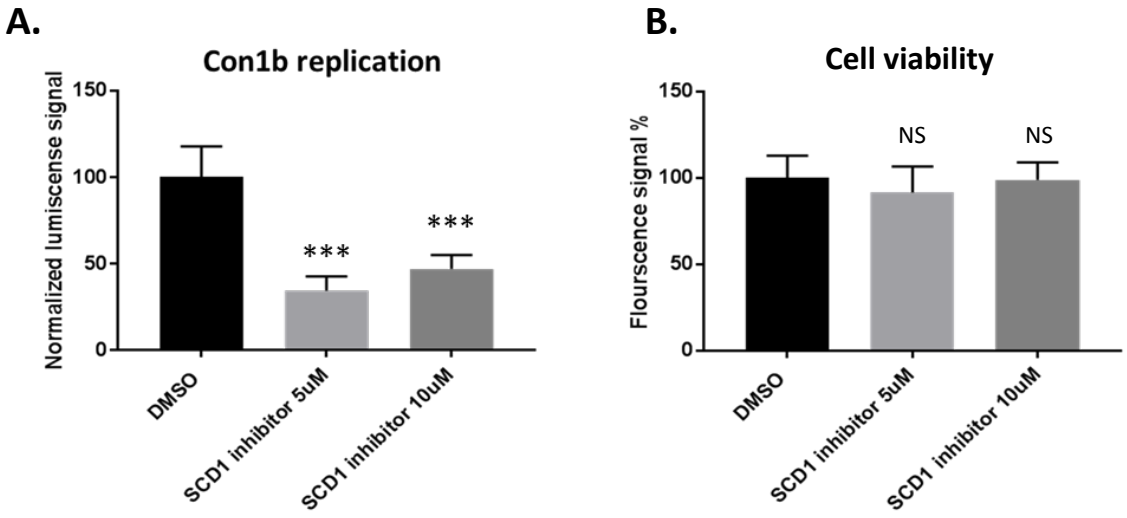

Figure 3.6. Effects of SCD1 inhibition on HCV Con1B genome replication.

A) The effects of pharmacological inhibition of stearoyl CoA dehydrogenase-1 (SCD1) using CAY10566 at 5 and 10 μM for 4 days on HCV replication-dependent luciferase activity. As a control, DMSO-treated cells were used. B) Cell viability of different treatments was determined using an Almar Blue assay. Data are representing 3 experiments (mean± SD). One-way ANOVA with Tukey's multiple comparisons test were used. P values <0.001 (***) and non-significant (NS) are indicated in comparison with DMSO treatment.

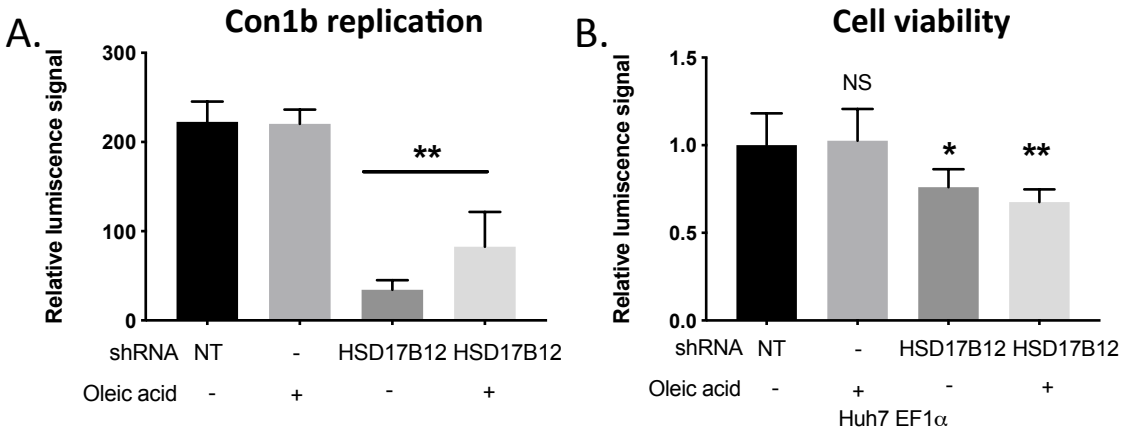

Figure 3.7. Effects of oleic acid treatment on HSD17B12 KD-mediated decrease of HCV Con1B replication.

A) Rescue experiments of HCV replication were performed in HSD17B12 KD cells by addition of BSA-oleic acids (10 μM) to the cell culture media using replicon-dependent Luc signal as readout. B) Cell viability of different treatments was determined using an Almar Blue assay. Data are representing (mean± SD) of 2 experiments including 8 biological replicates. One-way ANOVA with Tukey's multiple comparisons test were used. P values <0.01 (**) are indicated in the comparison between shHSD17B12 and shHSD17B12+ oleic acid in figure A. P values <0.05 (*), and non-significant (NS) are indicated in cell survival in comparison to NT/BSA treatment in figure B.

