“Cellular mRNA decay factors involved in the Hepatitis C Virus life cycle”

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a mis Viejos

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Summary

The genome of positive-strand RNA [(+) RNA] viruses acts as messenger for translation of viral proteins and as template for replication. As these two functions are mutually exclusive, the transfer of the viral genomes from the translation machinery to the replication complexes needs to be highly regulated. In the plant brome mosaic virus (BMV) it has been shown that the cellular LSm1-7 complexes and the Dhh1 and Pat1 proteins play a key role in such regulation. These proteins function in the deadenylation-dependent mRNA decay pathway, as activators of decapping mediating the transfer of cellular mRNAs from a translating to a non-translating state. In this work we show that HCV translation and replication are dependent on the human homologues of LSm1-7, Dhh1 and Pat1. Additionally, LSm1-7 rings bind specifically to HCV sequences linked to the regulation of viral translation and replication. Given the common replication strategies of (+) RNA viruses and the functional conservation of LSm1-7, Pat1 and Dhh1 from yeast to humans, our findings points out to these proteins as promising targets for the generation of broad-spectrum antiviral drugs in the future.

Resum

El genoma dels virus ARN de polaritat positiva actua com a missatger per la traducció de proteïnes virals i com a motlle per la replicació. Com que aquestes dues funcions son mútuament excloents, la transferència dels genomes virals de la maquinària traduccional als complexes de replicació necessita ser altament regulada. En el virus del mosaic del brom s'ha demostrat que els complexes cel.lulars LSm1-7 i les proteïnes Dhh1 i Pat1 juguen un paper important en la regulació esmentada. Aquestes proteïnes estan involucrades en la ruta de degradació dels mARNs cel.lulars que depèn de la deadenilació, com activadors del *decapping* mitjançant la transferència dels mARNs cel.lulars d'un estat de traducció a un de no traducció. En aquest treball demostrem que la traducció i la replicació del virus de l'hepatitis C són dependents dels homòlegs humans del LSm1-7, Dhh1 i Pat1. Addicionalment, els anells del LSm1-7 s'uneixen específicament a seqüències del HCV associades a la regulació de la traducció i replicació viral. Tenint en compte les estratègies de replicació en comú als virus ARN de polaritat positiva i la conservació funcional del LSm1-7, Dhh1 i Pat1 de llevats a humans, els nostres resultats apunten a aquestes proteïnes com a possibles dianes per la futura generació de drogues antivirals d'ampli espectre.

Preface

The hepatitis C virus (HCV) chronically infects over 170 million people worldwide, causing progressive liver damage and hepatocellular carcinoma. Furthermore, as HCV infection is generally asymptomatic for decades its victims often are unaware of the infection until it is too late for therapy. The main difficulties in eradicating HCV are the lack of an effective vaccine and limited treatment options.

A deep understanding of the viral and cellular factors involved in HCV infection is essential for the development of novel antiviral strategies. The main hurdle of these studies was the lack of a reliable system. However, the past decade has seen tremendous progress that started with the first establishment of subgenomic replicons and culminated recently in the development of an efficient system for production of infectious virus particles in cell culture. Taking advantage of these systems, this thesis has been focused on the identification of a group of cellular proteins with key roles on the HCV life cycle, with a promising use in therapeutic research. Two annexes have been included. The first one contains the article published with the results of this work. The second, the results obtained with a recombination system developed in our group that demonstrated for the first time the existence of recombination of HCV *in vitro*.

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I. Introduction

1. Positive-Strand RNA Viruses

The group of positive-strand RNA [(+) RNA] viruses constitutes an extensive group that infects a wide range of host species including bacteria, plants, animals and humans. It includes numerous pathogens with clinical and economical importance, and thus, has a great impact on global health, agriculture and economy. The most relevant pathogens of the animal and human (+) RNA viruses are members of the families *Coronaviridae* (Severe Acute Respiratory Syndrome or SARS-coronavirus), *Picornaviridae* (Foot-and-Mouth Disease Virus, Poliovirus, Hepatitis A), *Flaviviridae* (West Nile Virus, Hepatitis C Virus), and *Togaviridae* (Venezuelan Equine Encephalitis Virus, Sindbis Virus, Rubella Virus). From the medical point of view, the (+) RNA virus infections are usually associated with syndromes such as colds, febrile illness, meningitis, paralysis, hepatitis and hemorrhagic fevers.

The different families of (+) RNA viruses have evolved diverse genome organizations, virion morphologies and life cycles that exploit the biology and biochemistry of their hosts in many different ways. However, they have conserved features in their replication process. The entire life cycle of all (+) RNA viruses occurs in the cytoplasm and their genomes encode for an RNA-dependent RNA polymerase (RdRp) used in genome replication. However, unlike other RNA viruses, (+) RNA viruses do not encapsidate the polymerase within the virion. Thus, upon infection of a new cell, viral replication cannot begin until the genome is translated to produce the viral polymerase and additional replication proteins. After

translation, the viral RNA has to be recruited out of translation and, together with the replication factors, into the replication complex (RC). For all (+) RNA viruses that have been investigated, these RCs seem to associate with virus-induced membrane structures that are derived from different cellular compartments (90, 105, 176, 208, 240). This association ensures high local concentrations of replication components, protects the genomes from degradation by cellular nucleases and therefore increases replication efficiencies (177).

Once targeted to these membrane-associated RCs the genomic RNA serve as template to produce negative-strand RNA replication intermediates, which in turn are used to generate more positive-strand genomic RNAs. No natural DNA phase occurs during this process or any other step of (+) RNA virus life cycles. A successful infection occurs when viral genomic RNA is efficiently copied within the infected cell in order to provide both genomes for assembly into progeny virions and messenger RNAs (mRNAs) for the synthesis of viral proteins.

One of the most interesting aspects of (+) RNA virus life cycles is that they use the same genomic RNA as a substrate to perform different processes as translation, replication and encapsidation (6). As these processes can not occur simultaneously, mechanisms are required to separate packaging and replicative events away from translation of the RNAs, thereby avoiding competition between elongating ribosomes and the packaging or replicative machineries. Presently, there is clear evidence that numerous interactions between viral *cis*-acting elements and host-encoded replication factors facilitate the appropriate use of the genomic RNAs, although these regulatory mechanisms are not totally understood. The *cis*-acting signals that have been characterized in viral genomes mostly comprise not linear nucleotide sequences but RNA secondary

structures such as bulged stem-loops, tRNA-like cloverleaves, and pseudoknots, which are thought to create distinctive molecular environments that interact with the viral and host factors (82, 169). These interactions permit to specifically target viral molecules from among thousands of cellular RNAs and selectively drive them to replication and assembly complexes.

2. Hepatitis C as a global health problem

Among (+) RNA viruses and with more than 170 million people infected worldwide (269), the *Hepatitis C Virus* (HCV) is a leading cause of chronic liver disease and the main cause of liver transplantation (41). Primary infections are often asymptomatic or initially associated with mild and rather non-specific symptoms, but progression to chronic active hepatitis occurs in approximately 80% of infected individuals (206). The most severe manifestations of these chronic infections are liver cirrhosis and hepatocellular carcinoma (41). The main difficulties in eradicating HCV are the lack of an effective vaccine and limited treatment options. Combination therapy using ribavirin, a synthetic guanosine analogue, and polyethylene glycol-conjugated interferon (peg-IFN) results in sustained viral clearance rates only in approximately 50% of treated patients (59). Furthermore, this therapy is costly, prolonged, associated with significant adverse effects, and not considered suitable for many HCV-infected patients (197). Although alternative compounds that target key enzymes essential for the virus are in clinical trial phases (3, 218, 219), there is a clear and pressing need for the development of additional therapeutic strategies and potential novel antiviral target to enhance response rates of

treated patients. Another attractive approach would be to target cellular proteins that the virus will required at a specific step of the viral life cycle.

3. The Hepatitis C Virus

With the development of specific diagnostic methods for hepatitis A and B viruses in the ´70s, it became apparent that most cases of hepatitis that occur after blood transfusion tested negative for A and B. The identity of these non-A, non-B agents (NANB) remained elusive for many years. In the late ´80s a California biotechnology company was able to isolate a cDNA clone from chimpanzees infected with serum from a patient with post-transfusion NANB hepatitis. They identified the agent responsible for the vast majority of cases of NANB hepatitis and named it *Hepatitis C Virus* (147). Subsequently, the complete genomes of various HCV isolates were cloned and sequenced by several groups.

3.1. Classification and Nomenclature

The HCV has been classified as a member of the genus *Hepacivirus* within the family Flaviviridae (266). The family encompasses nearly 80 enveloped viruses with a single (+) RNA genome and gets its name from the type virus of the group, the Yellow Fever Virus (Lat. *flavus*, yellow). The Flaviviridae family contains numerous members of major global concern that cause a variety of diseases in humans and animals. It includes three genera, *Flavivirus* (Yellow Fever Virus, West Nile Virus, Dengue Fever Virus), *Pestivirus* (Bovine Viral Diarrhea Virus) and *Hepacivirus* (HCV).

The HCV strains show extensive heterogeneity and have been classified into six genotypes and more than fifty subtypes. The genotypes represent six distinct genetic groups defined by phylogenetic analysis of complete genomes sequences or subgenomic fragments (233). These isolates can differ in their nucleotide sequence by 31 to 33%, compared with 20 to 25% between subtypes within these genotypes (231). The most common subtypes in Western countries are 1a and 1b in genotype 1 and 2a, 2b, and 2c in genotype 2, nowadays widely distributed. Further epidemiology studies revealed the existence of more specific geographical variants, such as infections produce by subtypes of genotype 2 in Western Africa whereas those in Central Africa are by genotypes 1 and 4. Genotypes 3 and 6 present similar diversity in South and Eastern Asia (232). A complete knowledge of the genotype-specific responses to the different treatments is a major goal in clinical research.

The most representative variants belong to the genotypes 1 and 2 for which most information has been collected on disease progression and response to IFN-based treatment. Furthermore, two of the most common systems used to study HCV, the replicon system and the production of infectious HCV in cell culture (HCVcc), are based on subtypes 1b and 2a respectively. These systems, discuss in detail bellow, were used along this work to study different steps in the viral life cycle.

3.2. Viral Particle

The HCV viral particle is approximately 50 nm in diameter (129, 228) and is composed of an icosahedral nucleocapsid bearing the genome, surrounded by a host cell-derived membrane envelope, where the

envelope glycoproteins 1 and 2 (E1-E2) are anchored forming heterodimers (70, 210). The plasma HCV-containing particles is a very heterogeneous population that vary in density (4), probably as a result of the formation of complexes with low-density and very-low-density lipoproteins (LDL and VLDL). This association gives the virus the opportunity to use lipoprotein receptors to bind the target cells (15), which may modulate infectivity (256, 257) in conjunction with other HCV-specific receptors.

3.3. HCV Genome

HCV is an enveloped virus with a single linear (+) RNA genome. Its length of approximately 9.6 Kb is composed of a highly structured 5´ and 3´ non-translated regions (NTRs), essential for RNA replication and translation, and a large open reading frame (ORF) encoding a single polyprotein precursor of just over 3000 amino-acids residues (23, 24, 216) (Fig. 1A). The 5´ NTR has a length of about 340 nucleotides (nt) and contains an internal ribosome entry site (IRES) mediating translation of the polyprotein with the structural proteins, core (C), envelope 1 and 2 (E1 and E2) located in the N terminus and the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) in the remainder. The structural proteins are separated from the nonstructural proteins by the short membrane peptide p7, which is thought to be a viroporin (215).

The NTRs are among the most conserved parts of the viral genome due to their multiple functions in viral translation and replication. Different processes within the viral life cycle are regulated by "*cis*-acting" or "*cis*-replication" elements (CREs) in the NTRs of the genome (198, 261).

Among these elements we found the IRES that mediate cap-independent translation initiation of the HCV genome (Fig. 1B). The HCV IRES encompasses RNA structures within the 5´ NTR and the core protein coding sequence. It is composed by three stem-loop (SL) structures named SLII, SLIII and SLIV. The SLIII is further divided in sub-domains, SLIIIa, -b, -c, -d, -e and -f, which form a four-way junction and a pseudoknot. These RNA elements can mediate 48S complex formation without a 5´-cap structure or scanning, and requires only a small subset of the canonical eukaryotic translation initiation factors (eIFs). The high affinity interaction between the 40S ribosomal subunit and the HCV IRES pseudoknot drives the positioning of the polyprotein start codon in the 40S P site. Assembly of active 80S ribosome still requires canonical eIF3, eIF2/GTP/Met-RNA^{met} and, following GTP hydroloysis, release of these factors and joining of the 60S subunit. These steps also involves functional interactions of the IRES with the 40S subunit (162, 200).

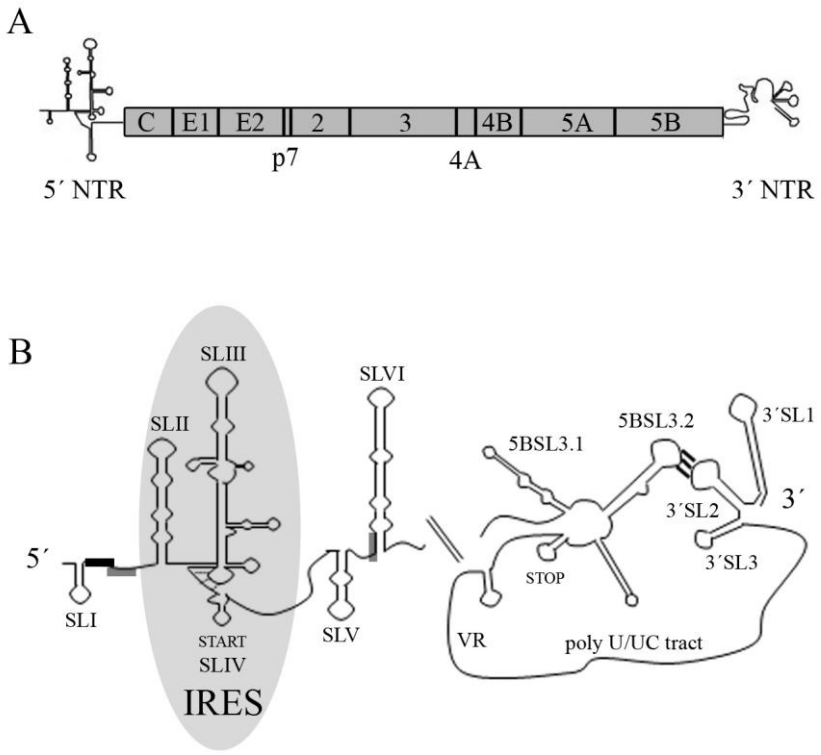

Fig. 1. HCV genome organization. (A) The HCV coding region is shown in grey indicating the positions where polyprotein cleavage occurs. The functions of viral proteins are described in the text. C, core; E1 and E2, envelopes proteins 1 and 2; p7, p7 protein; 2, NS2; 3, NS3; 4A, NS4A; 4B, NS4B; 5A, NS5A; 5B, NS5B. (B) Schematic diagram of the 5´ and 3´ HCV NTRs. The 5´ NTR contains six stem loop structures (SLI to SLVI). The IRES and the start site of polyprotein translation are shown within a light-grey shade. The miR122 biding site is shown with a black rectangle. The sites of interaction between the spacer region and the SLVI of the core coding region are shown with dark-grey rectangles. The 3´ NTR contains a tripartite structure, with a variable region (VR), a poly U/UC tract and the 3´ X region divided in three stem loops (3´SL1 to 3). The kissing loop interaction between 3´SL2 and the 5BSL3.2 stem loop within the NS5B coding region is shown with three parallels black lines.

Besides the IRES, additional CREs required for translation and replication are found in the 5´ NTR. Upstream from the IRES there is a spacer region (nt 21 to 43) between the SLI and SLII that contains two target sequences for a liver-specific microRNA (miR122) (Fig. 1B). Contrary to the role expected to play being part of the RNAi machinery, miR122 exert a

positive effect on HCV genome accumulation (127). Later on, revisiting previous results with a more accurate system, it has been shown that miR122 stimulates HCV translation by enhancing the association of the small ribosomal subunit with the HCV RNA (114). Furthermore, a recent study demonstrated that miR122 is involved in the dynamic modulation between two different HCV IRES conformational states, suggesting a correlation between this role of the miR122 with the described positive effect on translation (62). The miR122 was found to be abundant in liver cells and in the hepatoma cell line Huh7 (127, 149) which could explain why HCV replicates more efficiently in Huh7 than in other hepatoma cell lines and may contribute to the hepatotropism of the virus. It was also demonstrated that not only the spacer region between SLI and SLII is important for HCV replication, but also the SLs by themself (89). Finally, it has been reported that a long-range RNA-RNA interaction between the spacer region upstream the IRES (nt 22 to 40) and the SLVI of the core coding region exert an inhibitory effect on IRES-dependent translation (139) (Fig 1B).

The 3´ NTR, located downstream of the HCV coding sequence, consists of a proximal variable region that varies among genotypes, an internal poly(U/UC) tract of heterogeneous length, and the highly conserve sequence termed 3´ X region compose of three SLs named 3´SL1, 3´SL2 and 3`SL3 (34, 140, 244) (Fig 1B). The 3´ NTR functions in initiation of negative-strand synthesis and also as translation enhancer. The variable region is involved in RNA replication and, together with the poly(U/UC) tract and a portion of the 3´ X region enhance translation from the IRES (122, 123, 172, 295). Moreover, within the NS5B coding sequence it has been identified an additional SL structured, named 5BSL3.2, that participate in a long-range RNA-RNA interaction (kissing-loop) with the 3´SL2, essential for HCV RNA replication (88, 291, 292). The

poly(U/UC) tract not only functions as spacer for the kissing-loop interaction partners, i.e. 5BSL3.2 and 3´SL2, but also its length and sequence have been found to be important requirements for RNA accumulation (19, 291) and to be involved on activation of RIG-I-mediated innate immune response (263). The highly conserved 3´ X region is compose of two metastable SLs (3´SL-2 andg 3´SL-3) and a highly stable 3´-terminal SL (3´SL-1), and is absolutely required for replication in cell culture (87, 287, 288) and was found to be also of importance for infectivity *in vivo* (285).

3.4. HCV proteins

The expression of functional HCV proteins from the monocistronic genome is achieved by co- and post-translational processing of the polyprotein precursor. Cellular and viral proteases proteolytically cleaved the polyprotein into the structural proteins (core, envelope proteins E1 and E2), the hydrophobic peptide p7 and the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (22).

The structural proteins are released by host endoplasmic reticulum (ER) signal peptidases (214) and, afterwards, a cellular signal peptide peptidase process the core protein into its mature form (173). The core is an RNA-binding protein which forms the viral nucleocapsid (40) and it has been reported to be involved in regulating HCV RNA translation, particle assembly, generation of complete virion, cell signalling, apoptosis, carcinogenesis and lipid metabolism (251). The envelope proteins, E1 and E2, are type I transmembrane proteins targeted to the ER by aminoterminal signal peptides, where they are modified by extensive N-

linked glycosylation (61, 70, 192). These glycans have been shown to play a major role in protein folding, virus entry and protection against neutralizing antibodies (78, 102, 113). E1 and E2 interact to form non-covalent heterodymers, where the transmembrane domains of HCV envelope glycoproteins are essential for E1-E2 heterodimerization (152). These heterodymers are believed to be the basic units of the viral envelope.

The region located between the structural and the NS proteins codifies for the p7 polypeptide. The p7 ion channel of HCV is sensitive, in a genotype-dependent manner, to several classes of inhibitors *in vitro* (109, 110, 196, 207). It was also shown to be critical for infection and secretion of infectious viral particles in culture (126, 238).

The NS proteins are release from the polyprotein after cleavage by HCV NS2-3 autoprotease and, afterwards, NS3-4A is the viral protease involved in the cleavage of the remaining NS proteins (106, 107). The amino terminus of NS2 is cleaved from the adjacent p7 by cellular signal peptidases in a membrane-dependent manner. The NS2 protein by itself is not strictly required for genome replication (166) but it has been shown to be involved in virus assembly and release (201, 276, 289). The rest of the NS proteins are able to form the replication complex (RC) and undergo replication, as NS3-3´-NTR subgenomic replicons efficiently replicates in Huh7 cells (166). The NS3 is a bi-functional protein that contains a serine-type protease located in the N-terminal third and an RNA helicase domain in the C-terminal two-thirds (243). NS3 by itself has no transmembrane domain, but NS4A anchors the protease to intracellular membranes and contributes to the complete folding of the protease domain, enhancing its activity (21, 77, 137, 157, 245). The C-terminal helicase domain of NS3 is able of unwinding RNA-RNA duplexes (243)

removing RNA secondary structures and displacing RNA-binding proteins, thus, helping to the processivity of the RC. NS4B is a high hydrophobic protein that localizes to an ER-derived membranous compartment (118). Overexpression of NS4B induces the formation of a membranous web (105), and possibly it is involved, in conjunction with viral and cellular proteins, in the formation of the scaffold for the RC (72). NS5A is a phosphoprotein that has been shown to function in HCV RNA replication and assembly (16, 171, 249, 250). NS5A has been found in two different phosphorilation variants, a basal and a hyperphosphorilated form, and it was demonstrated that this modification strongly impaired HCV replication (75, 186). The phosphorilation of NS5A is a conserved feature among hepaci-, pesti-, and flaviviruses, supporting the concept that NS5A plays an important role in the regulation of viral replication (75, 186, 213). Finally, NS5B is the RNA-dependent RNA Polymerase (RdRp) which is the ultimate responsible of the synthesis of the HCV RNA genome (22).

An alternative reading frame protein (ARFP) or frameshift (F) protein encoded within the core region was reported by several groups (37, 267). These alternate ORF is present in all HCV genotypes (271). It has been recently shown that expression of the ARFP/F plays no role in HCV replication and virus production in cell culture and xenografted mice, however, the core stem loops within this region are important *cis*-acting RNA elements required for genome translation (268).

4. HCV Life Cycle

As for all (+) RNA viruses, the HCV RNA genome is the main actor in the life cycle, performing essential, mutually exclusive functions as mRNA for translation of viral proteins, serving as template for minus-strand synthesis during the replication process and as progeny genomes for assembly of viral particles (Fig 2). Thus, the coordination of these functions has to be a highly regulated process to ensure the efficacy of each step.

4.1. Entry

This process involves specific interactions between viral particle components and several host factors, starting with the binding of the virion to one or more host cell receptors until the delivery of the genome to its replication site. Using HCV pseudotype particles (HCVpp), i.e. lentiviral particles that incorporate unmodified HCV glycoproteins into the lipid envelope, it was demonstrated the importance of the heterodimer E1-E2 in viral entry (25, 69). A high affinity binding between E2 and two cellular receptors, CD-81 and Scavenger Receptor Class B Type I (SR-BI), was demonstrated (204, 220) and functionally linked to HCV entry. Further analysis have been carried out to identified the different E2 regions involved in these interactions (26, 42, 193). Some studies suggested that E1 plays a role in the fusion process (97, 153, 217), but detail research need to be conducted in order to elucidate E1 function in HCV entry.

