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Molecular characterization of the hepatitis C virus core protein

PRIYA DEVI

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Abstract

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Hepatitis C virus (HCV) is an RNA virus that causes chronic infection, which can lead to hepatocellular carcinomas in humans. Besides liver diseases, the chronic HCV infection causes a broad spectrum of extrahepatic complications such as lymphoproliferative, metabolic and autoimmune disorders. Notably, HCV encoded core (C) protein is the major virion component that is involved in the oncogenesis and immune subversion. Therefore, detailed molecular characterization of the C protein provides a rational starting point for identification of novel countermeasures against pathogenic HCV infections. In this thesis we have investigated the suppressive effect of the C protein on T cell functions in immortalized cell lines and clinical samples.

In paper I, we found that the expression of the C protein enhanced overall tyrosine phosphorylation in immortalized T cells. Interestingly, stable expression of the C protein specifically reduced accumulation of the tyrosine phosphatase SHP-1 mRNA. Our detailed bisulfite sequencing (BS) studies revealed that the SHP-1 P2 promoter was particularly hypermethylated at CpG1 and proximal islands in these cells. In paper II, we presented a new high-throughput next generation bisulfite sequencing (NGS-BS) protocol for the analysis of locus specific CpG methylation in HCV-infected cells using SHP-1 P2 as a model promoter. In line with our data from the BS, the NGS-BS method showed similar methylation profile at CpG1 island in immortalized cells. Strikingly, peripheral blood mononuclear cells (PBMCs) isolated from healthy controls and HCV-positive (HCV^+) patients, showed much lower levels of methylation at the CpG1 island with no significant difference in DNA methylation pattern. In paper III, we investigated the mechanism of the C protein-mediated release of Ca^{2+} from intracellular stores. We identified two distinct regions in the N- and C-terminal parts of the protein that were essential for activation of the Ca^{2+}/NFAT pathway. Of these, the N-terminal region was required for self-association of the C protein into nucleocapsid-like structures whereas the C-terminal part is essential for anchoring the protein to the ER-membrane. In paper IV, we presented a PCR based diagnostic method for the specific detection of positive and negative strand HCV RNA using primers with a non-viral tag. The method was evaluated by analysing the plasma and PBMC samples from chronic HCV^+ patients.

Taken together, our studies provide more detailed molecular characterization of the HCV C protein functions in immortalized as well as in HCV^+ T cells. Importantly, specific DNA methylation pattern of the SHP-1 gene promoter may function as a potential prognostic marker for the disease progression in HCV-induced tumors. In addition, our updated PCR-based HCV diagnostic method may provide a more specific tool to monitor HCV infections in minor reservoirs.

Keywords: HCV, core protein, SHP-1, CpG methylation, bisulfite sequencing, NGS, PBMC, Ca2+/NFAT, strand-specific RT qPCR

Priya Devi, Department of Medical Sciences, Infection medicine, Akademiska sjukhuset, Ingång 30, våning 1, Uppsala University, SE-751 85 Uppsala, Sweden.

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Dedicated to my parents.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Devi**, **P**., Ota, S., Punga, T., Bergqvist, A. (2021) Hepatitis C virus core protein down-regulates expression of src-homology 2 domain containing protein tyrosine phosphatase by modulating promoter DNA methylation. *Viruses*, 2021;13(12):2514.

II. **Devi**, **P**., Engdahl, K., Punga, T., Bergqvist, A. (2021) Next generation sequencing analysis of CpG methylation of a tumor suppressor gene SHP-1 promoter in HCV-positive patients. (*Submitted to Plos One*).

III. **Devi**, **P**., Punga, T., Bergqvist, A. (2022) Activation of the Ca^{2+}/NFAT pathway by the assembly of hepatitis C virus core protein into nucleocapsid-like particles. (*Submitted to Viruses*).

IV. **Devi**, **P**., Engdahl, K., Bergqvist, A. (2022) Development of a strand-specific RT qPCR assay for specific detection of genomic and antigenomic hepatitis C virus RNA. *Manuscript*.

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Contents

Abbreviations

HIV	Human immunodeficiency virus
nM	Nano meter
UTR	Untranslated region
U	Uridine
Kb	Kilo base
Bp	Base pair
ER	Endoplasmic Reticulum
pH	Potential of Hydrogen
dsRNA	Double stranded ribonucleic acid
G	Guanine
miR-122	Micro RNA-122
g/ml	Gram/microliter
c-myc	Cellular myelocytomatosis
HepG2	Hepatoma G2
p53	Protein 53
Huh 7	Human hepatoma 7
FasL	Fas ligand
CD95	Cluster of Differentiation 95
CD4/CD8	Cluster of Differentiation 4/ Cluster of Differentiation 8
c-Flip	Cellular FADD like IL-1β converting enzyme-inhibitory protein
CIDE-B	Cell death inducing DFFA-like effector B
RIG-1	Retinoic acid inducing gene 1
Bcl-2	B cell lymphoma 2
PI3/Akt	Phosphatidylinositol 3/Ak strain transforming
Th1/Th2	T helper1/ T helper2
RIBA	Recombinant Immuno Blot Assay
IU/ml	International Unit/ microliter
SH2	Src Homology 2
ZAP-70	Zeta chain associated protein 70
PLC γ	Phospholipase γ
LAT	Linker for activation of T cells
Lck	Lymphocyte kinase
Grb2	Growth factor receptor bound protein 2
SLP-76	Src Homology 2 domain containing leukocyte protein 76

Syk	Spleen tyrosine kinase
Ras/MAPK	Rat sarcoma/Mitogen activated protein kinase
IL-2	Interleukin 2
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
Ap-1	Activator protein 1
IRF3/7	Interferon regulatory factor 3/7
STAT3	Signal transducer and activator of transcription 3
HOXA10	Homeobox protein Hox-A10
CagA	Cytotoxin associated gene A
JAK1/3	Janus kinase 1/ Janus kinase 3
qPCR	Quantitative Polymerase Chain Reaction
ATP	Adenosine Triphosphate
Sp-1	Specificity protein 1
RT	Reverse transcription
kDa	Kilo dalton

Introduction

Hepatitis C virus (HCV)

In 1960s and early 1970s viral hepatitis was mainly associated with hepatitis A virus (HAV) and hepatitis B virus (HBV), which caused infectious and serum hepatitis [1]. The third form of viral hepatitis was observed in patients who received the blood transfusion and was not caused by HAV and HBV, and therefore for the time being appropriately named as non-A, non-B viral hepatitis (NANBH). Alter and colleagues collected plasma from patients who received a blood transfusion and developed NANBH and injected it to the chimpanzees that developed chronic hepatitis, and confirming that the bloodborne NANBH was transmissible and caused by a small and enveloped infectious agent [2]. In 1989 Michael Houghton and colleagues succeeded to isolate the virus from the blood of infected chimpanzees and identified its genome by screening a generated lambda library against pools of human sera from NANBH patients. The viral genome of the previously unknown agent NANBH was sequenced and found to the be a positive sense RNA virus that was named hepatitis C virus [3]. This discovery enabled generation of serologic tests that could immediately be used in clinical laboratories for the screening of human blood donors [4]. Rice and colleagues identified the uncharacterized region in the 3'-end of HCV genome, which is required for the virus replication, and generated an infectious clone that caused hepatitis in chimpanzees [5, 6]. Later in the year 2020, the trio scientists Harvey J. Alter, Michael Houghton and Charles M. Rice won the noble prize for the discovery of HCV in the field of medicine and physiology.

HCV is a member of *Flaviridae* family which is further divided into four genera: *Flavivirus*, *Pestivirus*, *Pegivirus* and *Hepacivirus*. The genus *Hepacivirus* includes several species that infect the mammalian species (both domestic and wild), such as cows, dogs, donkeys, horses, colobus monkeys, bats and rodents. HCV is the only known member within the *Hepacivirus* genus that can infect humans [7]. Based on the evolutionary data and differences in their genomic sequences, HCV is divided into 8 distinct genotypes (GTs) and 90 subtypes [8]. Except GT 5, 7 and the recently identified GT 8 [9, 10], all genotypes are classified into several subtypes. The nucleotide sequence between each genotype differs by 30 % whereas the subtypes of the same strains differ by 15 % [11]. The HCV GTs have different geographical locations and their distribution may associate with mode of

transmission, migration and available epidemiological data. GT 1, 3 and 2 are the most common HCV GTs respectively, and is present worldwide whereas GT (4-8) have more specific geographical locations [12-14].

HCV infection

The HCV in humans is spread through blood. Injection drug use is the major source of new HCV infections in the industrialized world. In the developing world however, transfusion of unscreened blood and the use of unsterilized injections or needles in medical facilities are the major risk factors for HCV transmission. Other modes of HCV transmission involve occupational exposure through accidental injury of medical personnel with an infected needle, mother to fetus transmission and sexual encounter mainly via HIV-positive men who have sex with men (MSM) [15, 16]. HCV is known to cause acute and chronic infections in humans and chimpanzees [17]. Acute infection is mostly asymptomatic and develops 1-3 months after initial infection. In 30 % (15-45 %) of infected individuals, the acute infection resolves naturally whereas the remaining 70 % (55-85 %) fail to clear the infection after initial exposure and develop chronic infection. In chronic patients, the virus persists in the blood for longer than six months and often leads to the development of hepatitis marked by infiltration of inflammatory cells in the liver, steatosis marked by an accumulation of fats in hepatocytes leading to fibrosis, which is marked by an accumulation of extracellular matrix protein such as collagen, and cirrhosis, which is an advanced stage of fibrosis associated with decreased liver function. The World health organization (WHO) in their global hepatitis report 2017, estimated that 71 million people globally are infected with chronic hepatitis [18]. The virus can persist in the liver for 30 years or more without any clinical symptoms before progressing to life threatening complications such as cirrhosis and liver cancer. In the absence of treatment, around 20 % of chronic infection progresses to liver cirrhosis where the annual incidence of developing HCV-related liver cancer is < 5 % [19]. The WHO report suggests that HCV infection is the leading cause of liver transplantation [20]. Currently, the mechanism of HCV-related hepatocellular carcinoma (HCC) is unclear but increasing evidence suggests an important role of HCV core (C) protein. The multifunctional C protein alters different signaling pathways by interacting with cellular proteins and induces oxidative stress and is also directly involved in steatosis [21, 22]. Although HCV infection is the major risk factor for the development of HCC in low-rate HCC areas due to cirrhosis, other factors such as age, alcohol consumption, obesity, diabetes, genotype as well as coinfection with HBV and HIV are also important factors contributing to cancerogenesis [23].

Although HCV is primarily a liver pathogen, several studies have reported the presence of viral nucleic acid and antigen in the peripheral blood

mononuclear cells (PBMCs) and liver-infiltrating lymphocytes [24-26]. Both strands of HCV RNA, positive (genomic) and negative (replication intermediate), were found in the PBMCs of chronic HCV patients reflecting a possible viral lymphotropism [27]. The use of extrahepatic proliferation could therefore be a virus strategy to escape the host immune response, a reservoir for persistent infection and a source of resistance to antiviral therapy [28]. Mixed cryoglobulinemia (MC) is one of the extrahepatic disorders that are best associated with HCV chronic infection. Cryoglobulinemia is the presence of abnormal circulating immunoglobulins (I_g) in the serum that precipitate in the blood below 37 °C. Based on their I_g composition and its association with the rheumatoid factors (RF), cryoglobulins are categorized into three types: I, II and III. Whereas type I is characterized by a single antibody of monoclonal origin, type II is characterized by polyclonal I_gG with monoclonal RF and type III by polyclonal I_gG and polyclonal RF. Type II and III are known as mixed cryoglobulinemia [29]. The clinical manifestation of MC affects the skin (purpura), joints (arthralgia), kidney (glomerulonephritis) and peripheral nervous system (neuropathy) [29, 30]. HCV infection is also associated with malignant lymphomas, especially with B cell non-Hodgkin lymphomas (B-NHLs), that may link it to MC [31, 32]. The most common B-NHLs associated with HCV chronic infection are marginal zone lymphomas (MZLs) and diffuse large B cell lymphomas (DLBCLs). Although the mechanism associated with HCV induced lymphogenesis is poorly understood, three possible mechanisms may involve 1) the continuous stimulation of the lymphocyte receptors by a viral antigen that might trigger consecutive proliferation 2) direct expression of oncogenic proteins such as core and NS3 may induce transformation by inducing oxidative stress in the cell, and 3) permanent B cell damage caused by the chronic inflammation that may lead to the mutation of proto-oncogenes and tumor suppression genes [33-36].

HCV structure

HCV is a small, enveloped virus and the genome contains single-strand positive sense RNA as genetic material (Fig. 1). The HCV virus particle is pleomorphic and the serum of HCV infected individuals carries three forms of virus particles including free virions, virions with envelope glycoproteins and virions associated with very-low-density and low-density lipoproteins. Depending on whether the virus particle is associated with the cellular lipoprotein which is the hallmark mark of HCV, the virus particles differ in size, buoyant density and infectivity. The enveloped virus particle is 56-65 nm in diameter while the size of the viral core is 45 nm [37].

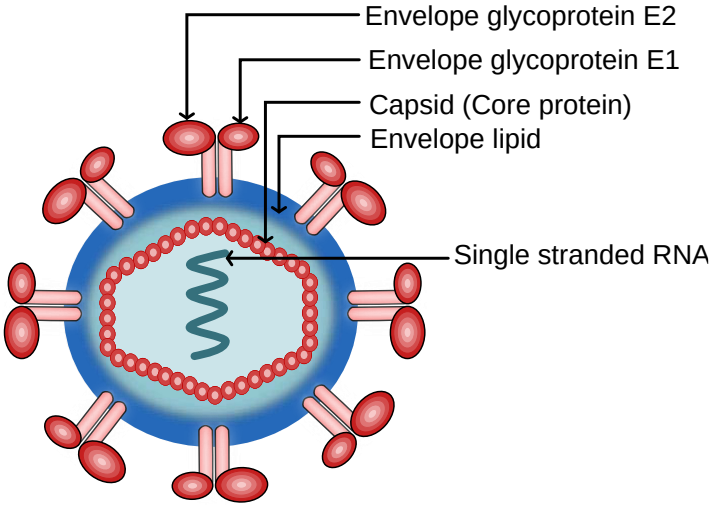

Fig. 1. Structure of HCV. The heterodimers of envelope glycoproteins E1 and E2 are embedded in the lipid membrane. The core protein forms the viral capsid around the single stranded RNA genome.

The viral genome is approximately 9,600 nucleotides long and is flanked by highly conserved non-coding 5´ UTR and 3´ UTR regions. The UTR regions contain tertiary RNA structures and play a role in replication and translation. The ribosome binds to the internal ribosome entry site (IRES) at 5´ UTR and the translation occurs in a cap-independent manner [38]. The 3´UTR plays a role in initiation of RNA replication and consist of three regions, a variable 40 nucleotide sequence, a poly (U/UC) pyrimidine tract followed by a highly conserved 98 nucleotide X-tail with stable stem loop structures [39]. The coding region of 9,600 bp encodes a single polyprotein of about 3000 amino acids (aa) that are processed by viral and host proteases. Processing of the polyprotein produces 3 structural proteins, which forms the virion structure, core (C) and the envelope protein E1 and E2, and 7 non-structural (NS) proteins that are vital for replication and assembly: p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 2).