Chapter 4. Discussion

4.1. Elucidating the contribution of HSD17B12 and VLCFA in *Flaviviridae* virus replication

The synthesis of fatty acids has long been recognized as a requisite for the replication of a wide range of viruses including HCV, DENV, YFV, WNV, and JEV. Moreover, antiviral effects have been described with the inhibitors of ACC and FASN [187, 190, 224, 317]. However, the requirement for the elongation of fatty acids to very-long-chain fatty acids (VLCFA) is a poorly understood aspect of *Flaviviridae* biology.

We demonstrated, for the first time, that the gene silencing of HSD17B12 is reducing the expression of viral proteins and inhibiting the replication of three *Flaviviridae* viruses. Accordingly, our studies showed that the elongation of fatty acids to VLCFA, which necessitate the enzymatic activity of HSD17B12 [318], is required for HCV, DENV and ZIKV replication and particle production.

Moreover, we provided substantial evidence that the antiviral effect achieved by the gene silencing of HSD17B12 was due to the inhibition of its enzymatic activity and not off-target effects. For this purpose, we performed multiple integrated experiments including (i) the testing of HSD17B12 KD on selected cellular lipid species and levels, (ii) comparing the antiviral effect with the precursor shorter fatty acid synthesis, (iii) probing the antiviral activity of HSD17B12 pharmacological inhibitor, (iv) evaluating the KD of related VLCFA synthesis enzymes such as ELOVLs, (v) characterizing HSD17B12 KD on the integrity and function of the HCV membranous replication site, and (vi) lipid droplets (LDs) as assembly sites. Consequently, from these studies, we established the pro-viral requirement of HSD17B12 enzymatic activity for the replication of HCV and related flaviviruses, which will be discussed in the following sections.

4.2. A role of HSD17B12 in the transition of HCV RNA genome from replication to assembly sites

The transition between virus replication and assembly needs an efficient and controlled viral RNA genome trafficking to LDs to be integrated in the encapsidation process. Moreover, the process requires a separation of the HCV core from the multi-enzymes replication complex to avoid competition for the HCV RNA binding [72, 312]. Accordingly, in resolving this problem, the HCV core is sequestrated and accumulated at LDs. Furthermore, NS5A is essential for HCV assembly and localizes with LDs [362]. The co-localization of NS5A and the core promotes the transfer of HCV RNA from replication to nucleocapsid assembly sites [363].

As a result, the promotion of HCV RNA transition from replication to assembly requires a high regulation of membrane phospholipid components in the interface between the HCV membranous web and LDs [364]. For example, the presence of phospholipids will provide the required fluidity and desired curvatures of the membrane-associated HCV RNA replication sites. Our previous study showed that the HCV core physically interacts with HSD17B12 [308]. We now showed that HSD17B12 overlaps HCV core cytoplasmic distribution, supporting the presence of the enzyme at core trafficking sites. HSD17B12 might contribute to the process of ER-derived membrane phospholipids regulation through the synthesis of VLCFA products as phospholipids precursors to these subcellular viral replication compartments. Hence, HSD17B12 has the ability to modify the membranous web and LDs associated to different cytoplasmic RNA virus life cycles.

4.2.1. HSD17B12 contributes to HCV-induced membranous replication sites

Our data draw attention to the link between HSD17B12-mediated VLCFA synthesis and the biology of HCV replication sites (membranous web) as the intracellular levels of lipids are critical factors in virus replication. Indeed, many single-stranded positive RNA viruses, including those of the *Flaviviridae* family, depend on the enrichment of specific and newly synthesized phospholipids at virus-induced membranous replication sites. The specific lipid enrichment not only contributes to the formation of viral RNA replication sites, but also provides protection from the sensing of viral RNA by the pathogen recognition receptor (PRR) of the antiviral response [192, 323, 324, 365, 366]. Recent studies describe a cellular increased level of phospholipids harboring C18:1 acyl chains and polyunsaturated fatty acids upon HCV infection [192]. Electron microscopy imaging studies further revealed that HCV-induced membrane rearrangements are predominantly protrusions of the ER [121]. Remarkably, the half-live of phospholipids at ER membranes is relatively short (60 hours) [367]. Hence, *de novo* synthesis of VLCFA is undoubtedly needed to replenish the phospholipids at the ER components [368]. Thus, the maintenance of HCV specialized membranes (membranous web) must necessitate the HSD17B12 enzymatic activity in close proximity to replication sites to continuously replenish its lipid components.

