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Reticulon-3 Modulates the Incorporation of Replication Competent Hepatitis C Virus Molecules for Release Via Exosomes

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RÉSUMÉ

Nombreuses études soutiennent l'importance des voies exosomales dans l'établissement de la persistance virale chez l'hôte. Cependant, les principaux régulateurs et mécanismes moléculaires par lesquels les virus interviennent dans la composition et la libération des exosomes restent inconnus. Dans notre étude, nous avons observé une augmentation significative de l'expression des isoformes courtes et longues de Reticulon-3 (RTN3S&L), une protéine résidente du réticulum endoplasmique (ER), était associée à une infection chronique par le Virus de l'Hépatite C (VHC) sur les cellules Huh7. Ainsi, la microscopie confocale et l'analyse RNA-ChIP ont révélé que RTN3S&L co-localisaient et interagissaient avec l'ARN viral et NS3. En plus, le knockdown du RTN3 dans les cellules au réplicon de pleine longueur du VHC était associé à une réduction significative de la sécrétion des exosomes independamment de la réplication virale. De plus, des exosomes sécrétés par les cellulles au réplicon de pleine longueur du VHC étaient moins infectieux lorsqu'ils étaient co-cultivés avec les cellules naïves. De manière remarquable, l'expression de RTN3L n'a augmenté que lorsque le mutant en c-terminal de RTN3S a été surexprimé, suggérant une surexpression compensatoire possible de RTN3L avec ce mutant. Finalement, le sofosbuvir, un inhibiteur du NS5B du VHC, peut significativement réduire l'expression de RTN3L&S induite par le VHC et atténuer la libération des exosomes infectieux. En conclusion, nos résultats suggèrent que RTN3 pourrait être un nouveau régulateur spécifique de la composition et de la libération des exosomes au moins dans l'infection par le VHC.

Mots-clés : Exosomes, VHC, RTN3, Réticulum endoplasmique

ABSTRACT

Evidence supports the importance of exosomal pathways in the establishment of viral persistence in the host. However, little is known about the key regulators and molecular mechanisms through which viruses mediate the selective sorting of viral and host molecules for loading and release via exosomes. In our study, we observed by western blotting analysis a significantly increased expression of both short and long isoforms of Reticulon-3 (RTN3S&L), an endoplasmic reticulum (ER) resident protein, during chronic Hepatitis C virus (HCV) infection of Huh7 cells. Confocal microscopy and RNA-ChIP analysis revealed that RTN3S&L co-localized and interacted with dsHCV RNA and HCV NS3. Subsequently, we revealed that RTN3 knockdown in HCV full-length (FL) replicon cells were associated with a reduction in the release of infectious exosome independent of viral replication. Further, exosomes from RTN3 knockdown HCV FL replicon cells were less infectious when co-cultured with naïve. Strikingly, RTN3L expression only increased when C-terminal deleted RTN3S mutant was overexpressed, suggests a possible compensatory overexpression of RTN3L with the c-terminal deletion of RTN3S. Moreover, we found that Sofosbuvir, an HCV NS4B inhibitor, could significantly reduce HCV induced RTN3L&S expression and also attenuated cell released infectious exosomes. Taken together, our results suggest that RTN3 might be a novel specific regulator of exosome loadings and release at least in HCV infection.

Keywords: Exosomes, HCV, RTN3, Endoplasmic Reticulum

SOMMAIRE RÉCAPITULATIF

Un nombre croissant d'études a montré que presque tous les virus profitent de la communication exosomale pour favoriser la propagation virale et entraver les réponses antivirales de l'hôte. La raison ultime pour exploiter les voies exosomales semble être un moyen d'établir une persistance virale chez l'hôte. De plus, ce qui reste frappant, c'est la composition moléculaire des exosomes impliqués dans l'infection virale et la persistance de la maladie est très différente de celle de l'état physiologique. Compte tenu de ces observations, nous supposons que les molécules impliquées dans la physiopathologie ne sont pas chargées au hasard à l'intérieur des exosomes. Cependant, les principaux régulateurs et mécanismes moléculaires par lesquels les virus interviennent dans le tri sélectif de ces molécules virales et de l'hôte pour le chargement et la libération dans les exosomes restent inconnus. Décrypter en détail ce processus de la génération d'exosomes infectieux est crucial pour développer de nouveaux traitements et mesures préventives pour certaines maladies chroniques associées aux infections virales, par exemple les maladies du foie liées à l'infection par le virus de l'hépatite C (VHC).

Les réticulons (RTNs), y compris RTN1-4, sont une famille de protéines conservées au cours de l'évolution et se trouvent principalement dans le réticulum endoplasmique (ER). L'ER est un site contenant un nombre abondant des ARNs viraux du VHC, qui interviennent activement dans la réplication du virus. En plus, les rôles que jouent ces réticulons dans la formation des vésicules ont été déjà montrés. Dans notre étude, on s'intéresse particulièrement à la protéine RTN3, puisque RTN3 est impliqué non seulement dans la génération des vésicules endosomales, mais aussi dans l'autophagie, sachant que les voies autophagique et exosomales sont reliées. De ce fait, l'objectif de notre étude est de déterminer le rôle de RTN3, dans la modulation de molécules spécifiques (cellulaire et virale) pour le chargement et la libération des exosomes lors de l'infection par le VHC.

Nous avons émis l'hypothèse que différentes isoformes de RTN3 seraient impliquées dans la modulation du chargement des molécules spécifique à l'intérieur des exosomes et/ou de la sécrétion des exosomes.

Dans notre étude, nous avons purifié et analysé les exososmes issus de cellules naïves, de cellules infectées par JFH1 (génotype 2a du VHC) et de cellules au réplicon de pleine longueur du VHC (FL-Réplicon). Une augmentation significative de l'expression de isoformes courtes et longues de RTN3 (RTN3S&L) dans les cellules infectées par JFH1 et dans les cellules FL-

réplicon du VHC par rapport à cellules naïves, a été observée. En plus, cet accroissement de l'expression de RTN3 était également observé dans les échantillons cliniques du foie et du sérum provenant des patients infectés par le VHC par rapport à des sujets sains. La microscopie confocale et l'analyse RNA-ChIP ont révélé que RTN3S&Lco-localisaient et interagissaient avec l'ARN et NS3 du VHC. Le knockdown (KD) de RTN3 médié par CRISPR/Cas9 ainsi que la surexpression de RTN3 et ses mutants dans les cellules infectées par VHC n'ont pas affecté l'expression de la protéine NS3 du VHC, laissant suggérer que KD et la surexpression de RTN3 n'ont pas d'impact sur la réplication du virus. De plus, le nombre des exosomes libérés par les cellules FL-réplicon du VHC était considérablement augmentés lorsque RTN3 étaient surexprimés, tandis que la sécrétion des exosomes était significativement diminuée lors d'une sous-expression de RTN3. En outre, l'expression de RTN3L n'a augmenté que dans les cellules où il y avait une surexpression du mutant ΔC36-RTN3S, suggérant une possible compensation fonctionnelle de RTN3L avec ce mutant de RTN3S portant une délétion en c-terminale. Par la suite, des expériences de co-culture a révélé que les exosomes secrétés par les cellules dans lesquelles il y avait une sous-expression de RTN3 étaient moins infectieux, suggérant que la capacité de la transmission de l'ARN viral médié par ces exsosomes était significativement réduite. Finalement, nous avons également constaté que le traitement par Sofosbuvir, un inhibiteur de la polymérase NS5B du VHC, réduisait de manière significative l'expression de RTN3 dans les cellules infectées par JFH1 et dans les cellules FL-réplicon du VHC. Ainsi, Sofosbuvir est capable de diminuer la sécrétion des exosomes infectieux induits par l'infection de VHC.

Pour conclure, RTN3 pourrait être un nouveau modulateur qui intervient dans la régulation de la composition et de la sécrétion des exosomes, au moins dans le contexte d'infection par le VHC. De ce fait, notre travail non seulement apporte une meilleure compréhension sur le mécanisme impliqué dans la régulation de la composition et de la libération des exosomes, mais aussi ouvre des pistes pour développer des nouvelles stratégies préventives et curatives contre les infections virales.

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LIST OF ABBREVIATIONS

- Ago2: Argonaute 2
- APC: Antigen Presenting Cell
- APP: Amyloid Precursor Protein
- Aβ: β-amyloid peptides
- BACE1: β-site Amyloid precursor protein Cleaving Enzyme 1
- BMV: Brome Mosaic Virus
- CCN2: Connective tissue growth factor 2)
- CNS: Central Nervous System
- DAA: Direct-Acting Antiviral
- DC: Dendritic Cell
- DENZ: Dengue Virus
- DNA: Deoxyribonucleic acid
- EIA: Enzyme ImmunoAssay
- EM: Electronic Microscopy
- ER: Endoplasmic Reticulum
- ESCRT: Exosomal Sorting Complexe Required for Transport
- EV: Extracellular vesicles
- EV71: EnteroVirus 71
- GDF: GDP displacement factor
- GDF: GDP Displacement Factor
- GT: GenoType
- HCC: Hepatocellular Carcinoma
- HCV: Hepatitis C Virus
- HIV: Human Immunodeficiency Virus
- HSC: Hepatic Stellate Cell
- HSP90: Heat Shock Protein 90
- ILV: Intra-Luminal Vesicle
- IncRNAs: long non-coding RNA
- IRES: Internal Ribosomal Entry Site
- LDL: Low-Density Lipoprotein
- LVP: LipoViral Particle
- MHC: Major Histocompatibility Complex

- miR: Micro RNA
- MVB: Multi-Vesicular Body
- NI: Nucleotide Inhibitors
- NS: Non-Structural
- nSMase: neutral SphingoMyelinase
- NTA: Nanoparticle Tracking Analysis
- NTR: Non-Translate Region
- ORF: Open Reading Frame
- PBMC: Peripheral Blood Mononuclear Cells
- PC: PhosphatidylCholine
- pDC: plasmacytoid dendritic cells
- PE: PhosphatidylEthanolamine
- PM: Plasma Membrane
- RHD: Reticulon Homology Domain
- RIBA: Recombinant Immunoblot Assay
- RIG-I: Retinoic acid-inducible Gene-I
- RISC: RNA-induced silencing complex
- RNA: RiboNucleic acid
- RT-PCR: Reverse Transcription Polymerase Chain Reaction
- RTN: Reticulon
- SNAP: Soluble NSF Attachment Protein
- SNARE: SNAP REceptor
- SR-B1: Scavenger Receptor B1
- TLR: Toll-Like Receptor
- VLDL: Very Low-Density Lipoprotein
- WHO: World Health Organization
- WNV: West Nile Virus
- ZIKV: Zika Virus

1 LITERATURE REVIEW

CHAPTER 1: PRESENT-DAY HEPATITIS C

1.1 Epidemiology

Hepatitis C Virus (HCV) infection is a major cause of liver cancer which represents the second leading cause of cancer mortality worldwide (Jefferies et al., 2018). According to the World Health Organization (WHO), there are an estimated 71 million individuals with active chronic HCV infection globally(Jefferies et al., 2018).

Although HCV infection is a global issue, its prevalence varies widely across countries and within regions (Figure 1.1). The most recent global estimates indicate that the viremic prevalence of HCV infection is <1.0% in most developed countries, including the United States, Canada, Australia, and most of the European countries. The prevalence is considerably higher in some countries in Eastern Europe (3.3% in Russia), certain countries in Africa (6.3% in Egypt, 7.0% in Gabon), the Middle East (3.0% in Syria), and some in Asia (3.8% in Pakistan, 6.4% in Mongolia) (Resat Ozaras and Hakan Leblebicioglu,2017).

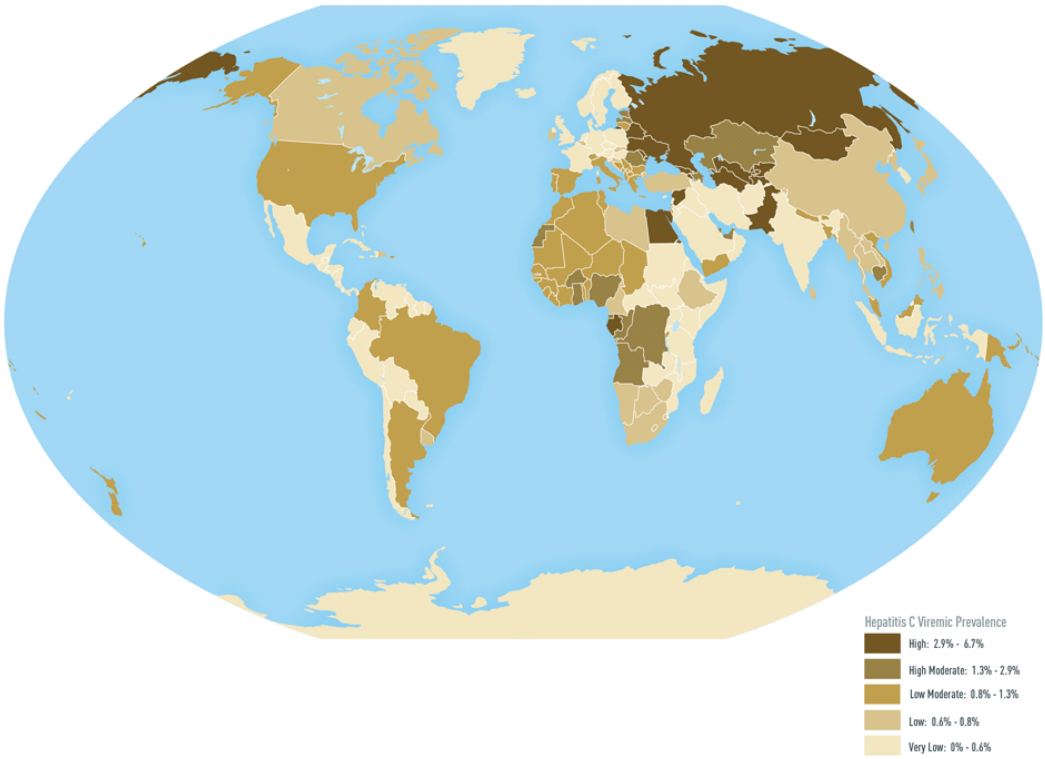

Figure 1.1 Global epidemiology of HCV infection (Global Hepatitis Report 2017, Geneva).

It should be noted that the prevalence assessment is undoubtedly underestimated because studies are conducted from blood donors, constituting a selected population probably different from the general population. Additionally, the silent evolution of the disease and the high frequency of transition to chronicity explain the existence of a vast reservoir of undiagnosed infected individuals. If not properly managed clinically, persistent HCV infection can cause progressive chronic inflammatory hepatic disease characterized by steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) in some individuals (Adejumo et al.; Pawlotsky, 2011). Further, chronic progressive HCV infection represents the leading cause of liver transplantation in the United States and Canada. Since there is no effective preventive vaccine against HCV infection, HCV infection remains a major public health problem.

1.2 Transmission mode

HCV transmission is mostly through direct exposure of bodily fluids infected individuals. Today, transfusion of unscreened blood and use of blood products in less developed medical settings are 2 main ways for HCV transmission. Unsafe injection practices, especially with multi-using syringes and needles for drug abusers is another important source for HCV transmission (Al-Moshary et al., 2019). Besides, HCV rarely transmits from mother to fetus or within sexual contact, except for sexual practices that lead to exposure to blood (Tovo et al., 2000). However, it seems that co-infection with Human immunodeficiency virus can increase the HCV transmission rate, but the identified risk factors remain unknown in over 30% of the cases of infection (Dominguez-Rodriguez et al., 2020).

1.3 Diagnosis

Currently, the diagnosis of HCV infection can be performed by direct and indirect methods, which are also called molecular and serological tests respectively (Chevaliez and Pawlotsky, 2009). Serological tests are based on detecting anti-HCV antibodies including anti-HCV IgM for the early infection, anti-HCV IgG for the late infection, and anti-HCV NS5 antibodies. Molecular biology tests by way of Reverse Transcription Polymerase Chain Reaction (RT-PCR), directly detects HCV-RNA (Chevaliez and Pawlotsky, 2009; Li and Lo, 2015).

There are 2 types of serological tests, known as an enzyme immunoassay (Adam et al.) and recombinant immunoblot Assay (RIBA) (Forns and Costa, 2006). EIA is an immune-enzymatic assay that can detect the specific HCV proteins in more than 95% of chronically infected individuals. The effective rate of this detection method is between 50% to 70% in acute HCV

infections in humans (Krajden, 2000; Warkad et al., 2018). A RIBA test is often performed to confirm a positive result obtained by EIA titration and highlights the antibodies reacting with specific HCV antigen (Chevaliez and Pawlotsky, 2009). A molecular test to detect viral RNA can also be performed to confirm the results of serological tests. This technique is also used for the diagnosis and monitoring of patients because of its many advantages (simplicity, speed) helping to establish the better treatment of infected patients. Moreover, HCV genotyping which can be conducted either by direct sequencing of the 5 'non-coding region of the viral RNA or a reverse hybridization is also encouraged to be implemented for better adaptation to treatment, even though the specificity of the test is very good for determining genotypes but slightly lower for subtypes (Forns and Costa, 2006).

1.4 Treatments

New infection with HCV does not require any treatments since the immune system has been proven to be capable of eradicating the virus in over 25% of exposed individuals (Gower et al., 2014). However, it is necessary to treat chronic HCV infected individuals to prevent the development of advanced forms of liver disease which can result in death in some individuals. WHO's updated 2018 guidelines recommend therapy with pan-genotypic DAAs (Li and Lo, 2015; Pawlotsky, 2011). Currently, the therapy is based on the combination of interferon-free treatment with DAA, which demonstrates high efficiency of cure in more than 95% persons in about 12 weeks to 24 weeks, depending on the absence or presence of cirrhosis (Grottenthaler et al., 2018; Zeuzem, 2017).

Up to now, there are 3 targets of DAAs which are NS5A, NS5B, and NS3/4A. As NS5A is an especially important protein involved in HCV replication and cellular function modulation, various NS5A inhibitors whose generic names ending in "-asvir" have been developed. NS5B, an RNA dependent RNA polymerase, plays a role in viral RNA poly chain construction, inhibitors of NS5B could also lead to virus eradication. NS5B inhibitors are also categorized as either nucleotide inhibitors (NI) or non-nucleoside inhibitors, their generic names end in "-buvir". At least but not last, inhibitors of the serine protease NS3/4A, which takes part in the splitting of the HCV polyprotein, has been proven to efficiently destroy the virus, their generic names end in "-previr (Table 1.4) (Li and Lo, 2015; Zeuzem, 2017).

Nevertheless, the potential global effect of therapeutic advances is currently limited as a result of under-diagnosis, and the fact that HCV treatment is not readily available for all the patient worldwide due to its high cost. Furthermore, Interferon-ribavirin therapy which is widely used

worldwide for HCV treatment is associated drug resistance and significant side effects limiting patient compliance to therapy (Manns et al., 2006). Setbacks are also being observed in the form of treatment drug resistance to some DAA agents (Szymanek and Krzysztof, 2013). Besides, HCV exosomes can mediate host receptors independent cell to cell transmission of infection, resulting in inefficiency of therapies that target host/viral proteins interations et the level of cell entry (Bukong et al., 2014). Our previous work also showed that infectious HCV exosomes contain replication competent HCV RNA in complex with miR-122-Ago2-HSP90 to a higher extent in patients resistant to interferon-ribavirin therapy (Bukong et al., 2014). As such, there is still an urgent need to develop new ways of HCV infection prevention and treatment.

Table 1.1: Antiviral drugs for HCV infection

Substance class	Drug	Daily dose	HCV. genotype activity (in vitro and as approved)
Nucleotide polymerase inhibitors	sofosbuvir	400mg	1a,b;2a,b;3a;4a;5a;6a Approval:1 through 6
Non-nucleoside polymerase inhibitors	dasabuvir	2x250mg	1a,b Approval:1
NS3/4A protease inhibitors	simeprevir	150mg	1a,b;2;4;5;6 Approval:1,4
	paritaprevir (ritonavir-boosted)	2×75mg (2×50 mg ritonavir)	1a,b;2a;4a;6a Approval:1,4
	Grazoprevir	100mg	1a,b;4 Approval:1,4
NS5A inhibitors	daclatasvir	60mg[*5]	1a,b;(2a);3a,4a,5a,6a Approval:1,3,4
	Ledipasvir	90mg	1a,b;2a,b;3a,4a.d;5a;6a,e Approval:1,4
	Ombitasvir	2×12.5mg	1,(2a),2b,(3a),4a,5a Approval:1,4
	Velpatasvir	100mg	1a,b;2a,b;3a;4a,d,r;5a;6a,e Approval:1 through 6
	Elbasvir	50mg	1a,b;4 Approval:1,4

CHAPTER 2: HEPATITIS C VIRUS (HCV)

2.1 Classification and genomic variability

HCV is a small envelope positive-sense single-stranded RNA virus belonging to *Flaviviridae* and genus *Hepacivirus*. It's a virus with a restrained tropism, since it principally infects the hepatocytes, but its proliferation can be also detected in extrahepatic tissues including peripheral blood mononuclear cells (PBMC) such as B cells, monocytes/macrophages, and dendritic cells (Forns and Costa, 2006).

HCV is divided into 8 genotypes (GT), with 30%-35% of the variation in nucleotide sequence) and 67 subtypes (designed by letters, with less than 15% of the difference in nucleotide sequence) to date (Chevaliez and Pawlotsky, 2006; Welzel et al., 2017). More than one subtype can be found in one genotype, for example, GT1-4 and 6 contain multiple subtypes that typically differ by 15%–25% and display high genetic variability, whereas GT5 has 1 subtype identified so far. Besides, a great genetic heterogeneity can be found in the same individual, resulting in the existence of quasi-species.

Even though GT1, 2, and 3 seem to have a global distribution, their relative prevalence differs from one region to another (Figure 2.1) (Gower et al., 2014; Lanini et al., 2016; Welzel et al., 2017). At the global level, subtypes 1a and 1b are relatively most common in high-income and upper-middle-income countries such as the USA and European countries, followed by genotype 3 and 4 which was common in lower-middle-income countries and low-income countries, respectively (Jefferies et al., 2018; Welzel et al., 2017).

Moreover, specific genotypes of HCV have been implicated in rapid disease progression in some individuals, although there are some conflicting data on genotypic associations and HCC development. Infection with HCV genotype 3 has been linked with the rapid progression of fibrosis compared to other genotypes (Gower et al., 2014; Lanini et al., 2016; Welzel et al., 2017). Further, it has been revealed that infection with different HCV genotypes affects treatment efficacy and resistance to various antiviral agents.