In addition to CD81 and SR-BI, several membrane proteins have been proposed to be essential HCV receptors or co-receptors, as the low density

lipoprotein receptor (LDL-R) (5) and the components of cellular tight junctions Claudin-1 (CLND1) (76) and occludin (161). However, human cell lines expressing these proteins remain HCV resistant, suggesting that HCV particles bind *via* a specific interaction of the viral E1-E2 proteins with a cellular receptor complex with no precise composition (294). As HCV particles in patients are associated with LDL and VLDL, recent studies have highlighted the importance of these associations on HCV infection (51, 68, 117). Finally, it has been proposed that fusion of the attached particles could be *via* receptor-mediated endocytosis in a clathrin-dependent manner (31).

4.2. Replication

Once the HCV RNA genome is release into the cytoplasm of the infected cell, HCV proteins are generated *via* IRES-mediated translation at the rough endoplasmic reticulum (ER). Viral proteins remain associated to the ER membrane, and after translation and processing, the viral replication machinery is formed. The replication process of HCV RNA genome to occurs in a ER-associated RC, composed of viral proteins, replicating RNA and additional host factors (71, 105). The crucial role of intracellular membranes and the lipid environment for HCV replication have been subjects of several studies (39, 50, 131, 286).

After assembly of the replication machinery, synthesis of the HCV RNA genome takes place in two steps catalyzed by NS5B protein, the HCV RdRp. By analogy with other (+) RNA viruses, HCV replication is thought to be semiconservative and asymmetric (52). First, NS5B in cooperation with additional viral, e.g. NS3/4A, NS5A, and cell factors

assembles at the 3'end of the (+) RNA molecule to initiate *de novo* synthesis (24). A genome-length negative-strand RNA [(-) RNA] is synthesize, and both strands remain base-paired, and are used to generate multiple copies of the viral (+) RNA genomes (209, 246). These new synthesize RNAs are either used for translation, a new round of RNA replication, or are packaged into virus particles. Although the regulation of this key step in HCV life cycle is still poorly understood, a number of host factors that influence HCV RNA replication *via* interaction with different viral proteins have been identified (see section 6.2).

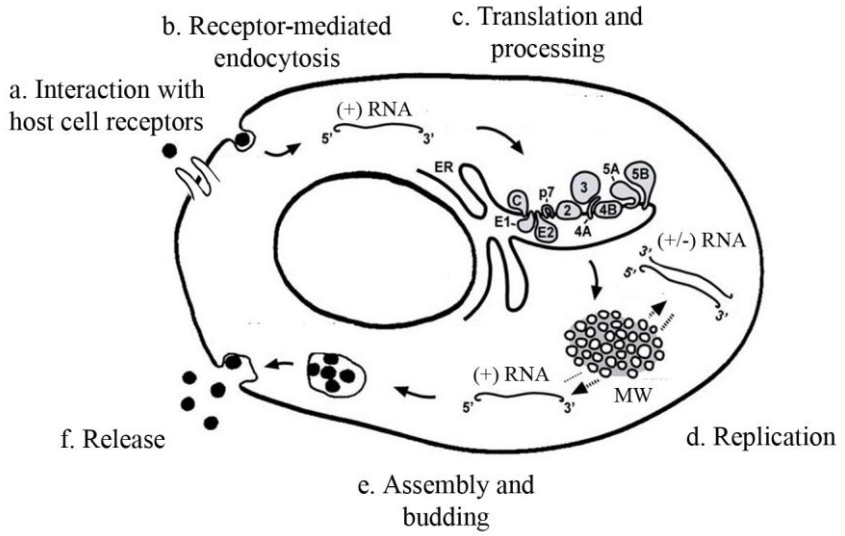

Fig. 2. HCV life cycle. The life cycle of HCV is similar to that of other members of the *Flaviviridae* family. Extracellular HCV virions interact with receptor molecules at the cell surface (a) and undergo receptor-mediated endocytosis (b). Then, the viral RNA is released into the cytoplasm. The genomic RNA is translated to generate a single polyprotein that is processed into the 10 mature HCV proteins in association with the membranous web (MW) (c). The mature HCV proteins replicate the RNA genome produce progeny RNA (d). A portion of this newly synthesized RNA is packaged into nucleocapsids and associated with the HCV glycoproteins, leading to budding into the ER (e). Virions follow the cellular secretory pathway and, during this transit, maturation of particles occurs. Mature virions are released from the cell, completing the life cycle (f). Adapted from (40)

4.3. Assembly and Exit

Genome packaging, virion assembly and secretion of infectious particles are the last stages in the HCV life cycle. Thanks to the development of HCV cell culture systems (HCVcc) (158, 270, 299), which allows to reproduce the complete viral cycle in culture, interesting studies have been conducted about the final steps of HCV infection.

The limitation of the HCVcc systems to produce infectious particles to one cell line or its derivatives exemplified the close relationship existing between the last stages of the cycle and the cellular environment (158, 299). A first clue about a virion-maturation process occurring before the exit of the infectious virions was the difference in density found between intracellular and secreted particles due to the association with lipoproteins (98), association later demonstrated of importance for infectivity *in vitro* and *in vivo* (159). Consistently, a recent study showed that impairment of VLDL production pathway decrease HCV infectivity (117).

Not surprisingly, it has been found that HCV core protein plays significant roles in release of viral particles. It has been demonstrated that a cellular signal peptide peptidase cleaved the core protein to its mature form, and that this process is required for virus assembly (7). Furthermore, the association between HCV core protein and lipid droplets, i.e. intracellular phospholipids storage and metabolically active organelles, was functionally linked in several studies to viral production and function *via* the recruitment by the core protein of NS proteins and RC to lipid droplet membranes (35, 178). This association of HCV core protein and lipid droplets could be related also to the formation of particles bound to lipoproteins, called lipo-viro-particles (LVPs) found in chronically infected individuals (35, 189).

5. HCV Systems

The main hurdle in the progression of understanding the viral and cellular factors involved in HCV replication was the lack of a reliable cell cultured system. However, the last decade has seen tremendous progress that started with the first establishment of HCV subgenomic replicons (166) and culminated in the development of efficient systems for production of HCV infectious virus particles in cell culture (158, 270, 299).

5.1. Replicons

Subgenomic replicons are *in vitro* transcripts of cloned DNA copies of the HCV genome where the sequences of the structural proteins, that are not required for RNA replication, were deleted and replaced by selectable markers or reporter genes (Fig 3A). These replicons replicate efficiently in the human hepatoma cell line Huh7 (166), however, although the introduction in the HCV replicon of cell culture-adaptive mutations allowed the generation of efficiently replicating full-length HCV genomes, virus production has not been observed (32, 119, 203). This limitation has been overcome by using the JFH1 isolate (Japanese Fulminate Hepatitis 1), a cloned 2a consensus genome which was isolated from a japanese patient with fulminant hepatitis (133).

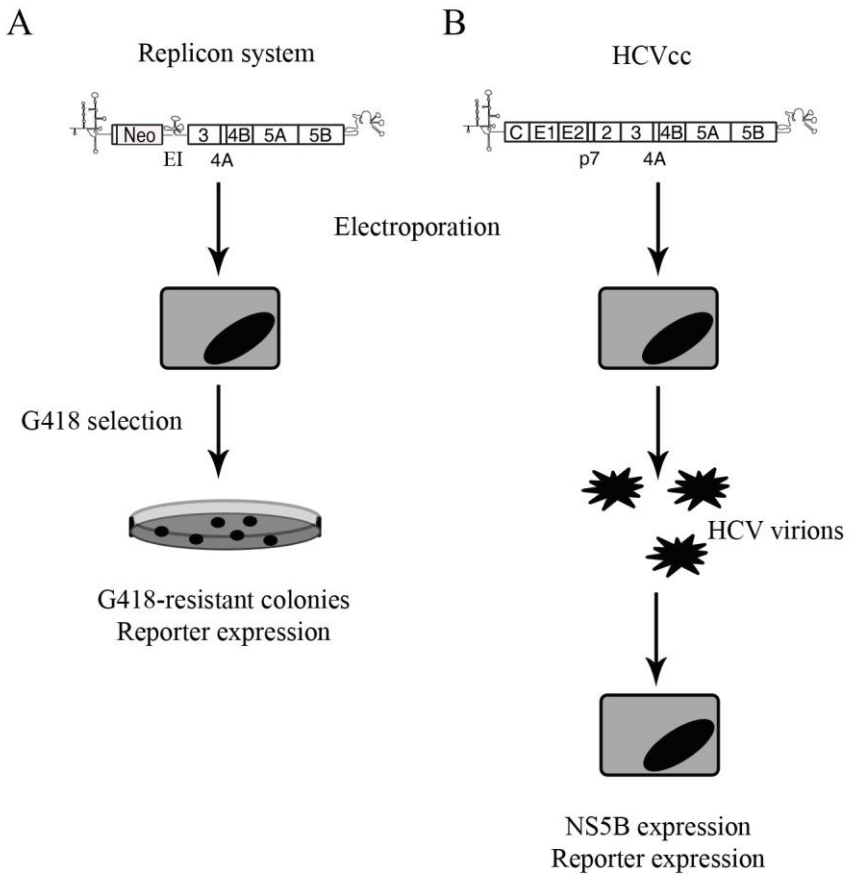

Fig 3. Systems for the study of HCV. (A) HCV replicon system, bicistronic replicon RNAs, encoding a selectable marker (Neo^r) under control of the HCV IRES in the first cistron and the HCV replicase proteins (NS3-NS5B) under the encephalomyocarditis virus (EMCV) IRES (EI) control in the second cistron. Replication of these RNAs leads to production of the selectable marker and allows for selection of colonies containing active RNA replication. Replicons expressing a number of reporter genes have been developed, as have methods to efficiently measure HCV proteins and RNA from these systems. (B) The HCVcc infectious virus system uses either JFH-1 HCV genomic RNA. These RNAs yield infectious HCV virions that can be used to infect naïve cells or animal models. Productive infection can be monitored by detection of the expression of NS5A, by a number of reporter genes, or by direct measure of viral RNA. Adapted from (248)

5.2. Virus

Taking advantage of the JFH1 isolate, three groups (158, 270, 299) have reported the production of infectious HCV particles in cell culture. The JFH1 isolate replicates to very high levels in Huh7 cells without requiring cell culture-adaptive mutations (132). Moreover, when Huh7 cells are transfected with genomic JFH1 RNA, infectious particles are released, that are infectious both for näive cells and for chimpanzees (270, 299) (Fig 3B).

These HCV cell culture systems have made possible to better characterize HCV proteins and to develop functional tests to study HCV-host proteins interactions affecting translation, RNA replication, particle assembly and release. Functional importance can be probed by a variety of methods, including RNA interference (RNAi) to downregulate specific gene products in mammalian cells. Accordingly, several host genes that modulate HCV replication and infectious virus production have been recently identified, however their specific role is mostly unknown (212).

6. Antiviral Drug Discovery

In the field of antiviral drug discovery, viral proteins have been the prime targets for viral treatment. The advantage of drugs that inhibit viral proteins are more likely to be virus-specific and, theoretically, less toxic, but due to the high error rate of viral polymerases is more likely the development of escape mutants, a main problem in antiviral treatment of RNA viruses. As an alternative, the development of drugs that target cellular proteins required for viral replication are predicted to be less likely to mutate in response to therapy but are expect to be more toxic.

However, it is reasonable to predict that there are cellular proteins unnecessary for cellular well-being, but critical to replication of viruses. Thus, as an alternative, a currently leading line of research in HCV field is the identification of host factors that HCV will required at some step of its life cycle (212, 248).

As mentioned above, current treatment consists in the combination of IFN and ribavirin, however, a sustained viral response is achieved only in 50% of treated patients. Presently, a variety of compounds are being under development or clinical trials as future alternatives to the conventional HCV standard of care (SOC) therapies. Among drugs design to target viral proteins we can distinguish between protease and polymerase inhibitors, targeting the NS3 protease and the NS5B polymerase, respectively (218). The NS3 inhibitors impaired the viral enzyme responsible of the HCV polyprotein processing. Moreover, as it has been shown that NS3 also disrupts the signalling pathway that lead to IFN production, it was suggested that this type of compounds could also function as immunity enhancers (218, 234). Although triple combination of a protease inhibitor together with peg-IFN and ribavirin showed an improve outcome compared with SOC therapies (218), the emergence of resistant mutations *in vitro* and *in vivo* have been reported (136, 146, 197, 219). Polymerase inhibitors, in combination with peg-IFN plus ribavirin, also showed an improvement outcome in comparison with SOC treatments, with no evidence of generation of viral resistance. However, several trials have been halted for diverse side effects, e.g. neutropenia, gastrointestinal problems or heptotoxicity (81, 154).

On the other hand, a distinct and novel approach for the treatment of HCV is to target host genes that may modulate different steps of the viral life cycle. As (+) RNA viruses share common features in their replication

process, it is possible that they rely on common host factors. This strategy impose a barrier to the generation of resistant mutants, although the principal disadvantage of using cellular proteins as targets for antiviral treatments is toxicity due to lost or impairment of its function. However, a clear example that the use of cellular proteins as antiviral has a promising future is the current state of clinical trials. Several host-targeted drugs are being tested and showed an improve sustained viral response (SVR) compared with SOC therapies or placebo, and include, among others, new interferon formulations, immune modulators, anti-steatosis drugs and cyclophilin inhibitors (60, 84, 218, 284). Nevertheless, these new antiviral therapies need to be administered in combination with peg-IFN and ribavirin and the SVR is still not optimal. Furthermore, a complete understanding of the interactions between the virus and the host is a prerequisite for the development of these new therapies. The identification of host factors that play important roles in common steps of (+) RNA virus replication open an extensive area of investigation that might serve as a cornerstone for the discovery of compounds that would have a inhibitory activity against many viruses.

6.1. Identification of Host Factors. Strategies

The identification of cellular proteins and the characterization of interactions with viral components have been hampered due to the complexity of cellular environments found in higher eukaryote cells. In order to outsmart these difficulties and gain insights into the interactions and roles existing between the virus and the host machinery, different systems have been developed. Mainly, the establishment of viral replication systems in genetically modified, easy-to-grow organism and, more recently, the development of the RNA interference (RNAi)

technique that allows transient depletion of specific cellular proteins in mammalian cells. With these approaches, the understanding of virus-host interactions, the identification of potential targets and the evaluation of the functional impact for antiviral therapy has seen tremendous progress.

6.1.1. RNA interference

A recent and powerful tool to evaluate virus-host interactions is the sequence-specific gene silencing using RNA interference (RNAi). The highly effective use of short-interference RNAs (siRNAs) to target either host or viral RNAs, demonstrates the potential of this technology to characterize gene functions, virus-host interactions and reveal novel antiviral targets. Recently, the development of genome-wide screening technologies allowed to systematically examine the human genome for the genes and pathways that are required by human pathogens such as *Human Immunodeficiency Virus* (HIV) (38), HCV (188, 212), WNV (143) and *Influenza A Virus* (112).

In spite of their advantages, the off-target effects and the different levels of depletion achieve are two of the main problems arise of the use of RNAi technology. Moreover, the efficiency and consistency of the transfection methods and the sensitivity of the reporter system are variables that must be taken into account at the moment of evaluate results. However, these disadvantages could be subdued whenever the studies are support by additional biochemical, virological and bioinformatics studies in order to confirm and validate gene function, providing a reliable approach for the identification of putative drug-targeted factors.

6.1.2. Yeast System

The establishment of systems that allow the replication of higher eukaryotic viruses in yeast have made grand contributions to the understanding of viral life cycles and the host factors involved. Reproducing known features of virus replication in their natural hosts, these systems permit to address relevant aspects of the viral life cycles like translation of viral proteins, template selection, formation of the replication complex, genome replication, encapsidation and recombination (10, 93).

The facility to grow and to be genetically modified of the yeast *Saccharomyces cerevisiae*, together with the high level of conservation of many biochemical pathways from yeast and humans has made this organism a fruitful system for understanding fundamental cellular processes (93). Moreover, the availability of gene-deletion mutant collections covering all the nonessential genes (100, 282), the down-regulatable essential gene-library (179), gene expression libraries with fluorescent or affinity tags that allow the study of subcellular localization and protein purification, respectively (64, 99), DNA microarrays chips that allow to assess the level of expression of thousands of genes in parallel (163, 223) and protoarrays with approximately 4100 purified yeast proteins (301) has been successfully used to identified host factors required for viral RNA replication and recombination of (+) RNA viruses (10). Genome-wide screenings identified over one hundred yeast genes affecting replication and recombination of different (+) RNA virus, as Brome Mosaic Virus (BMV) and Tomato Bushy Stunt Virus (TBSV) (125, 194, 226). Of note, these studies also link viral replication with previously unconsidered cellular pathways, e.g. mRNA turnover and stress response. Taking into account that (+) RNA viruses have common

replication strategies, it would be possible to translate the results obtained with this system to other members of the same viral group.

6.2. HCV and Host Factors

Within the last years, the number of research articles that connect cellular proteins with different steps in the life cycle of HCV has grown exponentially (199). These studies could have important implications for the future development of alternative and more effective treatments against HCV infection. Several host-targeted drugs, as mentioned before, are currently under preclinical phases. However, these new drugs could show incomplete effectiveness or the need of using them in combination therapies (218). In addition, clinical testing is an expensive and time-consuming process, hence, searching for more potent but less toxic HCV-modulators is required for the development and improvement of anti-HCV agents.

First works in this field have reported the *in vitro* interaction of several HCV proteins with a variety of cellular factors (94, 260, 272). Nevertheless, in most of the cases it was not clear if these interactions occur in the course of a normal viral infection or are observed as a result of protein overexpression. Various proteins have been found to bind to the HCV 5´ NTR, e.g. nucleolin (124), eukaryotic initiation factor 2B gamma subunit (eIF2Bγ) (145), to the 3´ NTR, e.g. Hu antigen receptor (HuR) (237), heterogeneous nuclear ribonucleoprotein C (hnRNP C) (104), the far upstream element of the *c-myc* proto-oncogene (FUSE) binding protein (FBP) (298), or with both NTRs, e.g. nuclear factors NF90, NF45 and RNA helicase A (121), La autoantigen and polypyrimidine-tract-binding protein (PTB) (58, 104, 122, 236, 259). In addition to their

interaction, it was also demonstrated a direct regulation in HCV replication, translation or both. Interestingly, starting with the observation that cyclosporine A (CsA) potently suppresses replication of HCV in cell culture (273), it was identified the target of its action, cyclophilin B (274). Cyclophilin B is a peptidyl-prolyl cis-trans isomerase that may alter the conformation of NS5B. It was shown that cyclophilin B specifically interacts with NS5B around the ER of the HCV replicon cells to promote association of the RdRp with the viral RNA (274). Nowadays, clinical trials with DEBIO-025, a cyclosporine analogue, showed high SVR in short-term monotherapy (84) and that combination with peg-IFN resulted in faster decline of the viral load (83), demonstrating that each one of these host-virus interactions represents potential antiviral targets.

To prove the involvement o a certain host factors on the HCV life cycle the RNAi-mediated knock-down of each of these cellular genes and/or over-expression of dominant negative mutants decreases viral replication have been extremely useful (94, 272, 296). Moreover, systematic RNAi screening have been carried out to promote the identification of HCV co-factors to a high throughput level (212, 241, 242). The proteins identified along these studies belong to a wide range of cellular processes, including intracellular vesicle trafficking, membrane biogenesis, lipid transport and metabolism, the unfolded protein response, immune response, RNA binding and translation and RNAi machinery. The demostration of the implication of the RNAi pathway itself as a proviral regulator of HCV replication adds strong evidence to the hypothesis that mammals, in contrast to plants and invertebrates, might not use RNAi as an innate antiviral defense mechanism (47, 48). A likely explanation for this proviral effect of RNAi components could be their use in miRNA biogenesis (20, 47, 49), given the demonstrated dependence of HCV on the liver-specific miRNA miR122 (127, 212).

7. Cellular RNA quality control

Eukaryotic gene expression is a highly-regulated process that contains many control checkpoints during mRNAs lifespan. From transcription, through post-transcriptional modifications to translation and degradation these control systems regulate normal mRNAs, prevent accumulation of non-functional RNAs and repress viral RNAs (168).

An important aspect of the regulation of eukaryotic gene expression is the cytoplasmic control of mRNA translation and decay. The mRNA turnover process functions setting the basal level of gene expression and as modulator of regulatory responses. Other quality-control processes involve the recognition and rapid degradation of aberrant mRNAs when translation termination occurs too early (non-sense mediated decay) (168), fails to occur (non-stop decay) (80) or when translation elongation stalls (no-go decay) (65). More recently, it has become clear that mRNA decay mechanisms play an important role in antiviral defenses, including basic mRNA decay machinery as well as specialized systems, such as RNAi (265, 275).

Interestingly, previous results from our laboratory linked the replication of positive-strand RNA viruses to mRNA turnover processes. The replication of the plant Brome Mosaic Virus (BMV) in the yeast *Saccharomyces cerevisiae* has been a fruitful model system for the identification and characterization of host factors involved in (+) RNA virus replication (10). By using this model it has been shown that the cellular activatgors of decapping Dhh1, LSm1-7 heptameric ring and the Pat1 proteins are required for translation and replication of BMV RNA suggesting a key function in the regulation of the switch of BMV RNA from the cellular translation machinery to the replication complex (63, 148, 170, 191).

7.1. mRNA Degradation pathways

The cellular mRNAs, transcribed by the RNA polymerase II, are characterized by a 7-methylguanosine (m^7G) cap in their 5´-end and a poly-A tail in their 3´-end, both elements confer stability to the mRNA molecule, preventing degradation of the transcript by cellular nucleases. It is possible to distinguish two different decay pathways regarding their dependence on the previous shortening of the poly-A tail, the deadenylation-dependent and the deadenylation-independent decay. A third degradation route is the endonucleolytic decay pathway, where the transcript is cleaved by cellular endonucleases, living two fragments without any protection susceptible to be degrade by exonucleases. However, the majority of the cellular mRNA transcripts are degraded by the deadenylation-dependent pathway.

In the deadenylation-dependent pathway degradation that have been identified in eukaryotic cells, the 5´ to 3´ and the 3´ to 5´ pathways. In both cases, the degradation of the transcript begins with the shortening of the poly(A) tail at the 3´end of the mRNA (182, 229). The deadenylation, which is a reversible step, results in the production of oligoadenylated mRNAs that can be readenylated and return to translation, or be destroyed. Once fated to be destroyed, the deadenylated mRNAs will follow two irreversible processes; they can be degraded either by the 3' to 5' by the exosome or by the Xrn1 exonuclease in the 5'-to 3' direction.

In the 3´-to 5´ decay, the exosome is the exonuclease machinery responsible of processing the mRNA. It is a 10 to 12 subunit complex that consists of six proteins with significant homology to 3´ to 5´ phophorolytic exoribonuclases and several accessory proteins including factors with homology to hydrolytic 3´ to 5´ exonucleases, RNA helicases

and proteins of the SuperKiller (SKI) family (116). The exosome has several roles in addition to cytoplasmic mRNA decay, including 3´ processing of non-coding RNAs in the nucleus. Following 3´ to 5´ decay, the 5´ cap on the remaining oligomer is metabolized by the scavenger decapping enzyme DcpS (160).