The membrane curvatures are vital to HCV replication membranous web formation [123] and involved the presence of oleic acid [241, 242]. Indeed, many studies reported the capacity of oleic acid to provide exclusive membranous characteristics to support viral RNA replication [241, 242, 330, 369, 370]. Based on the biology of membrane curving, there is a common way for membrane layers to adopt a curved

geometry. The curves occur by the asymmetric organization of the phospholipids of different shapes and physical features [370, 371 , 372]. Phosphatidylethanolamine (PE) has a smaller head group and a larger hydrophobic tail, and most probably induces negative membrane curvature when tightly packed with phosphatidylcholine (PC), which has a cylindrical geometric shape with a tendency to form a planar bilayer [371-373]. The induction of membrane curvatures also requires modification in membrane fluidity (how loose the packed membrane phospholipids are), which is governed by the structure of unsaturated fatty acyl chains (e.g. oleic acid) of its phospholipids [372, 373]. In accordance with the foregoing, our data demonstrate that the activity of HSD17B12 contributes to the supply of activated acyl chains (acyl-CoA) of greater than 16 carbons, which are requisite to the induction of membrane curvatures at HCV replication compartments, namely oleic acid and phosphatidylethanolamine (PE) [121, 374, 375].

The studies of Lyn *et al.* and Nguyen *et al.* [241, 242] further support our data and showed that blocking *de novo* synthesis of oleic acid by small-molecule SCD1 inhibitor and SCD1 gene silencing inhibits HCV RNA replication and viral particle production. Moreover, the effects are associated with defective DMVs (membranous web) synthesis, as revealed by electron microscope imaging [241, 242]. More recently, Hofmann *et al*. [192] showed that the inhibition of the polyunsaturated fatty acids (PUFA) synthetic pathway via the gene silencing of the rate-limiting Δ6-desaturase enzyme FADS2 (that desaturates palmitic acid and oleic to PUFA of equivalent carbon lengths), or by treatment with a small-molecule inhibitor, impaired HCV viral particles production [192]. Therefore, altogether, our data strongly support a role of HSD17B12 and production of VLCFA species at HCV replication sites, and possibly other related flaviviruses.

HCV also interacts with host membrane proteins to maintain replication site curvatures. These proteins include karyopherins and nucleoporins [123, 376]. The role of membrane proteins in membrane curvatures may include wedging themselves between the phospholipid layers [369, 377]. Additionally, membrane proteins can interact with the large surface of the facial side of the membrane and facilitate the induction of membrane curvature [378]. Interestingly, the combination of membrane lipids re-organization and of protein-induced curvature has been reported in several flaviviruses life cycles to establish the virus-induced replication specialized membranes [121]. For instance, DENV requires the activity of SCD1 to produce oleic acid to support the curvatures of its membranous compartment [194]. Furthermore, DENV NS4A protein, through the insertion of it predicted transmembrane segment 2 (pTMS 2) into the luminal leaflet of the ER membrane may act as a wedge resulting in a curvature toward the cytosol [379].

The structure of HCV-induced membranous web is enriched in PI(4)P molecules (contains C18:0 and C20:4 VLCFA tails) that require VLCFA synthesizing activity. Our study showed a co-localization between HSD17B12 and dsRNA at HCV replication sites. Hence, the proposed link between VLCFA synthesis and PI(4)P synthesis can give more insight into the dependence of the HCV replication compartment on HSD17B12 catalytic activity. Additional studies are required to elucidate the involvement of the VLCFA synthesis pathway for PI(4)P enrichment and PI(4)P-dependent recruitment of effector proteins (e.g., oxysterol binding protein (OSBP)) at replication sites, as well as for the requisite enrichment of important lipids such as cholesterol for membranous web integrity. Electron microscopy imaging of HSD17B12 KD cells could help to confirm

membrane alterations by the direct visualization of viral replication sites upon infection with HCV and related flaviviruses.

4.2.2. A role of HSD17B12 in LD biology and HCV assembly

HCV disturbs LD homeostasis by inhibiting LD lipolysis and increasing its stability [364]. This biology establishes a cellular microenvironment that is more favorable to viral infection. LD organelles have a unique structure with one layer of phospholipids covering a core of neutral lipids mainly formed of TGs and cholesterol esters (CEs). The composition of the LD phospholipid monolayer critically regulates their consumption and morphology [380]. The phospholipid monolayer is associated with LD-associated proteins such as adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and the family of PAT-domain proteins (perilipin, adipophilin, and TIP47). The ER hosts the enzymes needed for neutral lipid synthesis (TGs and cholesterol ester (CE)). From the ER membrane, the neutral lipid synthesis mediates LDs to undergo a series of well-organized processes and grow into larger and mature LDs [381]. Consequently, the growing of LDs requires very-long-chain acyl-CoA molecules, which are from a nutritional source or *de novo* synthesized [200, 381]. Hence, the deficiency in incorporating VLCFA, especially oleic acid, negatively affects the ability of TGs synthesis. In our study, we provided strong evidence that depleting HSD17B12 leads to the direct depletion of LD organelles by reduction of lipid species abundances such as oleic acid, TG and PE and/or by stimulation of hemostatic mechanisms including increased lipolysis gene expressions of HSL and PLA2. Accordingly, the increase in the expression of lipolysis genes is expected to lead to the digestion of lipid stores such as LDs and accessory lipid compartments. Importantly, the digestion of the lipid stores may