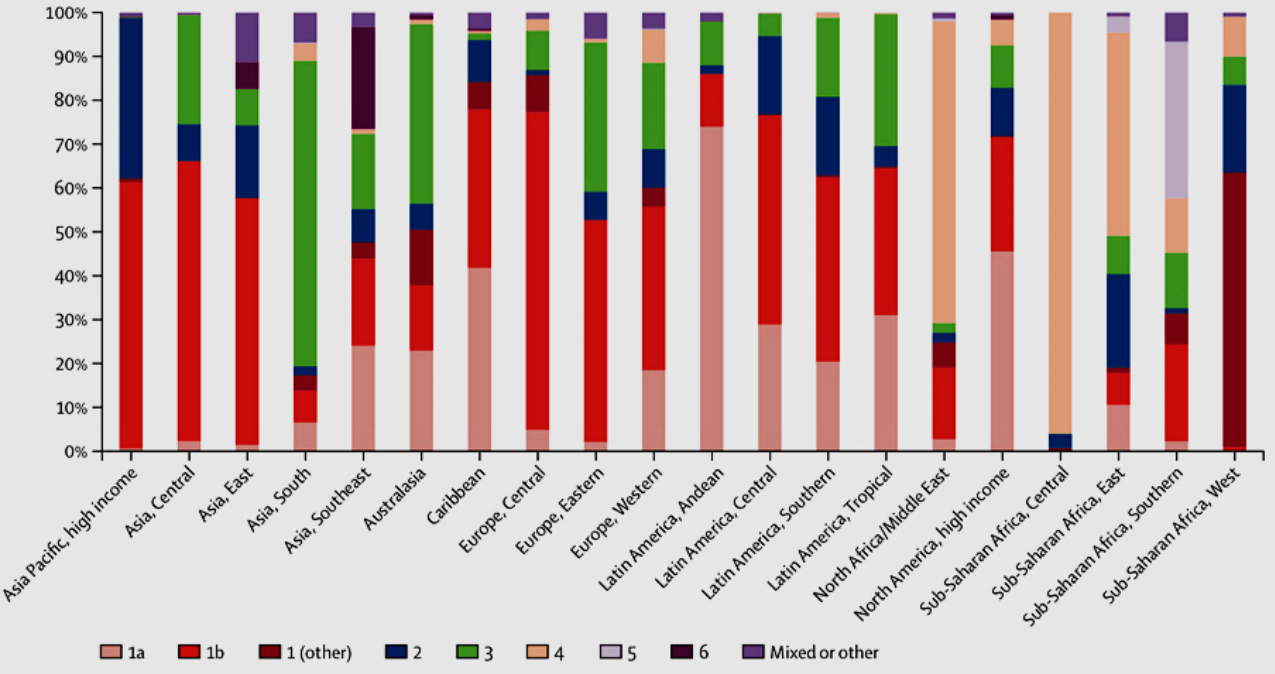

Figure 2.1 HCV genotypes distribution (Spearman et al., 2019).

2.2 HCV virion

HCV particles have been considered as the most structurally irregular members among the *Flaviviridae* family, they are spherical, with spike-like projections in size ranging from 55-65nm in diameter (Kaito et al., 1994). HCV particle is composed of the nucleocapsid (30nm-35nm in diameter) containing HCV genome, enveloped by ER-derived lipid bilayer where glycoproteins E1 and E2 are anchored (Figure 2.2) (Suzuki et al., 2010).

Evidence suggests that HCV circulates in the bloodstream as a hybrid lipoviral particle (LVP), which contain apoB and apoE on their surface to mask viral glycoproteins(Andre et al., 2002). Once in the bloodstream, HCV can be associated with different lipoproteins and form a density gradient heterogenic distribution(Nourbakhsh et al., 2013). The association of HCV with very-low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) (1.03 to 1.08 g/ml) exhibits higher specific infectivity compared to higher density fractions (1.17 to 1.25 g/ml) associated with IgG(Nourbakhsh et al., 2013; Suzuki et al., 2010). Moreover, given observations that HCV particles production by human hepatocytes depend on VLDL assembly and secretion, the association of HCV particles with VLDL has been suggested during HCV assembly and secretion (Gastaminza et al., 2008; Huang et al., 2007).

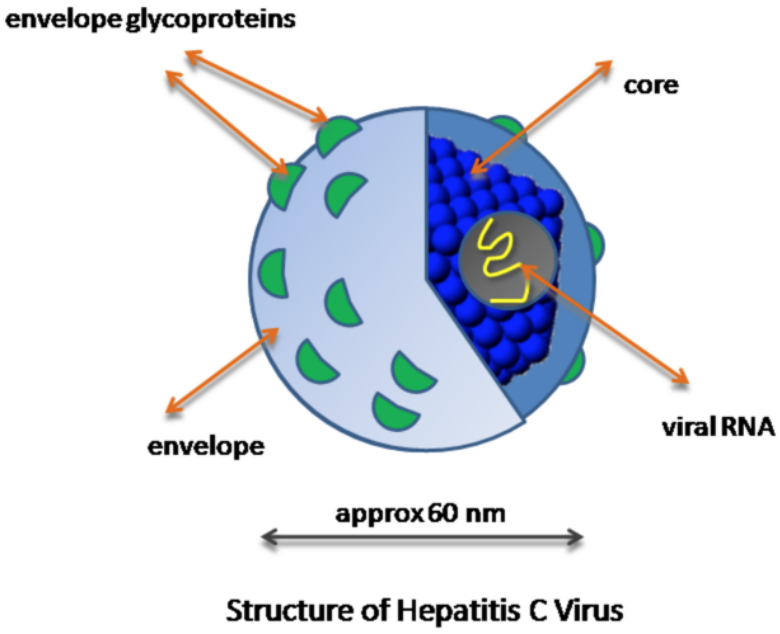

Figure 2.2 Schematic representation of HCV virion (Wikipedia).

2.3 HCV genome organization

The HCV genome RNA is composed of 3 distinct regions: the non-coding region at 5' also called 5' non-translate region (NTR), an open reading frame (ORF) of approximatively 9.6kb and a short 3'NTR (Figure 2.3-1) (Appel et al., 2006; Chevaliez and Pawlotsky, 2006). The 5'NTR containing 4 distinct domains (I to IV) is highly conserved and well structured, which assures ORF translation. Specifically, the domain II to IV as well as the beginning of the ORF constitutes the internal ribosomal entry site (IRES) which forms a stable pre-initiation complex by direct binding with the 40S ribosomal subunit for the HCV polyprotein translation. The importance of IRES in viral RNA replication and HCV replication has also been widely demonstrated (Catanese et al., 2013; Chevaliez and Pawlotsky, 2006). Besides, the 3'NTR region, which size varies within different genotypes, contains 3 distinct domains: domain I little conserved, domain II with polyU/UC and domain III also named region X where 3-rod loops are formed, plays an important role in negative-sense viral RNA synthesis (Chevaliez and Pawlotsky, 2006; Kato, 2000; Suzuki et al., 2010).

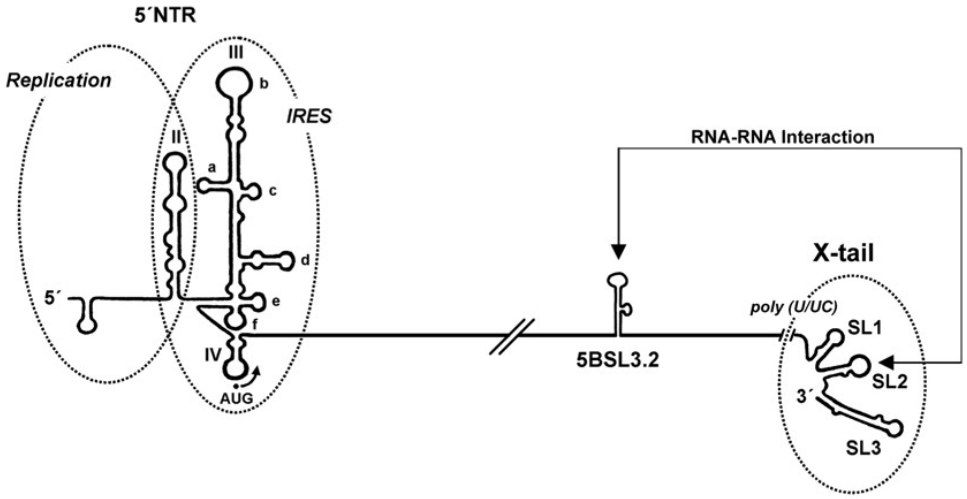

Figure 2.3-1 Secondary structure of 5' and 3'NTR of HCV(Appel et al., 2006).

Furthermore, the ORF encodes a polyprotein which will be further processed by the host and viral proteases into 3 structure proteins (core protein, E1, and E2) and 7 non-structure (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Figure 2.3-2). As their name indicates, the structural proteins are involved in viral particle construction, whereas NS proteins participle in viral maturation, replication, and assembly (Chevaliez and Pawlotsky, 2006; Kato, 2000).

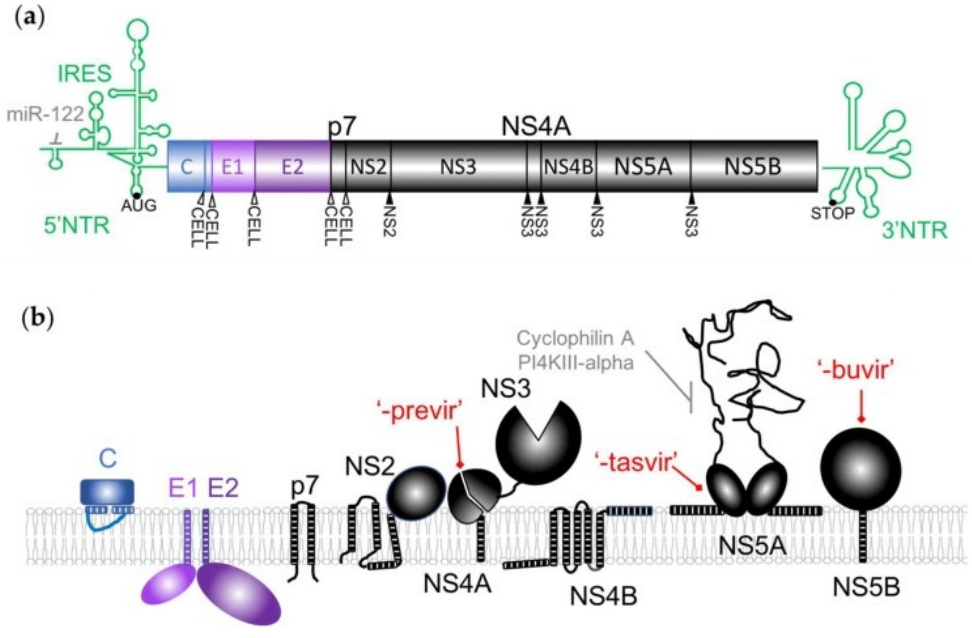

Figure 2.3-2 Schematic representation of the HCV genome (a) and HCV membrane-associated proteins (b) (Alazard-Dany et al., 2019).

2.4 HCV Life Cycle

The HCV life cycle has been extensively studied and is now well understood (Chevaliez and Pawlotsky, 2006; Dustin et al., 2016). In general, the multiple steps of the HCV life cycle (Figure 2.4) include:

1. Cellular attachment of HCV virion and Entry; HCV virion circulates in the bloodstream either as free-particle or as LVP, binds to the host cell by specific interactions with various surface receptors including LDL-receptor, the human Cluster of Differentiation 81 (CD81) or scavenger receptor B1 (SR-B1). It's thought longtime that both glycoproteins E1 and E2 play an essential role in attachment of HCV particle to cellular receptors, while studies have revealed that E1 and E2 act as complexes of disulfide-bound heterodimers and E2 is identified to be responsible for receptor binding (Cocquerel et al., 2003; Scarselli et al., 2002), whereas E1 seems to be an important component of atypical fusion machinery (Tong et al., 2018). Besides, it's believed that apoE, which is on the surface of LVP, plays a crucial role in initiating virus entry by interacting with cell surface heparan sulfate proteoglycans and/or SR-B1. Afterward, receptor-particle complexes are translocated at tight junctions where coreceptors Claudin-1 (CLDN1) and Occludin (OCLN) facilitate clathrin-dependent endocytosis to entry into the host cell.

2. Fusion; following endocytosis of HCV particles, E1/E2-mediated membrane fusion occurs in a low-pH dependent way, which further allows the release of the viral genome into the cytoplasm(Tong et al., 2017; Tong et al., 2018).

3. RNA translation, post-translational processing, and replication; The released positive-sense viral RNA is then translated in the ER into a single polyprotein which will be co- and post-translationally processed by cellular and viral proteases. The Core, E1, and E2 structural proteins, present in the virions, are produced from the N-terminus, whereas seven NS proteins are expressed from the C-terminus of the polyprotein. Specifically, NS2, a zinc-dependent metalloprotease that cleaves between NS2 and NS3; and NS3/4A, a serine protease that cleaves between the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions. NS4A, known as a cofactor for NS3, is also required for NS5A phosphorylation. It's been reported that NS5A is essential for viral replication even though the detailed mechanism remains unclear (Tripathi et al., 2013). On the other hand, the positive-sense RNA serves as a template to generate negative-sense RNA which in turn serves as a template to synthesize numerous positive-sense RNA and to be ultimately translated into a polyprotein. Moreover, the replication complexes are formed at the level of the "membranous web," an alteration of ER-membrane induced by the virus, in the

form of a vesicle allowing to concentrate all the viral and cellular factors necessary for the replication.

4. HCV assembly and secretion; Viral assembly occurs near the replication complexes at assembly sites associated with ER-derived membranes, in close proximity to lipid droplets where core proteins can accumulate. P7, a 63-amino acid polypeptide, has been demonstrated to act in concert with NS2 in recruiting core, E1, E2 as well as NS3, NS5A to the assembly sites, allowing the gathering of all viral proteins for assembly efficiently and correctly in the same places (Boson et al., 2011; Denolly et al., 2017; Stapleford and Lindenbach, 2011). NS4B was also showed to play a role in encapsidation of HCV assembly (Han et al., 2013). Besides, the involvement of host factors such as Exosomal Sorting Complexe Required for Transport (ESCRT) and Diacylglycerol O-Acyltransferase 1(DGAT1) in HCV virion production has also been described (Barouch-Bentov et al., 2016; Tamai et al., 2012). Following viral assembly, HCV acquires its membranes by budding at the ER where VLDL is enriched and finally secreted into extracellular space.

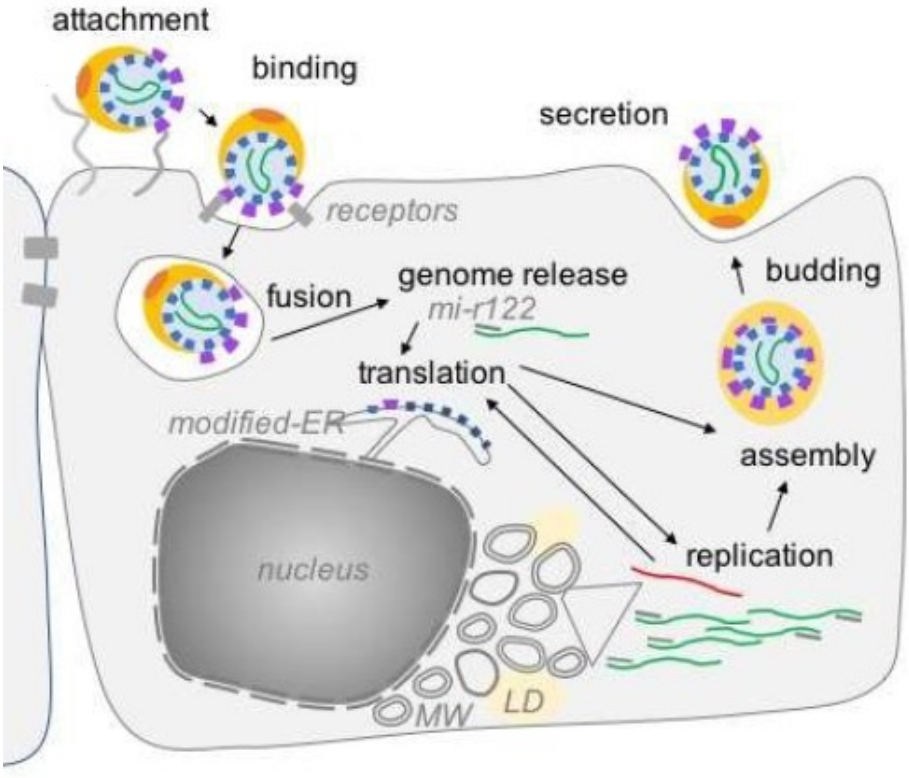

Figure 2.4 HCV Life Cycle (Dustin et al., 2016). HCV life cycle starts with attachment of HCV virions to cellular receptors, allowing the entry of the virus. Once in cells, the virus will replicate and translate its genome to polyprotein with support from ER. Then, les proteins and RNA viral will be packed together to form a virion release from host cells.

CHAPTER 3: EXOSOMES

Extracellular vesicles (EV) are classified into 3 main populations according to their size and origin: Microvesicles (MV) at 100nm-1µm, Exosomes at 30-150nm, and apoptotic bodies at usually greater than 1µm. Besides, as shown in figure 3, microvesicles and apoptotic bodies are formed by budding or blebbing of the cell membrane to pinch off new vesicles, whereas exosomes are produced via endosomal pathways and released by exocytosis (Charlotte Lawson 2017). Also, the components of these three main EVs differ from each other, microvesicles and exosomes contain proteins and nucleic acids, whereas apoptotic bodies contain nucleic fraction and cellular organelles (Elmore, 2007).

Since our study is focused on exosomes biogenesis regulation, we will specifically concentrate on exosomes in this chapter.

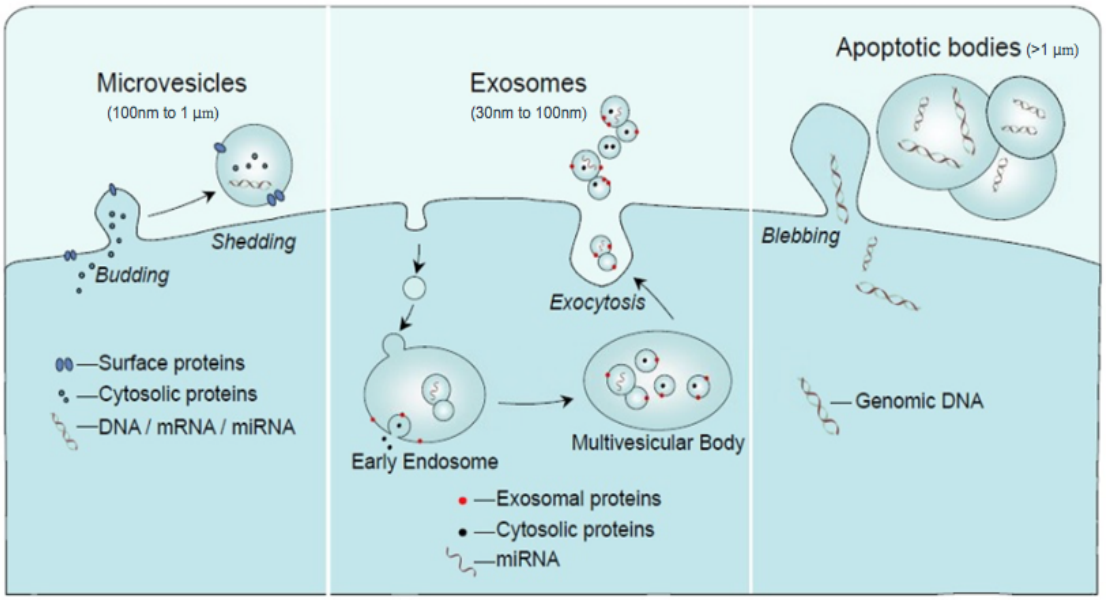

Figure 3 Schematic representation of 3 main populations of extracellular vesicles (Charlotte Lawson. 2017). Microvesicles (100nm to 1 µm), and Apoptotic bodies (>1 µm) are formed by budding or blebbing of the cell plasma membrane, whereas exosomes (30nm to 150nm) have an endocytic origin.

3.1 Discovery of exosomes

First described by the Tram's team as an "expansion" of the plasma membrane (Trams et al., 1981) then by Harding and Stahl (Harding et al., 1983), the notion of exosomes appeared in the 1980s during the time R. Johnstone's lab was studying the fate of transferrin receptor (TfR) when sheep reticulocytes differentiate into erythrocytes. Electron microscopy (EM) showed an endocytosis phenomenon of membrane protein, followed by an increasing concentration of

labeling within vesicles present in an endosomal compartment. Besides, the fusion of this endosome with the plasma membrane resulted in the release, into extracellular space, of the specific labeled protein of interest in nanovesicles with an average diameter of 50nm (Figure 3) (Johnstone, Adam, et al. 1987; Johnstone, Bianchini et al.1989; Johnstone 2005; Johnstone 2006). Finally, these small extracellular vesicles are termed as “exosome”, which comes from the association of two original Greek words: ‘exô’ (out of) and ‘sôma’ (the body). However, until 2000, exosomes were considered as cellular wastes resulting from by-products of homeostasis or cell damage. Interest in exosomes has intensively increased after the observation that they play an important role in cell communication (Camussi et al., 2010).

3.2 Morphological and physical features of Exosomes

Exosomes are small membrane-enclosed vesicles which display a spherical shape, observed by cryo-TEM (Transmission Electronic microscopy) (Figure 3.2) (Hyosun Choi and Ji Young Mun, 2017). These very small membrane-enclosed vesicles vary in the size range between 30nm and 150 nm and their densities are between 1.13 and 1.19 g/ml (Thery et al., 2009; Thery et al., 2002). Based on their ability to float in density gradients, exosomes can be isolated from other extracellular vesicles (EV) by a series of centrifugation and ultracentrifugation steps (Zhang et al., 2019). Other physical parameters of exosomes, such as light scatter, which is correlated to size but also geometry and composition, can be measured by Nanoparticle Tracking Analysis (NTA) which tracks the movement of laser-illuminated individual particles under Brownian motion and then calculates their diameter using statistical methods (Annexe1) (Thery et al., 2002; Zhang et al., 2019).

