CELLULAR PROCESSING BODIES AND THE HEPATITIS C VIRUS LIFE CYCLE: CHARACTERIZATION OF THEIR DYNAMIC INTERPLAY

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A la meva mare,

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SUMMARY

Processing bodies (P-bodies) are discrete and highly dynamic granules present in the cytoplasm of eukaryotic cells under normal growth conditions. They contain translationally repressed mRNAs together with proteins from the mRNA decay and miRNA machineries. Interestingly, multiple P-body components have been described to influence the replication of positive-strand RNA [(+)RNA] viruses, a group of viruses which genomes mimic cellular mRNAs. Recently, our group demonstrated that the P-body components PatL1, DDX6 and the complex LSm1-7, which function as decapping activators of cellular mRNAs, are required for hepatitis C virus (HCV) translation and replication. Remarkably, the corresponding homologs are also utilized by other (+)RNA viruses that infect different kingdoms, like the plant virus brome mosaic virus or the phage Qβ. Moreover, a genome wide screening in yeast determined that the exonuclease Xrn1, that also localizes in P-bodies, can affect the recombination rate in the (+)RNA plant virus tomato bushy stunt virus (TBSV) and, in consequence, viral evolution. It is unknown if similar mechanisms could be operating in other (+)RNA viruses. Thus, at the beginning of this work it was well-established that P-body components are intimately linked to (+)RNA viruses life cycles. However, whether P-bodies structures themselves play a role remained unknown.

In the present study, we explored the interface of HCV with P-bodies, demonstrating that HCV infection induces changes in P-body composition by specifically changing the localization pattern of P-body components required for viral replication. These alterations were associated with the HCV life cycle steps of translation and/or replication and could be reversed by inhibiting HCV replication with a polymerase inhibitor. Moreover, we demonstrated that P-body components but not P-body granules are required for HCV replication. Additionally, we set up a selectable HCV replicon-based recombination detection system in cell culture to analyze recombination events and to further characterize the possible involvement of P-body components in this evolution mechanism. With this system the first estimations of recombination frequencies were derived indicating that recombination in HCV seems rare. In contrast to the results observed in TBSV, Xrn1 depletion did not change recombination rates in HCV indicating that the use of the 5’-3’ exonuclease is not a general feature in the recombination of (+)RNA viruses. Together, these results

increase our understanding of basic aspects in HCV biology and its intimate relation with the host.

Els cossos de processament (cossos-P*)* són grànuls discrets i dinàmics que es troben presents en el citoplasma de les cèl·lules eucariotes en condicions normals de creixement. Contenen mRNAs represos en la traducció així com proteïnes involucrades en la degradació dels mRNAs i de la maquinària dels miRNAs. Curiosament, diversos components dels cossos-P influencien la replicació dels virus RNA de polaritat positiva [RNA(+)], el genoma dels quals imita els mRNAs cel·lulars. Recentment, el nostre grup ha demostrat que els components dels cossos-P PatL1, DDX6 i el complexe LSm1-7, que actuen com activadors del *decapping* dels mRNAs cel·lulars, són necessaris per la traducció i la replicació del virus de la hepatitis C (VHC). De fet, els corresponents homòlegs d'aquestes proteïnes són utilitzats per altres virus RNA(+) que infecten diversos regnes, com el virus del mosaic del brom, que infecta plantes, o el fag Qβ. A més a més, un *genome wide screening* en llevat ha determinat que l'exonucleasa Xrn1, que també es localitza als cossos-P, pot afectar la taxa de recombinació d'un virus RNA(+) de plantes conegut com el virus del nanisme ramificat del tomàquet (TBSV) i, en conseqüència, l'evolució viral. Es desconeix si l'existència de mecanismes similars podrien influenciar altres virus RNA(+). Així doncs, al començament d'aquest treball es coneixia que els components dels cossos-P estan estretament relacionats amb els cicles vitals dels virus RNA(+). No obstant, no estava clar si els cossos-P com estructures juguen un paper important en la propagació viral.