lead to the release of unbalanced amounts of free lipids intracellularly. For instance, the uncontrolled liberation of fatty acids species may distort and deform the HCV specialized membranes and the interface between the membranous web (MW) and LDs [225, 382]. Hence, inhibition of HSD17B12 activity induces a metabolic environment that disfavors LDs maintenance that promotes HCV infection. Moreover, the decreased oleic acid levels upon HSD17B12 inhibition may impact the triglycerides synthesis enzyme diacylglycerol O-acyltransferase 1 (DGAT1) as oleic acid is the main substrate for DGAT1 [383]. Two independent studies showed the significance of DGAT1 for HCV particle assembly. For instance, Herker *et al.* demonstrated that DGAT1 is required for the trafficking of the HCV capsid protein core onto the surface of LDs [144]. Another independent study showed that DGAT1 is critical for NS5A trafficking regarding LDs [384]. In addition, the NS5A trafficking needs an active DGAT1 enzyme as demonstrated by the ectopic expression of a catalytically inactive mutant of DGAT1 (H426A) that led to a blocking of NS5A recruitment to LDs, but not of core, and impaired the release of infectious viral particles [384]. Our observation that the inhibition of HSD17B12 catalytic activity leads to the deficiency of oleic acid underscores the potential inability of DGAT1 to in the trafficking of NS5A to the LDs for proper HCV particle assembly. More investigations are required to demonstrate an essential role of HSD17B12 catalytic activity for NS5A trafficking to LDs.

Interestingly, LDs also link the HSD17B12 catalytic activity to the very low-density lipoprotein synthesis [385] and to the HCV post-assembly particle secretion process [158]. VLDL synthesis occurs in two different stages. In the first stage, TGs are transferred from LDs by microsomal triglyceride transfer protein (MTP) to

apolipoprotein B (ApoB), which is critical for the integrity of the VLDL structure [386]. If sufficient source of TGs is not available, ApoB is ubiquitinated and degraded [387]. In the second stage, VLDL precursors containing ApoB fuse with ER/Golgi luminal triglycerides droplets [388] and is facilitated by apolipoprotein E (ApoE) [389]. Moreover, MTP transfers additional TG from LD to the growing VLDL particle at the luminal compartment. This activity promotes VLDL assembly and secretion [388]. Therefore, MTP is crucial for the proper secretion of VLDL, and genetic defects in the MTP gene severely reduce VLDL secretion [390, 391]. It has been shown that HCV secretion depends on the biology of VLDL assembly throughout the utilization of LDs triglycerides stores [158]. The study of Huang *et al.* [158] showed the significance of this process. When using the potent BMS-2101038 inhibitor of MTP involved in triglyceride transport from LDs to VLDL, HCV secretion is impeded by 80% but the inhibitor does not affect intracellular HCV RNA [158]. The study of Gastaminza *et al.* [392] took advantage of the accumulation of HCV infectious particle intracellular by treatment of cells with brefeldin A (BFA) (inhibits protein transport from ER to Golgi) in a manner reflecting the rate of production of HCV infectious particles. Furthermore, the authors studied the accumulation of intracellular infective particles in the presence of MTP inhibitor in cells treated with BFA. They demonstrated that the infectious particles accumulated only in BFA treated cells but not in cells treated with the combination of BFA and MTP inhibitor. They concluded that MTP inhibition impairs infectious maturation and secretion [392]. Altogether, these data demonstrate that the synthesis of VLDL from LDs is essential for HCV release. As the triglyceride components of LDs are mainly of VLCFA, especially oleic acid (C18:1) and stearic acid (C18:0) [200], it

strongly supports an important contribution of HSD17B12 catalytic activity for HCV post-assembly release. More studies are required to confirm a direct role of HSD17B12 on VLDL formation and HCV release, and to demonstrate the blockage of viral secretion with HSD17B12 inhibition.

4.3. Mode of action of HSD17B12 for the replication of flaviviruses

Previous studies showed a unified requirement of fatty acids upstream to the synthesis of VLCFA for replication of HCV and related flaviviruses. In this context, FASN and ACC are two key enzymes that have been explored for a broad-spectrum antiviral approach against flaviviruses [215, 224-226, 228, 393]. A recent study by Gullberg *et al.* [194] further demonstrated the essential requirement of SCD1 activity for DENV replication and infectivity. The study provided evidence of SCD1-mediated oleic acid synthesis in establishing replication compartments in tight coordination with sites of virion assembly. However, no studies have directly investigated host factors required for VLCFA synthesis that are critical to the replication of flaviviruses. The inhibitory effect of HSD17B12 depletion on HCV, DENV and ZIKV infection suggests similarity between HCV and flaviviruses in their requirement for establishing membranes rearrangement to (i) harbor their genome replication and (ii) coordinate the replication, assembly and release steps. Indeed, it has been demonstrated that VLCFA-containing lipid species are enriched upon flaviviruses infection [193, 394] and are involved in virus-induced membrane rearrangement [229]. The membrane rearrangement includes the formation of vesicles ~90 nm in diameter defined as vesicles packets (VPs) and formed by ER membrane layers invaginations. As such, the VLCFA pathway must be involved in VP formation. VPs have the same function as the DMVs of HCV replication

factories, as they are the sites of virus replication harboring the replicase components and dsRNA replication intermediates [395]. Electron microscope studies could help to confirm a direct role of HSD17B12 on DENV and ZIKV specialized membranes.

