ROMANIAN ACADEMY

INSTITUTE OF BIOCHEMISTRY

Ph.D. Thesis SUMMARY

Interaction between structural and non-structural Hepatitis C Virus proteins and biomedical applications

Scientific coordinator:

Dr. Andrei-Jose Petrescu

Ph. D. Candidate:

Ovidiu Constantin Vlaicu

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Research objectives

Hepatitis C virus (HCV) infection is a medical challenge and is a general public health problem, given the fact that over 170 million people are infected with this virus worldwide. Thus, understanding the molecular mechanisms of interaction between the virus and the host is important for identifying new treatment targets for HCV-induced hepatic cancer and new prognostic factors of the hepatic pathology.

This thesis aims to optimize the techniques for identifying the endogenous factors involved in the various stages of the life cycle of HCV. For this, I will use affinity purification chromatography followed by mass spectrometry analysis (AP-MS) and determination of endogenous interactome for different viral proteins involved in HCV morphogenesis such as NS2 and NS5A.

The first objective of the thesis will be the construction of functional recombinant viruses with appropriate inserts for AP in permissive positions within the structure of these viral proteins. Recombinant viruses will be used to characterize endogenous interactoms of viral proteins in a cell culture system that produces infectious virions.

The second objective of the thesis will be the realization of an inducible trans-complementation system for the HCV capsid protein which will allow for the temporary separation of the replication step from that of HCV assembly. A stable and inducible cell line for the capsid protein will be selected in the purpose of identifying viral protein-endogenous protein complexes, as well as for real-time imagistics of NS5A and core virus proteins.

The third objective of the thesis is the validation and functional characterization of endogenous factors previously identified by AP-MS. I will focus my efforts on proteins involved in lipid metabolism because lipid homeostasis is essential in liver pathology.

Chapter 1. Theoretical Aspects of Hepatitis C Virus

1.1 The structure and life cycle of Hepatitis C Virus

Hepatitis C Virus (HCV) infection is a serious public health problem, affecting 170-200 million people around the world. After a long evolution, it can cause steatosis, liver fibrosis, cirrhosis and hepatocellular carcinoma (Levrero, 2006). Discovered in 1989 (Choo et al., 1989), VHC is part of the *Flaviviridae* family. Its genome is made up of a positive polarity single stranded RNA. The *Flaviviridae* family has three genres: Flavivirus (Yellow Fever Virus, West Nille and Dengue Virus), Pestivirus (Bovine Diarrhea Virus, Classical Swine Fever Virus) and Hepacivirus. Together with HVG (Hepatitis G Virus), HCV is part of Hepacivirus genus (Kapour, 2011).

HCV infection affects lipid metabolism and in particular cholesterol homeostasis (Popescu et al., 2014). The association of HCV with changes in lipid metabolism has some particularities in the clinic. In liver biopsies of HCV-infected patients an increase in neutral lipids in the hepatocyte cytoplasm is observed. Also, viral infection is associated with hepatic steatosis, the frequent occurrence of hypobetalipoproteinemia and the reduction of cholesterol in circulating blood (Rodgers et al., 2012). Treatment with α-interferon and ribavirin balances cholesterol and lipoprotein levels in the serum of patients infected with the virus (Corey et al., 2009). Purified virus from the sera of infected patients has a low virion density but also an association with apolipoproteins (André et al., 2002). 25 years of HCV research have clarified some viral molecular mechanisms, including the association of the virus with lipid metabolism (Popescu et al., 2011). The development of a cell culture system that allows in vitro replication and assembly of the virus has led to the understanding of lipid involvement in the HCV life cycle (Lindenbach et al., 2005).

Prior to the development of a cell culture system which allows the in vitro study of the virus, the researchers characterized virions starting from viral isolates of chronically infected patients or animal models, such as the chimpanzee. Sucrose gradient studies have shown that the viral particle has a low density, between 1.03 g/cm^3 and 1.2 g/cm^3 and the particle density is inversely proportional to its infectivity (Pumeechockchai et al., 2002). The cell culture system has enabled the HCV particles to be purified and

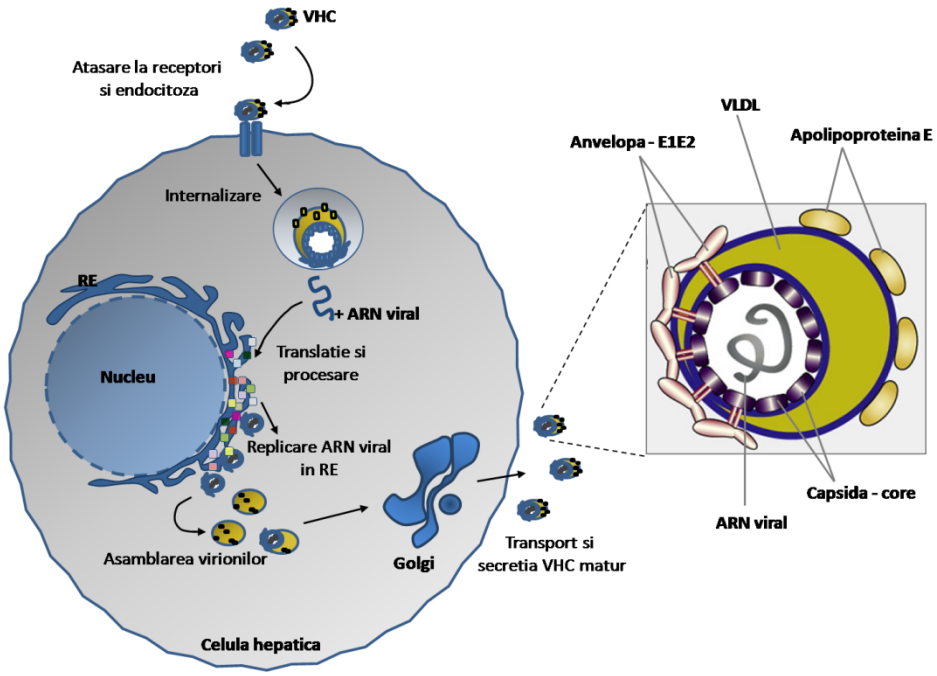

Figure 1. Life cycle and structure of HCV. The lipoviral particle attaches to the specific receptors present at the surface of the liver cell and internalizes through a clathrin-mediated endocytosis process. Acidification of the endosome causes the release of viral RNA into the cytoplasm. Host cell ribosomes recognize viral RNA and begin to translate it in the endoplasmic reticulum (ER). Following translation, a viral polyprotein is formed that will be proteolytically cleaved into ten distinct viral proteins. Viral proteins interfere directly with replication and assembling of the virus indirectly aided by cellular ones. Both replication and HCV assembly takes place at the ER level. The viral particles are transported and secreted by the Golgi apparatus and released into the extracellular environment where they can attach to another hepatic cell. From a structural point of view, the lipoviral particle has a lipid part consisting of VLDL and certain specific proteins, including ApoE. The viral component itself has a viral envelope consisting of E1E2 glycoproteins and a nucleocapsid composed of core protein and viral RNA (Adapted after Dubuisson and Cosset, 2014).

HCV particles have a diameter of 50-80 nm (Catanese, Loureiro, et al., 2013), consisting of a single-stranded positive polarity RNA. The RNA molecule together with the capsid protein (core protein) of the virus forms the nucleocapsid (Vieyres, Dubuisson and Pietschmann, 2014). It is surrounded by a lipid membrane, called viral envelope, where viral envelope glycoproteins E1 and E2 are anchored (Vieyres, Dubuisson and Pietschmann, 2014). A particularity of HCV is that during assembly, virions are associated with apolipoproteins such as apoE, apoB, apoA1, apoC1, apoC2 and apoC3 (André et al., 2002). Studies in cell culture systems have highlighted the presence of high density lipoproteins (VLDL) and low density lipoproteins (LDL) together with cholesterol esters (Merz et al., 2011) in the HCV composition (Figure 1).

HCV envelope glycoproteins are major determinants of virion internalization (Dubuisson and Cosset, 2014). They are involved in receptor binding and mediate the fusion process between the viral envelope and the endosome cell membrane.

1.2 The role of phosphatidylinositides in the life cycle of HCV

Replication but also HCV assemblage are dependent on lipid metabolism and in particular on the accumulation of LD in liver cells. Phosphoinositides (PI) represent a class of phospholipids located in different membranes and their level of expression is regulated by kinases and specific phosphatases. PI are involved in most cellular processes, such as cell cytoskeleton formation, cytokinesis, ER lumen formation, and in mechanisms involving transcriptional regulation. PI are phosphorylated derivatives of phosphatidylinositol. Phosphorylation and dephosphorylation of PI is mediated by different kinases or phosphatases, specifically distributed in the cell, which determine their targeting in different cell compartments. The process of formation of various PI (mono-, di-, triphosphate) in the mammalian cells is mediated by 19 kinases and 28 phosphatases (Balla, 2013). PI are secondary messengers responsible for transmitting and propagating the signal from cellular receptors to effector proteins, thus inducing cell response to a particular stimulus. PI interacts with effector proteins via specific binding domains, allowing interaction with different membranes and thus determining the response induced by the stimulus (Kutateladze, 2010). Another role of PI is the space and time regulation of several proteins involved in vesicular traffic (such as PI4P and PI3P), cytoskeleton rearrangement [PI(4,5)P2], polarity control, cell migration, proliferation and differentiation [PI(3,4)P2, PI(3,4,5)P3] (Brachmann et al., 2005).