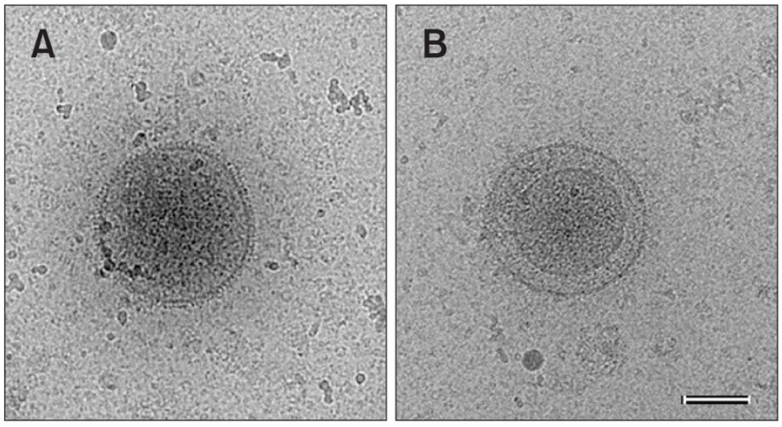

Figure 3.1 Cryo-transmission electron microscopy of fixed exosomes images. The bar represents 100nm. (Hyosun Choi and Ji Young Mun, 2017).

3.3 Exosomes biogenesis

Analysis of the exosome's protein cargo reveals that their biogenesis is linked to the different stages of maturation of endosomes, since protein of nuclear, mitochondrial, ER or the Golgi apparatus were found within the exosomes, confirming their endosomal origin (Colombo et al., 2014; Hessvik and Llorente, 2018; Zhang et al., 2019).

It is believed that most exosomes are released upon the fusion of multivesicular bodies (MVBs) with the cellular plasma membrane. MVBs also referred to as late endosomes, are components of endocytic pathways. After maturation of early endosomes into MVBs with concomitant intraluminal vesicles (ILVs) formation inside MVBs by inward budding of limited MVB membrane, MVBs fuse with the cell membrane and most ILVs are released into extracellular space as termed exosomes (Figure 3.3) (Hessvik and Llorente, 2018).

Since endocytic pathways is a highly dynamic cellular process involving in the internalization of cellular membrane components, their recycling to the PM, and/or their degradation. Newly formed MVBs can either fuse with lysosomes in which the lysosomal hydrolases degrade their components or fuse with PM to release their component as exosomes (Colombo et al., 2014). Even though the key mechanism that determines the fate of these MVBs remains unclear, it's believed that most of MVBs fuse with lysosome ending up with their content degradation, whereas organelles with hallmarks of MVBs, bearing the tetraspanin CD63, lysosomal-associated membrane proteins LAMP1 and LAMP2, and other molecules that are recruited during MVBs maturation such as MHC class I and II, might prefer to fuse with the PM, releasing their content into the extracellular space (Jaiswal et al., 2002; Raposo et al., 1996).

Moreover, one of the main strategies used by cells to maintain their homeostasis is the autophagy-lysosomal pathway. The autophagy-lysosomal pathway is an important cellular pathway to get rid of foreign, damaged, and unwanted molecules by targeting them into a tightly sequestered membrane-bound vesicles termed autophago-lysosomes. Importantly, it is also possible for autophagosomes to fuse with MVBs to further forme amphisome, which themselves fuse with PM to release their content as 'exosome'. Besides, autophagosomes could also fuse with PM allowing their cellular release which considered as 'pathogenic exosomes'.

Nevertheless, even though more studies are needed to determine the exosomes' biogenesis pathways, the main actor involved in exosome formation is MVB. MVBs and ILVs formation require the involvement of several molecular mechanisms, proteins belonging to Exosomal Sorting

Complexe Required for Transport (ESCRT) family are, by far, the best characterized. Thus, MVB and IVs biogenesis have been described in ESCRT-dependent and -independent ways.

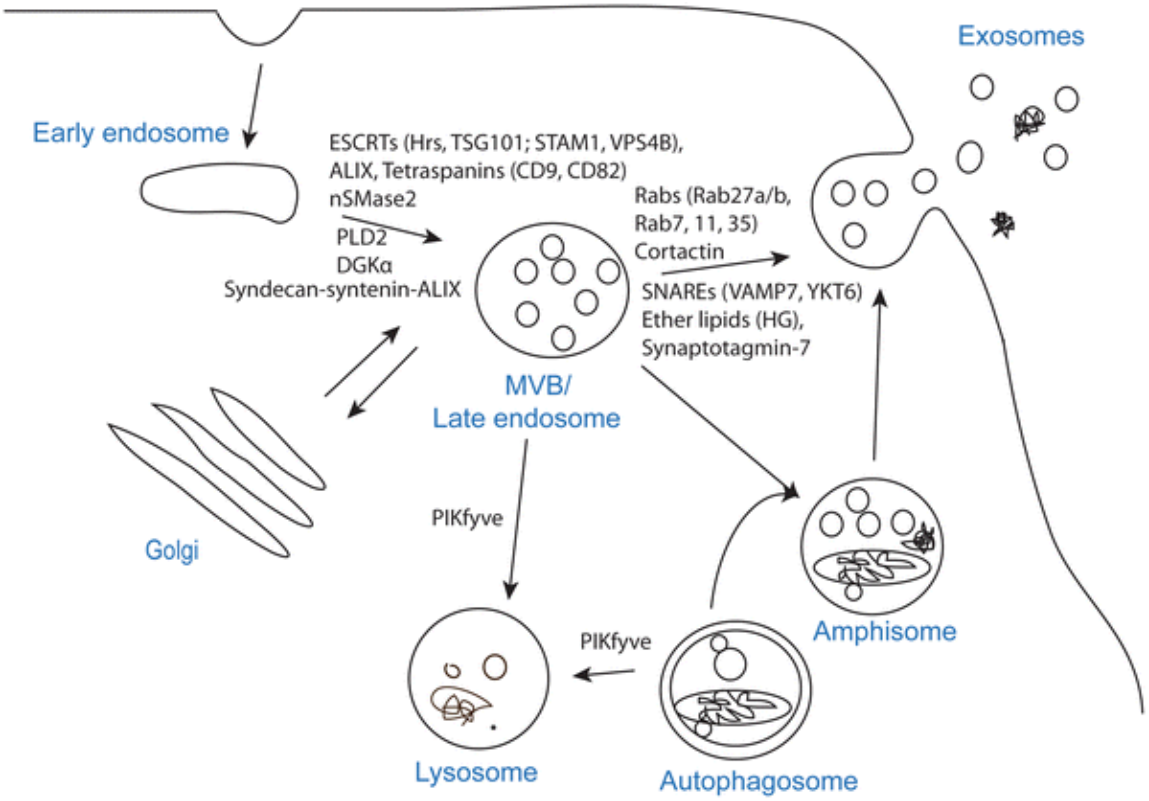

Figure 3.2 Exosomes biogenesis and a cross-link between autophagy and exosomal pathway (Hessvik and Llorente, 2018). The biogenesis of exosomes involves 4 different steps: (i) membrane invagination; (ii) MVBs formation; (iii) ILVs formation by inward budding of MVB membrane; (iv) the fusion of MVBs with the cellular plasma membrane, ILVs released as exosomes.

3.3.1 ESCRT-dependent mechanisms

The ESCRT family is consists of about 30 proteins that assemble into 4 ESCRT complexes: ESCRT-0, I, II, and III, with associated proteins such as VPS4, VTA1, and ALIX. The ESCRT-dependent MVB formation requires the coordination of all ESCRT complexes (Figure 3.3.1) (Hanson and Cashikar, 2012; Wollert and Hurley, 2010); The ESCRT-0 complex, composed of HRS (hepatocyte growth factor–regulated tyrosine kinase substrate, official gene symbol *HGS*), recognizes, ubiquitinates and sequesters ubiquitinated transmembrane proteins for MVB delivery and recruits the ESCRT-I complex through recruiting TSG101 of the ESCRT-I complex, which in turn recruits the ESCRT-II complex and the ESCRT-III complex. It has been revealed that the ESCRT-I and -II complexes appear to be responsible for membrane invagination into buds with

sorted cargo, whereas ESCRT-III components and accessory protein VPS4 subsequently drive vesicle scission, resulting in MVB membrane invagination and ILVs formation (Sahu et al., 2011).

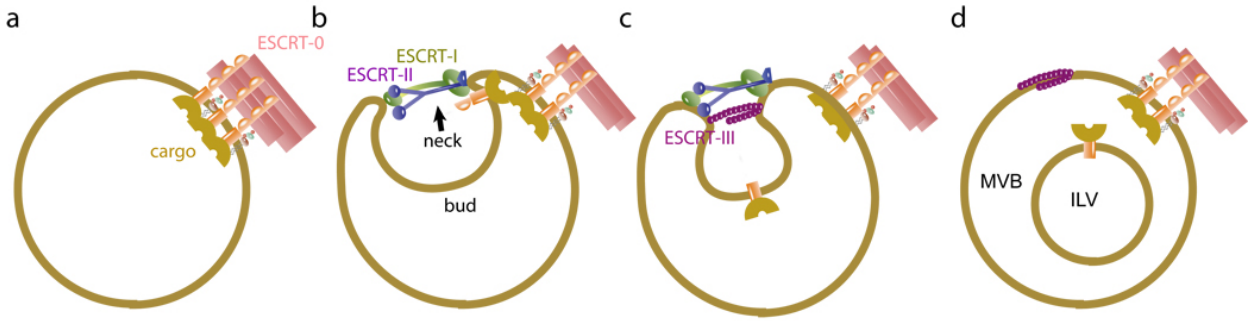

Figure 3.3.1 Molecule biogenesis of MVB in an ESCRT-dependent pathway (Wollert and Hurley, 2010).

3.3.2 ESCRT-independent mechanisms

The existence of ESCRT-independent mechanisms for MVE formation is supported by the finding that cells concomitantly depleted of four subunits of the ESCRT complex are still able to generate CD63-positive MVBs (Stuffers et al., 2009). Interestingly, the ESCRT-independent mechanism has initially been described in oligodendroglial cell lines (Trajkovic et al., 2008) in which exosomes biogenesis depend on nSMase (enzymes that hydrolyze sphingomyelin to ceramide). Moreover, observation of raft-based microdomains which is thought to be highly enriched in sphingomyelinase, emphasizes the key role of ceramide in exosome biogenesis. However, some studies showed that in human melanoma cells, by contrast, the depletion of neutral sphingomyelinases did not impair MVB biogenesis. Instead, some proteins such as tetraspanins, have been identified as actors in exosome biogenesis (van Niel et al., 2011). Additionally, a CD63-dependent mechanism, in which CD63 is instrumental in targeting the EBV-encoded LMP1 protein to ILVs and allowing its subsequent release, has been reported (van Niel et al., 2011).

3.4 Exosome secretion

Exosomes are constitutively secreted by most cells in the physiological state, however, their secretion is regulated by some factors such as Ca^{2+} intracellular concentration, ceramide concentration, cell depolarization, Rab family proteins activity, and extracellular pH. It's been reported that treatment with Ca^{2+} ionophores increases secretion of exosomes by the erythroleukemia cell line K562(Savina et al., 2003), oligodendroglial cells (Kramer-Albers et al., 2007), dendritic cells (DCs) (Montecalvo et al., 2012), and mast cells (Valadi et al., 2007). Moreover, a decreased exosome secretion has been observed when there was an increase in

sphingomyelin synthase (which synthesize sphingomyelin from ceramides) activity (Yuyama et al., 2012). Additionally, the family of Rab proteins is known as essentials regulators involved in vesicle transport between different compartments. Rab7, Rab11, Rab27, and Rab35 have been shown to increase the exosome secretion and may act in a sub-population of MVB-dependent manner (Bobrie et al., 2012a; Bobrie et al., 2012b). The pH in the extracellular environment also regulates the exosome release. Some studies demonstrated an increased exosome secretion in tumors, where acidic pH has been found in extracellular space (Kharaziha et al., 2012; Parolini et al., 2009).

3.5 Exosomes composition

As exosomes are originally from the endocytic pathway, their composition is quite similar to the composition of cytoplasm. However, relative to the composition of cytoplasm, exosomes are enriched in components such as proteins, lipids, and nucleic acids (Figure 3.5) (Colombo et al., 2014).

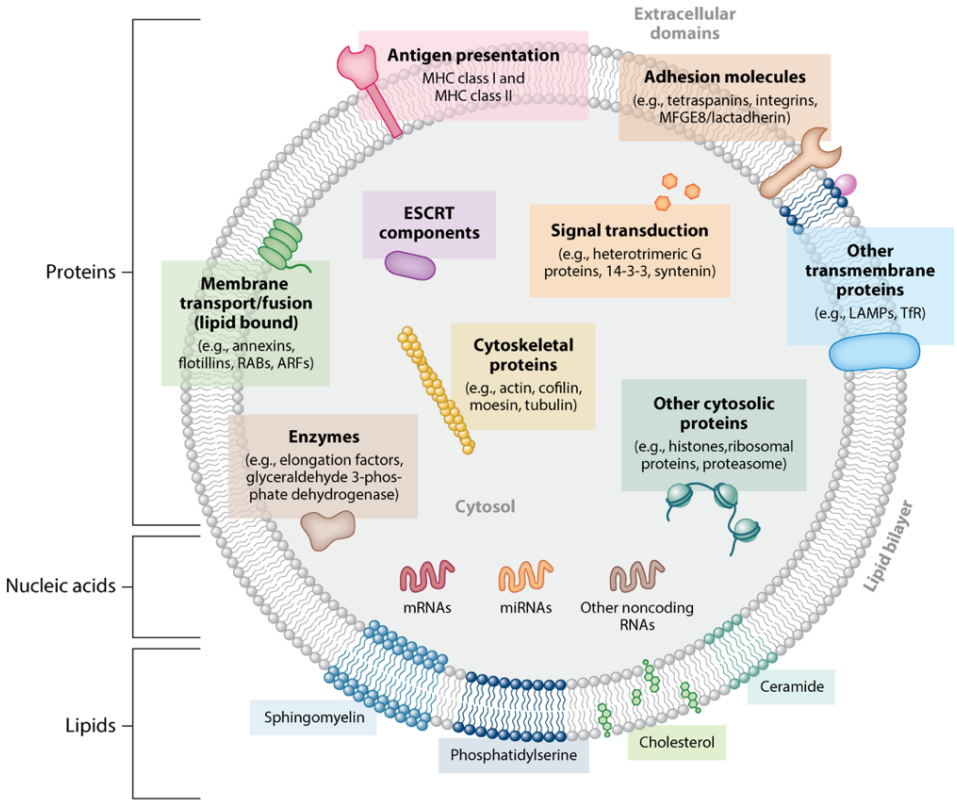

Figure 3.3 Schematic representation of exosome composition (Colombo et al., 2014).

Up to now, from the ExoCarta database (http://exocarta.org/index.html) including 286 studies, approximatively 9769 proteins, 4946 RNAs and 1116 lipids have been identified in exosomes from different sources, which represent a large diversity and complexity in their potential functions in cell-to-cell communications. Interestingly, among all the constitutive partners, some have been identified as ubiquitous elements that we found in all secreted exosomes, whereas others are cell-type and/or cellular physiological/pathological-state specific (Colombo et al., 2014; Zhang et al., 2019). Moreover, the cargo of exosomes often reflects the physiological and/or pathological state of the cells they originate from, thus, exosomes could also serve as biomarkers in a range of disease, such as chronic inflammation, cardiovascular disease, neurodegenerative disorders and tumors (Zhang et al., 2019).

- **Proteins in exosomes**

Typically, exosomes are highly enriched in proteins with a large variety of functions. Exosomes have a common protein composition which allows them to be characterized as vesicles derived from the endosomal pathway, and more particularly from the proteins involved in MVBs biogenesis (Alix, TSG101) as well as proteins responsible for membrane transport and fusion (Annexins A1,A2,A5,A6, Rabs, Flotillins, heat shock proteins HSP70, HSP90, Tetraspanins CD9, CD63, CD81) (Hessvik and Llorente, 2018; Zhang et al., 2019). Indeed, some of them exclusively take part in exosome biogenesis (Alix, flotillin, and TSG101) while others are specifically enriched in exosomes (CD63, CD81, TSG101). As such, CD63, CD81 and TSG101 are often used as exosomal marker proteins (Zhang et al., 2019). Other proteins are also commonly found in exosomes, such as adhesion molecules (ICAM-1, CD11/16, LFA-3, clathrin, integrins), cytoskeleton proteins (actin, tubulin, moesin), the major histocompatibility complex MHC-I, proteins involved in the process of apoptosis (thioredoxin peroxidase II, galectin 3) and various enzymes, elongation factors and proteins involved in cell signaling (Hessvik and Llorente, 2018; Thery et al., 2002; Zhang et al., 2019). Moreover, other proteins are specifically found in exosomes in a cell-type dependent way. Taken immune cells as examples, MHC type II and CD3 were found in APC (Antigen Presenting Cells)-derived exosomes; TLR (Toll-Like Receptor) proteins and cell-death linked proteins (perforin and granzyme) in cytotoxic T lymphocytes-released exosomes (Hessvik and Llorente, 2018; Thery et al., 2002; Zhang et al., 2019).

- **Lipids in exosomes**

As their endosomal origin and membrane-enclosed structure, the lipid composition in the exosomal membrane is therefore that of MVBs and ILVs, itself resulting from the PM and MVBs respectively, where membrane invagination has occurred (Zhang et al., 2019). Thus, the outer layer of PM which is enriched in phosphatidylcholine (PC) and sphingomyelin, while the inner layer is composed of aminophospholipid, phosphatidylserine (Kalra et al., 2013) and phosphatidylethanolamine (PE) have been observed in exosomes. However, if the transmembrane proteins have the same orientation between the PM and MVBs' membrane, the lipid asymmetry of the PM is not found at the exosome membrane (Zhang et al., 2019). Indeed, PE has been proven to be both in the cytosolic and luminal layer of the exosomal membrane, whereas other phospholipids also seem to be distributed randomly between the two lipid layers (Subra et al., 2007). Interestingly, poor in PC while rich in sphingomyelin and cholesterol has been shown in exosome membrane, suggesting the specific lipid composition of exosomes allows them to have a much larger membrane as rigid as that of the cellular PM (Zhang et al., 2019).

- **Nucleic acids in exosomes**

As important as proteins and lipid, RNA sequencing analysis revealed that exosomes are enriched with specific miRNAs, mRNAs, non-coding RNAs, tRNAs, and rRNAs. According to the ExoCarta database (http://exocarta.org/index.html), approximatively 3408 mRNAs and 2838 miRNAs have been identified so far, in a cell-type dependent and cell-state dependent manner. One of the most important characteristics of exosomes being in cell communication is the bioactivity of miRNAs. It has been widely reported the role of miRNAs in the establishment of an intercellular trafficking network, which, in turn, elicits transient or persistent phenotypic changes of the recipient, even though the exact sorting and loading mechanism is unclear (Valadi et al., 2007). Some studies showed the process of incorporating miRNAs into exosomes would be dependent on ceramides (Vickers and Remaley, 2012) and the RISC complex (RNA-induced silencing complex) (Pant et al., 2012). Although ESCRT machinery proteins do not seem essential for the incorporation of miRNAs into ILVs, it would suggest that these proteins can be associate with the effector proteins of the biosynthesis of miRNAs (Iguchi et al., 2010a; Iguchi et al., 2010b). Moreover, no DNA has been found in exosome, showed by several studies (Valadi et al., 2007). However, some studies have shown the presence of mitochondrial DNA (Guescini et al., 2010) and genomic DNA (Balaj et al., 2011) in murine myoblasts-derived exosomes.

3.6 Transfer of cell released exosomes

Once exosomes secreted, they can either directly stimulate the recipient cell by receptor-mediated interactions or transfer biologically active components to the target cells by fusion with their plasma membrane, which may ultimately reprogram the recipient cells and alter their functions in precise ways (Figure 3.6) (Camussi et al., 2010). Thus, exosome-mediated intercellular communication could also open some new paths with realistic hopes, in targeted therapy, thanks to their therapeutic potential as drug transport vehicles (Camussi et al., 2010; Cordonnier et al., 2017; Hessvik and Llorente, 2018; Zhang et al., 2019).

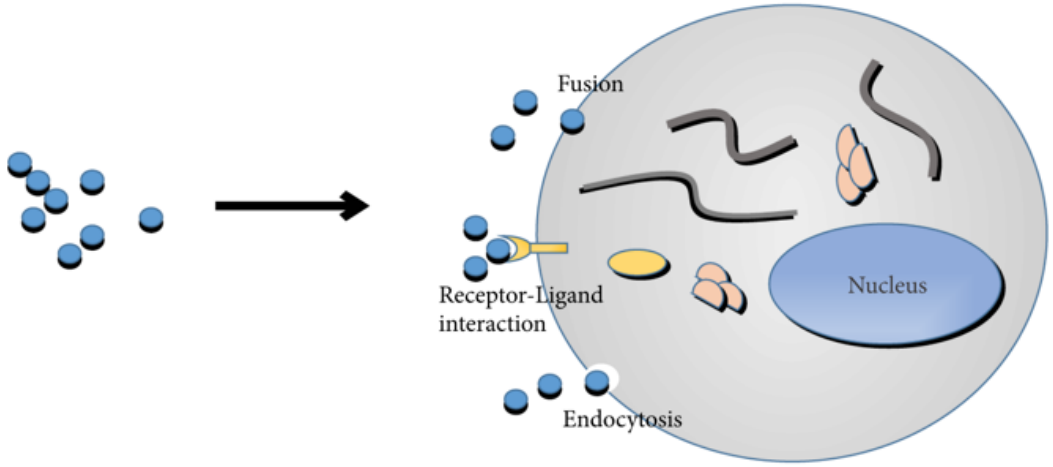

Figure 3.4 Schematic representation of exosome internalization(Cordonnier et al., 2017). The exosome may, (i) elicit transduction of the signal via intracellular signaling pathways by direct contact through adhesion molecules like integrin or through a ligand-receptor interaction, (ii) be endocytosed via phagocytosis, macropinocytosis or receptor-mediated endocytosis, or (iii) fusion with the plasma membrane and transfer its content into the cytoplasm of the recipient cell.

3.7 Exosomes in the context of HCV infection

3.7.1 HCV infectious exosomes composition

A previous study (Bukong et al., 2014) in our lab partially characterized HCV-infected hepatocytes-derived exosomes, showing that infectious HCV exosomes harbor negative-sense HCV RNA in complex with microRNA (miR-122), Argonaute-2 protein (Ago2) and heat shock protein90 (HSP90), as well as exosomal marker CD63 and CD9 (Figure 3.7.1). The presence of HCV RNA in exosomes renders these small vesicles infectious, however, it's unclear whether viral RNA in exosomes results from direct sorting or from hijacking the machinery for exosomes biogenesis, trafficking and/or release. Furthermore, an increased proportion of miR-122 in complex with Ago2

was found in exosomes from HCV infected patients with treatment-naïve and treatment non-responders and in exosomes from JFH1-infected 7.5 cells, compared to naïve cells (Bukong et al., 2014). Interestingly, other studies showed a higher level of miR-122 expression presented in HCV JFH1-infected Huh7.5 cells-derived exosomes compared to those from HCV infected individuals, suggesting by authors as a result of decreased interferon production since Huh7.5 cells contain a mutation in RIG-I (retinoic acid-inducible gene-I) involving in interferon production (Bukong et al., 2014; Hao et al., 2013; Sarasin-Filipowicz et al., 2009). Moreover, some studies demonstrated the presence of other components such as miR-214 and twist1 protein in HCV-related exosome plays an important role in liver disease evolution (Chen et al., 2015). Recently, increased miR-192 expression was also found in exosomes released by HCV-infected cells, thought to be significantly considerable in fibrosis progression (Kim et al., 2019).