The critical requirement of HSD17B12 to maintain the availability of LDs strongly supports a pro-viral role and represents a potential mechanism for the requirement of HSD17B12 activity in the life cycle of flaviviruses. DENV decreases the total LDs of infected cells due to the consumption of these organelles via lipophagy [396]. DENV uses the TGs reservoir of LD to release free fatty acids by activation of the lipophagy pathway. The increase in free fatty acid generates more ATP via cellular beta-oxidation and promotes viral replication [396]. Similarly, the impairment of cellular lipophagy decreases DENV replication [397]. ZIKV also shares its dependence on LDs. The two viruses exploit AUP1, a type-III membrane protein with dual localization signals for LDs and ER organelles [398]. AUP1 triggers LDs lipophagy, leading to the release of fatty acids for phospholipids synthesis and energy production. The $AUP1^{-/-}$ cells are resistant to ZIKV and DENV infection and virion production [399]. The LD homeostasis and requirement of a constant VLCFA synthesis for DENV and ZIKV replication are in strong support to the KD of HSD17B12 in mediating LDs depletion as a mechanism that is associated with inhibition of virus replication. LDs play a critical role in DENV assembly as suggested by evidence that DENV C (capsid) protein needs to associate with LDs [313]. The physical contact between DENV C protein and LD leads to the exposure of cationic facing the aqueous environment [275]. Moreover, a peptide drug spanning residues 14–23 from the C protein (pep14–23) was shown to inhibit C protein interaction with LDs [400], leading to the inhibition of viral morphogenesis [400]. This data also

support HSD17B12-mediated LDs depletion as one of the mechanism for inhibition of DENV particle production. Although LD is mainly composed of VLCFA-derived neutral lipids, our study is the first that highlights the link of HSD17B12 catalytic activity to the aforementioned LDs manipulation upon DENV and ZIKV replication. In the same context, it was reported that FASN is mobilized to LD in flaviviruses infected cells [401], suggesting that building up fatty acid is concurrent with lipophagy, emphasizing the availability of the palmitic acid substrate for HSD17B12-mediated VLCFA synthesis. Hence, HSD17B12 inhibition alone or in combination with FASN inhibition represents a promising targeting antiviral approach for the potent inhibition of flavivirus replication.

4.4. Broad-Spectrum Antiviral activity of specific HSD17B12 inhibitors

4.4.1. The pharmacological inhibition of HSD17B12 impedes replication of HCV and flaviviruses

Our study indicated that the inhibitor INH-12, which has a greater specificity for HSD17B12 than for other HSD17B enzymes [339], has a potent inhibitory effect on HCV RNA replication and viral protein levels. Furthermore, it showed a potent inhibitory effect on DENV and ZIKV replication, viral protein accumulation and virus particle production. This represents a novel example of the inhibition of enzymes acting on the *de novo* VLCFA synthesis, namely oleic acid synthesis. We showed that HSD17B12 inhibition reduced the synthesis of oleic acid levels (Chap 2), similarly to SCD1 inhibition [242], which correlates with the inhibitory effects on HCV replication and assembly. As evidence of the specificity of INH12, in addition to the direct and expected effect on oleic acid reduction, the concentrations range of the inhibitor that led to HCV

inhibition are in the same range of concentrations required for the *in vitro* enzymatic inhibition [339, 340].

4.4.2. HSD17B12 and FASN inhibition has comparable antiviral effects

The antiviral mechanism of FASN inhibition is not completely defined. Indeed, FASN has been reported to support palmitoylation activity [221]. Palmitoylation is the covalent attachment of palmitate (C16:0) to cysteine residues via a thioester bond [402]. The primary function of protein palmitoylation is to enhance membrane affinity, allowing the modified protein to interact with cellular membranes [402]. Besides, protein palmitoylation plays an essential role in protein stability [403]. The palmitoylation activity of FASN has been shown to promote the replication of several viruses [404-406]. Particularly the palmitoylation of HCV core and NS4B was shown to influence the efficiency of HCV replication and assembly [87, 141]. Accordingly, FASN inhibition hinders HCV RNA replication and protein accumulation [225], and involves the disturbance in viral proteins palmitoylation as shown with other viruses [404]. HSD17B12 KD hinders the viral replication in the presence of an increased abundance of palmitic acid levels, arguing that *de novo* VLCFA synthesis inhibition exerts a dominant antiviral activity downstream to the various FASN activities and bypassing FASN-mediated palmitic acid synthesis and its palmitoylation activity. The viral inhibition in the presence of increased palmitic acid levels suggests that FASN modulates HCV replication through two aspects: (i) providing palmitic acid for protein palmitoylation and (ii) providing palmitic acid as a precursor for further *de novo* VLCFA synthesis as proposed in the model depicted in **Fig. 4.1.**

