Hepatitis C Virus NS5A Targets the Nucleosome Assembly Protein NAP1L1 to Control the Interferon Response

Thesis

Hepatitis C Virus NS5A Targets the Nucleosome Assembly Protein NAP1L1 to Control the Interferon Response

Mia Cesarec

A Thesis submitted in fulfilment of the requirements of the Faculty of Life Sciences of the Open University for the degree of Doctor of Philosophy

International Centre for Genetic Engineering and Biotechnology (ICGEB)

Trieste, ITALY

Director of studies: Alessandro Marcello, Ph.D

External supervisor: Lawrence Banks, Ph.D

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ABSTRACT

Hepatitis C virus (HCV) is a hepatotropic virus affecting more than 150 million people worldwide. HCV establishes a chronic infection in the majority of cases, which leads to severe liver complications such as cirrhosis and hepatocellular carcinoma. Fortunately, recent potent direct acting antivirals can now cure the infection. However, such treatments can induce resistance, are extremely costly limiting their use to wealthier countries and are ineffective for the complications of the infection. Therefore, a better understanding of the interaction of HCV with the host cell remains a priority both to increase the armamentarium of antiviral drugs and to define the relationship between infection and malignant transformation.

My study focuses on the mechanisms governing the evasion of the innate immune system, which are required to establish a chronic infection. The non-structural viral protein NS5A has the capacity to interact with a large number of cellular factors involved in promoting viral replication/assembly and in the cell antiviral response to HCV. Interaction of NS5A with the nucleosome assembly protein NAP1L1 has been recently characterized in my laboratory. NAP1L1 is a histone chaperone protein with various functions related to nuclear chromatin remodelling that impact on the regulation of cell cycle, on cell differentiation and on transcription. I have confirmed the interaction of NS5A with NAP1L1 in the cytoplasm and demonstrated the NS5A-dependent impairment of NAP1L1 nuclear translocation. Whole genome transcription analysis performed in NAP1L1 depleted hepatocytes indicated that its nuclear function might be essential for the transcriptional control of several interferon stimulated genes and the function of key innate immunity pathways. Indeed, I was able to demonstrate that NAP1L1 is a novel factor involved in the interferon response and specifically modulates TBK1/IKKε mediated IRF-3 phosphorylation and NF-κB levels. Hence, both the TLR3 and RIG-I/MDA5 pathways are affected by NAP1L1 depletion. I could further demonstrate that NAP1L1 controls the basal transcription of genes involved in the immune pathway and that it interacts with the adaptor protein MAVS, which is required for RIG-I/MDA5 signalling.

In conclusion, by studying the interaction of the viral protein NS5A with the cellular factor NAP1L1 I could discover a novel mechanism of regulation of the innate response mediated by NAP1L1. These findings have wider implications for HCV and beyond, further highlighting the importance of studying viruses to uncover cellular functions.

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3’-UTR: 3’-untranslated region

5’-UTR: 5’-untranslated region

Ago2: Argonaute RISC catalytic component 2

AH: Amphipathic helix

Apo: Apolipoprotein

ApoE: Apolipoprotein E

CARDs: Caspase activation and recruitment domains

CD81: Cluster of Differentiation 81

CLDN1: Claudin 1

CKI: Casein kinase I

CKI-α: Casein kinase I alpha

CKII: Casein kinase II

CRE: Cis-acting replication element

CypA: Cyclophilin A

DAA: Direct Acting Antivirals

DCV: Declatasvir

DGAT1: Diacylglycerol acyltransferase 1

DMVs: Double membrane vesicles

dsRNA: double-stranded RNA

eIF2α: α subunit of eukaryotic initiation factor 2

EM: Electron microscopy

EMCV: Encephalomyocarditis virus

FADD: Fas-associated death domain

HAV: Hepatitis A virus

HBV: Hepatitis B virus

HCC: Hepatocellular carcinoma

HCVcc: HCV cell culture

HIV-1: Human immunodeficiency virus-1

HTLV-1: Human T-lymphotropic virus

HVR: Hypervariable region

IFNAR: Interferon alpha/beta receptor

IFN-α: Interferon-alpha

IFN-β: Interferon-beta

IFN-λ3: Interferon-lambda 3

IKKε: inhibitor of nuclear factor kappa-B kinase epsilon

IL28B: Interleukin 28B

IPA: Ingenuity pathway analysis

IRES: Internal ribosomal entry site

IRF-3: Interferon regulator factor-3

IRF-9: Interferon regulator factor-9

ISGs: Interferon stimulated genes

JFH1: Japanese fulminant hepatitis 1

LCS: Low complexity sequence

LD: Lipid droplet

LDL: low density lipoprotein

LGP2: Laboratory of genetics and physiology 2

LMB: Leptomycin B

LV: Lentivirus

LVP: Lipoviral particle

MAMs: Mitochondrial-associated membranes

MAVS: Mitochondrial antiviral-signalling protein

MDA-5: Melanoma differentiation-associated antigen 5

miRNA: Micro RNA

miR122: Micro RNA 122

MMVs: Multi-membrane vesicles

MyD88: Myeloid differentiation primary response gene 88

NANBH: non-A, non-B Hepatitis

NAP-1: Nucleosome assembly protein

NAP1L1: Nucleosome assembly protein 1-like 1

NES: Nuclear export signal

NF-κB: Nuclear factor κB

NLS: Nuclear localization sequence

NPC1L1: Niemann-Pick C1-like 1

OCLN: Occludin

ORF: Open Reading Frame

PAMPs: Pathogen-associated molecular patterns

PI4KIII α: Phosphatidylinositol-4 kinase III alpha

PI4P: Phosphatidyilinositol 4-phosphate

PHHs: Primary human hepatocytes

PKR: Protein kinase R

PRDs: Positive regulatory domains

PRRs: Pattern recognition receptors

REMs: Replication enhancing mutations

RdRp: RNA-dependent RNA polymerase

RLRs: RIG-I-like receptors

SEC14L2: SEC14-like lipid binding 2

SGR: Subgenomic replicon

RIG-I: Retinoic acid-inducible gene I

RLRs: RIG-I like receptors

SR-BI: Scavenger receptor class B type I

SSP: Signal sequence peptidase

ssRNA: single-stranded RNA

SVR: Sustained virological response

TBK1: TANK binding kinase 1

TLRs: Toll-like receptors

TMD: Transmembrane domain

TRAF3: Tumor necrosis factor receptor-associated factor 3

TRAF6: Tumor necrosis factor receptor-associated factor 6

TRIF: TIR-domain-containing adaptor-inducing interferon-β

VAP-A: Vesicle-associated membrane protein A

VLDL: Very Low Density Lipoprotein

WHO: The World Health Organization

y-NAP-1: Yeast NAP-1

1. INTRODUCTION

1.1 Hepatitis C virus – an overview

1.1.1 Hepatitis C virus discovery

In the early 1970s, the application of serological tests for the prevention of post-transfusion hepatitis caused by hepatitis A virus (HAV) and hepatitis B virus (HBV), indicated that 10% of transfusions produced a form of hepatitis characterized by persistent liver damage, implying the existence of another hepatitis causing agent (Feinstone et al. 1975). The unknown agent was named non-A, non-B hepatitis (NANBH). Despite the tremendous effort and intensive work, the identity of the agent remained unknown for more than a decade. In 1989, Houghton group used a blind immunoscreening method to identify the NANBH causative agent. A bacteriophage expression library was prepared with cDNA retro-transcribed from NANBH infected chimpanzee plasma and screened with serum from a patient with a chronic NANBH infection. With this approach they identified one positive clone, designed 5-1-1. Further experiments demonstrated that the clone was part of an RNA molecule of extra-chromosomal origin, encoding an antigen bound by antibodies of NANBH infected patients. These criteria were sufficient to identify the viral nature of the NANBH causative agent which was then re-named Hepatitis C virus (Choo et al. 1989). The identification of the clone 5-1-1 allowed the development of an enzyme immunoassay test for the detection of circulating antibodies that in following years enabled the production of blood screening tests for the prevention of transfusion related HCV infections (Kuo et al. 1989). In addition, the isolation of the clone led to the identification and sequencing of the whole viral RNA genome (Choo et al. 1991).

1.1.2 HCV classification, distribution and properties

Hepatitis C virus is an enveloped RNA virus and it is classified in the Hepacivirus genus of the Flaviviridae family. The Flaviviridae family is a group of viruses that currently consists of four different genera grouped on the basis of conserved motifs of the RNA-dependent RNA polymerase (Figure 1): Hepacivirus (from the Greek hepatos, liver), Flavivirus (from the Latin flavus, yellow), Pestivirus (from the Latin pestis, plague) and the recently included genus Pegivirus (an acronym derived from pe, persistent; g, GB or G) (Lindenbach et al. 2007; Stapleton et al. 2011).

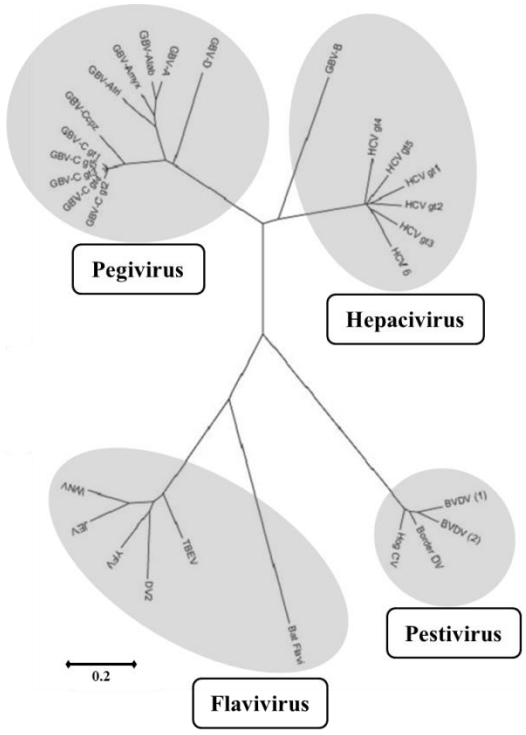

Figure 1. The Flaviviridae family. The phylogenetic tree shows a classification of the Flaviviridae family into four genera. The tree is constructed by neighbour joining analysis and based on conserved motifs of the RNA-dependent RNA polymerase (RdRp). A distant scale indicating the amino acid substitution per position is shown. Adapted from (Stapleton et al. 2011).

Recently, additional members of the hepacivirus genus, closely related to human Hepatitis C virus, have been discovered in dogs, horses, rodents and bats (Burbelo et al. 2012; Kapoor et al. 2011; Quan et al. 2013; Kapoor et al. 2013). These findings might radically revolutionize our knowledge about hepacivirus distribution and host specificity, with implication in drug and vaccine development.

The HCV particle is composed of a single copy RNA molecule surrounded by a nucleocapsid and a host cell derived lipid envelope containing E1 and E2 glycoproteins. Electron microscopy (EM) studies have determined a pleomorphic nature of the HCV particle with a diameter ranging from 40 to 80 nm (Gastaminza et al. 2010; Catanese et al. 2013) (Figure 2).

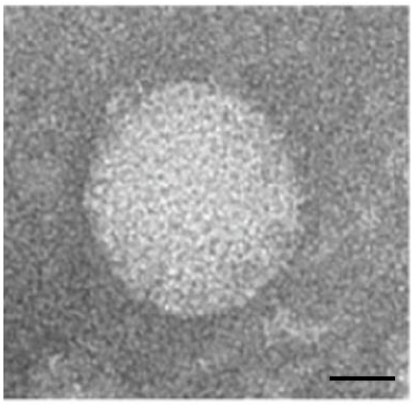

Figure 2. EM of highly purified HCV particle. Negatively stained HCV particle. Scale bar: 20 nm. (Catanese et al., 2013).

In comparison with other enveloped RNA viruses, HCV virions produced in cell culture or derived from serum of infected individuals exhibits a particularly low buoyant density, corresponding to 1,10 g/ml (Lindenbach et al. 2005). The HCV particle is defined as a lipoviroparticle (LVP) since it circulates in the blood of infected patients in association with low-density lipoproteins (LDL) and very low density lipoproteins (VLDL). Serum-derived HCV particles are found to be associated with Apo AI, ApoB, ApoC1 and ApoE, whereas cell culture derived HCV particles are found in association with ApoE and ApoC1 (Chang et al. 2007; Meunier et al. 2008; Merz et al. 2011). The unusually low buoyant density of the HCV particle could be due to its association with host lipoproteins. This interaction might facilitate the viral entry into the host cell and the evasion from the host immune response.

HCV shows great genetic diversity, and the variability of nucleotide sequences isolated from infected individuals allowed its classification in genotypes and subtypes. The current classification foresees 7 different genotypes comprising 67 subtypes (Smith et al. 2014). The high replicative nature of the virus, with a production of 10^{12} particles per day (Neumann et al. 1998) together with a lack of a proof reading activity of the viral RdRp is the base for the genetic heterogeneity present within the infected individual, leading to a number of quasispecies. The distinction of HCV isolates in genotypes and subtypes has important implications in the efficacy of treatment. Different genotypes respond differently to treatment and moreover show a distinct geographical distribution. The most resistant to IFN-α treatment genotype 1 has a worldwide distribution but dominates in Western countries together with genotype 2 and 3. Genotypes 4 and 5 are

most prevalent in Africa and genotype 6 is widespread in South and Southeast Asia. Genotype 7 was recently identified in a limited number of patients in South Africa.

1.1.3 HCV epidemiology and treatment

A recent report from the World Health Organization (WHO) indicates that approximately 150 million people, representing 3% of the world population, are chronically infected with HCV (http://www.who.int/mediacentre/factsheets/fs164/en/). HCV transmission occurs following direct exposure to infected blood. With the advent of screening tests for circulating antibodies or the presence of viral RNA (Kuo et al. 1989), transfusion-related HCV transmission was completely eliminated in developed countries and the main route of HCV transmission becomes associated with the use of unsterilized syringes and needles (Thomson 2009). HCV is considered to be a silent disease as the initial acute infection is usually asymptomatic and rarely accompanied by unspecific symptoms like fatigue, fever or malaise. During the acute phase of infection around 15% of infected patients are able to spontaneously eliminate the virus, whereas in the remaining 85% the virus establishes a persistent chronic infection. The chronic infection can progressively lead to severe complications of the liver such as cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997). At that stage of the disease, liver transplantation is suggested as a treatment option, although the recurrence of HCV infection occurs on a regular basis (Patel et al. 2006).

Until 2011, the standard therapy for HCV infection consisted of the administration of pegilated IFN-α and ribavirin for 24-48 weeks. This combinatorial therapy was associated with severe side effects which frequently led to the suspension of the treatment. The outcome of HCV infection depends on both viral and host genetic background. The sustained virological response (SVR), defined as the absence of detectable viral RNA in patients' blood after the termination of the therapy, show higher rates ranging from 50% to 90%, in patients previously infected with genotype 2, 3, 5, and 6, and below 50% in the presence of genotype 1 and 4. Moreover, a specific genetic polymorphism upstream the IL28B locus, which encodes for IFN-λ3 cytokine, is strongly associated with the spontaneous and treatment-induced clearance of HCV infection (Thomas et al. 2009; Ge et al. 2009). Interestingly, a SNP present in the IL28B locus gives rise to a new member of the IFN III family, termed IFN-λ4 and was strongly associated with a failure to clear HCV infection (Prokunina-Olsson et al. 2013).

In 2011, the first generation of direct acting antivirals (DAA) became available. In combination with IFN-α and ribavirin, the NS3/4A inhibitors, telaprevir and boceprevir, covalently blocking the enzyme active site, increased the SVR to 70% in naive as well as patients non-responsive to previous IFN treatment. Since the DAA therapy was aimed to treat only HCV infection caused by genotype 1 and had other limitations such as the interaction with other drugs, additional efforts have been implemented to improve the DAA therapy. Subsequently, compounds inhibiting NS5A or the polymerase activity of NS5B have been approved for the combinatorial therapy. Different combination of DAA composed of NS3/4A, NS5A, and NS5B inhibitors have been approved and released on the market over the following years mostly directed towards the most difficult to treat HCV genotype 1 and 4. Today's therapy is a combination of NS5B inhibitor sofosbuvir and next generation NS5A inhibitor velpatasvir, active against all genotypes with very few side effects (Foster et al. 2015; Feld et al. 2015).

DAA therapy can efficiently eradicate the virus but is not designed to cure end-stage liver disease. The treatment has been shown to limit the progression to most severe forms of liver damage, and therefore the patient should be monitored in the following years after the treatment. Other problems associated with DAA treatment is the eventual emergence of resistance to the drugs used, indicating a need for alternative forms of inhibitors used in a combinatorial regimen. Additionally, for a complete elimination of the virus, the DAA therapy will need to be affordable to all patients with HCV infection worldwide. Eventually the eradication of the virus might be achieved with the development of an efficient prophylactic vaccine (Dunlop et al. 2015).

1.2 HCV Genome organization and viral proteins

The HCV genome is a positive single stranded RNA molecule of 9.6 kb in length and contains a long open reading frame (ORF) (9024 to 9111 nucleotides depending on the genotype) encoding a polyprotein precursor of about 3000 amino acids. The ORF is flanked by a 5'UTR and 3'UTR regions with functions related to replication and translation of the viral RNA (Figure 3).

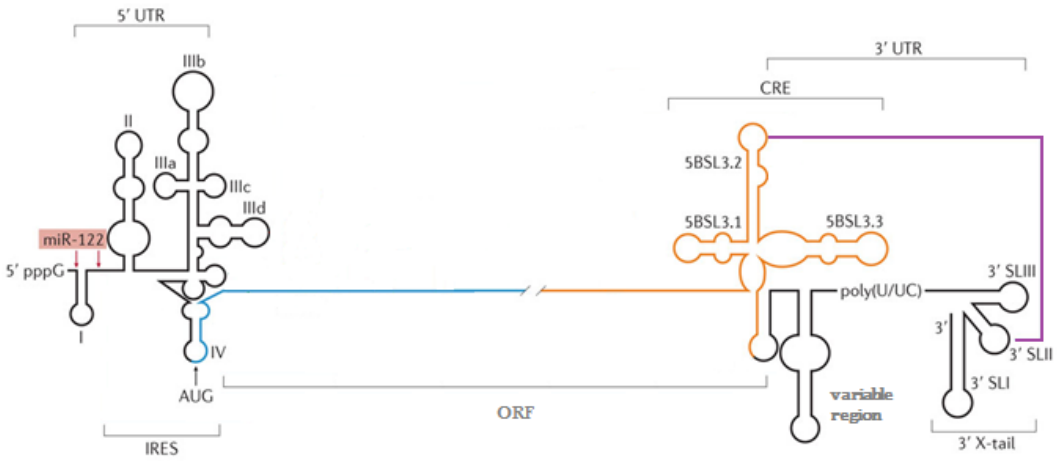

Figure 3. HCV genome organization. HCV RNA genome consist of a single open reading frame (ORF) which contains a region encoding structural (blue) and non-structural (orange) proteins. The ORF is flanked by highly structured 5' and 3' untranslated regions (UTRs). The 5'UTR is composed of four different stem loop (SL) regions (I-IV). SL II-IV and the initial sequence of core protein constitute the IRES. Two miR122 binding sites are present in region I of the 5'UTR. The 3' UTR contains a variable region, a poly (U/UC) region and the X-tail region. The X-tail stem loop II (SLII) interaction with 5BSL3.2 is show by the purple line. Additional cis acting replicative elements (CREs) present in the coding region of the NS5B (5BSL3.1-3) are shown. Adapted from (Li et al. 2015).

HCV 5'UTR is a 341 nucleotide long sequence composed of four different structural domains (domains I-IV) (Honda et al. 1996). The 5'UTR encompassing stem-loops II-IV together with a short core sequence is critical for translation as it constitutes the IRES that mediates the cap-independent translation of the viral RNA (Honda et al. 1999). Domain I and II have important roles in viral replication (Friebe et al. 2001). The interaction with the liver-specific microRNA miR-122 is necessary for the regulation of both viral replication (Jopling et al. 2005) and translation (Henke et al. 2008); Jangra et al. 2010). miR-122 could prevent the nuclease mediated degradation of the HCV RNA or the activation of the immune response (Machlin et al. 2011). Interestingly, another report proposed that the complex formed between Ago2 and miR122 protects the RNA from degradation (Shimakami et al. 2012).

The 3'UTR varies between 200 and 235 nucleotide in length and is composed of three different regions: a variable region, a poly (U/UC) tract and a conserved 98 nucleotide X region (Kolykhalov et al. 1996). The X region, together with 50 nucleotides of the poly(U/C) tract, is necessary for an efficient viral replication (Yi & Lemon 2003). Additionally, the viral replication is regulated by cis-acting replicative elements (CREs) present within the NS5B coding region. One particular NS5B stem loop, designated 5BSL3.2 was shown to interact with the 3'UTR to positively regulate RNA replication

(Friebe et al. 2005). In addition to regulating RNA replication the 3'UTR has important roles in RNA translation (Song et al. 2006).

The IRES mediated translation of the ORF yields a polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases into structural proteins (core and envelope glycoproteins E1 and E2) that form a viral particle, and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) involved in the viral life cycle (Figure 4).

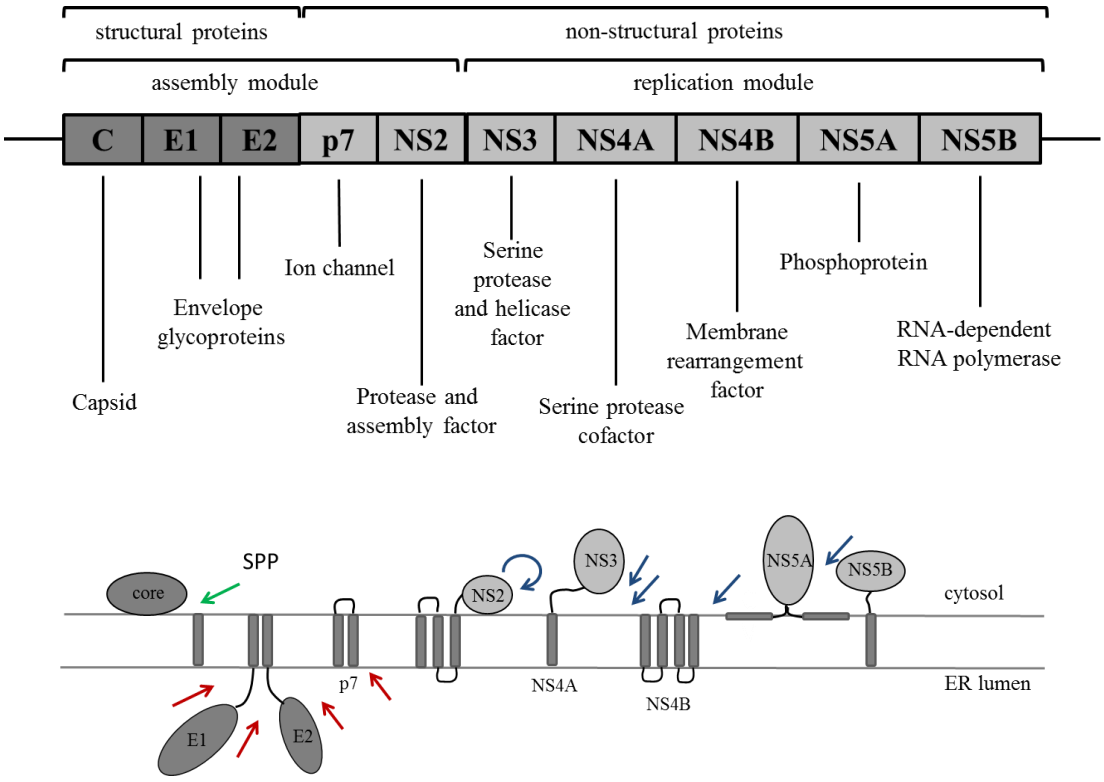

Figure 4. Hepatitis C virus genome organization and viral proteins. A) The HCV genome encodes 3 structural and 7 non-structural proteins. The capsid (C), the envelope glycoproteins (E1 and E2), the ion channel protein (p7) and the cysteine protease (NS2) are involved in assembly and constitute the assembly module, whereas the rest of the non-structural proteins (NS3-NS5B) are involved in replication and constitute the replication module. The main functions of all proteins are indicated. B) The polyprotein is co- and post-translationally cleaved by both cellular and viral proteases. The cleavage mediated by the cytoplasmic signal peptide peptidase (SPP) is indicated by the green arrow and the cleavage by the ER signal peptidase is indicated by red arrows. The blue cyclic arrows indicate the cleavage by the NS2 protease. Blue arrows denote the processing mediated by the NS3/4A protease.

1.2.1 Features of HCV structural proteins

1.2.1.1 Core

HCV Core (C) is the first structural protein encoded by the ORF and is primarily involved in the nucleocapsid formation. The signal sequence present between core and E1 translocate the newly formed polyprotein to the endoplasmic reticulum (ER). The signal peptidase mediated cleavage of the signal sequence produces the immature 191 amino acid core protein which is subsequently cleaved by a cytoplasmic signal peptide peptidase (SPP) to yield the mature 21kDa core protein able to translocate to the lipid droplets (LDs) (McLauchlan et al. 2002). The mature HCV protein consists of two domains. Domain 1 (D1) is highly hydrophilic and is involved in the interaction with RNA and self oligomerization, which are necessary activities to promote nucleocapsid formation (Majeau et al. 2004). Domain 2 (D2) is more hydrophobic and is involved in the interaction with LDs (Boulant et al. 2006). The encapsidation of the viral genome is thought to occur on the ER-LD interface with the assistance of NS5A that delivers the RNA to the core protein (Miyanari et al. 2007).

1.2.1.2 Envelope glycoproteins

The envelope glycoproteins E1 and E2 are essential for the assembly of the infectious viral particle, the viral entry, and the fusion with host cell membrane. E1 and E2 belong to the type I transmembrane protein family with the N terminal ectodomain located in the ER lumen and a short C terminal transmembrane domain (TMD) embedded in the ER membrane (Cocquerel et al. 2002). E1 and E2 ectodomains are highly glycosylated and stabilized by disulphide bonds which are believed to be essential for virus assembly and infectivity (Vieyres et al. 2010; Helle et al. 2010). It has been demonstrated that viral entry is mediated primarily by the E2 glycoprotein binding to cellular receptors CD81 (Pileri et al. 1998; Patel et al. 2000) and SR-BI (Scarselli et al. 2002).

1.2.1.3 P7

P7 is a small 63 amino-acid membrane protein consisting of two transmembrane α-helices connected through a cytoplasmic loop and both the N and C terminal region located in the ER lumen (Carrere-Kremer et al. 2002). It is a member of the viroporin family due to its capacity to assemble heptameric cation channels (Clarke et al. 2006).

P7 is dispensable for HCV replication but has essential roles in virus assembly. Although the exact function of these channels in the assembly of the virus particles is still unknown, some recent evidence suggests that p7 might limit the acidification of cellular compartments and facilitate virus production (Wozniak et al. 2010). Additional proof that p7 might be involved in the assembly process comes from studies showing the interaction of p7 with NS2, which acts in concert with other non-structural proteins such as NS5A, E1, E2, and core for efficient HCV production (Boson et al. 2011; Jirasko et al. 2010).

1.2.1.4 NS2

NS2 is a cysteine protease responsible for the cleavage of the polyprotein at the NS2-NS3 site. The catalytic site is represented by three amino acids: His143, Glu163 and Cys184 located in the C-terminal region. The protease activity is enhanced by the first N terminal region of NS3 (Schregel et al. 2009; Grakoui et al. 1993). Although the NS2 protease itself is dispensable for RNA replication *in vitro* (Lohmann et al. 1999), its activity is necessary to complete the viral life cycle by cleaving and liberating a fully active NS3 protein (Jirasko et al. 2008). Moreover, NS2 has a protease independent activity. Its interaction with other non-structural proteins results in the regulation of viron assembly as mentioned above (Jirasko et al. 2010; Boson et al. 2011).

1.2.1.5 NS3-NS4A

NS3 is a multifunctional protein with an N-terminal serine protease activity (Tanji et al. 1994) and a C-terminal NTPase/RNA helicase activity (Kim et al. 1995). NS4A is a cofactor for the NS3 serine protease consisting of an N-terminal hydrophobic region that links the complex to the membrane, and the C-terminal acidic region (Kim et al. 1995). The NS3-4A catalytic domain is represented by His57, Asp81 and Ser139 and its protease activity is essential for the HCV life cycle as it cleaves the polyprotein at NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions. Beside the role in replication, NS3-4A is also able to modulate the innate immune response by cleaving two adaptor proteins: TRIF (Li et al. 2005a) and MAVS (Cheng et al. 2006; Li et al. 2005b; Meylan et al. 2005; Loo et al. 2006). Not surprisingly, NS3-4A is one of the most popular viral targets for the development of novel antiviral therapeutics (Pawlotsky 2006). The NS3 NTPase/RNA helicase domain uses the hydrolysis of ATP

to unwind double stranded RNA or single stranded RNA secondary structure, which is critical for viral replication (Serebrov & Pyle 2004).

1.2.1.6 NS4B

NS4B is a hydrophobic integral membrane protein. Membrane association is thought to occur through its N terminal domain and four predicted transmembrane domains (Paul et al. 2011). Its main role in the HCV life cycle is to induce alterations in the ER membrane creating modified vesicles defined as membranous web required for HCV replication (Egger et al. 2002; Gosert et al. 2003). NS4B has been shown to interact with other HCV non-structural proteins as well as viral RNA (Einav et al. 2008). In addition it has been reported that it can form oligomers and that the oligomeric state induced by both N- and C-terminal regions is necessary for the establishment of functional replication complexes (Yu et al. 2006; Gouttenoire et al. 2010).

1.2.1.7 NS5B

The RNA-dependent RNA polymerase (RdRp) NS5B is a key enzyme involved in the synthesis of both positive and negative strands of the viral RNA molecule. It is anchored to the ER membrane through a C terminal transmembrane domain and exposes its functional domain to the cytoplasmic site. The crystal structure of NS5B shows a common "fingers, palm and thumb" structure. The interaction between the fingers and thumb domains produce an active catalytic site, harbouring the GDD domain in the palm region that can accommodate a single stranded RNA molecule and initiate transcription (Lesburg et al. 1999). NS5B is a critical target for the development of antiviral therapies, and several generations of NS5B inhibitors have been developed over the past several years (Pawlotsky et al. 2015).

1.2.1.8 NS5A

Structural features of NS5A protein

NS5A is a membrane associated protein with important roles in both viral replication and assembly. It contains a short N terminal amphipathic peptide (AH) (Brass et al. 2002) that anchors the protein to the ER membrane, a condition necessary for viral replication (Penin et al. 2004). Apart from the N terminal anchor peptide, NS5A is

composed of three other domains separated by serine or proline rich low complexity sequences (LCSs) termed LCS-I and LCS-II (Tellinghuisen et al. 2004). The domain organization of NS5A is shown in Figure 5. The crystal structure of the domain I (D1) has been resolved and it revealed the presence of a zinc binding site essential for RNA replication (Tellinghuisen et al. 2005). Moreover, the structure identified a dimeric form of D1 with a positively charged groove between the two monomers, possibly for the incorporation of an RNA molecule (Tellinghuisen et al. 2005; Love et al. 2009). Conversely, it was not possible to obtain the crystal structure for the other two domains, probably due to their disordered nature and absence of a defined secondary structure (Hanoulle et al. 2009b; Liang et al. 2007).

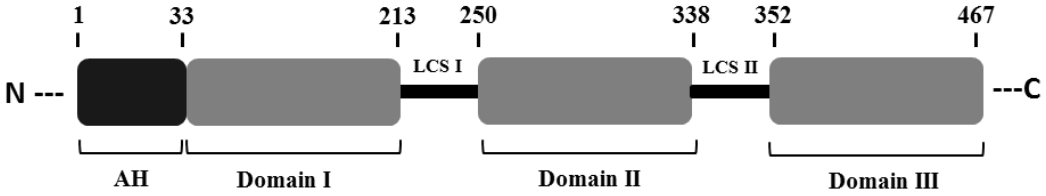

Figure 5. Schematic representation of the NS5A protein. NS5A is divided in three domains based on the presence of two low complexity regions designated as LCS-I and LCS-II. The N terminal domain contains an amphipathic helix (AH) that anchors the protein in the ER membrane leaving the three domains in the cytoplasm. Numbers refer to amino acid residues from the NS5A of the JFH-1 strain.

NS5A phosphorylation

NS5A is a highly phosphorylated protein and can be distinguished in a basally phosphorylated (p56) and hyper-phosphorylated form (p58) (Kaneko et al. 1994). Phosphorylation sites have been identified in domain I, LCS-I, LCS-II and domain III. Basal phosphorylation seems to occur in the central region of the protein as well as in domain III, whereas hyper-phosphorylated forms have been associated with phosphorylation of serines in the LCS-I. The hyper-phosphorylation of NS5A induces the dissociation from vesicle-associated membrane protein-associated protein A (VAP-A) and negatively regulates viral replication (Evans et al. 2004). Moreover, different adaptive mutations often embrace residues in the LCS-I. These observations led to the hypothesis that the phosphorylation status of NS5A might regulate the transition from replication to assembly with the involvement of different host factors (Evans et al. 2004; Appel et al. 2005). A various range of kinases have been identified as responsible for NS5A phosphorylation. The first in line is casein kinase I alpha (CKI-α) that was

shown to be responsible for the phosphorylation of NS5A (Quintavalle et al. 2007). Moreover, phosphorylation by CKI-α seems to be important for NS5A hyper-phosphorylation, localization to lipid droplets and particle assembly (Quintavalle et al. 2006; Masaki et al. 2014). Using both genetic and pharmacological approaches, casein kinase II (CKII) was identified as a responsible kinase for the phosphorylation of a serine residue in position 457 of the NS5A protein, which was demonstrated to be essential for virus assembly (Tellinghuisen et al. 2008). Another kinase involved in NS5A hyper-phosphorylation and RNA replication is the Polo-like kinase I (Plk1) (Chen et al. 2010). Additional kinases have been reported to mediate NS5A phosphorylation, including glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase (MAPK) (Reed et al. 1997).