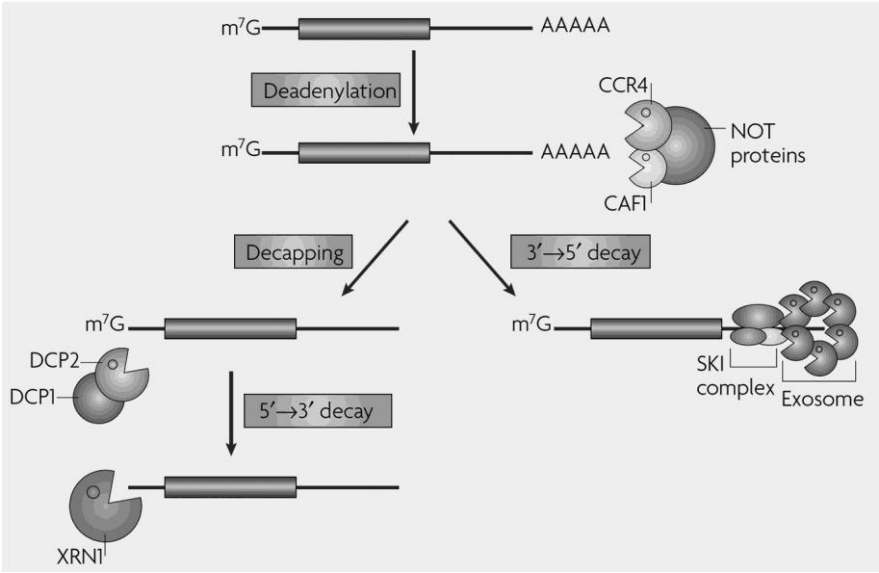

Fig 4. Degradation of bulk mRNA in eukaryotic cells by the deadenylation-dependent pathway. Degradation is initiated by deadenylation by the CAF1-CCR4-NOT complex. This complex consists of the deadenylases CAF1 and CCR and the NOT proteins. Following deadenylation, mRNAS can be degraded either 3' to 5' by the exosome and the SKI complex or 5' to 3' by the decapping-dependent pathway. In this last pathway, the cap of the mRNA is removed by the Dcp1 and Dcp2 enzymes and the RNA is then degraded by the exonuclease Xrn1. Extracted from (96)

In the 5´ to 3´ decay pathway, the poly(A) shortening triggers the removal of the 5´ cap structure in a process known as d ecapping performed by the dimer form by Dcp1 and Dcp2 proteins. In addition, the proteins Pat1, Dhh1 and the heteromeric complex Lsm1-7 have been shown to accelerate decapping. (discussed in detail below). Once decapped, the mRNA body is degraded by the Xrn1 exonuclease. All proteins involved in this

pathway have been found to localize in the so called cytoplasmic processing (P) bodies (12, 73, 222)

P-bodies are dynamic cytoplasmic granules that are formed by non-translating mRNAs together with enzymes of the basic decay machinery and the RNA-induced silencing pathway (65, 195). They have key roles in the switch of cellular mRNA from translation to another fate. The movement of mRNAs out of the translating polysome pool to P-bodies requires dramatic rearrangements in the state of the mRNA, including loss of ribosomes and translation factors. In yeast, the P-body proteins Dhh1, LSm1-7 and Pat1 function at this step acting as translation repressors and facilitators of P-body formation (45). Once in P-bodies, the mRNAs are either degraded or stored for subsequent return to translation.

7.2. The control of mRNA decapping

The decapping process is an important key and irreversible regulatory checkpoint, which represses gene expression by simultaneously shutting down mRNA translation and activating mRNA degradation by the 5´ to 3´ exonuclease Xrn1 (73, 195). This process is highly controlled and several factors are known to inhibit or to accelerate it. Among the proteins that inhibit the decapping are included the poly(A)-binding protein (Pab1) and components of the translation initiation complex as the cap-binding protein eIF4e (44). Several accessory factors are required to accelerate decapping although they are not absolutely required for decapping *per se*. In yeast, among these activators of decapping are found the LSm1-7 complex, a heptameric ring that binds to RNA, and the general translation repressors Dhh1 and Pat1 (45). These three proteins interact *in vivo* and are conserve from yeast to humans (29, 224).

The LSm1-7 complex is formed by seven Lsm proteins (Lsm1 to Lsm7) that belong to the highly conserved Sm/LSm family of proteins that exists in Archae, bacteria and eukaryotes. This family of proteins is characterized by two regions of conserved sequence termed the Sm motif, separated by a segment of variable length and composition (135). In eukaryotes, there are eight highly conserved LSm genes which form two distinct heptameric complexes, the cytoplasmic LSm1-7 and the nuclear LSm2-8. Both complexes form a ring structure suitable for RNA binding. The cytoplasmatic LSm1-7 has been shown to associate with mRNAs after deadenylation to activate decapping (36, 254, 255). This complex was shown to bind *in vitro* to single-strand RNAs (ssRNAs) at the 3'end and to be able to distinguish between oligoadenylated and polyadenylated RNAs, binding with much less affinity to the last ones (54). It is believed that the LSm1-7 complex function as an mRNA chaperon and by its binding to the mRNA would promote arrangements of the ribonucleoprotein structure (RNP) that would facilitate the access of the Dcp1/Dcp2 enzyme to the cap structure (55).

Pat1 is a protein with unknown biochemical properties and Dhh1 is a member of the DExD/H-box ATPases RNA helicase family. Although it is not fully understood yet how Pat1 and Dhh1 activate decapping, growing evidences suggest that they would act via promoting translation repression (45). Since translation and degradation are two competing processes, promoting translaton repression will accelerate decapping. In line with this, the decapping complex competes with the cytoplasmic translation initiation eIF4F complex for the mRNA cap (29, 224). Possibly, Dhh1 uses its ATPase activity to release eIF4F complex from the mRNP, which would simultaneously repress translation and stimulate decapping. However, Dhh1 also repress translation of reporter mRNAs that do not depend on eIF4F for translation (45). One possibility is that

translation repression by Dhh1 promotes a general destabilization of the eIF4F-mRNA cap complex in a more indirect manner, thus moving the balanced state toward decapping.

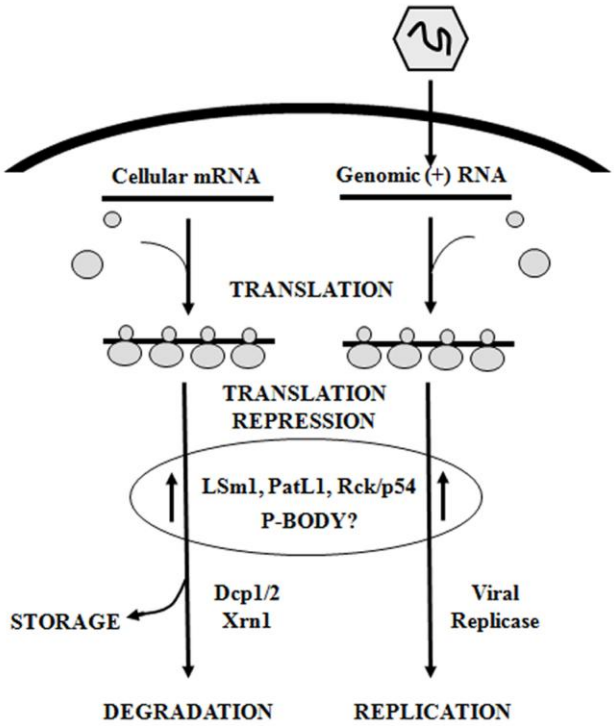

Fig 5. Common features determining the fate of cellular mRNAs and viral genomic positive-strand RNAs. The switch of cellular mRNAs from translation to storage or degradation requires similar host proteins as the switch of viral RNA genomes from translation to replication. Cellular mRNAs can be stored for future rounds of translation or be degraded. Viral mRNAs can also serve for future rounds of translation or used as template for replication. Ribosome subunits are symbolized as shaded circles.

8. Working Hypothesis

The genomes of (+) RNA viruses function as messenger RNAs for translation of viral proteins and as template for replication. Since these two functions, as cellular mRNA translation and degradation, are mutually exclusive, they need to be highly regulated. By using the ability of the brome mosaic virus (BMV) to replicate in yeast, it was shown that the Lsm1-7, Pat1 and Dhh1 proteins that transfer cellular mRNAs from translation to degradation regulate the transfer of the viral genome from translation to replication (Fig. 5).

Given the common replication strategies of (+) RNA viruses and the functional conservation of the cellular function of the Dhh1, Lsm1-7 and Pat1 proteins from yeast to humans, we hypothesize that also human (+) RNA viruses such as HCV use these proteins to control their own replication. Several observations support this hypothesis. First, the human homologues of Dhh1, LSm1-7 and Pat1, named Rck/p54, LSm1-7 and PatL1 respectively, have been identified and shown to localize in discrete cytoplasmic domains (12, 73, 222). Second, there is a functional conservation of yeast Dhh1 and human Rck/p54 in BMV RNA replication (11). Third, the LSm1 homologue in bacteria, Hfq, is required for the replication of the (+) RNA virus Qβ in *Escherichia coli* (180), thus, if this feature is conserved from prokaryotes to eukaryotes it is likely that is also conserved among eukaryotes. In my PhD I explored this possibility by testing the effects of these proteins in the human hepatitis C virus and other mammals positive-strand RNA viruses.

II. Specific Aims

1. Analysis of the effect of the decapping activators Rck/p54, LSm1 and PatL1 in the replication of HCV

Previously results in our laboratory have shown that the yeast decapping activators Dhh1, LSm1 and Pat1 play an essential role in translation and in the switch from translation to replication of the BMV genome. The functional conservation of these proteins from yeast to humans together with the common replication strategies of (+) RNA viruses suggest that they may be playing an important role in the replication of human (+) RNA viruses. With this in min dit was important to explore the function of the corresponding human homologues in HCV replication. The specif aims of this work were:

1.1. To establish silencing conditions of Rck/p54, LSm1, PatL1, Dcp2 and Xrn1 in hepatoma cell lines.
1.2. To evaluate the effect of the silencing on cell viability.
1.3. To set up conditions for efficient replication of HCV replicons.
1.4. To set up conditions for efficient HCV infection.
1.5. To test the effect of depletion of the decapping activators Rck/p54, LSm1 and PatL1 in the replication of HCV.
1.6. To test the effect of the silencing of Dcp2 and Xrn1 in the production of HCV replication.

2. Characterization of the mechanism involved in the effect of Rck/p54, LSm1 and PatL1 proteins on HCV translation

The decapping activators Rck/p54, LSm1 and PatL1 may affect HCV propagation either by acting in translation, replication or both. In order to elucidate their role in translation, here, we wanted to analyze the effect of Rck/p54, LSm1 and PatL1 silencing on HCV RNA translation using a replicative-defective HCV replicon. Since the major *cis*-signals controlling HCV RNA translation and replication are found in the 5` and 3´ NTRs of the genome, we will also address whether the function of these proteins in HCV propagation is linked with these areas. The aims were:

2.1. To establish a system to study the effect of Rck/p54, LSm1 or PatL1 silencing on HCV RNA translation.
2.2. To evaluate the effect of the silencing in the translation of HCV measure with HCV replicon and HCVcc derivatives.
2.3. To evaluate wheter the effect on translation inhibition depend on the HCV 5´ and 3´ NTRs.
2.4. To evaluate if a reconstituted human LSm1-7 complex is able to directly interact with HCV 5' and 3' UTRs.

3. Analysis of the effect of the decapping activators Rck/p54, LSm1 and PatL1 on the replication of other (+) RNA viruses

Besides HCV, other mammal (+) RNA viruses may also require Rck/p54, LSm1 and PatL1 for their life cycles. Given that 5´ and 3´ NTR regions of the positive-strand genomes present a variation of structures we selected those viruses that represent such variation. The aims of this part of the project were:

3.1. To test the effect of depletion of the decapping activators Rck/p54, LSm1 and PatL1 on the infection of a human Coronavirus.

3.2. To test the effect of depletion of the decapping activators Rck/p54, LSm1 and PatL1 on Poliovirus replication.

3.3. To test the effect of depletion of the decapping activators Rck/p54, LSm1 and PatL1 on BVDV replication.

III. Materials & Methods

Cell Cultures

Cell monolayers of the human hepatoma cell line Huh-7 (184), and its derivatives Huh7.5 and Huh7-Lunet cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, nonessential amino acids, and 10% fetal calf serum (FCS). Huh7-Lunet and Huh-7.5 cells represent cell clones that were generated by curing Huh-7 from HCV replicon with a selective drug or alpha interferon, respectively (33, 88), and show high permissiveness for HCV replication. HeLa cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, nonessential amino acids, and 5% fetal calf serum (FCS).

Plasmids

HCV replicons and viruses. Standard molecular biology practices were used throughout (297). All generated constructs used in this report were verified by sequencing. The plasmids pFKi389neoNS3-3'_ET, pFKi389neoNS3-3'GND, pFKi389FLucNS3-3'_ET and pFKi389FLucNS3-3'GND were kindly provided by Ralf Bartenschlager and used for generating the corresponding HCV Con1 (Genotype 1b) strain (GenBank accession number AJ238799) RNA replicons HCVrep-Neo, HCVrep-Neo-GND, HCVrep-Luc and HCVrep-Luc-GND, respectively. These constructs carry besides the reporter-genes neomycin phosphotransferase (HCVrep-Neo and HCVrep-Neo-GND) or firefly (*Photinus pyralis)* luciferase (HCVrep-Luc and HCVrep-Luc-GND) the highly adaptive mutations in NS4B (K1846T) and NS3 (E1202G+T1280I)

(1). The GND mutation in the active site of the NS5B gene in HCVrep-Luc-GND replicon impairs replication (167). The plasmids pFLJ6/JFH1/JC1 and pFL-J6/JFH/JC1p7Rluc2a (GNN) used to generate HCVcc and HCVcc-Luc-GNN RNA, were kindly provided by APATH LLC, ST. LOUIS, MO. Both contain the 5' NTR and non-structural regions NS3 through NS5B from the JFH1 (Genotype 2a) strain (GenBank accession number AB047639) and structural regions through NS2 and the 3' NTR from the J6 (Genotype 2a) strain (GenBank accession number AF177036). pFLJ6/JFH/JC1p7Rluc2a (GNN) contains a sea pansy luciferase (*Renilla reniformis*) gene fused in frame with the N-terminus of J6 NS2. The GNN mutation in the active site of the NS5B gene in this plasmid impairs replication (167).

BVDV replicon: The plasmid pBVDV/D9 was kindly provided by Sven-Erik Behrens and used for generating the corresponding DI9c BVDV replicon. This plasmid encoded for the ORF of the BVDV CP7 strain (GeneBank accession number U63479).

Luciferase-reporter derivatives. The luciferase reporter construct HCV-3`-NTR, kindly provided by Michael Niepmann (3), was used to generate HCV-NTRs-Luc and contains the Con1 5`-NTR and 3`-NTR flanking a firefly luciferase gene. The plasmid LUC cassette clone 2 was kindly provided by Fatima Gebauer and used to generate CAP-Luc-Poly(A), which contains a capped, non-viral 5´-NTR followed by the firefly luciferase gene and a poly(A) tail of 73 nucleotides. To generate the derivative HCV-5`NTR-Luc-Poly(A) containing the HCV 5´-NTR from genotype 1b and the firefly luciferase ORF followed by the poly(A) tail, the BamHI/ EcoRV fragment from the HCV 3`-NTR was treated with Mung Bean Nuclease and cloned into the LUC cassette clone 2 digested with SmaI and EcoRV. The derivative CAP-Luc-HCV-3`NTR was used

to generate the 5`-capped firefly luciferase transcript followed by the HCV 3` NTR and it was obtained by exchanging the HindIII/EcoRV fragment from the LUC cassette clone 2 by the HindIII/EcoRV fragment from the Con1 3`-NTR. The renilla luciferase reporter construct EMCV (A50) was kindly provided by Shelton Bradrick (4) and used to generate EMCV-IRES-Luc-Poly(A) which contains the EMCV-IRES followed by the renilla luciferase ORF and a poly(A) tail of 50 nucleotides.

Constructs used in electromobility shift assays. The sequence numbering of the HCV-RNAs used in this assay (Fig. 3) refers to the corresponding nucleotide of a complete Con1 HCV genome (GenBank accession number AJ238799). Fragments were amplified by PCR using forward primers with an EcoRI restriction site at the 5´ end followed by the T7 promoter and the HCV sequence. The HCV sequence specific reverse primers contained a BamHI restriction site at the 3´ end. The resulting EcoRI/BamHI PCR-fragments were subcloned into pUC18 (New England Biolabs, Ipswich, MA). To amplify the different areas of the 5`- and 3`-UTR of HCV, the following primers were used: 5'UTR: HCV pos1 fwd and HCV 400 rev; 1-129: HCV pos1 fwd and HCV 129 rev; 1-290: HCV pos1 fwd and HCV 290 rev; 290-400: HCV 290 fwd and HCV 400 rev; 129-400: HCV 129 fwd and HCV 400 rev; 9212-9605: HCV 9212 fwd and HCV 9605 rev; 9212-9375: HCV 9212 fwd and HCV 9375 rev; 9375-9605: HCV 9375 fwd and HCV 9605 rev; 9407-9605: HCV 9407 fwd and HCV 9605 rev; 9507-9605: HCV 9507 fwd and HCV 9605 rev. The DNA fragments Δ129-290 and ΔpolyU/UC were obtained by a two-step PCR procedure. For Δ129-290, the fragments 1-129 and 290-400 were amplified and then used as templates for another amplification round with the primers HCV pos1 fwd and HCV 400 rev. For ΔpolyU/UC, in the first step the fragments 9375-9407 and 9507-9605 were amplified, and then combined and reamplified in a second PCR with the oligonucleotides

9375fwd and 3'UTRrev. The oligonucleotides sequences used for each specific cloning are given in Table S1. The plasmid used to transcribe nt 9212-9356 of HCV RNA (pBSK-T7-3.1-3.3) was kindly provided by Ralf Bartenschlager. The plasmids used for expression and purification of individual LSm proteins and for transcription of the *Xenopus* U6 and U1 snRNA were already described (111, 293).

In vitro Transcription

In vitro transcripts of replicons were generated as previously described (165). In brief, plasmid DNA was digested with *Sca*I (HCV replicons) or *SmaI* (BVDV replicon) to linearized, extracted with phenol and chloroform, precipitated with ethanol, and dissolved in RNase-free water. *In vitro* transcription reactions were performed with RNAMaxx High Yield Transcription Kit (Stratagene) or MEGAScript kit (Ambion, Austin, TX) with T3 or T7 accordingly to manufacturer instructions. After 2 h at 37°C, additional 4 U of the corresponding RNA polymerase/µl were added, and the reaction was incubated for another 2 h at 37ºC. Transcription reaction was terminated by the addition of 2 U of RNase-free DNase per µg of plasmid DNA and 30 min incubation at 37°C. The transcription product was purified with the RNeasy Mini Kit (Qiagen). Capped RNA was generated using the ScriptCap m^7G Capping System (EPICENTRE Biotechnologies, Madison, WI). RNA concentration was determined by measurement of the optical density at 260 nm, and RNA integrity was checked by denaturing agarose gel electrophoresis. The transcripts used for electromobility shift assay were *in vitro* transcribed using T7 and SP6 polymerase (Fermentas GmbH, St. Leon-Rot, GER) and labelled with [α-^{32}P]UTP.

siRNAs and Antibodies

The hLSm1 and PatL1 siRNA duplexes were designed accordingly to the last recommendations of the manufacturer (Dharmacon) and synthesized as 21-mers with 3´dTdT overhangs. The Rck/p54 siRNA was purchased as pre-designed from Ambion. A BLAST search against the human genome sequence (NCBI UniGene database) was used to confirm that only the gene of interest would be targeted. The sense-strand sequences of the siRNAs are given in Table 1.

Table 1: Sense-strand sequences of siRNAs used in this study

Targeted gene	siRNA name	Sequence from 5`- to 3`end	Reference
LSm1	siL1	CAA ACU UAG UGC UAC AUC AdTdT	(239)
Rck/p54	si5076	GGA GGA GAG CAU UCC CAU UdTdT	(222)
PatL1	siPat #1	CUA GAA GAU CCA GCU AUU AdTdT	(222)
Xrn1	siXrn1	AGA UGA ACU UAC CGU AGA AdTdT	(239)
Dcp2	siDcp2	GAA AUU GCC UUG UCA UAG AdTdT	(239)
HCV-Con1-sequence	siHCV	CCU CAA AGA AAA ACC AAA CdTdT	(144)

As a negative control siRNA Non-Targeting # 1 (Dharmacon) was used, with at least 4 mismatches with all known human genes. Transfection efficiency was determined by FACS using a fluorescent-labelled siRNA (BlockIT, Invitrogen). For immunobloting, hLSm1 protein was detected with a polyclonal chicken anti-hLSm1 antibody (1:1000; Genway), Rck/p54 was detected with a polyclonal rabbit anti-Rck antibody (1:500; Biozol). The polyclonal rabbit anti-Xrn1 (1:1000) and anti-Dcp2 (1:200) were kindly provided by Dr. Lykke-Andersen and Dr. Kiledjian, respectively. The monoclonal anti-NS5A antibody (9E10) for detection of HCV NS5A protein in the infected cells was used in a 1:25000 dilution. The anti-NS3 antibody (code4) for detection of BVDV NS3 protein in transfected cells was kindly provided by Sven-Erik Behrens and was used in a 1:500 dilution. The protein loading control was done by detection of β-Actin with a monoclonal mouse anti-β-actin antibody (1:5000; Sigma) or piruvate kinase with a polyclonal goat anti-piruvate kinase (1:3000; Chemicon).

RNA Transfections

RNA transfections were performed by lipotransfection or electroporation. For lipotransfections, cells were cultivated as described above and transient transfections of siRNAs or HCV replicons were performed using Lipofectamine 2000 reagent (Invitrogen) accordingly with manufacturer instructions. Cells were assayed at different time points and efficient knockdown was confirmed by Western Blot. Transfection efficiency was determined by FACS analysis 4 hours post-transfection. Electroporation was carried out as described previously (164). In brief, Huh7 cells were trypsinized to detach, washed twice with phosphate-buffered saline (PBS), counted and resuspended at a density of 10^7 cells per ml in Cytomix (264) containing 2 mM ATP and 5 mM glutathione. Electroporation conditions

were 975 μF and 270 V using a Gene Pulser system (Bio-Rad, Munich, Germany). The viability of the silenced cells was assessed by quantification of propidium iodide (PI) (MBL International, Woburn, MA), by measurement of intracellular ATP-levels using CellTiterGlo (Promega, Madison, WI) or by growth rate, counting cells up to six days post-transfection. The ATP assay was used to analyze cell viability of the lipofectamine-transfected cells while growth rate and PI incorporation was used to analyze viability of the electroporated cells.