1.3 HCV complementation systems

Complementation is a genetic mechanism that naturally occurs in viruses, causing functional rescue of defective or mutant genomes. Due to the development of molecular biology techniques and cell culture systems, it has been possible to adapt this phenomenon for in vitro studies. In conventional experiments, which involve complementing a defective genome or a protein, salvation was achieved through the wild type genome - wt. In virology, complementation is a gene tool used to investigate the functionality of a protein or the replication and assembly of viral

particles. Also, this mechanism is a model for the construction of defective viruses that do not contain certain viral proteins.

A particularity of the *Flaviviridae* family is the replication of viral RNA in vesicular compartments ("membranous web"). Moreover, a series of viruses that have positive RNA genome, such as *Picornaviridae, Alphaviridae, Coronaviridae* and *Flaviviridae* have the ability to assemble viral particles by expressing structural proteins in trans, independent of proteins that interfere with the replication of RNA viral molecules (Puig-Basagoiti et al., 2005). These secreted viral particles are capable of a single round of infection. Also, this trans-encapsidation system can also be used in the production of vaccines (Zhou et al., 2011).

In conclusion, the trans-complement systems for HCV proteins showed the following aspects:

- mutations in certain regions of the NS5A protein or the entire deletion of domain III may be complementated by the NS3-5B sequence;
- the NS2 protein may be trans-complementated by its homologue wt;
- the HCV capsid protein may be trans-complementated by the wt form, with the mentioning that positions 170 and 174-177 of the protein structure are highly conserved.

Chapter 3. Novel replicons and trans-encapsidation systems for Hepatitis C Virus proteins live imaging and virus-host interaction proteomics

3.1 Validation of affinity purification tags in HCV full-length functional replicons for studying NS2 and NS5A proteins host interactions

To investigate the interaction between cellular and HCV non-structural proteins, several groups of researchers have opted for viral protein expression in a heterogeneous system, followed by purification of complexes by AP-MS or two-hybrid analysis (Ramage et al., 2015). The advantage of using these systems is to increase the probability of identifying direct interactors of viral proteins but, on the other hand, biological relevance is limited. The use of the cell culture system for HCV allows identification of the endogenous factors involved in particular in the steps of assembling and secreting the virus.

Since the late stages of the HCV life cycle are less well known, the interest of the NS2 and NS5A viral proteins, which are essential to the assembly process, is a priority. By molecular biology techniques, viable affinity purification (AP) insertions were introduced into permissive positions in the structure of two viral proteins (NS2 and NS5A). Together with other research groups, we noticed that the N-terminus region of the NS2 protein allows insertion of the tags, which leads to a thorough investigation of the HCV assembly process (Stapleford KA et al., 2011). Thus, we could insert a sequence of HA and one of StrepII to create the JFH-1^{HAStrepNS2} (HSNS2) virus. For the NS5A protein, we used the data previously reported in the literature that demonstrated that a 40 amino acid compensatory deletion of the viral domain III domain and a FLAG-Strep-Strep insert are optimal for the JFH-1^{FLAGstrepstrepNS5A} genome functionality (FSSNS5A) (Gottwein et al., 2011) (Figure 2A). The functionality of these modified viral genomes (HSNS2 and FSSNS5A) was tested in our HCV study system by comparison with the JFH-1^{wt} (CSA4) viral genome (Figure 2B). Functional infectivity results demonstrated that both viral genomes in which insertions are comparable in viral titer to control (CSA4).

To validate the utility of recombinant viruses in determining cell-viral protein interactions, Huh-7 cells were electroporated with viral RNA and cell lysis was performed at 72-96h post-electroporation. Cell lysates were subjected to IP technique by AP followed by WB analysis to identify viral interactors (Figure 2D). Thus, the NS2 viral protein was able to form protein complexes with other HCV proteins, including E2 and NS3, as previously described (Popescu et al., 2011,

Stapleford and Lindenbach, 2011). The 40 amino acid offset of the NS5A protein determined the faster migration of the protein into the PAA gel, as can be seen in Figure 2C, and the efficacy of the IP technique was demonstrated by the lack of this form in the post-IP lysate (Figure 2C).

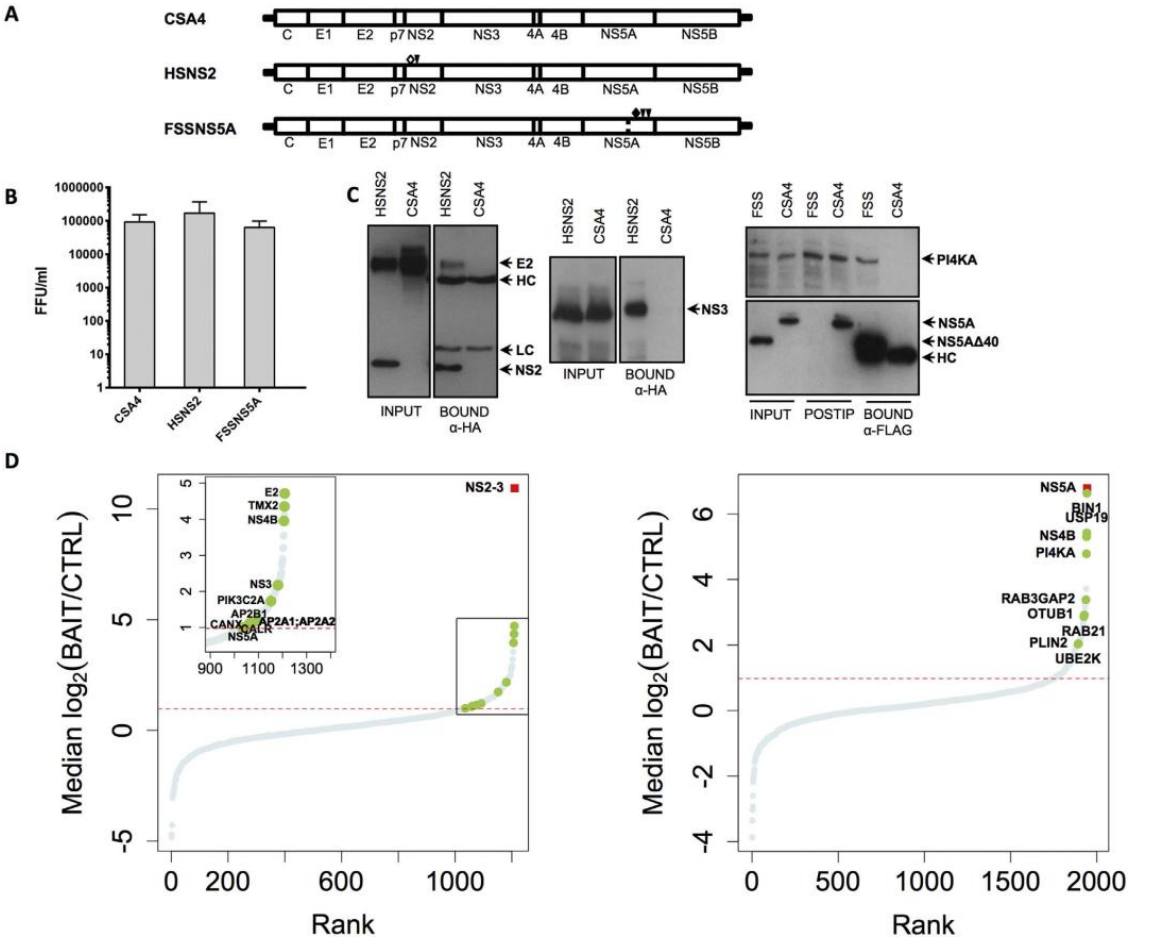

Figure 2. HCV NS2 and NS5A host interactomes. A) Schematic representation of the JFH-1 full-length RNA with different tag insertions (HA tag – white dimond; Strep II tag – black arrow; FLAG tag – black diamond). CSA4 was used as template for two full-length with tag insertions in NS2 (HSNS2) and NS5A viral protein (FSSNS5A), respectively; B) Huh-7 cells were electroporated with JFH-1 RNA and 72 h post-electroporation, the virus titre was determined by inoculating naive Huh-7 cells with serial dilutions of the cell supernatant. 48 h postinoculation, the infected cells were fixed in ice cold methanol and they were immunostained with anti-E1 antibody and the foci forming units (FFUs) were counted; C) At the same time, 72 h post-electroporation Huh-7 cell lysates were subjected to pull-down with different antibodies (α-HA, α-FLAG) followed by western blot interactors detection (E2, NS3, NS5A, PI4KA). LC – light chain, HC – heavy chain; D) NS2 and NS5A interactors were ranked as described in Material and Methods and they were represented as S-plots and a threshold was apllied (interrupted red line). There are represented the NS2 and NS5A baits as red squares, the highlighted interactors above the threshold as green dots and the other interactors as blue dots.