NS5A interactome

The unfolded nature of domain II and III makes NS5A an ideal candidate for the interaction with different viral and host proteins, that can regulate processes such as replication or assembly and manipulate the host-cell environment (De Chassey et al. 2008; Germain et al. 2014; Pichlmair et al. 2012).

To date there are more than 130 known NS5A interactors and the list is still growing (Ross-Thriepland & Harris 2015). Considering that a single protein cannot establish 130 interactions simultaneously, it is reasonable to think about a spatial and temporal distribution of these interactions in the infected cell. Some NS5A-host protein interactions are well established and described hereafter.

NS5A binds to proteins with an SH3 domain through a highly conserved polyproline (PxxPxR) motif present in LCS-II. Mutations that abolish the interaction have no effect on either replication or infectious virus particle production (Hughes et al. 2009).

NS5A interaction with the endoplasmic-reticulum isoform of the phosphatidylinositol 4-kinase (PI4K), PI4KIIIα, increases the amount of phosphatidylinositol 4-phosphate (PI4P) lipids required for the formation of the membranous web (Reiss et al. 2013). Apart from HCV, PI4P have been shown to play roles in the replication of a number of viruses (Delang et al. 2012). Different siRNA-screening studies identified PI4KIIIα as a positive regulator of HCV replication (Borawski et al. 2009; Berger et al. 2011; Reiss et al. 2011) and additionally it was reported to affect NS5A phosphorylation status (Reiss et al. 2013). Moreover, a direct interaction between NS5A and cyclophilin A (CypA)

was discovered. Cyclophilins are cis-trans isomerases of peptide bonds and facilitate protein folding. Indeed, the binding of domain II of NS5A to the active site of CypA led to its cis–trans isomerization (Hanoulle et al. 2009a) causing a conformational change that leads to HCV RNA binding (Foster et al. 2011). Additionally, host proteins annexin A2 and apoE were shown to modulate the formation of infectious viral particles through the interaction with NS5A (Benga et al. 2010; Backes et al. 2010).

NS5A and antiviral therapy

HCV NS5A does not possess any enzymatic activity, yet represents an excellent target for antiviral therapy together with NS3-4A and NS5B due to its role in both viral replication and assembly.

The first NS5A inhibitor, originally identified as BMS-790052 and subsequently renamed to daclatasvir (DCV) (Gao et al. 2010) was screened for the ability to block HCV replication with high specificity and potency. The discovery of DCV was followed by a number of alternative molecules, which have been approved or are still in clinical trials, active against all HCV genotypes with increased barrier to resistance when compared to first generation inhibitors (Pawlotsky et al. 2015). It was proposed that DCV and related molecules act by binding the dimeric form of domain I and inhibit the formation of HCV replication complexes (Gao et al. 2010; Berger et al. 2014), or alternatively reduce the amount of PI4P in the replication compartments (Reghellin et al. 2014).

1.3 Cell culture systems for HCV

1.3.1 HCV replicon system

After its discovery in the late 80's, scientists struggled for a long time to culture HCV and establish a robust HCV infection. Inspired by the idea that other RNA viruses efficiently replicate in cell culture in the form of sub-genomic replicons, they adopted this approach and created the first HCV sub-genomic replicon based on the genotype 1b of HCV (Con1 isolate). The first HCV replicon was a self-replicative bicistronic sub-genomic RNA that consisted of a neomycin-resistance gene, inserted in place of structural genes, under the control of the HCV IRES, and non-structural proteins, NS3 to NS5B, under the control of EMCV IRES (Figure 6). Upon transfection and neomycin

selection this replicon gave rise to high amounts of replicating viral RNA (Lohmann et al. 1999). Subsequently viral replication was increased by the identification and introduction in the parental genome of replication enhancing mutations (REMs) in the coding region of non-structural proteins, and particularly in the NS5A gene (Blight et al. 2000). Interestingly, while on the one hand these REMs increase viral replication on the other hand interfere with viral particle production *in vitro* and *in vivo* (Bukh et al. 2002; Pietschmann et al. 2009). Subsequently, the replicon system was also established for other HCV genotypes such as 1a (H77 isolate), 2a, 3a, and 4a (Blight et al. 2003; Kato et al. 2003; Saeed et al. 2013; Peng et al. 2013). The genotype 2a replicon was isolated form a patient with a fulminant hepatitis C virus infection (JFH-1) and it replicates efficiently in cell culture without the need for REMs (Kato et al. 2003).

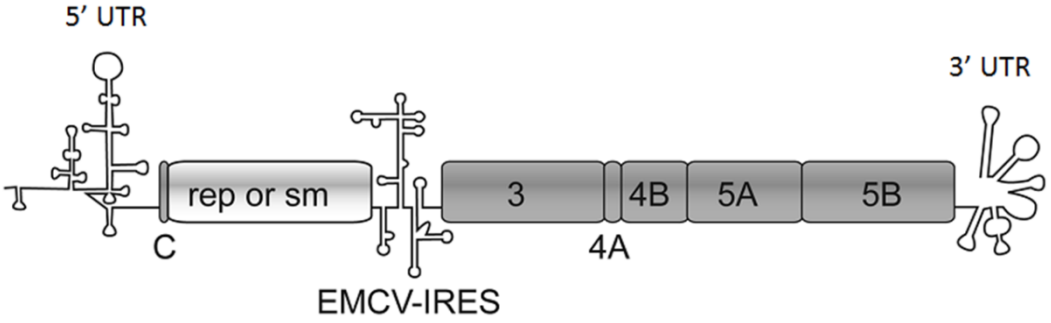

Figure 6. Scheme of a sub-genomic HCV replicon. A bicistronic replicon contains a reporter gene (rep) (i.e. firefly luciferase) or a selection marker (sm) (i.e. neomycin phosphotransferase) under the control of the IRES present in the 5'UTR, non-structural genes (NS3-NS5B) under the control of EMCV-IRES and 3' UTR regulatory elements. Taken from (Lohmann & Bartenschlager, 2014).

1.3.2 Cell culture infectious HCV system

After a series of attempts to produce a robust HCV infection from a cloned genome, the generation of a consensus HCV genome starting from a sequence of various clones of the same isolate derived from genotype 1a (H77 isolate), and its inoculation in chimpanzees, was sufficient to initiate an HCV infection (Kolykhalov et al. 1997). However, the replication and the formation of infectious viral particles were not robust enough for further studies. The major breakthrough came from the isolation of a genotype 2a virus from a patient with a fulminant hepatitis C infection, that replicated efficiently in Huh7 cells and produced high amounts of infectious viral particles both in a cell culture system and *in vivo* (Lindenbach et al. 2005; Zhong et al. 2005; Wakita et

al. 2005). These cell-culture derived HCV particles (HCVcc) allowed the study of the whole HCV life cycle, from viral entry to assembly and release. Unfortunately these studies were restricted only to the specific isolate JFH-1, as most other genotypes were unable to support the production of infectious viral particles. To overcome this inconvenience a series of chimeric viral genomes, representing all known genotypes, were generated. Generally the replication module is derived from the JFH-1 2a genotype, whereas the structural proteins and precisely the core to NS2 tract, is derived from the particular genotype of interest. The most efficient chimeric genome, created with a combination of two different genotype 2a isolates, J6 and JFH-1, and designated as Jc1, allowed the production of 10^6 infectious viral particle/ml (Pietschmann et al. 2006).

1.3.3 HCV permissive cell lines

Hepatocytes are the main target for HCV infection. Human hepatoma cell line Huh7 and its derivatives are permissive for HCV propagation and therefore used as a cellular model for the *in vitro* studies. A particular highly permissive subpopulation of Huh7 cells denominated Huh7.5 and Huh7-Lunet (Blight et al. 2002) (Friebe et al. 2005) have been selected by treating Huh7 replicon cells with IFN-α or HCV inhibitors. Despite REMs are important determinants that govern the outcome of HCV infection, the host cell background plays a decisive role as well. The lack of host factors stimulating viral replication or the abundance of restriction factors might govern the host cell permissiveness to viral infection. Huh7.5 cells are thought to be highly permissive due to a mutation in the RIG-I receptor that abolishes the activation of the innate immune response (Sumpter et al. 2005), although another study was not able to confirm this correlation (Binder et al. 2007). Other host factors such as the liver specific micro RNA, miR122 (Jopling et al. 2005), or apolipoprotein E, ApoE (Jiang & Luo 2009), are important host determinants for an efficient HCV replication and viral particle production. Recently, using a lentiviral based cDNA library in Huh7 cells, scientists were able to identified a host factor, named SEC14L2, that allowed the replication and infectious viral particle production of HCV genotypes other than JFH-1 without the need for REMs (Saeed et al. 2015). Alternatively, other cell lines of hepatic or non-hepatic origin have been used to propagate HCV (Narbus et al. 2011; Da Costa et al. 2012).

1.3.4 Primary cells models for HCV infection

Huh7 cells and its derivatives are a suitable *in vitro* system to study the entire HCV life cycle. However, they do not completely recapitulate the human hepatocyte status *in vivo* due to the lack of polarity and expression markers of mature hepatic cells (Decaens et al. 2008). This barrier was partially overcome by the use of primary human hepatocytes (PHH) representing the *in vitro* system that most closely resemble the HCV natural host cell. Although PHH show high variability between donors, easily lose their mature hepatocytes properties and are usually poorly available, which limit and complicate the experimental analysis, various studies have reported successful usage of PHH for HCV infection (Ploss et al. 2010; Podevin et al. 2010). In addition, induced pluripotent stem cells (iPSC) have been used to generate hepatocyte-like cells that supported HCVcc and serum derived HCV infection (Schwartz et al. 2011; Wu et al. 2012; Zhou et al. 2014).

1.4 The HCV life cycle

The HCV life cycle is a multistep process that involves viral entry and uncoating, RNA translation and polyprotein processing, followed by RNA replication, virus assembly and finally the release of the virus from the cell. These various stages of the process, schematized in Figure 7, are explained in detail in the following sections.

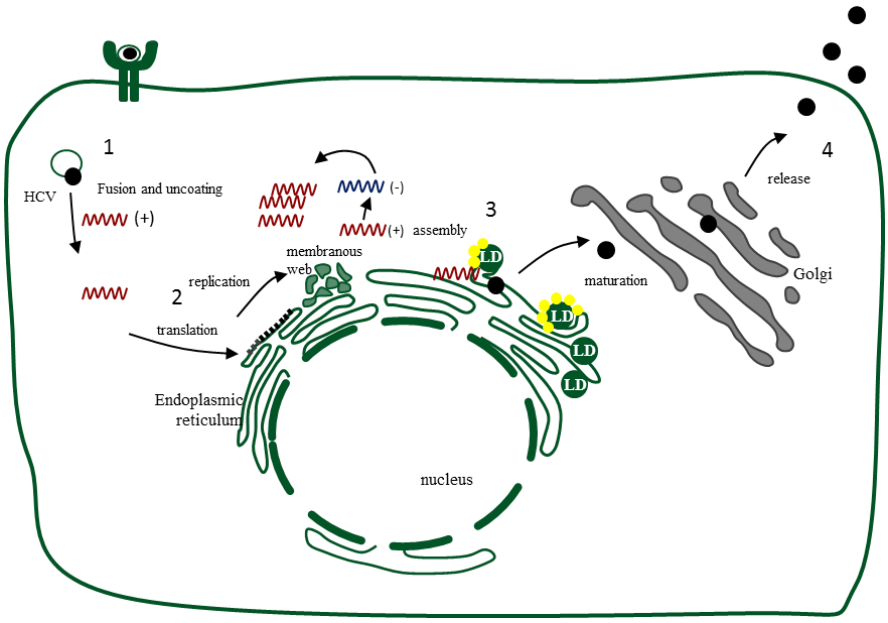

Figure 7. Schematic representation of the HCV life cycle. HCV binds to receptors present on the surface of hepatocytes and enters the cell through a clathrin mediated endocytosis. The acidification of the endosomes induces

the fusion of the membranes and the release of the viral RNA in the cytoplasm. The RNA gets immediately translated on the rough ER and the non-structural NS3-NS5B proteins of the replication complex replicate the viral RNA in the particular replication compartment called membranous web through a negative strand RNA intermediate. The positive sense RNA is re-used for translation of additional proteins or for the assembly of new viruses. The assembly take place on the surface of lipid droplets and ER membranes. The newly assembled particle buds into the ER and travels along the Golgi apparatus to complete the maturation process and is released from the cell following the exocytosis pathway.

1.4.1 HCV entry and uncoating

HCV circulates in the blood of an infected person in association with lipoproteins reaching the surface of hepatocytes (André et al. 2002; Nielsen et al. 2006). The first step of viral entry is mediated by the attachment of viral glycoproteins (Barth et al. 2003) or ApoE (Jiang et al. 2013) to the heparan sulphate proteoglycan (Lefèvre et al. 2014; Shi et al. 2013) and low density lipoprotein receptor (LDLR) (Agnello et al. 1999). Following attachment to the hepatocytes, HCV interacts with four specific cellular entry factors known up to date: scavenger receptor class B type I (SR-BI) (Scarselli et al. 2002), tetraspanin protein CD81 (Pileri et al. 1998), and tight junction proteins claudin 1 (CLDN1) (Evans et al. 2007) and occludin (OCLN) (Ploss et al. 2009). SR-BI interaction with E2 and viral lipoproteins is thought to prime the virus for its subsequent association with CD81. In particular, this is achieved by modifying the lipid composition of the virus (Thi et al. 2012) or interacting with the HVR1 region on E2 (Bankwitz et al. 2010), which are mechanism that eventually expose E2 site engaged in the interaction with CD81. Another cellular receptor involved in HCV entry is the tight junction protein CLDN1 and its association with CD81 is induced by EGFR signalling (Lupberger et al. 2011) or Rho GTPases signalling cascades (Brazzoli et al. 2008) that eventually promote virus internalization (Farquhar et al. 2012). Another tight junction protein that becomes available during a late step of HCV entry is OCLN (Benedicto et al. 2009). Together CD81 and OCLN determine HCV tropism for human cells (Dorner et al. 2011; Dorner et al. 2013). Since CLDN1 and OCLN are tight junction proteins it is reasonable to consider that after the binding to CD81 the virus would slide within tight junctions for its internalization. However, some reports suggest that this is not the mechanism adopted by the virus (Mee et al. 2008; Coller et al. 2009). Additional factors with specific roles in HCV entry have been described including Niemann-Pick C1-Like 1 (NPC1L1) (Sainz et al. 2012) and transferrin receptor 1 (TFR1) (Martin & Uprichard 2013). Following the attachment to the cell, HCV is

endocytosed in a clathrin dependent manner (Blanchard et al. 2006). The fusion occurs in early endosomes and is dependent on the acidification of the local environment (Coller et al. 2009; Hsu et al. 2003).

While the initiation of HCV infection is dependent on cell free viruses, the spreading within the infected liver can occur directly through viral cell to cell transmission, which seems to be a mechanism to escape from neutralizing antibodies and contributes to an evasion from the immune response and establishment of a chronic infection (Timpe et al. 2008).

1.4.2 HCV translation and replication

Once released in the cytoplasm, HCV RNA is translated exploiting the cellular machinery. The 5'UTR contains the IRES that drives the translation of the HCV RNA into a single polyprotein. The polyprotein is co- and post-transnationally processed by cellular and viral proteases, giving rise to 10 viral proteins. Host signal peptidase and SPP cleave the polyprotein to produce core, E1, E2 and p7 viral proteins. NS2 is the cysteine protease that cleaves the NS2/NS3 junction, whereas NS3/4A cleaves the downstream NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions, liberating the non-structural proteins. The mature proteins are associated with the ER membrane and the non-structural proteins, particularly NS4B, induce the rearrangement of intracellular membranes to create an intricate net of vesicles defined as membranous web, representing the viral replication compartment (Egger et al. 2002). Enzymatic digestion analysis of replication compartments identified them to be composed of 5% of total non-structural proteins, one RNA molecular of negative polarity and several RNA molecules of positive polarity (Miyanari et al. 2003; Quinkert et al. 2005). The replication compartment is represented mostly by double membrane vesicles (DMV) followed by multi-membrane vesicles (MMV) (Ferraris et al. 2010; Reiss et al. 2011). Viral non-structural proteins apart from being involved in replication by the formation of the membranous web have also a direct activity on HCV replication. NS4B have been shown to inhibit NS5A activity *in vitro* (Piccininni et al. 2002) and its interaction with NS3 might have important effects on HCV replication (Paredes & Blight 2008). NS3 helicase activity also contributes to the replication possibly by collaborating with NS5B for its enhanced activity (Piccininni et al. 2002). The viral RNA-dependent RNA polymerase NS5B replicates the HCV genome into a negative strand which is then used

to produce the positive sense RNA molecules used in part for the translation of new proteins and in part for the incorporation in virions. NS5A, as mentioned before, has a crucial role in modulating RNA replication and is specifically linked to its phosphorylation status (Appel et al. 2005). Apart from the non-structural proteins, several host factors have been identified as crucial for the morphogenesis of altered membrane vesicles, like phosphatidylinositol-4 kinase III alpha (PI4KIIIα) (Berger et al. 2009), or RNA replication including a vesicle-associated membrane protein-associated protein A, VAPA (Evans et al. 2004), a peptidyl-prolyl cis-trans isomerase, CypA (Liu et al. 2009a) and a liver specific microRNA, miR122 (Jopling et al. 2005).

1.4.3 HCV assembly and release

The HCV assembly process occurs at the surface of cellular lipid droplets (LDs) in close proximity to ER membranes and is strictly associated with the lipid metabolism. Following synthesis and maturation, the core protein is translocated from the ER to cytosolic LDs (Moradpour et al. 1996; Barba et al. 1997), with the help of cellular factors such as diacylglycerol acetyltransferase 1 (DGAT1) (Herker et al. 2010). Once translocated to lipid droplets, core is able to recruit other components of the viral assembly complex (Miyanari et al. 2007). It has been proposed that p7 and NS2 also regulate core trafficking (Boson et al. 2011) and that their coordinated interaction with E1 and E2 bring the glycoproteins in close proximity to lipid droplets for their subsequent incorporation in the viral particle (Jirasko et al. 2010; Popescu et al. 2011). Additionally, a role of NS5A protein in virus assembly has been established. It has been reported that the interaction between domain III of NS5A and core associated with LDs is necessary for efficient viral assembly (Appel et al. 2008; Masaki et al. 2008; Tellinghuisen et al. 2008)

HCV particle assembly and release are closely linked to the VLDL assembly pathway (Jones & McLauchlan 2010). Following assembly, the virion buds into the ER and follows the secretory pathway acquiring low buoyant density characteristics, to be finally released from the cell. P7 was shown to assist the secretion of the virus by neutralizing the acidic secretory compartments (Wozniak et al. 2010).

1.5 Innate immune response to RNA virus infection

Host protection against invading pathogens is regulated by two main type of immune responses defined as the innate and adaptive immune response. The innate immune response represents the initial defence and is mediated by germnline encoded pattern recognition receptors (PRR) that sense the presence of specific viral signature known as pathogen associated molecular patterns (PAMPs). The acquired immune response is involved in the elimination of a specific pathogen by T cells and by B cells producing antibodies. The innate immune response is a biphasic process consisting of a first phase of interferon production, initiated by the recognition of viral signatures and structures by host PRRs, and a second phase of IFN signalling and interferon stimulated genes (ISGs) expression. The sensing of PAMPs by PRRs, up-regulates the transcription of genes involved in the inflammatory responses such as cytokines and type I interferons (IFNs). Two PRR family involved in viral RNA recognition have been identified so far: the Toll-like receptor family (TLR) and RIG-I like receptor family (RLR).

1.5.1 Toll-like receptor family

Toll like proteins have been characterized as homologous to Toll protein, an important antifungal sensor in the Drosophila immune response (Rock et al. 1998). TLRs are expressed in most cell types but predominately in immune cells such as macrophages and dendritic cells (DCs) (Kawai & Akira 2007). TLRs are type I transmembrane proteins with leucine rich repeat (LRR) ectodomain for the recognition of viral or microbial signatures, a transmemabrane region and a cytoplasmic domain also known as Toll/IL-1 receptor (TIR) domain responsible for signal transduction (Akira & Takeda 2004). Various members of the TLRs family have been identified. Based on the localization in the cell, they are classified in those present on the surface of the cell (TLR1, 2, 4, 5 and 6) and involved in the recognition of bacterial, fungal and viral structures, and those present in intracellular compartments such as ER, endosomes, or lysosomes, and mainly involved in the recognition of viral nucleic acids (TLR3, 7, 8 and 9).

TLR9 recognizes unmethylated CpG DNA characteristic of bacterial genomic DNA (Hemmi et al. 2000). A specific recognition of viral agonist is mediated by TLR7, TLR8 and TLR3. TLR7 and 8 recognize ssRNA derived from many different viruses including

Influenza A virus (IAV) and Vesicular Stomatitis Virus (VSV) (Lund et al., 2004) among others. Since these receptors detect ssRNA present inside an endosome, a degradation of the endocytosed viral particle is necessary for their activation. TLR3 was described as a receptor involved in the recognition of dsRNA in endosomal compartments of macrophages and conventional dendritic cells (cDCs). The role of TLR3 in the antiviral response is still not clearly defined. Studies performed in TLR3 deficient mice suggest that TLR3 is required for type I interferon and proinflammatory cytokine production upon synthetic dsRNA treatment (Alexopoulou et al. 2001), while other reports showed that it does not influence the host adaptive immune response upon infection with different viruses (Edelmann et al. 2004). The different TLR3 requirement in the activation of the host immune response could reflect the type of cell infected, the amount and type of viral infection, the early or late stage of infection as well as the regulation of the adaptive immune response.

TLR signalling pathway

The binding of the ligand to the LRR domain of TLR receptors causes their dimerization and recruitment by the TIR domain of several adaptor proteins. This event culminates in the expression of type I interferon and pro-inflammatory cytokines (Figure 8). The type of the TLR receptor and the kind of adaptor protein engaged in the signalling cascade determines the nature of the innate immune response. TLR3 signals through the TIR-domain-containing adaptor-inducing interferon-β (TRIF) protein. TRIF interaction with the tumor necrosis factor receptor-associated factor 6 (TRAF6) results in the recruitment and activation of the receptor-interacting protein 1 (RIP1) kinase which is required for nuclear factor kappa-B (NF-κB) activation and consequent pro-inflammatory response. Alternatively, TRIF association with tumor necrosis factor receptor-associated factor 3 (TRAF3) results in the recruitment of IKK related kinases, TANK binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase epsilon (IKKε), for the phosphorylation of interferon regulatory factor 3 (IRF-3). Once activated, IRF-3 translocate to the nucleus and triggers the transcription of type I interferon genes. All other TLRs utilize myeloid differentiation primary response gene 88 (MyD88) as an adaptor protein for signalling transduction. Following receptor activation, MyD88 associates to the TIR domain and recruit interleukin-1 receptor associated kinase (IRAK) members. The interleukin-1 receptor associated kinase-4

(IRAK4) allows interleukin-1 receptor associated kinase-1 (IRAK1) auto-phosphorylation which then dissociates from MyD88 and interacts with tumour necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 promotes the recruitment and activation of TGF-β-activated kinase 1 (TAK1) which in turn mediates the downstream activation of the kinase complex IKKα/β/γ and the mitogen-activated protein kinase (MAPK). The coordinated activity of NF-κB and MAPK cascade results in the transcription of genes involved in the inflammatory response (Kawai & Akira 2007).

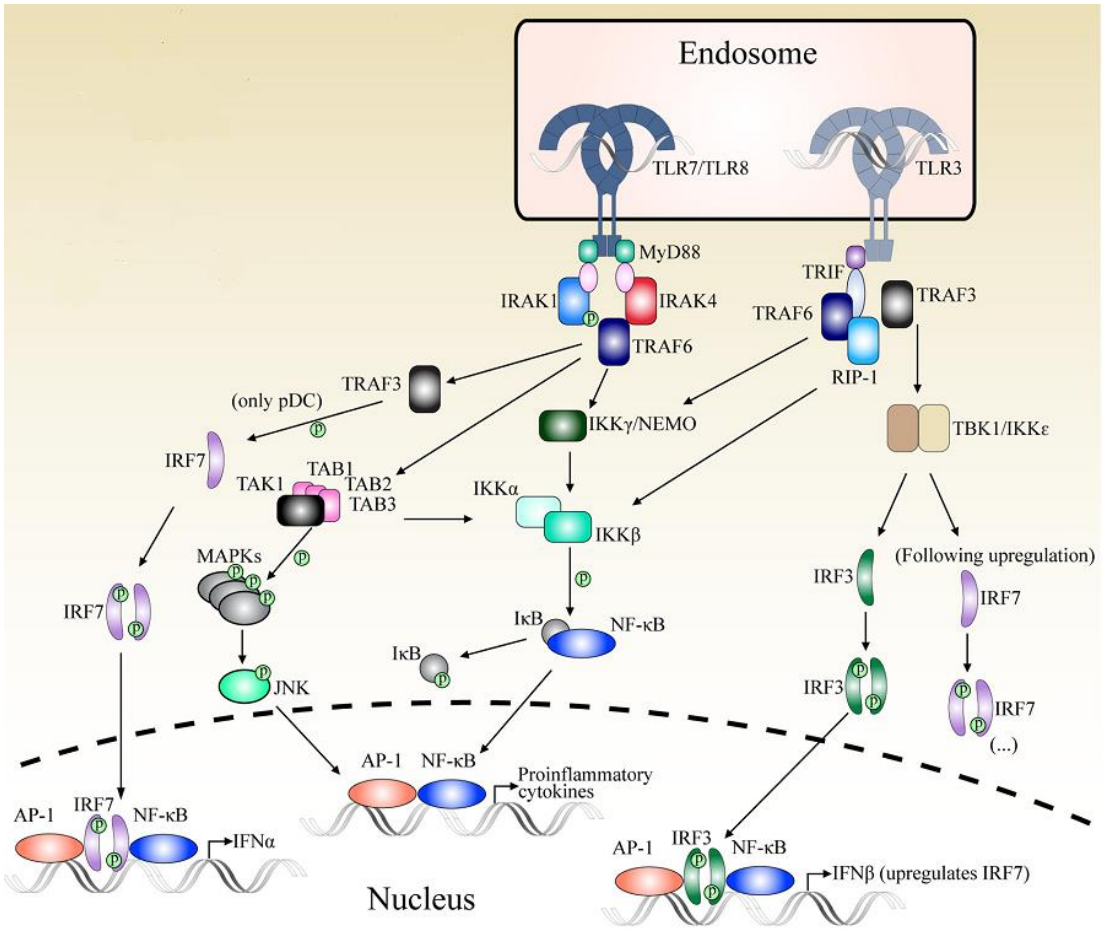

Figure 8. Endosomal TLR signalling pathway. TLRs present in endosomes are able to sense the presence of viral dsRNA or ssRNA molecules both in infected and non-infected cells. The signalling cascade is activated by the recruitment of MyD88 or TRIF adaptor proteins. TRIF, through the recruitment of TRAF6 and RIP1, as well as TBK1, IKKε and IKK kinase complex activates IRF-3 and NF-κB. MyD88 recruits TRAF6 and IRAK to activate NK-κB and MAPK signalling cascades. Adopted from (Jensen & Thomsen 2012).

1.5.2 RIG-I like receptor family

The RLR family members are a group of cytoplasmic receptors involved in the recognition of viral RNA in the cytoplasm of infected cells. The family consists of three

members: the retinoic acid-inducible gene I product (RIG-I), melanoma differentiation-associated antigen 5 (MDA-5) and laboratory of genetics and physiology 2 (LPG2) (Loo & Gale 2011). These sensors share some structural and functional similarities. The general structure is represented by the N-terminal domain with two tandem caspase activation and recruitment domains (CARDs), the central DExD/H box RNA helicase domain and a C terminal (CTD) repressor domain (RD). The CARD domain of RIG-I and MDA5 mediate the interaction with the CARD domain of the mitochondrial antiviral signalling protein (MAVS) to induce the activation of type I IFN and pro-inflammatory cytokines. On the other hand, the role of LGP2 in the modulation of the antiviral response is not yet well defined. Initially it was proposed that LGP2 since is deprived of the CARD domain and is therefore unable to signal through MAVS, had inhibitory functions on the RLR pathway (Yoneyama et al. 2005; Rothenfusser et al. 2005). Recent studies performed have showed that LGP2 acts by stimulating both the RIG-I and MDA5 activities (Satoh et al. 2010). The RD is involved in the recognition of dsRNA and 5'-triphosphate ssRNA viral signatures (Kato et al. 2006; Hornung et al. 2006) and the maintenance of the inactive state of the receptor in the absence of viral infection (Saito et al. 2007). The central DExD/H box RNA helicase domain has a dual function represented by the helicase/translocase activity and the ATPase activity.

RIG-I-like receptors signalling pathways

Upon binding of the viral agonist, RLRs expose their CARD domain following an ATP dependent conformational change. The CARD domain of both RIG-I and MDA5 interact with the CARD domain of the MAVS adaptor protein which triggers the recruitment of downstream signalling molecules for the activation of NF-κB and IRF-3 transcription factors. MAVS association with TRAF3 induces the activation of TBK1 and IKKε for the subsequent IRF-3 phosphorylation. Alternatively, the recruitment of Fas-associated death domain (FADD) and RIP1 triggers the NF-κB pathway. IRF-3 and NF-κB subsequent translocation to the nucleus triggers the transcription of the type I IFN and pro-inflammatory cytokine expression for the induction of the antiviral state (Yoneyama & Fujita 2009) (Figure 9).

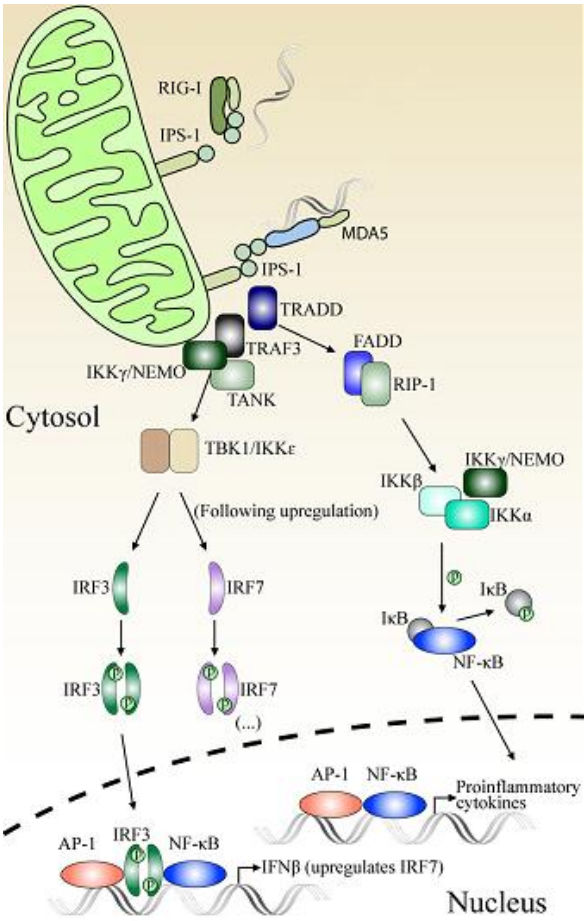

Figure 9. Scheme of the RLR signalling pathway. Viral RNA in the cytoplasm is detected by RIG-I and MDA5. Both receptors detect dsRNA, whereas RIG-I is also able to detect the 5'-triphosphate single-stranded RNA (ssRNA). Upon activation these two receptors interact with the adaptor protein IFN-β promoter stimulator 1 (IPS1), also known as MAVS, to induce the antiviral signalling cascade. TNF receptor-associated death domain (TRADD) is recruited to the adaptor protein and coordinates the recruitment of downstream signalling molecules. Association of FADD and RIP1 leads to the activation of NF-κB, whereas the association of TRAF3 together with TANK and IKKγ activates IRF-3. Adapted from (Jensen & Thomsen 2012).

1.5.3 Type I interferon expression and signalling

Interferon was originally identified in 1957 by Isaacs and Lindenmann as a molecule produced and secreted following infection with heat-inactivated influenza virus and able to restrict subsequent viral replication (Isaacs & Lindenmann 1957). Interferons are a large group of cytokines produced upon viral infection aimed to restrict viral replication by establishing an antiviral state in the cells. Interferons are grouped in three categories. Type I interferon consists of 13 different subtypes of IFN-α and only one IFN-β which are expressed by most cell types primarily upon viral infection. Moreover, IFN-ω, IFN-κ, IFN-ε, which present cell type and stimulus specific activation mechanisms, are also

members of type I IFN system. IFN-γ is the only member of the type II IFN system and is produced by NK cells and activated T cells. Type III IFN-λ1, IFN-λ2, and IFN-λ3, also known as IL-29, IL-28A, and IL-28B, respectively are ubiquitously expressed, but their activity is limited to epithelial cells and those at high risk of viral infection (Wack et al. 2015). Interestingly, type III IFNs similarly to type I INF are expressed upon viral signature recognition by cytosolic or endosomal bound receptors and induce an antiviral state in the cells by initiating a transcription of various ISGs. Studies performed on primary human hepatocytes infected with HCV identified a high mRNA levels of IL-28A, and IL-28B as well as a strong induction of ISGs expression (Thomas et al. 2012; Marukian et al. 2011) indicating that type III IFN plays a crucial role in the establishment of the antiviral response against HCV infection. Recently, a novel member of type III IFN system has been identified and named IFN-λ4 (Prokunina-Olsson et al. 2013). Intriguingly, although the protein signals through the same receptor as the other members of the family and has antiviral effects *in vitro* (Hamming et al. 2013), it negatively impacts HCV clearance (Prokunina-Olsson et al. 2013).