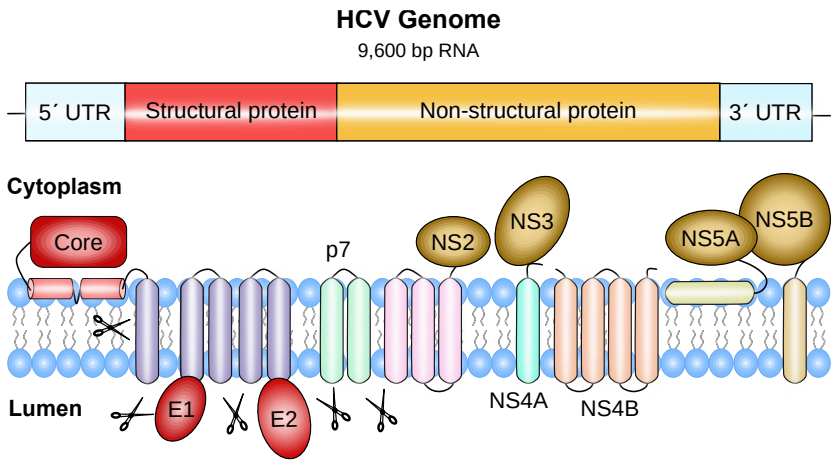

Fig. 2. Genetic organisation of HCV. The central positive strand RNA genome is flanked by non-coding 5´ UTR and 3´UTR regions. Single polyprotein of around 3000 amino acids is encoded by the RNA genome. The structural proteins are synthesized by the N-terminal part of the RNA and rest of the RNA encodes for non-structural protein. The polyprotein processing occurs in transmembrane domain and ER lumen by host and viral proteases. The location of structural and non-structural proteins is presented in the scheme. The scissors indicate the cleavage site for proteases.

The C protein is a highly basic protein, which is synthesized at the amino terminal part of the open reading frame. After processing by host specific proteases at ER, multiple copies of mature C protein form the nucleocapsid around the RNA genome [40]. The nucleocapsid is surrounded by a host derived lipid membrane in which the E1 and E2 glycoproteins are anchored through their transmembrane domains and interact with cellular receptors to gain entry inside the cell. The E1 glycoprotein has the fusion property whereas the E2 contains two hypervariable regions (HVRs), HVR1 and HVR2, that due to frequent mutations help the virus to escape the host immune responses and establish persistence [41, 42]. The p7 is a viroporin that possesses ion channel activity and enhances infectious virus production by translocating the NS2 to the assembly site [43]. NS2 is a hydrophobic transmembrane protein, which functions as cysteine protease by cleaving the polyprotein at the junction between NS2 and NS3. Although NS2 itself is not required for the replication, the efficient cleavage between NS2/3 is crucial for the HCV replication. NS3 is a bifunctional protein with an amino terminal protease and carboxy terminal helicase function [44]. Together with NS4A, NS3 cleaves the polyprotein downstream of NS3 through its protease domain and is required for the formation of replication complex, whereas the helicase domain helps in replication by unwinding the RNA secondary structures [45]. NS4A is a short transmembrane protein and functions as a protease cofactor for the NS3. NS4B is a small hydrophobic protein that is involved in the

formation of the membranous web, a replication site on ER by inducing the membrane alterations [46]. NS5A is a multifunctional phosphoprotein that interacts with both cellular and viral proteins. Similar to the NS4B, NS5A is one of the major proteins involved in the formation of membranous web. It not only binds to the viral RNA but also interacts with C protein on lipid droplets and has thus an essential role in virus replication and assembly [47, 48]. NS5B is an RNA-dependent RNA polymerase that synthesizes RNA by using an RNA template and lacks the proofreading and error correcting function commonly found in DNA polymerases [49].

HCV life cycle

The HCV particle is transported to the liver via the bloodstream. HCV entry is a multistep process involving a series of cellular receptors and attachment factors. The HCV particle associates with the lipoproteins to form infectious lipo-viroparticle of low-buoyant density, which compared to other enveloped viruses is a unique feature of HCV [50]. Apart from masking the virus glycoproteins and thus escaping the host immune system, viral lipoprotein also facilitates the virus entry via attaching to the low-density lipoprotein receptor (LDLR) and glycosaminoglycans present on the heparan sulfate proteoglycans (HSPGs). After the initial attachment, the four main cell surface receptors that are crucial for the entry of HCV particles are scavenger receptor class B type 1 (SR-B1), human cluster of differentiation (CD81), tight junction protein claudin-1 (CLDN1) and occludin (OCLN) [51]. SR-B1 is the first entry factor which interacts with the E2 glycoprotein. It is also a receptor for virus associated lipoproteins and changes the lipid composition of the virus particle to unmask the binding site for CD81 on E2 glycoprotein. The cell surface protein CD81, a member of tetraspanin family, interacts directly with the core sequence of E2 glycoprotein and primes the HCV envelope protein for low pH-dependent fusion. CD81 interaction with the CLDN1 forms CD81-CLDN1 coreceptor complex that activates the downstream signaling pathway to facilitate the virus entry. The tight junction proteins CLDN1 and OCLN are involved in the late stages of virus entry. Although their precise role is unclear, cell-to-cell infection likely via tight junctions is expected to be involved [52]. Similar to the *Flaviviruses* and *Pestiviruses*, HCV enters the cell by clathrin-mediated endocytosis in a pH-dependent manner [53]. The low pH inside the endosome triggers fusion of the viral envelope with the endosomal membrane. Although the fusion process is poorly characterized, evidences suggest that both E1 and E2 might be involved in the fusion process [52, 54, 55].

The viral non-structural proteins NS3-NS5B (NS3, NS4A, NS4B, NS5A and NS5B) together constitute the replicase complex and play an active role in virus replication. Since all HCV proteins are directly or indirectly associated with the intracellular membrane, the virus creates a special

compartment to replicate its RNA genome by massive rearrangement of ER membrane, the so-called membranous web (MW). The MW is predominately a double membrane vesicle (DMV) structure, an ER protrusion formed in cytoplasm close to the lipid droplets as observed by electron microscopy and 3D reconstruction [56-58]. Although DMVs are the major replication site in cultured cells, clusters of single membrane vesicle (SMV) have been observed in liver tissue of HCV infected patients [59]. The two viral proteins NS4B and NS5A are the major players in the biogenesis of DMVs. NS4B is proposed to form scaffold of MW due to its ability to self-oligomerize whereas NS5A is capable of inducing the DMV formation alone by remodeling the ER membrane through its amino terminal alpha helix and also by activating the phosphatidylinositol-4-kinase III (PI4KIII), which is required for the membrane integrity [60-62]. Formation of a separate replication compartment not only shields the dsRNA from the cytoplasmic innate immune sensors but also creates a platform to bring together the replicase proteins, virus genome and host proteins necessary for replication [59]. Inside the membranous web, NS5B, which is an RNA dependent RNA polymerase, copies the positive (+) strand genomic RNA to generate the negative (-) strand RNA. The (-) RNA is a replication intermediate and template for the synthesis of more (+) strand RNA copies for the packaging of progeny virus [51]. Due to the lack of proof-reading activity, the polymerase is highly error prone and has a high mutation rate of approximately 10^{-3} error/site for G: U/U: G mismatches [49]. During genomic replication, the lack of error correcting feature of polymerase is therefore responsible for the genetic heterogeneity and formation of quasispecies. Viral quasispecies are the presence of different viral sequences of a same genotype in an individual that are 90-99 % identical at the nucleotide level. Quasispecies provide the virus adaptive advantage over the parent strain and also serve to escape the host immune responses [63]. In addition to the viral proteins, certain cellular proteins also enhance the HCV RNA replication by either binding to the 5´ and 3´UTR, NS3, NS5A and NS5B [56]. Besides its role in translation, liver specific miR-122 also facilitates HCV replication by stabilizing the RNA from degradation by exonucleases [64].

Due to the overall low assembly efficiency and pleomorphic nature of the virus particle, very little is known about the assembly process and release of HCV virion. Initiation of assembly occurs by the synthesis of nucleocapsid, which is proposed to either occur at the surface of cytosolic lipid droplets (cLD), where the core protein is located, or at the ER membrane. In the latter case, the core protein is transported from cLD to the assembly site on ER membrane with the help of a microtubule, where it interacts with the replicase complex. Further, whereas the packaging event of HCV RNA into capsids is unknown [65], it may likely involve the interaction of HCV RNA with the highly basic domain (D1) of core and subsequent oligomerization of core to form the nucleocapsid around the viral RNA [66]. However, trans-encapsidation study suggests that the packaging of HCV may involve protein-

protein interaction rather than a protein-RNA interaction [67]. The envelopment of the HCV particle occurs by budding at lipid rich microdomain at ER through the interaction of domain 1 of core protein with E1 glycoprotein [68, 69]. The virus particle undergoes maturation by passage through the Golgi apparatus and secretory pathway before release into the circulation [37, 50, 70]. HCV particles are often bound to serum lipoproteins in the bloodstream, VLDL (very low-density lipoprotein) and LDL (low-density lipoprotein), and are called lipoviroparticles (LVPs) [71]. The association of HCV particle to the lipoproteins thus account for the low density, which is inversely correlated to the infectivity. HCV particles differ in their size and densities depending on the type of host cell they are produced. The densities of the circulating HCV particle in patient sera ranges from < 1.06 g/ml to 1.25 g/ml [72]. However, virus particles derived from the infectious cell culture system (HCVcc particle) have an average density of ~ 1.1 g/ml and are less infectious compared to the serum derived HCV particles. Although the association of virus particle to lipoproteins is unclear, the virus particle resembles the LDL and VLDL in their lipid composition with a high proportion of triglycerides and apolipoproteins (ApoB and ApoE) as the major components [71, 73].

HCV core (C) protein

Structure of C protein

Among all HCV proteins, C is the first protein to be synthesized from the HCV genome which is followed by E1 on ER. C is a highly basic RNA binding protein that constitutes the viral nucleocapsid by making multiple copies of mature C to package the viral genome [40, 74]. HCV structural proteins are stabilized by processing through host proteases. The first cleavage occurs between amino acid 191 and 192 by host signal peptidase (SP) to form 191 amino acid immature form of C protein, also called p23. The p23 is the precursor protein and contains the signal sequence that targets the E1 to ER. The exact position for the second cleavage is unknown but believed to take place between amino acid 173 to 179 by the ER-transmembrane resident signal peptide peptidase (SPP) to form mature C of approximately 177 amino acids, also known as p21. After SPP cleavage, the mature C is translocated to the surface of a lipid storage body, so-called lipid droplet to be used later during assembly process. The p23 and p21 are the two major C species where p21 is the predominant form detected in cultured cells and infected sera [75]. In addition, other alternative forms of C have also been detected, including the alternative reading frame protein (ARFP), also known as p17, and minicore, also known as p8 [76, 77]. Although the three-dimensional structure of core is unknown, but on the basis of distribution of charged amino acid residues

the precursor C (p23) protein can be divided into three domains. The N-terminal domain 1 (D1) consists of residue 1-117 and is rich in basic amino acid, domain 2 (D2) comprises amino acids 118-177 and is more hydrophobic than D1, and domain 3 (D3) spanning from residue 118-177, which contains the signal sequences for E1 protein and is the most hydrophobic (Fig. 3).

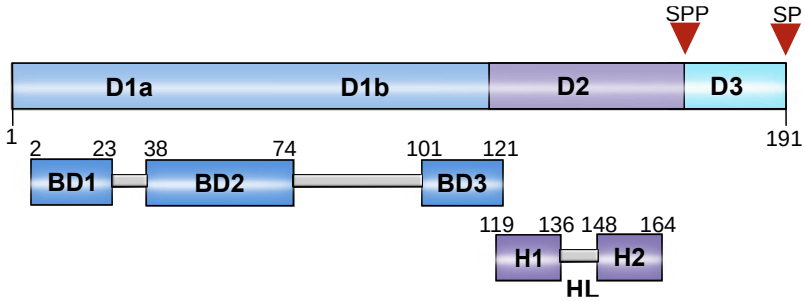

Fig. 3. Structural domains of HCV core (C) protein. The initial processing by signal peptidase (SP) in ER lumen generates an immature p23 form of C protein (1-191 amino acid), the second cleavage by transmembrane signal peptide peptidases (SPP) releases the mature p21 C protein (177 amino acids). The mature C protein lacks domain 3 (D3) and consist of a distinct N-terminal domain 1 (D1) and C-terminal domain 2 (D2). The immature C protein on the other hand contains an intact domain 3 (D3) region in addition to the domain (D1) and domain 2 (D2). The D1 is further divided into three basic domains (BD) as represented by BD1, BD2 and BD3. The D2 contains two helixes, helix 1 (H1) and helix 2 (H2) which are joined by a hydrophobic loop (HL).

The mature C (p21) is a dimeric alpha helical protein and contains domain 1 and domain 2. Domain 1 of mature C protein is involved in interaction with viral RNA and self-oligomerization to form the HCV-like particles. The C protein is mainly present on the ER membrane and the surface of LD but it is also found in the nucleus and mitochondria. The proteolytic cleavage by signal peptide peptidase is crucial for the association of C to the surface of lipid droplets, which is mediated by the alpha helixes present in domain 2 [78].

Function of C protein

Besides its function in synthesis of packaging material for the viral genome, C protein also has additional roles in HCV pathogenesis including apoptosis, cell growth, immune modulation, lipid metabolism and transformation. As HCV infection is the major risk factor for hepatocellular carcinoma (HCC), the C protein is directly involved in epigenetic changes in HCC. Although it is debatable whether C protein can function as an oncoprotein, this is indirectly implicated by its ability to modulate the expression of proto-oncogenes and tumor suppressor genes by dysregulation of different cellular signaling pathways [21, 79, 80]. One of the mechanisms responsible for the

inactivation of tumor suppressor function involves epigenetic modification of tumor suppressor genes through aberrant methylation of their promoters. Transactivation of the *c-myc* promoter and suppression of *c-fos* promoter activity by C protein was detected in HepG2 cells [81]. Studies from cultured cells indicate that the C protein suppresses the promoter activity of a well-known tumor suppressor gene p53 [82, 83]. However, compelling evidence supporting the role of C protein in oncogenesis came from the studies in transgenic mice. Development of HCC was detected between 16-19 months in C expressing transgenic mice with an incidence of ~ 30 % compared to control mice with 0 % incidence [84]. C protein suppressed the levels of a cell cycle regulator gene *p16* in HepG2 and Chang liver cells by hyper-methylating the p16 promoter and thereby increasing cell growth [85]. Independent studies from three different groups showed that C protein epigenetically silences genes that are involved in cell migration, such as *E-cadherin* and *SFRP1* (secreted frizzled-related protein), by hyper methylating the gene promoter in HepG2 and Huh7 cells [86-88]. The reduced levels of *E-cadherin* and *SFRP1* increased the epithelial to mesenchymal transition (EMT), a process associated with enhanced invasion property, and thereby increased the tumor aggressiveness and entry into the bloodstream. Apart from its involvement in hepatocarcinogenesis, another study showed that the C protein escapes its degradation by silencing the E6 associated protein (E6AP) expression, a ubiquitin ligase that ubiquitinates the target protein for proteasomal degradation, and thus facilitate virus propagation [89]. The methylation marks on the DNA are established and maintained through the action of DNA methyltransferases (DNMT) and HCV is known to interact with these enzymes [90-92].