3.2 Inducible trans-encapsidation of core deficient affinity purification tagged subgenomic replicons

To better understand the nature of the host-cell interaction, we have developed a system where viral protein-endogenous proteins complex can be identified in live or fixed cell samples. Thus, for real-time imaging of NS5A and core proteins, we adapted a trans-complementary system for defective core subgenomic replicons. The rationale for choosing real-time imaging of the NS5A protein is related to its involvement in a multifunctional complex by which HCV develops a mechanism against anti-viral therapy. The second viral protein to which we chose the dynamics is the core protein because it interacts with NS5A during HCV assembly (Masaki et al., 2008). Thus, we have constructed core defective subgenomic replicons (Δcore) that hase EGFP tag insertion in the NS5A protein (EGFPNS5A) or viral genomes that possess insertions of TC in the core protein but also EGFP in NS5A (TCcoreEGFPNS5A). Moreover, for the trans-complementation system, only the core protein with a TC insert susceptible to in vivo fluorescence labeling of transfected cells (Eyre et al., 2014) were fused into a DNA plasmid. We initially verified the expression of TCcore protein (pcDNA4-TCcore) as compared to core protein (pcDNA4-core) by transfection into Huh-7 cells. TCcore protein expression were detected by WB at 24h post-transfection with anti-core antibodies and Figure 3B shows the molecular weight difference between the fusion protein and the wt (Figure 3B).

The primary purpose of the trans-complementation system for the TCcore protein was to develop a stable and inducible cell line for the fusion protein (TCcore), but unfortunately no cell clones showed protein expression. Thus, all real-life imaging experiments for TCcore protein were performed in a transient expression system. As can be seen in Figure 3C, the infectivity of viral particles secreted by JFH-$1^{TCcoreEGFPNS5A}$ electroporated cells is comparative with both the CSA4 and JFH-$1^{EGFPNS5A}$ genome, demonstrating that the two inserts do not affect the virus life cycle and viral particle functionality.

Measurement of VHCtcp production implied electroporation of Huh-7 cells with the JFH-$1^{\Delta coreEGFPNS5A}$ replicon followed by transfection at 48 hours post-electroporation with the pcDNA4TCcore vector. At 72 hours post-transfection, the cell medium was harvested and virus infectivity titrated by FFU technique (Figure 3C right). As can be seen in Figure 3C, cells transfected with the capsid protein of the virus were able to secrete VHCtcp, compared to those transfected with DNA control plasmid. This experiment demonstrates that transient transfection can be used as

a trans-complementation system for real-time imaging of NS5A protein during replication (plasmid DNA transfection control) or assembly the virus (transfection with pcDNA4TCcore).

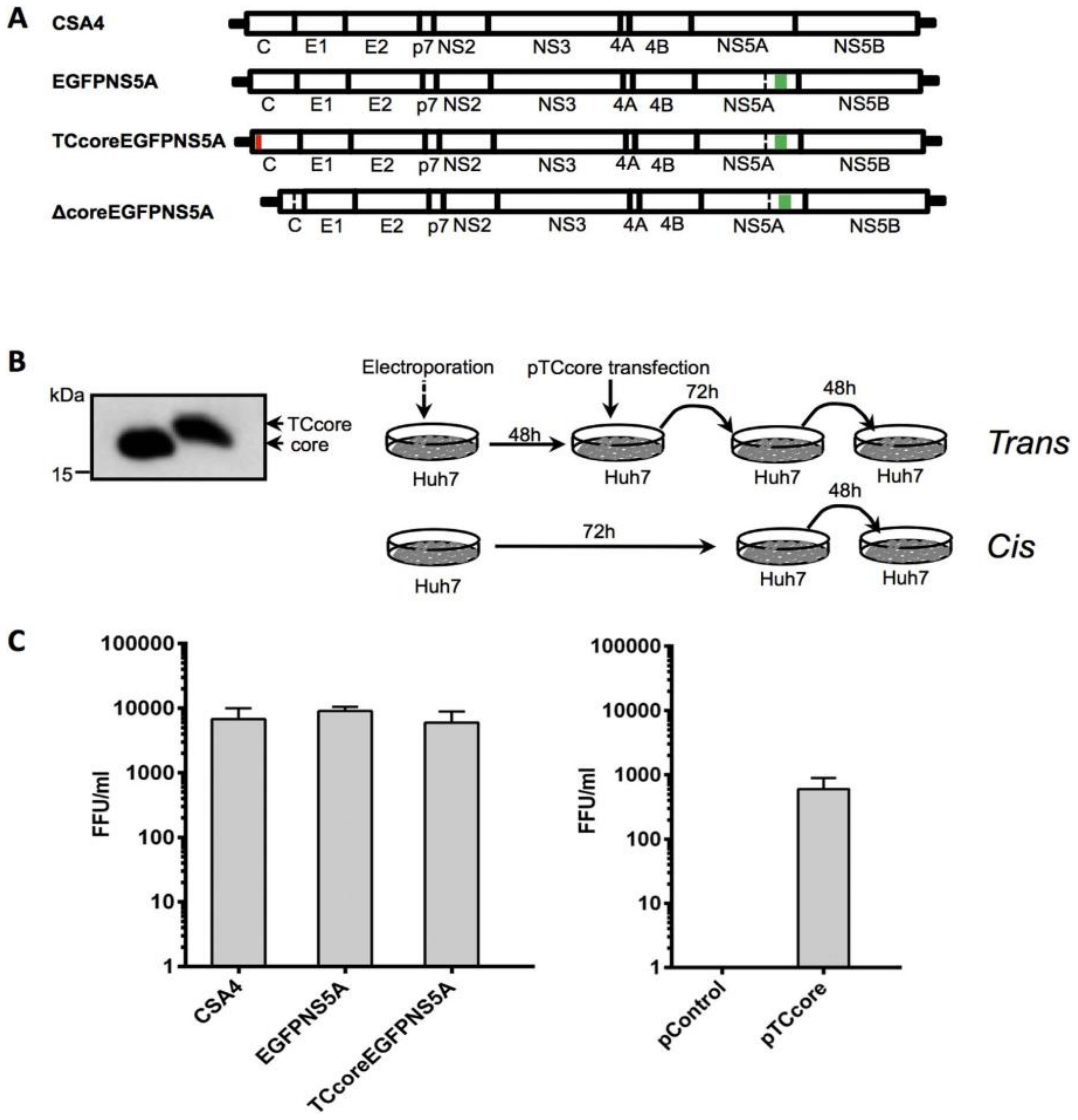

Figure 3. TCcore trans-encapsidation system for live imaging. A) Schematic representation of the full-length (CSA4, EGFPNS5A, TCcoreEGFPNS5A) or subgenomic replicons (ΔcoreEGFPNS5A) with different tags (EGFP – green rectangle; tetracystein tag (TC) – red rectangle); B) Huh-7 cells were transfected with pcDNA4core or pcDNA4TCcore and the core and TCcore protein expression were detected by western blot with anti-core antibody (left). TCcore cis- and trans-encapsidation systems were represented schematically (right). Huh-7 cells were electroporated with full-length replicons (CSA4, EGFPNS5A, TCcoreEGFPNS5A) and 72 h post-electroporation, viral titres were determined by the FFU method (cis). Huh-7 cells were electroporated with ΔcoreEGFPNS5A subgenomic replicon and 48 h post-electroporation, cells were transfected with pcDNA4TCcore (pTCcore) or the pcDNA4 plasmid (pControl). 72 h post-transfection, the infectivity of the trans-complementated particles was determined (trans); C) The titres of the particles produced in cis (left) and trans (right) complementation systems were determined by FFU assay.

3.3 Validation of the trans-encapsidation system for analysis of NS5A dynamics in different stages of the HCV life cycle

After checking the functionality of the trans-complemenatation system for TCcore, the next step was subcellular localization of viral proteins. Huh-7 cells were electroporated with JFH-$1^{\Delta coreEGFPNS5A}$ and 48 hours post-electroporation, transfection with pcDNA4TCcore plasmid was performed. At 48 hours post-transfection, the cells were in vivo labeled with ReAsH or fixed and incubated with anti-core antibody (Figure 4A). Fixed cells were analyzed by confocal microscopy. The results showed that expression of the TCcore fused protein in electroporated cells induces LDs relocation in the perinuclear zone by delimiting a 5 µm area around the nucleus (Figure 4B left). Moreover, the expression of the TCcore protein and its localization around LDs determines the recruitment of the NS5A protein in the vicinity of LDs (Right Figure 4B), a similar process to a direct interaction previously demonstrated by Miyanari in 2007 (Miyanari et al., 2007) and Masaki in 2008 (Masaki et al., 2008).