The importance of the interferon system was shown by an experiment performed in mice harbouring a deletion of the IFN-β gene or the IFN receptor (IFNAR) which rendered the mice more susceptible to viral infection (Müller et al. 1994; Deonarain et al. 2000). Therefore, the induction of the type I interferon expression must be tightly regulated. The PRR signalling cascades initiated upon viral infection results in the activation of transcription factors IRF-3, NF-κB and ATF2/c-Jun. Upon viral infection, the NF-κB inhibitor protein IκB-α responsible for its cytoplasmic localization, is phosphorylated and ubiquitinated, releasing NF-κB and allowing its nuclear translocation. IRF-3 becomes phosphorylated at particular serine residues which induces its dimerization and nuclear translocation. ATF2/c-Jun is already present in the nucleus and the MAPK signalling cascade activated upon PAMP recognition by PRR, induces the phosphorylation of its activation domain allowing the transcription factor to bind DNA and induce the expression of the IFN-β gene (Ford & Thanos 2010). The progressive and coordinated assembly of these transcription factors on the enhancer region of IFN-β gene, in cooperation with the high mobility group protein A1 (HMGA1), form a stable complex designated as enhanceosome (Yie et al. 1999). The IFN-β enhancer region consists of four positive regulatory domain (PRDs) known as PRDII, PRDIII-I and PRDIV which are bound by NF-κB, IRF-3 and ATF2/c-Jun

respectively. The assembled enhanceosome is recognized by PCAF and other unknown enzymes that induce the phosphorylation, acetylation or methylation of specific histone tails, forming a well-defined histone code, which is subsequently recognized by other factors involved in transcription (Agalioti et al. 2000; Agalioti et al. 2002). PCAF eviction is followed by CBP recruitment to the promoter region together with RNA Pol II, transcription factors TFIIE, TFIIH, TFIIF and SWI/SNF chromatin remodelling complex. The SWI/SNF activity induces the binding of TFIID transcription factor that in turn recruits TATA Binding Protein (TBP), responsible for the nucleosome sliding and unmasking of the TATA box, to initiate the transcription of IFN-β gene (Agalioti et al. 2000).

The second phase of the innate immune response is characterized by the IFN signalling through the JAK-STAT pathway. The secreted IFN-α/β acts in a paracrine and autocrine manner to induce an antiviral state in the infected cell as well as in the neighbouring non infected cells. The signalling transduction is initiated by the binding of the IFNα/β to a common heterodimeric trans-membrane receptor (IFNAR) formed of two subunits IFNAR1 and IFNAR2. The ligand induces the activation of the receptor associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). Jak1 and Tyk2 then phosphorylate the signal transducer and activator of transcription 1 (STAT1) and STAT2. Upon phosphorylation, these two molecules dimerise and translocate to the nucleus where their interaction with interferon regulatory factor-9 (IRF-9), to form the ISG factor 3 (ISGF3), leads to the activation of hundreds of ISGs. ISGs like myxovirus resistance 1 (MX1), IFN-inducible double-stranded RNA-dependent protein kinase (PKR), 2′-5′-oligoadenylate synthetase (OAS), IFN induced transmembrane proteins (IFITMs) along with many others, act in concert to establish an antiviral response in the cell (Ivashkiv & Donlin 2014). In addition to ISGs expression, type I interferons have a role in the adaptive immune response. It has been shown that IFNs play a crucial role in the maturation of DCs by activating natural killer NK cells and presentation of viral antigens for CD8+T cell activation (Stetson & Medzhitov 2006).

1.6 HCV induction and evasion of the host immune response

The innate immune response triggered by HCV infection represents a prerequisite for the functional activation of the adaptive immune response. Both the innate and adaptive arms of the immune response contribute to the outcome of HCV infection (Rehermann

2013). Interestingly, an acute HCV infection usually becomes chronic in about 70-80% of the cases, which eventually results in severe liver complications like fibrosis, cirrhosis or hepatocellular carcinoma. The outcome of the infection is probably related to a complex interplay between viral and host factors that regulate the innate immune responses to HCV infection (Figure 10).

HCV infection is detected by both cytosolic and endosomal PRRs. Among the cytoplasmic sensors, RIG-I is known to detect the HCV 3' UTR, enriched for polyU/UC, as well as the 5' triphosphate region in the 5'UTR (Saito et al. 2008; Uzri & Gehrke 2009). RIG-I was shown to sense HCV very early during infection and activate the signalling cascade before viral proteins accumulate in the cell (Loo et al. 2006). Once activated, RIG-I transduce the signal through the adaptor protein MAVS and induce the transcription of type I interferon and pro-inflammatory cytokines (Loo & Gale 2011). A late stage of HCV infection is sensed by TLR3. TLR3 is expressed in various cell types in the liver including hepatocytes and macrophages (Wang et al. 2009; Seki & Brenner 2008), where it detects the dsRNA produced during viral replication (Li et al. 2012a). It is considered that the uptake of dsRNA from the extracellular space or the dsRNA accumulated in endosomes and autophagic compartments is able to induce a late TLR3 signalling and cytokine production (Dansako et al. 2013; Dreux et al. 2009).

In order to subvert the RIG-I and TLR3 mediated immune responses, HCV has evolved different strategies. The most powerful strategy is represented by the viral NS3/4A protease that was shown to interfere with RIG-I induced signalling by disrupting MAVS association with the mitochondria (Foy et al. 2005; Baril et al. 2009). Although primarily localized on the mitochondria outer membrane, HCV NS3/4A is known to cleave MAVS that is anchored to the mitochondrial associated membranes (MAMs), an extension of ER membrane present in the ER-mitochondria junction (Horner et al. 2011), indicating that MAVS localized on the MAMs is responsible for the activation of the signalling pathway upon HCV infection. Moreover MAVS cleavage has also been observed in patient with chronic HCV infection and is correlated with the observed low level of IFN expression (Bellecave et al. 2010). Additionally, NS3/4A has also the ability to interfere TLR3 pathway activation through the cleavage of TRIF adaptor protein *in vitro* (Li et al. 2005a). However, this finding is still controversial as some

reports were unable to identified the cleaved fragment but confirmed the cleavage by reduced TRIF protein expression (Wang et al. 2009) while another report was unable to corroborate the data (Dansako et al. 2007).

HCV infection can be also sensed by the cytoplasmic dsRNA binding protein kinase (PKR), which contributes to the virus induced innate immune response. HCV IRES structure was identified as the ligand for PKR (Shimoike et al. 2009). The binding induces PKR kinase activity which results in the phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2α) and blocks the host mRNA translation, although it has no effect on IRES mediated HCV translation (Arnaud et al. 2010; Garaigorta & Chisari 2009). Additionally, IRES recognition by PKR induces its kinase independent activity consisting of MAVS mediated early activation of ISGs and IFN-β expression (Arnaud et al. 2011).

Surprisingly, PRK activity seems to have divergent roles in the HCV induced immune response. PKR kinase activity cause the translational arrest of host mRNA including those of ISGs and IFN-β, supporting in this case HCV replication. On the contrary, the stalled translation of host factors needed for an efficient HCV replication support the antiviral function of PKR (Arnaud et al. 2010; Garaigorta & Chisari 2009). Moreover, PKR kinase independent ISGs production seems to enhance the host response against viral replication, although an abundant production of ISG15 at early time points negatively regulates RIG-I activity (Arnaud et al. 2011). Nevertheless, HCV NS5A and E2 are able to interfere with PKR activities (Gale et al. 1997; Taylor et al. 1999). A recent report shows that PKR kinase activity can be switched off and on during HCV life cycle to guarantee the production of cellular factors and to maintain an appropriate environment for HCV replication (Arnaud et al. 2011; Ruggieri et al. 2012).

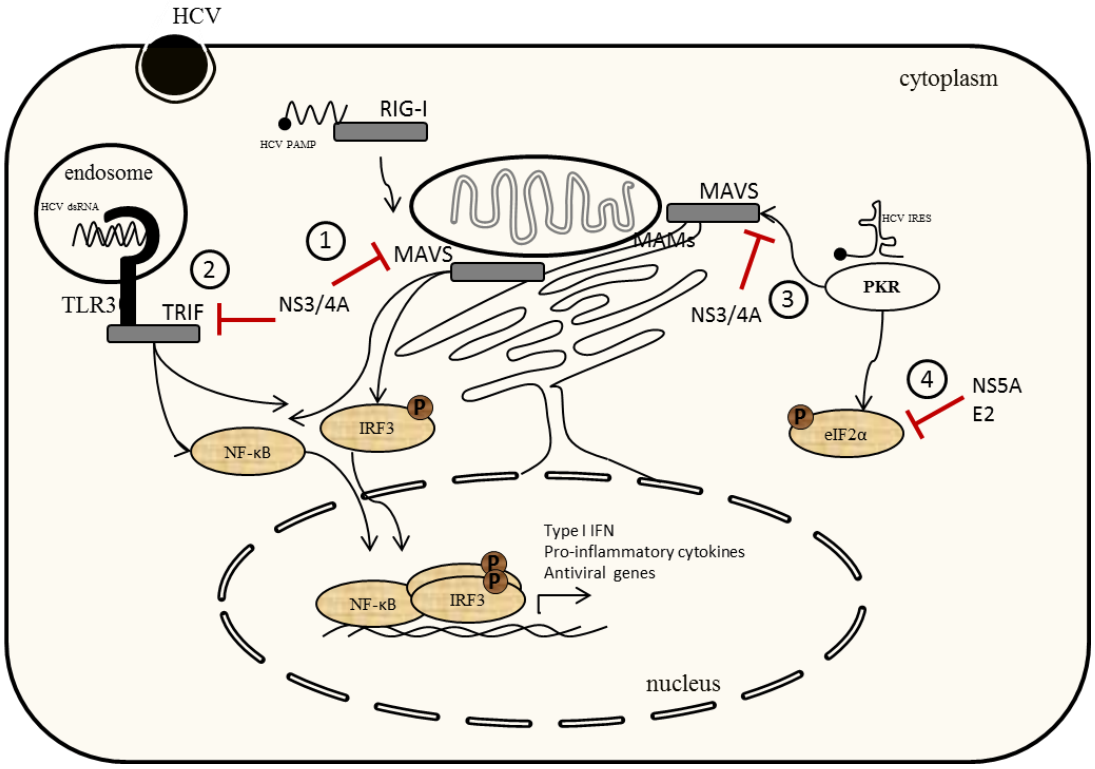

Figure 10. HCV activation and evasion of innate immune responses. HCV infection activates the innate immune response by different mechanisms. HCV PAMP is sensed by RIG-I in the cytosol, which activates a signalling cascade through MAVS that culminates in the transcription of type I interferon and pro-inflammatory cytokines. The HCV IRES is sensed by PKR which phosphorylates eIF2-α to block the host mRNA translation, whereas its kinase independent MAVS signalling induces the expression of IFN-β and various ISGs. In a late stage of virus infection the accumulation of dsRNA in endosomes is sensed by TLR3 which mediate a signalling transduction through TRIF adaptor protein to induce the type I IFN expression. HCV have evolved various strategies to counteract the host response to infection. The NS3/4A protease cleaves MAVS adaptor protein localized on the MAMs (1) and the TRIF adaptor protein present on endosomes (2) blocking both the RLR and TLR3 mediated responses. The NS3/4A protease is also blocking the PKR signalling through MAVS (3). Additionally, NS5A and E2 bind PKR and inhibit its kinase activity (4). Adapted from (Horner & Gale 2013).

1.7 NAP-1 family of histone chaperones

The NAP-1 family consist of highly conserved histone chaperone proteins with roles in both transcription and DNA replication. Apart from humans, NAP-1 proteins have been identified in yeast (Ishimi & Kikuchi 1991), Drosophila (Ito et al. 1996) and plants (Dong et al. 2005). NAP-1 was originally identified in mammalian cell extracts as a protein that fosters nucleosome assembly in coordination with other factors (Ishimi et al. 1987). NAP-1 family of histone chaperons are all characterized by a highly conserved central domain and an acidic C terminal domain. Both regions are involved in histone binding (Fujii-Nakata et al. 1992; Park & Luger 2006a). A crystal structure

for yeast NAP-1 has been resolved and it allowed the identification of two different domains of the protein. Domain I, involved in the dimerization process, consists of two short alpha helices flanking a longer one. Domain II is involved in protein interactions and consists of antiparallel β-sheets surrounded by α-helices (Park & Luger 2006b). A ribbon diagram is shown in Figure 11. The human NAP-1 is represented by five different paralogs: NAP1L1, NAP1L2, NAP1L3, NAP1L4, and NAP1L5. NAP1L1 and NAP1L4 are ubiquitously expressed while NAP1L2, NAP1L3 and NAP1L5 are specifically expressed in neurons (Attia et al. 2011).

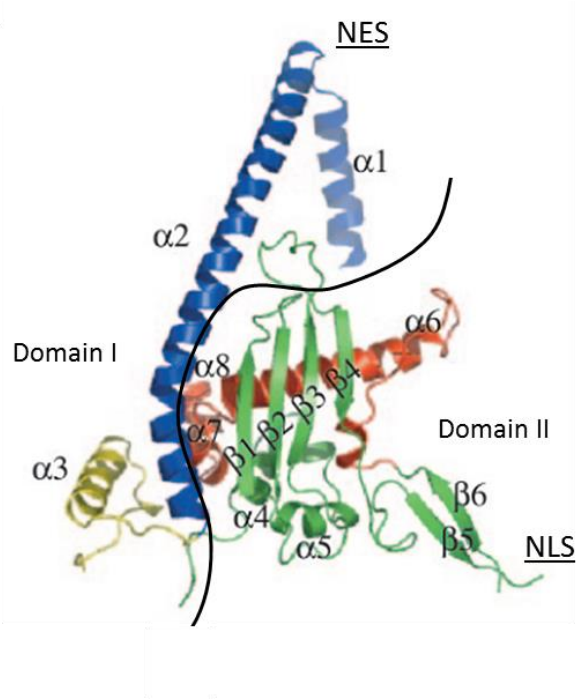

Figure 11. Ribbon diagram of y-NAP-1. y-NAP-1 monomer consists of two domains. Domain I (blue and yellow coloured) which is responsible for the dimerization of the protein consists of one long alpha helix flanked by two shorter alpha helices. It can be additionally divided in subdomain A (blue) involved in dimerization and subdomain B (yellow) which modulates the accessibility of the NES present in subdomain A. Domain II (green and red coloured) is involved in protein-protein interaction and consists of alpha helices and β-sheets. The subdomain C (green) of domain II, represented by an amphipathic β-sheet is protecting the alpha helix of subdomain D (red). Domain II contains also the NLS. Adapted from (Park and Luger, 2006b).

1.7.1 Nucleosome assembly protein 1-like-1

NAP1L1 is a 391 amino acids long protein with three highly acidic regions probably involved in the interaction with histone proteins (Simon et al. 1994) (Figure 12). Human NAP1 proteins are able to form dimers by pairing of the long α-helix present in domain I of each monomer (Shikama et al. 2000). NAP1L1 is a nucleo-cytoplasmic shuttling

protein as suggested by the presence of a nuclear localization signal (NLS) located in domain II (Simon et al. 1994) and a nuclear export signal (NES) located in domain I of yeast NAP-1 (Miyaji-Yamaguchi et al. 2003) and has a predominant cytoplasmic localization (Marheineke & Krude 1998). NAP1L1 as well as NAP1L4 can be phosphorylated by casein kinase II (CKII) and the phosphorylation state is associated with their subcellular distutibution during the cell cycle (Rodriguez et al. 2000). While CKII phsohorylation of y-NAP-1 induces its nuclear translocation (Calvert et al. 2008), the phosphorylation of NAP1L1 occours at the G_0/G_1 transition phase followed by dephosphoryaltion that triggers its nuclear translocation in the G_1/S phase of the cell cycle (Rodriguez et al. 2000). NAP1L1 is subjected to other post translational modification not yet characterized. It was shown to be ubiquitinated (Bish & Myers 2007; Xu et al. 2010), farnesylated (Kho et al. 2004) as well as polyglutamylated, a modification consisting in the addition of up to nine residues of glutamic acids in two putative glutamic acid position present in the C-terminal domain of the protein (Regnard et al. 2000).

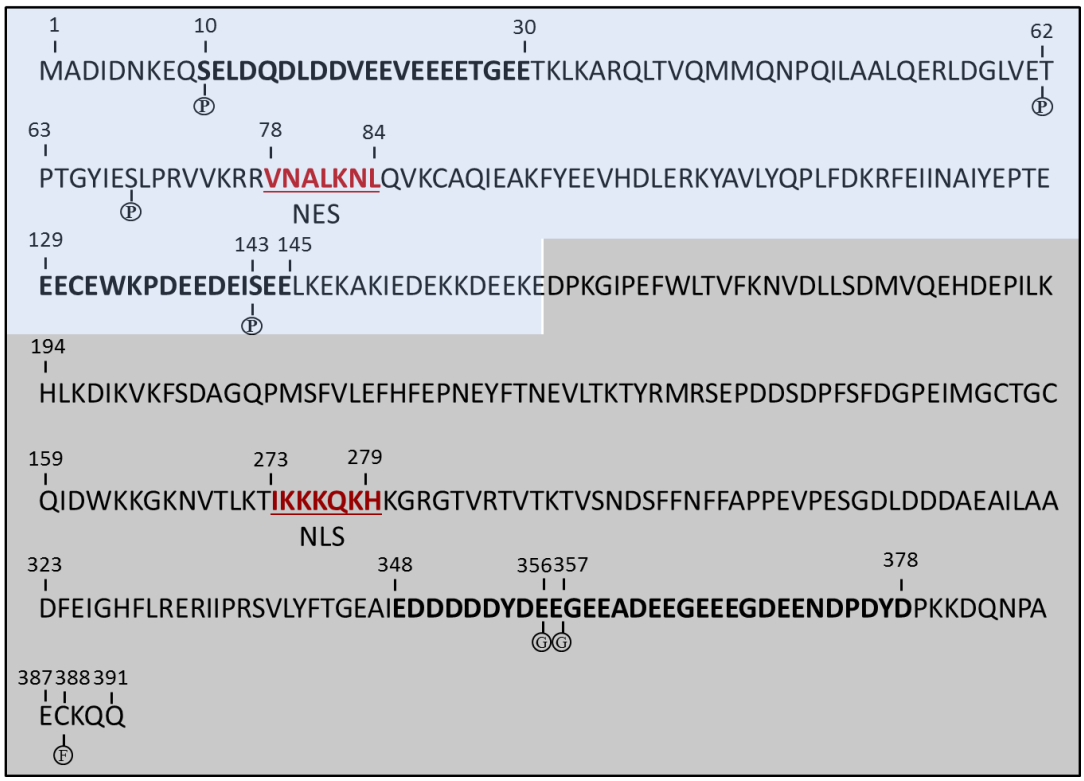

Figure 12. NAP1L1 Sequence. NAP1L1 contains two domains. Domain I (in light blue) contains the nuclear export sequence (NES) and domain II (in light grey) contains the nuclear localization sequence (NLS). Throughout the sequence, NAP1L1 is characterized by three acidic regions (black bold sequence) and various post-translational

modifications. Four phosphorylation site (P), two glutamylation sites (G) and one pharnesylation site (F) have been identified. Numbers refer to an amino acid sequence.

1.7.2 NAP-1 functions

Yeast NAP-1 interacts with H2A and H2B in the cytoplasm and mediates their transport to the nucleus by interacting with a karyopherin protein Kap114, a specific H2A and H2B nuclear importin protein (Mosammaparast et al. 2002) indicating a NAP-1 role in histone trafficking to the nucleus.

From its identification as a histone chaperon protein, NAP-1 function in chromatin assembly has been extensively studied, mostly *in vitro* (Fujii-Nakata et al. 1992; Ishimi & Kikuchi 1991; Ishimi et al. 1987). NAP1 interacts with all four histones *in vitro*: the histone dimer H2A/H2B, the $(H3/H4)_2$ tetramer and the histone H1 (Andrews et al. 2008; Bowman et al. 2011; Kepert et al. 2005). NAP-1 has been shown to assist the deposition of histones on DNA to assemble nucleosomes in a three step process. Initially, H3/H4 tetramers are loaded to the naked DNA. This process is followed by the deposition of one H2A/H2B dimer to form a hexasome and then by another H2A/H2B dimer to form a complete nucleosome complex (Nakagawa et al. 2001). The mechanism governing NAP-1-mediated nucleosome assembly *in vivo* are still not well defined but a recent study performed in yeast NAP-1 suggests that nucleosome assembly *in vivo* occurs through the elimination of non-nucleosomal histones from DNA as NAP-1 deletion significantly increases the atypical histone-DNA interaction (Andrews et al. 2010).

NAP-1 can also form interactions with histone variants such as H2A.Z and H3.3 (Park et al. 2005; Okuwaki et al. 2005). Considering the fact that these histone variants are synthetized outside the S phase of the cell cycle, it is thought that they can be used for nucleosome assembly uncoupled from DNA synthesis. In Drosophila, H3.3 histone variant is incorporated in sites of active gene transcription (Ahmad & Henikoff 2002). Walter and collaborators have showed that NAP-1 is involved in gene transcription by promoting nucleosome displacement and binding of transcription factors (Walter et al. 1995). In this case, NAP1L1 eviction of H2A/H2B promotes the binding of transcription factors. Subsequent reports have demonstrated that NAP-1 interacts with the histone acetyl-transferase co-activator p300 which may stabilize the transcription complexes. In this case NAP-1 assists the binding of transcription factors to the promoter region of genes. Subsequently the co-activator p300 is recruited and acetylates

the histone tails. At that point H2A/H2B dimers are removed from nucleosomes and translocated to NAP-1, activating gene transcription (Shikama et al. 2000; Ito et al. 2000). In addition, Xue and co-workers showed that y-NAP-1 promotes H3K9 acetylation by interacting with transcription elongation factors (Xue et al. 2013). The above mentioned experimental evidences indicate a possible role of NAP1L1 in gene transcription. In fact, a genome wide expression study showed that 10% of the yeast ORFs was differentially modulated in the absence of NAP-1 (Ohkuni et al. 2003). Moreover, NAP-1 was shown to be present on the promoter regions of genes, and deletion of NAP-1 was shown to increase nucleosome density at the promoter levels suggesting that NAP-1 could promote nucleosome eviction (Walfridsson et al. 2007).

In relation with its role in nucleosome assembly and disassembly, NAP-1 has been engaged in many other related functions. NAP1L1 was shown to have a fundamental role in the regulation of cell cycle and proliferation (Simon et al. 1994). Additionally, yeast NAP-1 participates in mitotic events through the interaction with cycline B. (Kellogg & Murry 1995).

Ultimately, a role for NAP1L1 in the cell differentiation has been described. NAP1L1 together with SWI/SNF and INO80 chromatin remodelling complexes promotes nucleosome depletion during embryonic stem cell differentiation (Li et al. 2012b). A report form Gong et al., shown that the depletion of NAP1L1 increase the differentiation into cardiomyocytes (Gong et al. 2014).

1.7.3 NAP1L1 as target of different viruses

An increasing number of reports indicate that viruses target NAP1L1 to control their own replication or modulate the expression of specific host genes. The HIV Tat protein has been found to interact with NAP1L1 to regulate Tat mediated activation of viral genes which results in an increased viral infectivity (Vardabasso et al. 2008). The interaction of Tat with NAP1L1 was detected in the perinuclear area by a FRET assay, suggesting a role in the nuclear import of Tat (De Marco et al. 2010). NAP1L1 interaction with Rev, another HIV protein, was found to influence Rev function (Cochrane et al. 2009). E2 protein form the Papillomavirus (PV) was found in association with NAP1L1 and p300 and mediated the transcription of target genes (Rehtanz et al. 2004). Moreover, always in collaboration with p300, NAP1L1 was shown to promote the eviction of histones from the promoter of human T-lymphotropic virus (HTLV-1) increasing its transcription rate (Sharma & Nyborg 2008). Recently it

has been shown that Kaposi's sarcoma-associated herpesvirus (KSHV) protein LANA recruits NAP1L1 for the expression of viral genes and the establishment of a latent infection (Gupta et al. 2016). Ultimately, a number of proteomic analyses showed that HCV NS5A and Core proteins can bind NAP1L1, but the functional relevance of this interaction is awaiting further elucidation (De Chassey et al. 2008; Pichlmair et al. 2012).

1.8 Aim of the thesis

Hepatitis C is a blood borne virus that in almost 90% of the cases causes a chronic disease, which if not treated can lead to sever forms of liver disease, like fibrosis cirrhosis or hepatocellular carcinoma. It is thought that the chronic infection is caused by the ability of the virus to supress the host immune response. HCV polyprotein is processed to give rise to 10 mature proteins and among them NS3/4A or NS5A are the major players in subverting the innate immune response. NS5A, due to its unfolded nature of domain II and III, interacts with hundreds of host proteins and perturbs many cellular pathways.

In this work I have investigated the interaction of NS5A with a histone chaperone protein NAP1L1, with the aim to understand the functional relevance of the interaction. In the first part of the work I performed an RNA-Seq analysis of NAP1L1 depleted cells to gain insight into NAP1L1 regulated genes and then analysed a role of NAP1L1 in the RLR and TLR3 pathway. In the second part I made an attempt to correlate NS5A-NAP1L1 interaction to a reduced IFN-β expression during viral replication in an immuno-competent HCV permissive cell line.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cells

Bacteria

- **XL10-Gold Ultracometent Cells** (Stratagene; cat.num. 200315)
  Genotype: Tet^rΔ(mcrA)183Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB $lacI^qZ$ΔM15 Tn10 (Tet^r) Amy (Kan^r)]
- **MAX Efficiency Stbl2 Competent Cells** (Invitrogen; cat. num. 10268-019)
  Genotype: F^- mcrA Δ(mcrBC-hsdRMS-mrr) recA1 endA1lon gyrA96 thi supE44 relA1 λ^- Δ(lac-proAB)

Mammalian Cells

- **HEK 293T** – Human embrionic kidney cell line containing the SV40 T-antigen
- **U2OS -** Human osteosarcoma cell line (ECACC No. 92022711)
- **Huh7 Lunet –** Human hepatocellular carcinoma cell line derived from drug treated Huh7 replicon carrying cell line (gift from R. Bartenschlager – University of Heidelberg)
- **Vero -** African green monkey kidney (ECACC No. 84113001)

2.1.2 Media

Bacteria

- **Luria Bretani (LB) Medium –** 10 g bacto-trypton, 5 g bacto-yeast extract, 10 g NaCl per 1 liter medium. Where needed LB was supplemented with ampicilin or kanamycicn at a concentration of 50-100 μg/ml. Solid medium was prepared by supplementing LB medium with 1,5% agar-agar.
- **SOC Medium –** is obtained by supplementing SOB medium (20 g bactotrypton, 5 g bacto-yeast extract, 0.5 g NaCl per 1 liter medium) with glucose at a final concentration of 20mM.

Mammalian Cells

- **DMEM Complete Medium –** Dulbecco's Modified Eagle's Medium (Gibco – cat.num. 31885-023) was supplemented with 10% Fetal Bovine Serum (FBS) (Euroclone – ECS0180L). For selection Puromycine dehydroclorate (Invitrogen – A1113803) or Blasticidin (Gibco – A1113902) were added at a concentration of 0,5-3μg/ml and 10μg/ml respectively.
- **OptiMEM –** Reduced Serum Media (Gibco – 31985-070)
- **Cryo Medium –** was prepared with 90% FBS and 10% DMSO and used to freeze cells in liquid nitrogen for long storage.

2.1.3 Antibodies and Antiserum

Primary antibodies

Reactivity	Species	Subtype	Source	Comments
HCV NS5A	Sheep	Polyclonal	M.Harris (University of Leeds)	1:200 IF
Human NAP1L1	Rabbit	Polyclonal	Abcam	1:200 IF 1:1000 WB
Human IRF-3	Rabbit	Monoclonal	Cell Signaling	1:100 IF 1:500 WB
Human Phospho-IRF-3(Ser396)	Rabbit	Monoclonal	Cell Signaling	1:500 WB
Human Phospho-NF-κB-p65	Rabbit	Monoclonal	Cell Signaling	1:100 IF 1:1000 WB
Human Phospho-NF-κB-p65 (Ser536)	Rabbit	Monclonal	Cell Signaling	1:1000 WB
Flag-M2	Mouse	Monoclonal	Sigma-Aldrich	1:1000 WB

Table 1. Primary antibodies used in this study

Secondary Antibodies

Reactivity	Species	Source	Comments
Alexa Fluor 594 Anti-Sheep IgG	Donkey	Molecular Probes	1:500 IF

Alexa Fluor 488 Anti-Rabbit IgG	Donkey	Molecular Probes	1:500 IF
Alexa Fluor 594 Anti-Rabbit IgG	Donkey	Molecular Probes	1:500 IF
Anti-Rabbit immunoglobulins/HRP	Goat	DakoCytomation	1:10000 WB
Anti-Mouse immunoglobulins/HRP	Rabbit	DakoCytomation	1:10000 WB
Anti-Sheep immunoglobulins/HRP		DakoCytomation	1:10000 WB
Human β-Actin Peroxidase		Sigma-Aldrich	1:10000 WB
HA-Peroxidase	Rat	Sigma-Aldrich	1:10000 WB

Table 2. Secondary Antibodies used in this study

2.1.4. Vectors

Expression and cloning vectors		
Plasmid	**Characteristics**	**Reference**
HA-NAP1L1	Expression vector for human NAP1L1; Amp^R	Asahara et al., 2002
pFLAG-CMV2	Cloning vector; Amp^R	Sigma-Aldrich
pIFN-b Luc	It carries the Firefly Luciferase gene under control of the IFN-b promoter	Jae Jung
pRL-CMV	Renilla expression vector; Amp^R	Promega
pFLAG-human IPS1	Expression vector for human IPS-1; Amp^R	Kindly provided by Dr. Takashi Fujita (Kyoto University)
pFLAG-human TBK1	Expression vector for human TBK1; Amp^R	Kindly provided by Dr. Takashi Fujita (Kyoto University)
pFLAG-human IKKε	Expression vector for human IKKε; Amp^R	Kindly provided by Dr. Takashi Fujita (Kyoto University)

pFLAG-human TRIF	Expression vector for human TRIF; Amp^R	Kindly provided by Dr. Takashi Fujita (Kyoto University)
pIRF-3(5D)	Expression vector for human phosphomimetic form of IRF-3	Kindly provided by Dr. Takashi Fujita (Kyoto University)
pHA-NF-κB p65	Expression vector for human p65 subunit of NF-κB	Kindly provided by Dr. Francesca Demarchi (LN-CIB, Italy)
pcDNA3.1+	Mammalian expression vector with the CMV promoter	Invitrogen
Lentiviral vectors		
PMD2.G	It expresses VSV-G envelope protein	Addgene; 12259
psPAX2	Lentiviral packaging plasmid	Addgene; 12260
pLKO.1 Scramble	Non-targeting shRNA	Addgene; 1864
pLKO.1 shNAP1L1	shRNA targeting NAP1L1	Produced by Dr. Emrah Cevik (Molecular Virology, ICGEB)
pTRIP-CMV-TLR3	Lentiviral vector for TLR3 overexpression	(Schoggins et al. 2011)
pTRIP-CMV-EGFP	Lentiviral vector for EGFP overexpression	(Schoggins et al. 2011)
pLVX-MAVSR	Lentiviral vector for the overexpression of the NS3/4A cleavage resistant form of MAVS	(Cao et al. 2015)
pTRIP Bsd 122	Lentiviral vector for the overexpression of miR122	Kindly provided by Dr. Matthew Evans
HCV replicons and NS5A plasmids		
pSGR-JFH1/Luc	Reporter replicon for the expression of NS3-NS5B proteins of genotype 2a HCV	Kato et al., 2005

	(JFH1)	
pSGR-JFH1/Luc GND	Reporter replicon for the expression of NS3-NS5B proteins of genotype 2a HCV (JFH1) with a mutation in the polymerase	Kato et al., 2005
pSGR-JFH1/m2	Reporter replicon for the expression of NS3-NS5B proteins of genotype 2a HCV (JFH1) with a mutation in NS5A gene	Masaki et al., 2008
pFLAG-NS5A/JFH1	Expression vector for NS5A (JFH1)	Produced by Dr. Emrah Cevik (Molecular Virology, ICGEB)
pFLAG-NS5A/m2	Expression vector for mutant NS5A; using P1 and P14 primers	Produced in this study
pFLAG-NS5A/m2E	Expression vector for mutant NS5A; using P1 and P14 primers	Produced in this study
pFLAG-NS5A/2m2	Expression vector for mutant NS5A; using P1 and P14 primers	Produced in this study
pFLAG-NS5A/2m2E	Expression vector for mutant NS5A; using P1 and P14 primers	Produced in this study

Table 3. Vectors used in this study

2.1.5 Oligonucleotides

Name	**Sequence 5' > 3'**
NS5A P1	CTA TAA GCT TTC CGG ATC CTG GCT CCG CGA C
NS5A P14	CTA TAG ATC TCT AGG TGG TAT CGT CCT CCT C
ADAM9 fw	ATA AGT TGG TGG ACG CTG GG
ADAM9 rev	CTC CTG GAA GGA ACC GAC AG
ADH1B fw	GAG CAC AGC AGG AAA AGT AAT CA
ADH1B rev	CCA CGT GGT CAT CTG TGT GA
ARHGEF2 fw	GCC GCT TGA AAG AAA GTT GC

ARHGEF2 rev	ACA TTG CAG GTT GGG CAG AT
ARHGEF12 fw	CCG ACA GGT TTC CCC TCA AA
ARHGEF12 rev	GCA ACG CTG AAC AAG ACC AT
ARHGEF40 fw	CTC ACT GGA AGA GCC GCC
ARHGEF40 rev	TTG CTT GAC GTC CAG ATC CC
BA1	CAT GTG CAA GGC CGG CTT CG
BA4	GAA GGT GTG GTG CCA GAT TT
BA promoter fw	CAA AAG GAG GGG AGA GGG GG
BA promoter rev	GCG GTC TCG GCG GTG
C3 fw	CCA AGA GCT CAA GGT GAG GG
C3 rev	GGA ACG GAC AAC GAG GAC TT
CASP7 fw	AGT GAC AGG TAT GGG CGT TC
CASP7 rev	CGG CAT TTG TAT GGT CCT CT
CCND1 fw	GAT CAA GTG TGA CCC GGA CTG
CCND1 rev	CCT TGG GGT CCA TGT TCT GC
CDK2 fw	GCA TCT TTG CTG AGA TGG TGA C
CDK2 rev	CAC TTG GGG AAA CTT GGC TTG
CFL2 fw	TGC TTT GTA CGA TGC CAC AT
CFL2 rev	TGC CAC TCA TGT TTA ATA CCT GTA A
CLDN1 fw	CCA GTC AAT GCC AGG TAC GAA
CLDN1 rev	CAC ACG TAG TCT TTC CCG CT
CROT fw	TGG CAG TCT CAA GCA GTT CA
CROT rev	GTT CTG GTG ACT GAT TCA AGG TA
E2F2 fw	GGC CCA TCG AAG TCT ACC TG
E2F2 rev	GGT GCT GGC ACT GAG GAT G
GAPDH fw	CAT GAG AAG TAT GAC AAC AGC C
GAPDH rev	AGT CCT TCC ACG ATA CCA AAG T
GAPDH promoter fw	TAC TAG CGG TTT TAC GGG CG
GAPDH promoter rev	TCG AAC AGG AGG AGC AGA GAG CGA
GBP2 fw	GGA AGG GGA TAC AGG CCA AA
GBP2 rev	TTC CAT CAT CTC CTC ATT CTT CTT
HEPACAM2 fw	AGC TGC TGG TCC AAG TTA CC
HEPACAM2 rev	GCA AGC ACC GAA GAG AAG GA
IFIT1 fw	GAA ATA TGA ATG AAG CCC TGG A
IFIT1 rev	GAC CTT GTC TCA CAG AGT TCT CAA
IFIT3 fw	CTG ATG CGT GCC CTA CTC TC

IFIT3 rev	GAC CTC ACT CAT GAC TGC CC
IFITM3 fw	CCG TGA AGT CTA GGG ACA GG
IFITM3 rev	TCC ATA GGC CTG GAA GAT CAG
IFN-β fw	AGG ACA GGA TGA ACT TTG AC
IFN-β rev	TGA TAG ACA TTA GCC AGG AG
IFN-β promoter fw	CTAAAATGTAAATGACATAGGAAAACTGAA
IFN-β promoter rev	GGCCTATTTATATGAGATGGTCCTC
NAP1L1 fw	GTT GAC TTG CTC AGT GAT ATG GTT C
NAP1L1 rev	CTG ACC TCA TCC TGT ATG TCT TTG
NF-κB 2 fw	AGG AGG CGG GCG TCT AA
NF-κB 2 rev	GGC TCC TTG GGT TCC ACA AT
OASL fw	TAC CAG CAG TAT GTG AAA GCC A
OASL rev	GGT GAA GCC TTC GTC CAA CA
RelA fw	CCA GAC CAA CAA CAA CCC CT
RelA rev	TTG GGG GCA CGA TTG TCA AA
RelA promoter fw	GCAGCGGCTGTGCGT
RelA promoter rev	TGCACTACAGACGAGCCATT
RelB fw	CGT TTC CAG GAG CAC AGA TGA
RelB rev	CAG GGT GAC CGT GCT CAG
TGFB1 fw	CTA TTG CTT CAG CTC CAC GG
TGFB1 rev	AGA AGT TGG CAT GGT AGC CC
TLR-3 fw	GCG CTA AAA AGT GAA GAA CTG GA
TLR-3 rev	TTG CGT GAA AAC AAC CTG GA
UBD fw	CCT CTG TGT GCA TGT CCG T
UBD rev	GAG GCT TCT CCG TGG CTT TA
UBD promoter fw	CTTAGGTTTGGGCTGTGGGT
UBD promoter rev	CTTCTCTCCTCAAGCAATCTATTTG

Table 4. Oligonucleotides used in this study

2.2 General procedure

2.2.1 Cell culture

Monolayers of cells were grown at 37°C, 5% CO_2 in DMEM complete medium. Cells were passaged after treatment with 0.05% Trypsin – 0.02% EDTA and seeded at the appropriate dilution.