HCV pathogenesis

Apoptosis

Apoptosis is an important cellular process that takes place during development and aging in the physiological state to eliminate the infected cell. However, in pathological conditions, such as virus infection many viruses prevent the apoptotic pathways to facilitate their own survival. Two major pathways involved in apoptosis are 1) intrinsic or mitochondrial apoptosis pathway, and 2) extrinsic or death receptor pathway. Mitochondrial apoptosis pathway involves the release of cytochrome c through the Mitochondrial Permeability Transition (MPT) pore followed by activation of initiator (caspases-9) and effector caspases (caspase-3, -6 and -7) that executes the cell death program. The extrinsic apoptosis pathway occurs through the interaction of extracellular ligands such as FasL/CD95 and TRAIL to the death receptor in the transmembrane that transfer the death signal through the death domain and

triggers the caspases (initiator caspases-8 & effector caspases-3, -6, -7) to mediate the cell death [93, 94]. In chronic HCV infection, liver pathology is associated with increased apoptosis and higher levels of immune related death ligands CD95/Fas, Tumor Necrosis factor α (TNF α) and Tumor Necrosis Factor Related Apoptosis Inducing ligand (TRAIL) [95-99]. Despite the increased apoptosis in the liver, it is still unclear whether the virus infected hepatocyte or the uninfected bystander cells are undergoing apoptosis [100]. Direct involvement of HCV in inducing apoptosis is controversial but studies showed that depending on type of cell line, HCV strain and experimental conditions, the HCV proteins have both pro-apoptotic and anti-apoptotic effects [95]. Effects of C protein on apoptosis show somewhat conflicting results, whereas C in hepatoma cell line inhibits apoptosis induced by CD95 and TNF α , in other studies including transgenic mice C cannot prevent CD95-induced apoptosis [100, 101]. C can also block apoptosis by upregulating the expression of a negative regulator of apoptosis c-Flip by inhibiting the caspase-8 [102]. On the other hand, an increased amount of reactive oxygen species (ROS) generated by C-mediated oxidative stress resulted in enhanced apoptosis by sensitizing the cells to other apoptotic stimuli [103]. In addition to C, other HCV structural proteins E1 and E2 inhibit the TRAIL-induced and caspase-dependent apoptosis, respectively [104]. Whereas NS2 prevents CIDE-B induced apoptosis, NS3 can prevent RIG-1-induced apoptosis by cleaving the adaptor protein Cardif, while NS4A can induce caspase-8 independent apoptosis [105-107]. Among the HCV NS proteins, NS5A was shown to exert both proapoptotic effects by blocking the bax-mediated apoptosis and antiapoptotic effect by mimicking the apoptosis regulator bcl-2, inhibiting p53-mediated apoptosis and activating the cell survival PI3K/Akt pathway [108-110]. Although data from *in vitro* experiments suggest that the virus proteins have important but complex functions in apoptosis, data on *in vivo* situation are lacking due to the very low expression of viral proteins in infected patients and therefore might not reflect the *in vitro* situation.

Immunopathogenesis

Liver damage caused in HCV infection is primarily attributed to local immune responses to virus infection mainly involving the participation of CD4+ and CD8+ T cells. Although the increased liver injury is associated with local expression of CD4+ T cell, which is composed mostly of IFN-γ producing Th1 phenotype, an important role of non-interferon γ producing cytotoxic CD8+ T cells (CTLs) response is also witnessed by killing both infected and bystander cells through Fas/FasL, TNF-α and perforin mediated mechanism [98, 111-113]. Also, increased recruitment of regulatory T lymphocytes to the liver of chronic HCV patients that suppress the HCV specific CTL responses

supports a dual role of CTLs in controlling infection and liver pathology [114, 115]. In addition, the CD4+ natural killer T (TNK cells) and T cell receptor (TCR) γδ cells might also be involved in controlling the infection and disease progression [116]. The direct cytopathic effect of HCV is controversial due to the lack of correlation between the viral load and severity of the disease. However, increased apoptosis and accumulation of fats in liver, so called steatosis, is directly linked to HCV and therefore suggests the direct role of virus in liver pathogenesis. The virus induced steatosis is directly linked to genotype 3 infection only (which upon treatment is reversible), whereas it with infection of other HCV genotypes are associated with other metabolic conditions, such as obesity and alcohol consumption [116, 117]. The role of viral proteins in liver diseases is obtained from *in vitro* studies and their role in natural infection has yet to be explored. The ability of C protein to associate with lipid droplets and regulation of lipid accumulation might serve its role in steatosis [118, 119]. HCV interferes with antiviral signaling through the NS3/4A protease, which shuts off RIG-1-dependent interferon production by cleaving the mitochondrial antiviral signaling protein (MAVS) [106].

Together, both the local immune response and direct viral cytopathic effect are responsible for the pathobiological changes caused in HCV infection.

HCV diagnostic and therapy

Diagnosis

The three markers used for diagnosis of HCV infection are 1) HCV specific antibodies 2) HCV RNA detection 3) HCV genotype identification. The HCV specific antibodies in the blood start to appear after a window period of 60 days characterized by the presence of detectable HCV RNA without HCV antibodies. The HCV specific antibodies can be detected after acute infection (after 60 days) and persist lifelong in chronic patients. HCV specific antibodies can be detected in the body fluids using enzyme linked immunoassay (EIA) and recombinant immuno blot assay (RIBA). In EIA, the antigen is coated onto the wells of microtiter plate or microbeads attached to the automated device. The presence of HCV antibodies is determined by the anti-antibody labeled with an enzyme that converts the substrate into a colored compound. The optical density of the reaction is directly related to the amount of antibodies present in the sample. As being cheap, easy, fully automated and applicable to larger volumes EIAs are preferred test in HCV serology [19, 120, 121]. However, the detection of HCV antibodies does not differentiate between acute or chronic infection from resolved infection as the antibodies in resolved person also last for long. In addition, individuals with immune suppression and coinfection such as HIV infection with HCV RNA are negative for the HCV antibodies. Detection and quantification of HCV RNA

is necessary for confirmation of infection and also for monitoring the virus load during antiviral treatment. HCV genome becomes detectable 1-2 weeks after infection and can be detected by target amplification using polymerase chain reaction and signal amplification using branched chain DNA assays. However, these classical techniques are now replaced with more sensitive real time PCR assays. The real time PCR is fully automated with lower detection limit of 10-15 IU/ml and upper range of quantification of 7-8 $\log_{10}$ IU/ml [120]. HCV has a high genetic diversity due to the presence of error prone RNA polymerase and a high level of replication [122]. Genotype identification is important due to the varying resistance to antiviral therapy and for effective treatment. Therefore, it should be determined before treatment. According to the recent study genotype 1 has the highest HCV cases (46.2 %) followed by genotype 3 (30.1 %), genotype 2, 4 & 6 (22.8 %) and genotype 5 comprising 1 % of all HCV cases [123]. HCV Genotyping is based on direct sequencing or reverse hybridization. Direct sequencing is the gold standard method and it identifies viral variants representing 20-25 % circulating in virus populations [120]. Reverse hybridization is a more sensitive method and detects minor variants as few as 5 % of viral population [124].

Treatment

Therapy for HCV began with the use of interferon α, a compound with broad antiviral effects. Interferons (IFNs) are cytokines that exerts antiviral effects by either targeting the cell surface receptors and thereby blocking the virus replication or by activating the innate and cellular arm of immunity to destroy the infected cell by various mechanisms [125]. The aim of the therapy is to achieve sustained virological response (SVR), which is defined as undetectable HCV RNA in the plasma 24 weeks after the end of treatment. Although IFN administration required parenteral injection and had adverse side effects, it substantially lowered the HCV RNA and serum transferase levels in a significant proportion of the patients. Further, the combination therapy of pegylated (PEG)-IFN α with a nucleoside analogue ribavirin (RBV) yielded higher sustained virological response (SVR) with even serum drug concentration by coupling the IFN to polyethylene glycol (PEG) to extend its half-life. Treatment with combination therapy of PEG-IFNα-RBV eliminated the virus in approximately 50 % of patients infected with genotype 1 after 48 weeks whereas the infection is cleared in 80 % of the patients infected with genotype 2 and 3 with a treatment duration of 24 weeks or even less [126]. With the recent development in HCV replicon and cell culture infectious system, new antiviral HCV treatments with reduced toxicity and shortened duration have been introduced. Currently, several HCV-specific antivirals are in clinical usage and are mainly targeted against three HCV

proteins, 1) NS3/4A serine protease inhibitors 2) phosphoprotein NS5A inhibitors and 3) NS5B (RdRp) inhibitors. The first approved NS3/4A protease inhibitors (PIs) were telaprevir (TVR) and boceprevir (BOC), which both are peptidomimetic and block the polyprotein processing by binding to the serine residue in the enzymes active site. Due to the low resistance barrier nature of monotherapy, the first PIs were administered as triple therapy in combination with PEG-IFN-RBV. The triple therapy yielded high SVR rates in approximately 62 % of patients infected with genotype 1 in phase 2 studies [127]. The first approved NS5A inhibitor was daclatasvir (DCV), which targets the N-terminal domain 1. The inhibitors mode of action is unknown but is proposed to reduce hyperphosphorylation and oligomerization of NS5A and thereby preventing replication and virus assembly [1, 128]. Based on their mode of action, the NS5B inhibitors are grouped into two classes 1) nucleoside and nucleotide inhibitors (NIs) and 2) non-nucleoside inhibitors (NNIs). The NIs are chain terminators that block further RNA synthesis by mimicking the natural nucleotide and incorporating defective analogs into the growing RNA chain during replication. Sofosbuvir, a uridine analogue, is the most advanced nucleotide inhibitor and effective against genotypes 1-6 together with PEG-IFNα-RBV but also effective as IFN free monotherapy against genotype 2 and genotype 3. The second category of NS5B inhibitors, the NNIs, binds to the allosteric sites of the enzyme and inhibits the polymerase activity by preventing conformational change. The approved NNIs against NS5B, such as dasabuvir, have a low resistance barrier and are mostly specific for genotype 1 [1, 129].

Protein phosphorylation and protein tyrosine phosphatase SHP-1

Phosphorylation is the modification of a protein by the addition of phosphate group that occurs after protein synthesis. Protein phosphorylation is a reversible process and one of the most extensively studied post translational modification in eukaryotic cells [130]. The phosphorylation modifies the protein by changing its conformation and has a fundamental role in controlling various cellular processes such as cell growth, metabolism, cell cycle, immune responses, gene expression, development and transformation. Two enzymes, protein kinases (PKs) and protein phosphatases (PPs) are the main switches in the event of protein phosphorylation. The PKs activate the target protein for relay of a signal by adding phosphate group whereas the PPs turn off the activation signal by removing phosphate from the protein molecule. Therefore, equal and balanced action of PKs and PPs are essential for proper control of phosphorylation [131]. In mammalian cells, protein phosphorylation occurs mostly at amino acid threonine, serine and tyrosine

respectively [130]. Although tyrosine phosphorylation is relatively less common but is fundamental for the signal transduction [132]. There are 107 protein tyrosine phosphatase (PTP) genes in humans of which 81 encodes for active phosphotyrosine specific phosphatases. T cells express approximately 50-60 PTPs, which are further divided into transmembrane, receptor PTPs (RPTPs) and cytoplasmic, non-receptor PTPs (NRPTPs) [133]. The RPTPs, such as CD45, CD-148 and SHP-2, are relatively less expressed in T cells and mostly have a positive role in T cell receptor (TCR) signaling [132, 134-136]. The Src-homology region 2 domain-containing tyrosine phosphatase (hereafter as SHP-1, but also known as PTPN6, SHP, SHPTP-1, HCP, PTP1C) is a NRPTP, which negatively regulates the TCR, cytokine and inflammatory signaling by dephosphorylating receptor-associated kinases as well as other kinases [137, 138].

SHP-1 expression and protein structure

The SHP-1 is encoded by the *PTPN6* gene that is present on chromosome 12 in region p12-p13 and consists of 17 exons spanning approximately 17 kb DNA. Two different promoters, promoter 1 (P1) and promoter 2 (P2) are present in SHP-1, which is responsible for distinct expression in different cell types. The transcription start site for P1 is present on exon 1 and controls the SHP-1 expression in epithelial cells [139]. The second transcription start site is present on exon 2, which is approximately 7 Kb apart from the P1 and governs the SHP-1 expression predominately in hematopoietic cells and at low levels in epithelial cells [140]. The two promoters contain the recognition sequences for different transcription factors. Two forms of SHP-1 mRNA transcripts, (I) and (II), which differ at their 5′ UTR and first few coding nucleotides (MLRSG are the first 5 aa of (I) SHP-1 and MVR are the first 3 aa of (II) SHP-1) are produced from promoter 1 and promoter 2, respectively, in humans [141-143].

The SHP-1 protein is a 595 amino acid protein composed of two N-terminal SH2 domains (SH2-N and SH2-C), a single central phosphatase (PTP) domain and a C-terminal tail with two potential tyrosine phosphorylation sites at Y536 and Y564 (Fig. 4) [144]. The SH2 domains interacts with the target proteins in a sequence-specific manner and are essential for localization and regulation [145]. The catalytic function is mediated by its central phosphatase domain. The C-terminal tail is important for its enzyme activity and regulation occurs through the phosphorylation of tyrosine residues [146]. It has been reported that tyrosine phosphorylation of Y536 by insulin through insulin receptor kinase and phosphorylation of Y564 by Lyn increases the phosphatase activity of SHP-1 [145]. A bipartite nuclear localization signal (NLS) is also located in the C-terminal tail [147, 148].

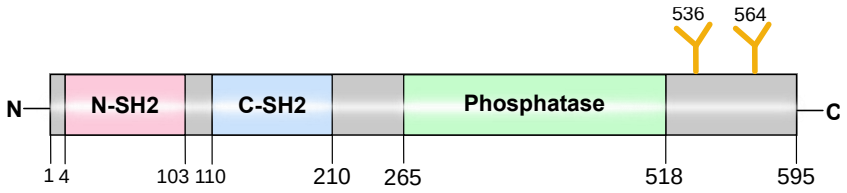

Fig. 4. Structure of SHP-1 protein. Defined domains within the proteins corresponding to the amino acid numbers (bottom) are represented in the scheme. Two potential tyrosine phosphorylation sites at amino acid position 536 and 564 at the C-terminal tail are represented in yellow.