After checking the functionality of the trans-complementation system and identifying subcellular localization of viral proteins similar to CSA4, the next stage of the study consisted of real-time imaging of the EGFPNS5A protein in determining its dynamics in non-transfected or transfected cells with the TCcore fusion protein. To compare the trans-complementation system with CSA4 virus infection, we chose electroporation of Huh-7 cells with the JFH-$1^{EGFPNS5A}$ viral genome. In order to maintain the same imaging conditions, Huh-7 cells were electroporated with JFH-$1^{\Delta coreEGFPNS5A}$ and transfected with pcDNA4-TCcore (core +) or untransfected (core-) in the same cell population. Cells that do not express the TCcore fusion protein can be identified due to the fact that the ReAsH staining agent does not cause protein labeling. Moreover, cells that do not express the HCV capsid protein exhibit a perinuclear LDs and NS5A relocation relative to those expressing the core protein (Figure 4).

In conclusion, the methods presented in this part of the thesis can be used for the identification of HCV protein interactors in a biologically relevant system. Moreover, optimization of the trans-complementation system allows the analysis of interactor dynamics at different stages of the HCV life cycle. Because of the HCV characteristics, these systems can be applied to other RNA viruses with a high potential for identifying molecular biomarkers that interfere with the life cycle of the virus.

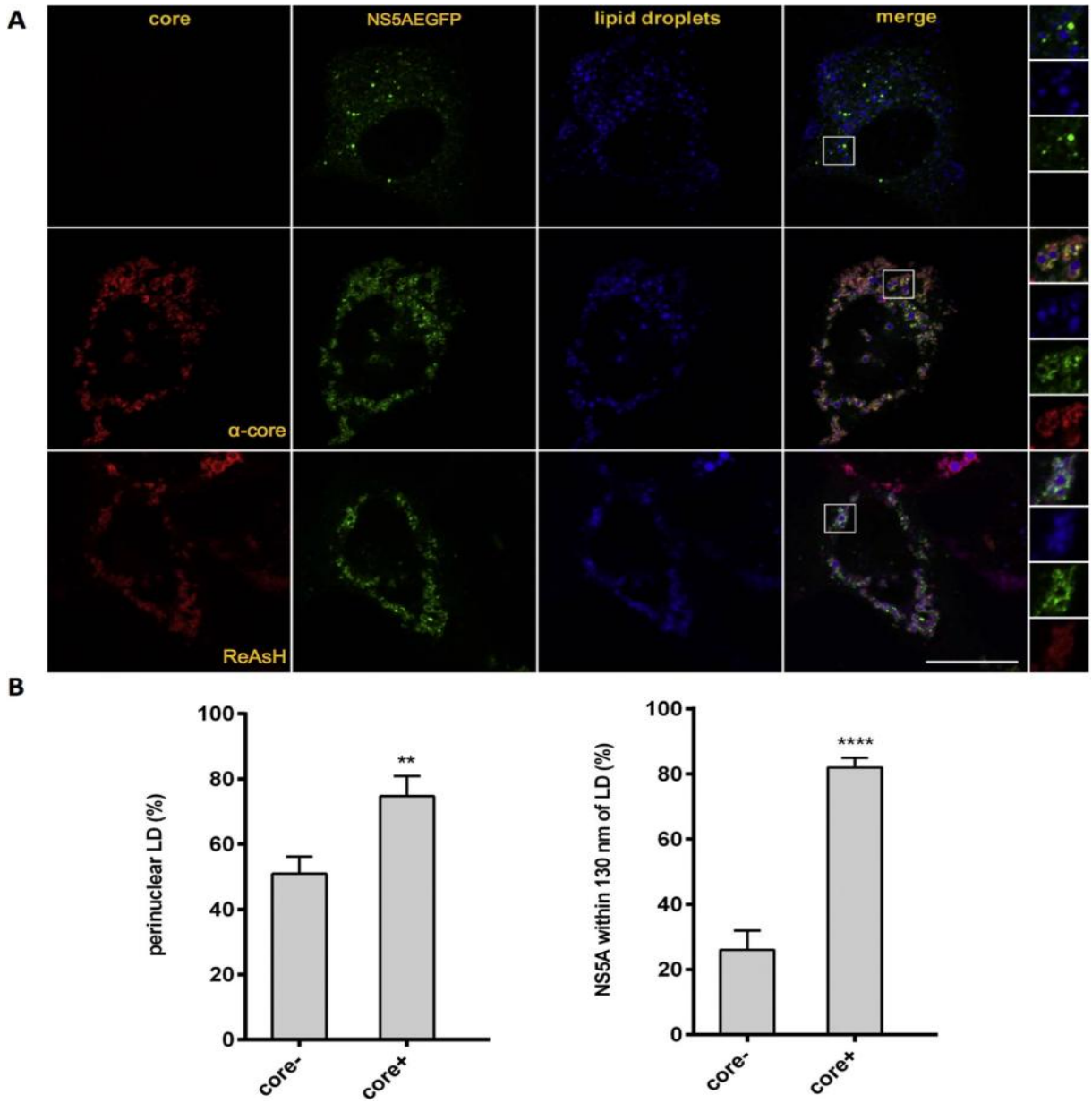

Figure 4. HCV proteins subcellular localization in the trans-encapsidation system. A) Huh-7 cells were electroporated with ΔcoreEGFPNS5A RNA (green) and 24 h post-electroporation, cells were transfected with the pcDNA4TCcore plasmid. 48 h post-transfection, cells were labeled either with anti-core antibody or ReAsH (red) and LipidTOX Deep Red Neutral Lipid stain for lipid droplets (blue) and imaged by confocal microscopy. Zoomed views of the indicated areas are shown in the right column. Bar, 20 µm; (B) The perinuclear LD surface was measured in two groups of cells expressing TCcore (core+) or not (core−) (left). The EGFPNS5A signal was measured in an area of 130 nm around the LD which was segmented using ImageJ software (right). Error bar represents SEM of 14 (core−)/17 (core+) measurements (** $p < 0.01$, **** $p < 0.0001$ for unpaired two-tailed t-test).

Chapter 4. Identification of PI3KC2 as a regulator for the HCV life cycle

4.1 PI3K Class II isoforms are involved in the HCV life cycle

From the same reasoning presenting in Section 3, using molecular biology techniques we introduced insertions such as HA, Strep II and FLAG into permissive positions at the N-terminus of the NS2 viral protein. Starting from the JFH-1^{CSA4} viral genome, we created three viral genomes with different insertions in the NS2 protein (HSNS2, HFNS2 and FNS2) (Vlaicu et al., 2017). The functionality of these modified viral genomes is presented in Part 3 of the results. Data obtained from the identification of endogenous interactors for the NS2 viral protein revealed the involvement of PI3KC2α in the HCV life cycle (Vlaicu et al., 2017).

To determine the importance of PI3K class II in the HCV life cycle, we optimized a protocol that involves the transfection of Huh-7 cells with siRNA for 48 hours in order to significantly reduce the expression of the protein of interest. At 48 hours post-transfection, the cells were inoculated with HCV (CSA4) for 4 hours (MOI = 0.5), followed by a second transfection with siRNA (see Materials and Methods, Transfection and RNA, Protocol 1). At 72 hours post-inoculation, the cell media was harvested, determining the extracellular titer and the cells lysed, analyzing by WB protein expression and FFU intracellular titer (Figure 5A). Results obtained from WB showed that the decrease protein expreson for α and γ isoforms of class II PI3K significantly reduced the expression of E2 and NS5A viral proteins, while isoform β showed no alteration in the expression of the two viral proteins compared to siCTR (Figure 5B). Two endogenous proteins (PI4KA and ApoE) used as control. This proteins significantly influence two of the HCV life cycle steps. Were used PI4KA as a replication control and ApoE for assembling of the virus. As can be seen in Figure 5B, reduction of the PI4KA protein inhibits virus replication while the absence of ApoE inhibits its assembly (Figure 5B).

The next step of the study was to determine the involvement of these isoforms in the internalization / replication steps as well as the secretion / infectivity of HCV. To study the internalization / replication step, 48 hours post-inoculation, Huh-7 cells transfected with siRNA and infected with CSA4 virus (see Protocol 1) were fixed with methanol then incubated with anti-E1 antibody and antibody secondary fluorescence according to IF technique. As can be seen in Figure 5C, all PI3KC2 isoforms affect differently the two stages of the HCV life cycle. Thus, reducing of

PI3KC2 isoforms expression decreased the HCV internalization / replication process compared to siCTR (Figure 5C). On the other hand, quantification of HCV assembling / secretion steps has shown that reduction of α and γ isoforms of PI3KC2 causes an increase in HCV secretion by approximately six fold compared to siCTR (Figure 5C). Instead, siRNA isoform β of PI3KC2 does not exhibit a significant change in this step of HCV replication (Figure 5C). RNA control was also used for each stage of the life cycle of the virus. Thus, for the internalization step, siRNA for the CD81 protein was used, representing the primary virus binding receptor and siPI4KA for replication of the viral genome while siApoE was used for the virion assembly step. The profiles of flow cytometry analysis of virus infected cells for the internalization / replication step as well as assembling / secretion are shown in Figure 5D.