2.2.2 Plasmid construction

All pFLAG-NS5A plasmids were constructed using pFLAG-CMV-2 plasmid as a backbone. pFLAG-CMV-2 was digested with BglII and HindIII restriction enzymes. Inserts were obtained by PCR amplification of the NS5A region from the corresponding HCV plasmid. Primers P1 and P2 were used in a following thermal cycling reaction: 95°C - 3min; 95°C – 30sec; 60°C – 30sec; 72°C – 30sec; 72°C – 3min. The PCR product was purified using QIAquick PCR Purification Kit (Qiagen - 28104,) and digested with BglII and HindIII restriction enzymes. Size and integrity of both vector and inserts was verified on 1% agarose gel and then excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen - 28704). Ligation of the inserts and linearized pFLAG-CMV-2 was performed at 16°C over-night. The authenticity of produced plasmids were analysed by enzymatic digestion and sequencing.

2.2.3 Plasmid transformation

XL10-Gold Ultracompetent cells (Stratagene) and Stbl2 Competent Cells (Invitrogen) were used for transformation of parental and produced plasmids. Cells were incubated with plasmids on ice for 30 min. Afterwards, a heat shock procedure at 42°C for 30 seconds was applied and cells were left for 2 more minutes on ice. Then, SOC medium was added and cells were incubated at 37°C or 30°C for 1-2 h. Finally, cells were plated onto LB agar with the desired antibiotic and grew over-night (O/N) at 37°C.

2.2.4 *In vitro* transcription

Template DNA for *in vitro* transcription was prepared from large-scale preparations of plasmid DNA performed using NucleoBond Xtra Midi Kit (Macherey-Nagel - 740410.50) according to the manufacturer's protocol. Parental HCV replicon plasmids were linearized by digestion with XbaI. All linearized plasmids were purified with phenolchloroform extraction and ethanol precipitation. The pellet was resuspended with RNase-free water. The 1-2 μg of template was transcribed using the T7 Quick High Yield RNA Synthesis Kit (New England Biolabs - E2050S). The reaction mixture (20 μl) was incubated for 4 h at 37°C. At the end of the reaction, 2U of DNase I (Life Technologies - 18068-015) was added and the mix was incubated for 15 min at 37°C to remove the template DNA. RNA was purified using the NucleoSpin RNA II Kit

(Macherey-Nagel - 740955.250). The RNA was eluted in RNase free water and the integrity of the *in vitro* transcripts was confirmed by denaturing agarose gel electrophoresis. The yield of RNA was determined by the Nanodrop.

2.2.5. RNA transfection by electroporation

Cell suspensions were prepared by trypsinization of monolayers and subsequent resuspension with DMEM complete medium. Cells were counted. Aliquots of 2 x 10^6 cells were suspended in DMEM, and washed with phosphate buffered saline with salts (PBS+). Cell were then resuspended in 800 µl of PBS+ and pipetted into a 0.4 cm Gene Pulser cuvette (Bio-Rad) with 10 µg of RNA. Cells were electroporated with a Bio-Rad Gene Pulser apparatus applying single pulse at 0.27 KV, 960 µF. After electroporation, cells were washed once in DMEM complete medium and seeded with the same medium.

2.2.6 Transfection with Lipofectamine

Poly(I:C) (Invivogen) was delivered into cells using Lipofectamine LTX and PLUS reagents (Life Technologies) according to the manufacturer's instructions

2.2.7 Luciferase assay

In order to assess the replicative capacity of HCV replicons or monitor IFN-β promoter activity, cells were harvested with 1X passive lysis buffer (Promega) and lysates were incubated for 15 min at 4°C. Following a centrifugation step, supernatants were plated into white 96-well-plates in triplicate. Measurements of luciferase activities in cell lysates were performed using the Envision Multimode Plate Reader (Perkin Elmer). For normalization of HCV replication/translation, 4h firefly luciferase activity was used, whereas the luciferase activity from the IFN-β promoter was normalize to a renilla luciferase activity.

2.2.8 Real-time quantitative reverse transcription PCR (q-PCR)

Total cellular RNA was extracted at the indicated time points by using isol-RNA lysis reagent (5 Prime) according to the manufacturer's instructions, treated with DNase I (Life Technologies) and then quantified. 500ng of RNA were used as a template to

synthesize cDNA using random primers (Life Technologies) and M-MLV Reverse Transcriptase (Life Technologies) according to manufacturer's protocol. Quantitative real-time PCR using Kapa Sybr Fast qPCR Kit (Kapa Biosystems) was performed to detect mRNA levels of different human genes. Signals of inducible cellular mRNAs were normalized to the α-actin mRNA signal. Amplification and detection were carried out on a CFX96 Real Time System (Bio-Rad).

2.2.9 Production of Lentiviral particles

HEK293T cells were used to produce lentiviral (LV) particles. Briefly, 2 x 10^6HEK 293T cells were plated in 10 cm dishes and the following day transfected with a calcium phosphate method. 5µg of expression plasmid (specified in table 2.3 – lentiviral vectors), 3.75µg of packaging plasmid (psPAX2) and 1.25µg of envelope plasmid (pMD2.G) was used for transfection. The day after, the media was replaced with fresh DMEM + 10% FBS. Cells were then incubated at 37 °C, 5% CO2 for 24 h. The following day, media containing the lentiviral particles were collected and centrifuged at 2250 rpm (Eppendorf Centrifuge 5804R) for 10 min at 4 °C. The supernatant was filtered with 0.45 µm sterile filters. The filtered lentiviral stocks were aliquoted and kept at -80 °C until needed for transduction experiments.

2.2.9 Lentiviral transduction

In order to produce cell lines with a knock down or overexpression of the desired protein, Huh7-Lunet or U2OS were plated in 10cm dishes and the next day incubated with 1ml of appropriate lentiviral particles. One or two days after, medium was replaced with fresh DMEM + 10 % FBS additioned with blasticidin (10µg/ml) or puromycin (0,5-3µg/ml). Cells under blasticidine selection were kept in culture for 1 week whereas cells under puromycin selection were kept in culture for 3 days with an exception of Huh7-Lunet MAVSR cell line that was kept in culture at least one month before proceeding with the electroporation.

2.2.10. Indirect immunofluorescence analysis

In order to detect the expression of host and viral proteins by immunofluorescence (IF) staining, cells were seeded into 6-wells or 12-wells plates containing microscope

coverslips and supplied with complete growth medium. At the indicated time, cells were washed three times with PBS and fixed in 3.7% paraformaldehyde (PFA) solution for 15 min at room temperature (RT). Afterwards, cells were again washed three times with PBS containing $CaCl_2$ and $MgCl_2$ (PBS+S) and incubated 5 minutes with 100 mM Glycine in PBS+S in order to saturate excesses of PFA and to stop the fixation reaction. Cells were permeabilized for 5 minutes with 0.1 % Triton X-100 in PBS and washed three times, 5 min each. Before incubation with antibodies, a blocking step was performed at 37°C for 30 min with blocking solution (1% bovine serum albumin (BSA) and 0.1% Tween 20 in PBS+). Primary antibodies were diluted to the desired concentration in blocking solution and cells were incubated for one hour at 37°C in a humidified chamber. Coverslips were rinsed three times with PBS+S 0.1 % Tween 20 (washing solution) and incubated with secondary antibodies for 1 hour at 37°C again in a humidified chamber. At the end of incubation, coverslips were washed three times with washing solution and mounted on slides using Vectashield mounting medium with DAPI (Vector Laboratories).

In order to detect endogenous IRF-3 intracellular localization, U2OS cells were fixed and permeabilized as previously described. The blocking was instead performed at 37°C for 1 h with different blocking solution (0.5% BSA and 0.04% Tween 20 in PBS+). Cells were incubated O/N at 4°C with anti-IRF-3 antibody diluted in the blocking solution described above. Next day, coverslips were washed twice with PBS+ for 20 min at RT and finally incubated with the secondary antibody for 1 h at 37°C with the same blocking solution used before. After two washes at RT for 20 minutes, coverslips were mounted on slides as already described.

2.2.11 Imaging of fixed cells

Fluorescent images of fixed cells were captured with two different confocal microscopes, the Live imaging – Nikon Ti Eclipse and the LSM 510 META (Carl Zeiss Microimaging, Inc.).

2.2.12 Immunoprecipitation (IP)

In order to study ectopic NAP1L1 with NS5A or other components of the PRR signalling pathway HEK293T cells were transfected using calcium-phosphate method

with HA-NAP1L1 plasmid and FLAG tagged plasmids (NS5A and NS5A mutants, MAVS, TBK1 and IKKε). Next day, cells were lysed with RIPA lysis buffer (50 mM TRIS HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSF, 1 mM EDTA, protease inhibitors and phosphatase inhibitors, centrifuged, and part of the supernatant was kept as input sample while the rest was incubated with equilibrated anti-FLAG M2 agarose beads (Sigma-Aldrich) for 3h at 4°C under rotation. After centrifugation the beads were spin down and washed five times with RIPA lysis buffer to eliminate unbound proteins. Finally, the beads were eluted with 2X Laemmli buffer and kept at -20°C.

2.2.13 Chromatin Immunoprecipitation analysis (ChIP)

In order to assess NAP1L1 occupancy at the IFN-β promoter Chromatin Immunoprecipitation was performed with the EZ-Magna ChIP ™ A/G Kit (Millipore - 17-10086). U2OS cells were plated in a 150mm dish and infected with VSV at a moltiplicity of infection (MOI) of 3 or left uninfected for 5h. Afterwards, the cells were treated with 1% high grade formaldehyde (Sigma Aldrich) for 10 minutes at RT to crosslink proteins and DNA. Following fixation cells were lysed using the cell lysis and nuclear lysis buffer provided by the Kit. The cross linked DNA was sonicated, 1% of the material was saved as "input" sample and the rest was incubated with protein A/G magnetic beads and the desired antibody. The immunoprecipitation was performed O/N at 4°C under constant rotation. The next day the protein/DNA complexes were eluted and reverse cross-linked to free the DNA fragments with an elution buffer and proteinase K. The purified DNA was used to perform a q-PCR analysis.

2.2.14 SDS-PAGE

Whole cell lysates were resolved by SDS–PolyAcrylamide Gel Electrophoresis (SDS-PAGE) at the appropriate acrylamide percentages. Initially, the protein lysates were boiled at 95°C for 10 min, and centrifuged for 1min at RT at 1000g, and subsequently loaded into the acrylamide gel. Gels were run in SDS electrophoresis buffer (25 mM Tris, 190 mM glycine, 0.1% SDS), initially at 90 V into the stacking gel and later at 140 V into the running gel.

2.2.15 Western blot analysis

For Western blotting, nitrocellulose membrane (GE Healthcare - 10600015) was used and membranes were blocked for 1 hour in 4% milk followed by incubation with the appropriate primary antibodies diluted in 4% milk/0,5% Tween-20 at 4°C O/N. After three washing with TBS 0.5% Tween-20 secondary antibodies conjugated with HRP (DakoCytomation – P0447/8) were diluted in 4% milk / 0,5% Tween-20 and incubated for 1 hour. Blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore – WBKLS0500) according to manufacturer's instructions.

2.2.16 Interferon-alpha (IFN-α) and Leptomicin B treatment

Huh7-Lunet cell transduced with LV-Scramble or LV-shNAP1L1 were treated with 1000 units/ml of IFN-α (ICGEB Biotechnology Development Group) for 8 hours. Cells were lysed with isol-RNA lysis buffer and total RNA was extracted for q-PCR analysis.

To analyse NAP1L1 nuclear translocation Huh7-Lunet cells electroporated with HCV sub-genomic replicons were treated with 150mM Leptomycin B (Santa Cruz Biotechnology - sc-358688) at 63 hours for transfection for 9 hours. At 72 hour post-transfection cells were fixed and nuclear translocation was assessed by immunofluorescence analysis.

2.2.17 ELISA

The quantification of IFN-β in the surnatant was measured with the VeriKineTM Human IFN Beta ELISA Kit (PBL Assay Science - 41410) according to the manufacturer's instructions.

2.2.18 Plaque Assay

Vero cells were seeded to form a monolayer. The day after cell were infected with a 10-fold serial dilution of TBEV or VSV in a total volume of 200 µl of serum-free medium. After 1 hour incubation at 37°C with 5% CO2, the inoculum was replaced with medium consisting of 1 volume of 6% carboxymethyl cellulose (CMC) and 1 volume of maintenance medium (DMEM supplemented with 4 % decomplemented FBS). The plates were incubated for 5 days (TBEV) or 3 days (VSV) before fixation with 4 % PFA dissolved in PBS. Infected cells were stained with 300 µl of 1% crystal violet solution

in 80% methanol/20% PBS. After 30 minutes the staining solution was removed and cells were washed 3-4 times with water. Viral titres were determined by multiplying the number of plaques for the dilution factor.

2.2.19 ImageJ quantification analysis

Acquired images were analysed by ImageJ software. Every single band was selected and band intensity was measured with ImageJ. Band intensity value of any band was normalized with the band intensity of other corresponding band.

2.2.20 Whole genome transcriptome analysis

Huh7-Lunet cells were seeded into 10-cm dishes. Next day, cells were transduced either with LV-Scramble or LV-shNAP1L1. Following day, medium was changed with a fresh medium containing 3μg/ml puromycin. At 3 days post-transduction, cells were collected with isol-RNA lysis reagent. Total RNA was harvested. Integrity and concentration of total RNA were measured with denaturing agarose gel electrophoresis and nanodrop, respectively. Three independent experiments were performed. Total RNA samples were sent to the company (IGA Technology Services, Udine, Italy). RNA integrity number (R.I.N.) was measured. RNA samples were run with HiSeq 2000 sequencing system (Illumina). About 30M reads (50 bp reads) were performed for each sample. Afterwards, the company performed alignment and expression analysis. Raw data was analysed by Dr. Danilo Licastro (CBM, Trieste, Italy). Briefly, bioconductor packages DESeq2 version 1.4.5 and EdgeR version 3.6.2 in the framework of R software version 3.1.0 were used to perform differential gene expression analysis of RNA-Seq data. Both packages are based on the negative binomial distribution (NB) to model the gene reads counts and shrinkage estimator to estimate the per-gene NB dispersion parameters. Specifically, rounded gene counts were used as input and the per-gene NB dispersion parameter was estimated using the function DESeq for DESeq2 while, for EdgeR the function calcNormFactors with the default parameters was used. To detect outlier data after normalization, R packages arrayQualityMetrix were used and before testing differential gene expression all genes with normalized counts below 14 were eliminated to improve testing power while maintaining type I error rates. Estimated p-values for each gene were adjusted using the Benjamini-Hochberg method. Genes with adjusted $P<0.05$ and absolute Logarithmic base 2 fold change > 1 were selected. Finally, some of

the down-regulated and up-regulated genes were validated by qRT-PCR analysis in order to prove the accuracy of the whole-genome transcriptome analysis.

2.2.21 Ingenuity Pathway Analysis

Significantly changed genes (up-regulated, down-regulated, or both) were analysed separately by using online bioinformatics tool Ingenuity Pathway Analysis (Qiagen). Settings for the analysis as following: Direct relationships were included with experimentally observed or highly predicted confidence from human species. Canonical pathways, diseases and disorders, and molecular and cellular functions were analysed.

3. RESULTS

3.1 HCV NS5A interacts with NAP1L1 in the cytoplasm

3.1.1 Characterization of the HCV NS5A-NAP1L1 interaction

NS5A is a phosphorylated protein composed of three domains linked together by two low complexity sequences (LCSI and LCSII). While domain I is organized and structured, domain II and III, with their intrinsically disordered nature, have a potential to interact with viral and many host proteins to regulate not only viral replication and assembly but also various cellular pathways in order to create an adequate environment for virus propagation. Various independent reports have shown, by both proteomic and genomic approaches, that the HCV NS5A protein binds the Nucleosome Assembly protein 1 like 1 (NAP1L1) (Ramage et al. 2015; De Chassey et al. 2008; Pichlmair et al. 2012). This interaction was confirmed in our laboratory and mapped to the C terminal region of NS5A, as re-addressed in Figure 13B, column 1. The casein kinase II (CKII) recognition site, represented by the motif –SEED-, present in the C terminal domain, was shown to be necessary for NAP1L1 binding (Cevik et al., unpublished data). Therefore we hypothesised that phosphorylation of serines S2428, S2430 and S2433, present in the surrounding region, could be required for NAP1L1 binding. In order to study the phosphorylation-mediated interaction between these two proteins I took advantage of a phospho-ablatant and a phospho-mimetic form of NS5A in which these three serines were mutated to alanine or glutamic acid (Figure 13A). Ectopically expressed wild type NS5A, serine to alanine mutant NS5A (from here on indicated as m2) or the mutant serine to glutamic acid NS5A (from here on indicated as m2E) together with NAP1L1, were immunoprecipitated with anti-Flag agarose beads. Immunoblot analysis shown in Figure 13B column 1 and 2 clearly indicates that the triple phospho-ablatant mutant has lost the ability to interact with NAP1L1. This result suggests that the phosphorylation of these three serines might be necessary for this interaction. I have confirmed this result by using an HCV replicon system schematized in Figure 13C. I could show that these two proteins also co-localize in the cytoplasm at sites which could represent replication complexes. The co-localization was lost with a replicon carrying a mutant version of the NS5A protein unable to bind NAP1L1 (Figure 13D).

Surprisingly, as shown in Figure 13B column 3, the triple phospho-mimetic form of NS5A, m2E, could not rescue the interaction. In order to analyse more in detail the role of phosphorylation I took advantage of a mutant with only two substitutions of serine to alanine or serine to glutamic acid at position S2428 and S2430 (Figure 13A). I could show that the double phospho-ablantant form did not interact with NAP1L1 while the double phospho-mimetic mutant re-established the interaction as shown in Figure 13B column 5 and 6. Overall, the result suggests that the phosphorylation or the negative charge of amino acids in position 2428 and 2430 are necessary for the interaction with NAP1L1. However, the interaction is lost by the presence of the negative charge in position 2433 indicating that the phosphorylation of serine 2433 might be the priming event for the phosphorylation of other serines involved in the interaction as discussed more in detail in the discussion section.

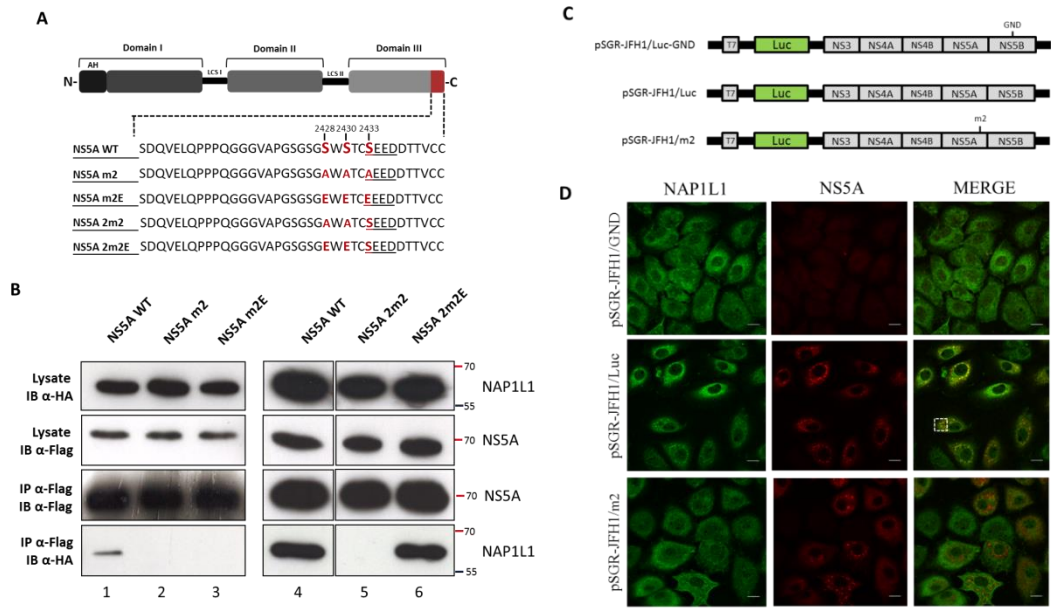

Figure 13. The extreme C terminal region of NS5A mediates the interaction with NAP1L1. A) Schematic representation of HCV NS5A and mutants used in this work. Full-lenght wild type NS5A is shown with the N-terminal amphypatic helix (AH) followed by domain I, II and III separated by low complexitiy sequences I and II (LCSI and LCSII). The C-terminal amino acid sequence of the wild type NS5A and mutants is also shown. The –SEED- motiv is highlited. B) HEK293T cells were transfected with Flag-NS5A, Flag-NS5A/m2, Flag-NS5A/m2E, Flag-NS5A/2m2 or Flag-NS5A/2m2E togerther with HA-NAP1L1. Lysates were immunoprecipitated using anti-FLAG agarose beads and immunobloted against NAP1L1 and NS5A using α-HA and α-Flag antibodies. C) Schematic representation of the HCV replicon used in this study. pSGR-JFH1/Luc-GND contains a replication deficient mutation in the RNA-dependent RNA polymerase; pSGR-JFH1/Luc is the wild type sub-genomic replicon; pSGR-JFH1/m2 contains a triple serine to alanine mutation in the C-terminal region of NS5A. D) Huh7-Lunet cells were electroporated with pSGR-JFH1/Luc-GND, pSGR-JFH1/Luc and pSGR-JFH1/m2. Cells were fixed at 72h post electroporation and stained with anti-NAP1L1 (green) and anti-NS5A (red) antibodies. Merge represents the overlap

of the two channels. Colocalization analysis was performed with ImageJ software and the Person's correlation coefficient for the ROI in white is 0,7. Scale bar is set to 30μm.

3.1.2 HCV NS5A restricts NAP1L1 nuclear translocation

We have observed that cellular NAP1L1 is sequestered by NS5A in specific cytoplasmic aggregates, most likely corresponding to HCV replication/assembly complexes. At steady state NAP1L1 has been shown to retain a cytoplasmic localization through the whole cell cycle, though only a small amount was observed in the nucleus (Marheineke & Krude 1998). However, the activity of NAP1L1 as histone chaperone is essentially nuclear and tightly controlled to avoid unscheduled chromatin remodelling. Nuclear shuttling of proteins can be studied by using an inhibitor of nuclear export such as Leptomycin B (LMB), which acts by blocking CRM1 mediated export of NES containing proteins. In fact, cells treated with LMB retain NAP1L1 in the nucleus (De Marco et al. 2010). In order to investigate if the interaction with NAP1L1 of NS5A inhibits its nuclear translocation, I transfected Huh7-Lunet cells with the HCV replicon pSGR-JFH1/Luc or with pSGR-JFH1/m2 carrying a mutant form of NS5A. Cells were then treated with LMB for 9h. Afterwards, cells were fixed and approximately 600 NS5A positive cells were analysed for nuclear NAP1L1.

In Figure 14A a representative immunofluorescence image of NS5A positive cells and NAP1L1 localization upon LMB treatment is shown. The graph in Figure 14B indicates that 60% of cells replicating the wild type HCV sub-genomic replicon show a nuclear NAP1L1 localization and the percentage is increased to almost 80% in cells replicating the HCV sub-genomic replicon with a mutant version of the NS5A protein, which is unable to bind NAP1L1. These results are suggesting that the interaction with NAP1L1 of NS5A inhibits its nuclear translocation, which could ultimately prevent NAP1L1 activity in the nucleus.

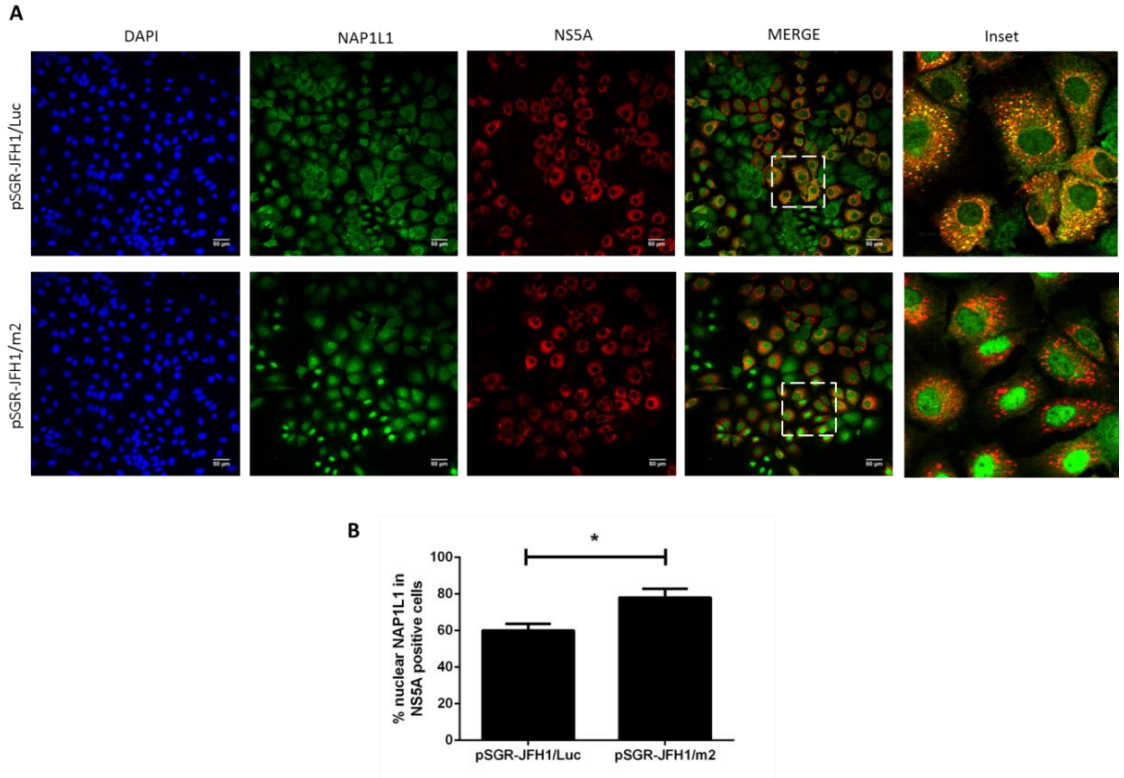

Figure 14. HCV NS5A inhibits NAP1L1 nuclear localization. Huh7-Lunet cells were electroporated with HCV pSGR-JFH1/Luc and pSGR-JFH1/m2. At 63h post electroporation cells were treated with 150nM LMB for 9h. A) At 72hpe cells were fixed and stained with α-NAP1L1 (green), α-NS5A (red) and incubated with DAPI (blue). Scale bar: 50µm. The green and the red channels were merged and the inset is shown. B) Approximately 600 NS5A positive cells were counted and assessed for NAP1L1 nuclear staining. The percentage of nuclear NAP1L1 is represented for each condition. The graph indicates as an average of three independent experiments with standard deviation. Statistical significance was analysed using Student's t-test; P-value: *P<0.05.

3.2 High-throughput transcriptome analysis for the identification of NAP1L1 regulated genes.

High throughput screenings represent an unbiased and powerful tool to gain insights into the biological complexity of the virus-host interactome. Over the past few years, several proteomic and genomic screening methods, including yeast two hybrid system (De Chassey et al. 2008) and AP-MS approaches (Pichlmair et al. 2012) have been performed to identify host proteins with a key role in HCV infection. As already mention above, a detailed interactome analysis of one of the most promiscuous HCV protein, NS5A, revealed a recurrent interaction with a host chaperone protein NAP1L1. I have confirmed the interaction and showed that it restricts NAP1L1 nuclear localization.

NAP1L1 is a histone chaperone protein with different functions related to gene transcription and replication. Its nucleosome assembly and disassembly activity, the incorporation of histone variants and the transferring of histones from the cytoplasm to the nucleus results in the transcriptional regulation of host genes, the regulation of the cell cycle, cell proliferation and differentiation.

3.2.1 RNA-Seq analysis of NAP1L1 depleted cells

Taking into consideration the results described above and the role of NAP1L1 as a transcriptional regulator, we decided to perform a transcriptome analysis of NAP1L1 depleted hepatocytes to identify genes regulated by NAP1L1, which will eventually shed light on the functional relevance of the NS5A-NAP1L1 interaction.

Huh7-Lunet cells were transduced with either Scramble or shNAP1L1 lentiviral vectors. After 3 days of puromycin selection, cells were lysed and total RNA extraction was performed. The RNA quality was checked by spectrophotometric analysis and denaturing agarose gel electrophoresis. RNA was shown to be free of contaminants with a 260/280 ratio higher than 1.8 (260/280>1.8) and RNA integrity was confirmed by the observation of two intese bands corresponding to 28S and 18S ribosomal RNA (Figure 15A). The efficiency of NAP1L1 KD was confirmed on both the RNA and protein level as shown in Figure 15B and 3C. Samples, with three biological replicates for each condition, were sent for RNA sequencing (RNA-Seq). Analysis of the raw data performed in collaboration with Dr. Danilo Licastro (CBM, Trieste, Italy) using two different software analysis (DESeq2 and EdgeR) revealed around 600 differentially expressed genes in the absence of NAP1L1. Specifically, we found 392 down-regulated (fold change ≤ 2) and 174 up-regulated (fold change ≥ 2) genes with a False Discovery Rate (FDR) of less than 0,05 (Figure 15D). Interestingly, only 1% of the total reads of the analysis (46623 reads) is represented by differentially modulated genes, which is indicative of a transcriptional specificity of NAP1L1 for certain set of genes.