In its physiological state, SHP-1 remains inactive as the binding of the SH2-N domain to the PTP domain blocks the access of substrates to its active site. However, SHP-1 is activated by conformational change upon binding of a phosphotyrosine residue (ligand) to the SH2 domain by disrupting the interaction between SH2-N and the phosphatase domain [144]. Besides having a regulatory role in enzymatic activity, the C-terminus is also involved in the nuclear localization of SHP-1. In hematopoietic cells, SHP-1 is a 68 kDa cytosolic protein but may translocate to the nucleus upon cytokine stimulation [148]. However, in epithelial cells the SHP-1 has a nuclear localization. The C-terminal alternate splice isoform of SHP-1 called SHP-1L is also detected in hematopoietic cells. The splice isoform SHP-1L (70 kDa) is the minor variant, which is 29 aa longer than the major variant SHP-1(68 kDa). SHP-1L lacks a potential tyrosine phosphorylation site at Y564 but retains the Y536 site at its C-terminus [149].

SHP-1 and signal transduction

SHP-1 controls the proliferation, differentiation and function of immune cells by regulating intracellular signaling. SHP-1 is predominantly a negative regulator that negatively regulates the signaling events induced by antigens, cytokines and growth factors [137, 150]. An important insight into the *in vivo* biological function of SHP-1 is explained by the studies conducted on Motheaten mice. In mice, two mutations in the SHP-1 gene generated two phenotypes: 1) *motheaten* (*me/me*) or Me, and 2) viable motheaten (me^v / me^v) or Mev. The Me phenotype demonstrates no SHP-1 protein expression due to the splice mutation in SH2 domain whereas a different splicing mutation causes insertion and deletion of a few amino acids in the phosphatase domain resulting in a Mev phenotype with reduced SHP-1 activity [151, 152]. The Me mice with motheaten allele (*me/me*) are more severe and display many phenotypic defects such as patchy hair loss, skin lesions, arthritis, interstitial pneumonia and death 3 weeks after birth. However, the Mev mice with viable allele (me^v / me^v) displayed similar but less severe defects and eventually died 8-12 weeks after birth. Expansion of myeloid cells due to increased

proliferation and inadequate activation caused pneumonitis and their early demise [145].

Activation of B and T cell takes place downstream of their surface receptors, the B and T cell antigen receptor (BCR and TCR respectively). Antigen stimulation of these receptors generates the signaling cascade that leads to proliferation, differentiation and other biological outcomes [137]. The role of SHP-1 in the negative regulation of TCR signaling is apparent from the *motheaten* mice, where the thymocytes derived from the SHP-1 deficient *me/me* mice hyperproliferate in response to the TCR stimulation and display hyperphosphorylation of different phosphotyrosyl proteins [153, 154].

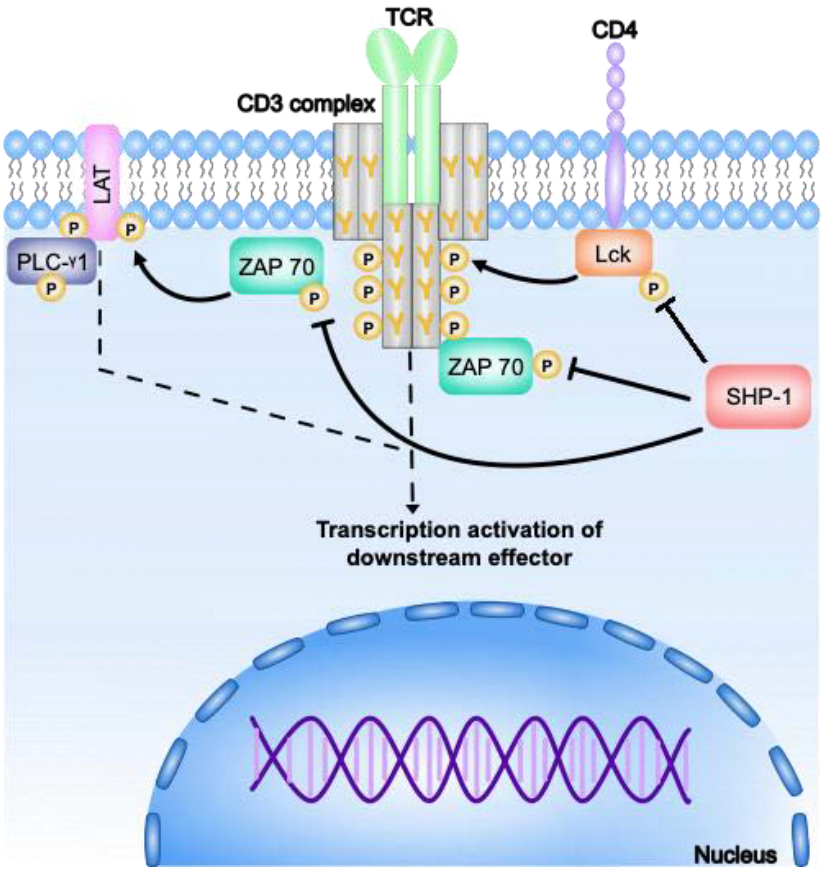

Fig. 5. Regulation of proximal T cell receptor (TCR) signalling cascade through SHP-1. Stimulation of TCR activates the associated kinases which phosphorylate the protein on tyrosine residue (Y). SHP-1 regulates the TCR signalling by dephosphorylating the phosphotyrosine. Arrow and bar represent the activation and inhibition of substrate molecule respectively.

TCR is made up of protein complexes, which have an antigen recognition module composed of α and β chain and a signalling module consisting of

several invariant chains (γ, δ, ε and ζ) of CD3 complex (Fig. 5). The binding of antigen to the αβ chain of the TCR activates the Src-family tyrosine kinases, such as Lck and Fyn, that phosphorylates the CD3 chains at their immunoreceptor tyrosine-based activation motifs (ITAMs). The phosphorylation of ITAMs provides the docking site for other SH2 domain-containing proteins including ZAP-70. Activation of ZAP-70 is a crucial event that is required for the amplification of downstream signalling targets [155]. Intriguingly, activation of TCR by phosphorylation is well characterized but relatively less is known about the negative regulation of TCR by deactivation or dephosphorylation. Presumably, SHP-1 facilitates the negative regulation by dephosphorylating either the phosphorylated ζ or ε chains of CD3 component and several effector proteins including Vav, Grb2 and SLP-76, either directly or indirectly via inactivation of Src-family kinases [153, 154, 156, 157]. SHP-1 also associates with CD5, an inhibitory receptor of TCR through immunoreceptor tyrosine-based inhibitory motifs (ITAMs) present in the cytoplasmic tail of CD5. Likely engagement of CD5 with TCR results in the downregulation of the TCR-induced CD3ζ, ZAP70, Syk and PLCγ tyrosine phosphorylation [158]. Association of SHP-1 with effectors like SLP-76, Vav and Grb2 has a role in the modulation of Ras/MAPK, calcium mobilization and cytoskeletal architecture [159]. SHP-1 controls T cell proliferation by dephosphorylating the β chain of activated IL-2 receptor under mitogen stimuli. This association results in the decreased phosphorylation of associated Janus PTK kinases, JAK1 and JAK3. Under certain pathological conditions such as infection, loss of SHP-1 expression may deregulate the IL-2 receptor signalling and may lead to the transformation of T cells [160].

SHP-1 expression and pathogenic deregulation

SHP-1 is proposed to be a candidate tumor suppressor gene due to its ability to maintain homeostasis by terminating the signaling pathways controlling cell proliferation, survival, migration and invasion [161]. Dysfunction of SHP-1 regulation can cause abnormal growth due to the sustained activation signal from growth promoting tyrosine kinases that ultimately results in different kinds of cancers. Since SHP-1 is highly expressed in hematopoietic and epithelial cells, its expression levels are tightly associated with hematopoietic and non-hematopoietic malignancies. The alterations such as genetic mutations and epigenetic modifications are associated with dysregulation of SHP-1 expression and function in both solid and hematological human cancers. Epigenetic silencing of SHP-1 due to the hypermethylation of CpG island in its promoter is the most common phenomenon in hematopoietic cancers such as leukemia and lymphomas [162-

169]. In addition to CpG hypermethylation, histone modification has also been detected in SHP-1 promoter regulation [170]. Both epigenetic processes CpG methylation and chromatin modification, silences the gene expression by repressing the transcription, either by interfering with transcription factor binding or rearranging the chromatin structure by histone modification.

However, in non-hematopoietic cancers the SHP-1 levels are either increased, as in the case of ovarian, prostate and breast cancer, or decreased, as in some prostate, colorectal and breast cancers with negative expression of estrogen receptors [171]. Therefore, it is conceivable that SHP-1 can either serve as a positive or negative regulator depending on the type of cell it is expressed in or its interaction. Although all three domains of SHP-1 protein are prone to mutations, the phosphatase domain (42.27 %) being the highest, most of these mutations (81.4 %) do not alter SHP-1 function [161]. In addition to the hematological malignancies, the loss of SHP-1 expression due to hypermethylation has also been detected in solid cancers such as hepatocellular carcinoma, esophageal squamous cell carcinoma, gastric adenocarcinoma, breast and endometrial carcinoma [172-177]. Together, these observations indicate that the epigenetic silencing of the SHP-1 is the main underlying mechanism associated with both hematological and solid malignancies.

SHP-1 and virus infection

Many pathogens such as *Leishmania,* bacteria and viruses have been found to exploit SHP-1 for its survival through various mechanisms [178, 179]. However, the role of SHP-1 in virus infection is relatively less known compared to its well characterized role in signal transduction and immune responses against bacterial infection. A recent *in vitro* study on murine primary macrophages has shown that SHP-1 suppresses the antiviral signaling pathway and type 1 IFN production in a DNA virus, herpes simplex virus (HSV 1) as well as RNA virus, vesicular stomatitis virus (VSV) infection, by targeting tumor necrosis factor receptor-associated factor 3 (TRAF3), which is required for the activation of downstream targets such as NFκB, AP-1, IRF3 and IRF7. SHP-1 inhibited the K63-linked ubiquitination (required for theTRAF3 activation) by dephosphorylating the Tyr116 and Tyr446 on TRAF3 and inhibited the IRF3 activation for interferon production [180]. An upregulation of SHP-1 expression with downregulation of TNF-α, a proinflammatory cytokine was detected in chikungunya virus infection in muscle cells [181]. In Theiler´s murine encephalomyelitis virus (TMEV) infection of muscle in mice, SHP-1 is involved in virus-induced myopathy by promoting differentiation of inflammatory macrophages [182]. Infection with this neurotropic virus and associated cytokines *in vitro* and *in vivo* induces

SHP-1 expression by upregulating the specific SHP-1 transcripts encoded from the P1 and P2 promoters in an IRF1 and STAT3-dependent manner [183, 184]. Strikingly, loss of SHP-1 in the central nervous system (CNS) cells developed the myelin pathology probably by chronic inflammation and production of reactive oxygen species [185]. A recent study has shown that during the late hepatitis B virus (HBV) infection, SHP-1 is involved in the regulation of its replication by repressing the p38/MAPK/STAT pathway. HOXA10 interacts with p38/MAPK and recruits SHP-1, which dephosphorylates MAPK and inhibits signal transduction [186]. Epstein-Barr virus (EBV) has been shown to increase gastric carcinogenesis by epigenetically silencing SHP-1 and thereby sustaining CagA phosphorylation in *Helicobacter pylori* infection [187]. SHP-1 also negatively regulates the IL2-IL2R signaling pathway by dephosphorylating the IL2βR, JAK1 and JAK3, which are required for the proliferation of T cells. However, infection with human T cell lymphotropic virus type 1 (HTLV) suppresses SHP-1 expression, which leads to oncogenic growth by activation of the JAK/STAT pathway [160]. Another study has shown that in HTLV-1-induced adult T cell leukemia (ATL), the HTLV-1 Tax protein silences SHP-1 expression by inducing CpG methylation and histone deacetylation of SHP-1 promoter 2 [163, 188].

DNA methylation

DNA methylation is one of the major and most well-studied epigenetic mechanisms and involves the direct chemical modification to the DNA. Modification to the DNA occurs by the addition of methyl group ($-CH_3$) to the 5^{th} carbon of cytosine (5mC) that precedes the guanine nucleotide, or so called CpG site, by the action of DNA methyltransferases (DNMTs) (Fig. 6).

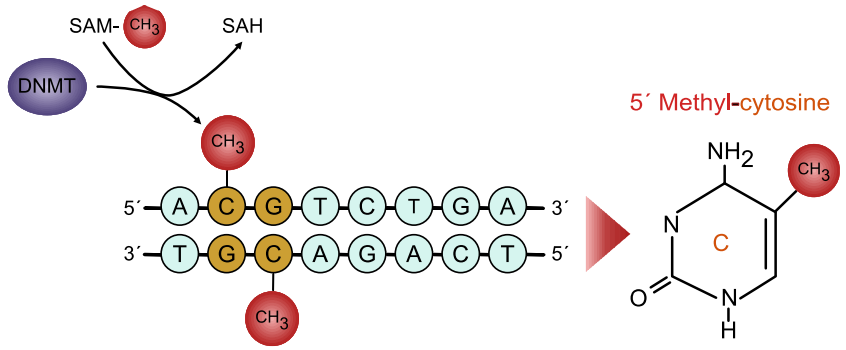

Fig. 6. DNA methylation. The double strand DNA with methylation at both strands is presented on the left. The DNA methyltransferases transfer the methyl group from the methyl donor S-adenosine methionine (SAM) to the cytosine (C) nucleotide at DNA that is preceded by a guanine (G). The methylated cytosine (5´methyl-cytosine) with methyl group at 5th carbon of the cytosine ring is represented in the right.

The “p” in CpG site stands for the phosphate bond between cytosine and guanine nucleotide. In mammals, cytosine methylation is mostly found on palindromic CG dinucleotide, meaning that the identical sequence is present on both DNA strands and therefore 5mC can be maintained by the maintenance enzyme DNMT 1 during DNA replication. However, 5mC can also occur at non-CpG sites, predominantly CpA sites, as observed in oocytes, brain and embryonic stem cells [189].