Next we quantified the number of infectious viral particles present both inside the siRNA transfected cells and inoculated with the virus but also secreted into the extracellular medium. Compared to siCTR, no isoform of PI3KC2 shows significant changes in the intracellular titer. On the other hand, when the extracellular titer was measured, reducing the expression of α and γ isoforms of PI3KC2 caused an increase of approximately five-fold the number of infectious viral particles secreted in the culture medium compared to siCTR (Figure 5E). Interestingly, the RNA isoform β of PI3KC2 causes a decrease of approximately one hundred times the extracellular viral titer (Figure 5E). This result suggests a possible involvement of PI3KC2 isoforms in the later stages of the HCV life cycle.

Results published by Maehama T et al. in 2013 showed that PI3KC2 isoform β affects the internalization of HCVpp (Maehama et al., 2013). Thus, using the viral pseudoparticle production system (VHCpp, Genotype 2a) and Protocol 2, we highlighted that reducing the expression of α and γ isoforms of PI3KC2 leads to a more pronounced decrease in viral internalization compared to siCTR, while the β isoforme of kinase only a slight decrease (Figure 5F). These differences can be explained by the subcellular localization of PI3KC2 isoforms. It can be argued that isoforms α and γ, which are located in the cell membrane, significantly affect the internalization of the virus while the isoform β, located in the ER lumen, does not influence this process. siCD81 was used as a positive control of receptor-dependent viral internalization and VSVG as a control of pseudoparticle transfection and secretion. There is no statistically significant difference between the VSVG chemiluminescent signal in any of the VHCpp production conditions, which demonstrates that this process has been carried out uniformly and the results obtained are true (Figure 5F).

To observe the involvement of PI3KC2 isoforms strictly in HCV replication, we followed protocol 3 presented in the Materials and Methods section. Huh-7 transfected with siRNA were electroporated with JFH-$1^{SGR\_RLuc\_3\text{-}5B}$ (genotype 2a), followed by quantification of luciferase activity at 48 hours post-electroporation. RLU analysis revealed a decrease in chemiluminescent signal for all siRNAs corresponding to kinase isoforms, but more pronounced for α and γ isoforms, consistent with low expression of NS5A protein (Figure 5G). siPI4KA was used as a virus replication control, completely inhibiting this process. These results suggest that reducing expression of α and γ isoforms of PI3KC2 inhibits virus replication by approximately 50%, while decreasing isoform β expression results in a slight inhibition of replication by approximately 15% compared to siCTR (Figure 5G). A possible explanation of different levels of expression of luciferase activity is correlated with the subcellular localization of isoforms of this protein. From analysis of NS5A protein expression it can be seen that siPI3KC2α and γ causes a significant reduction of the viral protein, while siPI3KC2β has a lower influence compared to siCTR (Figure 5G).

From the results we can conclude the following:

• significant reduction of intracellular expression of PI3KC2α and γ proteins results in a marked decrease in viral protein expression, correlated with decrease in viral RNA replication;

• siPI3KC2β does not appear to influence the expression of NS5A and E2 viral proteins;

• HCV secretion activity shows a significant increase in siRNA transfected cells for the α and γ isoforms of PI3KC2, but the β isoform appears to inhibit this step;

• the intracellular titer was not affected for any of the transfections with siRNA for PI3KC2, instead the number of infectious viral particles secreted in the extracellular medium increased approximately five-fold for the cells transfected with siPI3KC2α, respectively siPI3KC2γ;

• siPI3KC2β negatively influence the extracellular titer, causing a decrease in the virus secreted in the culture medium;

• Cells transfected with siRNA for α and γ isoforms of PI3KC2 inhibit HCV internalization while isoform β does not influence this process;

• replication of viral RNA is significantly affected by siPI3KC2α and siPI3KC2γ, respectively, compared to isoform β which does not exert a significant effect;

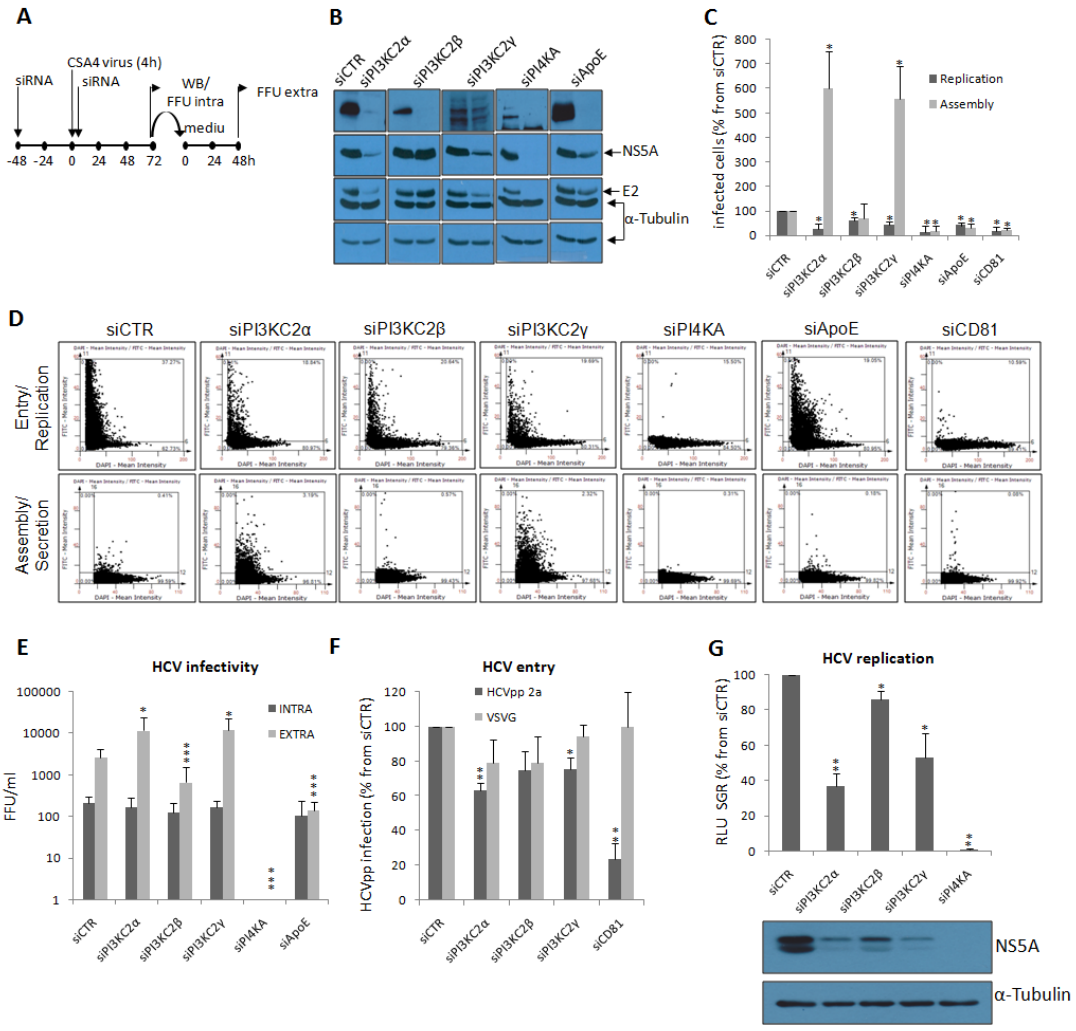

Figure 5. PI3KC2 differently regulate the HCV life cycle. A) Schematic representation of the protocol used for the HCV internalization / replication and HCV assay / secretion step analysis. Huh-7 cells were transfected with the siRNA followed 48 hours post-transfection by a CSA4 virus inoculation for 4 hours. Immediately after inoculation, the cells were subjected to a new round of siRNA transfection. At 72 hours post-inoculation, cells and cell medium were harvested for WB and FFU analysis. B) WB analysis of the expression of endogenous and viral proteins in Huh-7 cells subjected to the protocol of Figure A. C) Analysis by flow cytometry quantification of siRNA-transfected cells and inoculated with virus at 48 hours post-inoculation for the internalization / replication and assembly / secretion steps according to the protocol shown in Figure A. D) Huh-7 cell profiles subjected to the protocol scheme of Figure A and analyzed by flow cytometry. E) Quantitation by FFU of the intracellular and extracellular titer of Huh-7 cells under the protocol scheme of Figure A. F) Quantitation by VHCpp assay of internalizing viral pseudoparticles in Huh-7 cells transfected protocol 2 and normalized to siCTR and VSVG. G) Quantitation of luciferase activity of HCV replication in Huh-7 cells subjected to transfection protocol 3 and normalized to siCTR (top). WB analysis of NS5A viral protein expression in the same Huh-7 cells subject to protocol 3 having α-tubulin protein control (bottom). These results are presented as mean ± SD (n≥3 independent experiments, * p <0.05, ** p <0.005, *** p <0.0005).