En l'estudi que aquí es presenta hem explorat la relació del VHC amb els cossos-P, demostrant que el VHC promou canvis en la composició dels cossos-P a través de l'alteració de la localització d'aquells components que són necessaris per a la replicació viral. Aquestes alteracions s'han relacionat específicament amb la traducció i/o replicació del VHC, podent-se revertir després d'inhibir la replicació del VHC amb un inhibidor de la polimerasa viral. A més a més, hem demostrat que els components dels cossos-P però no els grànuls *per se* són necessaris per a la replicació del VHC. Addicionalment, hem posat a punt un sistema de detecció de recombinació en cultiu cel·lular basat en replicons del VHC que permet analitzar successos de recombinació i caracteritzar la possible participació dels components dels cossos-P en aquest mecanisme d'evolució. Amb aquest sistema s'han establert les primeres estimacions en la freqüència de recombinació del VHC, indicant que la recombinació en aquest

virus no és gaire comú. Contràriament als resultats observats en TBSV, la reducció del nivell d'expressió de Xrn1 no va alterar la taxa de recombinació del VHC indicant que la utilització de l'exonucleasa no és una característica general en la recombinació dels virus RNA(+). En conjunt, aquests resultats incrementen el nostre coneixement sobre els aspectes bàsics de la biologia del VHC així com de l'estreta relació que aquest virus estableix amb l'hoste.

PROLOGUE

Infection with the hepatitis C virus (HCV), a positive-strand RNA [(+)RNA] virus, represents a global public health threat as 130-200 million people are chronically infected. Without any protective vaccine and treatment options still limited, this pathogen has become the principle cause of hepatocellular carcinoma and one of the main indications for liver transplantation.

Deeper knowledge of HCV biology is required to understand its viral life cycle and, in turn, to control the virus. Since the HCV life cycle completely depends on host factors (HFs), their characterization is essential to understand HCV biology and can also provide novel targets for antiviral treatment. Recently, our group and others described that multiple components of the cellular 5'-3' deadenylation-dependent mRNA decay pathway play essential roles not only in replication but also in recombination of (+)RNA viruses. These components, and translationally repressed mRNAs, localize in cytoplasmic granules named processing bodies (P-bodies). They are discrete and highly dynamic foci present under normal growth conditions, characterized for being present in a wide range of organisms from yeast to humans. Whether P-body granules *per se* play a role in viral infection remained unknown. In this thesis we have explored the interface of P-bodies and HCV and established a model system that allows to analyze HCV recombination events and to characterize the putative involvement of HFs in this important evolution mechanism.

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INTRODUCTION

1. POSITIVE-STRAND RNA VIRUSES

The group of positive-strand RNA [(+)RNA] viruses encloses viruses with single-stranded RNA genome of positive polarity. (+)RNA viruses comprise over one third of all virus genera and infect a wide range of host species, including bacteria, plants, animals and humans. The most relevant animal and human pathogens within this viral group are members of the families *Coronaviridae* (e.g. severe acute respiratory syndrome virus), *Flaviviridae* (e.g. West Nile virus, yellow fever virus, dengue virus, hepatitis C virus) and *Picornaviridae* (e.g. foot-and-mouth disease virus, poliovirus and hepatitis A virus).

Despite their diversity in genome organization, virion morphology and life cycles, the different families of (+)RNA viruses share fundamental common features in their replication process (Figure 1). Their entire life cycle occurs in the cytoplasm, where (+)RNA viruses release their genome [2]. Since (+)RNA viruses do not encapsidate the RNA dependent RNA polymerase (RdRp), early at infection (+)RNA viral genomes perform two essential functions, they first serve as mRNAs for translation of the viral proteins and then as templates for replication. Since these two functions are mutually exclusive, the transfer of the genomic RNA from the cellular translation machinery to the replication complex must be highly regulated. Another important feature is that their viral replication complexes are associated with intracellular host membranes which proliferate and rearrange in response to the