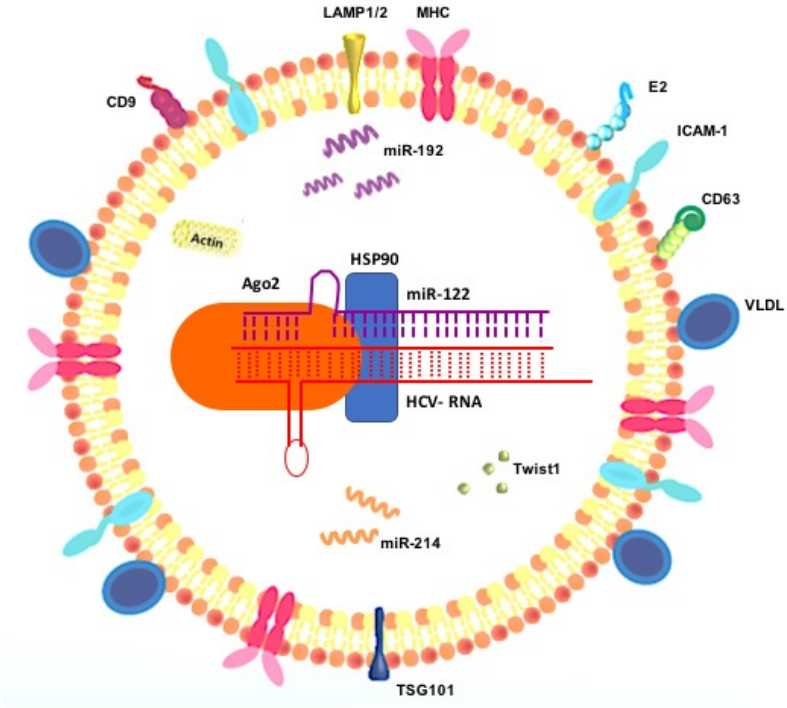

Figure 3.5.1 Schematic representation of HCV-infected hepatocytes-derived exosomes.

3.7.2 Exosomes in HCV replication and transmission

Reports have consistently demonstrated exosome can transfer viral RNA to neighboring cells (Figure 3.7.2-1) (Shen et al., 2017), and the association of Ago2 and miR-122 with HCV RNA in exosomes ensure the stability of viral RNA, suggesting that exosomes-mediated viral RNA could lead to a higher level of replication and infectivity, compared to RNA carried by HCV (Bukong et al., 2014; Cosset and Dreux, 2014; Shen et al., 2017). Indeed, given evidence that Ago2/miR-122

association binds to IRES at 5' of HCV RNA (Johnston et al., 2010), which is essential for viral RNA replication, it's believed that the presence of Ago2 with miR-122 enhances HCV replication. Additionally, HSP90 was also shown to stabilize the RISC complex and could potentially increase HCV replication (Johnston et al., 2010). Remarkably, HSP90 and miR-122 inhibition, associated or separately, can decrease the viral replication (Bukong et al., 2014). Moreover, miR-192, found recently in exosomes from HCV-infected cells, is also capable of increasing viral replication in HSC (Hepatic Stellate Cells) leading to rapid fibrosis progression (Kim et al., 2019).

Observations support that infectious HCV exosomes exhibit a higher level of HCV transmission to hepatocyte than the same multiplicity of infection of free HCV particles (Patman, 2014; Urbanelli et al., 2019). Also, this exosome-mediated HCV cell-to-cell transmission was found to be in a receptor-independent and neutralizing antibody-independent pathway (Bukong et al., 2014). Inhibition of CD81, SR-B1, and APOE showed no impacts neither on exosome secretion nor on HCV transmission. Furthermore, studies showed the presence of negative-sense RNA in exosomes is extremely important in HCV replication and transmission, given the fact that none of HCV treatment responder patients contained detectable HCV RNA in their sera exosomes, and only some of the treatment-naïve patients with both negative- and positive sense RNA detected in their exosomes, whereas most of them harbored only negative-sense HCV RNA (Bukong et al., 2014). Moreover, inhibition of host factors in exosomes, such as HSP90 and miR122 could significantly suppress exosomes-mediated HCV replication and transmission. Strikingly, attenuation of HSP90 or miR-122 expression in target cells was not sufficient to inhibit HCV transmission via exosomes (Bukong et al., 2014). This could be because HCV exosomes contain all the necessary viral and host protein factors that are otherwise not present in the endoplasmic reticulum and can thus mediate effective replication once the cellular entry is accomplished by exosome uptake.

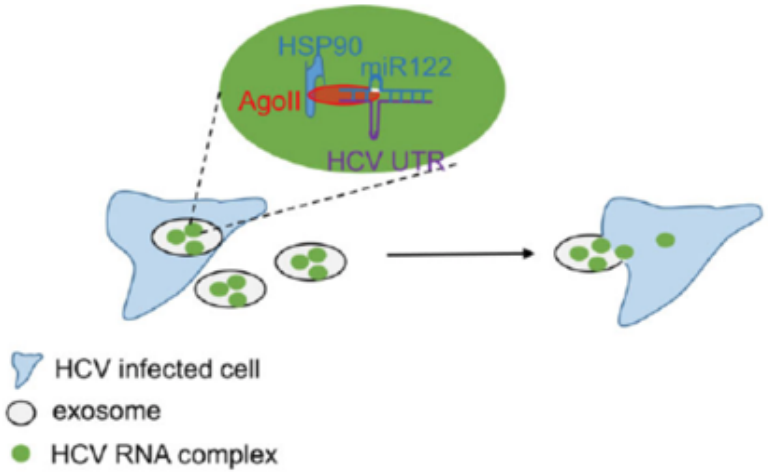

Figure 3.7.2-1 Exosomes-mediated HCV RNA to naïve hepatocytes(Shen et al., 2017).

Studies showed that exosomes-mediated enhanced HCV transmission could be due to their act on immune escape, leading to immune dysfunction (Ji et al., 2013; Rabinovich and Croci, 2012; Rabinovich and Toscano, 2009). Increased Galectin-9 production by monocytes has been revealed following coculture with exosomes isolated in HCV patients compared to healthy individuals. The higher level of Gal-9 can efficiently inhibit cytotoxic T cells by interacting with Tim-3 ligand, therefore, HCV infectious exosomes could inhibit adaptive immunity and enhance viral replication/transmission, resulting in viral persistence (Figure 3.7.2-2A) (Shen et al., 2017). However, some studies showed exosome-mediated viral RNA transfer could also activate IFNα-producing cells, such as plasmacytoid dendritic cells (pDCs), thus inhibits HCV transmission (Figure 3.7.2-2) (Shen et al., 2017) .

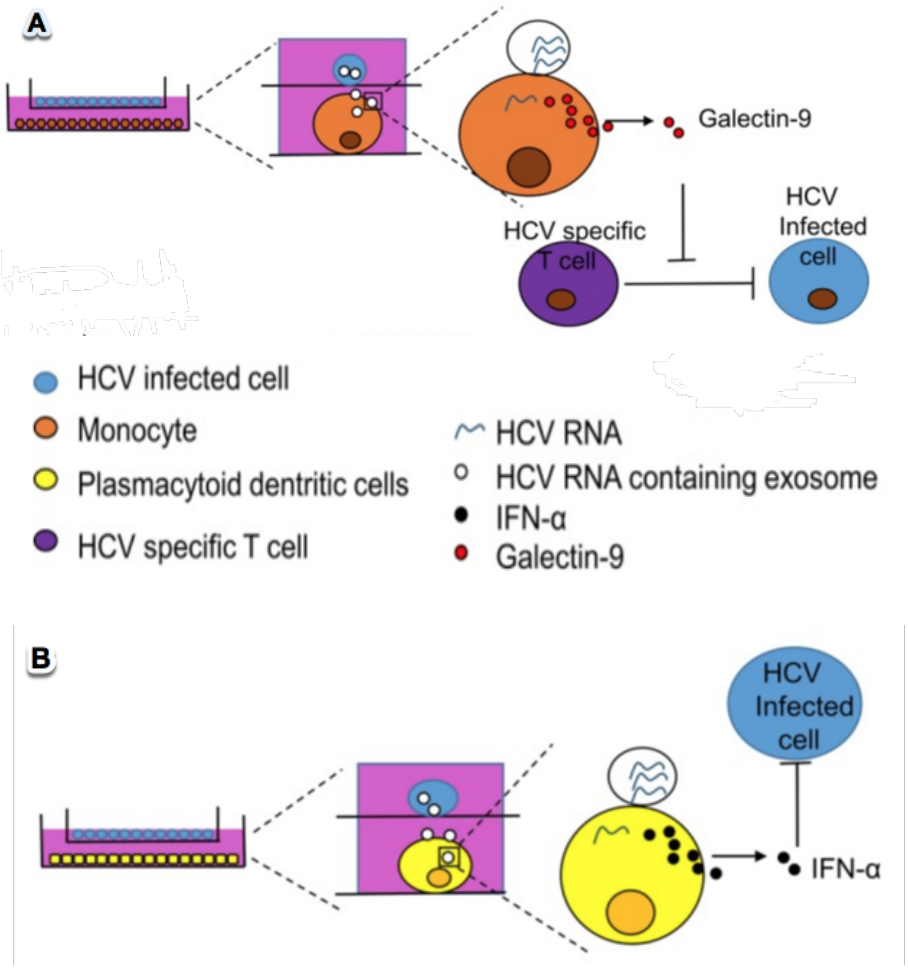

Figure 3.7.2-2 Roles of HCV-related exosomes on immune response(Shen et al., 2017). (a) HCV-RNA could be presented to PDCs *via* exosomes and activate the initial immune response (b) HCV-RNA could be presented to T cells *via* exosomes and inhibit the special immune response.

3.7.3 Exosomes in liver diseases

Some reports gave evidence that exosomal miR-214, which negatively regulated CCN2 (Connective tissue growth factor 2) expression in HSCs, has a considerable impact on liver fibrogenesis inhibition in activated HSCs (Chen et al., 2011a; Gressner et al., 2007). unlike miR-214, exosomal miR-19a and miR-192 involved in activation of the STAT3–TGF-β pathway in HSC, resulting in quicker fibrogenesis in liver cells have recently been demonstrated (Kim et al., 2019). It still seems so far unclear the exact role of HCV-related exosomes involved in liver fibrosis progression; Thus, further related studies will be needed to be done (Shen et al., 2017).

Studies investigated in the elucidation of the relationship between exosomes and HCC, showed exosomes derived from HCC harbor various miRNAs, which mainly promote tumor growth via modulating TAK1 expression. Moreover, lncRNAs (long no coding RNA)-containing exosomes

secreted from HCC has been proven as a mechanism of intercellular signaling pathways in HCC (Zhang et al., 2019). In fact, lncRNA H19, found in HCC cells-derived exosomes, impairs endothelial cells to promote angiogenesis in tumors (Conigliaro et al., 2015). Indeed, the most highly expressed lncRNA TUC339 has also been shown in exosomes secreted from HCC cells, resulting in altered tumor microenvironments involved in tumor growth (Sadri Nahand et al., 2019). Therefore, targeting there lncRNA-harboring exosomes might a new insight in HCC progression. Furthermore, some studies showed that HCC derived HSP90-containing exosome can increase the anti-tumor activity of NK (Natural killer) cells (Wang et al., 2014), thus HSPs expressing tumor exosomes may represent a promising alternative approach to the treatment of HCC.

CHAPTER 4: THE RETICULON FAMILY

4.1 Generality

The reticulon family is a group of evolutionary conserved ER membrane-associated proteins found throughout the eukaryotic kingdom (Yang and Strittmatter, 2007). Their presence only in eukaryotes suggests that RTNs evolved along with the eukaryotic endomembrane system. In mammals, 4 reticulon genes encoding for RTN1-4 each of which can have variable isoforms share a highly conserved c-terminal region called reticulon homology domain (RHD) with approximatively 73% of sequence identity among RTN1,3 and 4, whereas only 52% of identity is found between RTN2 and RTN4 (Yang and Strittmatter, 2007). Moreover, the deletion of RHD of RTNs completely abolishes their association with ER thus alters their cellular localization and ER-associated functions. In contrast to conserved RHD, the N-terminal region of RTNs display no sequence similarity and divergent expression even in the same organism, consistent with evolution- and cell-type-specific RTN functions. Reports showed that RTN2, 3, and 4 are ubiquitously expressed, while RTN1 expression can be only detectable in neural tissues (Di Sano et al., 2012; Yang and Strittmatter, 2007).

4.2 Reticulons functions

The RTN family is a large group of proteins displaying diverse cellular functions due to their numerous isoforms and divergent expression in mammals (Figure 4.2) (Yang and Strittmatter, 2007). Although most reticulon research pays lots of attention to reticulon (RTN4 in particular) functions in the central nervous system (CNS) and associated nervous disorders such as Alzheimer's, as our study focuses on exosomes biogenesis regulation in the context of HCV infection, we will particularly focus on the roles of RTNs in ER-shaping, in ER-Golgi trafficking and vesicles formation.

RTNs, known as ER-associated proteins, are involved in ER-membrane curvature (Chen et al., 2020; Diaz et al., 2010; Gao et al., 2019). Surprisingly, cells lacking reticulons expression do not exhibit any major defects in ER structure (Yang and Strittmatter, 2007). Moreover, the involvement of RTNs in material trafficking from the ER to the Golgi apparatus has been described (Wakana et al., 2005). In yeast Saccharomyces cerevisiae, RTN1B has been found in complex with Yip3p which is known to bind to multiple Rabs and act as a GDF (GDP displacement factor) for Rabs (Geng et al., 2005; Ortiz Sandoval and Simmen, 2012). Interacting with RTN1B and Yip3p thus can positively regulate Rab-dependent intracellular trafficking. Also, it's shown that RTN1 and

RTN4 take part in nuclear envelope assembly (Audhya et al., 2007) and nuclear pore complex formation (Kiseleva et al., 2007) respectively, each of which involves the ESCRT complexes (Pieper et al., 2020) resulting in the trafficking of materials within different compartments in cells. Furthermore, RTNs interacting with SNAREs and SNAPs, are involved in vesicle formation and recycling. RTN1C has been co-precipitated and found in the complex with syntaxin 1/7/13 and VAMP2 (SNAREs) involved in vesicle formation (Steiner et al., 2004). Moreover, some studies were also showed that RTN1/2/4 interacts with SNAP, which is known as an adaptor in SNAREs activation and recycling, promoting membrane trafficking and vesicle formation and recycling (Martin et al., 2006).

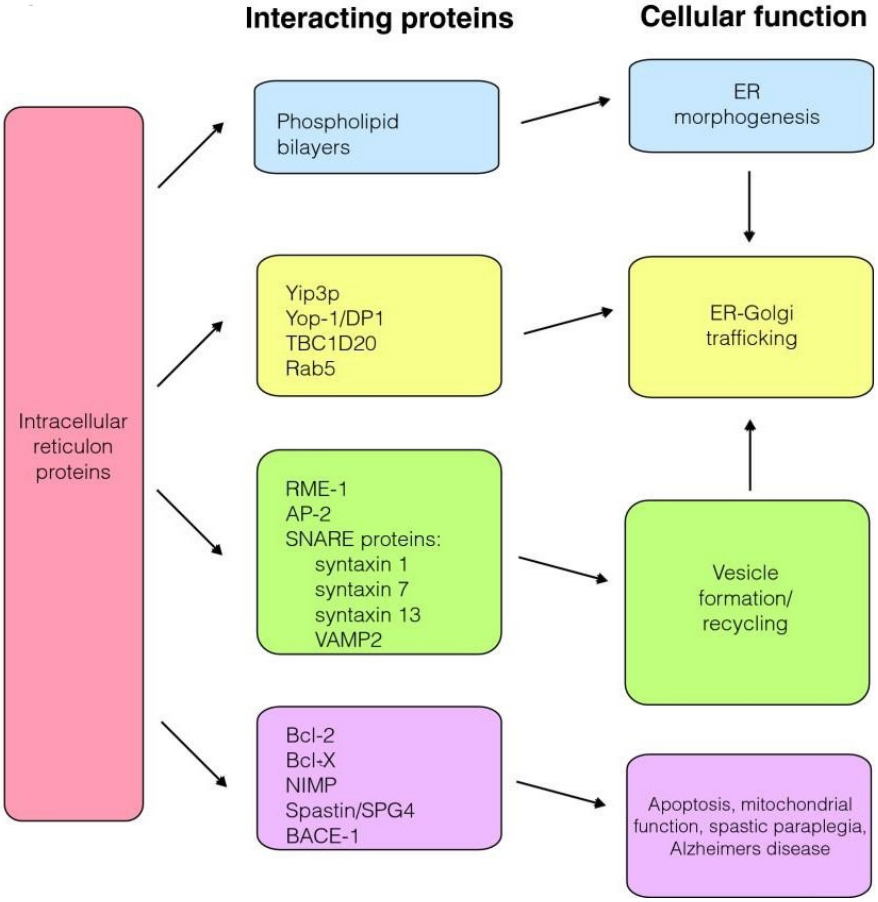

Figure 4.2 Interaction between ER-associated reticulons and different proteins exhibits diverse cellular functions(Yang and Strittmatter, 2007).

4.3 Reticulon 3 (RTN-3)

4.3.1 Human RTN3 gene and RTN3 isoforms

As shown in Figure 4.3.1-1, the human RTN3 gene has a size of approximately 76kb constituted by total of 9 exons under control of 3 different promoters (Di Scala et al., 2005). RHD is encoded exon 3, which is distant from exon 1. Evidence supports that exons found in the human RTN3 gene can be alternatively spliced, resulting in the generation of diverse RTN3 isoforms. Exons 1 and 2 are skipped in several human ESTs, whereas two 5'-splicing acceptor sites (3.1 and 3.2) have been identified in exon 3, strongly supporting alternative splicing mechanism in these exons.

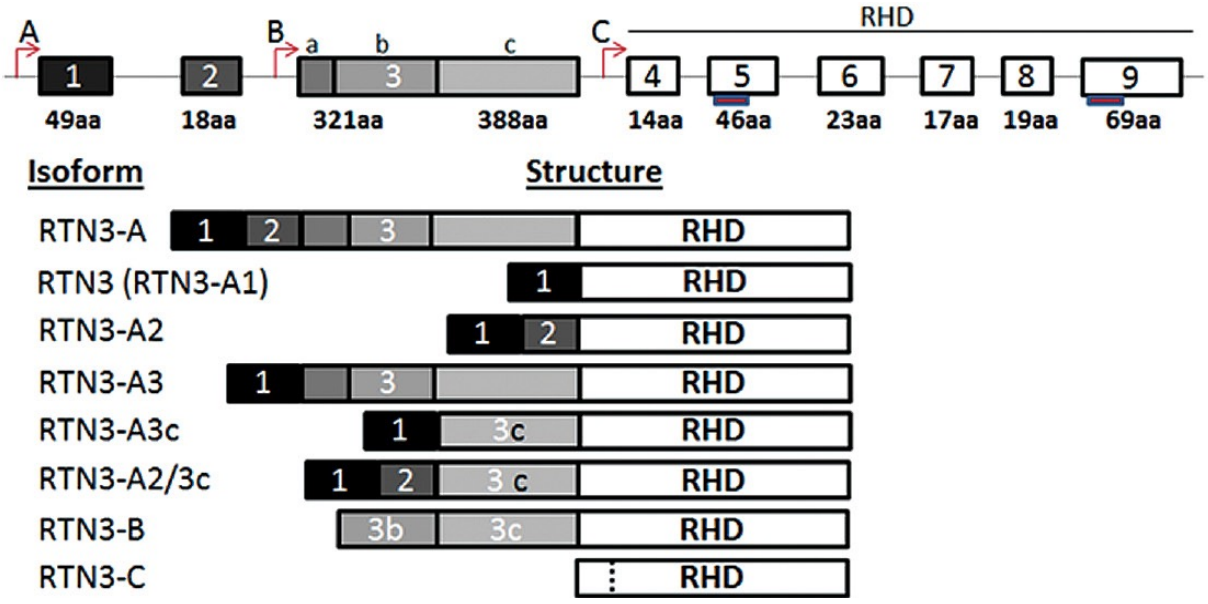

Figure 4.3.1-1 Schematic representation of the human RTN3 gene and spliced isoforms(Di Scala et al., 2005).

RTN3 RHD hydrophobic has been found to span into the ER membrane, whereas C- and N-terminal sequence projects into the cytoplasm (Figure 4.3.1-2) (Grumati et al., 2017). In fact, RHD of RTN3 is constituted by nearly 190 amino acids (aa), which has 2 trans-membranous domains forming a cytoplasmic 66 residue-loop. Moreover, it's believed that RHD is involved in basic cellular function, while the diversity displayed at N-terminal carry out isoform-specific functions.

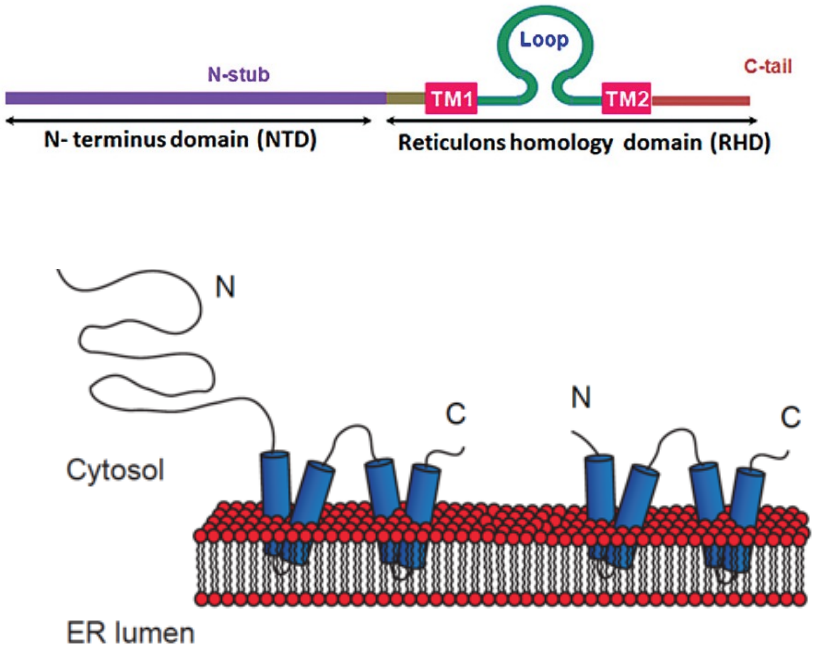

Figure 4.1.1-2 Schematic representation of RTN3 structure and its isoforms(Grumati et al., 2017).