4.2 The N-terminal region of PI3KC2α is involved in HCV assembly / secretion steps

The results obtained from the analysis of the PI3KC2α isoform domains revealed the involvement of this protein in the later stages of the HCV life cycle. In order to identify the region of this protein that interacts with the NS2 viral complex and implicitly regulates the activity of virus assembly / secretion processes, we constructed chimeric and mutant PI3KC2α proteins that we inserted into a DNA plasmid (pLNCX2). Thus, using molecular biology techniques, we fused the GFP protein to the N-terminal region of the PI3KC2α protein thereby constructing DNA plasmid pLNCX2-GFP-PI3KC2aWT (WT). Due to the fact that this protein exhibits enzymatic activity, we constructed a pLNCX2-GFP-PI3KC2αM1 (M1) mutant protein in which the enzyme site was modified to produce only PI3P. The purpose of using the M1 protein was to determine whether the PI(3,4)P2 enzyme product is involved in the HCV assembly / secretion process. Moreover, by DNA plasmid pLNCX2-GFP-PI3KC2aM2 (M2), where the enzymatic site is neutralized, we wanted to determine whether the enzymatic activity of PI3KC2α, regardless of the product obtained, is required in late stages of the viral cycle. A PI3KC2α mutant protein in which the N-terminal region was deleted [pLNCX2-GFP-PI3KC2αΔ (Δ)] was constructed in order to identify the region required for negative regulation of HCV assembling / secretion. As can be seen in Figure 6A, four fusion proteins were constructed, each exhibiting a particularity (Figure 6A).

Since the expressions were performed in a transient expression system, we optimized a co-transfection protocol for siRNA and DNA plasmid presented in the Materials and Methods Section. Thus, Huh-7 cells were seeded for 24 hours before inoculation with CSA4 for 4 hours, followed by co-transfection with siRNA and DNA plasmid, according to protocol 4 (Figure 6B). Huh-7 cells and extracellular medium were harvested 72 hours post-inoculation, assayed by WB expression of PI3KC2α, NS5A, E1, α-tubulin proteins and by IF for extracellular titer.

Normalization of transfection efficiency of DNA plasmid was performed by IF for GFP protein as well as for E1 viral protein. Thus, 72 hours post-inoculation, Huh-7 cells subjected to transfection protocol 4 were fixed with 4% PFA and incubated with anti-E1 antibody and secondary fluorescent antibody according to the IF protocol presented in the Materials and Methods Section. In Figure 6C it can be seen that all samples were transfected under the same conditions (fluorescent marker GFP) and infected in the same manner (E1 antigen) (Figure 6C).

The WB analysis of M1 and M2 revealed that the enzymatic activity of PI3KC2α is not involved in the HCV life cycle. Thus, Huh-7 cells undergoing transfection protocol 4 were harvested 72 hours

post-inoculation and lysed with a lysis buffer according to the method described in the Materials and Methods Section. As can be seen in Figure 6D, Huh-7 cells were co-transfected with siCTR or/and PI3KC2α and DNA plasmid for each mutant PI3KC2a protein. pLNCX2-GFP was DNA control plasmid (Figure 6D). Incubation of the WB membrane with anti-GFP antibody reveals that all plasmids were transfected into Huh-7 cells. Since protein Δ lacks the N-terminal region, it presents a molecular weight difference compared to WT, M1 and M2 proteins. Incubation of the WB membrane with anti-PI3KC2α antibody highlights the decrease in endogenous expression of PI3KC2α (right column) compared to siCTR transfected Huh-7 cells (left column). It can be seen that the expression of the endogenous PI3KC2α protein in cells transfected with siPI3KC2α is absent, while the PI3KC2α mutant protein (GFP-PI3KC2α) is present (Figure 6D). By analyzing the NS5A viral protein expression, it can be seen that the replication step of the virus is not severely impaired (Figure 6D). Comparing the expression of the E1 viral protein in both CTR / GFP (co-transfection and CTR and pLNCX2-GFP) and siCTR / WT (co-transfection and CTR and pLNCX2-GFP-PI3KC2αWT), a considerable decrease was observed in the second sample suggesting a negative impact on the HCV assembly / secretion processes (Figure 6D). Moreover, by comparing the expression of the E1 viral protein in the samples of PI3KC2α / WT (co-transfection and PI3KC2α and pLNCX2-GFP-PI3KC2αWT) with the other mutant proteins (co-transfection and PI3KC2α / M1, M2 and Δ) and PI3KC2α / Δ increases the level of tachy protein expression, which may suggest involvement of the N-terminal domain of the PI3KC2α protein in the regulation of HCV assembling / secretion steps.

Exogenous expression of the PI3KC2αWT protein in siCTR transfected Huh-7 cells causes a decrease in viral particle secretion detected by quantitation of extracellular titer compared to siCTR / GFP (Figure 6E). Although transfection protocol 4 was used, Figure 6E shows an increase in viral titer in cells co-transfected with siPI3KC2α / GFP, which correlates with the results obtained in Huh-7 cells which underwent transfection protocol 1. Rescue of viral titer was obtained in Huh-7 cells co-transfected with siPI3KC2a / WT, as compared to siCTR / GFP (Figure 6E). These results highlight that although the cell no longer expresses the endogenous form of PI3KC2α (cells transfected with siPI3KC2α), its exogenous introduction (WT-transfected cells) determines the function of the protein, not affecting the HCV life cycle.

Measurement of the extracellular titer of Huh-7 cells in which endogenous expression of PI3KC2α was inhibited and exogenous expression was expressed by co-transfection of the M1, M2 and D2 proteins (and PI3KC2α / WT, M1, M2, Δ) confirmed the result of the assay by WB. As can be seen in Figure 6F, the number of viral particles was not affected when the M1 and M2 proteins

were introduced into the cell compared to siPI3KC2α / WT (Figure 6F). An increase in viral secretion by approximately 50% was observed in Huh-7 cells co-transfected with siPI3KC2α / Δ as compared to siPI3KC2α / WT, which strengthens the hypothesis of N-terminal protein involvement of the protein in the later stages of the HCV life cycle (Figure 6F).

The results obtained after analysis of PI3KC2α mutant proteins revealed the following:

• exogenous expression of PI3KC2α, regardless of mutations present, does not affect the expression of NS5A protein and, implicitly, virus replication;

• decrease of viral titer in Huh-7 co-transfected cells with siCTR / WT indicates that over-expression of PI3KC2α protein inhibits the late stages of HCV life cycle;

• rescue of the viral phenotype was obtained in Huh-7 cells in which endogenous protein inhibition and its exogenous expression (co-transfection and PI3KC2α / WT) were performed;

• the enzymatic function of PI3KC2α is not involved in viral cycle regulation, but the N-terminal domain of the protein appears to influence this process.