DNA methylation can be removed by passive and active demethylation. Passive demethylation occurs in dividing cells by inhibition of the DNMT during DNA replication, a process that can be blocked by pharmaceutical drugs. The two cytosine analogues 5-Azacytidine and 5-Aza-2-deoxycytidine both use the same strategy by trapping the DNMTs and targeting them for degradation [190, 191]. The drugs at low doses do not inhibit proliferation and induce demethylation whereas at higher doses the drugs prevent proliferation and cause cytotoxicity. Active DNA demethylation occurs in both dividing and non-diving cells by the action of ten eleven translocation (TET) methylcytosine dioxygenases enzyme. The TET enzyme oxidizes 5mC to several intermediate compounds including 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). All the oxidized forms induce DNA demethylation during replication [192]. DNA demethylation is also recognized and corrected in case of 5fC and 5caC by excision base repair pathway.

DNA methyltransferases and their function

DNMTs transfer the methyl group from S-adenyl methionine (SAM) to the cytosine to form the 5mC [193]. DNMT catalyzes the DNA methylation in

two parts, 1) *de novo* methylation establishment and 2) maintenance methylation. *De novo* methylation is the establishment of a new methylation pattern that occurs by the addition of a methyl group to the previously unmethylated cytosines. It is catalyzed by DNMT3 family enzymes that includes DNMT3A, DNMT3B, DNMT3C and DNMT3L [194]. DNMT3A and DNMT3B are the major *de novo* DNMTs that establishes methylation pattern during embryonic development [195]. DNMT3C is formed by duplication of DNMT3B and active in rodents. DNMT3L is catalytically inactive but serves as a cofactor for DNMT3A/3B during *de novo* methylation in germ cells. Once the patterns are established, these methylation patterns are maintained during the DNA replication by the maintenance enzyme DNMT1. To preserve the epigenetic memory generated during the development, DNMT1 therefore copies the DNA methylation pattern from the parental strand to the newly synthesized daughter strand [194]. Therefore, DNMT1, DNMT3A, DNMT3B and DNMT3L are the key enzymes that plays important role in DNA methylation in mammals.

DNA methylation role in cells.

There are around 28 million CpG sites present in the human genome. These CpG sites are not evenly distributed across the genome, they tend to be present in clusters at certain regions referred to as CpG islands (CGIs). The CGIs are the CpG rich stretches on the DNA of approximately more than 200 base pairs length and a CG content higher than 50 %. Approximately 70 % of the gene promoters are present in the CGIs [196]. The promoters of all housekeeping genes in mammals contain CGIs. Most inactive CGIs are silenced by polycomb repressive complex-2 mediated methylation of lysine 27 of histone 3 (H3K27), a softer form of methylation compared to DNA methylation and thereby providing the gene more flexibility to rapidly express or repress [197]. DNA methylation is prevalent throughout the genome but it has a distinct function in different genomic regions. However, it is considered as a repressive regulator of promoters. Although how exactly the DNA methylation silences the gene transcription is unclear but two theories have been proposed: either by 1) hindering the binding of a transcription factor to the promoter or by 2) recruiting the repressive complex, such as methyl binding protein (MBD), and creating the repressed chromatin environment. In addition to the basal gene expression regulation, DNA methylation play role in the inactivation of X-chromosome, genomic imprinting and also silences the transposable elements (TE) particularly the retrotransposons [189].

DNA methylation and viruses

DNA methylation serves as an antiviral defense mechanism by suppressing the viral gene transcription and replication as occur in the case of endogenous retroviruses and retrotransposons [198]. However, to subvert the host antiviral responses as well as to establish the viral persistence, viruses have evolved counter mechanisms including hijacking the host epigenetic machinery. Viruses utilize the host DNA methylation machinery to modulate the immune related gene expression by induction of DNA hypermethylation. Immune suppression caused by the aberrant methylation over time may lead to tumorigenesis as occur in the case of RNA viruses HTLV and HCV, and the DNA viruses Kaposi´s sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV), hepatitis B virus (HBV) and human papillomavirus (HPV) [199].

Three HCV proteins C, NS3 and NS5A have been shown to have oncogenic properties in *in vitro* studies [22, 200]. A link between HCV infection and aberrant methylation of HCC related genes such as CDKN2A (cyclin dependent kinase inhibitor 2A), CDH1 (cadherin 1), SOCS1 (suppressor of cytokine signalling 1) RASSF1A (Ras associated domain family member 1), APC (adenomatous polyposis coli protein), STAT1 (signal transducer and activator of transcription 1) has already been established [201, 202]. Alteration of these genes induces cancer by promoting cell proliferation, mobility, invasion and immune evasion. Hypermethylation of E-cadherin (function altered in most carcinomas) promoter, has been detected in hepatocytes expressing the C protein from genotype 1b [86, 87]. The C protein has been shown to upregulate the DNMT1 and DNMT3B expression in HepG2 and Huh-7 cells, suggesting a role of C protein in HCV-induced DNA methylation [86, 91]. Although the mechanism by which the C upregulates DNMT expression is not clear, activation of STAT3 is required. A similar study has also shown that the C-mediated activation of DNMT is genotype dependent [91]. Another study has shown that downregulation of DNMT1 and DNMT3B hampers the production of HCV upon infection in cell culture (HCVcc). Knockdown of DNMT1 and DNMT3B in Huh-7 cells inhibited virus subgenomic replication but had no effect on virus entry and translation [92].

Methods to detect DNA methylation

There are several methods available for the determination of DNA methylation status of a specific gene/targeted region. These includes the direct sequencing, pyrosequencing, methylation specific PCR, bead array, PCR with high resolution melting and co-amplification at lower denaturation temperature (COLD) PCR for detection of unmethylated islands [203]. However, methylation specific PCR, pyrosequencing and direct sequencing are the most common methods used routinely for analysing the methylation

status of targeted regions [204]. Hence, only these methods will be described here in detail. Bisulfite modification is still the gold standard for most of the methods and is the first step before downstream processing. In bisulfite treatment, methylated cytosine in the gene is unchanged whereas the unmethylated cytosine is converted to uracil, which will be converted to thymine in the PCR amplification (Fig. 7). As a result, the methylation information of a gene is transferred to sequence information in the form of C (methylated) and T (unmethylated) [205].

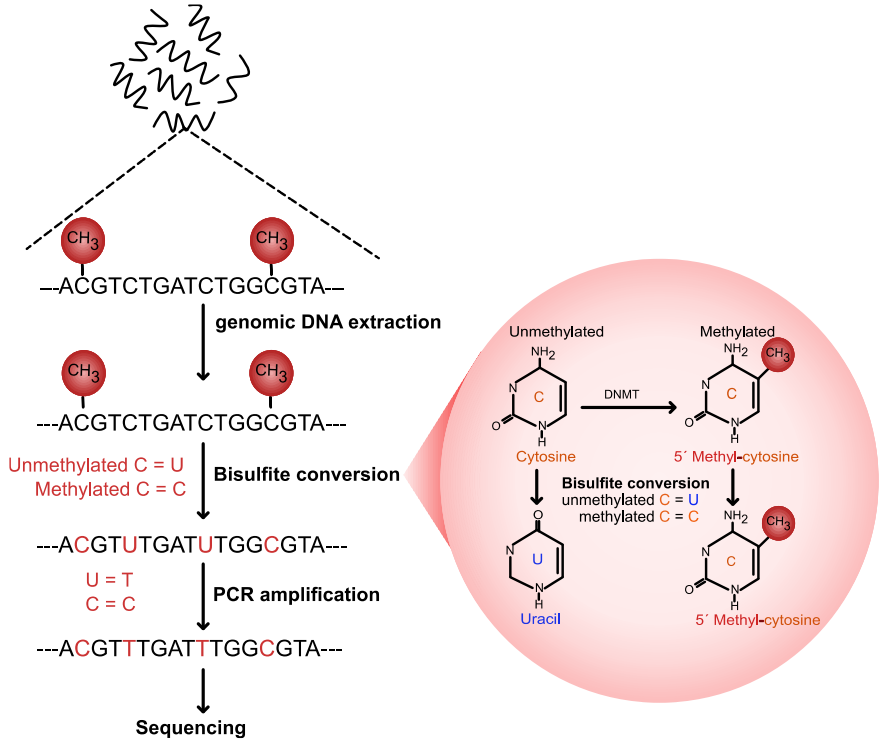

Fig. 7. Overview of modification of DNA sequences by bisulfite conversion method. The genomic DNA is modified by bisulfite treatment to convert the unmethylated cytosines to uracil (U), whereas the previously methylated cytosine does not change upon treatment and remains cytosine. In the PCR step, the uracil's are changed to thymine (T) and the amplicons are sequenced by genomic sequencing.

Methylation specific PCR (MSP)

MSP is one of the classical methods that uses bisulfite converted DNA as a template. The workflow uses two sets of primers, one pair of primers that are targeted against the methylated DNA template and another pair that favors amplification of unmethylated DNA. Two qPCR reactions are required for each sample and relative methylation levels are calculated by the difference in threshold cycle (Ct) values. The major disadvantages of this method are that

only one or two CpG sites can be analyzed at a time and the lack of quantitative estimation.

Pyrosequencing

Pyrosequencing is another low-throughput method used for assessing the DNA methylation at single nucleotide level [206]. The method also utilizes bisulfite conversion of the genomic DNA prior to PCR amplification of the target sequence. The downstream sequencing reaction requires single stranded PCR amplicon as template and four different enzyme mixture including DNA polymerase, ATP sulfurylase, luciferase and apyrase. It also uses adenosine 5´phosphosulfate (APS) and luciferin as substrate for the enzymes [207]. The light is detected from the release of pyrophosphate on nucleotide incorporation into the complementary strand by DNA polymerase. The methylation level at a particular CpG site is determined by comparing the peak light emission of cytosine or thymine. The advantage of the method is that it gives a quantitative measure of methylation levels. The disadvantage is that the method is sensitive for only short stretches of DNA (~ 100 base pairs) [203].

Direct sequencing

Direct sequencing is also called bisulfite sequencing (BS). Similar to the aforementioned techniques, BS also utilizes bisulfite converted DNA as template. The primers are designed against the bisulfite modified DNA and the region of interest is amplified by PCR reaction. The PCR products are then cloned in a bacterial expression system and sequenced. Sequencing results from several clones are used to determine the methylation level at individual CpG sites. In the past, it was the method to deduce the methylation status of individual sites. Alternatively, it is also possible to skip the cloning step and directly sequence the PCR product, either by Sanger or next generation sequencing (NGS). The methylation status of individual CpG island is determined by comparing the ratios of peak heights of cytosine verses peak heights of thymine [205, 208, 209]. The limitation of this method is that amplification of excessive thymine rich regions is difficult due to the reduced complexity of DNA after bisulfite treatment. However, direct sequencing is simple, inexpensive and high throughput compared to the bisulfite-cloning sequencing and pyrosequencing [210]. Compared to pyrosequencing, relatively long DNA regions can be analyzed for methylation status.

Aims of the current thesis

Hepatitis C virus (HCV) is a persistent virus that establishes a chronic infection in the infected host. The underlying mechanism of virus persistence is less characterized due to high virus diversity (both within and between the host), complex life cycle and insufficient knowledge of host immune responses.

Contrary to many other persistent viruses such as human immunodeficiency virus type 1, HCV does not integrate into the host genome and unlike hepatitis B virus does not form the viral minichromosome to establish persistence. Instead, HCV evades or surpasses the host antiviral responses by causing significant changes in host gene expression and function or by directly targeting the immune cells. Although new therapeutic regimens with direct acting antivirals have greatly changed the treatment of HCV, the lack of a safe and effective vaccine poses challenges to eradication of the virus. Further, the failure of recent vaccine trials highlights the gaps in our understanding of protective immune responses and a need to understand the host and viral factors that determine the fate of HCV infection and immunity.

One of the viral proteins that is implicated in various aspects of HCV pathology such as oncogenesis and immune subversion and therefore needs a better characterization is the core (C) protein. The C protein is an important structural component that forms a protective shell around the virus genome. It has a diverse cellular function and therefore plays an important role in HCV pathogenesis. Hence, the multifunctional nature of the C protein makes it an important protein to dissect and understand the details of HCV life cycle.

Paper I

The aim of paper I was firstly to elucidate the effect of HCV C protein on intracellular signaling in a T cell model. The second aim was to investigate the molecular mechanism associated with HCV C protein induced suppression of SHP-1, a key regulator of immune signaling pathway.

Paper II

One of the aims of the paper II was to extend our *in vitro* finding of paper I, the suppressive effect of HCV C protein on SHP-1 and to verify the methylation profiling by a high throughput next generation bisulfite sequencing (NGS-BS) method. The additional aim was to extend the finding from established cell lines to the clinical samples.

Paper III

The aim of paper III was to study the role and to investigate the mechanism of HCV C protein induced Ca^{2+} release by activation of Ca^{2+}/NFAT signaling and its role in nucleocapsid assembly.

Paper IV

The aim of paper IV was to establish a PCR based method for the specific detection of genomic/positive-sense single strand ((+)ssRNA) and antigenomic/negative-sense single strand ((-)ssRNA) HCV RNA.

Methods

Samples

Paper II and IV

In paper II and IV, the study was conducted on 23 PBMC samples. The blood samples were obtained from healthy blood donors (11) and HCV-positive patients (12) at Akademiska hospital, Uppsala. The PBMCs were isolated from whole blood at the department of Clinical Chemistry and Pharmacology, Akademiska Hospital, Uppsala. All the patients have the informed consent and the study was approved by the Local Ethics Committee of Uppsala (Dnr 2016/238). The samples were obtained from the patients prior to start of antiviral treatment.

Paper IV

In addition to the 23 PBMC samples, the study in paper IV was also conducted on 22 plasma samples from HCV patients that had been fully anonymized prior to use in this study. The plasma samples had previously been genotyped and subsequently stored with informed consent in the local branch of Uppsala Biobank located in the department of Clinical Microbiology, Akademiska Hospital, Uppsala. The patient samples had the three genotypes, GT1a, GT1b and GT3a.

Methylation analysis based on direct bisulfite sequencing (Sanger) and next generation sequencing (Illumina)

Identification of CpG islands and *in silico* bisulfite conversion

Three CpG islands on SHP-1 P2 promoter of *PTPN6* gene (NC_000012.12) was identified using Methyl Primer Express software v1.0 (Applied Biosystem). The CpG islands with a GC percentage greater than 60 % were selected. The DNA sequence of the CpG islands were modified by *in silico* bisulfite conversion and primers were designed against the modified sequences using the same software. The rationale behind the bisulfite

conversion is that the treatment does not change the methylated cytosine (5mC) whereas the unmethylated cytosines are converted to Uracil, which in subsequent PCR reaction are amplified as thymine (Fig. 8).