Up to now, 7 isoforms of RTN3 (RTN3A to RTN3G) have been identified. Among them, RTN3E is the longest, which is composed of 1032aa with a size of 112 kDa, whereas the shortest isoform, RTN3A, consists of 236aa with a size of 25kDa (Table 4.3.1).

Table 4.3.1 RTN3 isoforms.

PubMed annotation	RefSeqs	Isoform lengths	Isoform size	Mutation in RHD
RTN3A	NM_006054.3	236aa	25KDa	-
RTN3B	NM_201428.2	1013aa	110KDa	-
RTN3C	NM_001265589.2	255aa	27KDa	-
RTN3D	NM_201430.3	241aa	26KDa	+
RTN3E	NM_001265589.2	1032aa	112KDa	-
RTN3F	NM_001265590.2	920aa	100KDa	-
RTN3G	NM_001265591.2	214aaa	23KDa	+

4.3.2 RTN3 functions

Inconsistent with other RTNs, RTN3 is involved in ER bending and shaping (Grumati et al., 2017), in apoptosis (Zhu et al., 2007), in intracellular trafficking (Wakana et al., 2005) and vesicles

formation (Siddiqi et al., 2018). Overexpression of RTN3 in Hela cells prevents retrograde transport of proteins from the Golgi complex to the ER. The knockdown of RTN3 is associated with VLDL secretion through reducing VTV (VLDL transport vesicles) biogenesis (Siddiqi et al., 2018). Moreover, RTN3 has also been identified as an interactor as well as a down-regulator of BACE1, an amyloid precursor protein (Appel et al.,2006) protease required for β-amyloid peptides (Aβ) generation (Shi et al., 2014). Mechanistically increased RTN3-BACE1 interaction prevents BACE1 from interacting with its cellular components as well as their trafficking to the endosomes and other compartments such as axons. Interestingly, this reduction of BACE1 interaction network is also correlated with the reduced anterograde transport of BACE1 in axons(Borgia et al., 2018; Deng et al., 2013), suggesting RTN3 might be served as a therapeutic target of Alzheimer's disease. Interestingly, Interacting with Bcl-2, RTN3 can also upregulate apoptosis, suggesting its promising role in tumor suppression (Zhu et al., 2007).

In summary, numerous functions of RTN3 have been demonstrated in different study contexts. As we took advantage of HCV infection to study the regulatory role of RTN3 in exosomes biogenesis, more detailed functions displayed by RTN3 in viral replication will be described below. Moreover, it's possible that autophagosomes (the main player in autophagy) fuse with MVBs, instead of lysosomes, to form amphisomes. One of the fates of these amphisomes is to fuse with PM allowing their components released as exosomes. Thus, RTN3 functions in autophagy seem also need to be discussed in detail.

- **RTN3 in viral replication**

RTN3 has been proven as a very important host molecule involved in viral replication. Reports showed that direct interaction between RTN3 with N-terminal EV71 (*Enterovirus 71*) 2C protein efficiently enhance EV replication (Li et al., 2015; Tang et al., 2007), whereas siRNA-mediated RTN3 knock-down attenuated Brome mosaic virus (BMV) replication (Diaz and Ahlquist, 2012).

Infection by West Nile virus (WNV), one of the Flavivirus, promotes a redistribution of RTN3 in ER, facilizing the NS4B recruitment to the viral replication complex and enforcing WNV replication (Aktepe et al., 2017). However, this NS4B-RTN3 interaction has not been considered as a conserved mechanism in Flavivirus since neither Dengue virus (DENZ) nor Zika virus (ZIKV) NS4A recruitment via RTN3 has been observed. Critically, siRNA-mediated knockdown of RTN3.1A expression attenuated WNV, DENV, and ZIKV replication and severely affected the stability and abundance of the NS4B protein, coinciding with a significant alternation and reduction of viral membrane structures in the ER (Aktepe et al., 2017).

In the context of HCV infection, RTN3 has been identified as an interactor of HCV NS4B protein by yeast-two hybrid screen (Liu et al., 2005). Self-oligomerization of NS4B protein is required for the membranous web formation that is essential for viral replication. Known that ER is the site containing abundantly replication competent HCV RNA actively mediating HCV replication, evidence provided by Wu MJ et al., in 2014 (Wu et al., 2014), has shown that RTN3 can disrupt NS4B self-interaction thus negatively regulates HCV replication, whereas another study showed that RTN3 has no impact on HCV replication (Lin et al., 2017; Wu et al., 2014). Moreover, RTN3 has been identified as a novel regulator of HCV replication by interacting with HCV NS5A protein, demonstrated by NS5A interacting network analysis (Tripathi et al., 2013).

- **RTN3 in autophagy**

The relationship between ER and autophagy has been demonstrated in at least 3 manners; i) the ER-membrane is thought to be one of the sources of phagophores (Lamb et al., 2013; Zhuang et al., 2017); ii) autophagy is triggered upon ER stress to maintain cellular homeostasis; and iii) the ER, the site responsible for cellular quality control, is itself a target of autophagy, termed as 'ER-phagy' or 'reticulophagy' (Loi et al., 2018). Therefore, autophagy is believed to be involved in ER homeostasis, even if the mechanisms that control ER homeostasis and restore ER upon ER stress are not completely understood (Loi et al., 2018). Study in yeast showed Atg8 directly interacts with the RHD-containing protein Atg40 to mediate ER-phagy upon starvation conditions, giving rise to a new insight of the important involvement of HRD in autophagy pathway (Mochida et al., 2015). Other studies revealed that RTN3, whose activation is linked to ER stress, responsible for the impairment of the ubiquitin-proteasome system (UPS) in ER and induction of autophagy (D'Eletto et al., 2020; Grumati et al., 2017; Ling et al., 2019). However, others demonstrated inhibition of RTN3 promotes autophagy inhibition by favorizing the interaction between Bcl-2 and Beclin 1(Chen et al., 2011b). Moreover, Grumati et al.,' work in 2017 (Grumati et al., 2017) identified in mammals, RTN3L (the long isoform of RTN3) as a novel receptor of ER-phagy involving in selective degradation of ER tubules (Grumati et al., 2017). Overexpression of RTN3L can sufficiently induce ER-fragmentation in an autophagy-dependent pathway. In fact, RTN3L which is in the complex with proteins lacking RHD domains, bind to ATG8 through GABARAP and MAP1L3-interacting motifs ultimately inducing a selective degradation of specific cargo via ER-phagy (Lystad and Simonsen, 2019).

2 HYPOTHESIS AND OBJECTIVES OF RESEARCH

- **PROBLEMATIC**

An increasing number of studies have demonstrated that almost every virus takes advantage of exosomal communication to aid viral spread and hinder host antiviral responses. The ultimate reason for exploiting the exosomal pathway seems to be a means of establishing viral persistence and immune escape in the host.

Exosomes are small extracellular vesicles with a size range of 30-100nm in diameter with an endocytic origin. Sequential invagination of the plasma membrane ultimately results in the formation of MVB (multivesicular bodies), which can intersect with other intracellular vesicles and organelles, contributing to diversity in the constituents of exosomes. Furthermore, evidence supports the importance of exosomes in cell communication since their bioactive components could be easily transferred to neighboring cells and ultimately modulates the cellular function in a precise way. Also, what remains striking is the molecular composition of exosomes involved in viral infection and disease persistence is quite different from those in physiological conditions. Our previous study showed that HCV RNA is associated with Ago2/HSP90 in exosomes released from HCV-infected Huh7 (Bukong et al., 2014). Moreover, exosome-mediated HCV RNA could be efficiently transferred to naïve cells and maintains a considerable viral persistence in the host. Given these observations, we surmise that the molecules in exosomes involved in pathophysiology are not randomly loaded inside. However, the key regulators and molecular mechanisms through which viruses including HCV, mediate the selective sorting of viral and host molecules for loading and release into exosomes remain still unclear.

- **HYPOTHESIS**

Reticulons (RTNs), including RTN1-4, are a group of conserved proteins found predominantly in the ER. The ER is a site containing highly abundant replication-competent HCV RNA, which actively mediates viral replication. Numerous studies showed RTNs involvement in ER morphogenesis, intracellular trafficking, and vesicle formation. Our preliminary findings revealed a significant increase in RTN1-4 expression in HCV JFH1 infected cells compared to controls. Strikingly, the increased RTN1-4 expression was also observed in HCV full-length replicon (FL-Con1) and sub-genomic replicon (BB7) cells. These replicons bypass early endosomal formation, which is different from the classical HCV-JFH1 entry and infection. However, these replicons are still capable of producing infectious exosomes. Furthermore, the siRNA mediated knockdown of RTN1 significantly reduced the number of cell-released exosomes and the amount of negative-

sense viral RNA inside exosomes. Given these observations, we surmise that RTNs might play a role in determining the quantity and cargo of infectious exosomes, through both the endosomal-derived dependent and independent pathway.

- **OBJECTIVES**

The main objective of our study is to specifically determine the role of RTN3, in modulating specific cellular and viral cargo for loading and release of exosomes during the HCV infection. Moreover, 7 isoforms of RTN3 have been found so far with diversity in the N-terminal region, which is thought to be capable of exhibiting cell-type specific and/or cellular state-specific functions. Given these perspectives, we decided to take advantage of the shortest and the longest isoforms of RTN3, named RTN3S and RTN3L respectively, to separately investigate their role in exosome biogenesis regulation in the context of HCV infection.

Objective 1: To evaluate RTN3S&L expression at the cellular and exosomal level in HCV infection. RTN3S&L expression in HCV JFH1-infected hepatocytes, in HCV FL-replicon cells as well as in HCV-infected patients' liver samples, along with their proper controls have been analyzed by western-blot. Further, HCV FL replicon cell-derived exosomes and HCV-infected patients' serum samples have been isolated, purified, and quantified by Nanosight NS3000. Then, RTN3S&L expression has been analyzed and normalized by using extracted total exosomal proteins of an equal number of exosomes.

Objective 2: To decipher the role played by RTN3S&L in exosomes loading and release. Exosomes from siRNA-mediated and CRISPR-Cas9 based knockdown of RTN3S&L HCV FL replicon cells have been isolated, purified, and quantified by Nanosight NS3000. The capacity of transmission of ARN viral-mediated by exosomes has also been evaluated by co-culturing with naïve Huh7 cells. Similar experiments have also been carried out in the context of different mutants of RTN3S&L overexpression conditions. To rule out the possibility that RTN3S&L facilize HCV replication which could ultimately enhance exosome secretion, the role of RTN3S&L on HCV replication has also been demonstrated.

Objective 3: To identify the possible interactors of RTN3S&L involved in infectious HCV exosomes loading.

3 ARTICLE

Title of the article: Reticulon-3 Modulates the Incorporation of Replication Competent Hepatitis C Virus Molecules for Release Inside Infectious Exosomes

Titre en français : Reticulon-3 module la composition et la libération des exosomes lors de l'infection par le virus de l'hépatite C

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Authors contribution statements

Jingjing Li carried out experiments with support from Terence Ndonyi Bukong and Patrick Labonté. She also performed analysis for the experimental data and drafted the manuscript.

Terence Ndonyi Bukong was involved in planning and supervised the work. He also participated in processing the experimental data, in performing the analysis, drafting the manuscript, and in designing the figures.

Carla S. Coffin provided human clinical samples. Carla S. Coffin and Patrick Labonté aided in discussing the results and in commenting on the manuscript.

Ebtisam Abosmaha carried out western-blot experiments for overexpression of RTN3L in HCV FL-replicon cells.

PLOS ONE

RESEARCH ARTICLE

Reticulon-3 modulates the incorporation of replication competent hepatitis C virus molecules for release inside infectious exosomes

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Abstract

Background

Cell released microvesicles specifically, exosomes, play an important role in mediating immunologic escape, treatment resistance, and disease persistence of Hepatitis C virus (HCV) infection. Reports on the molecular compositions of exosomes released by cells under diverse conditions, especially during viral infections, suggest that their cargo contents are not randomly loaded. However, the precise molecular mechanisms directing the selective cargo sorting and loading inside infectious viral exosomes remains elusive.

Aim

To decipher the role of Reticulon 3 (RTN3) in the selective molecular cargo sorting and loading inside infectious viral exosomes during HCV infection.

Methods

We used Huh7 cells–JFH1 HCV infection and HCV Full-Length (FL) replicon systems. Additionally, we analyzed human liver and serum exosome samples from healthy and treatment naïve HCV infected individuals. Our experiments made use of molecular biology and immunology techniques.

Results

HCV infection (JFH1-Huh7 or HCV-FL replicon cells) was associated with increased RTN3L&S isoforms expression in cells and cell released exosomes. Accordingly, increased expression of RTN3L&S was observed in liver and serum exosome samples of HCV infected individuals compared to healthy controls. RNA-ChIP analysis revealed that RTN3L&S interacted with dsHCV RNA. Lentiviral CRISPR/Cas9 mediated knockdown (KD) of RTN3 and plasmid overexpression (OE) of wild type, C- and N-terminal deletion mutants of RTN3L&S in HCV- infected Huh7 cells differentially impacted the cellular release of

infectious viral exosomes. RTN3L&S KD significantly decreased, while RTN3S OE significantly increased the number of Huh7 cell-released infectious exosomes. The C-terminal domain of RTN3 interacted with and modulated the loading of dsHCV RNA inside infectious exosomes. Antiviral treatment of HCV infected Huh7 cells reduced virus-induced RTN3L&S expression and attenuated the release of infectious exosomes.

Conclusion

RTN3 constitutes a novel regulator and a potential therapeutic target that mediates the specific loading of infectious viral exosomes.

Introduction

Hepatitis C virus (HCV) infection is an important cause of morbidity and mortality globally. HCV is an envelope positive-sense single-stranded RNA Flavivirus with a genome size of approximately 9.6kb [1]. The HCV genome contains an open reading frame (ORF) encoding a single polyprotein which is cleaved by cellular and viral enzymes into ten mature proteins [1]. There is currently no effective HCV vaccine and the World Health Organization estimates that there are over 71 million [2] individuals worldwide with active infection. If untreated, approximately 70–80% of HCV infected individuals will develop complications with progressive liver fibrosis, cirrhosis, and hepatocellular carcinoma [3, 4]. The use of pan-genotypic direct-acting antiviral (DAAs) regimens is curative in >95% of HCV infected individuals [5]. However, access to diagnosis and treatment in some countries is very limited and resistance to some DAA treatment regimens is continuously being reported [6–8]. The propensity of HCV to establish persistent infection stems from multiple remarkable strategies by the virus to evade host immune and therapeutic strategies [9, 10]. In 2013–2014 groundbreaking reports including our study revealed that viruses can hijack cell-released extracellular vesicles (EVs), precisely, exosomes, to evade host immune and therapeutic strategies leading to persistent infection [11, 12]. Specifically, these studies revealed that exosomes can harbor replication-competent viral material and can bypass classical receptor-mediated viral entry mechanisms to facilitate active viral infection of naïve cells [11, 12]. Strikingly, recent scientific reports have also implicated exosomes in the pathomechanism of several viral infections including HBV [13], the Human Immunodeficiency Virus (HIV) [14], Human T-cell Lymphotropic Virus (HTLV) [15], Ebola Virus [16] and Zika Virus [17].

Exosomes are cell-derived microvesicles that are continuously produced by almost all cell types into the extracellular space and range in size from 30 to 150 nm. The molecular composition of exosomes most often reflects the physiological/pathological state of the cells they originate from [18]. In addition to their pathogenic role, exosomes also carry out important cellular communication functions by interacting and /or transferring their cargo contents to target recipient cells altering their function in precise ways [19]. Even when released by the same cell, each exosome is composed of a specific repertoire of proteins, lipids, and nucleic acids while others are excluded [20]. These observations suggest that the molecular cargo found inside a specific exosome is not randomly loaded. Numerous mechanisms have recently been proposed on how specific cellular molecules by utilizing specialized cellular mechanisms can modulate the molecular composition of exosomes in both normal, stress, and infection conditions [21]. Studies have proposed that exosomal protein composition can be controlled by endosomal complex required for transport (ESCRT)-dependent and -independent mechanisms [22].

Notwithstanding, most described mechanisms seem to act differently depending on the cell type and stimuli/infection which ultimately results in the production of different subsets of exosomes even by the same cell. While exosome biogenesis was suggested to originate from multivesicular bodies (MVB) [20], observations of different types of exosomes from the same cell suggest the possible existence of different MVB subsets. Taken together, the mechanisms by which specific cellular molecules are selectively loaded inside exosomes while others are excluded especially during viral infections remain poorly understood.

Here, we explore for the first time the role of cellular Reticulons (RTNs), specifically RTN3, in modulating the specific incorporation of host and replication-competent viral molecules for release inside exosomes. Reticulons (RTNs) are a large family of evolutionarily conserved proteins predominantly located at the endoplasmic reticulum (ER) of cells. They are most often associated with membrane morphogenesis, intracellular trafficking, and microvesicle formation [23, 24]. This family of proteins contains four main gene products (RTN1, RTN2, RTN3, and RTN4) [23]. Genes encoding for reticulons contain many introns and exons, and most are alternatively spliced into multiple protein isoforms [25]. Morphologically, RTNs contain a highly conserved C-terminal Reticulon Homology Domain (RHD) and an N-terminal domain which is highly variable [23, 24]. RTN proteins are mostly enriched in the nervous tissue, however, RTN3 and RTN4 are expressed ubiquitously [23]. Primarily RTNs have been associated with promoting membrane curvature development, nuclear pore complex formation, vesicle maturation, autophagy, and inflammatory functions [23, 26, 27]. In the context of viral infections, RTNs have been associated with the replication of single-stranded RNA viruses and membrane trafficking of early secretory proteins [23, 28]. Recent reports have revealed that RTN3 can mediate viral remodeling of host cell membranes and the stabilization of viral proteins within the endoplasmic reticulum during flavivirus replication [28]. Furthermore, RTN3 has been associated with direct modulatory role during HCV viral replication [29, 30]. However, the role of RTN3 in modulating the selective cargo sorting associated with infectious viral exosomes release from HCV infected cells has not been evaluated.

Here, we reveal RTN3 as a novel regulator that mediates the specific loading of infectious cell-released viral exosomes. Our observations also suggest that RTN3 represents a novel infection biomarker and therapeutic target that can be exploited to prevent the cellular release of infectious viral exosomes associated with HCV infection.

Material and methods

Cell lines and hepatitis C virus

Huh7 cells (a gift from Dr. Charlie Rice, Rockefeller University, New York) and HCV FL replicon [HCV genotype 1b] (a gift from Takaji Wakita) were cultured as previously described [11] with slight modification, using exosome depleted FBS (System Bioscience cat. #Exo-FBS-50A-1). Infectious HCV genotype 2a (clone JFH1) virus was generated as previously described [31, 32].

Exosome isolation from cell culture supernatants and human serum samples

Cell culture supernatants of Huh7, JFH1 infected Huh7 and HCV FL replicon cells were collected and centrifuged at 800× g for 5 minutes to remove cell debris. To concentrate microvesicles in cell culture supernatants, cleared supernatants were then transferred into Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-100 membrane (Millipore, cat. #UFC910024) and followed by series of centrifugations at 3,500× g for 20 minutes. Concentrated culture

supernatants were mixed with the appropriate volume (50μl—150μl) of Exoquick (System Bioscience cat. #EXOQ5A-1) according to the manufacturers' specifications and centrifuged at 6000×g for 20min. The exosome pellet was washed 2 times with PBS by centrifugation at 800x g at 4˚C for 5 minutes and re-suspended in 1X phosphate buffer saline (PBS). For JFH1 and serum exosomes from human samples, further positive anti-CD63 immuno-selection was performed as described previously [11].

Transfections of siRNA and over-expression plasmids

Cellular transfections were done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Small interfering RNA (siRNA) targeting Reticulon (RTN3) or control (scrambled) (Life Technologies). Overexpression plasmid with a flag-CMV-2 backbone was used for expressing RTN3S full-length (FL), N-terminally or C-terminally truncated RTN3s mutants (i.e., ΔN11, ΔN35, ΔN45, and ΔC36) (gift provided Prof. Mitsuo Tagaya, Tokyo University of Pharmacy and Life Science) [24], and pMRX-INU-FLAG-RTN3L (gift from Noboru Mizushima, Addgene plasmid # 128264; http://n2t.net/addgene:128264; RRID: Addgene_128264) [33].

Lentiviral CRISPR-Cas9 knockdown, transfections, and Co-culture experiments

Specific Reticulon 3 target guide RNA sequences for CRISPR-Cas9 were designed using the website (http://www.e-crisp.org/E-CRISP/). Specific oligonucleotide primer pairs were used to amplify the long and short isoforms of human Reticulon 3: RTN3L Forward: `5'-CACCGCCATGTGTTAGGGAGCCAGCCT-3'`; RTN3L Reverse complement: `5'-AAACAGGCTGGCTCCCTAACACATGGC-3'` and RTN3S Forward:`5'-CACCGGGAGATGGAATGGGACTGAG-3'`, and RTN3S Reverse complement: `5'-AAACCTCAGTCCCATTCCATCTCCC-3'`. The complementary oligonucleotides for guide RNAs (gRNAs) were annealed and cloned into the LentiCrispr V2 vector [LentiCrispr, a gift from Feng Zhang; Addgene plasmid # 52961; http://n2t.net/addgene:52961; RRID: Addgene_52961)] [34]. The human GeCKOv2 CRISPR knockout pooled library was a gift from Feng Zhang (Addgene catalog #1000000048) [34]. Hemagglutinin (HA) lentivirus vector was used as a control. The LentiCrispr V2 vector was transfected into HEK-293T cells using Lipofectamine 2000 and media exchanged after 12h. The lentivirus was harvested from the cell culture media after three days, and viral particles were concentrated by ultracentrifugation. Huh7-JFH1 infected and FL Replicon cells were co-cultured with the RTN3L&S CRISPR-Cas9 lentiviruses or the HA lentivirus control. Cellular RTN3L&S expression in target cells was analyzed by western blotting.