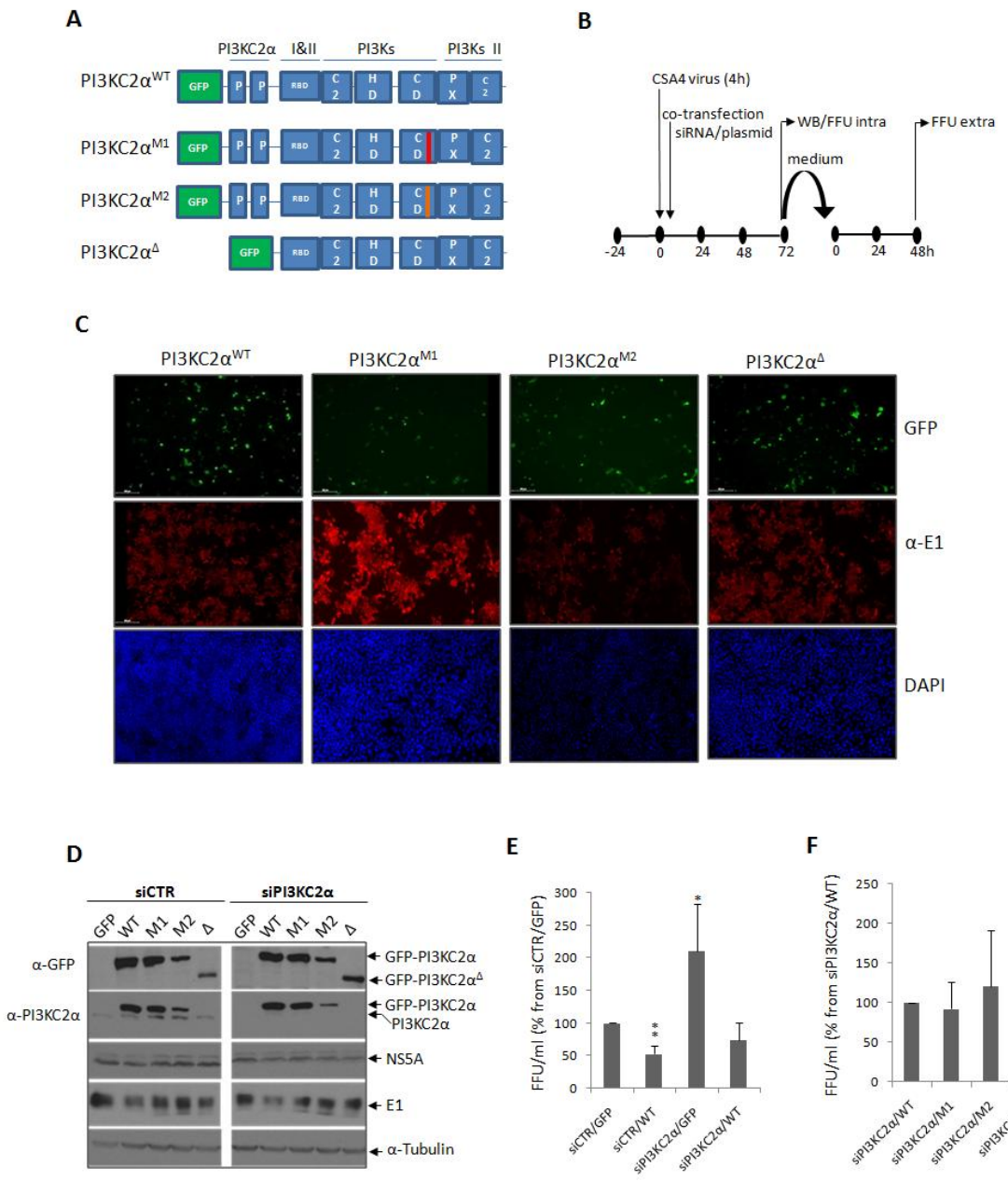

Figure 6. PI3KC2α interacts with NS2 protein and the N-terminus of PI3KC2α is involved in the later stages of the HCV life cycle. A) Schematic representation of PI3KC2α mutant proteins. B) Schematic representation of the protocol used to rescue the viral phenotype. Huh-7 cells were inoculated with CSA4 virus for 4 hours followed by a co-transfection with siRNA and DNA plasmid. At 72 hours post-transfection cells and medium were harvested for WB and FFU assay. C) IF analysis of expression of GFP and E1 proteins in Huh-7 cells subjected to co-transfection and viral inoculation using protocol 4. Cells were labeled with DAPI. D) WB analysis of the expression of endogenous and viral proteins in Huh-7 cells subjected to the protocol scheme of Figure B. E, F) Quantification by FFU of the extracellular titer of Huh-7 cells subjected to the protocol scheme of Figure B. These results are presented as mean ± SD (n≥3 independent experiments, * p <0.05, ** p <0.005).

Chapter 5. Discussion

This work is made up of two major parts. The first part concerns the construction and verification of the functionality of viral genomes showing different insertions in a trans-complementary HCV system (Vlaicu et al., 2017). The construction of viral genomes were used to identify endogenous proteins or fluorescence imaging allowed their characterization in a cis-complementary system (Figure 2 A, B). Confirmation of several proteins identified by other research groups by IP / WB, followed by LC-MS / MS, (Miyanari et al., 2007, Popescu et al., 2011, Stapleford and Lindenbach, 2011) of the HCV cellular system. Following LC-MS / MS results analysis, a list of endogenous proteins was obtained that could interact with viral proteins and regulates the HCV life cycle. Among these proteins was also PI3KC2α (Figure 2D).

The development of an inducible trans-complementary system for HCV capsid protein has allowed the study of the replication and assembly mechanisms of the virus (Figure 3). The trans-complementary cell line (Huh-$7^{Tetcore}$) allowed HCVtcp to be obtained through various defective core genomes having inserts in the NS2 or NS5A viral proteins (Figure 3).

For the study of real-time imaging of various viral proteins (core and NS5A), we compared two complementary systems (cis and trans) (Figure 4). The production of viral genes exhibiting different inserts susceptible to *in vivo* labeling of proteins led to the secretion of HCVtcp for the TCcore fusion protein (Figure 4C). These results are the first to demonstrate the trans-complementation of the TCcore fusion protein into a transient transfection system.

Real-time imaging on Huh-7 cells revealed an increase in number of LD and NS5A in cells which the expression of the capsid protein is present (Figure 4). Moreover, the results revealed an increase in the dynamics of viral complexes in cells where the expression of the capsid protein is absent (core -) compared to cells in which the core protein exerts its function (core +). These data are closely related to the localization pattern of the NS5A protein but LD also in Huh-7 cells which the core protein expression is absent (Figure 4).

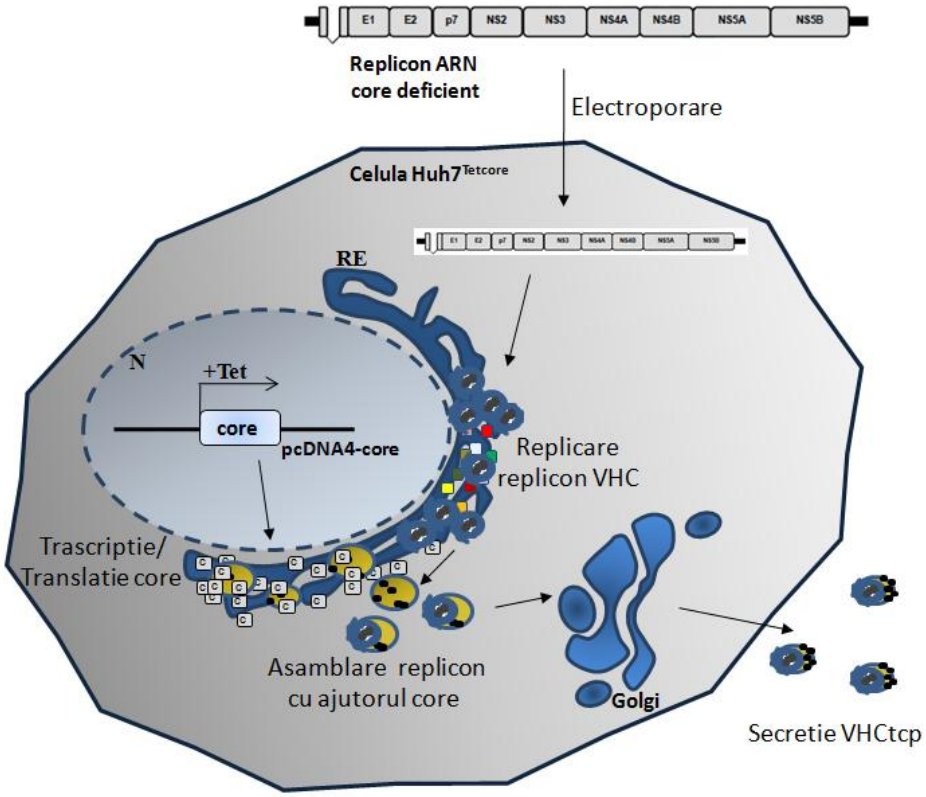

Figure 7. Inducible trans-complementation system for HCV capsid protein using defective core subgenomic replicons. The Huh-7^{Tetcore} cell line has stably inserted in the nucleus of the gene that encodes the HCV core protein. Tetracycline added to the culture medium represses the gene promoter causing transcription / translation of the capsid protein. Electroporation of subgenomic core defective replicons into the cell determines their assembly by inducible expression of the core protein and secretion of HCVtcp outside the cell. The secreted viral particles are capable of a new round of infection in Huh-7 cells, but the lack of the core gene in the viral genome structure does not allow for viral assembly and secretion.

All the results generated in Chapter 3 of this paper have led to the development of a system that allows the differential study between HCV replication and assembly steps. Thus, it was possible to highlight a kinase-function protein (PI3KC2α) that regulates the viral life cycle. Huh-7 transfected with siRNA for kinase isoforms have demonstrated that replication of the virus is inhibited differently when protein expression is profoundly impaired, instead, the assembly process is regulated according to the cellular localization of each kinase. Thus, due to localization of the α and γ isoforms of PI3KC2 at the cell membrane, the internalization of the virus is facilitated, probably

due to interaction with clathrin. This aspect was also observed by other research groups that focused on PI3KC2β (Maehama et al., 2013).