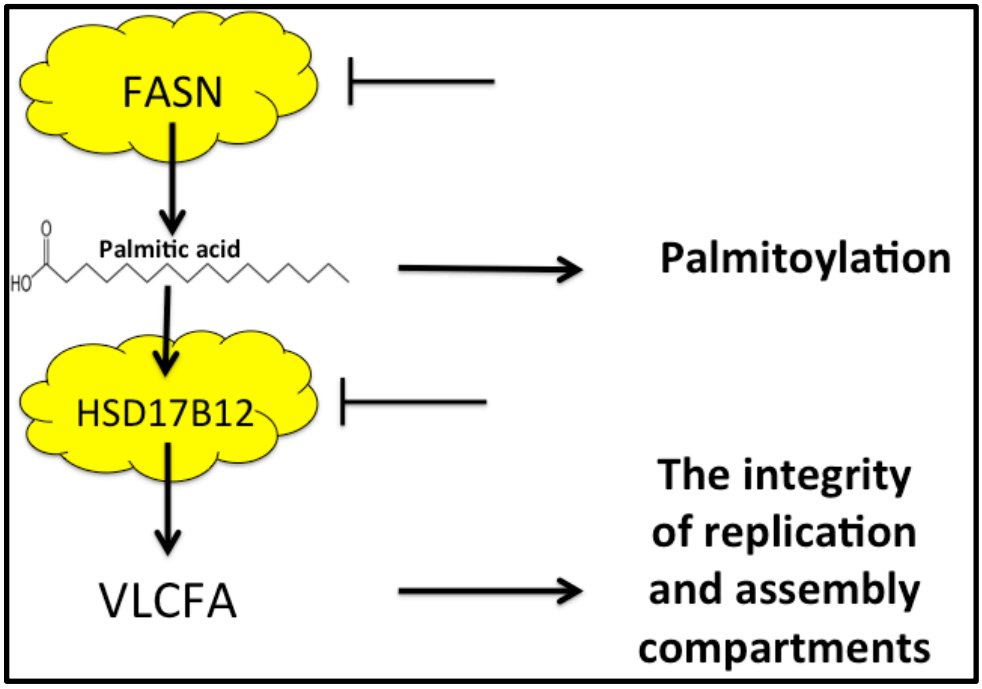

Figure 4.1. FASN pathway provides HSD17B12 catalysis with palmitic acid for elongation.

Inhibition of FASN (pharmacological or genetic knockdown) leads to downstream deprivation of palmitic acid, which is a major precursor for the *de novo* synthesis of VLCFA.

4.5. HSD17B12 targeting as a broad-spectrum antiviral approach

The understanding of the regulation of host lipid pathways controlling replication of HCV and flaviviruses would enable the tailored design of host-targeted antiviral molecules directed against VLCFA metabolic enzymes with safety margin. Several approaches have been used to discover antivirals that depend on small-molecule high throughput screening (HTS) against phenotypic viral replication assay (not target based). Furthermore, a major obstacle that has emerged from antiviral drug discovery with antiviral targets is the emergence of drug resistance. This phenomenon favor the targeting of host proteins that are dependence factors for viral replication and that are less critical for cell biology. From the present study, we explored the VLCFA synthesis pathway for promising targets against flaviviruses replication. Our results suggest that it would be essential for antiviral design to focus on the key enzymes of the pathway and specific lipid moieties that can generate effective antiviral responses by depriving the virus of the replication membranous compartments (membranous web and lipid droplets). In addition, combining inhibitors of several host enzymes that target the synthesis of these specific VLCFA and derived lipids that hinder the stability of the virus replication compartments may maximize the success of therapy to eliminate the virus and to reduce cytotoxicity effects. Moreover, such combination is expected to inhibit the virus from acquiring the ability to manipulate the patient's immune response. Such combination should also cover the most effective VLCFA species in inhibiting a broad spectrum of flaviviruses from ensuring the achievement of the maximum response in infected patients.

4.6. Future perspective

In order to gain more insight of targeting HSD17B12 activity as an antiviral approach against flaviviruses, we propose to further validate the requirement of HSD17B12 activity for DENV and ZIKV replication using a gene knockout (KO) approach. The CRISPR-Cas9 technology will be applied to human cell lines, primary hepatocytes, and monocyte-derived macrophages (MDM) [407]. In order to evaluate the level of dependency of virus replication on HSD17B12 expression, we plan to implement a CRISPR interference (CRISPRi) methodology, in which a doxycycline-inducible deactivated Cas9 is fused to a KRAB repression domain, which specifically, reversibly and dose-dependently inhibit gene expression [408] **(Fig. 4.2)**. If cell viability of HSD17B12 KO cells is occurring, the CRISPRi technology will allow evaluating the balance between the minimal gene depletion required for maximal antiviral potency in the absence of cytotoxicity.