Real-time quantitative PCR for detection of HCV RNA from cells and exosomes

Total RNA was extracted by initially lysing cells with Trizol (Invitrogen, CA), and purified using RNeasy micro kit (cat. #74004, Qiagen) according to the manufacturers' instructions. Briefly, 1μg RNA was subjected to reverse transcription using the iScript reverse transcription supermix (Bio-Rad, California, United States). The obtained cDNAs were used for qPCR using a Bio-Rad CFX96 system with SYBR green. The target primers/reaction conditions used methods described previously [35]. The comparative delta-delta ct method was used to analyze specific genes using 18s RNA as the normalizing gene, similar to previous studies [11, 36].

Western blotting to detect specific proteins

Total cellular proteins from Huh7.0, JFH1-infected Huh7.0, and HCV FL replicon cells were extracted using RIPA buffer (cat. #BP-115, Boston Bioproducts) as previously described. Proteins resolution was done on an 8–15% gel SDS-PAGE denaturing gel. Resolved proteins in acrylamide gels were transferred onto Polyvinylidene fluoride (PVDF) membranes and membranes were blocked in PBS containing 5% dry milk for 1h, then probed with specific primary antibodies in the recommended dilutions at 4˚C for overnight. The following primary antibodies were used: anti-HCV NS3 (Abcam cat. #ab13830 and cat. #ab65407); anti-HCV NS5A (Abcam cat. # ab13833 and BioFront cat. # HCV-2F6); anti-HSP90 (Abcam cat. # ab13492); anti-FLAG (Abcam cat. #ab1162); anti-CD63 (Abcam cat. #ab8219 and Santa Cruz Biotechnology cat. #sc-15363); RTN3 antibody (Abcam cat. Ab68328 and Thermofisher cat. # PA5-53360); TSG101 (Abcam cat. # ab125011); normal rabbit IgG-AC antibody (Santa Cruz Biotechnology cat. # sc-2345); anti-beta actin [Ac-15] (Abcam, cat. #ab6276). Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology cat. # sc-2004 and sc-2005) and clarity TM Western ECL blotting substrate (Bio-Rad) was used for visualization with the ChemiDoc XRS+ system (Bio-Rad, California, United States).

Exosome quantification in HCV infected vs. control in vitro and Ex-Vivo clinical samples

Quantification of cell released microvesicles were done using Nanoparticle Tracking Analysis (NTA) with NanoSight NS300 (Malvern, UK) and NanoSight NTA software v3.2. Briefly, cell culture supernatants and human serum samples were pre-cleared to remove any cellular debris by centrifugation at 5000 Revolutions Per Minute (RPM) for 5 minutes. The cleared cell culture supernatants and serum samples were filtered through a 0.22 μM membrane into sterile tubes. The quantification of exosomes was done using the NTA measurement system as follows: 25˚C, 25 frames per second, 3 measurements per sample.

RNA chromatin immunoprecipitation (ChIP) and co-immunoprecipitation analysis

Cell samples were fixed at room temperature with 4% formaldehyde buffered saline. Cells were subsequently lysed in SDS ChIP lysis buffer (Millipore cat. # 20–163) supplemented with protease and RNase inhibitor. Total cellular proteins were pre-cleared with protein G beads. 100 μg of total protein was incubated with anti-dsRNA, RTN3S, and RTN3L antibodies. Immunoprecipitation was performed overnight at 4˚C using 10 μg/ml primary antibody and normal rabbit/mouse IgG (Santa Cruz cat #sc-3877 and sc-69786) non-specific antibody serving as IP control. A mixture of Protein A/G PLUS-Agarose beads (Santa Cruz cat. #sc-2003) was added, and the incubation was continued for an additional 60 minutes. The samples were washed with SDS ChIP lysis buffer supplemented with protease inhibitor and RNase inhibitor. The immunoprecipitants (protein-RNA complexes) were either used for Western blot analysis or RNA purification using the Trizol reagent and RNeasy kit. Purification of RNA included spiking all samples with 1uL of cel39RNA before total RNA extractions following RTN3L and RTN3S pull-downs. Negative -sense HCV RNA and miR122 quantification were done using RT-qPCR with CFX Connect Real-Time PCR Detection System (Philadelphia, USA), using methods described previously. RT-qPCR data were normalized to miR Cel39 and fold change was calculated using the delta-delta ct method as previously described [11].

Human samples and ethics statement

Human serum samples were collected at the University of Calgary Liver Unit under an approved ethics protocol for use of human samples for research [Conjoint Ethics Research Board (CHREB) ID: REB14-1965_REN1]. All healthy control subjects had no evidence of systemic disease, HCV infection, or other liver diseases. All subjects who donated samples for this project provided signed written informed consent. Human liver biopsy specimens were obtained from the liver tissue cell distribution system (LTCDS), [Minneapolis, Minnesota] [Pittsburgh, Pennsylvania] [Richmond, Virginia], which was funded by NIH contract # N01-DK-7-004/HHSN26700700004C.

Data analysis

Data were presented as mean +SEM. Statistical analysis was carried out by using the Mann-Whitney U test on GraphPad Prism version 8.1.2. All data presented are representative of at least 3–4 independent repeat experiments. A p-value of <0.05 was considered significant.

Results

HCV infection is associated with increased Reticulon-3 expression in Huh7 cells and exosomes

A recent report revealed increased intrahepatic RTN3 expression which correlated with higher HCV genotype 1 RNA levels [37]. Further, conflicting reports have indicated that RTN3 knockdown in cells can lead to either an increase [29] or no change [30] in intracellular HCV RNA levels. Also, RTN3 has been shown to modulate intercellular molecular trafficking and linked to microvesicles biogenesis and secretion by cells [24, 27, 38]. Total protein was extracted from Huh7 and HCV JFH1 infected Huh7 cells. We found that HCV JFH1 infection of Huh7 cells was associated with increased RTN3L&S isoform proteins compared to control (uninfected) cells by western blotting analysis (**Fig 1A**). Similar to our previous report [11], we found that HCV JFH1 infection of Huh7 cells was associated with the increased cellular release of exosomes (**Fig 1B**). We used exosome isolation and NanoSight NS300 quantification methods as described previously [11]. We found that cell released exosomes from HCV JFH1 infected Huh7 cells were significantly enriched with RTN3L, based on western blot analysis (**Fig 1B**). We did not detect Calnexin protein in our analyzed exosome samples (**Fig 1B**), suggesting that our methodology (as described previously [11]), successfully excluded microsomal or cellular debris contamination [39]. Next, we evaluated whether RTN3L&S expression in HCV FL-replicon cell-released infectious exosomes but not free virions. By western blotting analysis, we found increased expression of RTN3L&S isoforms in HCV FL-replicon cells compared to parent Huh7 control cell line (**Fig 1B**). Additionally, compared to control (uninfected) cells, HCV FL-replicon cells released significantly more exosomes (**Fig 1C**). Similar to JFH1 infection (**Fig 1B**), the released exosomes from HCV FL-replicon cells was associated with increased RTN3L protein expression (**Fig 1D**).

We used human clinical samples from healthy and HCV infected individuals (**Tables 1 and 2**) to enhance the translational relevance of our study and the potential role of RTN3 in HCV infection. We found that HCV infection was associated with increased hepatic RTN3L&S protein expression (**Fig 1D**). Specifically, we found by western blotting analysis an increase in RTN3L&S protein expression in HCV infected individuals compared to healthy controls (**Fig 1E**). We also detected, as previously reported [11], that serum from persons with HCV infection showed significantly increased exosomes compared to control subjects (**Fig 1F**). Strikingly, we found by western blotting analysis that serum exosomes samples of HCV infected

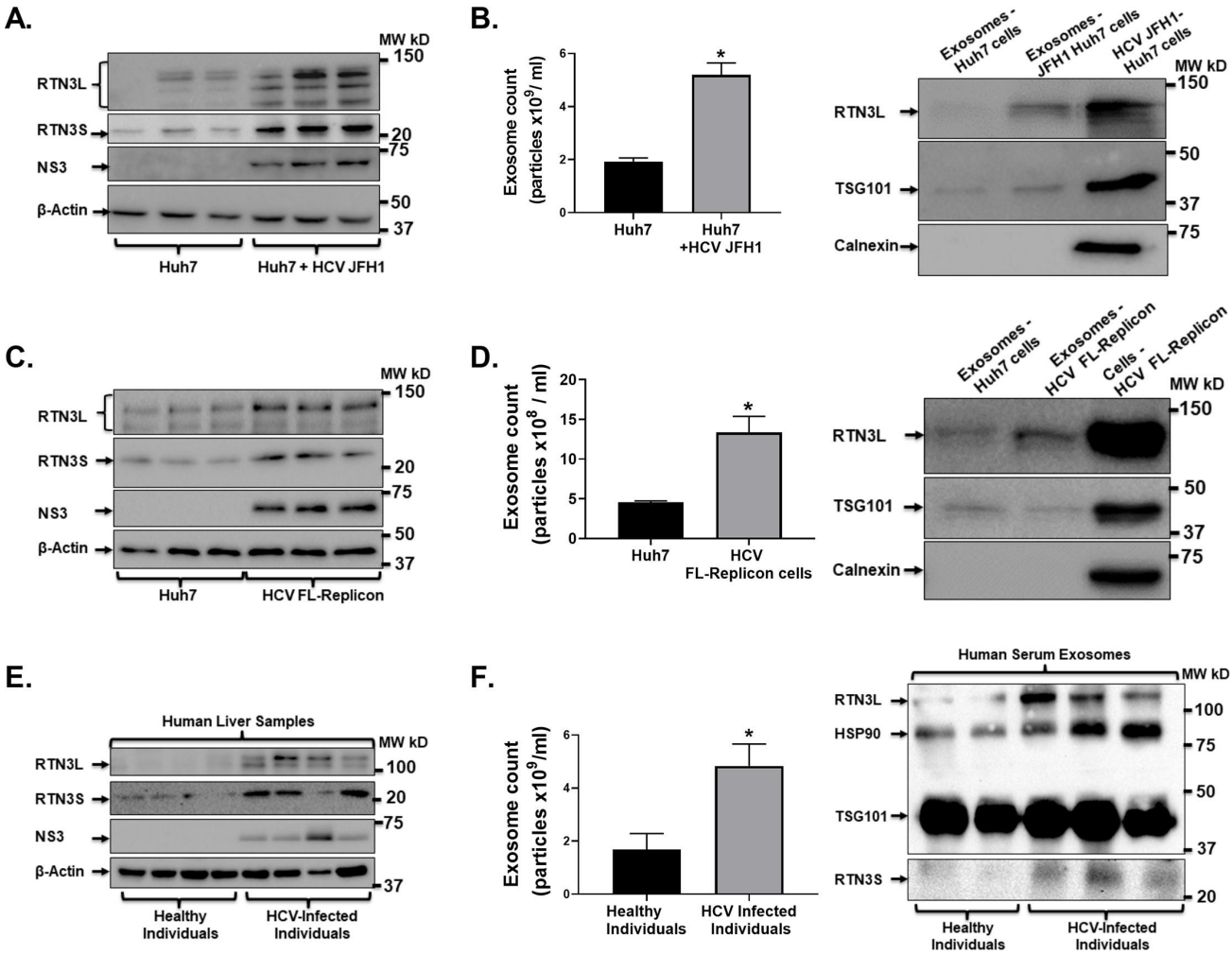

Fig 1. HCV infection induces increased RTN3L&S expression in Huh7 cells and cell released exosomes. (**A**) Total cell proteins were extracted from Huh7 and HCV JFH1 infected Huh7 cells and analyzed by western blotting probing for RTN3L&S and HCV NS3 with β-actin serving as a control for equal protein loading. (**B-Left panel**) Cell released exosomes from Huh7.0 and JFH1 infected Huh7 cells and quantified by NanoSight NS300. (**B-Right panel**) Total proteins were extracted from cell released exosomes in cell culture media of Huh7.0 cells and HCV JFH1 infected Huh7.0 cells, with total cell proteins from HCV JFH1 infected Huh7.0 cells serving as a positive control. Extracted total proteins from exosomes were analyzed by western blotting probing for RTN3L, TSG101, and Calnexin. (**C-Left panel**) Total cell proteins were extracted from Huh7.0 and HCV FL-Replicon cells and analyzed by western blotting probing for RTN3L&S and HCV NS3 with β-actin serving as a control for equal protein loading. (**C-Right panel**) Cell released exosomes from Huh7.0 and HCV FL-Replicon cells were quantified by NanoSight NS300. (**D-Right panel**) Total proteins were extracted from cell released exosomes in cell culture media from Huh7.0 cells and HCV FL-Replicon cells with total cell protein from HCV FL-Replicon cells serving as a positive control. (**D-Left panel**) Total protein was then analyzed by western blotting probing for RTN3L, TSG101, and Calnexin. (**E-Left panel**) Total cell proteins were extracted from liver samples from healthy and HCV infected individuals and analyzed by western blotting probing for RTN3L&S and HCV NS3 with β-actin serving as a control for equal protein loading. (**F-Left panel**) Serum exosomes were isolated from healthy individuals and HCV infected individuals were quantified by NanoSight NS300. (**F-Right panel**) Total proteins were extracted from human serum exosomes as indicated and analyzed for RTN3L&S, HSP90, and TSG101. (In-vitro data is representative of 3 independently repeated experiments. Human tissue samples are representative of 5 healthy and 8 HCV infected individuals. *$p<0.05$ was considered significant by Mann–Whitney U test.

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individuals were also enriched with RTN3L&S (Fig 1F), and HSP90 (Fig 1F), consistent with our previous observations [11]. We used β-actin as a loading control for total cellular protein and TSG101 as a loading control for exosomal analysis by western blotting (Fig 1).

Table 1. Clinical parameters of serum from HCV infected individuals.

Parameters	Distribution
Gender: Male/Female	4/3
Age	50.2 (±2.89)
Genotypes	1, 1a, 2, 4
ALT (IU/mL)	110.6 (±31.3)
Treatment Status	Untreated or Harvoni Treatment Failure

5 Samples from healthy uninfected subjects served as controls.

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Reticulon-3 interacts with and is in complex with dsHCV RNA and HCV NS3

RTN3 has been shown to interact with HCV NS4B required for the formation of the membranous web that is obligatory for HCV replication [29, 40, 41]. However, the interaction between RTN3 and dsHCV RNA the predominant form in human liver and interferon-treated cells [42] has not been demonstrated. Given our interest in deciphering the role of RTN3 in infectious exosomes loading, we assessed if RTN3 interacted with dsHCV RNA. Our RNA ChIP analysis revealed that dsHCV RNA interacts with RTN3L&S which is also in complex with HCV NS3 (**Fig 2A**). However, RTN3L&S isoform showed differential levels of interactions with dsRNA (**Fig 2B**) and miR-122 (**Fig 2C**) in HCV JFH1 infected Huh7 and HCV FL-replicon cells. While dsHCV RNA interacted more with RTN3L in JFH1 infected Huh7 cells, interactions in HCV FL-replicon cells involved mainly RTN3S (**Fig 2C**). miR-122 interaction with RTN3L&S was also much lower in FL-replicon cells compared to uninfected and JFH-1 infected Huh7 cells (**Fig 2C**).

Knockdown of Reticulon-3 impacted the cellular release of replication-competent viral-exosomes from HCV infected cells

The presence of infectious viral material inside exosomes is a classical feature of hepatitis C, well recognized in human infection and cell culture systems [11]. How replication-competent viral material gets loaded into exosomes in general currently remains ill-defined. To address

Table 2. Clinical parameters for HCV human liver samples.

Parameters	Distribution
Gender: Male/Female	5/6
Age	56.4 (±2.57)
Genotypes	1, 2 and 4
AST (IU/mL)	99.36 (±21.0)
Albumin (g/dL)	2.76 (±0.13)
Alkaline Phosphatase (U/L)	124.18 (±15.80)
Creatinine (mg/dL)	1.64 (±0.40)
Total Bilirubin	8.27 (±3.56)
Prothrombin Time (INR)	2.56 (±0.20)
Histopathology Report Summary	Chronic inflammation with Cirrhosis or end-stage Cirrhosis secondary to active Hepatitis C virus infection.

10 Samples from uninfected donors with intracranial hemorrhage, cerebrovascular accident, and accident victims served as controls.

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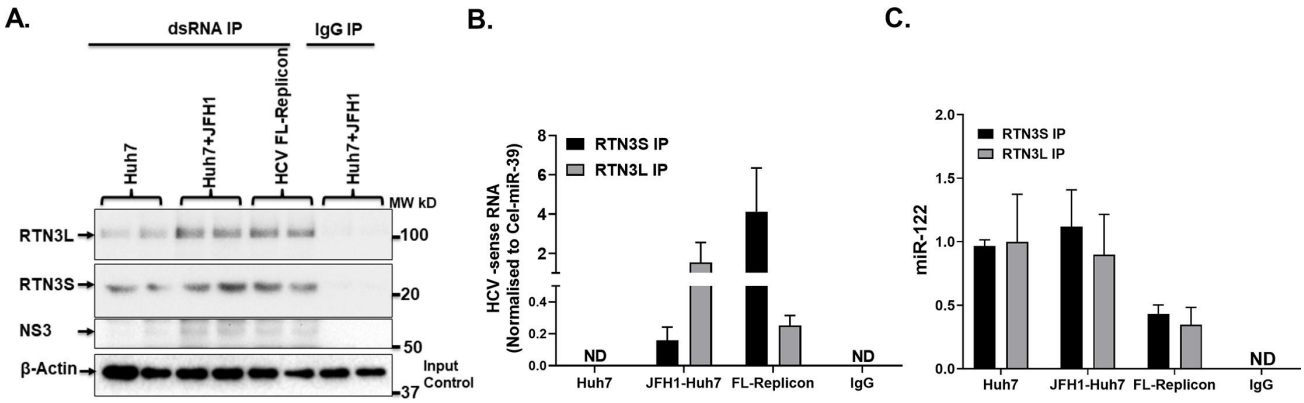

Fig 2. RTN3L&S interact with double-stranded viral RNA in HCV infected Huh7 cells. (**A**) RNA ChIP analysis was performed from cell lysates from Huh7.0, Huh7.0 +JFH-1 infected, and HCV FL-Replicon cells. Immuno-precipitations of cell lysates were performed using specific dsRNA and non-specific IgG antibodies using protein A/G pulldown. (**A**) Pull-down proteins interacting with dsRNA were probed by western blotting targeting for RTN3L&S and HCV NS3. Input total lysates were subjected to western blot analysis and probed for β-Actin. (**B**) Total RNA was isolated from Immuno-precipitations of cell lysates following the pulldown of RTN3L&S and non-specific IgG antibodies. Total RNA extracted was subjected to RT-qPCR targeting (**B**) negative-sense HCV RNA and (**C**) miR-122. miR-Cel39 RNA served as an exogenous loading control. Data is representative of 3 independently repeat experiments with $p<0.05$ was considered significant by the Mann–Whitney U test.

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this knowledge gap, we investigated whether any relationship existed between infectious exosome generation and RTN3. First, we transiently knockdown RTN3L&S isoforms by siRNA methods in JFH1 infected Huh7 cells (Fig 3A). We observed by western blotting analysis that RTN3L&S knockdown in HCV JFH1 infected Huh7 cells did not impact HCV NS5A protein levels (Fig 3A) Similarly, RTN3L&S knockdown in HCV FL-replicon cells using a lentiviral CRISPR/Cas9 did not impact HCV NS3 protein expression (Fig 3B-**Upper panel**). However, the was a modest but not statistically significant reduction of HCV RNA in FL-replicon cells following RTN3L&S knockdown (Fig 3B- **Lower panel**). These observations suggested that knockdown of RTN3L&S did not impact viral protein translation but might modestly impact cellular viral RNA replication. Strikingly, both RTN3L&S knockdowns were associated with a significant reduction of cell released exosomes from HCV FL-replicon cells (Fig 3C). Also, knockdown of RTN3L&S significantly reduced the amount of HCV RNA loaded inside cell released exosomes (3C-Upper panel). Notably, Knockdown of RTN3L in HCV infected cells resulted in a modest but not statistically significant enrichment of negative-sense HCV RNA in cell released exosomes suggesting possible compensatory effects of RTN3S (Fig 3C-**Lower panel**). Further, the co-culture of cell released exosomes from RTN3L&S knockdown cells with naïve uninfected Huh7 cells was associated with less robust infection as revealed by HCV NS3 protein expression compared to control conditions (Fig 3D). These observations suggest that RTN3L&S directly modulates the incorporation of replication viral material packages inside infectious HCV exosomes.