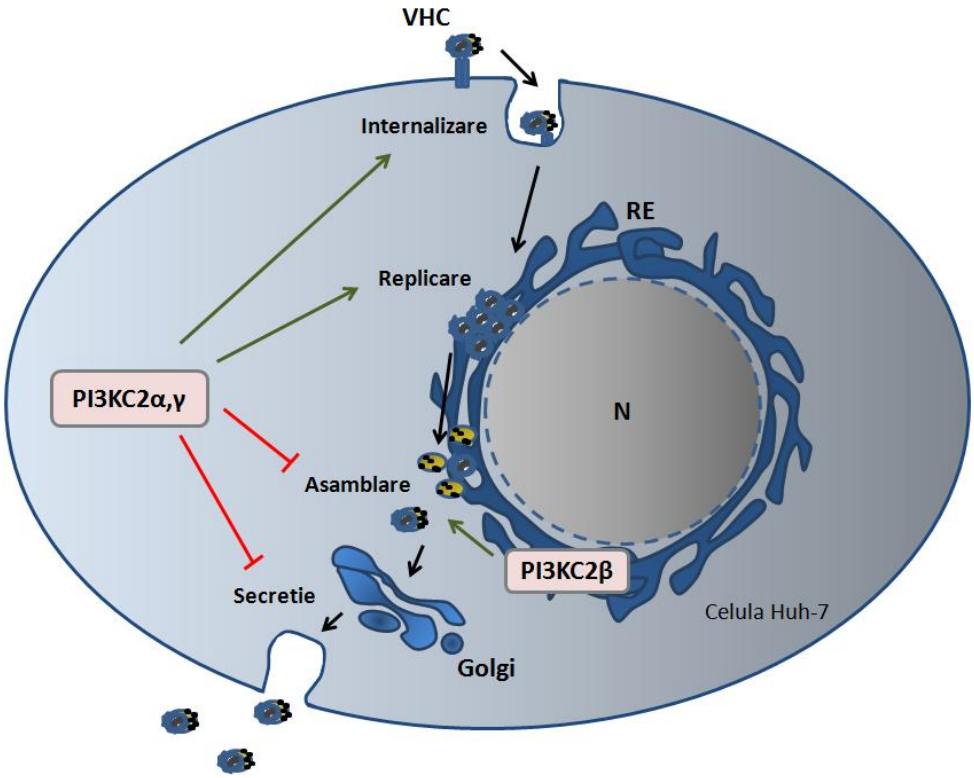

Figure 8. Mechanism by which PI3KC2 regulates different stages of the HCV life cycle. The cytosolic location of PI3KC2α and γ facilitates the internalization of the virus and replication in the cell. The HCV assembly and secretion processes are inhibited by these two isoforms but stimulated by PI3KC2β due to its localization at the ER compartment.

Viral RNA replication analysis reinforced the hypothesis that early life cycle processes (internalization, replication) are favored by α and γ isoforms of PI3KC2 while assembly and viral particle secretion processes are inhibited. On the other hand, the presence at the ER of PI3KC2β facilitates the later processes in the viral life cycle, leading to a more efficient virion assembly / secretion (Figure 8).

Mutagenesis studies performed on PI3KC2α led to the hypothesis that the N-terminus domain of the kinase appears to interact with the NS2 protein, thus regulating the assembly process. It seems that the enzymatic activity of kinase does not seem to influence the life cycle of the virus.

However, further analyzes are needed to accurately determine whether only the N-terminus domain of PI3KC2α is involved in clathrin binding and implicitly favoring the internalization of the virus or there are other endogenous factors that are involved.

On the other hand, detailed studies are needed to elucidate the interaction with viral proteins. The hypothesis that PI3KC2α interacts indirectly in a protein complex with the NS2 protein has to be demonstrated by identifying the protein binding therebetween. If indeed the N-terminus domain is responsible for this link or there are other factors that influence the HCV assembly process.

Within this thesis several cell culture systems of HCV-based on JFH-1 have been optimized which allow:

a) sequential investigation of HCV life cycle stages under reduced biosecurity conditions;

b) identification of endogenous factors involved in the various stages of the HCV life cycle.

The trans-complementation of the HCV capsid protein presented in Chapter 3 allows tandem use of proteomics and imaging techniques to investigate molecular events associated with HCV infection (Vlaicu et al., 2017).

In order to identify new endogenous factors involved in HCV infection with a potential role in the virus-induced carciogenesis process, a discovery proteomome approach based on de novo identification of viral protein interactors was used in a cellular system that produces infectious particles being biologically relevant. After identification of proteins by affinity chromatography followed by mass spectrometry analysis, a broad functional characterization of PI3KC2α was performed, optimizing loss of function and gain of function tests that could be applied to any identified targets.

In conclusion, the present paper describes for the first time the differentiated role of PI3KC2 isoforms in the HCV life cycle, which brings novelty in the understanding of the link between the endogenous pathway and the later stages of the HCV life cycle (assembly and secretion). In addition, HCV can be used as a model for understanding the class II PI3K function, which are less characterized.

List of publications

1. Ovidiu Vlaicu, Tudor Selescu, Florin Pastrama, Cristian Munteanu, Laura Riva, Jean Dubuisson, Yves Rouille, Costin-Ioan Popescu; Novel replicons and *trans*-encapsidation systems for Hepatitis C Virus proteins live imaging and virus-host interaction proteomics. Journal of Virological Methods 246, 2017, *42–50*. ISI: 1.7 (2016)

2. Anca Coman, Codruta Paraschivescu, Niculina Hadade, Andrei Juncu, Ovidiu Vlaicu, Costin-Ioan Popescu, Mihaela Matache; New acyloxymethyl ketones: useful probes for cystein protease profiling. Synthesis 48, 2016, 3917-3923. ISI: 2.6 (2016)

3. Costin-Ioan Popescu, Laura Riva, Ovidiu Vlaicu, Rayan Farhat, Yves Rouillé and Jean Dubuisson; Hepatitis C virus life cycle and lipid metabolism (Review). Biology, 2014, *3*, 892-921.

In preparation:

4. Ovidiu Vlaicu, Florin Pastrama, Leontina Banica, Simona Paraschiv, Dan Otelea, Claire Gondeau, Charles-Henry, Tarik Asselah, Yves Rouilles, Jean Dubuisson, Costin-Ioan Popescu; Phosphoinositide 3-kinases class II regulate Hepatitis C virus assembly and secretion.

International conferences

1. Functional JFH1 based recombinant viruses with insertions suitable for “in vivo” imaging and proteomic analysis; Ovidiu Vlaicu, Sandrine Belouzard, Leontina Banica, Dan Otelea, Jean Dubuisson and Costin-Ioan Popescu; Poster, ANRS, Paris, 23-24 jan. 2014

2. Hepatitis C cell culture system - a valuable tool for understanding viral resistance mechanisms; Ovidiu Vlaicu, Leontina Banica, Simona Paraschiv, Dan Otelea and Costin-Ioan Popescu; Poster, “Al VII-lea Congres National HIV/SIDA cu participare internationala”, Sibiu, 29-31 mai 2014;

3. Inducible *trans*-encapsidation system for Hepatitis C Virus allows temporal separation between the different stages in the viral life cycle; Ovidiu Vlaicu, Leontina Banica, Dan Otelea, Andrei-Jose Petrescu and Costin-Ioan Popescu; Poster, The Annual International Conference of the Romanian Society of Biochemistry and Molecular Biology and Workshop “Viral hepatitis-from cell culture to clinic”, Baile-Felix, 05-07 iunie 2014;

4. Interactomics and live imaging as tools for identification and characterization of endogenous factors involved in Hepatitis C Virus life cycle; Ovidiu Vlaicu, Florin Pastrama, Cristian Munteanu, Georgiana Toma, Simona Paraschiv, Dan Otelea, Andrei-José Petrescu, Costin-Ioan Popescu, Poster, Symposium Nicolae Cajal, Bucharest, 2015;

5. Live imaging of Hepatitis C Virus NS5A and CORE proteins in a *trans*-complementation system; Ovidiu Vlaicu, Tudor Selescu, Andrei Juncu, Yves Rouille, Andrei-Jose Petrescu, Costin-Ioan Popescu, Poster, The Annual International Conference of the Romanian Society of Biochemistry and Molecular Biology, Bucharest, 17-19 sept. 2015;

6. Investigation of hepatitis c virus-host interaction using interactomics and functional genomics techniques in infectious virions producing cells; Ovidiu Vlaicu, Florin Pastrama, Cristian Munteanu, Leontina Banica, Simona Paraschiv, Laura Riva, Yves Rouille, Jean Dubuisoon and Costin-Ioan Popescu, Oral presentation, The Annual International Conference of the Romanian Society of Biochemistry and Molecular Biology, Bucharest, 17-19 sept. 2015;

7. Genomic telaprevir resistance and phenotyping of the resistance mutantions in hepatitis c virus cell culture system; Emil Neaga, Andrei Juncu, Ovidiu Vlaicu, Leontina Banica, Simona Paraschiv, Dan Otelea and Costin-Ioan Popescu, Poster, The Annual International Conference of the Romanian Society of Biochemistry and Molecular Biology, Bucharest, 17-19 sept. 2015;

8. Hight throughput screening assay for identification of new chemical scaffolds which inhibit HCV NS2 cystein protease activity; Andrei Juncu, Ovidiu Vlaicu, Alina Bora, Liliana Pacureanu, Costin-Ioan Popescu, Poster,The Annual International Conference of the Romanian Society of Biochemistry and Molecular Biology, Bucharest, 17-19 sept. 2015;

9. Temporal analysis of the hepatitis C NS5A interactome in infectious virions producing cells; Ovidiu Vlaicu, Florin Pastrama, Cristian Munteanu, Costin-Ioan Popescu; Poster, 22nd International Symposium on Hepatitis C Virus and Related Viruses, Strasbourg, 9-13 oct. 2015;

10. Identification of PI3KC2α as a hepatitis C NS2 complex interactor which is involved in the viral replication; Ovidiu Vlaicu, Florin Pastrama, Cristian Munteanu, Leontina Banica, Simona Paraschiv, Dan Otelea, Yves Rouille, Jean Dubuisson, Costin-Ioan Popescu, Poster, 22nd International Symposium on Hepatitis C Virus and Related Viruses, Strasbourg, 9-13 oct. 2015;

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