In addition, we plan to characterize compounds of the HSD17B12 inhibitor series in collaboration with Dr. Poirier from the University of Laval. An important control is the testing of compounds with similar structure that have no inhibitory effects on purified HSD17B12 enzyme using *in vitro* enzymatic assay that correlate with the absence of antiviral activity. Complete dose-response curves will be performed with INH-12 to provide antiviral potency against ZIKV, and DENV and cytotoxicity. More direct investigations using LC-MS/MS analysis of INH-12 treated cells will unambiguously identify VLCFA species and derived lipids that are reduced, and correlates with significant decrease of DENV and ZIKV infectious particle production. These studies will validate the direct inhibition of HSD17B12 in controlling *Flaviviridae* infection *in*

vitro, and provide strong support to initiate *in vivo* pre-clinical studies with animal models.

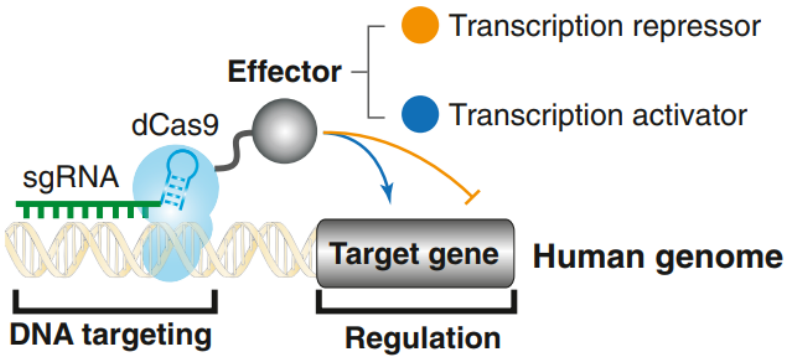

Figure 4.2. A modular RNA-guided genome regulation.

The fusion of catalytically inactive Cas9 protein (dCas9) to effector domains with specific regulatory characteristics enables stable and efficient transcriptional activation or repression in human cells. Furthermore, A co-expressed short guide single-guide (sg) RNA solely determines the site of delivery. The fusion of dCas9 to a transcriptional repressor domain can robustly silence the expression of multiple endogenous genes. Adapted with permission from (Gilbert, L.A., et al., CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell, 2013. **154**(2): p. 442-51.)[409].

We plan to evaluate targeting multiple VLCFA synthesis enzymes in a combinational approach. A good rationale is the testing of HSD17B12 inhibitor in combination with ELOVL6 inhibitor that block the elongation reaction from palmitate (C16:0) to stearic acid (C18:0) [410, 411]. Furthermore, we could combine various levels of HSD17B12, ELOVL6 and stearoyl-CoA desaturase 1 (SCD1) inhibitors, which completely block the synthesis of oleic acid (C18:1) [242]. These combination studies are

unprecedented in literature and can provide pharmacological evidence for a safer combination approach based on synergistic inhibition and partial blockage of selected VLCFA metabolizing enzymes that control *Flaviviridae* infection but at levels of enzyme inhibition for which there is an acceptable or no appreciable target-based toxicity.

The dose-dependent antiviral effects of INH-12 or of any inhibitor combination identified previously would be tested in a pilot *in vivo* study. INH-12 and newly synthesized compounds from the same series and optimized for antiviral potency and adequate pharmacokinetic properties and safety in animal studies would be tested for their efficacy in established DENV- and ZIKV- infected AG129 and Ifnar1$^{-/-}$ mice model [412, 413].

4.7. Potential limitations

All experiments were performed on Huh7.5 cell lines that have different lipid metabolism compared to primary cells. Indeed, cell lines are more dependent on *de novo* fatty acid synthesis to meet the need for expanding their biomass and continuous cell division. Such metabolic difference may alter the homeostatic events of HSD17B12 KD in cell lines compared to primary cells or *in vivo* studies. Therefore, this highlights the importance of using primary cells for *in vitro* viral infections. Importantly, as factors regulating cellular lipid metabolism *in vivo* are more complicated than *in vitro* conditions, *in vivo* validation studies are required.

The JFH-1 strain is unique among the HCV strains and makes it not necessarily representative of HCV biology [414]. Furthermore, different HCV strains have different interactions with cell lipids. For example, a study by Weng *et al.* [415] demonstrated that sphingomyelin (SM) binds and enhances the template binding activity of genotype (1b) RNA-dependent RNA polymerase (RdRp). However, this effect varied according to HCV genotypes. For instance, SM did not activate J6CF (2a) RdRp. Further, SM binds to RdRps of genotype 1a and 2a (JFH1) but did not activate them [415]. In another example, the HCV of genotype 3 induces the expression of lipid *de novo* synthesis genes and contributes to the development of hepatic steatosis more aggressively than other genotypes [50]. While the distinction in how HCV genotypes interact with lipids should be considered when we generalize the conclusions of the present study, the fact that we showed antiviral effects for genetically distinctive viruses of the flaviviruses tempered the potential differences within HCV genotypes.