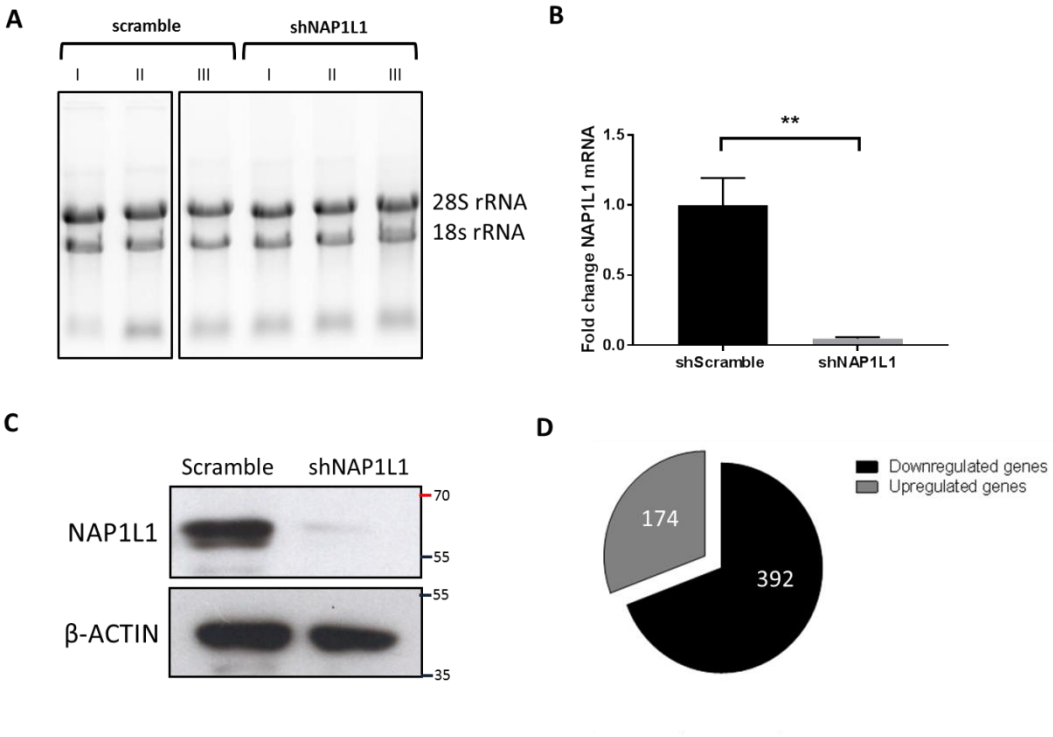

Figure 15. RNA-Seq analysis of NAP1L1 depleted hepatocytes. Huh7-Lunet cells were transduced with LV-Scramble or LV-shNAP1L1. Next day cell were put under puromycin selection (3μg/ml) for three days. A) Cells were then lysed to extract RNA. Gel electrophoresis was performed for Scramble and shNAP1L1 samples in triplicate (I-III) loading 2μg of each sample. B) mRNA levels of NAP1L1 were measured by qRT-PCR and normalized to β-actin levels and represented as fold change over Scramble. Average and Standard deviation of three biological replicates are shown. Statistical significance was analysed using Student's t-test; P-value: **P<0.01 C) Cells were lysed and western blot analysis was performed for Scramble and shNAP1L1 samples using α-NAP1L1 and α-β-actin antibodies. The experiment was repeated three times and a representative picture is shown. D) Raw data were analysed and a number of down-regulated (grey slice of the cake) and up-regulated (black slice of the cake) by NAP1L1 is shown.

3.2.2 Functional interpretation of the data by the Ingenuity Pathway Analysis (IPA) software

IPA is a commercial software for the analysis and interpretation of "omics" data. I took advantage of the IPA program for an in depth analysis of the experimental results. In order to understand which are the molecular pathways and biological functions regulated by NAP1L1, a list of differentially expressed genes were divided in categories of down-regulated, up-regulated or both up-and down-regulated genes, uploaded in the IPA program and the alteration of the top canonical pathways was evaluated.

IPA analysis of all the differentially expressed genes indicated that the most significantly affected pathway is represented by the Aryl-Hydrocarbon Receptor

Signalling pathway. Other pathways that follow apply to Pancreatic Adenocarcinoma Signalling, Molecular Mechanism of Cancer and Cyclins and Cell Cycle Regulation (Figure 16A).

A detailed analysis of down-regulated genes indicated that canonical pathway such as Molecular Mechanism of Cancer, Pancreatic Adenocarcinoma Signalling and Signalling by Rho family GTPases were mostly affected as presented in Figure 16B. Canonical pathways such as Aryl-Hydrocarbon Receptor Signalling pathway and Pancreatic Adenocarcinoma Signalling pathway are mostly affected by NAP1L1 up-regulated genes as shown in Figure 16C.

In addition to the affected pathways, we were able to address disease and disorders associated with the knock-down of NAP1L1 and find that cancer and hepatic system disease are linked to up-regulated genes, while down-regulated genes are associated with inflammatory responses.

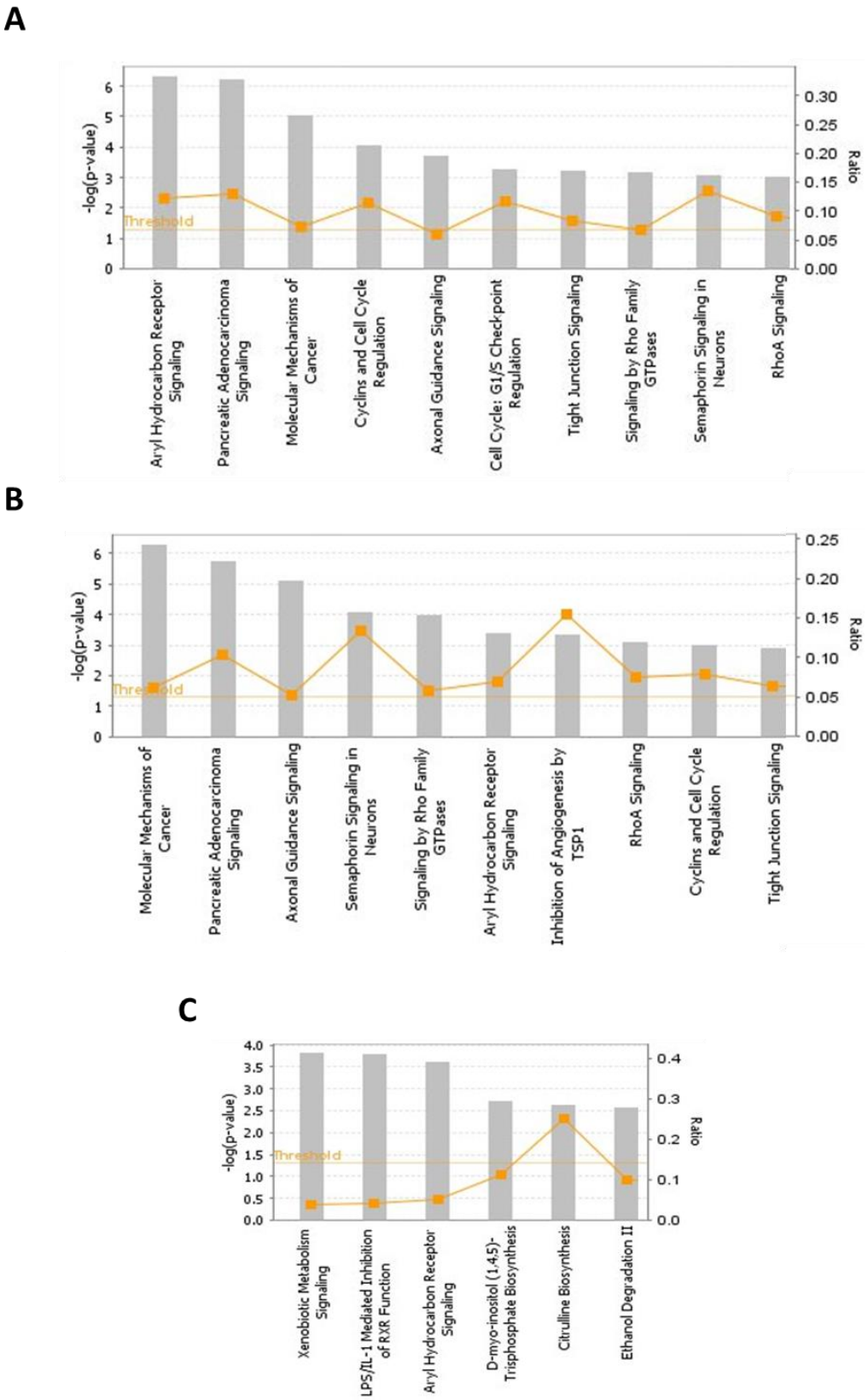

Figure 16. Ingenuity Pathway Analysis of differentially modulated genes in the absence of NAP1L1. The most highly affected canonical pathways are shown, divided as follows: A) both up- and down-regulated genes, B) down-regulated genes and C) up-regulated genes, found in cells depleted of NAP1L1 compared to control Scramble cells. The x-axis is referred to the affected canonical pathway; the left y-axis indicates the statistical significance and the

"threshold" is indicated as a yellow dotted line. The ratio represented on the right y-axis and by an orange square for a single pathway, indicates the number of molecules present in the RNA-Seq dataset divided by the number of molecules that belong to the pathway and are present in the IPA database.

3.2.3 Validation of RNA-Seq analysis

A fundamental task of the RNA-Seq analysis is an identification of differentially expressed transcripts among a group of samples. Although stringent criteria are used to provide a statistically significant result, a probability of encountering a false discovery remains very high. Therefore, I decided to validate the accuracy of the transcriptome analysis. For this purpose, I designed primers that span the exon-exon junction of selected genes in order to avoid the transcription from genomic DNA, and re-measure the expression of 22 selected genes (2 up-regulated and 19 down-regulated genes and NAP1L1 as positive control) by qRT-PCR as shown in Figure 17A. Genes were selected in order to cover the most affected pathways of the IPA analysis and the number of validated genes is chosen in proportion of the up- and down-regulated genes of the RNA-Seq analysis. The expression levels of all genes tested by qRT-PCR were comparable to that obtained by RNA-Seq analysis indicating the reliability of sequencing data.

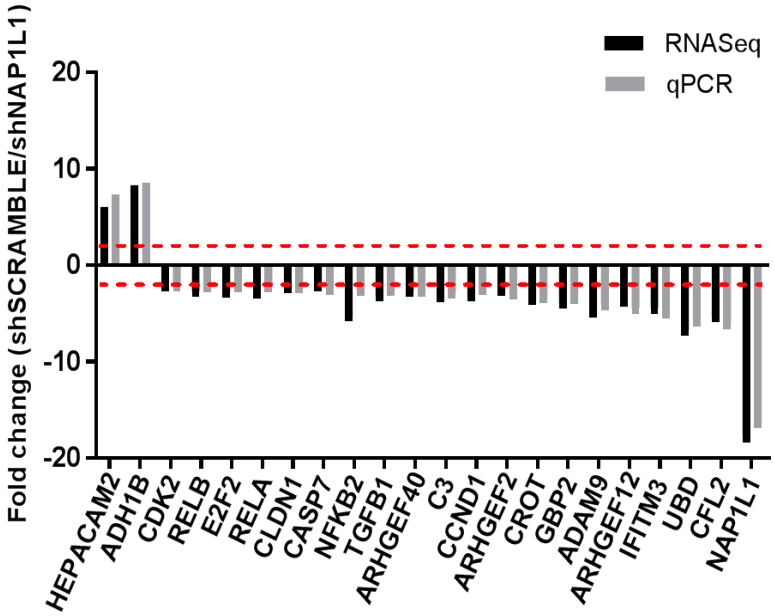

Figure 17. Validation of the differentially modulated transcripts. A total of 2 up-regulated and 19 down-regulated genes together with NAP1L1 gene as positive control were analysed by qRT-PCR. Relative mRNA down-regulation

or up-regulation was calculated by taking the ratio of Scramble to shNAP1L1 for each gene. RNA-Seq data (black) was compared with qRT-PCR data (grey). The red dotted line indicates the 2 fold induction threshold for both the up and down-regulation. The result represents the average of three independent experiments.

3.3 NAP1L1 regulates the antiviral state in the cell

The innate immune response to viral infection is a biphasic process that culminates in the induction of an antiviral state in infected as well as in bystander cells. The initial phase is characterized by the transcriptional activation of type I interferons (IFN-α and IFN-β) and pro-inflammatory cytokines, followed by type I IFN signalling, which leads to the expression of interferon stimulated genes (ISGs).

3.3.1 ISGs induction is not regulated by NAP1L1

A more detailed investigation of genes belonging to the most affected pathways and networks in the IPA analysis and a careful inspection of genes used to validate the RNA-Seq data combined with literature search led us to the identification of a number of interferon stimulated genes (ISGs). Genes, such as IFITM3, UBD, GBP2 and many others, were found to be down-regulated in the absence of NAP1L1. Therefore we speculated that NAP1L1 might be involved in IFN mediated ISGs expression. To test this hypothesis I treated control and NAP1L1 depleted Huh7-Lunet cells with 1000 U/ml of IFN-α for 8h. Following IFN-α induction cells were lysed and a total RNA extraction was performed to measure the mRNA levels of UBD, GBP2, IFITM3 and GAPDH (negative control) by quantitative RT-PCR. Although the basal expression of these genes seems to be regulated by NAP1L1, when the stimulus was introduced they showed an equal expression in Scramble and shNAP1L1 treated cells (Figure 18A).

Moreover, I also tested a set of ISGs that were not present in the RNA-Seq results due to their low basal expression levels in an un-stimulated environment such as IFIT1, IFIT3 and OASL. As shown in Figure 18B, neither the expression analysis of highly induces ISGs upon IFN-α treatment could demonstrate a significant difference between control and shNAP1L1 treated cells. These results suggest that NAP1L1 does not participate in INF-α mediated ISGs induction, but rather controls basal transcription of certain ISGs.

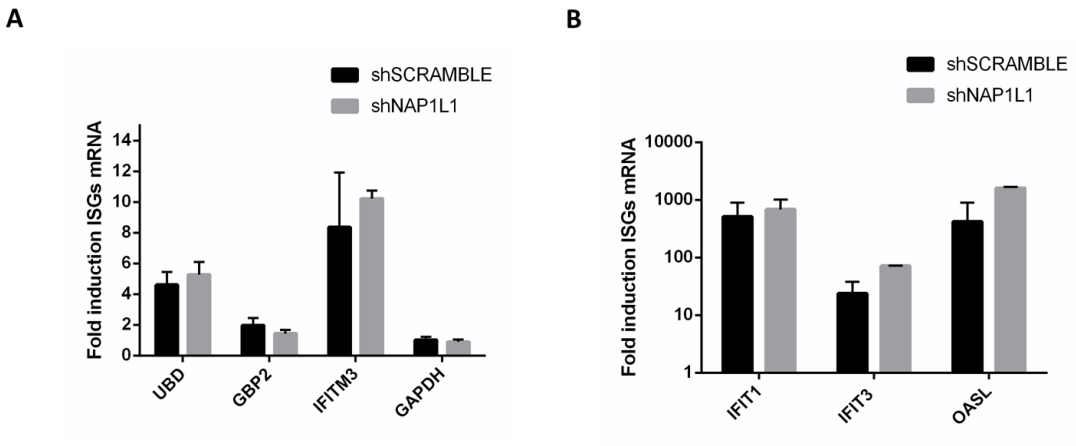

Figure 18. NAP1L1 does not participate in the induction of ISG genes. Scramble or NAP1L1 depleted Huh7-Lunet cells were treated with 1000U/ml of IFN-α or mock treated for 8 h. Cells were lysed and total RNA was extracted. mRNA levels of A) UBD, GBP2, IFITM3, GAPDH and B) IFIT1, IFIT3, OASL were measured by qRT-PCR, normalized to β-actin levels and representated as fold induction relative to basal non induced expression. The result represents the average and standard deviation of three independent experiments

3.3.2 NAP1L1 controls RIG-I/MDA5 induced IFN-β expression

In type I IFN system, the two cascades i.e. IFN induction and IFN signalling, are strictly associated. Along with the identification of NAP1L1 down-regulated ISGs I identify genes involved in type I interferon transcriptional regulation such as RELA (p65 subunit of NF-κB), c-Jun or the recently identified GEF-H1 (Chiang et al. 2014) showing decreased expression in the absence of NAP1L1. Moreover, driven by the observation that NAP1L1 is not engaged in IFN-α mediated ISGs expression, I aimed to further investigate a possible role of NAP1L1 in the innate immune response focusing the attention on the IFN-β expression pathway. In order to address this question I needed to switch to another cell line as Huh7-Lunet are defective for IFN expression (Keskinen et al. 1999)(Keskinen et al. 1999)(Keskinen et al. 1999)(Keskinen et al. 1999)(Keskinen et al. 1999)(Keskinen et al. 1999)(Keskinen et al. 1999)(Keskinen et al. 1999)(Keskinen et al., 1999). Indeed, I benefited of the U2OS cell line which has been used to study the interferon response following TBEV infection and hence possess an active innate immune system as shown by Miorin et al. (Miorin et al. 2012). The induction of interferon was achieved artificially through the use of a long double-stranded RNA analogue known as Polyinosinic-polycytidylic acid or poly(I:C). Poly(I:C) was initially discovered as an extracellular ligand for TLR3 (Alexopoulou et al. 2001), but it is also able to activate the RIG-I/MDA5 pathway when transfected into

cells (Kato et al. 2008). I proceeded by transducing U2OS cells with lentiviruses targeting a control (Scramble) or NAP1L1 (shNAP1L1) RNA, and transfecting either 1μg of poly(I:C) complexed with lipofectamine or just lipofectamine alone as negative control. Experimental results indicate that IFN-β expression is strongly affected at both the mRNA (Figure 19A) and protein level (Figure 19B) in the absence of NAP1L1. Altogether the data suggests that NAP1L1 is an essential player in the poly(I:C) induced RIG-I/MDA5 mediated interferon response.

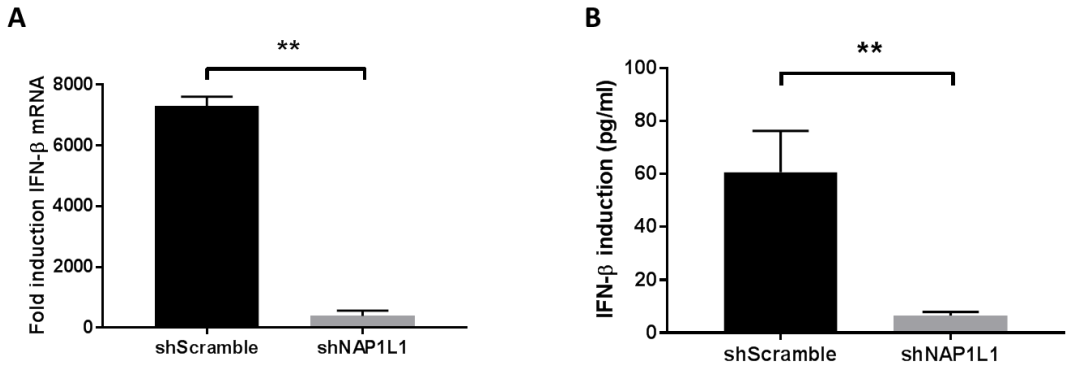

Figure 19. Poly(I:C) mediated RIG-I/MDA5 induced IFN-β expression is affected by NAP1L1 depletion. U2OS cells were transduce with LV-Scramble or LV-shNAP1L1 and transfected with 1 μg of poly(I:C) or mock transfected (lipofectamine alone) for 8h. A) Cells were lysed and total RNA was extracted. IFN-β mRNA levels were measured by qRT-PCR, normalized to β-actin levels and the result is represented as fold change relative to mock. An average of two independent experiments with respective standard deviations is shown. B) Cells supernatant was collected and the secreted IFN-β protein levels were measured by a commercial ELISA Kit. The result shows an average of 3 independent replicates and standard deviations. Statistical significance was analysed using Student's t-test; P-value: **P<0.01.

3.3.3 NAP1L1 controls TLR3 mediated IFN-β expression

Besides cytoplasmic recognition by RIG-I/MDA5, a virus derived dsRNA or its synthetic analogue can also be sensed by TLR3 receptors present in endosomal compartments. Binding of dsRNA by either RIG-I/MDA5 or TLR3 induces a signalling cascade that leads to a transcriptional activation of type-I interferon and pro-inflammatory cytokines.

Since I have already demonstrated a role of NAP1L1 in the regulation of RIG-I/MDA5 mediated IFN-β transcription, I wanted to further investigate a possible involvement of NAP1L1 in the TLR3 pathway. Since TLR3 recognizes dsRNA present only inside endosomes, stimulation with exogenous poly(I:C) is required. Although some reports

have shown that U2OS cell lines possesses a relatively abundant amount of TLR3 transcripts (Paladino et al. 2006), it seems not to be sufficient for a complete activation of the TLR3 response when treated with poly(I:C) (Laredj & Beard 2011). Therefore, using LVs, I overexpressed TLR3 receptor in U2OS cells already depleted of NAP1L1 or Scramble as control. Subsequently I challenged the cells with 50μg/ml of poly(I:C) by direct addition in the medium for 24h. TLR3 was highly overexpressed both in Scramble and shNAP1L1 cells with respect to control EGFP samples as confirmed by qRT-PCR analysis and shown in Figure 20A. In order to ascertain that the overexpression of TLR3 does not induce an interferon response *per se,* which could compromise the readout of the result, I measured by qRT-PCR the expression of IFN-β and the IRF-3 dependent gene, IFIT1. A shown in Figure 20B, I could confirm that TLR3 overexpression does not activate the type I interferon signalling pathway.

Next, I focused the analysis on the involvement of NAP1L1 in the TLR3 pathway. Even though poly(I:C) stimulation of Scramble TLR3 expressing cells did not induce detectable levels of IFN-β mRNA (Figure 20C line 3), the TLR3 pathway was shown to be active for the expression of IFIT1 mRNA which showed a 40 fold induction compared to control EGFP expressing cells (Figure 20D line 3). IFIT1 is a member of the ISG56 family of genes and is most potently induced by IFN-α/β. Nevertheless, it can also be induced by direct activation of IRF-3 in response to dsRNA or virus infection independently of IFN activity. For this reason, IFIT1 expression is usually used as readout of IRF-3 activity (Zhu et al. 2010) and PRR activation pathways. In fact, when IFN-β mRNA levels were measured I could not appreciate a NAP1L1 mediated effect on the TLR3 pathway as IFN-β was not induced to a statistically significant level (Figure 20C). Conversely, when I examined IFIT1 mRNA levels I was able to observe that the shNAP1L1 TLR3 expressing cells displayed a marked reduction of IFIT1 expression when compared to Scramble TLR3 expressing cells (Figure 20D). These data indicate a role for NAP1L1 in the TLR3 mediated immune responses.

In conclusion, I showed that NAP1L1 is a novel player in both the RIG-I/MDA5 and TLR3 mediated innate immune responses.

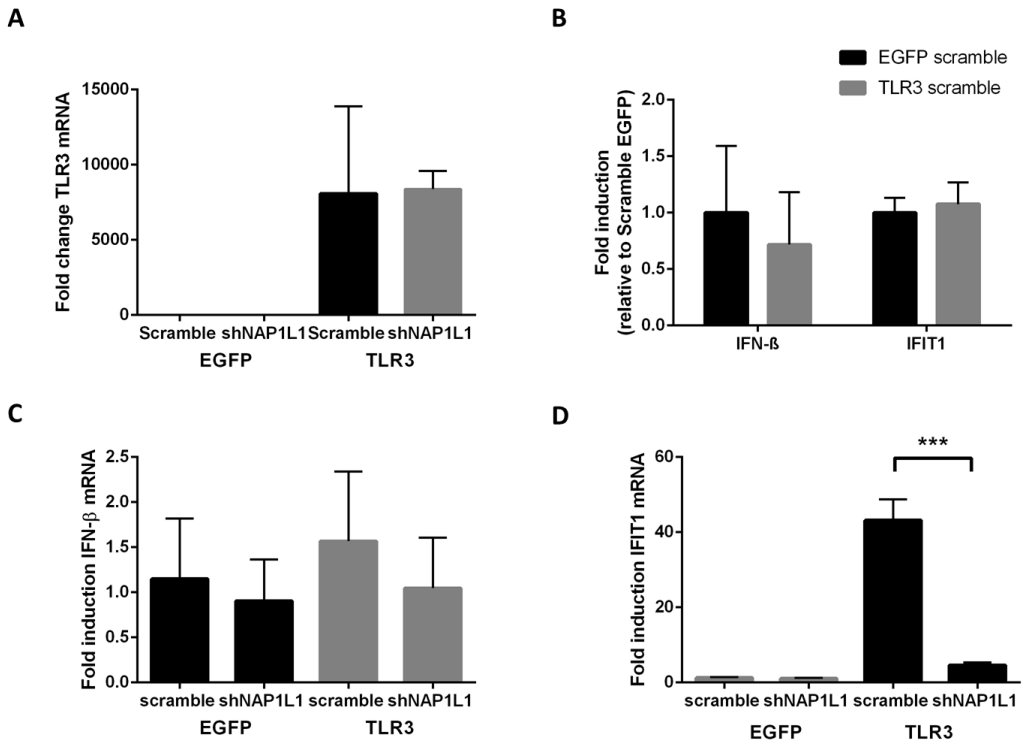

Figure 20. Poly(I:C) induced TLR3 mediated IFIT1 expression is affected by NAP1L1 depletion. Scramble or NAP1L1 depleted U2OS cells were transduced with LV for the expressing of TLR3 or EGFP as control and incubated with 50μg/ml of poly(I:C) by direct addition to the medium for 24h or left untreated. Cells were lysed and total RNA was extracted. qRT-PCR analysis was performed to check A) TLR3 mRNA levels B) IFN-β and IFIT1 mRNA levels in untreated conditions, C) IFN-β mRNA levels and D) IFIT1 mRNA levels upon poly(I:C) treatment. In all the experiments mRNA levels were normalized to β-actin levels and results are represented as fold change relative to mock EGFP condition. An average of three independent experiments with respective standard deviations is shown. Statistical significance was analysed using Student's t-test; P-value: **P<0.01.

3.3.4 NAP1L1 controls IFN-β induction during virus infection

RNA virus replication gives rise to dsRNA intermediates, a by-product which is recognized by RIG-I/MDA5, TLR3 or other PRR such as PKR to induce a potent interferon response. In order to understand if NAP1L1 has a more general role in the regulation of the innate immunity, I investigated the NAP1L1 mediated interferon response following a viral infection. To this end, I used as models two RNA viruses, VSV and TBEV, which induce IFN-β expression through the RIG-I/MDA5 pathway. In brief, U2OS Scramble and NAP1L1 depleted cells were infected with TBEV or VSV at a MOI of 1 for 24h and 8h, respectively. The induction of interferon was analysed by qRT-PCR. As shown in Figure 21A and Figure 21B, both viruses induced a potent interferon response in U2OS control cells, whereas in NAP1L1 deficient cells the

interferon response was profoundly affected. These results confirmed a role for NAP1L1 in dsRNA mediated interferon response. Although the viral RNA levels seem to decrease upon KD of NAP1L1 (Figure 21C and D) the overall viral yields are not affected (Figure 21 E and F).

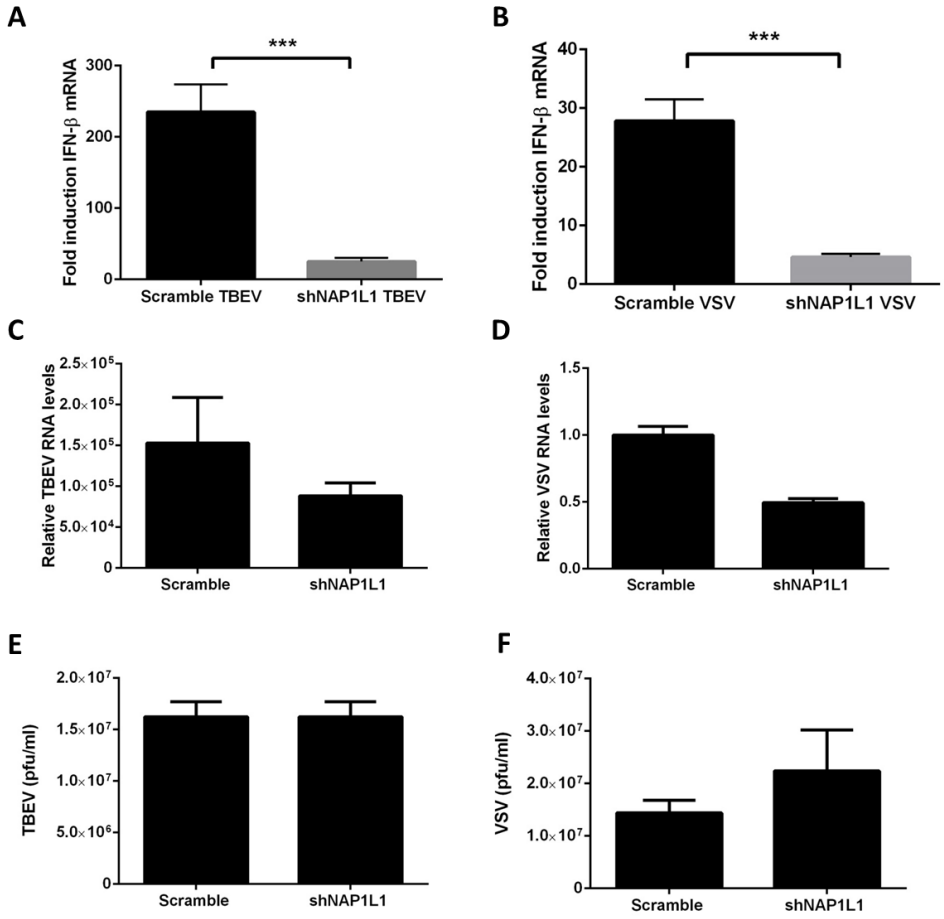

Figure 21. Virus induced IFN-β expression is affected by NAP1L1 depletion. U2OS cells were transduced with LV for shNAP1L1 or Scramble as control and infected with A) TBEV and B) VSV at a MOI of 1 for 24h and 8h respectively. Cells were lysed and RNA was extracted. IFN-β mRNA levels were measured by qRT-PCR and results are representes as fold induction over mock infected cells. The experiment was performed in triplicate and the average with the corresponding standard deviation is shown. Statistical significance was analysed using Student t-test; P-value: ***P<0.001. C) TBEV and D) VSV RNA levels were measured by qRT-PCR and results are representes as fold induction over mock infected cells. Supernatants from E) TBEV and F) VSV infected cells were used to determine viral yields (pfu/ml).

3.4 NAP1L1 mechanism of action

IFN-β expression is a multistep process that starts with the recognition of dsRNA or other virus-related signatures. The identification of the agonist induces the activation of RIG-I/MDA5 and TLR3 that subsequently recruit adaptor proteins such as MAVS or TRIF, respectively. The pathway then signals to NF-κB and IRF-3 transcription factors that translocate to the nucleus and mediate the transcription of type I interferon genes.

NAP1L1 can potentially modulate any segment of this pathway and contribute to the expression of interferon, early ISGs such as IFIT1 and pro-inflammatory cytokines. I hypothesized that either NAP1L1 could be involved in: (1) the transcriptional regulation of the IFN-β and other related genes; (2) the modulation of the cytoplasmic signalling cascade by direct interaction with one or more components of the pathway; (3) the transcriptional activation of genes that are required for the correct activation of the pathway (Figure 22).

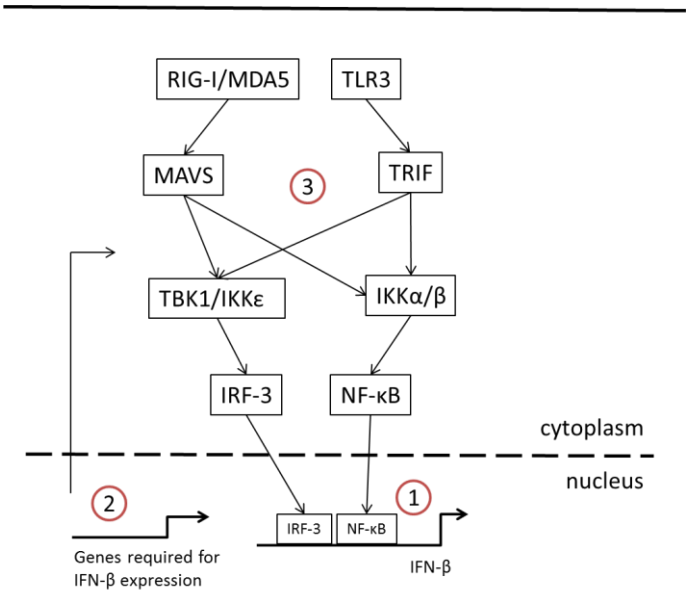

Figure 22. Schematic representation of the RIG-I/MDA5 and TLR3 pathways and hypothetical steps of NAP1L1 intervention. In order to modulate IFN-β expression NAP1L1 engages with chromatin remodelling complexes and transcription factors to initiate the transcription of IFN-β and related genes (1); modulate the transcription of other currently unknown genes whose function is associated with activation of the IFN-β promoter (2); or interacts with adaptor proteins, kinases, or signalling molecules to activate the signalling cascade leading to an efficient IFN-β expression (3).

3.4.1 NAP1L1 is present on the promoter of different genes.

Experimental evidence suggests that NAP1L1 functions as a histone assembly and disassembly protein in both DNA replication and transcription regulated processes, whereas at steady state its localization is mainly confined to the cytoplasm. As long as transcriptional regulation is concerned, a number of reports are in favour of NAP1L1 acting at the promoter level (Walfridsson et al. 2007).

Until now I have observed that IFN-β expression requires the presence of NAP1L1. Considering the role of NAP1L1 in transcriptional regulation I speculated that it could directly affect IFN-β promoter activity.

Based on literature evidence and our experimental results, the leading hypothesis is that NAP1L1 occupancy of the IFN-β promoter should increase when the gene is expressed. To test if NAP1L1 induces IFN-β expression by modulating the nucleosome density at the promoter level I performed a ChIP analysis. VSV infection was used for a potent and natural inducer of IFN-β expression. In order to ensure that the time and multiplicity of infection induced IFN-β expression, a qRT-PCR for IFN-β expression was performed. As indicated in Figure 23A, at 5h post infection with a MOI of 1 IFN-β expression was induced by 40 fold with respect to mock infected cells, while a higher MOI (MOI=4) only slightly increased IFN-β expression. Consequently, U2OS cells were infected with VSV at a MOI of 3 or mock infected for 5h, DNA fragments were immune-precipitated using an antibody against NAP1L1, RNAPol II as a positive control, IgG as a negative control, and analysed by qRT-PCR with primers specific for promoter regions of IFN-β, housekeeping genes GAPDH and β-actin, and other two genes involved in the first and second phase of the IFN response, p65 and UBD respectively.