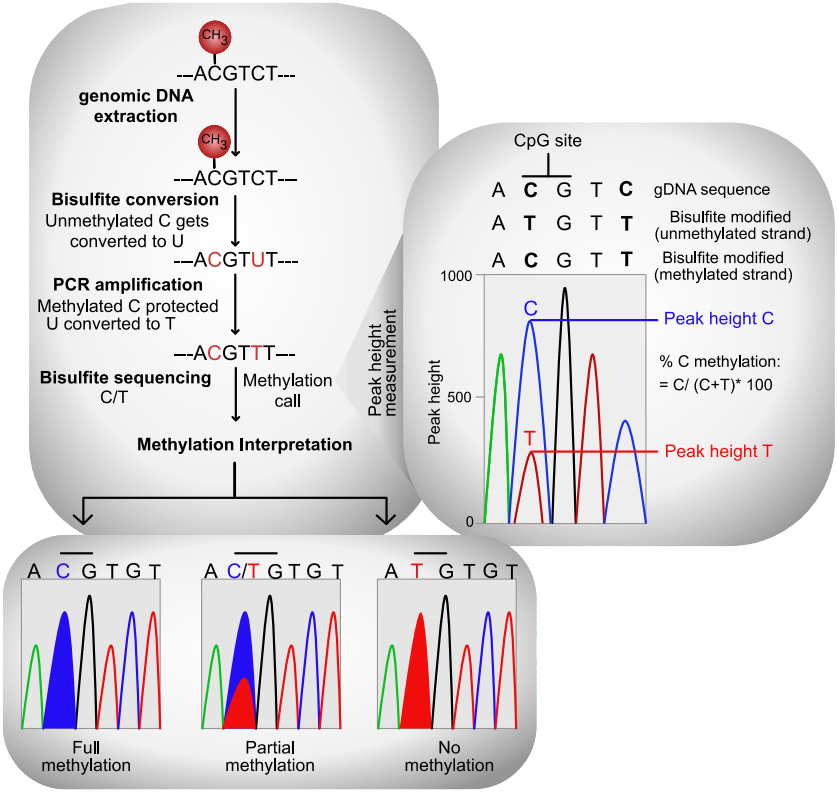

Fig. 8. Schematics showing the bisulfite modification, analysis of sequencing data and interpretation of cytosine methylation. After bisulfite treatment the unmethylated cytosine (C) changes to uracil (U) and in the PCR, step amplified as thymine (T). The amplicons are sequenced and the methylation at C is calculated by the peak height measurement. The single peak of C at CpG site indicates the full methylation, double peaks of C and T indicates the partial methylation and single peak of T indicates no methylation.

Bisulfite sequencing

In paper I and II, about 500 ng of purified gDNA extracted from the cell lines and HCV-positive PBMC samples were treated with sodium bisulfite using EZ DNA methylation-Gold kit™ (Zymo Research Corporation, Täby, Sweden). The bisulfite converted templates were amplified with bisulfite specific forward and reverse primers (Paper I**)** using HotStar Taq DNA polymerase (Qiagen). The second PCR was performed using 3 µl of input from the first PCR and the amplicons were verified by agarose-gel

electrophoresis. The PCR products were treated with Affymetrix EXOSAP-IT enzyme (Thermo Fisher Scientific). In paper I, the sequences were sent to Eurofins Scientific (Konstanz, Germany) for sequencing by capillary electrophoresis (Sanger) method.

Primer design, PCR and library preparation using dual barcoding

In paper II, to construct the sequencing library for bisulfite-based amplicon sequencing, Illumina-specific adapter sequences were added to the 5´ ends of the template-specific forward and reverse primers (Paper II). The five NNNNN in the adapter sequences are random nucleotides to improve the cluster generation during sequencing. The adapter sequences are part of the sequencing primers and also needed for the hybridization of amplicon to the surface of flow cell as well as cluster generation by bridge amplification (Fig. 9) [211]. In the first (Inner) PCR, the target region was amplified by adapter-tagged, template-specific forward and reverse primers in first PCR by using HotStar Taq DNA polymerase (Qiagen). The amplicon product was verified for accurate size and primer dimer formation by agarose gel electrophoresis. In the second (Outer) PCR, the amplicon from the first PCR was barcoded with unique dual indexes (tagged with Illumina adapters) at both ends of the DNA for multiplexing. The quality of the PCR product was tested by agarose gel electrophoresis and treated with Affymetrix EXOSAP-IT enzyme (Thermo Fisher Scientific) before sequencing.

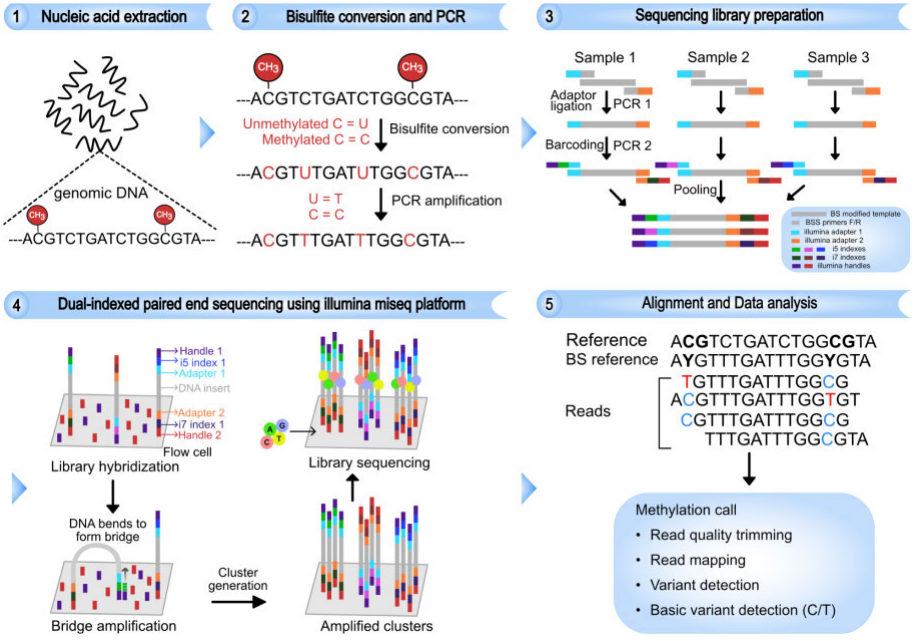

Fig. 9. Schematics layout of the next generation bisulfite sequencing (NGS-BS) method. The image is from paper II, Fig. 1.

Next generation sequencing (NGS)

After preparing the sequencing libraries and PCR cleanup, the DNA concentration of individual libraries were measured using a Qubit fluorometer (Thermo Fisher Scientific) and the fragment sizes were confirmed with Agilent 2200 TapeStation system (Agilent Technologies) using high sensitivity D1000 Screen Tape. The libraries were diluted with EB buffer and denatured. Paired end sequencing was performed on Miseq (Illumina Inc) using the Miseq® Reagent Nano Kit v2 at Clinical Genomics Uppsala (Science for Life Laboratory, Sweden).

Sequence analysis for methylation

The bisulfite sequencing data for the forward and reverse nucleotide sequences of all samples were aligned to the reference sequence using MEGA6 software. The efficiency of bisulfite conversion was evaluated by mapping the sequences to *in silico* bisulfite converted reference sequences. Almost all the cytosine bases that were not followed by a guanine nucleotide were represented by thymine indicating the high efficiency of the bisulfite conversion. The methylation status of cytosine residue at each CpG site was recognized as double peaks, of cytosine (C) and thymine (T) in the case of forward sequences or guanine (G) and adenine (A) in case of reverse

sequences. The methylation levels at individual CpG sites were calculated by using the peak height measurement approach as described previously by others [205, 208, 209].

The NGS sequencing data (paper IV) for methylation analysis was evaluated on CLC genomic workbench software v20 (Qiagen). The sequences were demultiplexed and adapter and indexed sequences were removed from the sequencing reads by using Long Run Manager at the sequencing facility. The trimmed sequencing reads were then aligned to the *in silico* generated bisulfite reference sequences. The methylation call at individual CpG site was analyzed by basic variant detection.

The methylation analysis of CpG1 island on SHP-1 P2 promoter performed on cell lines by bisulfite sequencing in paper II was reproduced by the next generation sequencing.

Results

Paper 1. Hepatitis C virus core protein down-regulates expression of src-homology 2 domain containing protein tyrosine phosphatase by modulating promoter DNA methylation

HCV infection modulates the host cell immune responses by altering intracellular signaling pathways. One of the mechanisms by which HCV evades the immune surveillance is by disrupting the T cell signal transduction that is crucial for the T cell development. Proper T cell signaling is maintained by the balanced action of tyrosine kinases and tyrosine phosphatases. Kinases activates the protein by phosphorylating tyrosine residues whereas phosphatases terminate the activation signal by dephosphorylation and therefore maintain a feedback control loop in T cell signaling [212]. Accumulation of phospho-tyrosine proteins in the absence of tyrosine phosphatases disturbs the feedback control loop and leads to abnormal cell growth and proliferation. The graphical representation of the feedback regulation of TCR signaling and the summary of the paper I is shown below (Fig. 10).

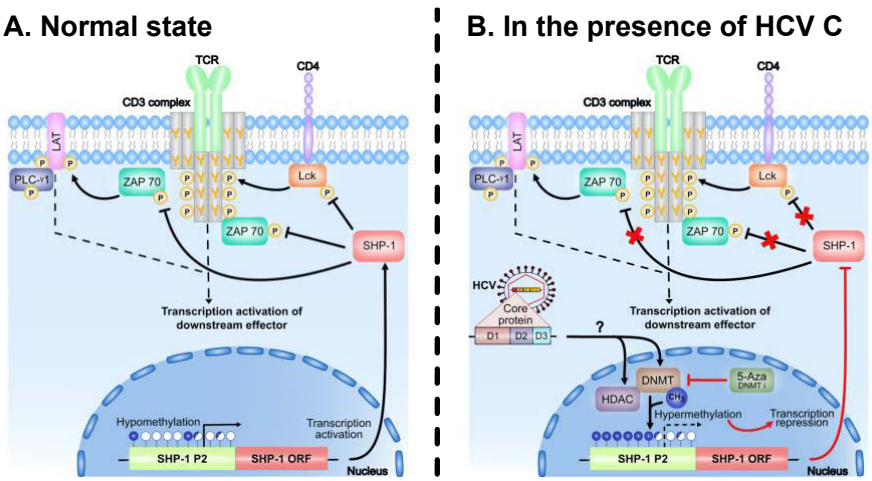

Fig. 10. Feedback regulation of T cell receptor signalling by negative regulator SHP-1. Image is from paper I, Fig. 8.

Effect of HCV C protein on T cell receptor (TCR) signaling pathway

To explore the effect of HCV C protein expression on TCR signaling, we evaluated the general tyrosine phosphorylation (p-Tyr) levels in parental Jurkat and three HCV C protein expressing JHC cell lines (JHC.d, JHC.g and JHC.h). Immunoblot assay revealed an enhanced general p-Tyr levels in JHC cell lines compared to the Jurkat cell, whereas the kinase deficient J. Cam cell showed no p-Tyr, as expected (Fig 1A, paper 1). This observation suggested that the C protein might modulate the p-Tyr, either by increased activation of tyrosine kinases or by inhibition of tyrosine phosphatases. Further investigation of p-Tyr status of the three signaling proteins PLC-γ1, ZAP-70 and LAT showed enhanced p-Tyr levels in JHC cells compared to the parental Jurkat cell upon stimulation with anti-CD3 (Fig 1B, paper 1). Notably, among the three targets, ZAP-70 showed the highest p-Tyr levels in JHC cells.

The upregulation of p-Tyr levels of SHP-1 targets (PLC-γ1, ZAP-70 and LAT) suggested that SHP-1 might be non-functional in C expressing cell lines. Therefore, we decided to focus on SHP-1 protein and its role in C mediated modulation of T cell signaling pathway.

Specific reduction of SHP-1 expression in C expressing cells

In addition to the SHP-1, we evaluated the accumulation of two additional TCR-related tyrosine phosphatases, CD45 and SHP-2, in Jurkat and JHC cell lines. Although the expression levels of CD45 and SHP-2 were unchanged in both cell lines, a significant reduction in SHP-1 protein accumulation was detected in JHC cells compared to the Jurkat cell (Fig 2A, paper 1). Further, no effect on basal accumulation of PLC-γ1, ZAP-70 and Lck further strengthened the hypothesis that the HCV C protein interferes with proximal TCR signaling by specifically inactivating SHP-1. To further validate the effect of the C protein on SHP-1 accumulation, we performed flow cytometry experiment on Jurkat and JHC cell lines. Notably, similar reduction of SHP-1 protein accumulation was found in JHC cell lines when compared with the Jurkat cell (Fig 2B, paper 1). Owing to the possibility that the reduced SHP-1 protein accumulation can be due to a reduction of SHP-1 mRNA synthesis, we quantitated the SHP-1 mRNA levels by real-time PCR. The quantitative analysis showed a significant reduction of SHP-1 mRNA levels in individual C protein expressing JHC cell lines compared to the parental Jurkat cell (Fig 2C, paper 1).

Together, these data indicate that the constitutive expression of C protein blunts the SHP-1 mRNA levels in T cells.

Reduced SHP-1 expression corresponds to the SHP-1 P2 promoter hypermethylation

Apart from its role in negative regulation of TCR signaling, SHP-1 also function as a tumor suppressor gene and loss of SHP-1 expression is relatively a common abnormality in blood-related cancers. A plethora of evidences shows that the SHP-1 expression is epigenetically regulated through the promoter CpG methylation in hematological [162-169] and solid malignancies [174-177]. The C protein is involved in HCC by directly altering the CpG methylation pattern of the tumor suppressor genes *p16*, *E-cadherin* and *RASSF1A* [85, 87, 213]. Based on these findings, we hypothesized that the observed suppression of SHP-1 could be result of epigenetic modification of the SHP-1 promoter caused by the C protein.

To test our hypothesis, we evaluated the methylation status of three previously studied CpG islands (CpG2, CpG1 and proximal island) on the SHP-P2 promoter in Jurkat and JHC cell lines by bisulfite sequencing. Parallelly, to validate our experimental approach, we evaluated the aforementioned CpG island methylation in two distinct cell types of hematopoietic (THP-1) and a non-hematopoietic (293TT) cell origin where the SHP-1 expression is governed by the two different mutually exclusive promoters P2 and P1, respectively. Bisulfite sequencing data revealed a clear difference in the methylation patterns between different cell lines. Whereas high methylation levels were found at all CpG positions in 293TT cells, only CpG positions (4, 5, 6 and 7) at CpG1 island displayed increased methylation in THP-1 cells (Fig 4C, paper 1). Further analysis of the CpG1 island in Jurkat and JHC cells revealed that the two-cell lines differed in their methylation patterns at the first three CpG positions (1-3), where JHC cells were significantly hypermethylated compared to the parental Jurkat cell (Fig 4D, paper 1). Analysis of CpG2 island showed hypermethylation status in both Jurkat and JHC cell lines without any significant differences between the two cell lines (Fig 5B, paper 1). Similar to the CpG1 island, 293TT cell line showed hypermethylation at all analysed CpG motifs in proximal island whereas in THP-1 cell only CpG motif 6 possessed high methylation levels (Fig 6B, paper 1). Whereas the CpG motif 6 at the proximal island was solely hypermethylated in JHC cells, a marginal level of methylation was detected at the same CpG position in Jurkat cells (Fig 6C, paper 1). Intriguingly, the CpG position 6 in the proximal island overlaps with the binding sequence of the transcription factor Sp-1.