Finally, we used a ribozyme-based JFH-1 infectious system (DNA plasmid) developed by Kato *et al.* [321]. Two independent studies from our lab have validated this infectious system [309, 338]. Accordingly, the validation studies showed that the transcription from the transfected JFH-1 DNA plasmid generates low viral RNA (vRNA) levels and vRNA increases as a function of time due viral replication and propagation by reinfection (and is blocked by the HCV protease inhibitor BILN2061 validated in human) [309, 338]. As control, cells were transfected with the replication-defective mutant JFH-1 GND (containing a point mutation in the HCV polymerase NS5B), which indicated the basal levels of HCV RNA (plasmid-dependent) detected at day one to six, upon the lack of RNA polymerase activity [309]. The data revealed that the plasmid-dependent viral RNA (vRNA) transcription minimally contributes to the observed levels of intracellular vRNA, especially at four days post-transfection (intact JFH-1 plasmid has 35-fold vRNA more than the one with defective polymerase (GND)). Moreover, the vRNA in the supernatant was quantified, and the supernatant of cells transfected with JFH-1 plasmids was used to infect naïve cells, also the resultant infectivity was successfully assessed by qPCR. These validation studies confirm that using the JFH-1 infectious DNA plasmid allow to monitor HCV vRNA replication and viral infectivity in order to evaluate the effects of proteins KD and newly discovered host-targeted antivirals.

DENV replicates predominantly in human cells of the innate immune system including monocytes, macrophages, immature dendritic cells and mature dendritic cells [416-421]. On the other hand, human studies and animal models (mice and non-human primates) have detected ZIKV in placenta [422] and neuronal cell types including neural progenitor cells and mature neurons and astrocytes [423], ocular tissues including the

cornea, neurosensory retina and optic nerve, as well as the aqueous humor of the anterior chamber [424], and cells of the reproductive tract including testis [425] and the vaginal epithelium and the uterus [426]. These target cells of DENV and ZIKV replication have different lipid metabolism requirement in comparison to Huh7.5 hepatoma cells that was used in our experiments. The difference in metabolism should be identified and taken in consideration regarding the *in vivo* role of HSD17B12 and its targeting to block DENV and ZIKV replication.

We also used commercially available inhibitors to probe several lipid metabolic pathways and relayed on the literature for the specificity and cytotoxicity of these compounds. This can be a limitation because as we did not characterize the compounds in depleted cell for the target enzyme using shRNA and validating the phenotype achieved by each inhibitor, providing more confidence regarding the results obtained with those inhibitors in our cell models for virus replication.

4.8. Conclusion

This project was based on the discovery of the HCV core interacting protein HSD17B12 that plays a pro-viral role in HCV replication. The interaction led us to focus on studying the importance and mechanisms of HSD17B12-mediated VLCFA synthesis for HCV replication. We then investigated the significance of the requirement of HSD17B12 to the replication of related flaviviruses ZIKV and DENV. We provided *in vitro* evidence that the modulation of HSD17B12 catalytic activity has potential broad-spectrum antiviral effects against *Flaviviridae* infections.

Our mechanistic studies demonstrated that HSD17B12 contributes to the formation of functional viral replication compartments and to the homeostasis of LDs that are essential as viral assembly platform. Moreover, the lipidomic shotgun analysis of total extracts of HSD17B12 KD cells directly showed a decreased abundance of essential VLCFA-containing PE required for specific membrane characteristics that promote virus replication. Additionally, we observed metabolic conditions that disfavor availability of LD required to promote virus assembly including (i) decreased TG abundance and (ii) increased expression of lipolytic enzyme hormone-sensitive lipase (HSL). Altogether, our studies support the fact that HSD17B12 contributes to the flux of *de novo* VLCFA synthesis in proximity to subcellular compartments that are required for promoting replication and assembly/release of flaviviruses.

The research in this thesis has provided novel evidence for the critical requirement of *de novo* synthesis of VLCFA to *Flaviviridae* infection by demonstrating i) HSD17B12 KD cells can not form proper replication or assembly lipid compartments, and have a deteriorated viral proteins accumulation ii) the inability to produce HCV viral

particles by the use of HSD17B12 KD iii) hindering DENV and ZIKV replication and viral particles production by the use of HSD17B12 KD and inhibitor. These results are a valuable stepping-stone in understanding the dynamics of VLCFA synthesis for *Flaviviridae* infections, as well as a starting point to study the effects of targeting HSD17B12 host enzyme and other of the VLCFA synthesis pathway to block flaviviral infections with the long-term goal of discovering novel pan-viral therapeutics.

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