Replication assays

For the HCV colony-formation assays, 1 μg of *in vitro*-transcribed RNA and 10 μg of total RNA (HCVrep-Neo and HCVrepNeo-GND), with or without 1 to 4 μM of each siRNA, was added to 400 μl of cell suspension in a cuvette with a gap width of 0,4 cm (Bio-Rad), co-electroporated, and immediately transferred to 8 ml of DMEM containing 10% FCS and 1.25% DMSO and seeded in 10-cm-diameter cell culture dishes. The medium was replaced 24 hours after electroporation with DMEM supplemented with 10% FCS and G418 (500 μg/ml). Medium was changed once a week, and 4 weeks after electroporation, colonies were stained with crystal violet solution (1% crystal violet in 50% ethanol) and quantified. The concentration of siRNA used is similar to the ones used in other publications that also use electroporation for Huh7 cells transfection with siRNAs (279, 280). To monitor luciferase activity in transient assays, Huh7-Lunet cells were co-electroporated as described above or transfected with lipofectamine 2000. For HCV replication assays, 5 μg of *in vitro*-transcribed RNA and 10 μg of total RNA, with or without 1 μM of each siRNA were mixed with the cell suspension. Electroporated cells were immediately transferred to 20 ml of medium and seeded at a density of 4.2 x 10^4 cells/cm^2 in 24-well plates. Quantification of the intracellular

expression of replicon-encoded luciferase (HCVrep-Luc and HCVrep-Luc-GND) at different timepoints was done using luciferase-reporter assay systems (Promega, Madison, WI) in a Berthold FB12 Luminometer (Pforzheim, GER).

For the BVDV replication assay, Huh7 cells were co-electroporated as described above with1 µg of *in vitro*-transcribed DI9c BVDV RNA, with or without 1 µM of each siRNA. Protein samples were subjected to western blot analysis against BVDV NS3 protein. RNA samples were analyzed by using the SYBR GREEN PCR Master Mix and the ABIPRISM 7900HT instrument (Applied Biosystems) for detection and real-time quantification, using BVDV-specific primers. As an internal control, changes in the expression of actin mRNA were monitorized (Table 2).

To investigate the effect of the knockdown of the analyzed proteins on HCVcc replication, silenced cells in 24-well plates were transfected by lipofectamine 2000 with 0,8 µg of HCVcc RNA per well at the time of most efficient silencing. To maintain the protein knockdown of LSm1 and PatL1, an additional transfection with siRNA was required 24 hours later. Intracellular HCVcc RNA levels as well as infectious HCVcc particles in the supernatant of transfected cells were quantified at various time-points up to 72 hours post-transfection. The obtained values were standardized to the amount of transfected RNA quantified 4 hours after transfection to equalize transfection efficiencies.

Translation assays

Analysis of HCV translation was performed with different luciferase constructs. After transfection by lipofectamine 2000 of the respective

RNAs in previously silenced cells, luciferase activities were measured 4 hours later and normalized to the total amount of protein. Then these values were corrected by the amount of HCV RNA that was obtained by qRT-PCR using specific Taq-Man primers and probes after normalization to internal 18S RNA (Table 2). Proteins were extracted with a lysis buffer supplied in the Dual-Luciferase Reporter Assay System (Promega), and luciferase activity was measured in a Berthold FB12 Luminometer (Pforzheim, Germany). At least, two independent experiments with replicates were performed for each assay. To test the levels of general cellular translation in silenced cells, cells starved for 1 hour in medium lacking methionine and cysteine (Invitrogen, Carlsbad, CA) were incubated for 30 minutes with [^{35}S]-methionine (200 µCi/ml). Then cells were harvested directly in sample-loading buffer and analysed by SDS-PAGE and autoradiography.

Titration of infectious HCVcc particles and RNA quantification

Titration of infectious particles in the supernatant of HCVcc RNA-transfected cells was performed in a limited dilution assay, as described (158). The NS5a protein in the infected cells used in this assay was detected by a 1:25000 dilution of anti-NS5A antibody (9E10) and a 1:200 dilution of Horseradish-Peroxidase-conjugated anti-mouse IgG (GE Healthcare, Amersham, UK).

For RNA quantification, total RNA was isolated from HCV RNA-transfected cells or luciferase reporter-transfected cells using the RNeasy Mini Kit (Qiagen, Hilden, GER) and subsequently treated with DNAse I (Ambion, Austin, TX). Total RNA was quantified using Nanodrop® ND-1000 - UV/Vis Spectrophotometer. For RNA quantification, 40 ng of total

RNA was reverse transcribe using random primers and Superscript III according to manufacturer´s recommendations (Invitrogen, Carlsbad, CA). The cDNA was amplified with specific primers and probes using an ABI Prism 900HT sequence detection system (Applied Biosystems, Foster City, CA) show in Table 2. The amplifications were standardized to an internal 18S control (ABI Taqman HS HS99999901_s1*; Applied Biosystems, Foster City, CA) using a relative quantification analysis from the SDS 2.3 software (Applied Biosystems, Foster City, CA).

Infections with HCoV-299E and Poliovirus

HCoV-299E: Wild-type Human Coronavirus E229 (HCoV-E229) stocks were used to infect previously silenced Huh7 cells at the time of most efficient silencing at different moi. Levels of replicating virus were titrated by limiting dilution by a standard plaque assay. Huh7 cells were grown to 90% confluence in 6-well plates using complete DMEM. Serial 10-fold dilutions of each sample were prepared in serum-free DMEM and added onto the monolayer and allow absorb for 1 hour at 33º C. A 1:1 overlay mixture consisting of 2 X DMEM supplemented with 10% FCS, 2X penicillin/streptomycin and 2% Methyl Cellulose was prepared. After incubation the medium was aspirated and replaced with 500 µl of overlay mixture and incubated O.N. at 33º C. Plates were stained with Crystal Violet and plaques were counted with the assistance of a light box.

Poliovirus: wild-type Poliovirus stocks (Mahoney strain) were produced in HeLa cells and used to infect previously silenced HeLa cells at the time of most efficient silencing at different moi. Levels of replicating virus in the supernatants were determined by TCID50. For that, HeLa cells were grown up to 90-100% confluence in 96-well plate using complete DMEM. Serial 10-fold dilutions of each sample were prepared in 1% FCS DMEM, added to the cell monolayer and allowed to absorb for 1 hour at 37º C.

After incubation, the medium was removed, the cells washed with PBS and fresh 1% FCS DMEM medium was added. After 24 to 48 hours post-inoculation, cells were examined under a light microscope for cytopathic effect.

Electromobility shift assays.

Expression, purification of individual LSm proteins and reconstitution of complexes were performed as previously described (293). 300 cpm of gel-purified *in vitro* transcribed HCV-RNAs were incubated with 10 pmol of LSm protein heptameric complexes in a buffer containing 20mM HEPES-NaOH pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 0.1U/µl RNasin and 0.1 µg/µl yeast tRNA in a 5 µl assay at 30°C for 1hr. Samples were loaded on previoulsy pre-run 5% native PAGE gels, and run at 4°C for 2 hours, 30 mA. Gels were autoradiographed on maximum sensivity films (KODAK Biomax MS). For assays that included RNA competition, the indicated amount of RNA competitor was added to the reactions. The assays for binding to *Xenopus* U1 and U6 snRNAs were performed as already described (293).

Table 2: Oligonucleotides and Probes used for quantitative RT-PCR

Quantified RNA	Targeted sequence	Function	Sequence from 5`- to 3`end with indicated modifications	Reference
HCVcc **HCVcc-Luc-GNN** **HCVrep-Luc-GND** **HCV-UTRs-Luc**	HCV 5`-UTR	Primer sense	TCTTCACGCAGAAAGCGTCTAG	this study
	HCV 5`-UTR	Primer anti-Sense	CGGGTTGATCCAAGAAAGGA	this study
	HCV 5`-UTR	Probe	CGGGAGAGCCATAGTG-6-FAM-MGBNFQ	this study
HCVcc **HCVcc-Luc-GNN** **HCVrep-Luc-GND** **HCV-UTRs-Luc**	HCV 3`-UTR	Primer sense	GGCTCCATCTTAGCCCTAGTCA	this study
	HCV 3`-UTR	Primer anti-Sense	CAGTATCAGCACTCTCTGCAG	this study
	HCV 3`-UTR	Probe	CTAGCTGTGAAAGGTC-6-FAM-MGBNFQ	this study
HCV-5`UTR-Luc-Poly(A)** **CAP-Luc-HCV-3`UTR **CAP-Luc-Poly(A)**	Firefly Luciferase ORF	Primer sense	CCGTTGTTGTTTTGGAGC	this study
	Firefly Luciferase ORF	Primer anti-Sense	CGATGACGCCGGTGAACT	this study
	Firefly Luciferase ORF	Probe	CTGGCGACGTAATCCACGAT-6-FAM-MGBNFQ	this study

EMCV-IRES-Luc-Poly(A)	Renilla Luciferase ORF	Primer sense	GCTGTTATTTTTTTACATGGTAACGC	(50)
	Renilla Luciferase ORF	Primer anti-sense	CGCGCTACTGGCTCAATATG	(50)
	Renilla Luciferase ORF	Probe	CCTCTTCTTATTTATGGCGACAT-6-FAM-MGBNFQ	(50)
BVDV	BVDV ORF	Primer sense	CCATGCCCTTAGTAGGACTAGC	
	BVDV ORF	Primer antisense	CGTCGAACCACTGACGAC	
ACTIN	Actin ORF	Primer sense	GCATCGTGATGGACTCCGGTG	
	Actin ORF	Primer antisense	CATGAGGTAGTCAGTCAGGTC	

Western Blots

Total cellular protein extracts were obtained with 1x Lysis Buffer (Promega). The lysates were then centrifuged at 12000 rpm, 2 min at 4ºC and protein concentration in the supernatant was quantified using the BCA protein assay kit (Pierce). For western blotting, 10-20 µg of total proteins were separated by SDS-PAGE gel electrophoresis at 100 V in 1X running buffer (25 mM Tris-base, 200 mM glycine, 0.1% [w/v] SDS). Proteins were transferred to a PVDF membrane (Immobilion P transfer membrane, Millipore) at 100 V for 1 h in 1X transfer buffer (25 mM Tris-HCl pH 8.3, 200 mM glycine, 20% [v/v] methanol). After the transfer, acrylamide gels were stained with Comassie brilliant blue (Sigma) and membranes were stained with Ponceau Red staining solution to confirm equal protein loading and correct transfer, respectively.

Transferred membranes were blocked with 5% skimmed milk diluted in TBS-0.1% Tween-20 for 1 h at room temperature (RT) and later incubated with the primary antibody diluted in 1% skimmed milk in TBS-0.1% Tween-20 for 1 hour at RT or O.N. at 4ºC. Four washes of 10 minutes in TBS-0.1% Tween-20 eluted non-bound primary antibody. Membranes were ncubated with the secondary antibody conjugated to horseradish peroxidise (HRP) diluted in 1% skimmed milk in TBS-0.1% Tween-20 for 1 hour at RT. After four 10-minutes washes in TBS-0.1% Tween-20, membranes were revealed with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Proteins were detected with the specific antibodies at specific dilutions described above.

IV. Results

1. Analysis of the effect of the decapping activators Rck/p54, LSm1 and PatL1 in the replication of HCV

Previous studies in our laboratory with the BMV/yeast system demonstrated that the decapping activators LSm1-7 complex and Dhh1 and Pat1 proteins are required for BMV RNA replication and translation (10, 11, 63, 120, 170). To evaluate whether the respective human homologues, human LSm1-7 complex, Rck/p54 and PatL1 proteins affect HCV replication in human hepatocytes, a gene silencing strategy was employed with two different HCV systems. These were HCV replicons (166), which allow efficient replication but do not result in virus production, and infectious HCV virus (HCV cell culture or HCVcc) (158, 270), which reproduces the entire virus life cycle.

Host factors can act in every step of HCV life cycle, such as translation, replication and encapsidation. The use of both the replicon and HCVcc systems has been of great help to define in which step is affected by specific host factors. Replicons can replicate in cell culture but due to the lack of structural proteins do not encapsidate. Thus, with this system one can separate effects on HCV translation and replication from effects on encapsidation. The HCV replicons belong to the Con1 strain of the 1b genotype and are composed of the HCV 5´ NTR followed by a neomycin phosphotransferase selection marker (Neo) (HCVrep-Neo) or the firefly (*Photinus pyralis*) luciferase gene (HCVrep-Luc), the internal ribosome

entry site (IRES) of the encephalomyocarditis virus (EMCV) and by the HCV genes for the non-structural proteins and the HCV 3´ NTR. In these systems, HCV replication and translation are coupled, as the replicon-encoded polymerase only supports replication in *cis*. However, it is possible to test effects on translation by using replication-deficient mutants of both replicons (HCVrep-Neo-GND and HCVrep-Luc-GND). These constructs carry a mutation in the NS5B polymerase active site (GDD) (Fig. 6).

The HCVcc system covers all steps of HCV life cycle, translation, replication and encapsidation. Measurements of infectious virus titers in infected cells depleted of the host factors of interest give therefore the maximum of information about the effect of these proteins on HCV life cycle. In addition, the parallel measurement of intracellular viral RNA levels or reporters encoded by this RNA can point out to an effect on a translation/replication versus later steps. For example, similar intracellular RNA levels but different extracellular titers would indicate an effect on the assembly/release processes. The HCVcc belongs to a 2a genotype and was used directly for the production of infectious particles (HCVcc) or with a luciferase reporter (HCVcc-Rluc) (Fig. 6). HCVcc contains the 5' NTR and non-structural regions NS3 through NS5B from the JFH1 (Genotype 2a) strain and structural regions through NS2 and the 3' NTR from the J6 (Genotype 2a) strain (202). The derivative HCVcc-RLuc contains a sea pansy (*Renilla reniformis*) luciferase gene which is fused in frame with the N-terminus of J6 NS2. In addition, as in the replicon system a replication-defective derivative (HCVcc-RLuc-GNN) is available that allows to test effects on translation. (Fig. 6) (167).

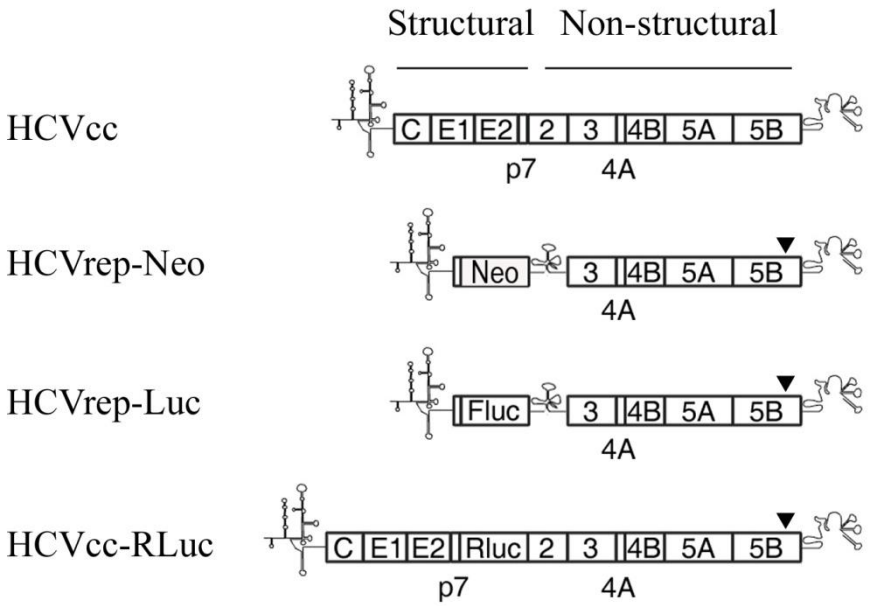

Fig.6. Schematic representation of the genomes of HCVcc, HCV replicons and derivatives used in this study. *Neo*, neomycin phosphotransferase gene; *Fluc*, firefly luciferase gene; *RLuc*, renilla luciferase gene. The arrowheads indicate the position where the replication-defective constructs contain the GND or GNN mutation.

The replicons and HCVcc systems require the use of HCV-permissive cell lines. These cell lines are human cells of hepatoma origin, specifically Huh7 cells and their derivatives. Currently, three cell lines were used for the assays, Huh7, Huh7-Lunet and Huh7.5, as these cells confer different properties regarding the systems for HCV replication. The Huh7-Lunet and Huh7.5 cells are subclones of the Huh7 cell line that were generated with a selectable HCV replicon and cure from HCV by treatment with interferon-α (33, 209). For the HCV replicons, we used Huh7 and Huh7-Lunet cells, as these cell lines support most efficient replication of HCV (88). In contrast to this, we used for HCVcc the Huh7.5, as these cells

express high amount of surface CD81 and therefore allow most efficiently release and infection of HCVcc (142).

1.1. Set up conditions for efficient replication of HCV replicons

To analyze the replication of HCV in cell culture, two different replicon constructs were used, the HCVrep-Neo and the HCVrep-Luc (Fig. 6). The replication of HCVrep-Neo confers neomycin resistance. Under selection with neomycin, cells containing replicating HCVrep-Neo propagate to neomycin-resistant cell colonies which can be quantified in a colony-formation assay. For this, Huh7 cells were electoporated with 1 µg of HCVrep-Neo or HCVrep-Neo-GND replicon and 24 hours later geneticin (G418) was added to the medium as a selective pressure. The cells were fixed with crystal violet three weeks later, and the number of colonies quantified. As shown in Fig. 7A we obtained approximately 5 x 10^3 colonies per µg of HCVrep-Neo electroporated. As expected, no colonies were found for cells transfected with the non-replicating HCVrep-Neo-GND.

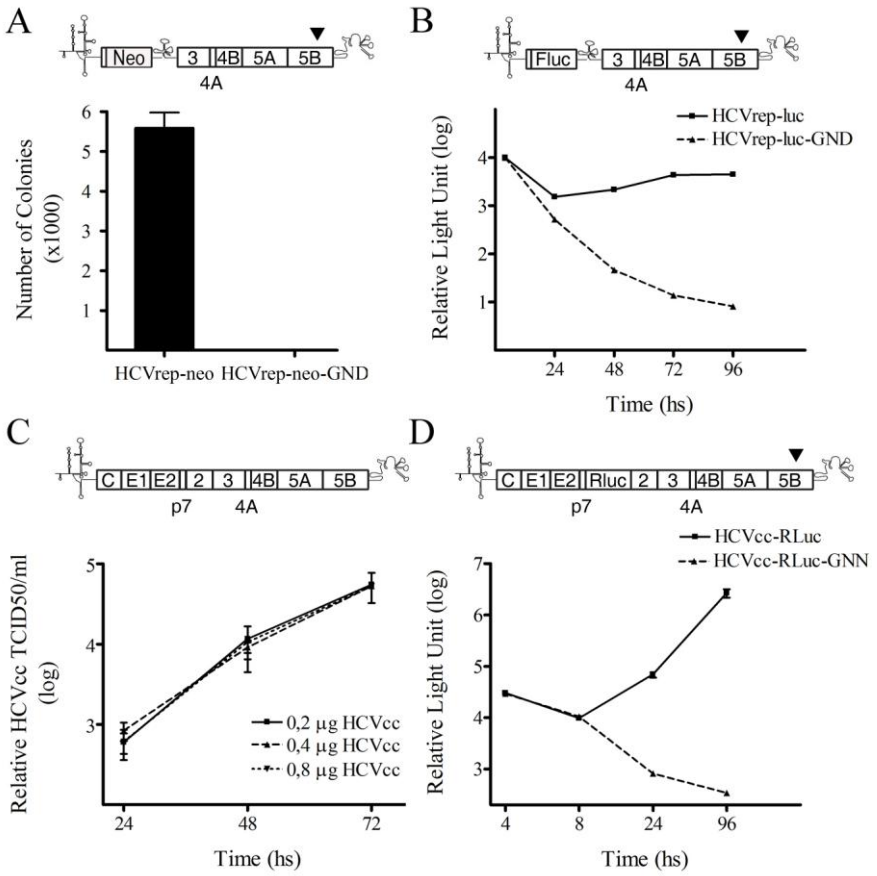

Fig. 7. HCV systems. (A) *HCV-Rep-Neo*. Mean number of G418-resistant colonies per 1 µg replicon RNA electroporated into Huh7 cells after three weeks of selection with G418. Error bars indicate the standard deviation of three independent experiments. (B) *HCV-Rep-Luc.* Percentage of luciferase light units after electroporation of 5 µg of replicon RNA in Huh7-Lunet cells. Data were normalized for transfection efficiency among identical replicons as determined by measurement of the luciferase activity 4 hours after transfection. Values were measured by triplicate and bars representing the standard deviation were too small to show. Note the logarithmic scale of the ordinate. (C) *HCVcc.* Titration of supernatants from Huh7.5 cells transfected with different amounts of HCVcc RNA. The titration was performed by mean of a limited dilution assay by immunostaining of the HCV NS5A protein and normalized by the qRT-PCR values of the 5´ NTR at 4 hours. (D) *HCVcc-RLuc.* Percentage of luciferase light units after transfection of 0,8 µg of the transcripts into Huh7.5 cells. Values were normalized by the qRT-PCR values of the 5´ NTR at 4 hours. In the top of each graph schematic representations of the genomes of HCV replicons, HCVcc and HCVcc-RLuc are shown.

The HCVrep-Luc replicon contains the firefly luciferase gene (*luc*) under the control of the HCV IRES (Fig. 6). The kinetic of the activity of the luciferase reporter mirrors the amount of intracellular HCV RNA and therefore the HCV replication. To measure HCV replication, 5 µg of HCVrep-Luc replicon were electroporated into Huh7-Lunet cells and samples were taken at 4, 24, 48, 72 and 96 hours after transfection. The obtained luciferase values were corrected by the total amount of protein. The luciferase values obtained at 4 hours after transfection reflected only the translated input RNA, as shown by similar luciferase values obtained with HCVrep-Luc and its derivative HCVrep-Luc-GND, which does not replicate. Therefore luciferase light units obtained with cells harvested 4 h after electroporation were used to normalize the values obtained with cells harvested at later timepoints to relative light units (RLU). The replication kinetic of the HCV-rep-Luc showed a marked decrease in luciferase values 24 hours after electroporation, with a gradual increase in the wt replicon until 96 hours (Fig. 7B). In contrast, the luciferase values obtained for the replication defective HCV-rep-Luc-GND decreased to background levels at 96 hours.