Interpretation of results in mock infected condition (Figure 23B) illustrates a high reliability of the ChIP data. As negative control, an unspecific IgG antibody that shows no enrichment on the promoters of tested genes, was used. Promoters of housekeeping genes such as β-actin and GAPDH show a high abundance of RNAPolII, detected with an antibody specific for a CTD region of RNAPolII, whereas the IFN-β promoter and promoters of UBD and RELA genes were occupied to a much lesser extent, indicative of a repressed transcriptional state. In comparison, NAP1L1 showed an equivalent

distribution on promoters of the tested genes as shown in Figure 23C. Surprisingly, VSV infection did not increase RNAPolII or NAP1L1 occupancy on the IFN-β promoter (Figure 23C). Instead NAP1L1 remained equally distributed on promoter of tested genes and the percentage of immunoprecipitated chromatin was reduced by approximately 2 fold following VSV infection for all genes taken in consideration in the ChIP analysis.

In conclusion, although the ChIP analysis favours the idea that NAP1L1 does not modulate IFN-β promoter activity, it does not clearly define the role of NAP1L1 at the chromatin level. Therefore this result will need further reinforcement by other experimental approaches (as described in section 3.4.2).

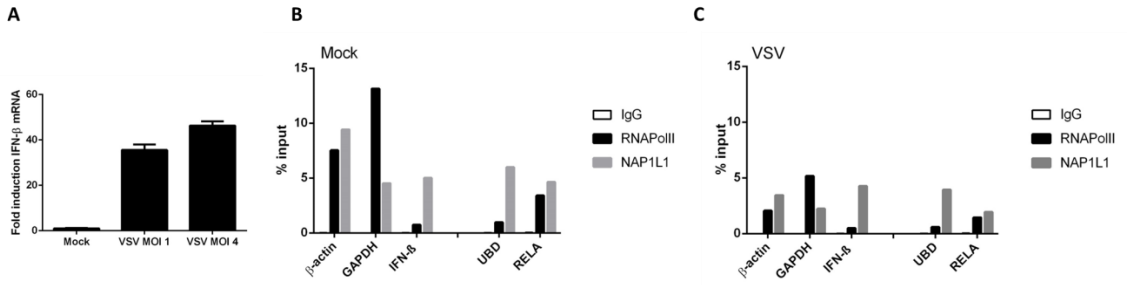

Figure 23. NAP1L1 is enriched on the promoter region of several genes. A) U2OS cells were infected with VSV at a MOI of 1, MOI of 4 or mock infected for 5 hours. Cells were lysed, RNA was extracted and mRNA levels of IFN-β were measured by qRT-PCR and normalized to β-actin mRNA levels. The result is represented as fold change over mock infected conditions. The experiment was repeated three times and an average with standard deviation is shown. B and C) U2OS cells were B) mock infected or C) VSV infected with a MOI of 3 for 5 hours. Cells were fixed, lysed and the DNA cross-linked with proteins was fragmented by sonication. IP was performed using α-NAP1L1, α-IgG and α-RNAPolII antibodies. The IP material was reverse cross-linked and used to perform qRT-PCR analysis for the promoter region of β-actin, GAPDH, IFN-β, UBD and RELA genes. The result is presented as percentage of the input sample.

3.4.2 NAP1L1 does not interfere with IRF-3 post-phosphorylation events.

Viral agonist or poly(I:C) is recognized by PRR which induces the phosphorylation of a cytoplasmic transcription factor IRF-3, followed by its dimerization and nuclear translocation. Once in the nucleus, phosphorylated IRF-3 is deposited on the IFN-β promoter and interacts with the transcriptional co-activator p300/CBP to mediate the transcription of IFN-β gene in association with other transcription factors (Yoneyama et al. 1998). Due to an ambiguous ChIP result, I wanted to address the question of whether

NAP1L1 contributes to the modulation of the IFN-β promoter by using a different approach.

To this end I performed an IFN-β promoter reporter assay with IRF-3 5D, a constitutively active phosphomimetic form of IRF-3. Hiscott and colleagues identified a Serine/Threonine cluster in the C terminal region of IRF-3, which, when mutated to a negatively charged aspartic acid resulted in a constitutively active form of IRF-3 (Lin et al. 1998). This mutant shows constitutive dimerization, nuclear translocation, association with p300/CBP and activation of IFN-β and ISRE containing promotes even in the absence of virus infection.

Huh7-Lunet cells were first depleted for NAP1L1 or Scramble as control and then transfected with an IFN-β Luciferase reporter together with IRF-3 5D. The efficiency of knock-down of NAP1L1 and transfection of IRF-3 5D was analysed by WB as show in Figure 24A. In Figure 24B the IRF-3 5D activity on the IFN-β promoter is represented as a direct measure of luciferase activity. IFN-β promoter was activated with the same intensity by IRF-3 5D in presence or absence of NAP1L1. Thus, I could conclude that NAP1L1 was not able to influence IFN-β transcription once IRF-3 was phosphorylated, meaning that IRF-3 nuclear translocation and recruitment to IFN-β promoter to activate the transcription was completely independent of NAP1L1.

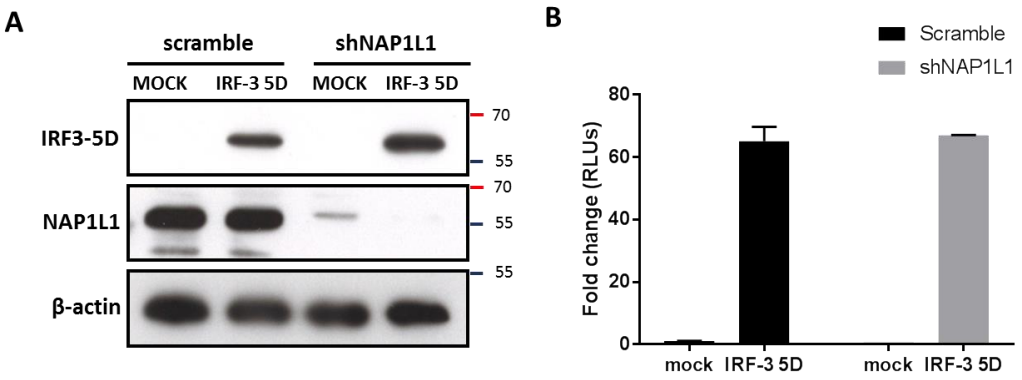

Figure 24. IRF-3 post-phosphorylation events are not modulated by NAP1L1. Cells were transduced with LV for shNAP1L1 or Scramble and subsequently transfected with an expression vector for IRF-3 5D, the reporter IFN-β-Luc and the Renilla. A) 24h post-transfection cells were lysed and WB was performed to check IRF-3 5D and NAP1L1 expression levels. B) Cell lysates were used to perform a luciferase assay. Luciferase activity in response to IRF-3 5D activity was normalized to Renilla and presented as fold change over un-stimulated condition (mock). All experiments are performed in triplicate and an average with standard deviations is shown.

3.4.3 Dissecting the role of NAP1L1 in the IFN-β pathway

Exploiting the activity of IRF-3 5D, I was able to demonstrate that NAP1L1 does not participate in IRF-3 post-phosphorylation events leading to IFN-β transcription. Since IFN-β expression is compromised in cells lacking NAP1L1, I inferred that NAP1L1 must directly or indirectly cooperate with other proteins of the cytoplasmic signalling pathway for the activation of transcription factors such as IRF-3 and NF-κB involved in IFN-β expression. Virus induced signalling cascades begin with RIG-I/MDA5 or TLR3 activation and subsequent recruitment of adaptor proteins MAVS or TRIF respectively. The association of other signalling and adaptor proteins by MAVS or TRIF results in the activation of the kinase complex IκB kinase, which phosphorylates IκB inhibitor and releases the transcription factor NF-κB that can be further phosphorylated to increase its nuclear translocation. In parallel the adaptors can activate the kinase complex TBK1/IKKε necessary for the phosphorylation of IRF-3. Each step of the two convergent signalling pathways could be modulated by NAP1L1 (Figure 25C). In order to analyse the putative NAP1L1 mediated mechanism of action on IFN-β activation, first I investigated which step of the cytoplasmic signalling pathway requires NAP1L1 for efficient IRF-3 phosphorylation and IFN-β expression. In brief, HEK293T cells were transfected with plasmids encoding MAVS, TRIF, TBK1, IKKε, p65 or IRF-3 5D together with an interferon-β luciferase and Renilla reporter plasmids in a Scramble or shNAP1L1 background. While the transfected components showed an equal expression in Scramble and shNAP1L1 condition (Figure 25A), the IFN-β promoter activity was significally affected by NAP1L1 depletion following the activation of the pathway by TRIF, MAVS, TBK1, IKKε or p65 (Figure 25B). Surprisingly, I was not able to detect the expression of the Flag-TRIF construct by WB using even stronger cell lysis methods (Figure 25A). On the contrary, NAP1L1 depletion could not inhibit IFN-β promoter activation induced by the IRF-3 5D (Figure 25B). Specifically, these results are indicative of NAP1L1 activity on TBK1/IKKε level and suggest that NAP1L1 is needed in the context of TBK1/IKKε directed IRF-3 phosphorylation.

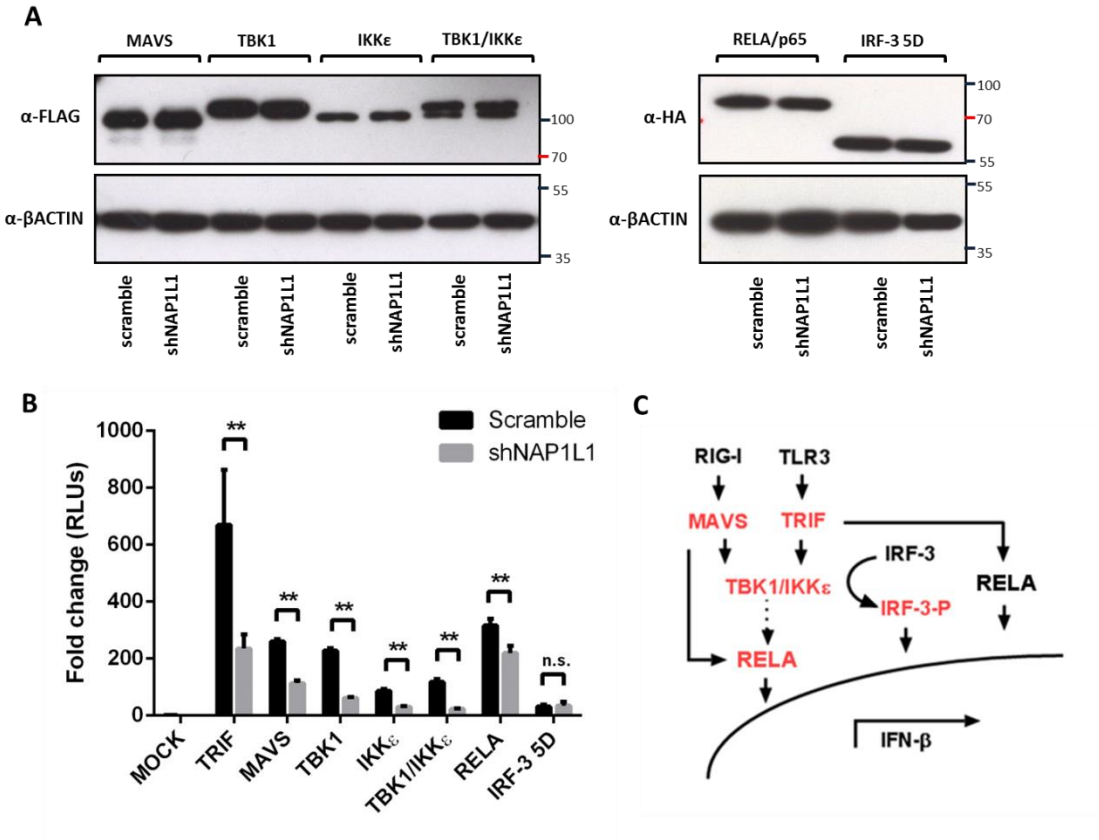

Figure 25. NAP1L1 is necessary for TBK1/IKKε mediated IRF-3 phosphorylation. HEK293T cells were transduced with LV for Scramble and shNAP1L1 and transfected with IFN-β Luc, Renilla, FLAG-MAVS, FLAG-TRIF, FLAG-TBK1, FLAG-IKKε, HA-p65, HA-IRF-3 5D or mock transfected for 24 hours. A) Cells were lysed and WB analysis was performed with α-Flag, α-HA and α-β-actin antibodies. B) Cell lysates were used to perform a dual-luciferase assay. Luciferase activity was normalized to Renilla and showed as fold change over un-stimulated condition (mock). Experiments were performed in triplicate and average with standard deviation is shown. Statistical significance was analysed using Student's t-test; P-value: **P<0.01. C) A schematic representation of the RIG-I and TLR3 pathway is shown. The constructs used for the transfection are highlighted in red.

3.4.4 NAP1L1 affects IRF-3 phosphorylation and its nuclear translocation

I have observed that TBK1/IKKε but not IRF-3 5D mediated activation of the IFN-β promoter is drastically reduced in the absence of NAP1L1, which could indicate that NAP1L1 acts in concert with TBK1/IKKε for the phosphorylation of IRF-3. These two kinases activate IRF-3 through a C-terminal phosphorylation of specific serine residues, which induces dimerization and nuclear translocation of the transcription factor and its consequent association with the co-activator p300/CBP. To address the ability of the kinases to activate IRF-3 I measured IRF-3 phosphorylation and nuclear translocation in the context of NAP1L1 depletion. Scramble and NAP1L1 depleted U2OS cells were

transfected with poly(I:C) and subsequently phosphorylation and nuclear translocation of IRF-3 were assessed by WB and indirect immunofluorescence, respectively. As shown in Figure 26A, IRF-3 is constitutively expressed and the levels are not affected by poly(I:C) treatment or NAP1L1 depletion. This evidence was also confirmed by quantification of WB bands with ImageJ, with no significant changes in protein levels between Scramble and shNAP1L1 treated cells (Figure 26B). Conversely, the phosphorylated form of IRF-3 was only detected following poly(I:C) treatment and it was induced in a time dependent manner. Interestingly, IRF-3 phosphorylation was profoundly affected in cells lacking NAP1L1 (Figure 26A). Quantification analysis showed that an attenuated IRF-3 phosphorylation in the absence of NAP1L1 is statistically significant at every time point measured (Figure 26C). To further validate these findings, I also analysed IRF-3 nuclear translocation in response to poly(I:C) induction. Scramble and shNAP1L1 U2OS cells were transfected with poly(I:C) or mock transfected for 8 hours, stained with IRF-3 antibody and analysed for nuclear IRF-3 staining. In mock treated cells IRF-3 displayed a preferentially cytoplasmic localization, whereas upon poly(I:C) treatment a notable number of cells showed nuclear IRF-3 staining. As expected, in condition of NAP1L1 depletion, a smaller number of cells exhibited IRF-3 nuclear translocation as indicated in Figure 26D. Furthermore, around 500 cells were analysed for nuclear IRF-3 in each condition and it was observed that in average 50% of the cells displayed a nuclear IRF-3 localization in Scramble condition whereas the percentage was reduce to 25% in the absence of NAP1L1 (Figure 26E). These results highlight the importance of NAP1L1 in the IRF-3 phosphorylation, nuclear translocation and consequently in IFN-β expression.

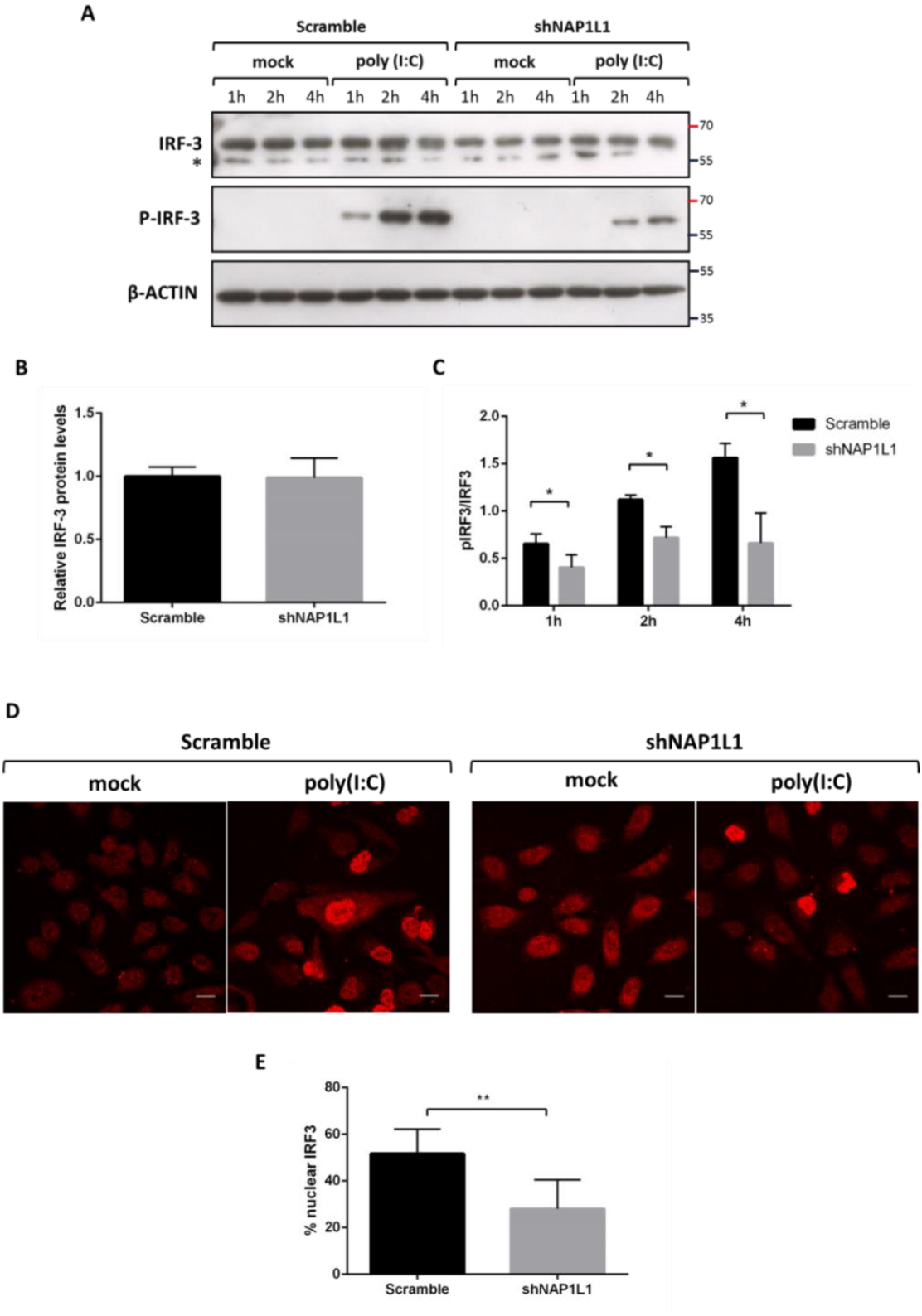

Figure 26. NAP1L1 depletion affects IRF-3 phosphorylation and nuclear translocation. U2OS cell were transduced with LV for Scramble or shNAP1L1 and transfected with 1μg of poly(I:C) or mock transfected only with lipofectamine. A) At the indicated time point post transfection (1, 2 and 4h post transfection) cell were lysed and a WB analysis was performed with α-IRF-3, α-P-IRF-3 and α-β-actin antibodies. The experiment was repeated three times and a representative picture is shown. * aspecific band. B) The intensity of the bands and the ratio of

shNAP1L1/Scramble were measured with ImageJ. The average of three independent experiments with standard deviation is shown C) The intensity of the bands was measured with ImageJ and the ratio of P-IRF-3/IRF-3 is shown. The average of three independent experiments with standard deviation is shown. Statistical significance was analysed using Student's t-test; P-value: *P<0.05. D) At 8 h post transfection cells were fixed and stained with antibody against IRF-3 and AF-549 secondary antibody. E) Approximately 500 cells were counted for each condition and the percentage of nuclear IFR3 is shown. The experiment was performed in triplicate and the average and standard deviation is shown. Statistical significance was assessed using Student's t-test; P-value: **P<0.01.

3.4.5 NAP1L1 affects NF-κB basal levels and its phosphorylation

IFN-β expression is a highly ordered process of deposition of transcription factors to the promoter region to assemble the enhanceosome, which drives transcription of the IFN-β gene. Besides IRF-3 phosphorylation, dimerization and nuclear translocation, the activation and nuclear translocation of NF-κB is equally important to fully activate IFN-β transcription.

The activation of NF-κB by a variety of extracellular stimuli is crucial for the control of immune and inflammatory responses. In resting condition NF-κB is localized in the cytoplasm as a heterodimer composed of p50 and p65 subunits bound to an inhibitory protein IκB. A key step in the activation of NF-κB is a stimulus induced phosphorylation of the inhibitor. This event targets IκB for ubiquitination and proteasomal mediated degradation which releases NF-κB heterodimer allowing it to translocate to the nucleus to cooperate with other transcription factors for the formation of the enhanceasome. The kinase complex responsible for the phosphorylation of the IκB inhibitor is the IKK complex defined by at least two subunits IKKα and IKKβ. However, an increasing number of evidence suggests that NF-κB pathway can be reinforced by the activity of two non-canonical kinases TBK1 and IKKε (Buss et al. 2004). As NF-κB signalling contributes to IFN-β expression, I investigated its activity in cells depleted for NAP1L1. I followed the same approach used to study IRF-3 activation. Briefly, U2OS cells transduced with a LV for shNAP1L1 or Scramble were transfected with poly(I:C) and then the phosphorylation status of NF-κB subunit, p65, and its nuclear localization were assessed by WB and immunofluorescence analysis. In order to analyse NF-κB/p65 protein levels cells were treated for 1h, 2h and 4h with poly(I:C), lysed and blotted against endogenous NF-κB/p65. WB analysis in Figure 27A and quantification analysis in Figure 27B indicated that p65 protein levels were reduced in cells depleted for NAP1L1. This finding is in agreement with previous results

showing a down-modulation of p65 mRNA levels in the absence of NAP1L1 (Figure 17). Therefore I could show that NAP1L1 is necessary for the maintenance of the basal NF-κB/p65 mRNA as well as protein levels. Although un-stimulated control U2OS cells displayed basal levels of phosphorylated p65, poly(I:C) stimulation increased p65 phosphorylation already at 1h post transfection and was maintained until 4h post transfection, whereas in cells depleted for NAP1L1 a slight reduction in p65 phosphorylation was consistently observed only at 4h post transfection (Figure 27A and quantification in Figure 27C).

Since I have observed a statistically significant alteration of p65 phosphorylation in NAP1L1 depleted only at 4h post poly(I:C) transfection, I proceeded by analysing p65 nuclear localization at that time point. Scramble and shNAP1L1 U2OS cells were transfected with poly(I:C) or mock transfected for 4 hours, stained with NF-κB/p65 antibody and approximately 500 cells for both Scramble and shNAP1L1 condition were analysed for nuclear NF-κB/p65. Un-stimulated cells presented a cytoplasmic p65 localization, whereas upon poly(I:C) treatment 30% of control cells displayed a nuclear p65 localization and this percentage was reduced to 20% in cells lacking NAP1L1 as shown in Figure 27D and Figure 27E.

In conclusion, these results suggest that NAP1L1 is important for the constitutive expression of NF-κB and seems to have a role in p65 transactivation activity, although this effect is evident only at later time points.

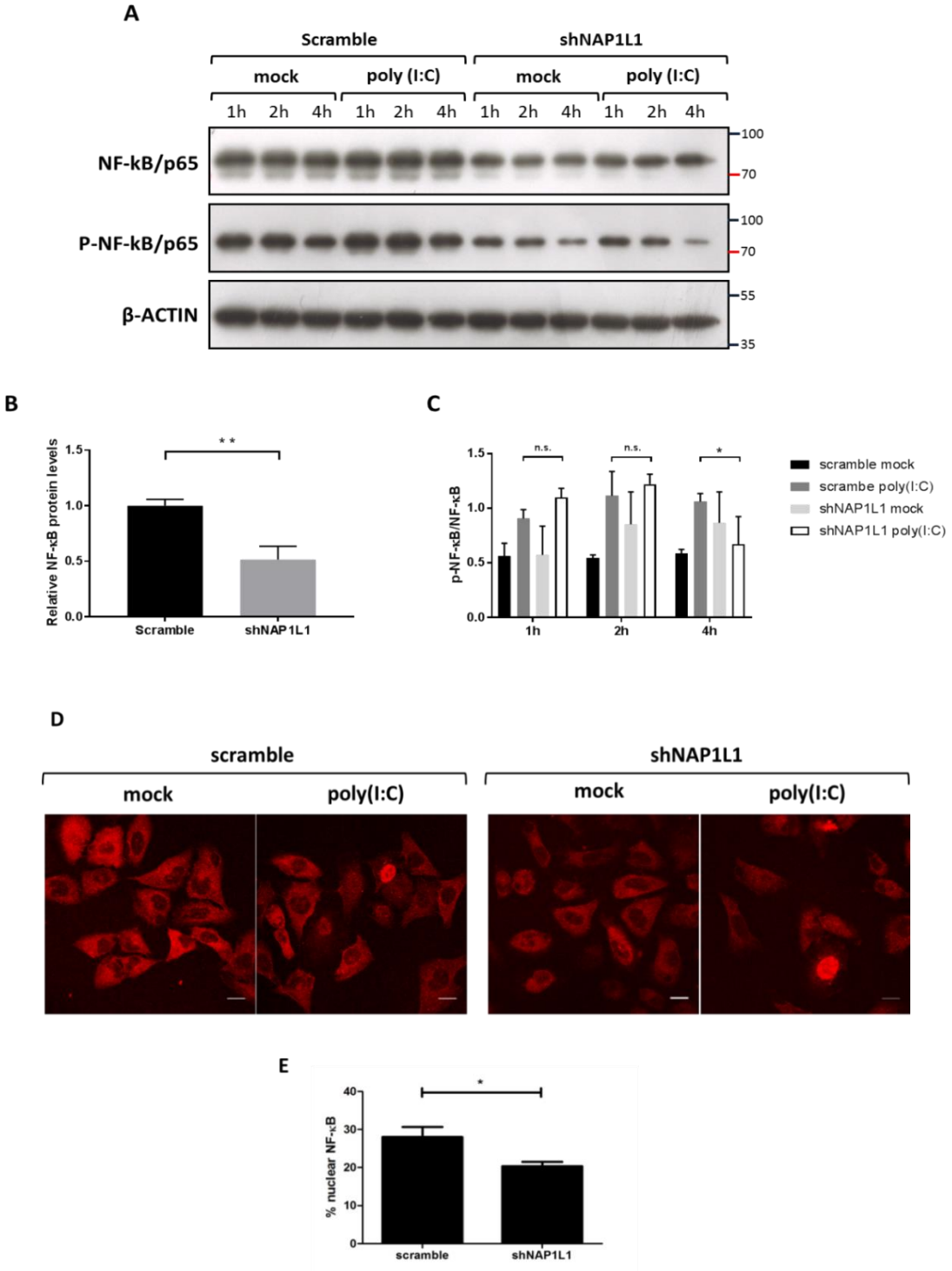

Figure 27. NAP1L1 depletion affects NF-κB basal expression and a late phosphorylation. U2OS cell were transduced with LV for Scramble or shNAP1L1 and transfected with 1μg of poly(I:C) or mock transfected only with lipofectamine. A) At the indicated time point post transfection (1, 2 and 4h post transfection) cell were lysed and a WB analysis was performed with α-p65, α-P-p65 and α-β-actin antibodies. The experiment was repeated three times and a representative picture is shown. B) The intensity of the bands in untreated condition was measured with ImageJ and the ratio of shNAP1L1/Scramble as an average of three independent experiments with standard deviation is shown. Statistical significance was analysed using Student's t-test; P-value: **P<0.01. C) The intensity of the bands was measured with ImageJ and the ratio of P-p65/p65 is shown as average of three independent experiments with

standard deviation. Statistical significance was analysed using Student's t-test; P-value: *P<0.05. D) At 4 h post transfection cells were fixed and stained with p65 primary antibody and AF-549 secondary antibody. E) Approximately 500 cells were counted for each condition and the percentage of nuclear p65 is shown. The experiment was performed in triplicate and the average and standard deviation is shown. Statistical significance was assessed using Student's t-test; P-value: **P<0.01.

3.4.6 NAP1L1 does not interact with TBK1 or IKKε

I have demonstrated that NAP1L1 is involved in TBK1/IKKε mediated IRF-3 phosphorylation and p65 late point phosphorylation. At this point NAP1L1 could act directly, interacting with the complex, or indirectly, regulating the transcription of yet unknown genes involved in the process. Reinecker and colleagues have shown that a guanine nucleotide exchange factor GEF-H1 is necessary for TBK1 mediated IRF-3 phosphorylation (Chiang et al. 2014). Interestingly, as shown in Figure 17, this particular factor together with other isoforms is down regulated by NAP1L1 depletion. This evidence is in favour of NAP1L1 acting at the transcriptional level. Alternatively, NAP1L1 activity could be exerted directly in the PRR signalling pathway which could be a faster mechanism of action since the chaperone is already present in the cytoplasm. Since the NAP1L1 site of action has been linked to TBK1/IKKε mediated phosphorylation of IRF-3, I immunoprecipitated the two kinases to investigate whether NAP1L1 constitute an integral component of the kinase complex. To this end, HEK293T cells were transfected with TBK1, IKKε, NS5A as positive and NS5A m2 as negative control together with NAP1L1. Flag tagged constructs were pulled down with anti-FLAG agarose beads and WB was performed to determine the presence of NAP1L1 in the complex. As indicated in Figure 28 NAP1L1 did not co-precipitate with TBK1 or IKKε, meaning that there is no direct interaction between NAP1L1 and the two IKK related kinases.

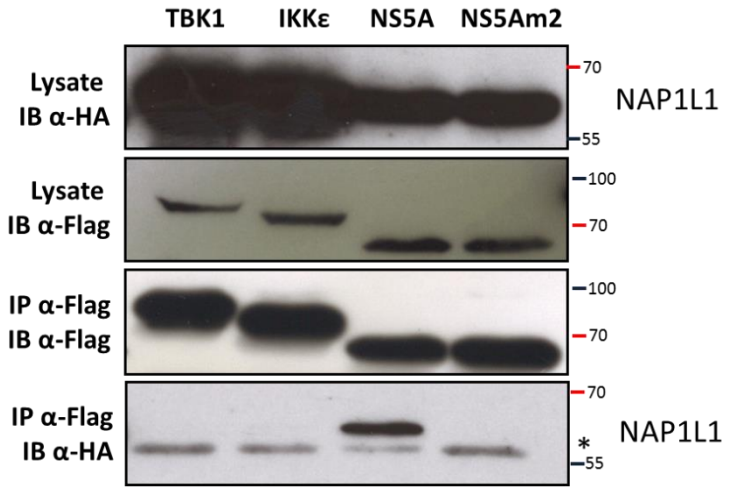

Figure 28. NAP1L1 is not a component of the TBK1/IKKε complex. HEK293T cells were transfected with HA-NAP1L1 and FLAG-tagged TBK1, IKKε, NS5A as positive control or NS5A-m2 as negative control. Cells were lysed and co-IP was performed with anti-FLAG agarose beads. Lysates and IP samples were blotted with α-FLAG and α-HA antibodies. The asterisk indicates the position of immunoglobulin heavy chain. The experiment was repeated three times and a representative image is shown.

3.4.7 NAP1L1 associates with MAVS

A very recent report proposed a model for MAVS and TRIF induced TBK1 mediated IRF-3 activation where adaptor proteins MAVS and TRIF recruit the kinase TBK1 for their own phosphorylation. Once phosphorylated, the adaptor proteins recruit IRF-3 for its phosphorylation by TBK1 (Liu et al. 2015). Therefore adaptor proteins, kinases, transcription factors and other associated scaffold proteins seem to be engaged in a complex signalling network. Although I did not find an association between NAP1L1 and the kinases, the interaction with other adaptor molecules remains and open possibility. For this reason I investigated NAP1L1 direct interaction with MAVS by immunoprecipitation analysis. As before, HEK293T cells were transfected with NAP1L1 together with MAVS or NS5A as positive control. Immunoprecipitation of FLAG-tagged constructs were pulled down with anti-FLAG agarose beads and a western blot was performed to check for the presence of NAP1L1. Interestingly, WB analysis against HA-NAP1L1 revealed its association with MAVS protein (Figure 29). This result indicates that NAP1L1 is engaged in larger MAVS containing signalling complex.

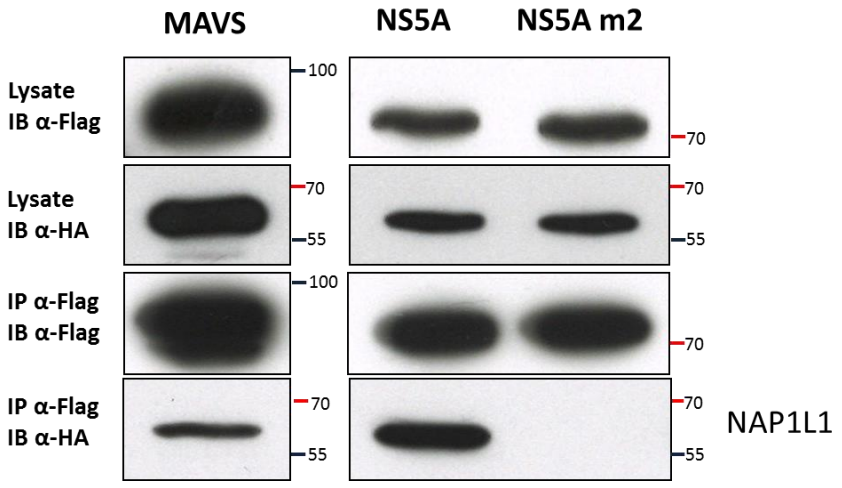

Figure 29. NAP1L1 interacts with MAVS adaptor protein. HEK293T cells were transfected with FLAG-MAVS or FLAG-NS5A (positive control) together with HA-NAP1L1. Cells were lysed and co-immunoprecipitation was performed with anti-FLAG agarose beads. Lysates and IP samples were blotted with α-FLAG and α-HA antibodies. The experiment was repeated two times and one representative image is shown.