Together, since the CpG methylation negatively correlates with the gene transcription, we anticipate that the C-induced specific methylation of CpG1 and proximal island may impact the SHP-1 gene transcription by dislocation of Sp-1 from the promoter region.

Paper II. Next generation sequencing analysis of CpG methylation of a tumor suppressor gene SHP-1 promoter in HCV-positive patients

The transformation event is a multistep process that involves changes at both genetic and epigenetic levels. Many viral infections manipulate the host epigenetic machinery through their encoded gene products [214]. Loss or reduced tumor suppressor function of tumor suppressor genes due to aberrant CpG methylation is relatively a common abnormality and often a first step in many blood-related cancers. Therefore, the identification of CpG methylation patterns in tumor cells will serve as a biomarker for disease diagnosis and progression. Cytosine methylation at the gene promoter is an important epigenetic modification as it is a dynamic regulator of gene expression. However, in the past, profiling of such epigenetic changes in clinical setting was limited due to the low quantitative accuracy and lack of high throughput sample processing.

Establishment of next generation bisulfite sequencing (NGS-BS) for CpG methylation profiling

For precise quantification of CpG methylation at specific locus such as specific gene promoter or CpG islands, we have combined the bisulfite conversion with next generation sequencing. The method is unique and differs from other existing methods in sequencing library preparation. The NGS-BS method involves following steps: bisulfite conversion of genomic DNA, PCR amplification of the target region using bisulfite specific primers, sequencing library construction using Illumina-specific adapters, dual barcoding and next generation sequencing (Illumina, Miseq). The method provided fast and accurate quantification of cytosine methylation with base specificity.

Since SHP-1 gene expression is frequently lost or reduced due to the CpG promoter methylation in lymphomas and leukemia, the CpG1 island on SHP-1 P2 promoter was used as a model promoter to analyze cytosine methylation in immortalized human T cell lines and HCV positive patients.

Characterization of CpG methylation pattern on SHP-1 P2 in cell lines using NGS-BS

To validate the NGS-BS method and to verify our previous observation obtained by bisulfite sequencing (Fig 4D, paper I), we evaluated the cytosine methylation in established cell lines. Our previous result from the bisulfite sequencing on Jurkat and JHC cell lines showed that the CpG1 island on SHP-1 P2 was hypermethylated and that the two-cell lines differed significantly in their methylation levels at the first three (1-3) CpG positions. Analysis of the

CpG1 island by NGS-BS method showed methylation of individual CpG positions (11/11) in both Jurkat and JHC cell lines. Whereas the methylation levels at CpG positions between 4 to 11 in JHC cell lines were indistinguishable from the control Jurkat cell line, significantly higher levels of methylation were found at CpG positions one to three (1-3) (Fig 2B, paper II).

Together, methylation analysis by the NGS-BS method showed that HCV C protein expression in immortalized T cells correlates with hypermethylation of the CpG1 island at the SHP-1 P2 promoter and also verified our previous observation assessed by bisulfite sequencing.

Characterization of PBMC cell population

To compare the cell populations of healthy controls (11) and HCV patients (12), the PBMCs were analyzed by flow cytometry technique. Cells were separated from the whole blood and sorted into distinct cell types on the basis of their differentiation markers. The major cell type population that comprised the PBMCs of healthy controls and HCV patients were T cell, NK cell, monocytes and B cells respectively. Although no significant difference was found for other cell types, an enhanced B cell population was found in HCV^+ patients (6.6 %) compare to the healthy controls (3.3 %) (Table 2, paper II).

Characterization of CpG methylation pattern on SHP-1 P2 in clinical samples using NGS-BS

In order to verify our *in vitro* findings on methylation pattern of CpG1 island on SHP-1 P2 in clinical samples, we tested the PBMC samples derived from healthy controls (11) and HCV^+ patients (12) by using the NGS-BS method. Surprisingly, PBMC samples from healthy controls and HCV^+ patients showed much lower levels of methylation at CpG1 island compared to the cell lines. Except the three CpG motifs, 2, 5 and 7, which showed methylation levels of ≥ 3 %, ≥ 10 % and ≥ 5 % respectively, all the other eight CpG motifs possess a methylation signal below 3 % in both healthy and HCV^+ PBMCs. Remarkably, the healthy control and HCV^+ PBMCs did not show any significant difference in the methylation levels at CpG1 island (Fig 2C, paper II).

Together, our method indicates that next generation sequencing of bisulfite amplicons is a sensitive and cost saving method and can be used to quantitatively characterize the CpG methylation status of individual CpG positions in cell lines, virus-associated and non-associated tumors.

Paper III. Activation of the Ca^{2+}/NFAT pathway by assembly of hepatitis C virus core protein into nucleocapsid-like particles

Calcium (Ca^{2+}) is a signaling molecule and its functions in lymphocytes involves the activation of nuclear factor of activated T cell (NFAT), a transcription factor which is present in phosphorylated form in the cytoplasm of resting cell. Influx of Ca^{2+} activates the phosphatase calcineurin, which dephosphorylates NFAT and causes its nuclear translocation. In the nucleus, NFAT cooperates with another unrelated transcription factor AP-1 (Fos/Jun) and mount a productive immune response by activating large number of cytokine genes [215]. However, many viruses disrupt the immune signaling by perturbing the Ca^{2+} homeostasis through its structural or non-structural proteins to favor its multiplication and release of infectious virion [216]. In this paper, we have further characterized our previous observation that HCV C protein can trigger the calcium-mediated signaling pathways.

Generation of C protein deletion mutants

In our previous report we demonstrated that the expression of C protein in human T cells promotes the release of Ca^{2+} from the intracellular stores and causes the ca^{2+} oscillation that induces the activation of NFAT-regulated cytokine gene (IL-2) promoter [217, 218]. To further unravel the mechanism of C-induced Ca^{2+}/NFAT signaling, a set of C deletion mutants were generated that targets all the three domains (D1, D2, and D3) of C (Fig. 11). The C deletion mutants Δ2-12, Δ26-57, Δ61-68, Δ70-79 and Δ81-123 was generated by deleting sequences in D1, a highly basic domain essential for the core protein oligomerization and RNA binding. The mutants Δ124-142, Δ147-161, 1-152, 1-161 and 1-173 were the D2 mutants, a hydrophobic region that contains alpha helices and essential for lipid droplet targeting.

The deletion mutant 1-182 and Spmt mutants, Spmt1-191 and Spmt1-194 were generated either by truncation (1-182) or introducing three amino acid substitution in the signal sequences in the transmembrane domain D3, a highly hydrophobic region required for maturation of the C protein. Finally, the mutants 1-152, 1-161 and 1-173 have partial deletions in D2 and lacks the entire D3.

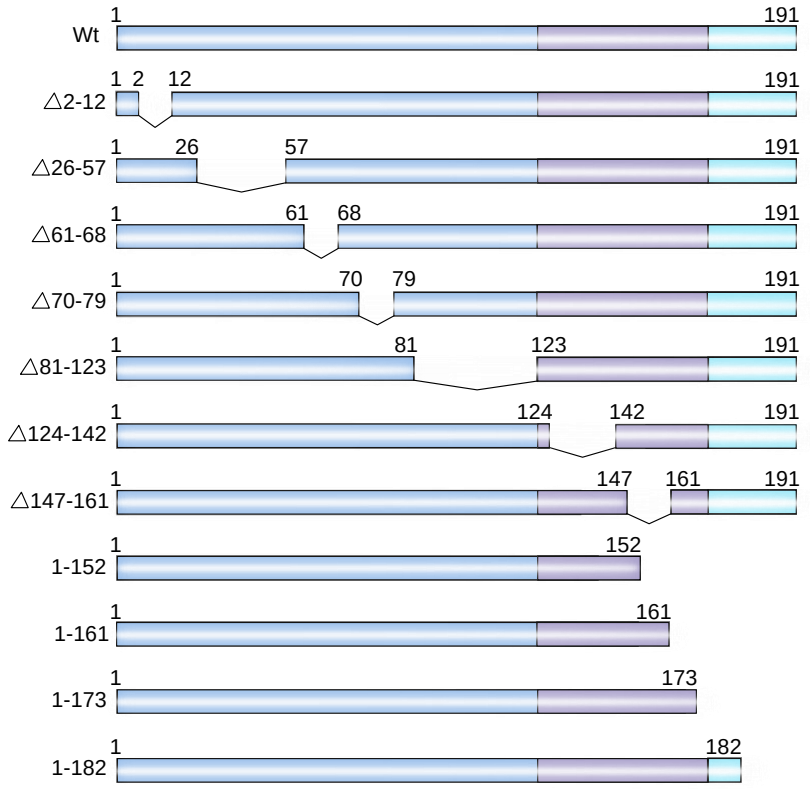

Fig. 11. Schematic map showing the construction scheme of HCV C deletion mutants. The three regions of HCV C protein are shown, domain 1 (blue), domain 2 (purple) and domain 3 (green). The triangle denotes the deletions whereas the number corresponds to the amino acid position.

Characterization of C deletion mutants for the activation of Ca^{2+}/NFAT signaling

To find out which region of the C protein is associated with activation of Ca^{2+}/NFAT signaling, a reporter system was generated by fusing the three copies of distal composite NFAT/AP-1 element from the IL-2 promoter with luciferase gene. The core deletion mutants were tested for the reporter expression that directly correlates with NFAT activity. The reporter assay revealed that deletion of sequences at the N-terminal half (Δ2-12, Δ26-57, and Δ61-68) and C-terminal half of C protein (1-152, 1-161, 1-173 and 1-182) abolished activation of NFAT. In contrary, deletion of sequences in the central domain (Δ70-79, Δ124-142 and Δ147-161) either had the similar reporter expression as the wild type C or displayed three-fold enhanced reporter gene activation (Δ81-123) (Fig 1B, paper III).

Taken together, our data indicates that two distinct regions in C protein, N-terminal half of D1 and the C-terminal half the ER anchoring domain is important for the activation of Ca^{2+}/NFAT signaling.

Correlation of Ca^{2+}/NFAT signaling with subcellular localization

Due to the notion that the expression of C triggers the accumulation of cytoplasmic Ca^{2+}, which in turn causes activation of NFAT, a colocalization assay to test the distribution of C mutants was performed by using indirect immunofluorescence. As markers for intracellular localization, ADFP for lipid droplet association and calnexin as an ER marker were used. Similar to what others had reported for wild type C [75], a ring like staining pattern around the lipid droplet was observed for wild type C as well as mutants Δ61-68 and Δ70-79 in our experiments (Fig 3A, paper III). Also, the mutants Δ2-12 and 1-173 were associated with lipid droplets although we in these cases were unable to observe any ring-like structures. In contrast, the deletion mutants Δ81-123 displayed a punctate like staining in the cytoplasm whereas the mutants Δ124-142 and Δ147-161 were predominantly present in the nucleus and associated with calnexin in cytoplasm. However, the mutant 1-152 lacking the ER anchor domain was found in nucleus (Fig 3B, paper III).

Together, these results indicate that activation of Ca^{2+}/NFAT signaling is independent of the C subcellular localization. However, deletion in D2 and D3 sequences alters the usual location of C from lipid droplet to the ER and nucleus.

Correlation of Ca^{2+}/NFAT signaling with C processing

Since our results from the NFAT assay indicated that the entire C-terminal transmembrane domain is required for the activation of Ca^{2+}/NFAT signaling, we tested the activity of intermediate p23 (unprocessed form of C) protein. For this, we utilized two D3 mutants, Spmt1-191 and Spmt1-194, which comprises a 3 amino acid substitution in the signal sequences at the transmembrane region and therefore are resistant to the protease cleavage to generate the mature form of C (p21). Intriguingly, transmembrane mutants Spmt1-194 and Spmt1-191 showed 5- and 10-fold enhanced activation of the NFAT compared to the wild type core in reporter assay (Fig 4B, paper III). Similar to the Spmt mutants, one of the domain 1 mutant Δ81-123, which failed to generate the mature C and retained in the ER, also showed an enhanced NFAT activation.

Together, these data indicate that intermediate p23 form of HCV C is responsible for the activation of Ca^{2+}/NFAT signaling.

Connection between Nucleocapsid-like particles (NLP) formation and NFAT signaling

The previous studies have reported that the distinct regions within the D1 (aa 36-91, aa 1-75, and aa 82-102) of C protein is essential for the self-association of C protein into the nucleocapsid-like particles (NLPs) [40, 219, 220]. Based on these previous observations, four C mutants with deletions in domain 1a and 1b were characterized with respect to their capacity to self-associate into NLPs. Whereas Δ70-79 and Δ81-123 were indistinguishable from the full-length core predominantly found in the intermediate fractions, Δ2-12 and Δ61-68 were distributed over the entire gradient (Fig 5A, paper III).

For the further analysis of their biochemical properties, two of the C mutants with adjacent deletions in D1 were selected, Δ61-68 and Δ70-79. These mutants had identical stability and intracellular localization but different effect on NFAT activation and sedimentation. Whereas wild type C and Δ70-79 displayed similar densities on the CsCl gradient and showed NLPs resembling HCV in electron microscopy, this was not observed for Δ61-68 (Fig 5D, paper III).

Taken together our result indicate that the aa61-68, which have an effect on NFAT activation, is also critical for the assembly into nucleocapsid-like particle formation (NLPs).

Paper IV. Development of a strand-specific RT qPCR assay for detection of genomic and antigenomic hepatitis C virus RNA

HCV is known to cause a short term acute and lifelong chronic infections in the infected host. The acute infections are usually asymptomatic and associated with elevated levels of liver transaminases. Relatively, minority of patients that develops the hepatitis symptoms such as jaundice, fatigue and fever happen to clear the infection than those with clinically silent infection and avoid the chronic diseases [221]. The chronic infection causes liver inflammation and progresses to silent development of liver fibrosis that over a period of time can lead to end-stage liver disease [222]. One of the major reasons behind disease progression is that the onset of virus infection is mostly asymptomatic and remain undetected due to low levels of HCV RNA in plasma below the detection limit of current detection system. HCV mainly replicates in liver by generating (-) RNA as a replicative intermediate, but it can also use extrahepatic sites such as PBMC as a second replication compartment although controversial. Therefore, accurate quantification of both strands of HCV RNA is important to understand the HCV life cycle and to monitor the silent infection in minor reservoirs. In this paper, we have established a PCR based diagnostic assay RT qPCR that allows strand-specific detection of genomic (+) and antigenomic (-) HCV RNA (ssRT qPCR).