1.2. Set up conditions for efficient HCV infection

Two different HCVcc systems were used to test the effect of host factors on the entire HCV life cycle in cell culture, the HCVcc and the HCVcc-RLuc (Fig. 6). For the establishment of the experimental conditions for infection with HCVcc, three different concentrations of *in vitro* transcribed RNA, 0,8; 0,4 and 0,2 µg per well of a 24-well plate, were

transfected using Lipofectamine 2000 into Huh7.5 cells. Then, from 24 to 72 hours later the supernatants were collected and used to infect new monolayers of Huh7.5 cells. Titration of the supernatants was performed by mean of a limited dilution assay by immunostaining of the non-structural protein NS5A, as described before (158). Differences in transfection efficiencies could result in differences in the ammount of HCV RNA trasfected. Moreover, the host factors under study are involved in the RNA degradation pathway, thus, their depletion might affect stability of HCV RNA in knocked-down cells. Thus, it is important to quantify the RNA input because differences observed in viral titer could solely be due to differences in the RNA transfected. Consequently, to normalize the titer values, intracellular HCV RNA levels were quantified by quantitative RT-PCR (qRT-PCR) at 4 hours post-transfection and used to normalized the titer values. Accordingly, total RNA was isolated from HCV RNA-transfected cells, reverse transcribe and amplified with specific primers and probes. For this, we focused on both HCV NTRs to ensure detection of the whole HCV genome instead of partially degraded molecules. As the relative values for both NTRs did not vary (Fig. 12A and B), for normalization of subsequent experiments were used the qRT-PCR values of the 5´ NTR at 4 hours. The titration of Huh7.5 cells transfected with the different HCV RNA concentrations show that no differences in titer were observed among the different concentrations assayed (Fig. 7C), thus, the lower concentration was chosen as experimental condition.

When the HCVcc-RLuc and its replication-defective derivative HCVcc-RLuc-GNN were used, cells plated in a 24-well plate were transfected with 0,8 µg of *in vitro* transcribed RNA per well by lipofectamine 2000, and luciferase activity was followed until 96 hours post transfection. The replication kinetic showed a significant increment in luciferase values

from 24 to 96 hours compared with the replication-defective transcript, indicating active replication throughout this timepoint (Fig. 7D). In contrast to this, at 4 up to 8 hours after transfection, the luciferase values of HCVcc-RLuc and HCVcc-RLuc-GNN are indistinguishable indicating that replication of HCVcc in this system is only detectable at timepoints later than 8 hours.

1.3. Establishment of the silencing conditions for Rck/p54, LSm1, PatL1, Dcp2 and Xrn1 in human hepatoma cell lines

In the 5´- 3´ deadenylation-dependent mRNA decay pathway, Rck/p54, LSm1-7 and PatL1 act in an early step by promoting the transfer of the mRNA from a translationally active state to a translationally repressed state. The translationally repressed mRNA is a substrate for decapping *via* the Dcp1/Dcp2 decapping enzyme and subsequent 5´ to 3´ degradation by the exonuclease Xrn1 (44). To study whether Rck/p54, LSm1 and PatL1 together with Dcp1 and Xrn1, late components of the same cellular pathway, play a role in HCV RNA replication, we set up the conditions for their transient knock-down in cells permissive for HCV replication by using specific siRNAs. With respect to the cytoplasmic LSm1-7 ring, we focused on the LSm1 subunit because it is the one that defines the role of the ring in decapping. The other six subunits when associate with LSm8 form a nuclear complex that is involved in splicing.

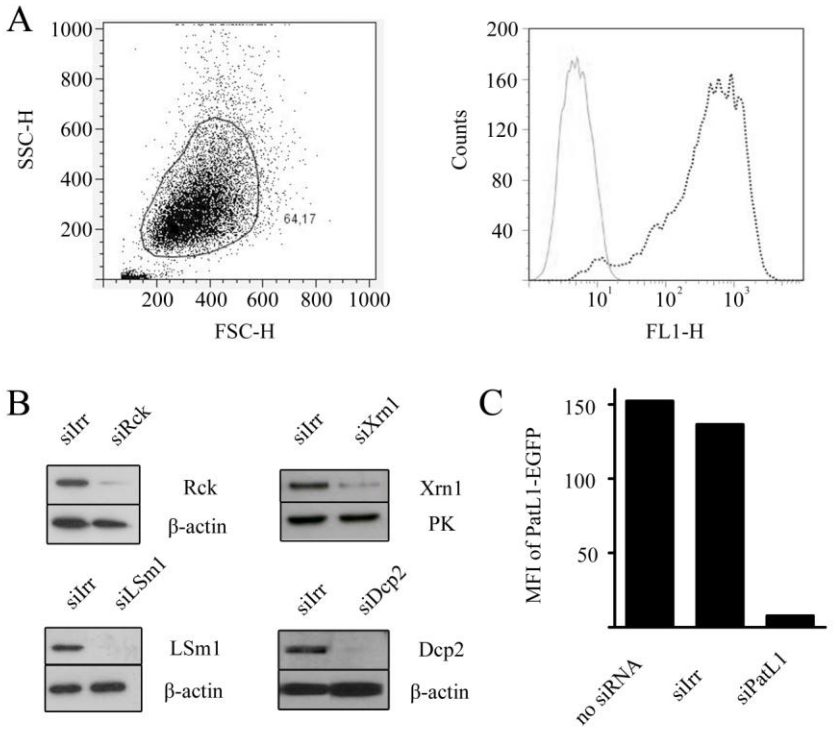

Fig.8. Depletion of Rck/p54, LSm1, PatL1, Xrn1 and Dcp2 proteins in hepatoma cell lines. (A) Representative FACS analysis showing the siRNA-transfection efficiency measurement. All the analyses were performed on viable cells represented in the encircled region (left panel), excluding debris and dead cells. The right panel shows the differences in fluorescence intensity observed between control cells (solid-gray line) and FITC-labeled siRNA transfected cells (dotted-black line). In this example, the transfection yielded more than 95% of positive cells. The same analysis was applied for both transfection methods. (B) Huh7-Lunet cells were transfected with 50 nM of siRNA irrelevant (siIrr) or siRNA targeting Rck/p54, LSm1, Xrn1 and Dcp2. Immunoblot analyses of Rck/p54, LSm1, Xrn1, Dcp2, β-actin or pyruvate kinase levels are shown. (C) Since no specific antibody is available for PatL1, to test PatL1 silencing, PatL1-EGFP expression plasmid and siRNAs were co-transfected in Huh7 cells, and fluorescence was analyzed one day later by flow cytometry. Values are expressed in mean fluorescence intensity (MFI). Similar silencing results were obtained for Huh7.5 cells.

For the transient knockdown of Rck/p54, LSm1, PatL1, Dcp2 and Xrn1 in these cell lines, we used two different transfection methods, lipid-based transfection and electroporation. In all cases, transfection efficiencies

were determined 4-6 hours post-transfection by cytometric measurement of FITC-labeled siRNA trasfected cells (Fig. 8A). The transfection efficiencies obtained in the experiments presented along this study for all cell lines were always higher than 80%. SiRNAs targeting the specific mRNAs were transfected by lipofectamine 2000 into Huh7-Lunet and Huh7.5 cells. Titration experiments perform previously in our group using a specific siRNA agains Rck/p54 in HeLa cells defined the optimal concentration for the assays to 50 nM.

An effective knockdown of Rck/p54, Xrn1 and Dcp2 was observed two days after a single transfection of 50 nM of the pre-designed siRNA into all cell lines tested. As an example, we are showing here the effective knockdown in Huh7-Lunet (Fig. 8B, upper-left, upper-right and lower-right panels). Using the same protocol for a siRNA against LSm1, we could not detect a decrease in LSm1 protein levels within the same period. This is a common observation for stable proteins. Thus, for Huh7 and Huh7-Lunet, we performed a second round of transfection on the third day post-transfection to extend the silencing on the selected target. With this strategy an efficient inhibition of LSm1 expression level was obtained five days after the first transfection (Fig. 8B, lower-left panel). The LSm1 protein levels in Huh7.5 were, in contrast to this, only efficiently depleted after three successive transfections, with the second and the third round on day two and four respectively. To keep the level of this protein during the time of the experiment knocked-down, an additional transfection of siRNA was applied on the day six past the initial siRNA-transfection. Due to the lack of specific antibodies against PatL1, the efficiency and specificity of the selected siRNA against this protein was tested by co-transfecting cells with this siRNA and a PatL1-EGFP fusion protein encoding plasmid, named Pat1L-GFP. Using this strategy we followed silencing effects by measuring the mean fluorescence intensity levels by

flow cytometry. The obtained results show that the siRNA against PatL1 was able to decrease the expression of Pat1L-GFP protein to 10% of the cells transfected with non-specific siRNA (Fig. 8C). It has been demonstrated in yeast that the Pat1 protein form a complex associated with the LSm1-7 ring (253, 255) and their integrity is essential for their mRNA binding properties and to promote decapping (56). Therefore, taking into account that this protein is found complex with LSm1, to ensure efficient knockdown of endogenous PatL1, the same silencing protocol as for LSm1 was used for each cell line. With these strategies, Rck/p54, LSm1, PatL1, Dcp2 and Xrn1 were silenced in HCV-permissive cell lines by 80-85% compared to the protein levels in cells transfected with the non-targeting siRNA (siIrr) as a control.

For the replication experiments where the HCV and BVDV replicons were used, co-transfection of viral RNA and siRNAs were performed by electroporation in Huh7 and Huh7-Lunet cells as described previously (164). The HCV colony-formation assay requires selection of the replicon-containing cells for over three weeks, however, after transfecting the siRNA silencing of the specific proteins lasted for 2 to 4 days post-transfection. Therefore, we evaluated the optimal amount of siRNA required by measuring its effect on the number of colonies. For this, we co-electroporated specific siRNA for Rck/p54 in a range from 250 nM up to 4 μM together with 1 μg of HCV-rep-Neo into Huh7 cells (211). We observed a dose-dependent effect (Fig 9), which was specific for the protein knocked-down, as judged by the co-electroporation of 4 μM control siIrr (siIrr). Consequently, a maximal concentration of 4 μM was used in the subsequent co-electroporation assays. For LSm1 and PatL1 silencing, an additional lipid transfection of 50 nM of the specific siRNA was performed on the day three past the initial co-electroporation to extend the effect of the silencing.

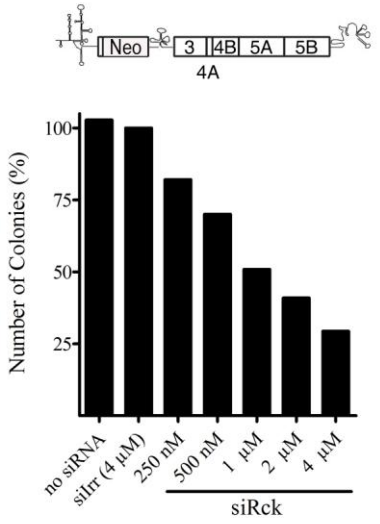

Fig. 9. Titration of Rck/p54 siRNA in hepatoma cell line. Coelectroporation of 1 µg of HCV-Rep-Neo RNA with 4 µM of siIrr or 250 nM to 4 µM of siRck showed a dose-dependent effect on HCV replication.

As we observed that subtle changes in the cell passage or confluence may have a big influence on the efficiency of transfection and silencing in Huh7 cells and its derivatives, we tested these two parameters in every experiment. Only experiments that showed siRNA-transfection efficiencies higher than 80% and protein levels lower than 20% compared with an irrelevant-transfected control were considered for this study.

1.4. Effect of protein depletion on cell viability and Interferon induction

Depletion of a host protein could have an indirect effect on HCV replication by affecting the cell metabolism or by leading to lethality. To exclude this possibility we evaluated the effect of the knockdown of Rck/p54, LSm1, PatL1, Dcp1 and Xrn1 on the cell by using three complementary methods that measure cell viability, cell metabolism and growth rate. To measure cell viability, silenced cells were stained with propidium iodide (PI), which allows separation of dead to live cells by flow cytometry. The ATP assay measures the intracellular levels of the universal energy unit of living organism. Finally, the growth rate of silenced cells was followed by sequential counting of the number of cells during the time of the experiment. Silencing of Rck/p54, LSm1 and PatL1 did not affect either viability, ATP values or growth (Fig. 10A-C). The evaluation of cell viability in siXrn1- and siDcp2-transfected cells was done solely by ATP assay and did not showed differences on the ATP levels compare to the control (Fig 10B).

Type I-interferons have a negative effect on HCV replication. Recently it has been described that transfection of some specific siRNAs can cause the induction of type I-interferons (115). To eliminate the possibility that type I-interferons induced by the siRNA used were nonspecifically repressing HCV replication, we tested MxA protein expression in Huh7.5 cells transfected with the siRNAs against Rck/p54, LSm1, PatL1, Dcp2 and Xrn1. MxA is an IFN-induced GTPase that has antiviral activity against various RNA viruses, and thus is a very sensitive marker for type I-interferon induction. Neither of the siRNAs used induced MxA protein expression in silenced cells (Fig 10D).

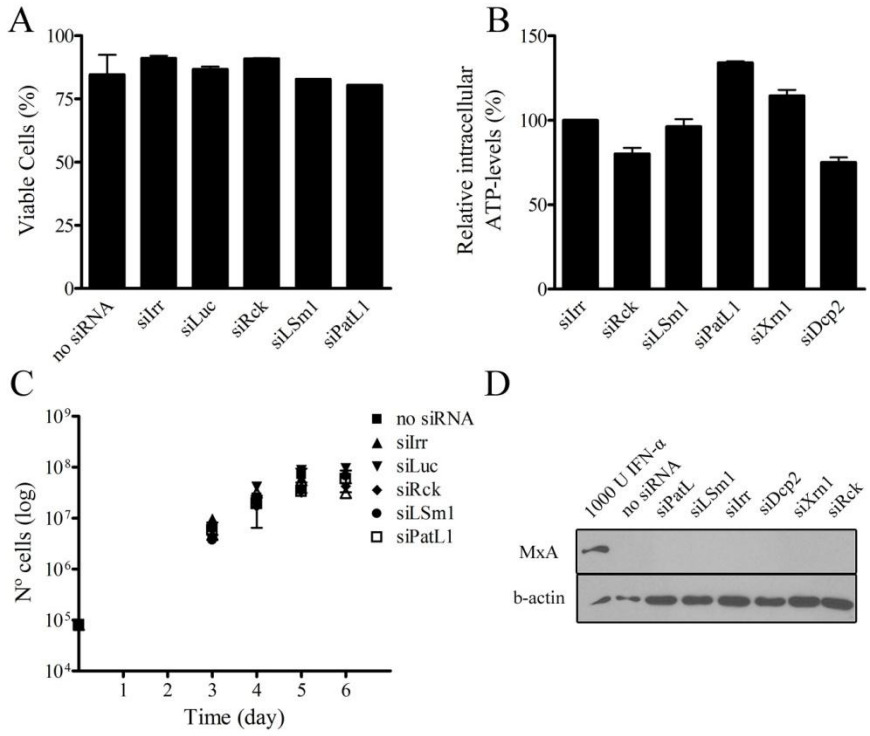

Fig. 10. Transient depletion of Rck, LSm1, PatL1, Dcp2 or Xrn1 does not affect viability, proliferation and MxA induction. (A) Silenced cells were stained with Propidium Iodide to check viability. Values are expressed as percentage of living cells. (B) Intracellular levels of ATP were measured at the time of most efficient knockdown of the different proteins. For PatL1, values were measured at the timepoint of most efficient knockdown of LSm1. ATP levels of control siRNA (siIrr) -transfected cells were set to 100%. (C) Cell growth was followed by counting the total number of cells throughout each experiment in cells coelectroporated with HCV replicon and specific siRNAs. Mean values are plotted, error bars indicate standard error of the mean of at least two independent measurements. (D) Huh7.5 cells were transfected with the corresponding siRNAs. At the time of most efficient protein knock-down levels of Rck,/p54, LSm1, PatL1, Dcp2 and Xrn1, intracellular MxA expression was visualized by immunoblot showing MxA and β-actin. Controls were left untreated (no siRNA) or were incubated 24 h with 1000 U/ml of IFN-α.

1.5. Effect of depletion of the decapping activators Rck/p54, LSm1 and PatL1 on the replication of HCV

Colony-formation Assay

To test the effect of Rck/p54, LSm1 and PatL1 proteins on HCV replication, we used the above describe replicon containing the neomycin resistance gene (HCVrep-Neo). Huh7 cells were co-electroporated with 1 and 4 μM of the specific siRNAs or control siRNA (siIrr) plus 1 μg of HCVrep-Neo and selected with G418 for 3 weeks. As control for the efficient transfection of siRNA, we used 1 μM siHCV, which targets the HCV IRES sequence and can efficiently suppress HCV replication, as described previously (211). Using this siRNA, we observed 4% of G418-resistant colonies compared with siIrr (Fig. 11A), indicating that siRNA was efficiently transfected into Huh7 cells under these experimental conditions. Importantly, co-electroporation of HCVrep-Neo with 1 μM of siRck, siLSm1 or siPatL1 reduced the number of G418-resistant colonies to 49, 45 and 19%, respectively when compared with siIrr. This inhibition increased when concentrations of 4 μM of Rck/p54, LSm1 or PatL1 siRNAs were used, reducing the number of G418-resistant colonies to 16, 9 and 9%, respectively (Fig. 11A).

Luciferase Assay

To exclude the possibility that the results observed with the HCV-rep-Neo were related to the reporter used, we confirm these results performing transient replication assay using the HCVrep-Luc replicon. Co-electroporation of Huh7-Lunet cells with 5 μg of HCVrep-Luc and 1 μM of siRck, siLSm1 or siPatL1 resulted in a marked reduction of the luciferase activity by approximately 80, 70 and 60%, respectively (Fig.

11B). As controls, siIrr and siLuc were used. As expected, the co-electroporation of the replicon with the siIrr did not affect the luciferase activity, and was similar to that obtained with no siRNA (Fig. 11B). The co-electroporation of siLuc, a siRNA targeting directly the luciferase sequence within the replicon, results in a decrease of approximately 84% in the luciferase activity, indicating that this reduction level is the maximum achievable with siRNA cotransfection in this system.

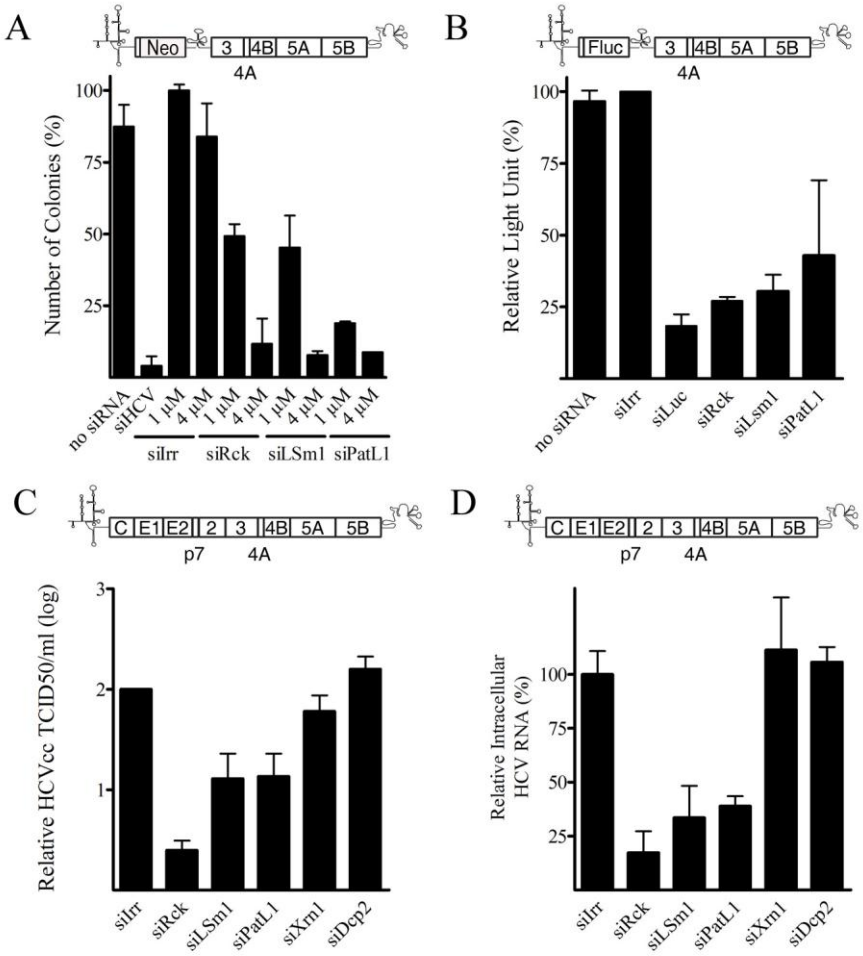

Fig. 11. Depletion of Rck/p54, LSm1 or PatL1 in hepatoma cell lines impairs HCV replication. (A) Huh7 cells were cotransfected with 1μg of *in vitro*-transcribed HCV-Rep-Neo replicon plus 1 μM of siHCV, or plus 1 or 4 μM of siIrr, siRck, siLSm1 or siPatL1. G418-resistant colonies were selected with 500 μg/ml G418, stained with crystal violet and quantified 3 weeks later. (B) Huh7-Lunet cells were cotransfected with 5 μg of HCV-Rep-Luc replicon plus 1 μM of each siRNA. A second round of siRNA transfection was required for LSm1 and PatL1 silencing. Values obtained at 4 hours postransfection were used to determine the transfection efficiency. Luciferase activity expressed as the percent RLU determined at the point of most efficient silencing compared with the activity of cells transfected with siIrr. (C) Three days after transfection of silenced Huh7.5 cells with HCVcc RNA, the HCVcc infectivity in the supernatant was titrated by a limited dilution assay. (D) The accumulation of intracellular HCVcc mRNA was analyzed by quantitative RT-PCR. Both values were normalized to the amount of transfected RNA and are shown relative to siIrr-transfected cells. Bars represent mean values and error bars indicate standard error of the mean of the results of at least two independent experiments. On the top of each graph a schematic representation of the used HCVcc and HCV replicon is shown. *Neo*, neomycin phosphotransferase gene; *Fluc*, firefly luciferase gene; *RLuc*, renila luciferase gene.

HCVcc system

To analyze whether Rck/p54, LSm1 or PatL1 are playing a similar role in the production of HCV infectious particles, we took advantage of the well-established HCVcc system. As HCVcc is a chimera of different isolates from the 2a genotype, we were analyzing furthermore, if the dependency on Rck/p54, LSm1 and PatL1 is conserved between genotypes. For this, at the time of maximum silencing, Huh7.5 cells were transfected with 0,2 μg of HCVcc RNA. Three days later, cellular supernatants were harvested for titration of infectious particles (Fig. 11C) while intracellular HCV RNAs were quantified by quantitative RT-PCR (Fig. 11D). In all cases, HCV production from Rck/p54-, LSm1- and PatL1- depleted cells was significantly reduced, the infectious titers being 50, 10 and 10 fold lower than in the siIrr control, respectively. Moreover, a comparable decrease in intracellular HCV RNA levels was observed. For defects in early steps of the viral life cycle such as translation and replication, it is expected that both particle production and viral RNA accumulation are inhibited.