Loading of replication-competent viral molecules inside exosomes is mediated by the C-terminal region of Reticulon-3

Our transient knockdown experiment revealed that RTN3 might directly impact the loading of replication-competent viral exosomes (Fig 3). To further delineate this role, we additionally overexpressed wild type, N and C-terminal deletion mutants of RTNL&S in HCV infected cells alongside appropriate controls (Fig 4A & 4B). Our experiments revealed that overexpression of either the wild type or mutant isoforms in JFH1 infected Huh7 or HCV FL-replicon cells did not impact HCV replication as revealed by HCV NS3/NS5A western blotting

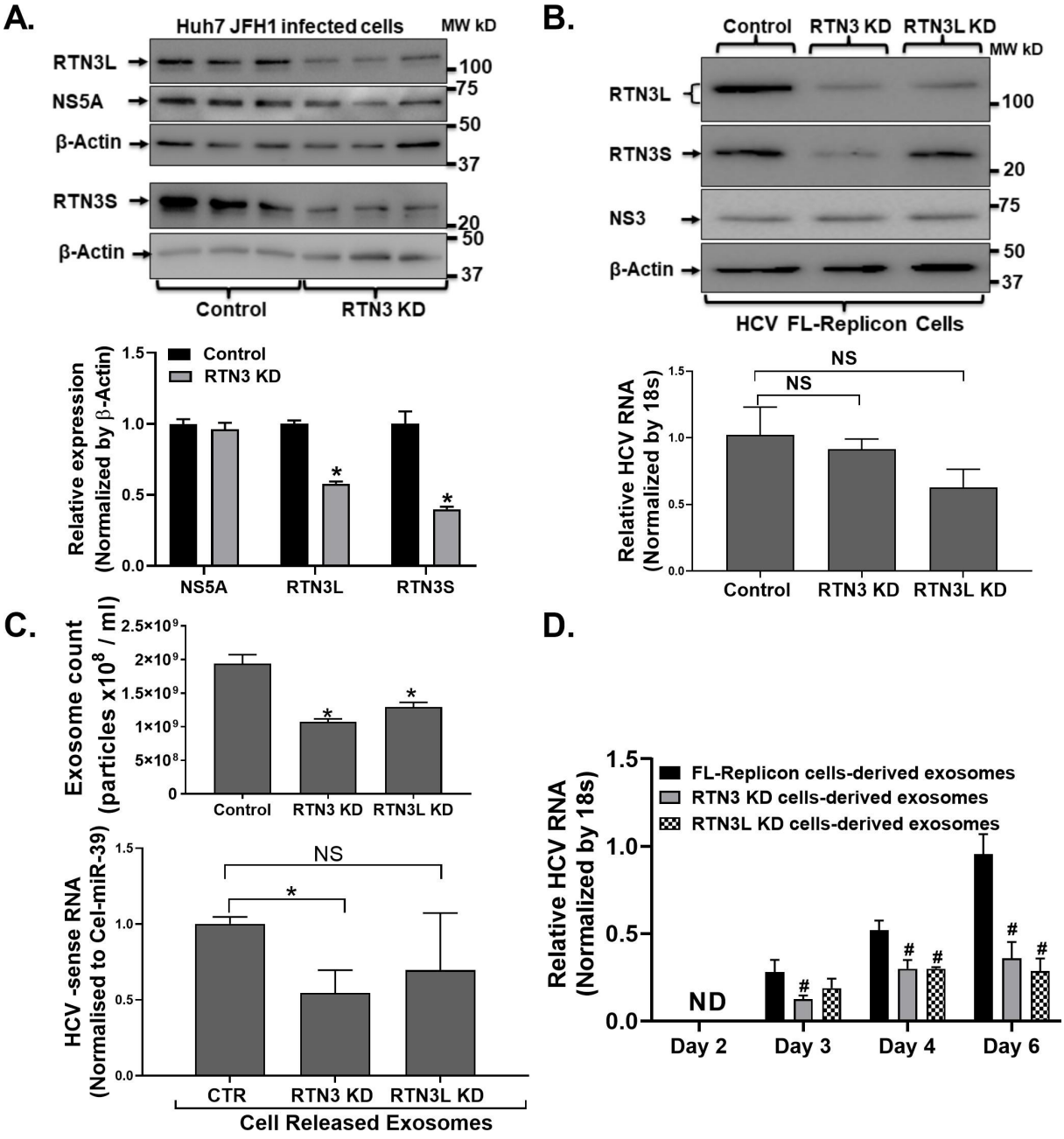

Fig 3. RTN3 modulates the loading of infectious viral molecules inside exosomes. (**A&B**) Reticulon 3L&S (RTN3L&S) was knockdown using specific siRNA(**A**) and LentiCRISPR lentiviral CRISPR/Cas9 (**B**) methods in HCV FL-Replicon cells alongside appropriate control as indicated. 48h post RTN3 knockdown, cell culture supernatants, and cells were harvested. Total proteins extracted from cells were subjected to western blotting probing for RTN3L&S, NS5A, and NS3 with β-actin serving as an equal loading control. (**C**) NanoSight NS300 was used to quantify cell released exosomes in cell culture supernatants. (**D**) Cell released exosomes from LentiCRISPR lentiviral CRISPR/Cas9 knockdown of RTN3L&S conditioning and was co-cultured with naïve Huh7.0 cells (5k Exosomes: 1 Huh7.0 cell) over 72h. Total RNA was then extracted from cells and analyzed by RT-qPCR for HCV RNA using 18s as a housekeeping gene. Data is representative of 3 independently repeat experiments with $^{*}/^{\#}p<0.05$ was considered significant by Mann–Whitney U test.

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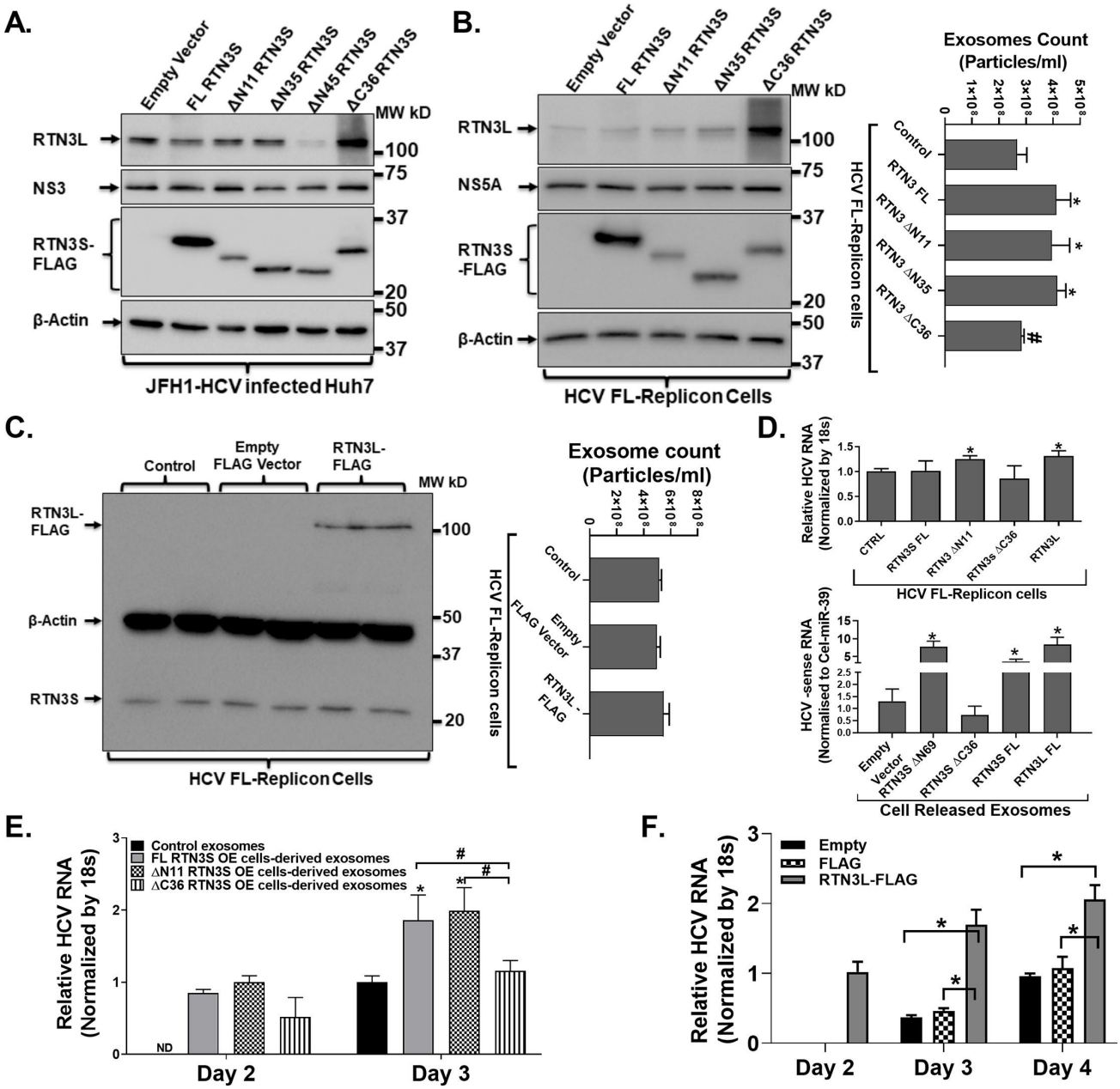

Fig 4. RTN3L&S carboxyl-terminal domain modulates the loading of replication-competent viral material inside infectious exosomes. (**A&B-Left panel**) Full-length Flag-tagged RTN3S, N-terminal deletion RTN3S, and C-terminal deletion mutants of RTN3 were overexpressed in HCV JFH-1 infected Huh7 and HCV FL-replicon cell. Total proteins were then extracted from cells 72h post-transfection and analyzed by western blotting probing antibodies targeting RTN3L, Flag, HCV NS3, and NS5A. β-actin served as a loading control. (**B-Right panel**) Cell released exosomes harvested from cell culture supernatants from HCV FL-replicon cells without and with the overexpression of full-length Flag-tagged RTN3S, N-terminal deletion RTN3S and C-terminal deletion mutants of RTN3 were quantified by NanoSight NS300. (**C**) Full-length Flag-tagged RTN3L were overexpressed in HCV FL-replicon cells. Total proteins were then extracted from cells 72h post-transfection and analyzed by western blotting probing antibodies targeting RTN3S and Flag. β-actin served as a loading control. (**D-Upper panel**) Total RNA was then extracted from cells for conditions described in B&C and quantified for HCV RNA using 18s RNA as a normalization control (**D-Lower Panel**) Total RNA was extracted from cell released exosomes from conditions described in (**B&C**) and analyzed for negative-sense HCV RNA by RT-qPCR using cel39 as an exogenous normalization control. (**E&F**) Cell released exosomes from conditions described in (**B&C**) were co-cultured with naïve Huh7 cells over a 3 to 4-days period. Total RNA was then extracted from cells and quantified for HCV RNA using 18s RNA as a normalization control. Data presented here are representative of 3 independently repeat experiments with $^{*}/^{\#}p<0.05$ was considered significant by Mann–Whitney U test.

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analysis (Fig 4A & 4B-**Left panel**). Strikingly, overexpression of C-terminal deleted RTN3L&S mutants was associated with a corresponding increase in the native cellular forms of RTN3L&S proteins (Fig 4A & 4B-**Left panel**). However, we found that overexpression of the wild type or N-terminal deletion mutants of RTN3S (Fig 4B-**Right panel**) was associated with the increased cellular release of exosomes compared to empty vector-transfected control (Fig 4B-**Right panel**). Further, overexpression of C-terminal deletion mutants of RTN3 was associated with significantly reduced cell released exosomes compared to overexpression with full length and N-terminal deletion mutants (Fig 4**B-Right panel**). Overexpression of RTN3L did not impact RTN3S expression (Fig 4C-**Left panel**) or the number of cell-released exosomes (Fig 4C-**Right panel**). Given that cell release exosomes are loaded with replication viral RNA, we next assessed in the overexpression of RTN3L&S as well as mutant isoforms impacted the amount of viral RNA in HCV infected cells. While overexpression of RTN3S FL and RTN3S C36 mutants in FL-replicon cells did not impact HCV RNA levels, we found that the overexpression of RTN3SΔN11 and RTN3L was associated with a modest but statistically significant increase in cellular viral levels (Fig 4D- **Upper panel**). We however found that the over-expression of full-length RTN3S, RTN3SΔN11, and RTN3L in FL replicon cells significantly increased the enrichment of negative-sense HCV RNA in cell released exosomes. Remarkably, the over-expression of RTN3SΔC36 mutant in FL-replicon cells was associated with significantly reduced negative-sense HCV RNA enrichment in cell released exosomes (Fig 4D-**Lower panel**). Further, suggest that the overexpression of RTN3L in FL-replicon cells significantly increased HCV RNA in cells (Fig 4D-**upper panel**) and also increased the loading of viral RNA inside cell-released exosomes(Fig 4D-**Lower panel**). In addition, co-culture of exosomes from RTN3S wild type, N and C-terminal deletion mutants revealed that C-terminal mutants of RTN3 were less infectious as revealed by real-time qPCR (Fig 4E). These findings suggest that the C-terminal domain of RTN3 protein potentially mediates critical interactions and trafficking of replication-competent viral RNA and specific cellular host molecules for loading and release inside infectious viral exosomes. Finally, co-culture of cell released exosomes from RTN3L overexpressing cells revealed that these cell released exosomes were more infectious compared to exosomes from control conditions (Fig 4F)

Treatment of HCV JFH-1 infected Huh7 cells reduced RTN3 expression and the number of cell-released infectious exosomes

Hepatitis C can be treated by effective direct-acting antiviral agents. The impact of these treatments on cell released infectious exosomes remain ill-defined. Therefore, we treated HCV JFH1 infected Huh7 and HCV FL-replicon cells. Our western blotting analysis revealed that sofosbuvir (an NS5B polymerase inhibitor currently used several approved combination anti-HCV regimens) treatment of HCV infected cells abrogated viral replication (with reduced HCV NS3 expression) (Fig 5A & 5B). Further, the increased RTN3 expression, typically induced by HCV infection, was significantly decreased after sofosbuvir treatment (Fig 5A & 5B). Subsequently, the co-culture of exosomes from HCV infected treated cells with naïve Huh7 cells reduced both the number (Fig 5C) and infection capacity (Fig 5D) of exosomes compared to appropriate controls. These observations suggest that available HCV treatments, specifically sofosbuvir can directly suppress the release of infectious exosomes.

Discussion

All viruses have developed sophisticated strategies to hijack host defense mechanisms using host cell molecules and pathways in unique ways to achieve effective infection. Several reports, including our studies, have revealed that HCV can hijack the host exosomal pathway to

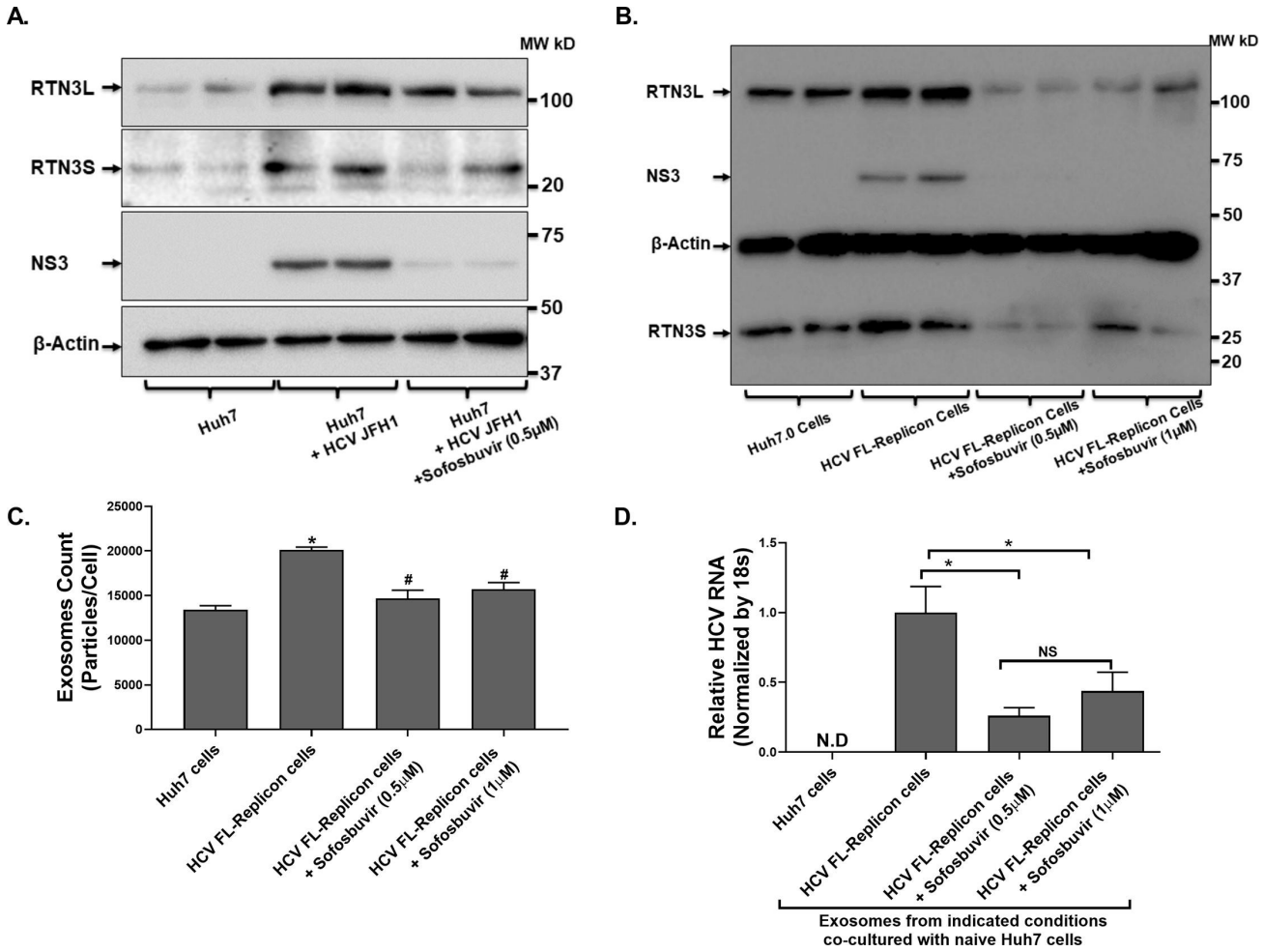

Fig 5. Treatment of HCV infected cells with Sofosbuvir significantly reduced HCV induced RTN3L&S and cell released infectious exosomes. (A) Huh7-JFH1 infected and (B) HCV FL-Replicon cells were treated with Sofosbuvir or not as indicated alongside non-treated Huh7.0 control cells. 10-days post-Sofosbuvir treatment, cell culture supernatant, and cells were harvested. (**A&B**) Total protein was extracted from cells and analyzed by western blotting probing for HCV NS3 and RTN3L&S with β-actin serving as a control for equal protein loading. (**C**) Cell released exosomes in cell-cultured supernatants were quantified by NanoSight NS300 and normalized per cell based on total cell counts at the end of treatment. (**D**) Cell released exosomes from treatment conditioning in (**B**) were co-cultured with naïve Huh7.0 cells (5000 Exosomes: 1 Huh7.0 cell) over 72h. Total RNA was then extracted from cells and analyzed by RT-qPCR for HCV RNA using 18s as a housekeeping gene. Data for 3 repeat experiments are presented with $^{*}/^{\#}p<0.05$ considered significant by the Mann -Whitney U test.

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mediate HCV immunologic escape and persistence [11, 12]. However, the mechanism by which the HCV can modulate the specific loading of replication-competent viral material inside infectious exosomes remains undefined.

In the current study, we reveal that RTN3 protein can mediate the specific loading of replication-competent HCV RNA and specific cellular molecules for release inside infectious viral exosomes. We found that HCV JFH1 infection of Huh7 cells was associated with increased cellular expression of RTN3L&S protein isoforms. Similarly, western blot analysis revealed increased expression of RTN3L&S protein isoforms in liver samples from HCV infected individuals compared to healthy control (HCV negative) subjects. Further, knockdown and overexpression of RTN3 in HCV infected cells, respectively, decreased and increased the number

of cells released infectious exosomes. Further, we demonstrate that RTN3L&S isoform proteins interacted with dsHCV RNA and that the c-terminal region is required for efficient loading of replication-competent viral material released inside infectious exosomes.

Reticulons are ER-located proteins (primary site for HCV replication) and despite their multiple isoforms, all share a common Reticulon homology domain (RHD) [23]. Reticulons have been shown to have differential effects on viral infections. In Flaviviruses, RNT3 was shown to be significantly enriched within membranous structural sites of virus replication [28, 43]. In the context of HCV infection, Lin et al revealed increased intrahepatic RTN3 levels which correlated with higher HCV genotype 1 RNA levels [37]. These observations are in concert with our findings which revealed increased RTN3L&S isoforms in HCV JFH1 infected Huh7 cells and liver samples from HCV infected treatment naïve individuals. Further, we revealed that cell released exosomes from in-vitro HCV infection and replication cell systems were enriched with RTN3L. Strikingly, serum exosomes from HCV infected individuals showed increased enrichment with RTN3L&S and HSP90 [11], which we revealed previously. There are reports that RTN3 can act as an anti-viral agent during HCV infection [29, 44]. Specifically, this study demonstrated that RTN3 interacts with HCV NS4B and negatively regulates viral replication [29]. The silencing of RTN3 in this study was associated with a significant increase in HCV replication which could be counteracted by the overexpression of recombinant RTN3 [29]. These observations are in contrast with our findings since we reveal here that knockdown or overexpression of RTN3S and RTN3ΔC36 did not impact HCV replication. However, some of our findings are in concert with reports by Tripathi *et al* who revealed that RTN3 knockdown did not impact HCV JFH1 replication [30]. Additionally, we found while overexpression of RTN3SΔN11 did not impact HCV NS3 protein in cells, it was associated with a modest but significant increase of cellular viral RNA. Together, our observations suggest that RTN3L&S knockdown and overexpression of wild type or the various isoform mutants might not impact viral gene translation but can certainly differentially impacts viral RNA turnover inside cells and the enrichment of viral RNA in cell released exosomes. Consequently, exosomes from RTN3 knockdown cells were less infectious compared to exosomes from released by RTN3 over-expressing HCV infected cells. Taken together, our findings support a notion that the various RTN3L&S isoforms perform different functions and can lead to opposite effects depending on their impact on viral replication and viral gene translation [29, 30].

For effective loading of infectious viral exosomes, efficient cellular trafficking and compartmentalization of specific molecules are required. As previously indicated, the biological function of RTN3 includes cellular trafficking most often involving its c-terminal domain interactions with other molecules [24]. The significance of this observation in the context of infectious exosome loading has not been exploited. Here, we show that RTN3 specifically differentially interacts with HCV dsRNA, miR-122 as well as in complex with HCV NS3 protein. Observed differential interactions of RTN3 with host and viral molecules in the JFH1 (HCV Genotype 2a) and FL-replicon (HCV Genotype 1b) infected cells can be attributed to the characteristic differences of the HCV strains, not to the parental Huh-7 cell line. Additionally, the trafficking and loading of exosomes involving RTN3 are most likely to be a very dynamic process and variabilities observed in our interaction experiments should not be uncommon. Further, we reveal that the wild type and N-terminal deletion mutants of RTN3L&S isoforms significantly induced the release of infectious viral exosomes. Strikingly, cellular overexpression of C-terminal deletion mutants of RTN3L&S resulted in a significant decrease in cell released infections exosomes. We also observed corresponding increased expression of RTN3L&S during cellular overexpression of c-terminal deletion mutants of RTN3L&S forms respectively. These novel observations suggest that the c-terminal domain of RTN3 plays an

active role in the loading and generation of infectious viral exosomes. Our observation of corresponding increases in cellular RTN3L&S proteins following respective overexpression of C-terminal deletion mutants of RTN3L&S suggests possible compensatory regulation between both proteins to maintain infectious exosome generation. However, the cellular knockdown of both RTN3L&S did not completely block the cellular release of infectious viral exosomes. These observations suggest the existence of additional mechanisms that can mediate infectious exosome loading associated with HCV infection.

There is currently highly effective therapy for HCV infection, hence we assessed the effect of treatment on infection-induced increased RTN3 and infectious exosome release. We found that Sofosbuvir, an HCV NS5B nucleotide polymerase inhibitor [45], significantly reduced increased RTN3 expression caused by HCV infection. Furthermore, Sofosbuvir significantly reduced the cellular release of infectious viral exosomes. Our observation of increased RTN3L&S expression in liver and serum exosomes of HCV infected individuals, that decreased with anti-HCV treatment may have future applications in terms of complementary viral or disease prognostic biomarker. Further, in an era of effective DAA treatments for HCV infection, assessing exosomal or hepatic expression of RTN3 may serve to assess treatment outcomes and potential drug resistance.