Evaluation of the assay parameters

In vitro generated, positive (+) and negative (-) strand of HCV RNA was used as standards. To determine the linearity of the assay, ten-fold serial dilutions of the positive and negative strand RNA were performed. The standard curve approach was used to determine assay parameters such as efficiency, linear range and reproducibility. For positive strand HCV RNA the following values were obtained, PCR efficiency = 1, slope = -3.223 and the R^2 = 0.994 (Fig. 2A, paper 4). For negative strand HCV RNA, PCR efficiency = 1,1, slope = -3.105 and the R^2 = 0.997 (Fig. 2B, paper IV). The specificity for the assay was tested by running known quantities of either strand of standard RNA in the presence of opposite strand and calculated by comparing the difference in Ct values between the two strands. The assay showed a specificity of 4.3 $\log_{10}$ and 4.7 $\log_{10}$ for the detection of positive and negative strand HCV RNA, respectively.

Together, for the positive and negative strand RNA the reaction efficiencies were close to 100 %. However, at lower concentration the assay was less reproducible and at higher concentrations of the opposite strand a background signal was observed.

Analysis of human sera for HCV positive and negative RNA

In order to evaluate the strand-specific RT qPCR assay in clinical settings, the plasma samples from 22 HCV patients were tested for the presence of positive and negative HCV RNA. All (22) plasma samples showed signals in the positive strand assay as expected. Quantitation of positive strand RNA indicated linearity when compared with total HCV RNA levels. In contrary, only four (4/22) samples showed signals in the negative strand assay (Table 2, paper IV). However, due to the high Ct values, the quantitation was not possible for the negative strand HCV RNA.

Analysis of PBMCs for HCV positive and negative RNA

Past studies have reported the presence of both positive and negative HCV RNA in PBMCs [223-225]. To evaluate the extrahepatic infection and replication, ssRT qPCR assay was performed on PBMC samples (12) derived from the HCV^+ patients for detection of positive and negative strand RNA. PBMC samples (11) derived from the healthy controls served as negative controls. By ssRT qPCR, all (11/11) PBMC samples from healthy controls were negative for the presence of either strands of HCV RNA. Notably, whereas nine (9/12) PBMC samples from HCV^+ patients showed signal for the positive strand HCV RNA, none (0/12) of the samples were found positive for negative strand HCV RNA. The PBMC samples were further verified by Abbott RealTime HCV assay that do not discriminate between positive and negative strand HCV RNA. The Abbott assay showed that whereas no (0/11) viral RNA was found in healthy controls, low levels of HCV RNA was quantitated for all (12/12) HCV^+ PBMCs (Table 4, paper IV).

Together, our data indicate that although the specificity and sensitivity could further be improved, it could nevertheless be used to verify the polarity of clinical samples.

Discussion and future perspective

Paper I

In this paper, we attempted to find out the mechanism behind one of our previous findings that stable expression of HCV C protein in human T cells alters TCR signaling [226]. Therefore, the present study is focused on investigating the effect of C expression on phosphotyrosine signaling in T cells. Firstly, we showed that the expression of C skews the TCR signaling by enhancing tyrosine phosphorylation levels of proximal signaling effectors, such as PLC-γ1, LAT and ZAP70. Of note, a maximum effect was seen in ZAP70 which itself is an important tyrosine kinase and is regulated by the tyrosine phosphatase SHP-1 [156]. Further, our immunoblot data confirmed that C expression selectively reduced SHP-1 and correlated with hyperphosphorylation of ZAP-70. This implies that the tyrosine kinases and phosphatases set up a fine threshold for TCR signaling where suboptimal levels of SHP-1 are unable to terminate the activation signal, resulting in a prolonged, phosphorylated state of the signaling molecules. Since both SHP-1 splice variants, SHP-1 and SHP-1L, was expressed in our cell lines, we concluded that the reduced levels of SHP-1 were not due to post-transcriptional modification. Quantitative analysis of SHP-1 mRNA confirmed that the reduced SHP-1 accumulation was due to defects at the level of gene transcription. Further exploration of the transcriptional event at the promoter level revealed that the SHP-1 P2 promoter possessed differential methylation status and that the CpG1 and proximal islands were hypermethylated in C protein expressing cells. Notably, the CpG methylation is negatively correlated with gene expression and previous reports have shown that HCV inactivates different tumor suppressor genes involved in HCC by modulating DNMT´s and histone deacetylase [85, 86, 88]. Whereas the exact mechanism by which HCV C regulates SHP-1 expression is unclear, it nevertheless suggests that promoter methylation together with histone modifications are involved. This scenario is in line with our experiment with a methylation inhibitor, where we did not observe any demethylation effect upon treatment with 5-Aza-deoxycytidine, although high cell toxicity associated with this drug was a major barrier that restricted a definite conclusive interpretation. For the future studies, it will be still worthwhile to use the combination of 5-Aza or decitabine with other histone modifying drugs such as trichostatin A (TSA), valproate (VPA) and LBH589 that will be

crucial in unraveling the SHP-1 regulation [88]. Future studies targeted on pinpointing the involvement of factors (DNMTs and histone deacetylases) involved in the C mediated silencing of SHP-1 will shed light on the virus perspective.

Paper II

Here, we have successfully described a method for quantitation of locus specific methylation at individual CpG motifs by using next generation sequencing protocol for bisulfite amplicons (NGS-BS). The protocol comprises sequencing library generation using Illumina specific adapters and a dual barcoding system and is compared to existing methods relatively simple and inexpensive [204, 211].

The NGS-BS method further confirmed our previous findings on SHP-1 P2 promoter methylation in cell lines obtained by bisulfite sequencing. Further, the methylation pattern on SHP-1 P2 promoter differed between transformed cells and clinical samples. In cell lines, where the P2 promoter was hypermethylated, much lower levels of methylation were found in PBMCs from healthy control and HCV^+ patients. We believe that several factors might be responsible for the observed discrepancies between cell lines and clinical samples. Based on a past study indicating that individual genes in different blood cell types may have distinct methylation patterns [227], we anticipate that the heterogenicity of the PBMCs comprising of T and B cells, monocytes and NK cells (Table 2, paper II) could be a major reason behind the opposite methylation pattern in clinical samples. Therefore, while using the whole blood, analysis of methylation pattern of individual cell type for specific gene beforehand would be informative and useful for the future studies. Further, since PBMCs used in this study was taken from the chronic carriers where the frequency of HCV positive cells is expected to be low, C expression may therefore present below the detection limit of NGS (Illumina Miseq). In that scenario, either enrichment of the virus infected T cell population or using other NGS platform with higher sensitivity may present a better picture. Of note, transformation is a late event and previous studies have shown a positive correlation between loss of SHP-1expression with clinical stages of leukemia [162, 228]. Using cells from patients with progressive HCV infection leading towards tumorigenesis may therefore better mimic the SHP-1 methylation status. Alternatively, upregulation of DNMT expression in the tumor cell lines could be an explanation behind the hypermethylation of the SHP-1 P2 promoter in cell lines [229].

Paper III

In this study, we have further characterized our previous finding that HCV C expression in T cells triggers Ca^{2+} signaling and alters the Ca^{2+} homeostasis [218]. Our aim here was to figure out how exactly the C protein causes Ca^{2+} release from the ER. Since the C-mediated release of Ca^{2+} was independent to the Ca^{2+} channel in the ER, the inositol-3-phosphate gated channels (IP_3), we hypothesized that C protein execute Ca^{2+} release by directly targeting the membrane integrity. Further, our mutational analysis the C protein showed that the part of N-terminal region that is also required for association of C into a nucleocapsid-like structure and the entire C-terminal domain containing the ER targeting sequence of is required for Ca^{2+} release. Although several studies have reported the involvement of N-terminal region in assembly of C protein into NLPs [40, 219, 220], our data that the amino acid residues 61-68 is required for Ca^{2+}/NFAT activation is a novel finding and therefore could be useful in understanding assembly and egress steps in the virus life cycle. Further, our observation that the entire ER targeting domain at the C-terminal is needed for Ca^{2+}/NFAT activation may link with ER stress, a downstream consequence of inhibition of C processing, and will aid into our understanding of disease mechanism of HCV. Based on our experimental data, we therefore propose that the C-mediated Ca^{2+} release is a consequence of damage caused to the membrane during budding of assembled NLPs through the ER membrane. An alternative model to the story could be that the C protein with intact transmembrane domain on the ER membrane forms pore-like structure as a result of C multimerization leading to increased Ca^{2+} permeability. Although, this possibility is not unlikely as the formation of viroporins have been detected for other viral proteins [216, 230, 231], it still remains to be verified for HCV C by future studies. The graphical representation of proposed model for activation of Ca^{2+} signaling by HCV C protein is summarized in Fig. 12.

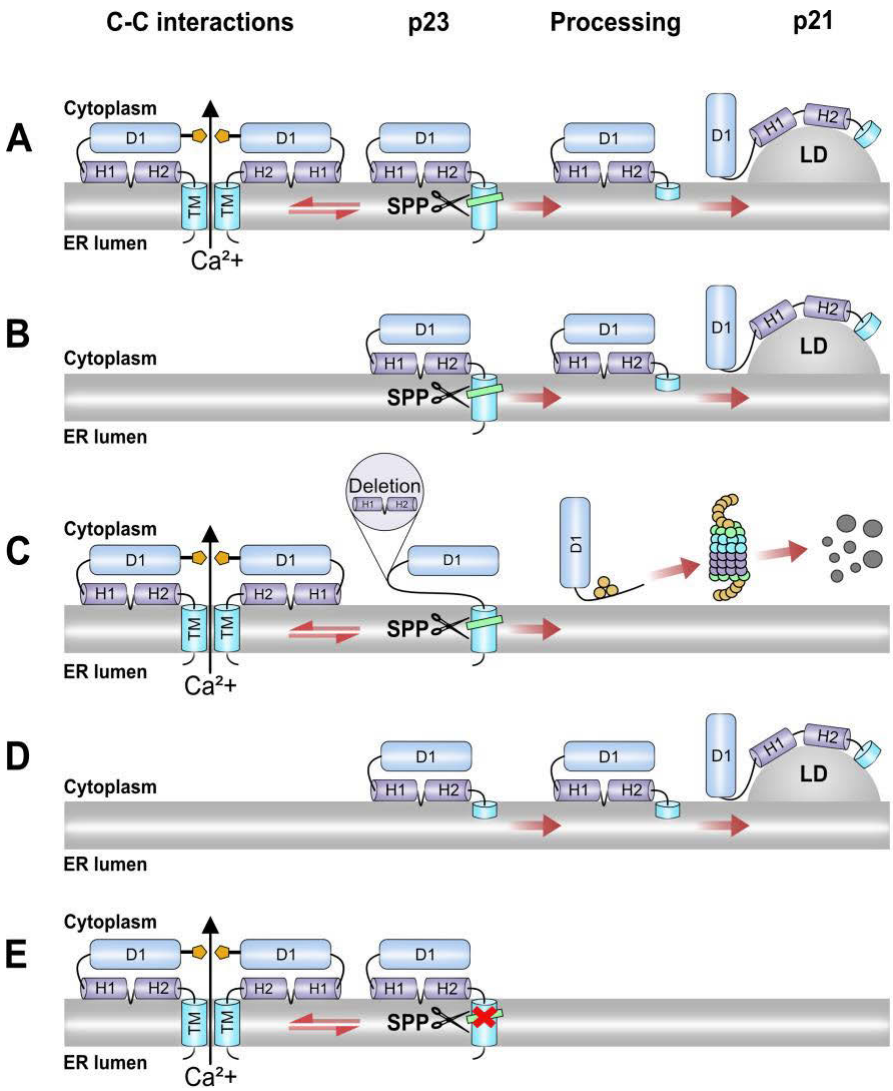

Fig. 12. Proposed model for activation of Ca^{2+} signalling by HCV C protein. Image is from the paper III (Fig, 6).

Paper IV

In this study, we attempted to set up a diagnostic, PCR based strand-specific RT qPCR (ssRT qPCR) assay for separate detection of positive and negative strands of HCV RNA. Apart from understanding the virus replication process, one of the purposes was to monitor occult infection as a potential source for virus persistence, drug failure and reinfection, particularly after transplantation. Although our assay showed a lower sensitivity in detecting

the specific HCV RNA, it correlated quantitatively with the total RNA levels as determined by commercial assays. The low sensitivity in our assay could be explained by low input RNA use and the dilution step that was incorporated to improve assay specificity. However, the sensitivity could be improved in future with careful consideration of other PCR parameters. While our method had limited specificity and would benefit from further improvement it should be noted that no other method has so far achieved complete specificity in discriminating the two strands. Further, the presence of negative strand HCV RNA in four plasma samples could occur due to false signal as a result of high viral titer or a consequence of release of replicative complexes from damaged hepatocytes. However, the former argument in this case seems less likely as the two of the plasma samples used in the study with high viral titer have no signal for the negative strand HCV RNA. Although the presence of positive strand RNA in PBMCs is not an absolute marker for HCV replication, the absence of negative strand RNA in PBMCs could be nevertheless be a result of low levels of virus replication. This interpretation is based on observation that no correlation existed between plasma viral load and amount of viral RNA in PBMCs and high Ct values observed for positive HCV RNA. At present we lack an additional control experiment and use of a replicon system to quantify the negative strand HCV RNA in future will improve the study.

Conclusions

Paper I

- HCV C protein expression enhances the tyrosine phosphorylation of TCR signalling molecules, PLC-γ1, ZAP-70 and LAT.
- HCV C protein confers the specific down-regulation of accumulation of SHP-1 at both RNA and protein level.
- HCV C protein in part, inactivates the SHP-1 expression by hypermethylating the SHP-1 P2 promoter.

Paper II

- Established a sensitive and cost-effective next generation sequencing method for bisulfite amplicon (NGS-BS) for the quantification of DNA methylation at specific locus.
- The SHP-1 P2 promoter methylation in cell lines obtained by bisulfite sequencing is confirmed by the NGS-BS method.
- Higher B cell population found in the PBMCs from HCV^+ patients than healthy controls.
- Cell lines and clinical samples showed distinct methylation pattern at SHP-P2 promoter.

Paper III

- Two distinct regions in C sequence, N-terminal half (aa 1-68) and C-terminal half (aa 152 onwards) is required for activation of Ca^{2+}/NFAT signaling.
- Wild type C and deletion mutants in domain 1 are localized in the perinuclear region on lipid droplets whereas internal deletions 81-161 is localized in the nucleus and/or ER.
- The immature p23 form of C has enhanced activation of Ca^{2+}/NFAT signaling than the mature p21 form
- The region in N-terminal half of domain 1 (aa 1-68) required for self-assembly of C into nucleocapsid-like particles.

Paper IV

- The strand-specific RT qPCR assay has a dose dependent response for quantitative analysis.
- Assay has a specificity of 4.3 and 4.7 $\log_{10}$ for detection of genomic (+) and antigenomic (-) HCV RNA, respectively.
- Genomic HCV RNA detected in all (22/22) whereas antigenomic RNA detected in four (4/22) plasma samples from HCV+ patients.
- Genomic HCV RNA detected in nine (9/12) whereas no (0/12) antigenomic RNA detected in HCV+ PBMCs.

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