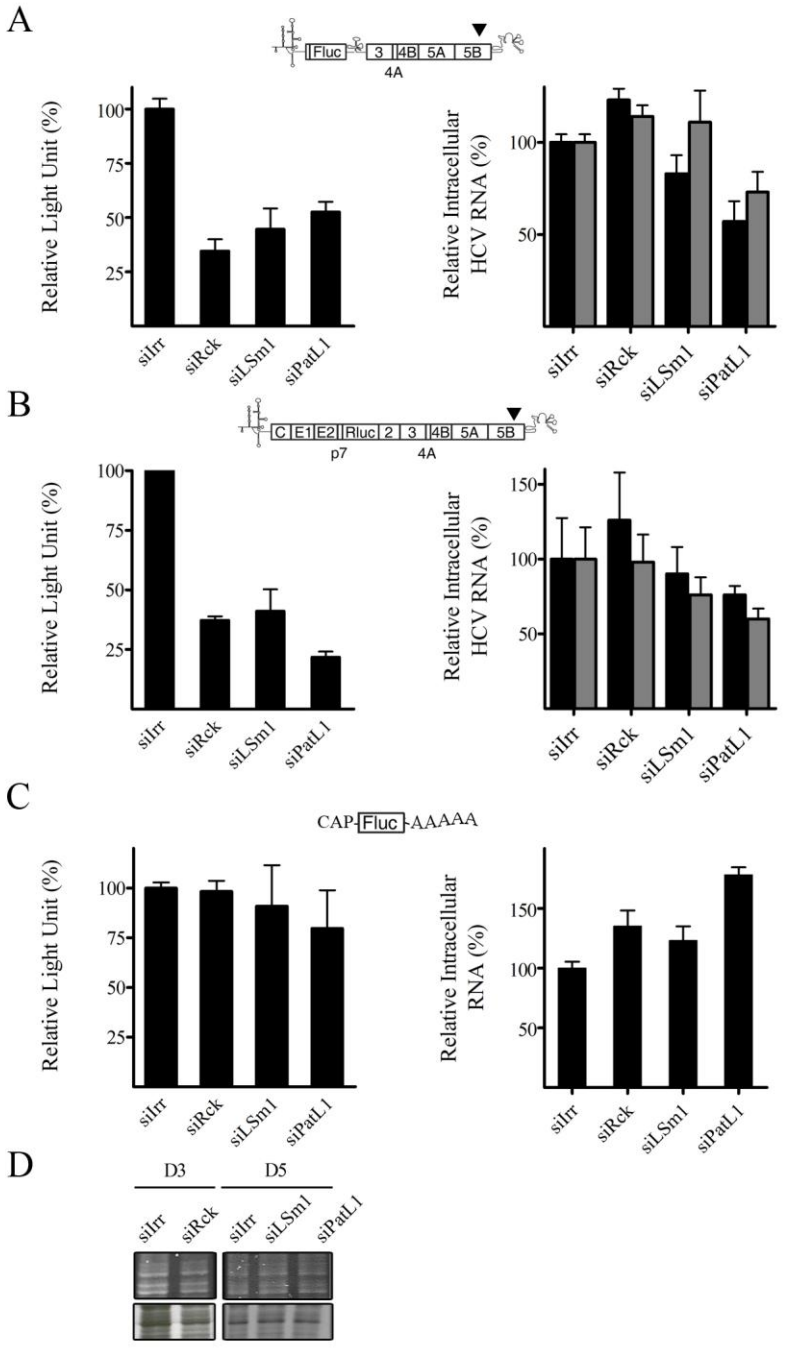
A
Fluc
3
4B
5A
5B
4A
Relative Light Unit (%)
100
75
50
25
siIrr
siRck
siLSm1
siPatL1
Relative Intracellular HCV RNA (%)
100
50
B
C
E1
E2
Rluc
2
p7
150
C
CAP-Fluc-AAAAA
Relative Intracellular RNA (%)
D
D3
D5

Fig. 12. Rck/p54, LSm1 and PatL1 silencing influences HCV RNA translation. Huh7-Lunet and Huh7.5 cells were transfected with siRNAs targeting Rck/p54, LSm1, PatL1 or a non-targeting siRNA, siIrr. The silenced cells were further transfected with (A) a non-replicating bicistronic luciferase replicon (HCVrep-Luc-GND), (B) a non-replicating luciferase-HCVcc (HCVcc-Luc-GNN), (C) a derivative (CAP-Luc-Poly(A)) containing the 5`capped, non-viral 5´UTR followed by the firefly luciferase ORF and a poly(A) tail. The luciferase activity was measured 4 hours post-transfection (A, B and C, left panels) The respective intracellular RNA levels were measured 4 hours after transfection by quantitative RT-PCR specific for the 5´ NTR (black bars) or the 3´ NTR (grey bars) of HCV (A and B, right panels) or specific for the a luciferase sequence (C, right panel). The 5´ NTR results were used to normalize the luciferase values (mean +/-SEM; n=3). (D) To examine the influence of Rck-, LSm1- and PatL1-silencing on the synthesis of cellular proteins, silenced cells were labeled with [^{35}S]-methionine for 30 min, separated on a denaturating polyacrylamide gel and visualized by autoradiography (lower panel). Gels were coomassie-stained to visualize protein-loading (upper panel). In the top of each graph is shown a schematic representations of the genomes of the reporter constructs. *Neo*, neomycin phosphotransferase gene; *Fluc*, firefly luciferase gene; *RLuc*, renilla luciferase gene. The arrowheads indicate the position of the GND or GNN mutation.

Taking together, in two different HCV systems we could show a dependence on the decapping activator proteins Rck/p54, LSm1 or PatL1. This suggests that translation and/or replication processes are dependent on these proteins, as we obtained similar results with HCVcc and HCV replicons. This dependence seems to be common for HCV, as this feature is conserved between systems from two different genotypes.

1.6. Depletion of the proteins Dcp2 and Xrn1 does not affect infectious HCV production

To evaluate the putative effect of some late components from the 5´- 3´ deadenylation-dependent mRNA decay pathway on HCV replication, we selected Dcp2 and Xrn1. The effect of these proteins on replication of HCV was assayed as before with the HCVcc system (Fig. 11C and D). No significant differences in the virus titer of the supernatants or in the

intracellular HCV RNA level between Dcp2- or Xrn1-depleted cells compared with the siIrr-transfected cells were observed. These results indicated that HCV does not depend on the decapping and degradation process itself but on the proteins acting upstream of it.

2. Characterization of the mechanism involved in the effect of Rck/p54, LSm1 and PatL1 on HCV replication

The proteins Rck/p54, LSm1 and PatL1 may affect HCV amplification by acting on HCV RNA translation, replication or both, as it is the case for BMV. The results described above using the replicons and HCVcc systems suggested that the decapping activators Rck/p54, LSm1 and PatL1 exerted a role on HCV translation or replication, because similar effects were observed for the replicon and the infectious HCVcc systems. Since the translation process is a prerequisite for late steps of the HCV life cycle to take place, we tested the effect of the depletion of Rck/p54, LSm1 or PatL1 on translation by using HCV non-replicative transcripts.

2.1. Establishment of a system to study the effect of Rck/p54, LSm1 or PatL1 silencing on HCV RNA translation

To investigate whether Rck/p54, LSm1 and PatL1 play a role in HCV RNA translation, we compared the expression of luciferase-reporters

driven by the non-replicative derivatives from a replicon, the HCVrep-Luc-GND, and from a virus, HCVcc-RLuc-GNN, at 4 hours after RNA transfection because at this time viral RNA replication has not started yet. In addition, these derivatives carry a mutation in the NS5B RdRp gene that impairs replication. Consequently, any luciferase activity of these derivatives at the timepoint indicated can be ascribed only to translation of the transfected HCV RNA (Fig 7B and D). To exclude the possibility that differences in luciferase values reflected differences in RNA accumulation instead of translation efficiencies, accumulation of intracellular HCV RNA was analyzed 4 hours after transfection by qRT-PCR specific and used to normalize the luciferase values.

2.2. Effect of the depletion of the activators of decapping Rck/p54, LSm1 and PatL1 on HCV RNA translation

HCVrep-Luc-GND or HCVcc-RLuc-GNN RNA was transfected into Rck/p54-, LSm1- or PatL1-depleted cells and luciferase activities as well as HCV RNA levels were measured 4 hours later. When normalized to the abundance of intracellular HCV RNA, luciferase activity decreased by approximately 65%, 55% and 48% for the HCV replicon in Rck/p54, LSm1 and PatL1 silenced cells (Fig. 12A), while the reductions with HCVcc were 63%, 59% and 79% (Fig. 12B), respectively.

To exclude the possibility that the values obtained were a generalized effect of Rck/p54, LSm1 and PatL1 silencing on cellular translation, we included two controls. We evaluated effects on the translation of a luciferase reporter flanked by a 5´ cap and a 3´ poly(A) tail and containing

a 5´ and 3´ NTR of non-viral origin that mimic cellular mRNAs and, on *de novo* protein synthesis by metabolic labeling with [^{35}S]-methionine. We did not observe a significant effect of the silencing of Rck/p54, LSm1 or PatL1 on translation of the reporter construct (Fig. 12C). In addition, the metabolic labeling did not show differences between silenced and control cells (Fig. 12D). Taken together these results suggest that the silencing of Rck/p54, LSm1 and PatL1 has a specific effect on HCV translation.

2.3. Inhibition of HCV RNA translation by Rck/p54, LSm1 and PatL1 depletion depend on the HCV 5´ and 3´ NTRs.

The major *cis*-signals controlling HCV RNA translation and replication are located in the 5´ and 3´ NTRs of the genome. To evaluate whether the translation inhibition of HCV constructs by depletion of Rck/p54, LSm1 and PatL1 is associated to these sequences, translation experiments in silenced cells were performed with a genotype 1b HCV RNA reporter construct containing the firefly luciferase ORF flanked by the HCV 5´ and 3´ NTR regions. This construct was transfected into previously silenced cells and reporter activities were measured 4 hours post-transfection. As before, the luciferase values were comparable to the ones obtained with the complete luciferase replicon HCVrep-Luc-GND (Fig. 13A). To elucidate the dependence of this effect on these NTRs sequences, luciferase-reporter derivatives were used in which either the HCV 3´ NTR was exchanged by a 3´ poly(A) tail or the HCV 5´ NTR by a capped-unrelated 5´ NTR. Silencing of Rck/p54, LSm1 or PatL1 had no

significant effect on the translation of any of these RNA constructs (Fig. 13B and C).

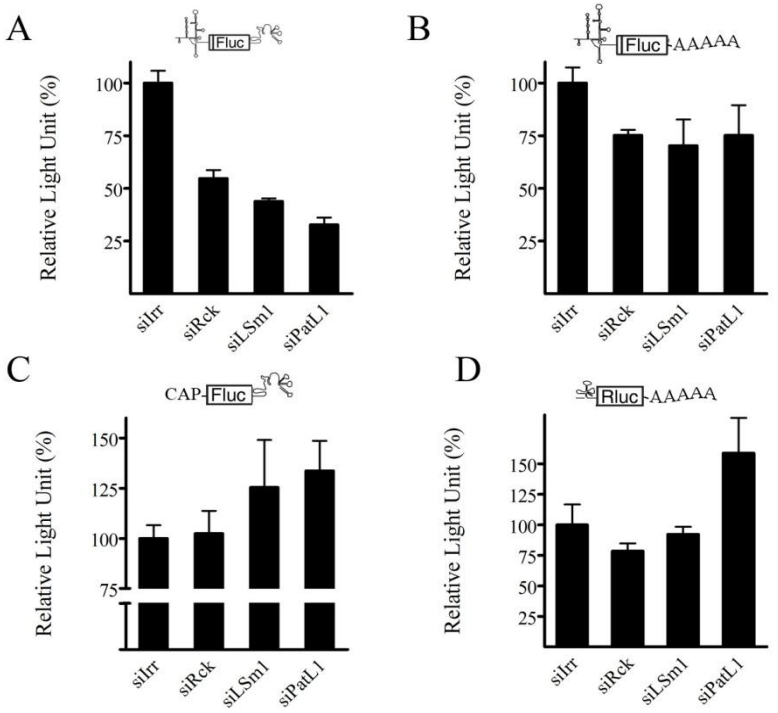

Fig. 13. Effect of transient knock-down of Rck/p54, LSm1 and PatL1 on EMCV IRES-, cap- and polyA-dependent translation. Huh7-Lunet cells were transfected with siRNAs targeting Rck/p54, LSm1, PatL1 or a non-targeting siRNA, siIrr. The silenced cells were then transfected with (A) a derivative construct (HCV-NTRs-Luc) containing the HCV 5´- and 3´ NTRs from genotype 1b flanking the firefly luciferase ORF (B) a derivative from HCV-NTRs-Luc in which the HCV 3´ NTR was exchanged by a poly(A) tail, (C) a derivative from HCV-NTRs-Luc in which the HCV 5´ NTR was exchanged by capped, non-viral 5´ NTR, and (D) a EMCV-IRES-Luc-Poly(A), a mRNA containing the EMCV-IRES follow by the renilla luciferase ORF and a poly(A) tail. The luciferase activity was measured 4 hours post-transfection and normalized to the respective intracellular RNA levels measured by quantitative RT-PCR (mean +/-SEM; n=3). On the top of each graph is shown a schematic representations of the reporter used.

In addition, silencing of Rck/p54, LSm1 or PatL1 did not significantly inhibit EMCV IRES-mediated translation, as shown by using a construct containing the EMCV IRES and a 3´ poly(A) tail (Fig 13D). This suggests that HCV RNA translation specifically depends on Rck/p54, LSm1 and PatL1 and that this dependence is linked to the presence of both HCV NTRs.

2.4. Reconstituted human LSm1-7 complex is able to directly interact with HCV 5' and 3' NTRs.

In yeast cells Dhh1, Pat1 and the LSm1-7 ring have been shown to interact *in vivo* (44), and there is evidence of a direct interaction of the LSm1-7 ring with deadenylated cellular mRNAs (54, 255). This renders the possibility likely, that the respective human homologues, Rck-, LSm1-7, and PatL1 act through LSm1 binding directly and specifically on HCV RNA. Considering a direct interaction model, it is likely that LSm1-7 ring interacts with the 5´ and 3´ NTRs of HCV since they are essential regions in the regulation of viral translation and replication (248), and our translation results suggest a functional link of Rck/p54, LSm1 and PatL1 to these sequences. To examine this possibility, we reconstituted functional human LSm1-7 rings according to a recently reported strategy (293), and performed electromobility shift assays with HCV RNA fragments (Fig. 14). Incubation of the LSm1-7 rings with the corresponding ^{32}P-labeled transcripts demonstrated a strong binding to both NTR regions reflected by a complete band shift (Fig. 14B and C). This binding was specific because addition of excess unlabeled 5´ or 3´

NTR sequences resulted in binding competition (Fig. 14D), whereas addition of excess unlabeled nonbinding HCV RNA sequences did not.

To identify the viral RNA motifs involved in the interactions with the LSm1-7 rings, we systematically deleted domains of defined RNA structure and function from the HCV 5´ and 3´ NTRs respectively. The HCV 5´ NTR contains four stem loops (SL) structures, SLI to SLIV. SLI is required in replication but is dispensable for translation. SLII, SLIII and SLIV form the internal ribosome entry site (IRES). From these, SLIII is proposed to interact with the 40S ribosomal subunits thus playing a key role in translation initiation. The SLII and SLIII function in replication as well. Electromobility shift analysis showed that the SLIII was both necessary and sufficient for binding of the LSm1-7 ring to the 5´ NTR region (fig 14B) as evidenced by binding to this RNA motif (positions 129-290) and by loss of binding upon removal of it.

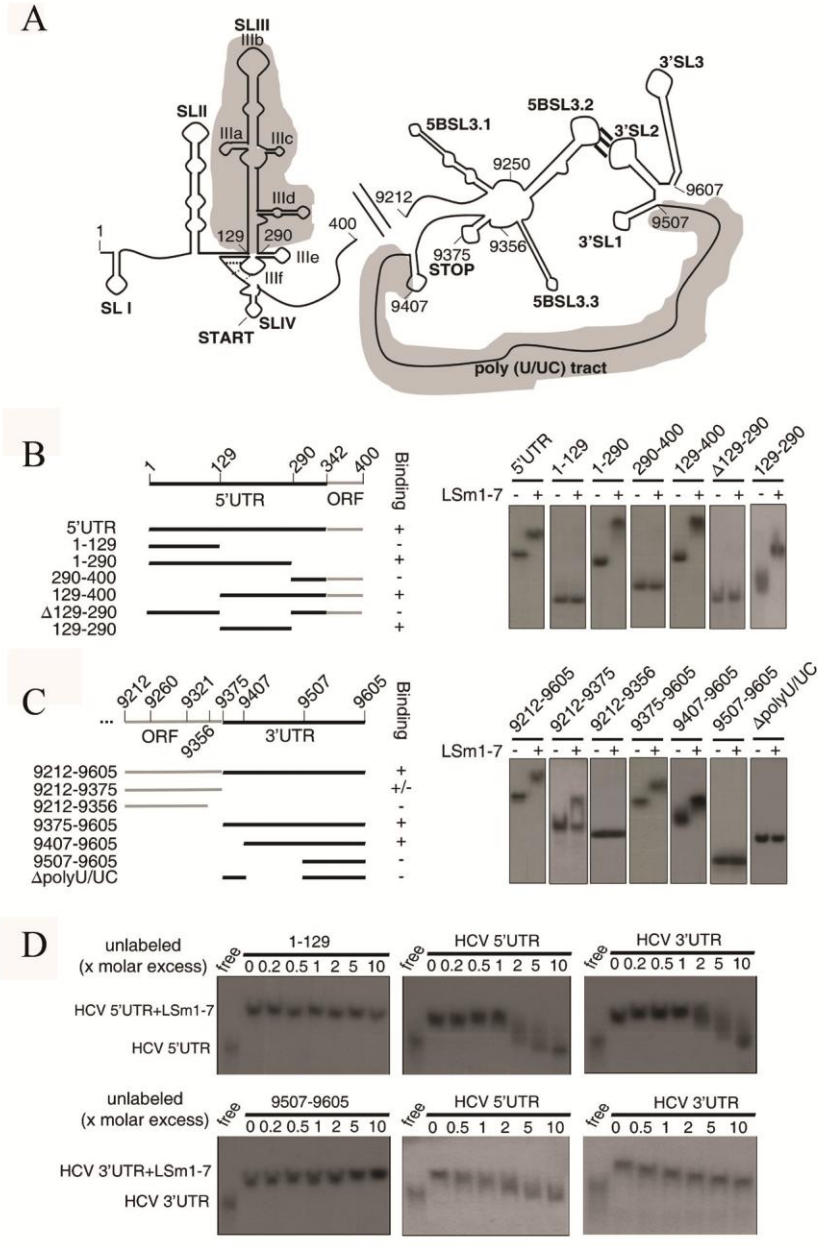
A
SLIII
IIIb
SLII
IIIa
IIIc
IIId
IIIe
IIIf
SL I
SLIV
START
STOP
1
129
290
400
9212
9250
9375
9356
9407
9507
9607
5BSL3.1
5BSL3.2
5BSL3.3
3'SL1
3'SL2
3'SL3
poly (U/UC) tract
B
5'UTR
ORF
Binding
1-129
1-290
290-400
129-400
Δ129-290
129-290
LSm1-7
C
9212
9260
9321
9375
9407
9507
9605
9356
3'UTR
9212-9605
9212-9375
9212-9356
9375-9605
9407-9605
9507-9605
ΔpolyU/UC
D
unlabeled
(x molar excess)
free
0 0.2 0.5 1 2 5 10
HCV 5'UTR+LSm1-7
HCV 5'UTR
HCV 3'UTR+LSm1-7
HCV 3'UTR
0 0.5 1 2 5 10

Fig. 14. Reconstituted LSm1-7 rings bind to specific HCV 5´ and 3´ NTR regions. (A) Schematic representation of the secondary structures of the HCV 5´ and 3´ ends. Upstream of the 3´ NTR, the NS5B coding sequence containing an RNA cruciform structure is shown. Shadowed regions hightlighted the binding sites of the LSm1-7 rings. (B and C left) The constructs used in the electromobility shift assays are shown. The numbers refer to the nucleotide positions in the genome of the HCV Con1 strain. (B and C right) Radiolabeled, gel-purified RNA transcripts were incubated with the reconstituted LSm1-7 rings. After complex formation, products were separated on a native polyacrylamide gel and visualized by autoradiography. (D) Labeled HCV 5´ and 3´ NTR RNAs (HCV sequences 1-400 and 9,375-9,605, respectively) were incubated with reconstituted LSm1-7 rings in the presence of increasing amounts of unlabeled HCV 5´ and 3´ NTR transcripts. As noncompeting controls, HCV RNA sequences 1-129 and 9,507-9,605 were used. After complex formation, products were treated as in B and C.

The HCV 3´ NTR consists of a variable region, a poly (U/UC) tract and a highly conserved terminal region termed 3´X tail that consists of three SL structures (248). Importantly, the 3´ NTR is not only required for replication but also for efficient translation (235). The electromobility shift analysis revealed a robust area of binding corresponding to the poly (U/UC) tract. Binding to the LSm1-7 ring was lost when this area was deleted (transcript ΔpolyU/UC) and gained when it was added (transcripts 9,507-9,605 and 9,407-9,605). The length and sequence composition of this region has recently been shown to have an important function in HCV RNA replication and the binding of host factors that could regulate this function has been suggested (291). An additional weak binding was observed to the NS5B coding sequence that includes a RNA cruciform structure (transcript 9,212-9,375). This binding was lost when the transcripts was reduced to the positions 9,212-9,356. Because this remaining HCV sequence still contained the cruciform structure, either this was not the target of binding or the complete sequence might be required for proper folding to allow binding. Thus, in summary, the LSm1-7 ring binds robustly and directly to two important motifs in the 5´ and 3´ NTR regions that are involved in the regulation of translation and replication of HCV. Interestingly, a direct and specific binding of LSm1-7

ring to specific translation/replication regulatory signals in the BMV genome has been recently reported in our laboratory (92).

3. Analysis of the effect of the decapping activators Rck/p54, LSm1 and PatL1 on the life cycle of other (+) RNA viruses

Our results showed that HCV translation and replication of HCV depend on Rck/p54, Lsm1-7 and PatL1 proteins. Given the conservation of (+) RNA virus replication strategies and the remarkable common use of these proteins by different positive-strand viruses, HCV in human cells, BMV in plants and the phage Qβ in bacteria, it is likely, that other members of the same viral group are regulated by these host factors as well.

The genomes of (+) RNA viruses mimic cellular mRNAs and are directly translated by the cellular translation machinery. In contrast to cellular RNA, which contain a methylated cap structure at the 5´ end and a poly(A) tail at the 3´ end, (+) RNA viruses show a variety of structure combinations at the 5´ and 3´ ends of their genomes. For example, the human Coronavirus (HCoV) genome is 5´ capped and 3´ polyadenylated, others such as HCV and Bovine Viral Diarrhea Virus (BVDV) genomes have 5´ and 3´ secondary structures. In contrast to this, the BMV genome is 5´ capped and presents a secondary structure at the 3´ end and the poliovirus genome presents a 5´ IRES and a 3´ poly(A) tail (Fig. 15). Since differences in the 5´ and 3´ structures might reflect differences in the regulation of translation and replication, we tested in addition to HCV

and BMV the effect of Rck/p54, LSm1 and PatL1 on the replication of Coronavirus, Poliovirus and BVDV.