In conclusion, our results provide evidence that RTN3 interacts with HCV NS3, replication-competent double-stranded HCV RNA, and miR-122. Reports have revealed increased endoplasmic reticulum (ER) blebbing in persistent HCV infected hepatocytes [46]. Taken together (**Fig 6**), we propose a model whereby HCV infection results in increased dsHCV RNA and specific cellular proteins interaction/binding to RTN3. Further, increased interactions/binding of dsHCV and specific cellular proteins coupled with cellular stress associated with chronic cellular infection results in ER blebbing to maintain cell fitness. This ER blebs containing dsHCV RNA and specific cellular proteins mature in multivesicular bodies and subsequently get released from cells as infectious exosomes. Given our novel revelations and

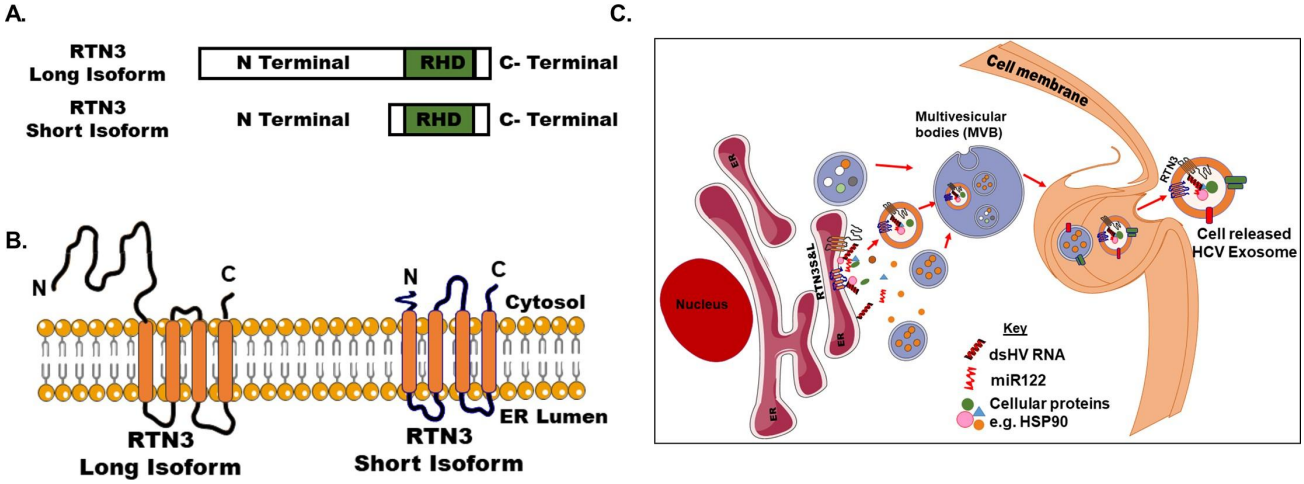

Fig 6. Schematics of RTN3 facilitates the loading of infectious HCV exosomes. (**A**) Schematic illustration of RTN3 long and short isoforms (RTN3&S) and (**B**) their cellular endoplasmic reticulum (ER) locations. (**C**) HCV infection is associated with increased expression of double-stranded (ds) HCV RNA in Huh7 cells. Double-stranded HCV RNA can interact with endoplasmic reticulum located RTN3L&S protein isoforms HCV infected Huh7 cells. Additionally, direct or indirect interactions also occur between other cellular proteins and nucleic acids with dsHCV RNA and RTN3L&S in HCV infectedHuh7 cells. RTN3L&S in HCV infected Huh7 cells can directly modulate the incorporation of replication-competent HCV dsRNA in association with other proteins inside membrane vesicles which are translocated to multivesicular bodies (MVB). This MVB in HCV infected Huh7 cells then fuse with the plasma membrane resulting in the release of infectious viral exosomes. Schematic illustration made use of some smart servier medical art templates (https://smart.servier.com).

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the critical role of infectious exosomes in almost all viral infections, further clarification of the biological roles of RTNs in modulating the specific loading of pathogenic exosomes in the context of other viral infections should be evaluated.

Supporting information

S1 Raw images.
(PDF)

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4 DISCUSSION AND CONCLUSION

DISCUSSION

The attachment/entry of viruses to the cell host, initiating the viral infection, depends on viral surface binding protein to target host membrane receptors. Notwithstanding, host organisms have developed formidable antiviral strategies, such as neutralizing antibodies blocking the viral entry and numerous RNA- and DNA-sensors to activate the immune system, resulting in viruses clearance (Carney and Gale, 2006). However, viruses can also fight back by developing sophisticated strategies to hijack host signaling pathways to escape from immune system surveillance and further to achieve their persistence in the host. Our previous study (Bukong et al., 2014) is in concert with other reports (Cosset and Dreux, 2014; Ramakrishnaiah et al., 2013; Saha et al., 2017), showing that HCV can take advantage of host exosomal pathways to mediate host receptor independent, as well as, neutralizing antibody-resistant cell to cell transmission (Bukong et al., 2014; Patman, 2014). Besides, exosomes are continuously produced by cells into the extracellular space and their composition most often reflects the physiological/pathological state of the cells they originate from. In addition to their communication roles, exosomes can transfer biologically active proteins, lipids and RNAs to target recipient cells, thus alters their functions in precise ways. Previous studies including ours have demonstrated a significant role of exosomes in modulating the establishment of productive infection or cellular response to viral infections(Arenaccio et al., 2014; Bukong et al., 2014; Jaworski et al., 2014; Pleet et al., 2017; Zhang et al., 2016). What remains striking is that the exosomes that module viral infection are composed of quite distinct cellular and viral molecules while others are excluded. Given observations, the major question becomes: How do viruses mediate the specific sorting and loading of specific viral and host molecules into exosomes? While it has been advanced that specialized mechanisms do exist that modulate the specific molecular composition of exosomes, the fact that we are currently in incapacity of generating exosomes with a specific molecular composition illustrates our knowledge of this process remains incomplete. Studies have proposed that exosomal protein composition can be controlled by ESCRT-dependent and -independent pathways (Babst, 2011). Exosomes biogenesis has been also suggested to originate through MVB formation, observations of exosomes with different molecular composition can be produced by the same cell, suggest the existence of different MVB subsets or other biogenesis pathways that are currently unknown. All these mechanisms seem to act differently depending on the cell type, stimuli which ultimately lead to the production of different subsets of exosomes even by the same cell.

In the context of HCV infection, stabilized and replication competent HCV RNA inside exosomes can efficiently mediate HCV transmission in targeting cells establishing viral latency in host. Besides, the infectious HCV exosomes contain replication competent HCV RNA which is in association with Ago2-HSP90-miR-122 complex. However, little is known about the key regulator of mechanism by which the replication-competent HCV RNA has been loaded into exosomes. In present work, we have identified RTN3 as a novel regulator in infectious exosomes biogenesis and release, at least in context of HCV infection.

♦ Exosome isolation and purification

All analyses in our study are based on exosomes, thus, a pure isolation of exosomes is required. To achieve this goal, we started by getting rid of exosomes already present in the cell culture media DMEM for the *in vitro* experiments. Fetal Bovine Serum (FBS) is the most commonly used in cell culture to ensure cell growth and proliferation. However, FBS contains also bovine exosomes which would ultimately contaminate our exosomes samples. Therefore, a bovine exosomes-depleted FBS was used in cell culture media all along in our study. Nevertheless, a slower but healthy cell growth has also been observed in DMEM containing exosome-depleted FBS. Moreover, centrifugation was further performed to remove cell debris and the isolated exosomes were re-purified using Exoquick density separation system.

Previous work from some research teams including ours (Bukong et al., 2014; Helwa et al., 2017; Kalra et al., 2013; Liga et al., 2015), took advantage of CD63-based immunopurification and a series of ultracentrifugation steps to isolate exosomes. Indeed, these isolation approaches are based on exosomes-specific biochemical and physical characteristics: 1-CD63 is a common exosomes maker. 2-exosomes exhibit a density gradient. However, using these isolation techniques cannot rule out the contaminations of other types of EVs since almost all cell-derived EVs contains CD63. Besides, a series of ultracentrifugation can lead to an intensive loss of exosomes.

The size of HCV virions is approximately 60nm in diameter (Chevaliez and Pawlotsky, 2006; Suzuki et al., 2010), coincidently in the range size of exosomes. Therefore, to avoid the HCV virions contaminations eventually present in exosome samples, we decided to take advantage of the HCV replicon system (Annexe 2), in which HCV virions assembly is blocked. In fact, up to 1999, transiently transfection of HCV gene is the only method to examine the HCV pathogenesis *in vitro*. However, this method is not the most appropriate way to study the effect of an RNA virus and cannot be used to illustrate the various aspects of the HCV life cycle. As such, the

development of the HCV replicon system by Lohmann et al. provided a new and important model to study HCV pathogenesis and replication (Lohmann et al., 1999): A subgenomic clone of genotype 1b HCV was developed that contained the 5'UTR, a neomycin resistance cassette, the ECMV IRES and the HCV NS3 to 3'UTR region. Once transcribed into RNA, the subgenomic replicon construct was electroporated into Huh7 cells and stably transfected cells were selected using G418. Later in 2002, FLHCV replicon has been developed by using the same strategies, however, low rate HCV replication and no assembly and production of infectious viral particles have been reported (Ikeda et al., 2002). Further optimizations have been made by mutating several specific regions of the FL replicon in order to allow efficient viral replication and adaptation to cell culture. As such, the advanced FL replicon system that we used in our project have certain mutations in NS5A. Mutations modified phosphorylated states of NS5A that is deleterious for HCV virions assembly and release. However, these mutations do not have any effects on HCV replication nor infectious exosomes release (Evans et al., 2004a; Evans et al., 2004b). Furthermore, HCV full length replicon cells with possibly no early endosome formation observed in classical JFH1 entry and infection still produces infectious exsomes (Ramakrishnaiah et al., 2013). These observations could support in part that proteins other than ESCRT and associated protein could be the regulators of infectious viral molecules loaded into exosomes.

- **RTN3 in HCV infection**

RTN3 is an ER-resident protein while ER is the primary replication site of all Flaviviruses where membranous web formed. In Flaviviruses, RNT3 was shown to be redistributed and significantly enriched within a viral replication site (Aktepe et al., 2017). In the context of HCV infection, the correlation between increased intrahepatic RTN3 expression and higher HCV genotype 1 RNA levels have been revealed (Lin et al., 2017). These observations are in concert with our findings which revealed increased RTN3L&S isoforms in HCV JFH1 infected hepatocytes and liver samples from HCV infected individuals. As showed by western-blot analysis, HCV JFH1-infected Huh7 cells as well as HCV-infected patients displayed higher RTN3S&L expression, compared to naïve Huh7 cells and healthy individuals, respectively. Moreover, divergent expression levels in both RTN3S&L are also observed in HCV-infected individuals, suggesting a large variability of RTN3 expression in HCV infection which might be due to different i) HCV genotype; ii) infection; iii) anti-viral treatment. Thus, a statistic on a large number of HCV-infected patients is required to further characterize the role of RNT3 variants in an HCV-related manner.

In our study, we also found that knockdown or overexpression of RTN3 did not change NS3 level suggesting RNT3 has no impact in HCV replication, which is in concert with a report that revealed that RTN3 knockdown did not impact HCV JFH1 replication (Tripathi et al., 2013). However, some reports showed that WNV infection induced a redistribution of RTN3 in ER, which facilitates NS4B recruitment to the replication site and enhances viral replication. Moreover, down-regulation of RTN3 attenuates ZEKV, WNV, and DENV replication (Aktepe et al., 2017). Besides, Wu et al., 2014 showed in HCV infection, RTN3 is an inhibitor of viral replication by preventing NS4B self-interaction (Wu et al., 2014). The controversy observed in the role of RTN3 during flaviviruses infection might be due to i) using different RTN3 isoforms with a high diversity sequence at the N-terminal region exhibiting diverse functions and ii) using different experimental approaches with a different sensitivity. To further validate the role of RTN3 in HCV infection, the extent clinical liver damage and virological response must be examined.

- **RTN3 in HCV infectious exosome loading**

Our previous study revealed that, HCV RNA associated with the Ago2/HSP90/miR-122 complex has been characterized in HCV infectious exosomes, supporting that exosomal cell-to-cell communication pathway enhances HCV transmission and persistence. In our current study, we reveal that ER-located RTN3 protein can mediate the specific loading of replication-competent material into exosomes. Specifically, we revealed that higher RTN3S&L expression was associated with higher HSP90 expression in serum exosomes samples from HCV-infected patients compared to those from healthy individuals. However, RTN3S was undetectable in exosomes isolated from both HCV FL-replicon cells and naïve Huh7 cells, whereas RTN3L was enriched in HCV FL-replicon released exosomes. Given these observations, we suggest that we might reach the limit of western-blot analysis due to the insufficiency of cell derived-exosomes collected. Also, it is possible that HCV favors some exosomes biogenesis pathways, leading to a large repertoire of sub-population of exosomes. It's also possible that one or some of these subpopulations of exosomes are specifically enriched in RTN3S&L.

Further, by using confocal Microscopy (not shown in published version) and ChIP assay, we demonstrate that RTN3L&S isoforms interacted with replication-competent dsHCV RNA and NS3 protein, supporting that RTN3-mediated HCV RNA loading into exosomes during HCV infection. Despite many efforts in testing antibodies used in IF experiments, the image quality is still needed to be improved (Annexe 3). Moreover, knockdown of RTN3 attenuates, while its overexpression increases the infectivity of exosomes, demonstrated by HCV RNA levels in receiving cells. Indeed,

the co-culture of exosomes from overexpression of C-terminal and N-terminal mutants of RTN3 HCV-infected cells and naïve Huh7 experiments gives evidence that C-terminal region is required for efficient loading of replication-competent viral material released inside infectious exosomes. As previously indicated, the biological function of RTN3 includes cellular trafficking most often involving its c-terminal domain interactions with other molecules, it is thus worthy to perform further assays to identify those molecular interactors of RTN3 that involved in exosome generation.

Although RTN3 has been showed its modulatory role in determining the selection of host molecules and replication competent viral for loading into infectious exosomes, it's possible that additional target exists. While HCV infection is used as model system in our study, it is possible that other viral infections might have distinct processes that mediate the generation of infectious exosomes.

- **RTN3 in HCV infectious exosomes release**

In our study, we demonstrate exosomes from siRNA-mediated and CRISPR-Cas9 based RTN3 knockdown of HCV FL replicon cells, are significantly less numerous than those from control condition, whereas overexpression of RTN3S&L significantly induced exosome secretion, suggesting that RTN3 is also involved in exosomes release. Interestingly, no impact on exosome secretion has been observed from overexpression of the C-terminal deletion mutants of RTN3S&L Huh7 FL replicon cells, confirming the importance of the C-terminal region of RTN3 in exosomes release. Surprisingly, overexpression of RTN3L was observed only when the C-terminal deletion mutant of RTN3S was overexpressed, suggesting a possible compensatory regulation between both isoforms of RTN3 to ensure infectious exosome generation. However, the cellular knockdown of both RTN3L&S did not completely block the cellular release of infectious viral exosomes. These observations suggest the existence of additional mechanisms that can mediate infectious exosome loading associated with HCV infection.

The autophagy and exosomal pathway are two important cellular homeostatic pathways used to degrade or eliminate unwanted/defective/pathogenic cellular molecules. Despite being quite distinct, the fact that autophagy is dysregulated during HCV infection as well as being associated with a significant increase in exosome release, suggests a crosstalk between these pathways. RTN3 has been recently proposed as a specific receptor for selective autophagy of ER (D'Eletto et al., 2020; Grumati et al., 2017), which could in part support the potential link between autophagic and exosomal pathways.

♦ RTN3 in HCV treatments

Sofosbuvir, an HCV NS5B nucleotide polymerase inhibitor, is used worldwide for efficient viral eradication in HCV-infected patients(Li and Lo, 2015; Zeuzem, 2017). However, its effect on infectious exosomes generation has not been described. In our current study, we found that Sofosbuvir could significantly reduce the higher-level exosomes secretion induced by HCV infection. Furthermore, we showed Sofosbuvir decreased a higher level of RTN3S&L expressions due to HCV infection. Given these observations, we suggest that cellular and/or exosomal RTN3 expression might be used as a potential index to assess antiviral treatment outcome and drug resistance. To further validate this idea, the effect of different DAA on RTN3 expression need to be done. Furthermore, RTN3-targeting strategies might also be applied in prognostic viral or disease and antiviral therapy, at least in HCV infection.

CONCLUSION

In conclusion, our results provide evidence that HCV hijacks exosomal pathway to generate infectious exosomes by C-terminal RTN3-mediated replication-competent viral material as well as associated Ago2-miR-122-HSP90 complex loading and release via exosomes.

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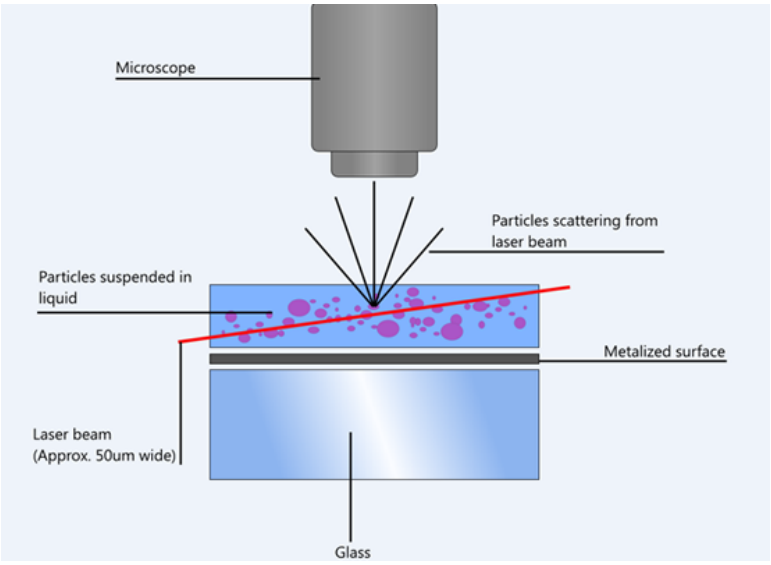
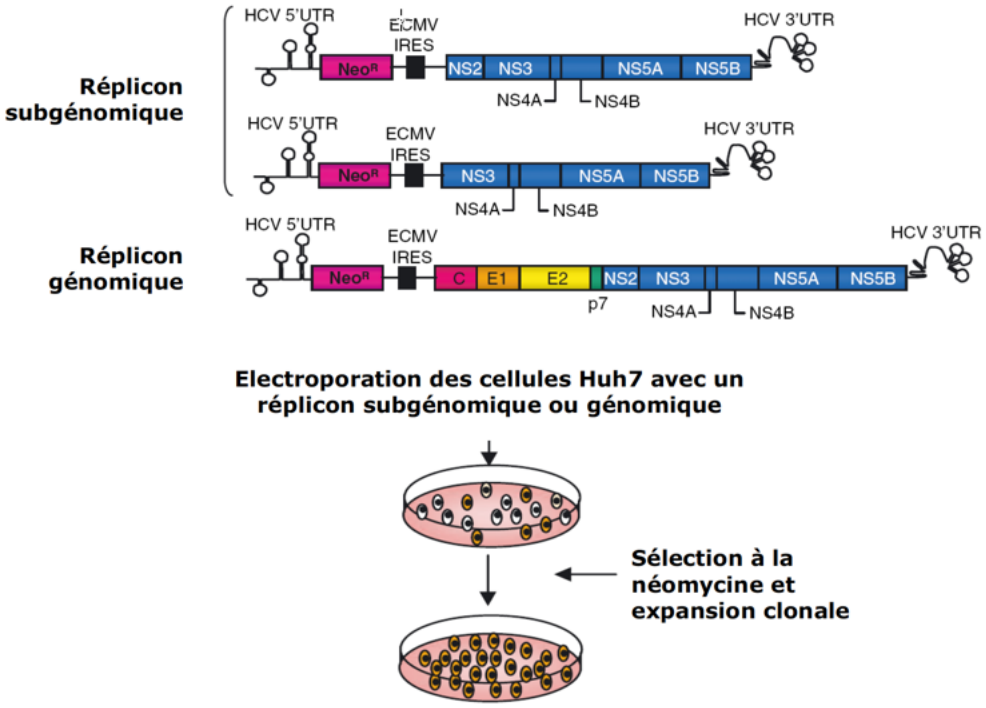
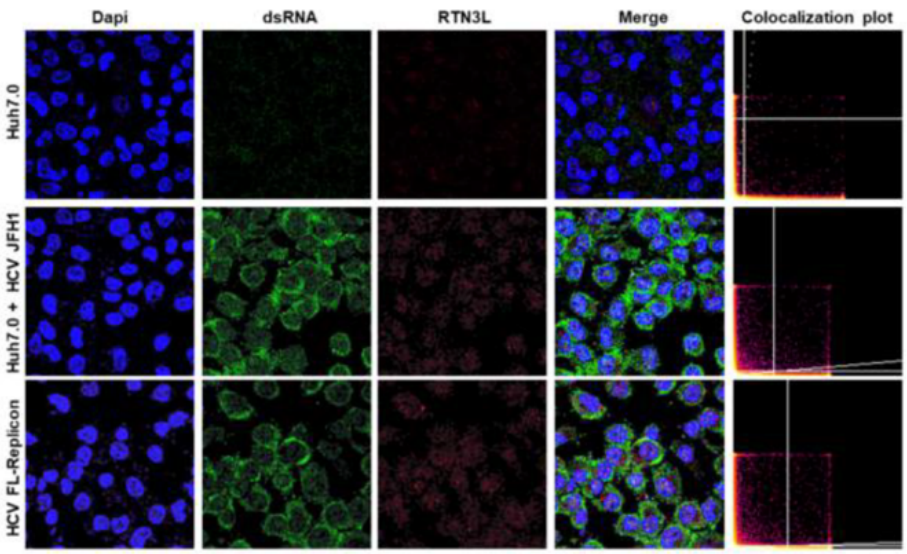
6 ANNEXES



Annexe 1. Nanoparticles Tracking Analysis (NTA).



Annexe 2. HCV replicon systems (Gegeard et al., 2017).



Annexe 3: RTN3L&S interact with double-stranded viral RNA in HCV infected Huh7 cells. Huh7.0, Huh7.0+JFH-1 infected and HCV FL-Replicon cells were fixed, permeabilized, and probed with dsRNA and RTN3L primary antibodies followed by specific secondary fluorescent conjugated antibody. DAPI was used for nuclei staining. Images were acquired by confocal fluorescence microscopy. The level of co-localization (upper outer quadrant) was determined using the NIH ImageJ software.