3.5 HCV NS5A interaction with NAP1L1 controls IFN-β expression.

HCV has various strategies to control the innate immune response. The most important player in subverting the interferon mediated antiviral activities is the protease NS3/4A. The protease is able to cleave both MAVS and TRIF adaptor proteins to control the interferon response. Nevertheless, other HCV proteins have also the ability to interfere with the host response to infection. NS5A for instance, operate at different levels to regulate type I interferon production.

3.5.1 HCV NS5A recapitulates NAP1L1 depletion phenotype.

Until now I have demonstrated that NAP1L1 has a fundamental role in the regulation of type interferon response and that the interaction of NS5A with NAP1L1 is critical to inhibit NAP1L1 nuclear translocation. These two observations led us to hypothesize that NAP1L1 activity might be restricted due to its interaction with NS5A leading to an impairment of the IFN response. In order to demonstrate this issue first I validated if NS5A expressed as an individual protein is able to modulate TBK1 induced IFN-β expression. To this end, I transfected HEK293T cells with a Flag tagged plasmid for TBK1 expression together with a Flag tagged NS5A wild type or m2, a reporter plasmid containing the IFN-β promoter upstream of a firefly luciferase gene and a plasmid for

the expression of Renilla luciferase gene to normalize the transfection efficiency. As shown in Figure 30A IFN-β promoter activity was highly induced by TBK1 expression, whereas the co-expression of NS5A or the mutant form, almost completely block the IFN-β promoter activation. In fact it is well known that NS5A has potent strategies in controlling the interferon response. In order to delineate the difference in IFN-β expression mediated by the interaction with NAP1L1 I proceeded by carefully titrating the amount of expression plasmid used for the transfection that would be sufficient to halve TBK1 induced IFN-β expression. Therefore, increasing amounts (0ng-50ng) of NS5A plasmid were co-transfected with a fixed amount of TBK1 expression plasmid. As shown in Figure 30B, 40ng of NS5A were sufficient to completely block the IFN-β promoter activity as a further increase of the amount of plasmid did not induce further attenuation of the IFN-β promoter activity. The amount of 15ng of NS5A was chosen to validate the hypothesis. As shown in Figure 30C, NS5A m2 inhibits TBK1 mediated IFN-β promoter activation less efficiently when compared to wild type NS5A. These results suggest that HCV NS5A sequester NAP1L1 to interfere with the interferon activation.

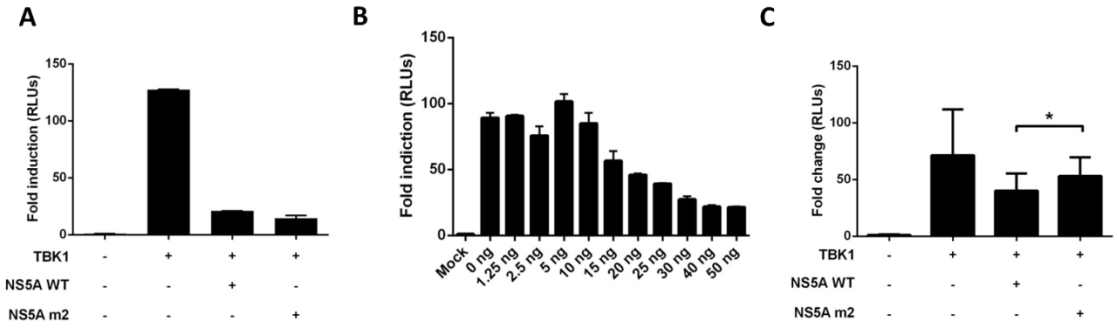

Figure 30. HCV NS5A inhibits TBK1 mediated IFN-β promoter activation. A) HEK293T cells were co-transfected with plasmids encoding for FLAG-TBK1 together with FLAG-NS5A or FLAG-NS5A/m2. 24h post transfection cells were lysed and the lysates were used to perform a luciferase reporter assay. B) HEK293T cells were transfected with a fixed amount of TBK1 expression plasmid and increasing amounts of FLAG-NS5A wild type expression plasmid. Values were normalized to mock transfected cells and plotted as fold induction over mock. C) 15ng of FLAG-NS5A or FLAG-NS5A/m2 was used for transfection as in A. The experiment was performed in quadruplicate and represented as average with standard deviation. The Student t-test was performed and the statistical significance was calculated based on the P-value: *P<0.05.

3.5.2 NAP1L1 mediated effects on IFN-β activation during HCV replication in U2OS miR122 cells

3.5.2.1. Establishment of the U2OS miR-122 cells

The contribution of NAP1L1 in HCV NS5A mediated inhibition of the IFN response was further examined in a context of HCV replication with the cooperation of other HCV non-structural proteins. The most powerful tool that was developed so far to study the HCV life cycle is given by the combination of Huh7 derived cell lines and the JFH1 genotype 2 HCV genome (Wakita et al. 2005). Although Huh7 cells and its derivative Huh7.5 or Huh7-Lunet cell lines, replicate the viral genome very efficiently, they are not suitable for the analysis of the HCV induced innate immune response as Huh7 are very inefficient producers of IFN and ISGs following viral infection. In order to overcome this inconvenience, I tried to generate a cell line that would support active HCV replication and mount and adequate and measurable IFN-β response. Historically, different strategies have been envisaged to render Huh7 cells more competent for IFN expression or render non hepatic cells with an active innate immune response more permissive for HCV replication. Several groups have shown that the expression of a liver specific microRNA, miR-122, enhances HCV replication in both hepatic and non-hepatic cell lines (Chang et al. 2008; Narbus et al. 2011). U2OS cell line possess and intact interferon signalling pathway and in addition have been shown to replicate the HCV genome, albeit at low levels when compared to hepatoma derive cell lines (Targett-Adams & McLauchlan 2005). The efficiency of HCV replication was further increased by exogenous expression of miR-122. Briefly, U2OS cells were transduced with a lentiviral vector expressing miR-122, selected with blasticidine for the enrichment of miR-122 positive cells and electroporated with a HCV SGR-JFH-1 expressing a luciferase reporter gene. HCV replication was monitored by measuring luciferase activity at 4, 24, 48 and 72h post electroporation. Although the replication in U2OS miR122 was relatively inefficient if compared to the replication in Huh7 cells (Kato et al. 2005), miR-122 overexpression in U2OS cells was able to augment viral replication (Figure 31). Therefore the U2OS miR-122 cells were used in subsequent experiments to study the involvement of NAP1L1 in HCV mediated IFN-β expression.

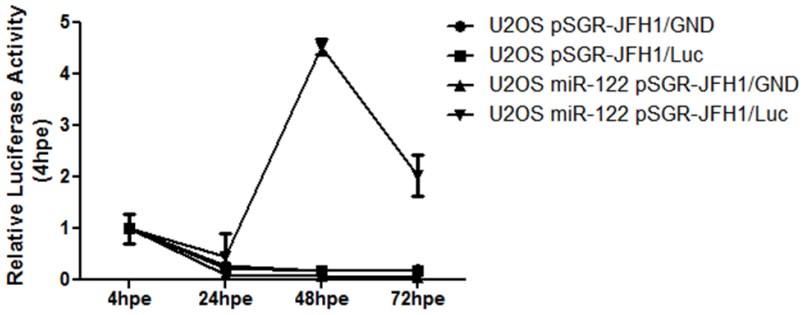

Figure 31. U2OS miR-122 cells are permissive for HCV replication. U2OS and U2OS miR-122 cells were electroporated with pSGR-JFH1/GND and pSGR-JFH1/Luc. Cells were lysed and collected at 4, 24, 48 and 72hpe. Luciferase activity was measured at every time point and normalized for 4hpe. The experiment was performed two times and an average with standard deviation is shown.

3.5.2.2. HCV replication in U2OS miR-122 does not induce IFN-β expression

As U2OS cells were already known to be competent for IFN-β expression (Miorin et al. 2012), and the ectopic expression of miR-122 was shown to increase its permissiveness for HCV replication, I transfected this cell line with the pSGR-JFH1/GND and pSGR-JFH1 carrying the wild type or mutant form of NS5A and monitor both the genome replication and IFN-β production. Following RNA electroporation luciferase activity was measured at 4h post electroporation, a time point used to determine the input levels of HCV RNA. The maximum peak of replication is achieved at 48hpe and then declined at 72hpe. Interestingly, serine to alanine substitutions in NS5A in a context of HCV genome did not affect HCV replication which went alongside with that of the wild type (Figure 32A). It is nevertheless important to point out that neither Masaki and colleagues noticed a difference in the replication of the mutant replicon, although they measured replication in Huh7 cells, lacking an active interferon system (Masaki et al. 2008). In fact, it was almost impossible to appreciate a difference in replication since, as shown in Figure 32B, HCV RNA replication did not induce measurable levels of IFN-β mRNA. A slight induction of IFN-β mRNA in cells replicating the RNA genome was observed only at 48h post electroporation albeit not sufficiently to appreciate the NAP1L1 induced effect of IFN-β expression.

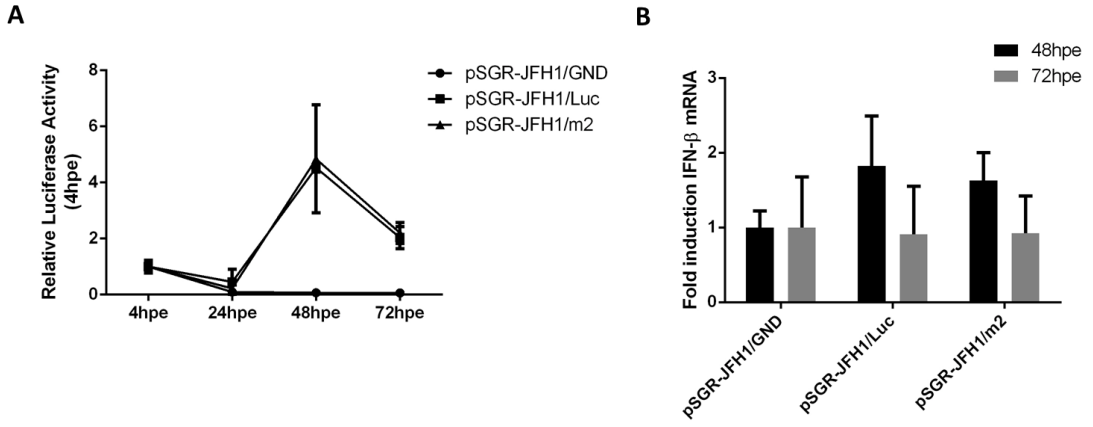

Figure 32. U2OS miR122 replicate the virus inefficiently and induce a poor IFN-β response. U2OS cells were transduced with LV for the overexpression of miR122. U2OS and U2OS miR122 were electroporated with pSGR-JFH1/Luc-GND, pSGR-JFH1/Luc and pSGR-JFH1/m2 RNA. A) at 4, 24, 48 and 72h post electroporation cells were lysed and luciferase assay was performed. Data were normalized to 4h post electroporation. B) at 48 and 74 h post electroporation cells were lysed and RNA was extracted. IFN-β mRNA levels were measured by qRT-PCR and normalized to β-actin levels. The result is represented as fold change over pSGR-JFH1/Luc-GND electroporated cells. The experiment was performed in triplicate and results are represented as average with standard deviations.

3.5.3 NAP1L1 mediated effects on IFN-β activation during HCV replication in Huh7-Lunet TLR3 cells

In addition to the cytoplasmic RNA sensing machinery, dsRNA is also recognized inside endosomes by a membrane bound pathogen recognition receptor, TLR3, which activates type I IFN and pro-inflammatory cytokine expression through the adaptor protein TRIF. The overall contribution of TLR3 signalling in HCV infected PHH is still poorly understood and remains an object of different studies. Wang et al., have demonstrated that PHH show high expression of TLR3 and the pathway is activated upon poly(I:C) induction. Moreover, they have demonstrated that reconstitution of the TLR3 signalling pathway in Huh7 cells deficient for TLR3 expression, is sufficient to establish an antiviral response to HCV infection (Wang et al. 2009).

As previously demonstrated, NAP1L1 depletion inhibits IFIT1 expression in U2OS cells reconstituted with TLR3 signalling or HEK293T cells overexpressing the TRIF protein, indicating that NAP1L1 is involved in TLR3 mediated immune responses. In order to demonstrate that NAP1L1 is required for an efficient HCV induced IFN response, I reconstituted Huh7 cells with TLR3 to generate a highly permissive Huh7-Lunet/TLR3 cell line with an active immune system.

3.5.3.1 Generation of Huh7-Lunet/TLR3 cells

In order to produce a cell line with an intact TLR3 signalling cascade, I took advantage of a bi-cistronic lentiviral vector that contains EGFP or TLR3 gene under the control of a CMV promoter and a RFP marker under the control of an EMCV IRES (Figure 33A). Once packaged, the lentivector was used to overexpress TLR3 in Huh7-Lunet cells by lentiviral transduction. The efficiency of lentiviral transduction was verified by immunofluorescence analysis, analysing the expression of selection markers GFP and RFP, as an antibody against TLR3 protein was not available in our laboratory. As shown in Figure 33B, the majority of Huh7-Lunet/EGFP cells showed the expression of both markers, whereas Huh7-Lunet/TLR3 show the expression only of the RFP marker as expected. Expression of TLR3 mRNA was verified by PCR (Figure 33C). Next, I investigated if TLR3 overexpression reconstituted the signalling pathway in Huh7-Lunet cells. TLR3 senses dsRNA present inside endosomes that has been endocytosed form the extracellular milieu. Therefore, in order to study the functionality of the pathway, I treated Huh-Lunet/TLR3 and Huh-Lunet/EGFP cells with 50μg/ml of poly(I:C) by direct addition to the medium for 24h and monitored the expression of IFN-β and IRF-3 responsive IFIT1. Interestingly, poly(I:C) stimulation of did not induce IFN-β mRNA expression in Huh7-Lunet cells harbouring TLR3 as shown in Figure 33D. Conversely, it highly induced IFIT1 mRNA expression in these cells which is indicative of an active signalling pathway (Figure 33E).

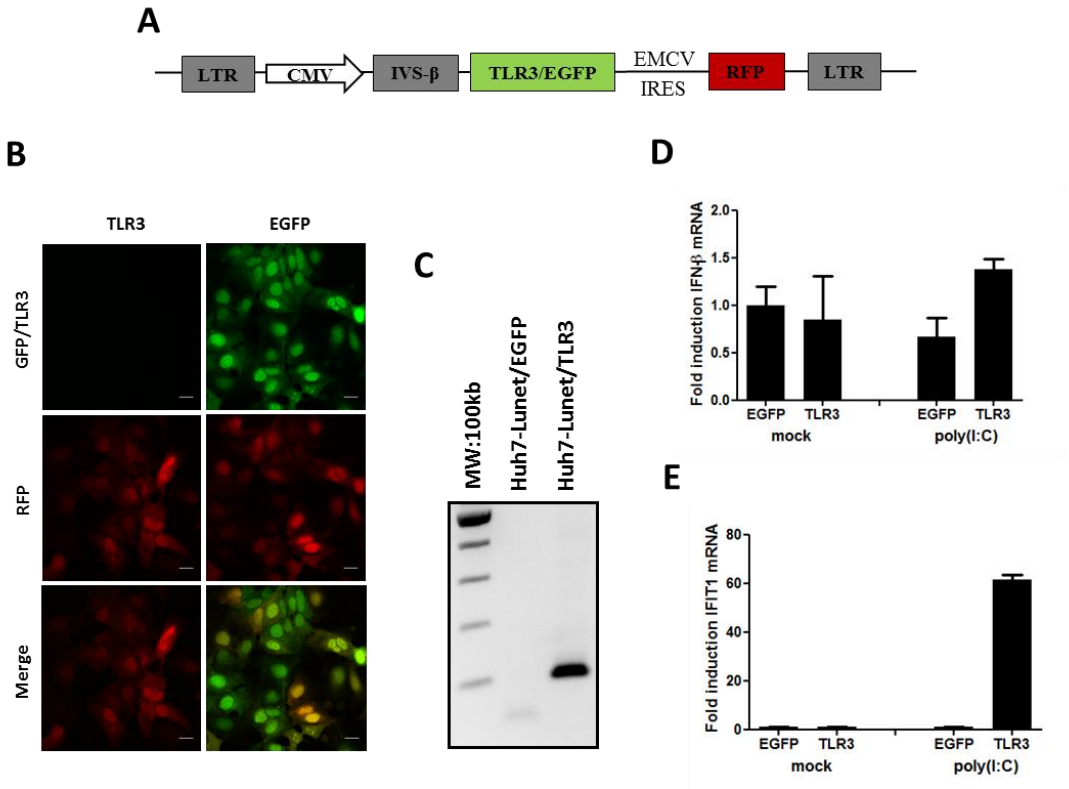

Figure 33. Reconstitution of a functional TLR3 pathway in Huh7-Lunet cells. Huh7-Lunet cells were transduced with a lentivector for the expression of TLR3 of EGFP as control. A) Schematic representation of lentiviral vector used in the study. B) Transduced Huh7-Lunet cells were fixed and analysed by fluorescent microscopy for the expression of EGFP (green) or RFP (red) signals. C) Transduced Huh7-Lunet cells were lysed, RNA was extracted, retro-transcribed and used to perform a PCR for TLR3 amplification. D) TLR3 or EGFP transduced Huh7-Lunet cells were treated with 50µg/ml of poly(I:C) by direct addition to the medium or left untreated. Cells were lysed and RNA was extracted. IFN-β and IFIT1 mRNA levels were measured by qRT-PCR. The results are represented as fold induction over non treated mock samples. The experiment was repeated in duplicate and an average with standard deviation is show.

3.5.3.2 HCV replication in Huh7-Lunet/TLR3 cells does not show a clear NAP1L1 mediated effect on IFN-β expression

Since NAP1L1 has been identified as a component regulating TLR3 signalling pathway and the ectopic expression of TLR3 in Huh7 cells was shown to be sufficient to trigger the antiviral signalling in response to HCV infection (Wang et al. 2009), I could assess NAP1L1 mediated contribution in the antiviral signalling pathway upon HCV replication. To this end, I electroporated Huh7-Lunet/TLR3 cells with an HCV replicon carrying a wild type or mutant form of the NS5A protein and collected the cells to measure both the virus replication and IFIT1 induction. As shown in Figure 34A HCV

replicated quite efficiently in this cell line if compared to a pSGR-JFH1/Luc-GND, a non-replicative form of the replicon. The replication increased up to 40 fold at 48 hours post transfection and then dropped drastically at 72 h post transfection. Concomitantly, as shown in Figure 34B, HCV replication activated the antiviral response as demonstrated by the induction of IFIT1 mRNA levels in cells carrying the functional but not the replication defective HCV replicon. Nevertheless the effect of NAP1L1 on the IFIT1 expression in the context of HCV replication and consequently NS5A interaction was not appreciated with this experimental approach, as both the wild type and mutant NS5A carrying replicons induced IFIT1 expression to the same extent.

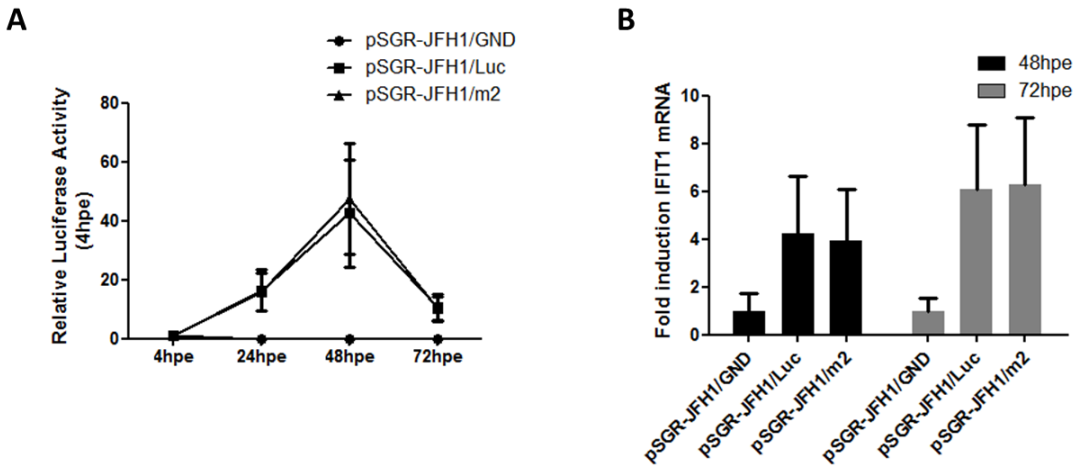

Figure 34. HCV replication induces a functional immune response in Huh7-Lunet cells reconstituted with TLR3. Huh7-Lunet cells were transduced with a lentivector for the expression of TLR3 of EGFP as control and electroporated with pSGR-JFH1/Luc-GND, pSGR-JFH1/Luc and pSGR-JFH1/m2 RNA. A) at 4, 24, 48 and 72h post electroporation cells were lysed and luciferase assay was performed. Data were normalized to 4h post electroporation. The result is represented as an average of three independent experiments with standard deviations. B) at 48 and 74 h post electroporation cells were lysed and RNA was extracted. IFIT1 mRNA levels were measured by qRT-PCR and normalized to β-actin levels. The result is represented as fold change over pSGR-JFH1/Luc-GND electroporated cells with standard deviations.

3.5.4. NAP1L1 mediated effects on IFN-β activation during HCV replication in Huh7-Lunet/MAVSR cells

3.5.4.1 Generation of Huh7-Lunet/MAVSR cells

The HCV encoded protease NS3/4A is able to cleave the MAVS at a particular cysteine residues located at position 508 (C508), releasing the adaptor protein from the MAMs

and subverting the host immune response. As a result, HCV replication does not induce detectable IFN-β mRNA levels. It has been shown that MAVS proteins with the substitution of the cysteine in position 508 with an arginine (C508R) are resistant to NS3/4A cleavage yet maintain an intact antiviral signalling cascade (Li et al. 2005b). With the attempt to define the role of NAP1L1 in the HCV mediated innate immune response, I generated a hepatic cell line stably expressing a Flag tagged version of the cleavage resistant form of MAVS protein designated as Huh7-Lunet/MAVSR. Next, I determine the expression levels of the ectopically introduced MAVS C508R. The mRNA and protein levels showed a weak yet detectable expression of MAVS C508R protein as indicated in Figure 35A and Figure 35B that did not significantly induce the expression of the IFN-β gene (Figure 35C).

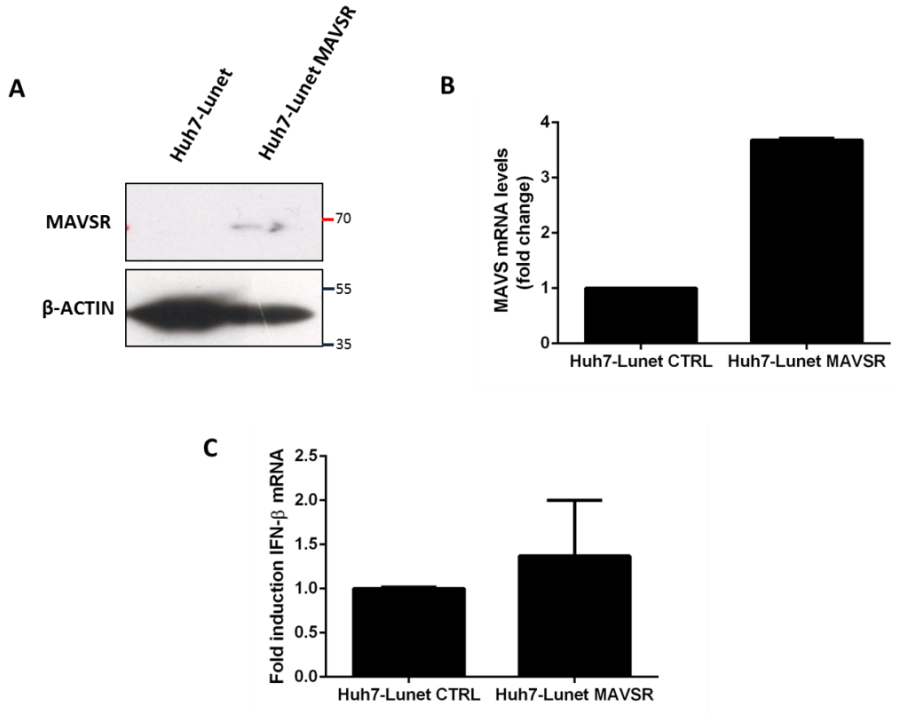

Figure 35. Characterization of Huh7-Lunet MAVSR cells. Huh7-Lunet cells were transduced with a LV for the overexpression of MAVS C508R. After transduction cell were put under puromycin selection (0,5μg/ml) for approximately one month. A) cells were lysed and lysates were used to perform a western blot analysis with an α-Flag and α-β-actin antibodies. B and C) cells were lysed and RNA was extracted, retrotranscribed and used to perform a qRT-PCR analysis. MAVS and IFN-β mRNA levels were measured and normalized to β-Actin levels. The result is presented as fold change over mock, non-transduced cells. The experiment was performed two times and the average with standard deviations is shown.

3.5.4.2 HCV replication triggers a potent IFN-β expression in Huh7-Lunet MAVSR

In order to outline the contribution of NAP1L1 in the IFN-β response following HCV replication, I electroporated Huh7-Lunet/MAVSR cells with the replication deficient HCV replicon and the replication competent replicon carrying the wild type or the mutant version of the NS5A gene. Next, I assessed the replication at 4, 24, 48 and 72 hours post electroporation as well as the induction of IFN-β by qRT-PCR. As shown in Figure 36A the replication of both wild type and m2 replicons was quite inefficient remaining below the input levels with an increase at only 72hpe. A slight advantage in the replication capacity was observed for the wild type replicon with respect to the mutant one. Despite low levels of replication both replicons were able to activate the IFN-β expression albeit with different intensity (Figure 36B). The difference in the IFN-β expression between the wild type and mutant replicon at both 48 and 72 h post electroporation could be a result of a slight variation of their replication capacity, as the system seems to be highly sensitive and respond to small variation in replication. Curiously, also with this strategy I was unable to detect an effect on IFN-β activation due to an interaction between NS5A and NAP1L1. Further repetition of the experiment will be necessary to confirm the data and other strategies will have to be implemented to appreciate the variation in the IFN-β response following HCV replication.

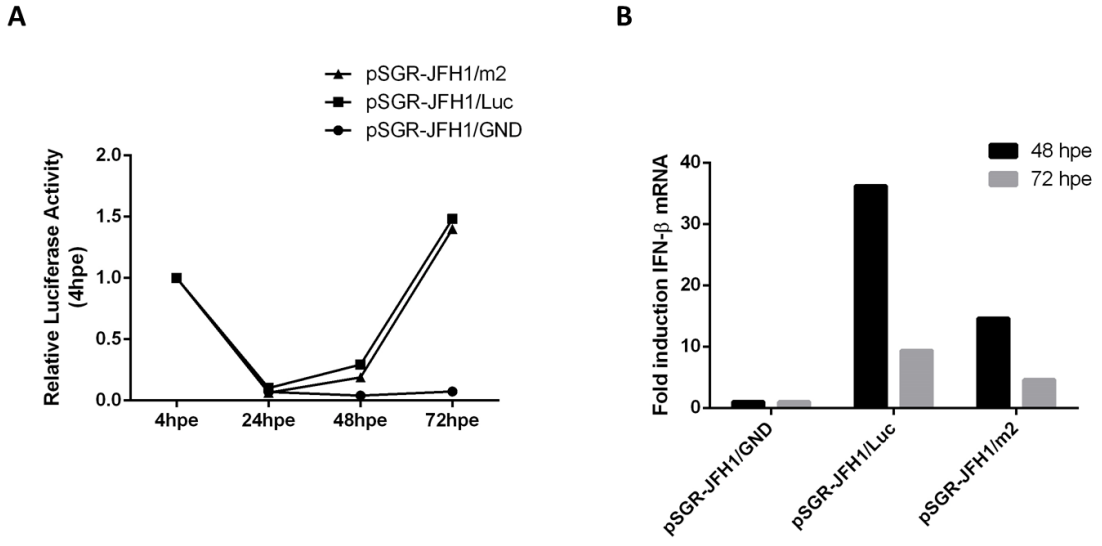

Figure 36. HCV replication induces a strong IFN-β response in Huh7-Lunet MAVSR cells. Huh7-Lunet/MAVSR cells were electroporated with pSGR-JFH1/GND, pSGR-JFH1/Luc and pSGR-JFH1/m2. A) At 4, 24, 48 and 72h post electroporation cells were lysed and a luciferase assay was performed. Data were normalized to 4h

post electroporation. B) At 48 and 72 h post electroporation cell were lysed, RNA was extracted and retroranscribed. IFN-β mRNA levels were measured by qRT-PCR and noramlized to β-Actin levels.

4. DISCUSSION

Hepatitis C virus is a leading cause of viral hepatitis and approximately 3% of the world population is chronically infected. In 85% of the cases the initial acute HCV infection is able to bypass the barriers imposed by the cell and evolve into a chronic infection. The mechanism governing the transition from the acute to a chronic state are not well established, but the complex interplay between the host immune system and the viral mechanism aimed to hijack the immune response, along with other factors, determine the outcome of the disease. In addition to the NS3/4A protease, well known for its ability to block the IFN response by cleaving MAVS and TRIF adaptor proteins, also NS5A is able to modulate several cellular pathways including the IFN signalling pathway. Recently, NS5A was repeatedly and independently found by different groups to interact with the nucleosome assembly protein 1 like 1, NAP1L1, although the functional relevance of this interaction was never investigated. NAP1L1 is a histone chaperone protein that has many functions strictly related to histone assembly and disassembly activities, such as gene regulation, transcription, cell cycle regulation and differentiation. NAP1L1 was found to be a target of many viruses that in this way control the transcription of their own genes or host genes to modify the cellular environment. The interaction and colocalization in viral replication complexes was confirmed in our laboratory. Taking advantage of a nuclear export inhibitor, I could demonstrate that NAP1L1 is retained in the cytoplasm during viral replication, which could compromise its nuclear activity. Performing a whole transcriptome analysis of NAP1L1 depleted cells I could demonstrate that NAP1L1 affects the transcription of specific genes and some of them were represented by ISGs or involved in inflammatory responses. Interestingly, when I analysed NAP1L1 involvement in the innate immune response I found that it is required for the poly(I:C) and virus induced IFN-β activation. Additionally, NAP1L1 has been found to be involved in IRF-3 and p65 phosphorylation. Moreover, I found that the overexpression of the mutant form of NS5A, which does not bind NAP1L1, was unable to reduce the IFN-β promoter activity to the same extent as the wild type NS5A. Unexpectedly, I was not able to recapitulate this effect during viral replication possibly due to inappropriate experimental model systems or a limited effect of NAP1L1 in the modulation of the innate immune response following its interaction with NS5A.

4.1 HCV NS5A interacts with NAP1L1

It is well established among the scientific community that HCV NS5A is a highly phosphorylated protein which gives rise to a basal- and hyper- phosphorylated form of the protein (Macdonald & Harris 2004). Although the functional relevance of NS5A phosphorylation has not been clarified in detail, a growing body of evidence indicate that it has important roles in regulating both viral replication and assembly and may represent a switch between these two activities. This post-translational modification seems to regulate also the interaction with other viral proteins as well as cellular factors for the modification of the host environment. The intrinsically disorder nature of domain II and III is the driving force for the engagement with more than 130 cellular partners. The panel of NS5A interactors is continuously growing (Ross-Thriepland & Harris 2015) and among them NAP1L1 has been repeatedly and independently found to interact with NS5A (Ramage et al. 2015; De Chassey et al. 2008; Pichlmair et al. 2012). However, in these reports the authors did not address the functional relevance of the interaction.

In this study we used biological and biochemical techniques to confirm the interaction both in the context of NS5A overexpression, performing co-immunoprecipitation analysis (Figure 13B), and in the presence of other HCV proteins during viral replication (Figure 13C). It was already established in our laboratory, by sub-mapping experiments, that NAP1L1 binds the last C terminal region of NS5A. Particularly, it was observed that only when the motif –EEDDTT- was intact the interaction with NAP1L1 occurred. NS5A phosphorylation is known to be mediated by different kinases including CKI-α, CKII (Masaki et al. 2014; Tellinghuisen et al. 2008; Kim et al. 1999) among others. The motif corresponding to the binding region of NAP1L1 is predicted to be an ideal recognition site for CKII. In fact, CKII minimal recognition site is represented by the sequence S/T–X–X–D/E, where X can potentially be any amino acid. Moreover, the group of Tellinguinsen et al., demonstrated that this exact serine residue at position 457 of NS5A from genotype 2 JFH-1 (corresponding to the position 2433 of the polyprotein) lies within a CKII recognition motif and becomes phosphorylated by CKII *in vitro* (Tellinghuisen et al. 2008). These findings, in addition to the fact that NS5A and NAP1L1 show the highest degree of co-localization only at 72h post electroporation (Cevik et al., unpublished data), favour the hypothesis that a post-

translational modification is required for the interaction with NAP1L1. Phosphorylation has a specific role in protein-protein interactions and therefore I reasoned that the phosphorylation of serines in the surrounding area might be the trigger for NAP1L1 interaction. Results indicated that serine 2428, 2430, and 2433 of NS5A represent the crucial cluster involved in the interaction with NAP1L1. Accordingly, serine to alanine mutation that disrupts these phospho-acceptor sites dissolves also the interaction with NAP1L1. Surprisingly, when these amino acids were substituted with glutamic acids, which, due to their negative charge, mimic a constitutive serine phosphorylation, the interaction could not be restored (Figure 13B). Various possibilities could be taken into account for the last observation: the negative charge derived from the incorporation of glutamic acids is not equivalent to that given by the phosphorylation of serines, or alternatively, the phosphorylation of one or more serines in that cluster could trigger a conformational change in the protein which will then allow the interaction with NAP1L1. Moreover, Tellinghuisen et al., have showed that serine 457 is phosphorylated by CKII and that this event is necessary for infectious particle production due to its interaction with Core protein (Tellinghuisen et al. 2008). Therefore, I analysed more in detail the contribution of these serines for NAP1L1 interaction. I found that serines 452 and 454 are involved in the interaction with NAP1L1, as the binding with NAP1L1 was abolished by alanine substitutions in these sites. Interestingly, when serine 452 and 454 were mutated to glutamic acid, leaving the serine at position 457 intact, the binding was restored. As Tellinghuisen et al., already hypothesised, the phosphorylation of serine 457 could represent a priming event for the consequent phosphorylation of other serines, and drive NS5A to a hyper-phosphorylated state.