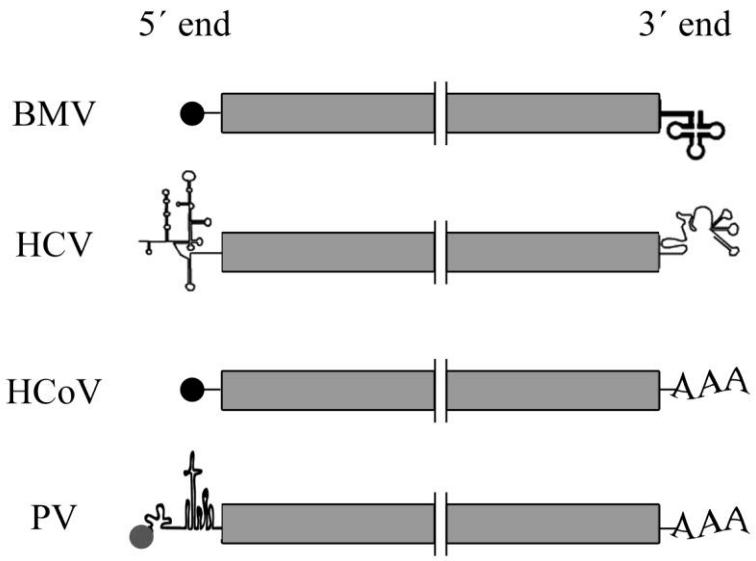

Fig. 15. Schematic representation of the distinct features of the 5´ and 3´ ends from three different groups of (+) RNA viruses. The BMV belongs to the Alphavirus Family and has a tripartite genome with each segment flanked by a 5´ cap (black circle) and a tRNA-like 3´ end. The HCV and BVDV belong to the Family Flaviviridae and are flanked by the 5´ IRES and the 3´ end with complex secondary structures. The human Coronavirus (HCoV) genome has a 5´ cap and a 3´ poly(A) tail. The Poliovirus genome is flanked by the VPg protein (grey circle) and the IRES in the 5´ and a poly(A) tail at the 3´ end.

3.1. Effect of Rck/p54, LSm1 and PatL1 depletion on HCoV-229E infection

The HCoV-229E belongs to the Family Coronaviridae. These viruses generally cause respiratory and/or intestinal infections, being not only of

economical importance as animal pathogens but also representing a risk to humans, as demonstrated by the emergence of the severe acute respiratory syndrome coronavirus (SARS-CoV). Coronaviruses have the largest known nonsegmented viral (+) RNA genome (about 30 Kb), with a 5´ cap structure, and a polyadenylated 3´ end.

Two HCoV-229E viruses were used, the wild-type (HCoV-229E-WT) and a derivative that express the Enhances Green Fluorescence Protein (EGFP) as a reporter (HCoV-229-EGFP). Effects on HCoV-229E-WT were measured by titration of the supernatant and effects on HCoV-229-EGFP by cytometric analysis of EGFP expression in infected cells. Since both viruses replicate efficiently in Huh7 cells, we were using the established silencing strategy for Rck/p54, LSm1 and PatL1. At time of maximum silencing, cells were infected at different multiplicity of infections (moi). In every case, protein levels were measured at the moment of infection and 48 hours later to confirm efficient knockdown of the proteins throughout the experiment (Fig. 16D). The levels Rck/p54 knockdown were between 80-90% at both timepoints tested. In the case of LSm1, we observed an efficient silencing at the moment of infection, but the protein levels were increased until approximately 50% at 48 hours. As shown in Fig. 16A and B, we did not observed any significant differences in titers between cells silenced for Rck/p54, LSm1 or PatL1 and control cells an a moi of 1. To eliminate the possibility that with the fast kinetic of HCoV growth is masking the effect of Rck/p54, LSm1 and PatL1, we decided to infect with a lower moi of 0,01. This strategy caused a delay of approximately 12 hours in the viral kinetic, however we did not observe differences between silenced and control cells.

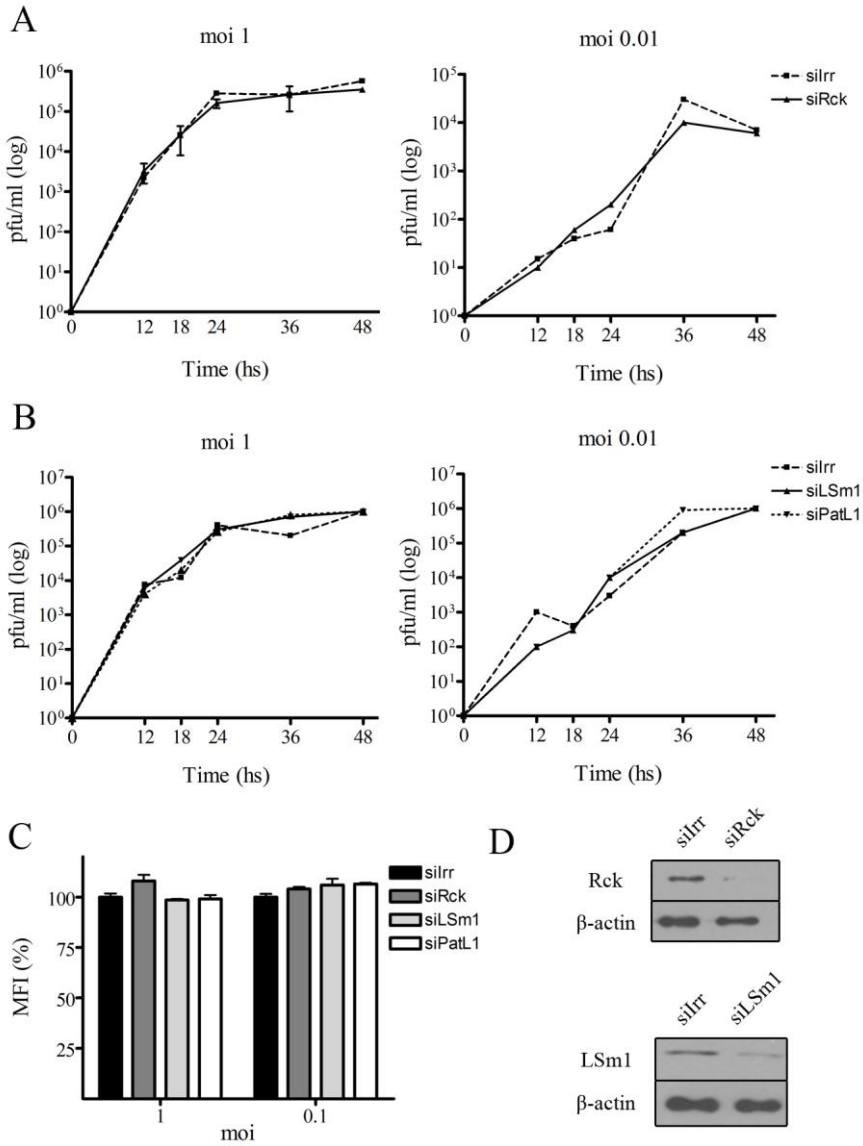

Fig. 16. Effect of Rck/p54, LSm1 and PatL1 silencing on HCoV-299E replication. Previously silenced Huh7 cells for Rck/p54 (A), LSm1 or PatL1 (B) were infected with wild-type HCoV-299E at moi 1 and 0,01. Supernatants were collected at the indicated time-points and titrated. (C) Previously silenced Huh7 cells for Rck/p54, LSm1 or PatL1 were infected with HCoV-299E-EGFP at moi 1 and 0,1. Cells were harvested 16 hours post-infection and the EGFP expression was analysed by flow cytometry and values are given as mean fluorescence intensity (MFI). The mean value of each point is represented. Error bars indicate standard error of the mean of the results for two independent experiments. (D) Huh7 cells were transfected with 50 nM of siRNA irrelevant (siIrr) or siRNA targeting Rck/p54 or LSm1. Representative immunoblot analyses of Rck/p54, LSm1 and β-actin levels are shown.

Next, previously silenced cells were infected with HCoV-229E-EGFP, harvested 16 hours post-infection and analysed by flow cytometry. In agreement with the above results, we did not observe with this construct any significant differences in the EGFP expression levels in cells silenced for Rck/p54, LSm1 and PatL1 in contrast to control-silenced cells (Fig. 16C). The depletion levels of Rck/p54 were higher than 90% at the moment of infection, but for LSm1 less than 50%.

In summary, the titration of HCoV-229E WT virus and EGFP expression levels of an EGFP virus, showed that knock-down of Rck/p54, LSm1 and PatL1 did not affect viral replication.

3.2. Effect of Rck/p54, LSm1 and PatL1 depletion on Poliovirus replication

The Family Picornaviridae includes many important pathogens of humans and animals (174). Members of this group cause a wide range of diseases from "common-cold"-like illnesses, to poliomyelitis and chronic infections in livestocks. They are divided in two main categories, enteroviruses and rhinoviruses. Poliovirus is a human enterovirus, and is the causative agent of poliomyelitis, one of the most dread childhood diseases, causing paralysis and death (174). Picornaviruses do not have a 5' cap structure but encode a protein known as VPg which attach to the 5' NTR and regulates translation (Fig. 15). However, like mammalian mRNA, the genome does have a poly(A) tail at the 3' end.

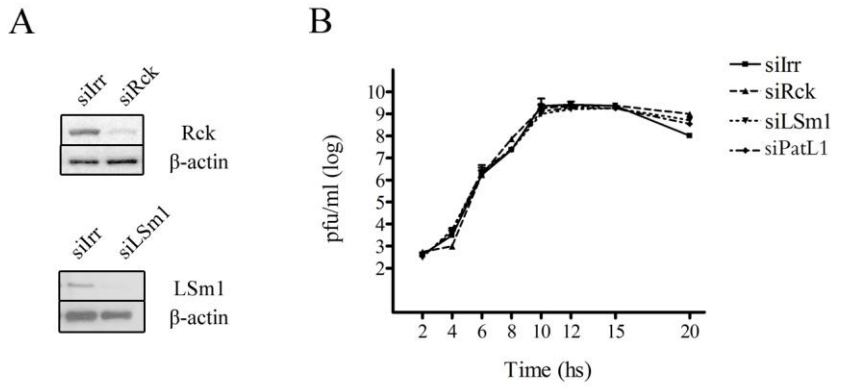

Fig. 17. Effect of Rck/p54, LSm1 and PatL1 silencing on Poliovirus replication. (A) HeLa cells were transfected with 50 nM of siRNA irrelevant (siIrr) or siRNA targeting Rck/p54 or LSm1. Immunoblot analyses of Rck/p54, LSm1 and β-actin levels are shown (B) Previously silenced HeLa cells for Rck/p54, LSm1 or PatL1 were infected with wild-type Poliovirus at moi 0,01. Supernatants were collected at the indicated time-points and titrated. The error bars indicate standard error of the mean of the results for three independent experiments.

We used for these experiments the Poliovirus type 1 Mahoney strain that infects and replicates efficiently in HeLa cells. Silencing conditions for this cell line was previously established in our group. Thus, silenced HeLa cells for Rck/p54, LSm1, PatL1 or the control siIrr were subjected to poliovirus infection at the time of maximum silencing. Samples were taken at different timpoints and effects on replication were measured by titration of the supernatants. Protein depletion levels of Rck/p54 and LSm1 was checked at the moment of infection. An efficient silencing was achieved for Rck/p54 and LSm1 protein, around 80-90% compared to the control, assuming a similar efficiency for PatL1. As shown in Fig. 17, knock-down of the decapping activators proteins Rck/p54, LSm1 or PatL1 did not affect viral replication compared with the transfection of control siRNA (siIrr).

3.3. Effect of Rck/p54, LSm1 and PatL1 depletion on BVDV replication

The bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* that, together with the *Flavivirus* and *Hepacivirus* constitute the Family Flaviviridae (247). The BVDV is associated with a range of economically important clinical diseases including reproductive disorders and acute fatal haemorrhagic disease in cattle industry. Vaccination is still the most important strategy for controlling BVDV infections in many countries of the world. As all members of this Family, their (+) RNA genomes encompass one large ORF flanked by 5´ and 3´ NTRs. Translation initiation is mediated by an IRES within the 5´ NTR. BVDV does not present a poly(A) tail.

According to the effects in tissue culture, two different BVDV types can be distinguished, cytopathogenic (cp) and noncytopathogenic (ncp) strains (101, 155). Molecular studies have been revealed that cp strains evolve *in vivo* from ncp BVDV (175). These cp strains replicate faster, than the ncp BVDV and express high amounts of NS3 proteins, which is not present in the ncp BVDV. For the experiments we take advantage of a cp BVDV isolate (DI9) that replicates efficiently in Huh7 cells. We therefore followed the same silencing strategy already described to silence Rck/p54. 48 hours after the first electroporation with siRNAs, silenced cells were coelectroporated with the DI9 transcript and the corresponding siRNA in order to prolong the silencing effect. To quantify DI9 replication, we performed immunoblot analysis using specific antibodies against BVDV NS3 protein. At the same time, intracellular levels of BVDV RNA were quantified by quantitative RT-PCR. Protein and RNA samples were taken at 4, 24 and 36 hours post co-electroporation.

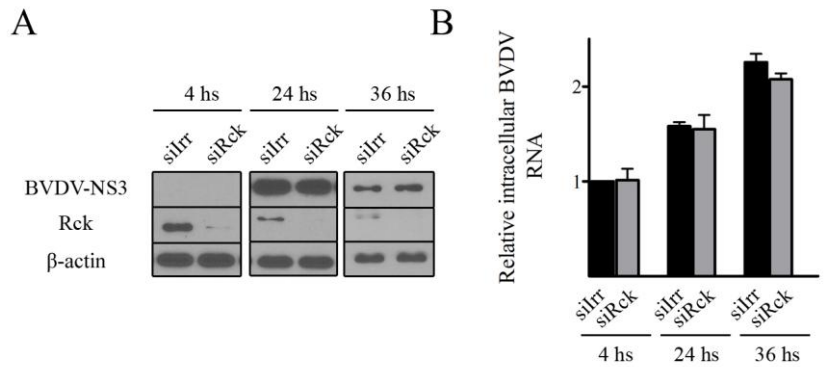

Fig. 18. Effect of Rck on BVDV RNA replication. Previously silenced Huh7 cells for Rck/p54 were co-electroparated with BVDV DI9 strain plus the corresponding siRNA. (A) Protein samples were taken at the indicated timepoints and subjected to immunobloting analysis. A representative WB of two independent experimentes is shown. (B) The accumulation of intracellular BVDV mRNA was analyzed by quantitative RT-PCR specific the BVDV RNA (mean +/-SEM; n=3).

After transfection, the BVDV RNA values are increasing, indicating active replication (Fig 18B). During the experiment, Rck/p54 was efficiently silenced in Huh7 cells (Fig. 18A) However, no differences in BVDV NS3 protein or BVDV RNA levels were detected between cells silenced for Rck/p54 and control siRNA (siIrr) silenced cells (Fig. 18A and B). The effects of LSm1 or PatL1 on BVDV replication were not analysed, as we expect similar effects with these proteins to Rck/p54 like observed before for BMV, HCV, CoV and Polio.

V. Discussion

Viruses are obligatory intracellular parasites that depend on their host organism to complete their life cycles. Despite differences, the group of (+) RNA viruses has conserved features in their replication process. A more detailed view of virus-host cell interactions and the identification of such host factors affecting common and key steps of (+) RNA virus life cycles will greatly help to gain insights in the biology of this group of viruses and have also practical implications since it can provide new targets for the development of effective broad-spectrum antiviral agents.

In this work, by using a gene silencing strategy in human hepatocytes, we have shown that HCV RNA genome translation and replication depend on the decapping activator proteins Rck/p54, LSm1-7 complex and PatL1. These proteins belong to the cellular 5´ to 3´ deadenylation-dependent mRNA decapping machinery. This is one of the main pathways of mRNA decay and is highly conserved from yeast to humans. In this pathway, two distinct processes are necessary for decapping to occurs (85). First, since mRNA translation and decay are two competing and mutually exclusive processes, translation needs to be repressed in order to remove the cap-binding translation factors from the mRNA. Rck/p54, LSm1-7 complex and PatL1 function at this step. Next, the decapping complex (Dcp1/Dcp2) removes the 5´ cap structure and the mRNA is degraded via the 5´ to 3´ exonuclease Xrn1.

Translation and replication of HCV RNA genome were impaired when Rck/p54, LSm1 or PatL1 protein levels were downregulated by RNA interference (RNAi) in human liver cell lines. Toxicity and biochemical assays of depleted cell lines demonstrated that the observed effects were

not indirect due to a general alteration on the cellular metabolism by lack of a particular protein, but were direct and specific. These results were obtained for two different HCV genotypes, 1b and 2a, suggesting that this dependence is conserved between genotypes. The analysis of late components of the 5´ to 3´ deadenylation-dependent mRNA decay pathway, i.e. Dcp2 and Xrn1, did not show any effect on HCV translation and replication. Thus, the decapping and degradation processes themselves are not important for the regulation of the HCV life cycle but proteins acting upstream of them in the translation repression step.

The observation that the downregulation of Rck/p54, LSm1, or PatL1 proteins affects HCV RNA translation and intracellular HCV RNA accumulation may be explained by two different scenarios. Silencing of these host factors would have an effect only at the translation level or by an independent effect on both translation and replication, as observed in the plant brome mosaic virus (BMV). Previous results from our group using this virus have shown that the LSm1-7 complex, and the Pat1 and Dhh1 proteins are required for the translation and the exit from translation to replication of the BMV genome (63, 170, 191). An apparent dual and antagonistic function is found for other cellular proteins as well. The biological advantage of the presence of bi-functional protein complexes in the cell is the possibility to respond rapidly to different and changing cellular environments regulating the abundance and activity of the same set of proteins. For example, the cellular protein CPEB depending on its phosphorylation state can behave as activator or repressor of cellular mRNA translation (2). Viruses could take advantage of Rck/p54, LSm1 and PatL1 proteins in the same way to achieve a tuned regulation of their life cycles.

The 5´ and 3´ nontranslated regions (NTR) of HCV, as for all (+) RNA viruses, are highly structured and multifunctional. These sequences constitute important signals that regulate translation, replication and encapsidation of the viral genome. By reconstituting functional human LSm1-7 complexes we were able to show that they interact directly and specifically with essential motifs in the HCV 5´ and 3´ NTR. As these motifs were already known to be involved in the regulation of HCV translation and replication, our observations were suggestive of a mechanistic link between these complexes and sequences in both, the 5´ and the 3´ regions. The LSm1-7 recognizes the SLIII of the HCV IRES, present in the 5' NTR of the genome, and also the poly(U/UC) tail in the HCV 3' NTR. Both motifs have been shown to regulate translation and replication of HCV RNA. Interestingly, LSm1-7 ring complex was shown to directly interact with two translation/replication regulatory signals in the BMV genome, the tRNA-like structure (TLS) region present in the 3' end of all three genomic RNAs, and to two internal single-stranded A-rich loop sequences in the intergenic region of BMV RNA3 (92). This suggests that the mechanism of action of LSm1-7 on (+) RNA viral genomes is conserved.

The LSm1-7 complex as other members of the Sm/LSm family are believed to act as RNA chaperones. Thus, the direct interaction of the LSm1-7 complex with sequences in both, the 5´ and the 3´ NTRs of the HCV genome, could lead to rearrangements in the viral RNP structure and composition, recruiting proteins such as Rck/p54 and PatL1 from the cellular mRNA repression/decay machinery and instead of promoting decay, might promote HCV RNA translation and subsequent transfer to replication. This view is consistent with a recent proposal made for the regulation of mRNAs generated by poxviridae. Viruses of this family generate viral mRNAs with an additional oligo(A) tract located at their 5´

ends. It has been shown that the binding of reconstituted LSm1-7 rings to such a tract in the 5´ end of reporter RNAs does not result in mRNA decay but rather in RNA stabilization through inhibition of decapping and degracation (30). This effect was proposed to be mediated by the simultaneous binding of LSm1-7 rings to the 5´ and the 3´ ends. A similar model is proposed to explain the requirement of the LSm1-7 complexes for the efficient translation of HCV, since our data showed a direct interaction of the rings with both the 5´ and the 3´ NTRs of the genome.

Interestingly, in the cytoplasm of eukaryotic cells there are sites of protein aggregation named processing bodies (P-bodies) and stress granules (SG). These cytoplasmic *foci* accumulate proteins involved in the regulation of cellular mRNA translation, storage or degradation. Under stress SGs are formed, while P-bodies increased in number. SGs assemble around stalled 48S pre-initiation complexes containing mRNA and translation initiation factors, like eIF4G, eIF4E, eIF3, eIF2 and PABP. Furthermore, they contain RNA binding proteins, which seem to determine the fate of the bound mRNAs in mRNP complexes (14, 277). Depending on the composition of the mRNP, they can be stored, directed to translation or to P-bodies (134). P-bodies contain proteins involved in mRNA decay processes and, on the contrary to SGs, no ribosomal proteins (134). Therefore, it is believed that P-bodies are involved in mRNA storage prior to degradation, although mRNA can be also redirected from these structures back to translation (13). In agreement with the proposed functions, it has been shown that both structures interact physically. They also share some common components such as mRNA derived from disasembled polysomes, components of the miRNA-mediated gene silencing machinery and other proteins such as eIF4E, TPP, BRF1/2, Xrn1, Rck/p54 and FAST (14). Our results suggest that P-bodies and SGs may provide the physical structures for dynamic viral genome segregation

within the cell. In agreement with this idea besides the proteins described in this work, some additional P-bodies and SGs components have been already described to act on the HCV life cycle, like G3BP (290), HuR (141), TRAF2 (188), DDX3 (18, 221). In addition, HCV RNA has been shown to physically interact with HuR and LSm1-7 (237). Interestingly, the respective yeast homologues play also a role in regulation of the translation and replication of the brome mosaic virus (BMV) (11, 63, 170, 191). Furthermore, the BMV RNA genome interacts directly with LSm1-7 and localize to P-bodies in yeast (27, 92). Two interesting issues to address are i) whether the individual components of P-bodies and SGs by themselves are the ones required for the HCV life cycle or the organization of these proteins in granules (28), and ii) whether HCV infection affects the formation of these structures and if so, how this might affect the HCV life cycle.

Our results showed that LSm1-7, PatL1 and Rck/p54 play an essential role on HCV replication. Moreover, results from our laboratory have demonstrated that these proteins have also a key role on the replication of the plant (+) RNA virus BMV and the animal (+) RNA virus Flock House Virus (FHV) (unpublished results). Interestingly, Hfq, the bacterial counterpart of LSm1, is required for the replication of the (+) RNA phage Qß. The common use of these cellular proteins by different (+) RNA viruses that infect bacteria, plants, animals and humans, strongly suggests that members of this viral group have hijacked host factors that are involved in the transit of cellular mRNAs from translation to degradation to regulate their own cycle. Why this might be the case? Cellular mRNAs need exit translation to be available to other fates, as storage or decay. The same is true for (+) RNA viruses, as only when translation factors are released from (+) RNA genomes the viral polymerase will be able to start the replication process.