An increasing number of reports are showing that NS5A hyper-phosphorylation negatively correlates with RNA replication, which is thought to be due to interaction with VAP-A, a cellular factor involved in RNA replication (Evans et al. 2004). Moreover Masaki et al., showed that a hyper-phosphorylated form of NS5A is enriched on the surface of LDs, which correlates hyper-phosphorylation with viral particle assembly (Masaki et al. 2014).

Surprisingly, the amino acids that were found to be responsible for the interaction with NAP1L1 as well as their phosphorylated status, mirror completely the interaction occurring between NS5A and core protein as described by Masaki and collaborators (Masaki et al. 2008). Additionally, NAP1L1 was identified as a binding partner of the

core protein (Pichlmair et al. 2012). The presence of NAP1L1 on the surface of LDs in association with core and NS5A was already observed in our laboratory. At this stage it is tempting to speculate that NAP1L1 might be involved in the assembly process, although experimental evidence conducted in absence or overexpression of NAP1L1 indicate that NAP1L1 does not influence HCV infectivity (Cevik et al., unpublished data). In summary, we learned that the same region of NS5A binds independently to core and NAP1L1. The putative requirement of core as an intermediate for NAP1L1 binding to NS5A is excluded by several observations showing binding and co-localization in the absence of core (Cevik et al., unpublished data). Since NAP1L1 binds NS5A also in the absence of core, it is possible that these two interactions are spatially and temporally separated, or that one fraction of NS5A protein targets NAP1L1 and the other is involved in the interaction with core. Additional experiments are required to address this query.

4.1.1. NS5A inhibits NAP1L1 nuclear translocation

As NS5A recruits NAP1L1 to specific aggregates, corresponding to replication or assembly complexes, we reasoned that this control of NAP1L1 localization might interfere with normal NAP1L1 function in the nucleus. NAP1L1 was shown to have a cytoplasmic localization at steady state. Previous studies in the laboratory demonstrated that NAP1L1 at steady state is present in the cytoplasm, but when the cells are incubated with an inhibitor of nuclear export, NAP1L1 gets retained in the nucleus (De Marco et al. 2010). I took advantage of this system to understand if NS5A retains NAP1L1 in the cytoplasm, thus precluding its nuclear activity. Leptomicin B treatment caused an increased accumulation of NAP1L1 in the nucleus in cells replicating the mutant sub-genomic replicon with respect to the wild type (Figure 14). This result suggests that NS5A destabilizes NAP1L1 and inhibit its nuclear translocation potentially preventing its nuclear activity.

4.2 Whole genome transcriptome analysis suggests NAP1L1 involvement in inflammation and innate immune responses.

NAP1L1 has different activities in the cell; it regulates nuclear histone trafficking, cell differentiation, histone assembly and disassembly and most importantly it was shown that NAP1L1 is involved in transcriptional regulation through the interaction with CBP or in the deposition of histone variants such as H2A.Z, known to have a role in transcriptional regulation.

Since I reasoned that NAP1L1 function might be prevented following its recruitment by NS5A to cytoplasmic loci, I opted to perform an RNA-Seq analysis of NAP1L1 depleted cells to shed light on the genes regulated by NAP1L1. RNA Seq data were analysed together with Dr. Danilo Licastro and with his support I was able to identify differences in gene expression in cells depleted of NAP1L1 compared to control cells. In fact, I could observe that only 1% of the total number of reads of the analysis (46623 reads) was differentially modulated in the absence of NAP1L1. Similarly, Ohkuni and colleagues have shown that 10% of all genes exhibited an altered expression profile following a deletion of yeast NAP1 (Ohkuni et al. 2003). Moreover, a simultaneous deletion of plant NAP1 genes with redundant activities, caused a differential expression of around 500 genes which represents approximately 2.5% of the plant genome (Liu et al. 2009b). Considering the amount of genes regulated by NAP1L1, it seems that the chaperone possess a specific role in transcriptional regulation of a subset of genes.

The solely discovery of differentially modulated genes does not provide any additional information unless inserted in a context of a biological process and assigned to a particular pathway or network. Accordingly, I proceeded to evaluate, using an appropriate bioinformatics analysis tool (IPA), which are the cellular pathways and molecular networks specifically affected by NAP1L1 depletion and potentially related to HCV infection.

IPA analysis of both up- and down- regulated genes is consistent with previous findings stating that NAP1L1 regulates cell cycle and differentiation processes (Kellogg & Murry 1995; Gong et al. 2014), considering that pathways related to Cyclines and Cell Cycle Regulation as well as other cellular functions like growth and proliferation, cellular development, cell death and survival and cellular movement were profoundly

affected in the absence of NAP1L1. These results suggest that NAP1L1 strictly controls both cell cycle and proliferation processes.

The differential expression of NAP1L1 regulated genes inferred also with the Molecular Mechanism of Cancer. Interestingly, IPA analysis of up-regulated genes indicated that gastrointestinal, hepatic system disease and lipid metabolisms were affected, arguing for a carcinogenic potential of NS5A. Although we did not focus our work on this aspect it will be interesting in the future to examine the role of particular oncogenes that showed a slight up-regulation by NAP1L1 depletion to understand if NS5A interaction with the chaperon protein can contribute to the progression to cirrhosis and hepatocellular carcinoma (HCC).

NS5A oncogenic potential is thought to be linked with an active yet inefficient host immune response to viral infection that causes liver degeneration and ultimately hepatocellular carcinoma. In the combined IPA analysis the highly affected canonical pathway is represented by the Aryl-Hydrocarbon Receptor (AHR) Signalling pathway. The aryl-hydrocarbon receptor is a transcription factor that for years has been studied exclusively for its ability to mediate toxic effects to various xenobiotics. Only recently a role of the AHR pathway in the innate immune response to viral infection has received more attention and it was demonstrated that virus induced type I IFN response is suppressed by constitutive AHR signalling (Yamada et al. 2016). Importantly, an increased expression of AHR pathway related genes has been showed in HCV induced HCC, (De Giorgi et al. 2009) implying that a suppression of the innate immune response during HCV infection through the modification of the AHR pathway is necessary for the progression to HCC. IPA analysis of up-regulated genes indicated that again the AHR pathway was affected.

This initial indication that NAP1L1 might contribute to the modulation of the IFN response during virus infection was further sustained by the observation that NAP1L1 down-regulated genes are related to inflammatory responses and that a great number of ISGs were down-regulated in the absence of NAP1L1.

Guided by the RNA-Seq results and IPA analysis, I analysed the role of NAP1L1 in the innate immune response, since its down modulation by NS5A could potentially lead to an establishment of a chronic infection.

4.3 NAP1L1 – a regulator of the innate immune response

A manually curated database of Interferon Regulated Genes-Interferome (Rusinova et al. 2013), an intense literature research and a careful inspection of the RNA-Seq data allow the identification of a great number of ISGs such as IFMT3, UBD and GBP2 among others, whose expression was decreased upon NAP1L1 depletion. Moreover, genes directly involved in the transcriptional activation of the IFN-β gene like c-Jun, NF-κB subunit RelA and the recently identified GEF-H1 factor, also showed a decreased expression in the absence of NAP1L1. The transcriptome and IPA analysis indicated a possible role for NAP1L1 in the host immune response, an assumption that needs to be corroborated in a cell culture system.

IFN-α treatment of Huh7-Lunet cells ruled out a possible involvement of NAP1L1 in the second phase of the IFN response relative to the activation of ISGs and the establishment of the antiviral state (Figure 18). Nevertheless it is important to mention that although NAP1L1 does not participate in the ISGs transcription once the pathway is activated by IFN-α, it does control the basal expression of some ISGs. In our RNA-Seq analysis we were only able to detect the most abundantly expressed ISGs that showed a 2 fold change difference with respect to control cells. The qRT-PCR analysis of ISGs showing a lower expression at steady state like IFIT1, IFIT3 or OASL confirmed that NAP1L1 controls basal expression but not induced expression of these ISGs (Figure 18B).

Next I concentrated the efforts to understand if NAP1L1 was involved in the initial phase of the IFN response relevant to the PRR sensing of a viral agonist.

HCV infection triggers the IFN response by activating RIG-I, TLR3 and PKR signalling pathways. The 5'triphosphate and the 3'polyU/UC sequence is recognized by RIG-I, whereas double stranded RNA corresponding to the intermediate of replication is the agonist for PKR and TLR3 activation. Once activated PKR and RIG-I transduce the signal through the adaptor protein MAVS, whereas TLR3 signals are transmitted through the adaptor protein TRIF. These signalling cascades induce the transcription of IFN-β, ISGs (ISG56, ISG60, ISG15 and others) and pro-inflammatory cytokines as a result of IRF-3 and NF-κB activation and their nuclear translocation.

Using the U2OS cell line, in which poly(I:C) transfection is able to activate the RIG-I/MDA5 pathway and induce IFN-β transcription, I could demonstrate that the first arm of the IFN system relative to the sensing mechanism was compromised in the absence of NAP1L1. In order to test if NAP1L1 is specifically involved in the RIG-I/MDA5 pathway or it has a role also in other signalling pathways, I used U2OS cells reconstituted for TLR3 expression. In this case poly(I:C) stimulation of the endosomal bound receptor did not lead to IFN-β transcription but it induced IFIT1 (ISG56) gene expression indicating that the pathway is nevertheless active. The abrogated IFIT1 expression in the absence of NAP1L1 indicated that the chaperone plays a role also in the immune response following TLR3 activation.

In conclusion, NAP1L1 appears to be a novel factor regulating poly(I:C) (Figure 19) and virus (Figure 21) induced type I interferon responses through both the RIG-I/MDA5 and TLR3 pathways.

4.3.1 Mechanism of action of NAP1L1 in the PRR pathway

Few hypotheses about the mechanism of NAP1L1 mediated control of the host innate immune response can be taken into consideration. Firstly, considering NAP1L1 nuclear function, it might regulate the promoter activity of genes like IFN-β or IFIT1, which are directly involved in the establishment of the innate immune response. Secondly, it could regulate the transcription of genes whose products are involved in the RIG-I or TLR3 pathways. Thirdly, as a cytoplasmic protein at steady state, it could modulate the pathway by direct interaction with one or more components of the signalling system.

I started by analysing NAP1L1 occupancy on the IFN-β promoter to understand if it can directly modulate its promoter activity.
Nucleosome assembly and disassembly activities of NAP1L1 have been linked to mechanisms uncoupled from DNA synthesis. In fact, 10% of yeast ORF showed an altered transcription level in cells deficient for NAP1 (Ohkuni et al. 2003). Moreover, a work from Asahara and collaborators showed that p300 associates with NAP1L1 to activate transcription of p300 regulated genes (Asahara et al. 2002). Active transcription is associated with reduced histone density at promoter regions. Walfridson and colleagues have demonstrated that actively transcribed genes recruit CHD chromatin

remodelling factors and NAP1 to promote nucleosome disassembly (Walfridsson et al. 2007). NAP1L1 chaperoning activity observed during gene transcription could be coupled with IFN-β expression following virus infection or poly(I:C) stimulation. Therefore the most reasonable approach was to analyse NAP1L1 occupancy on the IFN-β promoter gene during its activity. If NAP1L1 operate as a positive regulator of IFN-β gene expression it should be enriched on the promoter region following virus infection (i.e. pathway activation).

The formation of the IFN-β enhanceosome, required for the efficient transcription of the cytokine, is an ordered and progressive assembly of transcription factors on the promoter region of the gene. Within two hours of infection with Sendai virus, the p65 subunit of the NF-κB is recruited to the promoter region, followed by the recruitment of c-JUN/ATF2 at three hours post infection and IRF-3 at 4 hours post infection. The subsequent CBP association mediates histone acetylation which serves as a signal for the recruitment of SWI/SNF remodelling complex that uncover the TATA box for the efficient initiation of transcription (Agalioti et al. 2000). Based on the fact that NAP1L1 has been proposed to cooperate with SWI/SNF complex for the remodelling of chromatin structure (Li et al. 2012b), that Brg1, a component of the SWI/SNF complex was shown to be present on the IFN-β promoter from 6 hours to 9 hours post infection and that RNA Pol II was recruited slightly before 6 h post infection (Agalioti et al. 2000), I decide to perform ChIP analysis after 5 hours of VSV infection when the presence of IFN-β mRNA transcripts has been confirmed (Figure 23A).

Surprisingly, an equal NAP1L1 distribution was observed on the promoter of both highly and low expressed genes indicated by RNA Pol II occupancy. Moreover VSV infection only reduced but did not alter the distribution of NAP1L1 occupancy on promoter of tested genes. No correlation of NAP1L1 occupancy on promoters of tested genes with active transcription could be appreciated. Although the results from the ChIP analysis were not fully convincing, the suggestion that NAP1L1 was not involved in the modification of the IFN-β promoter activity was further corroborated with an experiment showing that IFN-β expression induced by a constitutively active form of IRF-3 (IRF-3 5D) was preserved in conditions of NAP1L1 depletion (Figure 24). Together these results indicated that NAP1L1 depletion affects a step in the pathway leading to IRF-3 phosphorylation but does not alter any step downstream of IRF-3 phosphorylation.

In order to understand which step of the RIG-I or TLR3 pathway is affected by NAP1L1 depletion I proceeded by dissecting the pathway. In this way I was able to demonstrate that the NAP1L1 activity was confined at the TBK1/IKKε level (Figure 25), which is a convergence point of both TLR3 and RIG-I pathways. These two PRR diverge for the adaptor protein they recruit, but they both utilize TBK1 to trigger the downstream signalling transduction. TBK1 activity results in a direct phosphorylation of its substrate IRF-3, for the establishment of the antiviral response. The kinase activity of TBK1 was demonstrated to be influenced by post translational modification such as phosphorylation or ubiquitination (Ma et al. 2012; Tu et al. 2014) and by the formation of functional TBK1 complexes (Goncalves et al. 2011). Particularly, I have noticed that NAP1L1 depletion reduces TBK1 kinase activity as demonstrated by a reduced IRF-3 phosphorylation at Serine 396 (Figure 26).

Apart from IRF-3, the IFN-β promoter is also regulated by NF-κB. Among various stimuli that trigger NF-κB activation, virus infection is also known to induce IKK-α and IKK-β mediated phosphorylation and consequent ubiquitination of the NF-κB inhibitor, IκB-α, which releases NF-κB dimer and makes it available for the translocation to the nucleus. The results demonstrated that not only the mRNA levels of the p65 subunit of NF-κB were reduced in the absence of NAP1L1 (Figure 17), but also protein levels were drastically diminished when compared to Scramble cells (Figure 27A and B). Thus, NAP1L1 controls p65 constitutive expression. The strength and duration of the NF-κB response is also regulated by post-transcriptional modifications such as phosphorylation of the p65 subunit of NF-κB. We have observed that S536 phosphorylation of p65, which maximizes its transactivation activity, does not seem to be affected by NAP1L1 depletion with an exception at 4h post-transfection, where a slight but significant decrease in p65 phosphorylation was consistently observed. Kinases IKKα, IKKβ, IKKε and TBK1 have all been shown to be involved in S536 phosphorylation of the p65 subunit of NF-κB (Fujita et al. 2003; Sakurai et al. 1999). Although the role of TBK1 and IKKε in NF-κB activation is still enigmatic, it was suggested that TBK1 and IKKε were involved in IL-1 induced p65 serine 536 phosphorylation (Buss et al. 2004). In line with this notion, I could formulate the hypothesis that TBK1 and IKKε, which have already been shown to reduce IRF-3 phosphorylation in the context of NAP1L1 depletion, could be responsible also for the decrease of p65 phosphorylation observed at later time points (4h post-transfection).

Additional experiment will be required in order understand the involvement of NAP1L1 in TBK1 or IKKε mediated NF-κB trans-activation.

Taken together these findings, I can conclude that NAP1L1 is indeed necessary for the establishment of the innate immune response by mediating IRF-3 phosphorylation and regulating NF-κB basal expression levels and late phosphorylation events.

How does NAP1L1 modulate the activity of these two transcription factors? Having confined NAP1L1 activity at the TBK1/IKKε levels, two possible mechanisms of action could be taken into consideration:

a) the direct interaction of NAP1L1 with components of the kinase complex as well as associated factors;

b) NAP1L1 mediated transcriptional regulation of genes encoding factors involved in the kinase complex activity.

Careful inspection of NAP1L1 down-modulated genes led to the discovery of GEF-H1 as a potential candidate for the latter hypothesis. GEF-H1 is a guanine nucleotide exchange factor whose GDP/GTP exchange activity promotes the activation of Rho GTPases. Besides already known to function in cell motility, cell cycle regulation or apoptosis, GEF-H1 has lately been studied for its capacity to mediate antiviral innate immune responses (Zhao et al. 2012). A recent report by Chiang et al., demonstrated that GEF-H1 contribute to a TBK-1 mediated IRF-3 phosphorylation for the enhancement of the antiviral host defence mechanism (Chiang et al. 2014). Interestingly, our RNA-Seq and qRT-PCR data indicated that GEF-H1 expression is highly down-modulated (more than 2-fold with FDR<0,05) by NAP1L1 depletion. If GEF-H1 was involved in RIG-I mediated IFN-β activation, as suggested by Chiang et al., I would expect that a rescue of GEF-H1 activity in NAP1L1 depleted cells would increase the antiviral host response at least for the contribution given by this factor. Surprisingly, overexpression of GEF-H1 in our studies did not enhance TBK1 induced IFN-β expression, precluding in this manner its subsequent usage in the rescue experiment (data not shown). In summary, although we were unable to confirm the contribution of GEF-H1 for the enhancement of RIG-I mediated IFN-β activation, the possibility that other genes not yet identified might be regulated by NAP1L1 and play a role in the pathway still remains. In fact, not only GEF-H1 was down-regulated by

NAP1L1, but we also noticed a down-modulation of other genes involved in the IFN-β expression like the already mentioned p65 subunit of the transcription factor NF-κB and c-JUN onco-protein, both necessary together with IRF-3 for the efficient IFN-β expression. Additionally, NAP1L1 might control the transcription of other genes involved in the pathway that were overlooked in the RNA-Seq analysis due to their increased expression exclusively following viral agonist recognition and pathway activation. ChIP-Seq analysis in poly(I:C) stimulated conditions or virus infection will be necessary to identify putative components of the pathway whose expression might be regulated by NAP1L1.

Unless stimulated to translocate to the nucleus, NAP1L1 is localized predominately in the cytoplasm where it could modulate IFN-β expression by direct interaction with components of the PRR signalling pathway. NAP1L1 does not possess any enzymatic activity to regulate the activation of the components of the pathway, but its various post-translational modifications such as phosphorylation or ubiquitination (Calvert et al. 2008; Bish & Myers 2007) could potentiate the interaction and modification of the signalling pathway. Although NAP1L1 activity has been linked to TBK1/IKKε levels, the kinase complex does not seem to interact with NAP1L1 (Figure 28). Surprisingly, a very preliminary result indicated that NAP1L1 is associated with the adaptor protein MAVS (Figure 29). Future experiment will be needed to determine the outcome of this interaction and establish if the binding to MAVS is required for an efficient IFN-β expression. For that it will be necessary to define the binding region of NAP1L1 to MAVS to create mutants that will reveal if NAP1L1 interaction with MAVS is necessary for an efficient downstream signalling transduction pathway. TLR3 and RIG-I pathways have in common different signalling intermediates. Nevertheless it is widely accepted that the convergence initiate at the TBK1/IKKε and IKKα/β level for the activation of IRF-3 and NF-κB respectively. The observed interaction between NAP1L1 and MAVS does not explain how NAP1L1 deficiency could affect TLR3 pathway since MAVS is supposed to be specific for the RIG-I/MD5A pathway. A possible link between these two pathways was already proposed in one of the pioneering work on MAVS as an adaptor protein where Xu and co-workers described MAVS (also called VISA) as part of the TLR3 mediated signalling (Xu et al. 2005). This concept was re-analysed in the following years by the group of Zhang et al., who recover the interaction between TRIF and MAVS in un-stimulated and poly(I:C) treated mDC (Zhang et al.

2011), and the group of Lakhdari et al., showing that a MAVS isoform is involved in the control of the TLR3 pathway (Lakhdari et al. 2016). Moreover, a recent report showed that the TBK1 and IRF-3 are recruited to the adaptor proteins MAVS and TRIF to form a larger signalling complex, and following an ordered phosphorylation events induce the IFN expression (Liu et al. 2015). Altogether, these results support the idea that MAVS and TRIF have intersecting activities in the dsRNA induced signalling pathway, which makes NAP1L1 a perfect candidate to modulate cellular responses directed by both RLR and TLR3 receptors.

To summarize, we propose that NAP1L1 has multiple strategies to regulate the IFN response albeit the exact mechanism needs still to be elucidated. In particular, the NAP1L1 mediated modulation of IRF-3 and p65 phosphorylation could be achieved by its direct involvement in the MAVS signalling complex for the activation of TBK1 and IKKε kinases or by a transcriptional regulation of genes involved in the signalling pathway such as GEF-H1. Additional strategies to control the antiviral signalling pathway concern the transcriptional regulation of the p65 gene and its consequent protein levels as well as a regulation of ISGs basal expression levels. Collectively these mechanisms promote the expression of IFN and pro-inflammatory cytokines for an efficient eradication of the virus.

4.4 Is NS5A able to modulate IFN expression through NAP1L1?

The above mentioned findings led us to hypothesize that HCV NS5A usurps NAP1L1 to interfere with the IFN expression during viral replication.

Innate immune responses are an essential prerequisite for an efficient HCV clearance. However, in 80% of individuals with an acute HCV infection, the inability to control virus replication leads to a chronic hepatitis infection that can ultimately progress to a most severe form of liver disease. The mechanism governing the high frequency of chronic infection is not well understood, but it is thought to be related to the ability of the virus to supress the host innate immune response.

HCV interferes with various aspects of the innate immune system. Besides modulating the PRR and interferon signalling pathways, it supresses ISGs mediated antiviral activities. The virus most powerful activity in regulating the innate immune evasion is

given by the NS3/4A protease action on MAVS (Meylan et al. 2005; Baril et al. 2009) and TRIF proteins (Li et al. 2005a). Apart from HCV, other hepatotropic viruses like hepatitis A (HAV), have been shown to target and cleave MAVS adaptor protein to control the host immune response (Yang et al. 2007). Unlike HCV, HAV infection does not progress to chronicity. This would implicate that mechanism beyond MAVS cleavage are necessary to establish a chronic infection. In line with this hypothesis, other HCV non-structural proteins are involved in the regulation of the innate immune response. Core has been show to interfere with the JAK-STAT signalling pathway preventing the induction of ISGs following HCV infection (Lin et al. 2006); NS4B was shown to suppress STING mediated interferon response (Nitta et al. 2013); NS5A induces IL-8 expression, a chemokine known to interfere with the antiviral activity of interferon (Polyak et al. 2001), interacts with IRF7 to supress the IFN-α activation (Chowdhury et al. 2014), blocks STAT-1 phosphorylation (Lan et al. 2007; Kumthip et al. 2012) and together with E2 suppresses PKR activity (Gale et al. 1997; Taylor et al. 1999),

Collectively all these strategies contribute in the maintenance of a depressed innate immune signalling system which ultimately leads to a chronic infection. Here I propose another strategy employed by HCV NS5A protein to subvert the innate immune response. In fact I could show that NS5A overexpression alone could inhibit IFN-β promoter activation to a higher degree when compared to NS5A protein unable to bind NAP1L1 (Figure 30C). These findings suggested that NS5A interaction with NAP1L1 prevent NAP1L1 mediated IFN-β activation. Our next approach was to validate this finding in a more physiological environment that would resemble what is occurring during the HCV life cycle. Unfortunately, studying HCV related innate immunity is a challenging task since a cellular model that is commonly used to replicate the virus, Huh7 cells and derivatives do not possess an active innate immune system. In order to understand if the replication of the virus with a mutant form of NS5A would be more prone in inducing IFN-β expression I had to generate an HCV permissive cell line with an intact PRR signalling pathway. To this end, I have adopted various strategies.

In a first attempt to demonstrate this hypothesis, I used as a background for the experiments Huh7-Lunet cells which are highly permissive for HCV replication but lack an antiviral signalling pathway. To obviate this inconvenience I reconstituted the cell line with an active TLR3 signalling pathway. While some reports suggest that NS3/4A

has the ability to cleave TRIF adaptor protein both in cell culture system and *in vitro* and attenuate IFN production (Li et al. 2005a), other results were unable to confirm this data (Dansako et al. 2007). In addition, TLR3 expression in Huh7 cells was shown to be sufficient to induce an antiviral activity upon HCV infection (Wang et al. 2009). These evidences make a TLR3 expressing Huh7-Lunet cell line an ideal candidate for testing the hypothesis. Surprisingly, both HCV replicons activated the TLR3 pathway to the same extent, as demonstrated by the expression of an IRF-3 dependent gene, IFIT1. One possible explanation could be that the activation of TLR3 pathway per se was not sufficient to appreciate the difference in IFIT1 genes expression upon a wild type and mutant HCV replication. Indeed, a crosstalk between RIG-I and TLR3 pathway could be necessary for an efficient IFN-β and pro-inflammatory response. The induction of a TLR3 signalling pathway by a parainfluenza virus 5 (SV5) was shown to up-regulate RIG-I expression and cytokine production (Manuse & Parks 2010).

Consequently, my next approach was to examine NAP1L1 activity on IFN-β expression following viral activation of the RIG-I pathway. To this end, I used U2OS cell line as it possesses an active RIG-I signalling pathway, and I increased HCV replication by overexpressing a liver specific miRNA, miR122, as various reports demonstrated that HCV replication in a hepatoma derived and in non-hepatic cell lines could be increased by ectopic expression of a liver specific miR122 (Fukuhara et al. 2012; Kambara et al. 2012). The results indicated that IFN-β was not induced upon HCV replication, which could be explained by the fact that viral replication in U2OS miR122 was insufficient to trigger the IFN response or by the fact that the virus NS3/4A protease interfered with the pathway activation by cleaving MAVS protein.

The activity of NS3/4A is extremely potent in blocking the IFN response. In order to delineate a difference in the NAP1L1 mediated IFN-β response following its recruitment by NS5A, I have adopted an alternative strategy. The overexpression of the cleavage resistant form of MAVS in Huh7-Lunet cells induced a strong antiviral response upon HCV replication, but it did not emphasize the role of NAP1L1 in the IFN-β activation.

To summarize, the strategies that were adopted to gain insights into NAP1L1 mediated IFN-β activation during HCV replication, were not successful, probably due to the modest effect of NAP1L1 on IFN-β expression. The interaction might not phenocopy

entirely the KD of NAP1L1. The virus may allow low levels of IFN-β signalling in order to control its own replication and prevent the spread to a fulminant state. This will allow the virus to establish equilibrium with the host that will subsequently lead to a chronic infection.
This strategy seems to be applied in a late time of infection as the highest frequency of interaction with NS5A was observed at 72 h post transfection, possibly by filling in the leakage of IFN-β derived from an incomplete cleavage of MAVS or TRIF.

Viral targeting of transcription factors is a common method to control the IFN response. La Crosse encephalitis virus was shown to suppress the interferon production by degradation of the RNA Pol II subunit RPB1 (Verbruggen et al. 2011). Moreover, NS1 protein form influenza A supresses PAF1 mediated transcription of a subset of antiviral genes (Marazzi et al. 2012).
In conclusion, additional experiments with alternative strategies and cellular models are required to demonstrate NAP1L1 mediated effect on the IFN expression during HCV replication. A growing body of evidence indicate a predominant role of type III IFNs in the response to HCV infection. It will important to investigate whether the interaction has an effect on the IFN-λ expression. Huh7-Lunet MAVSR cell seem a good starting model, in which I could down-regulate NAP1L1 and look for a difference in HCV replication and/or type I or type III induction. Alternatively the experiment could be performed in PHHs.

4.5 Conclusion and future perspectives

To summarize, I have investigated the role of NAP1L1 in the activation of the PRR pathway and its involvement in the induction of the innate immune response following HCV replication. HCV NS5A recruitment of NAP1L1 to replication complexes during viral replication limits NAP1L1 nuclear localization thus precluding its nuclear activity. A transcriptome analysis of NAP1L1 depleted cells revealed NAP1L1 involvement in inflammation, innate immunity related pathways and ISGs regulation. Indeed, NAP1L1 was found to modulate p65 basal expression levels and late phosphorylation events, TBK1/IKKε mediated IRF-3 phosphorylation and the consequent IFN-β expression, possibly by different mechanism. The relation between NS5A recruitment of NAP1L1 in the cytoplasm and the NAP1L1 function in the IFN-β expression suggest that the two

events might be linked. In fact, I have demonstrated that NS5A expression is able to inhibit the IFN-β expression in a NAP1L1 dependent manner. Although I was not able to corroborate this effect in cells replicating the viral genome, I hypothesise that this could be an additional strategy employed by the virus to lower the IFN response, which in the case of HCV could be important for a progression to a chronic state of infection. Thereby I propose a model which could explain the NS5A mediated IFN-β attenuation through NAP1L1. During HCV infection NS5A interaction with NAP1L1 in the cytoplasm restricts its nuclear translocation and ultimately the transcription of genes involved in the regulation of the RIG-I and TLR3 pathway for the induction of IFN-β. Alternatively the interaction with NS5A might prevent NAP1L1 translocation and interaction with MAVS for an efficient activation of the PRR signalling pathway. Nevertheless, additional experiments are needed to demonstrate this concept. In order to identify NAP1L1 regulated genes involved in RIG-I and TLR3 signalling pathway a ChIP-Seq experiment could be performed. Moreover, an RNASeq experiment performed in the context of HCV infection could reveal the different modulation of the components of the RIG-I/MDA5 or TLR3 pathway in the presence or absence of NAP1L1. Alternatively, the requirement of NAP1L1 on the adaptor protein MAVS will be investigated during HCV replication in immuno-competent cell line.

Additional experiments that are aimed to investigate more in detail NS5A interaction with NAP1L1 and the modulation of the IFN response will be required. Specifically the identification of NAP1L1 binding region to NS5A will allow me to create mutants that will escape from the interaction and study the related phenotype independently of core interaction.

The RNA-Seq analysis revealed the down-regulation of HCV entry factors such as CLDN1 and NPC1L1. Future experiments will be conducted to understand if during the course of HCV infection NS5A interaction with NAP1L1 causes the down-modulation of these factors possibly to prevent HCV superinfection.

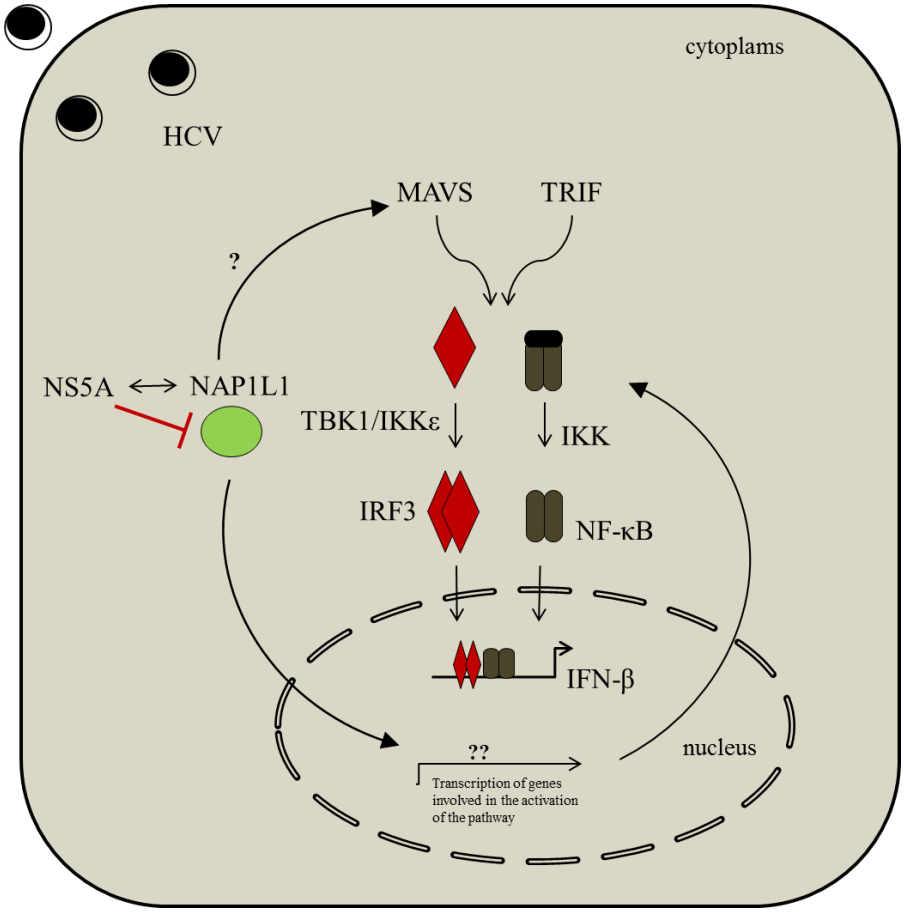

Figure 37. Schematic representation of the NAP1L1 mediated attenuation of the IFN response during HCV infection. During HCV infection (black circles) the NS5A protein interacts with NAP1L1 (green circle) in the cytoplasm. This interaction precludes its nuclear translocation and the transcription of genes involved in the activation of the RLR and TLR3 pathways. Alternatively the interaction might also prevent NAP1L1 recruitment to MAVS containing complexes. Collectively, these strategies impact the NF-κB and IRF-3 activation, their nuclear translocation and in turn block the interferon induction.

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