To explore how spread is the use of LSm1-7, Pat1 and Dhh1 we tested if these proteins affect the life cycle of other (+) RNA viruses. LSm1-7, Pat1 and Dhh1 role in cellular mRNA metabolism seems to be regulated by interactions with the 5´ and 3´ NTRs. Moreover, the dependence of HCV and BMV RNA translation on LSm1-7, Pat1 and Dhh1 is linked to sequences in the 5´ and 3´ NTRs of the their genomes. Thus, the 5´ and 3´ structures have a key role in the function of LSm1-7, Pat1 and Dhh1 in cellular and viral mRNAs. Within the group of (+) RNA viruses the 5´ and 3´ NTRs of their genomes show different characteristics. The 5´ ends are capped or have IRES. The 3´ends are polyadenylated or contain a secondary structure. Thus, we selected viruses that represent the different combinations of 5´ and 3´ NTR structures. The BVDV genome, like HCV, is flanked by 5´ and 3´ secondary structures. In contrast to BMV and HCV, the Coronavirus RNA genome is capped and polyadenylated and the Poliovirus genome present an IRES structure at the 3´ end and a poly(A) tail at the 5´ end (Fig. 15). We used these viruses to test whether Rck/p54, LSm1 or PatL1 are required for their life cycles.

When Rck/p54-, LSm1- or PatL1-depleted cells were infected with coronaviruses and polioviruses, no measurable differences on virus replication were observed between silenced and no silenced cells. In addition, downregulation of Rck/p54 did not affect the replication of the pestivirus BVDV. The observed results could be explained in two different ways, i) the knock-down of these proteins do not exert a measurable effect on the replication of these viruses and, ii) we are not detecting any effect due to problems in the experimental approach.

The first possibility could be related to specific translation requirements in different (+) RNA viruses. Translation initiation is either cap-dependent or

cap-independent. In viruses with capped-genomes, translation initiation occurs in the standard cap-dependent way and, as with cellular mRNAs, requires specific translation initiation factors which drive assemble of the ribosomal initiation complex. In the cap-independent pathway, initiation of protein synthesis through the use of an IRES structure could be directed in the absence or with a reduced number of cellular translation initiation factors. HCV and HCV-like IRES, a type of Picornavirus IRES, only requires eIF2 to assemble successfully the translation initiation complex. Recently, it has been shown that HCV IRES-mediated translation, under stress conditions when eIF2 is inactivated by phosphorylation, could be driven by a non-canonical pathway in an eIF2-independent manner (252). Other viruses, as Encephalomyocarditis virus (EMCV), with different IRES architecture failed to use this pathway. Interestingly, our results showed that translation of the reporter transcript that harbors an EMCV IRES in its 5´ end is not affected when Rck/p54, LSm1-7 or PatL1 were silenced. Thus, an attractive possibility would be that HCV-induced stress conditions and its IRES architecture could facilitate the use of this non-canonical translation mechanism and that Rck/p54, LSm1 and PatL1 proteins might act on this alternative pathway. If so, viruses as poliovirus and BVDV, that differ in the organization of the 5´ NTR or apply different mechanism to coordinate translation (108), would not be able to use this non-canonical pathway and, consequently, will not depend on Rck/p54, LSm1-7 or PatL1 proteins for translation. However, these proteins are required for BMV RNA translation, which has a capped genome with a tRNA-like structure on its 3´ end. Interestingly, PCBP2, a facilitator of IRES-mediated translation and a component of P-bodies and SGs (91), is also request for BMV translation (unpublished results). Possibly, coronaviruses having a capped genome but a poly(A) on its 3´ end, behave as cellular mRNAs and are translate independently of Rck/p54, LSm1-7 or PatL1 proteins.

There are several points to consider concerning the approaches used for the evaluation of the effect of Rck/p54, LSm1 and PatL1 on coronavirus, poliovirus and BVDV. A common feature of these viruses is their fast replication kinetic in cell culture. In addition, these viruses possess the ability to cause a strong cytopathic effect (CPE) to the host cells. On the contrary, HCV infection has a slow replication kinetic and does not show CPE. One possibility could be that we are not able to measure experimentally the effect with these systems due to the fast kinetic subjected the cells to a high viral load in short time. Three approaches could be used to overcome this experimental difficulty. First, due to differences in transfection efficiencies inherent to any of the particular transfection method used, i.e. lipid transfection or electroporation, a cell subpopulation of about 20% is not efficiently transfected and remains wild-type for the silencing induced by the transfected siRNA. Additionally, in transfected cells there are different levels of protein downregulation. Thus, we are always working with a heterogeneous population, in which the level of depletion for the targeted protein varies among individual cells. This variable silencing efficiency could lead to a situation where effects on viral replication are underestimated, especially if the virus replicates very aggressively. To rule out this possibility, an interesting strategy would be the establishment of stable-depleted cell lines for the proteins of interest, what would allow the use of a homogeneous knocked-down population. To avoid side effects due to the constant depletion of a protein, it would be worthy the generation of conditional knock-down cells. Second, a common observation in certain cellular pathways is that there are proteins which are redundant, therefore, knockdown of one protein is maybe compensated by the presence of others. Eulalio *et al.* have demonstrated that decapping activator proteins have partially redundant functions in miRNA-mediated mRNA decay

(74). Depleting single decapping activators, such as Dcp1, Edc3 or Me31B (the *Drosophila melanogaster* homologue of Rck/p54) stabilize a reporter mRNA only slightly. Strinkingly, co-depletion of two of these decapping activators restored mRNA levels more than single-protein depletion, arguing for a compensatory role of the proteins in this pathway. Thus, the lack of detectable differences between silenced and control cells observed in coronavirus, poliovirus or BVDV replication could be a result of the compensatory function of decapping activators that stayed at wild-type levels. The fact that an inhibitory effect was observed on HCV translation and replication could be due to the slow replication of HCV. Co-depletion experiments will be extremely useful to clarify this point. At the same time, this approach should be used in parallel with the HCV system to test whether the silencing of more than one protein of the mRNA decay machinery have an accumulative effect on HCV translation and replication. Third, to better understand the role of LSm1-7, Rck/p54 and PatL1 on the replication of other viruses that are very adapted to cell culture and does replicate very aggressively, the generation of virus mutants with impaired replication capacity may be useful.

The antiviral role of the miRNA pathway has been shown in early studies for plants and insects (12, 18), and later evidence suggested a similar function in mammals (17). Additionally, it has been demonstrated that miRNAs target cellular mRNAs to P-bodies, by promoting repression of their translation by the miRISC complex *via* Rck/p54 (57). Different groups have reported that replication and translation of HCV is enhanced by the interaction of a liver-specific miRNA (miR-122) with sequences on the 5´ NTR of the viral genome (114, 127). Recently, another human miRNA has been found to interact with a human virus, the specific cellular miRNA (miR29a) represses HIV-1 replication and infectivity by enhancing the interaction of the RISC complexes with the HIV-1 mRNA

leading to translation repression, and that silencing of Rck/p54 reverts the effect of the miR29a on the virus, enhancing its replication (185). An interesting possibility is that Rck/p54 regulates viral translation and replication through its role in the miRNA pathway. In agreement with this idea the depletion of Rck/p54 could impair this pathway what would result in a loss of the effect promoted by the specific miRNA. The regulation exerted by specific miRNAs on virus life cycles is poorly understood, however, the results showed on Rck/p54-depleted cells could be a first hint of how this regulation is controlled.

Despite continuous advances, HCV remains a major threat for human health with limited therapy options. Actually, several drugs that target key enzymes essential for HCV are in clinical evaluation (205, 218) to establish their usefulness for anti-HCV treatment. Besides the safety requirements common to all new drugs, the success of anti-HCV agents will be influenced by their ability to inhibit all genotypes, prevent the emergence of escape mutants and enhance response rates of treated patients. Although no drug can be considered totally safe from the risk of resistance development, agents targeting host factors are less likely to select for escape variants, as human genes are less likely to mutate in response to therapy. Our findings points out to Rck/p54, LSm1 and PatL1 as promising targets for anti-HCV treatment. Furthermore, the lack of toxicity of Rck/p54, LSm1, and PatL1 proteins downregulation in human cells and the fact that the respective yeast knock-out strains are viable, make the development of such approaches suitable as future antiviral treatments.

VI. Appendix

Scheller N, **L. Mina**, R.P. Galão, A. Chari, M. Giménez-Barcons, A. Noueiry, U. Fischer, A. Meyerhans, J. Díez. Translation and replication of hepatitis C virus genomic RNA depends on ancient cellular proteins that control mRNA fates. Proc Natl Acad Sci U S A 106: 13517-22

Scheller N, Mina L, Galao RP, Chari A, Giménez-Barcons M, Noueiry A, et al. Translation and replication of hepatitis C virus genomic RNA depends on ancient cellular proteins that control mRNA fates. Proc Natl Acad Sci U S A. 2009; 106(32): 13517-22.

Reiter J, Pérez-Vilaro G, Sheller N, **Mina L**, Díez J and Andreas Meyerhans. Hepatitis C Virus RNA Recombination in Cell Culture. *In preparation.*

Recombination is an integral part of RNA virus biology. Previous studies suggested that recombination between parental (+) RNA viruses replicating in the same cell occurs predominantly by a copy choice mechanism and that host factors can contribute to the magnitude of recombination. Recently, HCV recombinants have been detected phylogenetically, both, as novel circulating recombinant forms or within infected patients. To obtain first estimates about the extent to which recombination may play a role in HCV evolution, we have developed a selectable, HCV replicon-based recombination detection system (RDS) in hepatocyte cell lines. In this work, recombinants were readily detected with frequencies correlating to the distance between the mutations, demonstrating for the first time the existence of recombination of HCV *in vitro*. My contribution to this manuscript was to collaborate in the design of the system and the performance of the preliminar experiments.

Hepatitis C virus RNA recombination in cell culture

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Recombination is an important mechanism in virus evolution. To analyze and characterize recombination events during HCV replication, a neomycin-selectable, HCV replicon-based recombination detection system was established. Replicons were mutated within either the neomycin-phosphotransferase gene or the NS5B polymerase. Upon co-transfection of hepatic cells lines, recombination between the mutated sites was necessary to restore the selectable phenotype. Recombinants were readily detected with frequencies correlating to the distance between the mutations. The recombinant frequency per nucleotide was around $3.7x10^{-8}$. Thus, while recombination in HCV seems rare, it might become biologically important when strong selection pressures are operative.

The Hepatitis C virus (HCV) is a major threat for human health with around 170 million chronically infected people worldwide (151, 227). As a positive strand RNA virus of the family Flaviviridae, the viral RNA-dependent RNA polymerase NS5B lacks proof-reading activity (1, 8). Consequently, error prone replication of HCV in its host leads to the generation of related but genetically distinct virus variants collectively named viral quasispecies (17, 66, 67, 79, 103). It is this quasispecies nature of HCV infection that is a major hurdle for efficient antiviral treatment and the development of a vaccine.

Recombination is an integral part of RNA virus biology (150, 183, 230, 262, 283). Early, extensive studies with poliovirus and more recently with viral replication models in yeast suggest (i) that recombination between parental, (+) strand RNA viruses replicating in the same cell occurs predominantly by a copy choice mechanism (95, 138, 278) and (ii) that host factors can contribute to the magnitude of recombination (53, 226). For HCV, such studies are lacking and it was rather recent that HCV

recombinants were detected phylogenetically, both, as novel circulating recombinant forms or within infected patients (43, 46, 128, 130, 156, 181, 190, 225).

To obtain first estimates about the extent to which recombination may play a role in HCV evolution, we have developed a selectable, HCV replicon-based recombination detection system (RDS) in hepatic cell lines. Starting from the HCV genotype 1b replicons pFKi389neoNS3-3'-ET and pFKi389neoNS3-3'-ΔGDD that both carry the neomycin-phosphotransferase gene (npt-gene) (24, 166), three additional replicons were generated by mutagenesis (Fig. 1A; see Table S1 in the supplemental material). Replicons 5'm and 3'm have mutations that disrupt functional npt gene expression while Δ5B is derived from the NS5B-defective replicon NAΔ5B (=pFKi389neoNS3-3'-ΔGDD) and contains three cell-culture adaptative mutations in NS3, NS4A and NS4B (see Table S2 in the supplemental material for summary) (165). Transfection of Huh7-Lunet cells (164) with the individual replicons 5'm, 3'm, NAΔ5B or Δ5B did not lead to neomycin-resistent cell colonies due to defects of the npt gene or the lack of a functional NS5B polymerase. However, when different HCV replicons were co-transfected, cell colonies were readily selectable. As the HCV NS5B polymerase only functions in *cis* (258), *trans*-complementation of the two Δ5B replicons by 5'm or 3'm can be excluded and thus the appearance of neomycin-resistant cell colonies suggested replicon recombination between the mutated sites.

To determine the frequency of HCV replicon recombination, $4x10^6$ Huh 7-Lunet cells were electroporated in Cytomix (165) with 10µg carrier RNA plus 4µg of each replicon RNA. By differently combining the mutated replicons, the effective cross-over range for recombination was varied.

Combination of 5'm plus 3'm (RDS1), 3'm plus NAΔ5B (RDS2), 5'm plus NAΔ5B (RDS3), 3'm plus Δ5B (RDS4) and 5'm plus Δ5B (RDS5) resulted in cross-over ranges of 0,6 kb, 2 kb, 2,6 kb, 6 kb and 6,6 kb respectively (Fig. 1B). As controls, cells were electroporated with the wt replicon, the individual mutated replicons or with carrier RNA only. Immediately after electroporation, cells were transferred to 40 ml of complete DMEM medium, seeded into cell culture dishes and cultured at 37°C with 5% CO_2 (165). Twenty-four hours later, 500 μg/ml G418 (Sigma-Aldrich, St. Louis, MO) was added to select for replicon-containing, neomycin-resistant cell colonies. The medium was exchanged once per week. After three weeks in culture, cells were stained with 1% crystal violet and cell colonies were counted. A representative result for such an experiment is given in Fig. 2. Co-transfections of 3'm plus Δ5B (RDS4), as well as 5'm plus Δ5B (RDS5) resulted in the appearance of neomycin-resistant Huh 7-Lunet colonies whereas single transfections of mutated replicon RNA (5'm, 3'm and Δ5B) did not (Fig. 2A). Experiment 1 gave 23, 10 and 20880 colonies for RDS4, RDS5 and wt replicon, while experiment 2 gave 17, 12 and 8800 colonies respectively (Fig. 2B).

To verify that the G418-selected cell colonies were indeed the result of an HCV replicon recombination event, colonies from RDS4 and RDS5 were isolated using cloning cylinders (Sigma-Aldrich, St. Louis, MO) and expanded in G418-containing media. RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and reverse transcribed with SuperScript III RT (Invitrogen, Carlsbad, CA). Subsequently, the regions of npt and NS5B carrying the inactivating mutations were amplified by PCR with specific primers (see Table S3 in the supplemental material). PCR products were sequenced directly or ligated with the pGemT vector (Promega, Madison, Wisconsin) and cloned. Three individual plasmid clones were sequenced according to the

BIGDye 3.1 protocol (Applied Biosystems, Foster City, CA). Using both strategies, only wt sequences were detected for both regions, strongly suggesting that the selected cell colonies were indeed the result of recombinations between the inactivating mutations.

To exclude the possibility that accidental integration of replicon sequences into the Huh 7-Lunet genome had provided neomycin-resistance, we isolated 6 colonies obtained from an RDS1 experiment and cultivated them in the presence of 100 IU/ml IFN-α. This procedure was previously shown to cure cells from HCV replicons but would leave integrated DNA from replicons unchanged (86). After 2 weeks of culture, 500 μg/ml G418 was added. All expanded cell colonies were sensitive to G418 treatment, indicating that no replicon sequence was integrated into the genome.

The observed HCV recombinant frequency, which is the ratio of the number of neomycin-resistant cell colonies with recombined HCV replicons divided by the respective number with wt replicons, was dependent on the cross-over range (Fig. 3). With increasing cross-over length, the recombinant frequency increased accordingly suggesting that there was no major recombination hotspot between the mutated sites. Averaging over the whole assessable region, the mean recombinant frequency was calculated to be $3.7x10^{-8}$ per nucleotide or $3.5x10^{-4}$ per HCV genotype 1b genome. These numbers were derived from 4 to 8 independent experiments. The HCV recombinant frequencies are low when compared with the notoriously recombinogenic human immunodeficiency virus (HIV) that exhibits 3-30 RNA strand cross-overs during a single reverse transcription step or a poliovirus with a recombination frequency of around $6,84x10^{-3}$ per kb (281). Nevertheless,

given the large number of around $2x10^{10}$ HCV-infected hepatocytes in a chronic carrier, HCV recombination *in vivo* might become important when strong selection pressures are operative. This is to be expected in the near future when the novel protease and polymerase inhibitors reach the clinic. With the simplifying assumption that the recombination between the HCV replicons described here mimics recombination events within infected hepatocytes *in vivo*, then $2x10^{10}$ times $3.5x10^{-4}$ = $7x10^{6}$ HCV recombinants might be expected to be generated within one or few days (= the available time for the recombination event in our experiments) within a patient. Taken into account the distance between protease and polymerase resistant mutants as previously determined in clinical trials and cell culture (9, 219, 300), this number comes down to $2.6x10^{5}$ to $2.9x10^{6}$ recombinants for a distance of 360 nucleotides (Telaprevir-resistant mutants at amino acids 36 and 156 of NS3) and around 4000 nucleotides (distance between Telaprevir- and PS1-6130-resistance mutants) respectively. Clearly, a significant fitness advantage is necessary to enable such recombinants to compete with the estimated 10^{12} non-recombined viruses produced per day (187).

In summary, a neomycin-selectable, HCV genotype 1b replicon-based recombination detection system is described that enabled the first quantitative estimate for the recombination of HCV in a hepatic cell line. The recombinant frequency was low and dependent on the available cross-over range. This system will be of great help to better define the mechanism of HCV recombination, to study the involvement of cellular host factors in recombination and to elucidate the implications of HCV recombination in patients undergoing treatment.

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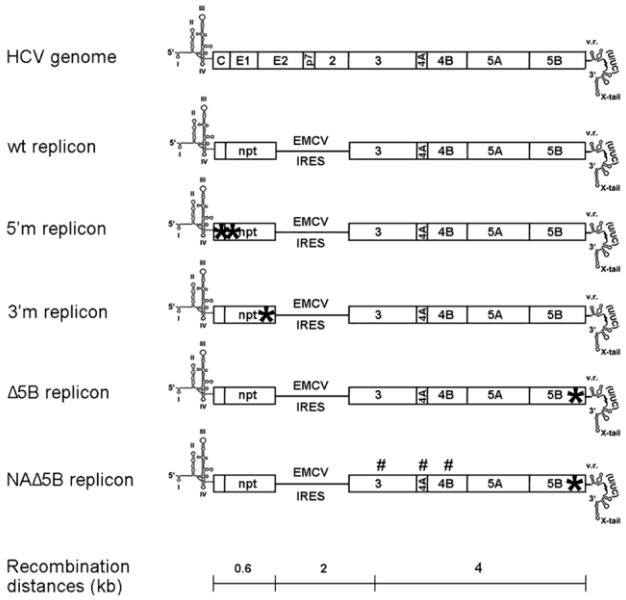
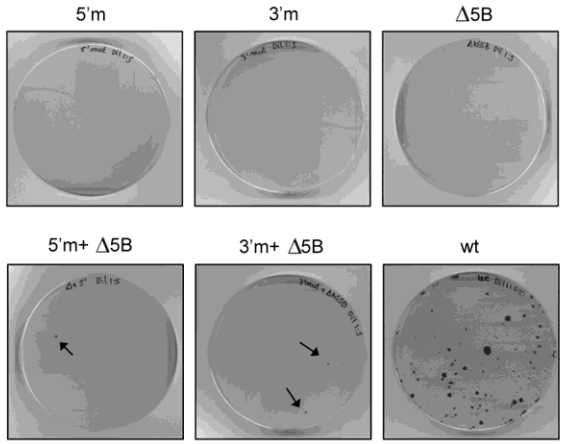


FIG. 1. Schematic representation of the HCV genome and derived genotype 1b replicons used for the neomycin-selectable, HCV replicon-based recombination detection system. Wt- and NS5B-defective replicon NAΔ5B were previously described as pFKi389neoNS3-3'-ET and pFKi389neoNS3-3'-ΔGDD and kindly provided by Ralf Bartenschlager. While the wt replicon carries cell culture adaptive mutations in NS3, NS4A and NS4B, the replicon NAΔNS5B does not. The other three replicons were generated by mutagenesis (see Table S1 in the supplemental material). 5'm and 3'm were made neomycin-phosphotransferase (npt)-defective by mutating the start codons for the core and the npt-gene or by mutations in the active site of the enzyme. The replicon ΔNS5B was generated from Wt by mutating the GDD encoding motif of the NS5B. An asterix indicates inactivating mutations while a cross point shows the lack of a cell-culture adaptative mutation. Nucleotide distances between the critical mutation sites are shown.

A



B

	Total number of cell colonies		
Experiment	3'm+Δ5B	5'm+Δ5B	wt
1	23	10	20880
2	17	12	8800

FIG. 2. Representative results of two recombination selection experiments. (A) Transfected cells were grown for three weeks in the presence of G418 and stained with cristal violet to visualize cell colonies. Only co-transfections with 3'mut + Δ5B (RDS4) and 5'mut + Δ5B (RDS5) resulted in cell colonies (indicated with an arrow) while cells transfected with 5'mut, 3'mut or Δ5B only were all sensitive to G418. (B) Total number of colonies obtained in each experiment. Transfection of wt replicons were performed as positive controls and used to calculate the recombinant frequency.

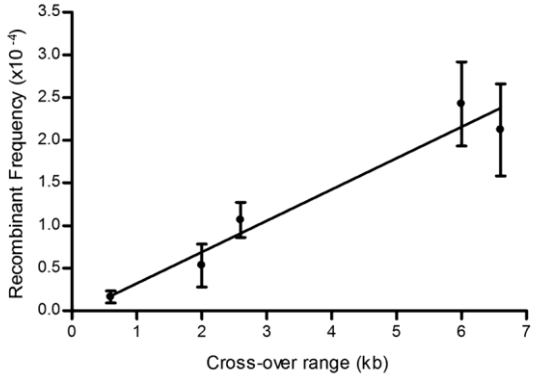


FIG. 3. Linear relationship between the HCV recombinant frequency and the distance between the inactivating replicon mutations. The recombinant frequency is given as a function of the cross-over length in kilobases (kb). The mean values ± standard error of the mean are the results of eight independent experiments of the 5'm+3'm combination, five independent experiments of the 3'm+NAΔ5B combination, six independent experiments of the 5'm+NAΔ5B combination and four independent experiments of the 5'm/3'm+Δ5B combination. The regression line follows the function y = 0,3673x-0,0468; r^2=0,9518.

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