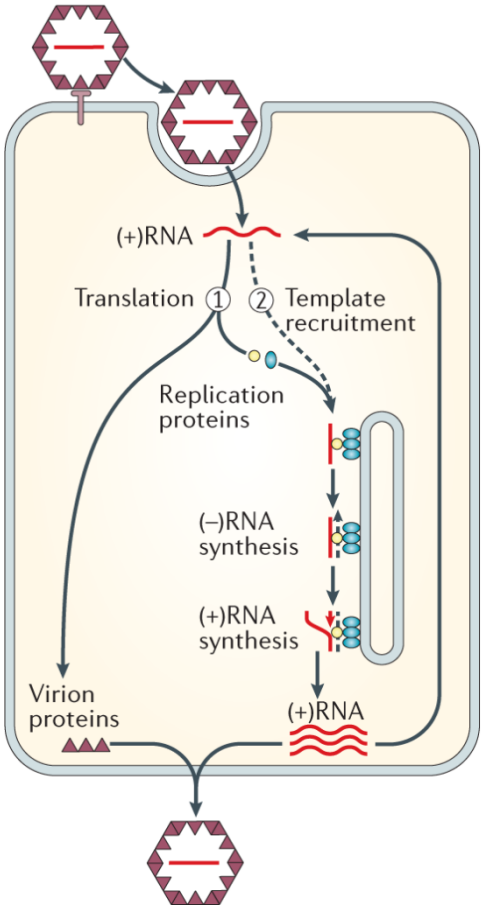

Figure 1. (+)RNA viruses life cycle. All viruses infect cells through a first attachment step to the cell surface. Once internalized, (+)RNA viruses release their genome, which initially will be used as mRNAs for translation of the viral proteins and later as templates for replication. New genomes will be encapsidated and after maturation the viral progeny will exit the cell. Adapted from Ahlquist, Nature 2007.

expression of viral proteins. This localized replication increases the efficiency of the process, as it ensures high local concentrations of replication components, protects the viral genomes from cellular degradation machinery and eliminates competing processes such as translation [2]. Once in these membranes the viral RNA is used as a template to generate negative-strand RNA intermediates that, in turn, will be used as templates for the generation of a new progeny of positive-strand genomic RNAs. The new (+)RNA genomes, together with the translated viral proteins, will be encapsidated into new progeny virions. Thus, viruses included in this group face the challenge to direct their (+)RNA genome in a highly regulated manner to translation, replication or encapsidation. Finally, since these viruses have very limited coding capacity, they highly depend on host proteins to regulate their life cycle [160].

2. HEPATITIS C VIRUS EPIDEMIOLOGY

2.1. Taxonomy and genotypes

Hepatitis C Virus (HCV) is a (+)RNA enveloped virus classified in the *Hepacivirus* genus within the *Flaviviridae* family. HCV, as other RNA viruses, is characterized by a high level of genetic variability. As a consequence, HCV isolates can be grouped into genotypes and subtypes. Six different genotypes have been described that differ in their nucleotide sequence by 30-35%. Each genotype comprises varying numbers of more closely related variants, named subtypes (designated a, b, c and so on), that differ from each other in their nucleotide sequence by 20-25% [116]. HCV genotypes are differentially distributed over distinct geographical areas and although they share their most biological features, there are some differences in their susceptibility to interferon (IFN)-based therapies. The most common variants found in Western countries include the genotype 1 (subtypes 1a and 1b), and genotype 2 (subtypes 2a, 2b and 2c) [206], being genotype 1 the most resistant to the IFN-based therapies against chronic hepatitis C [93].

2.2. Prevalence and transmission

Around 130-200 million people are infected with HCV worldwide, which encloses the 3% of the global population. In 75-80% of HCV infected patients the infection will evolve into chronic liver disease and cirrhosis, resulting one of the most common indications for liver transplantation in developed countries [34, 87]. Currently, there is no vaccine available. In addition, the limited response rate of the standard treatment and its side effects results in 350.000 people around the world dying from HCV-related liver diseases every year [173]. Moreover, the existence of different genotypes and subtypes with variations in treatment success, as well as the fact that different countries have to confront divergent challenges when dealing with HCV epidemic, complicate the scenario [234] (Figure 2).

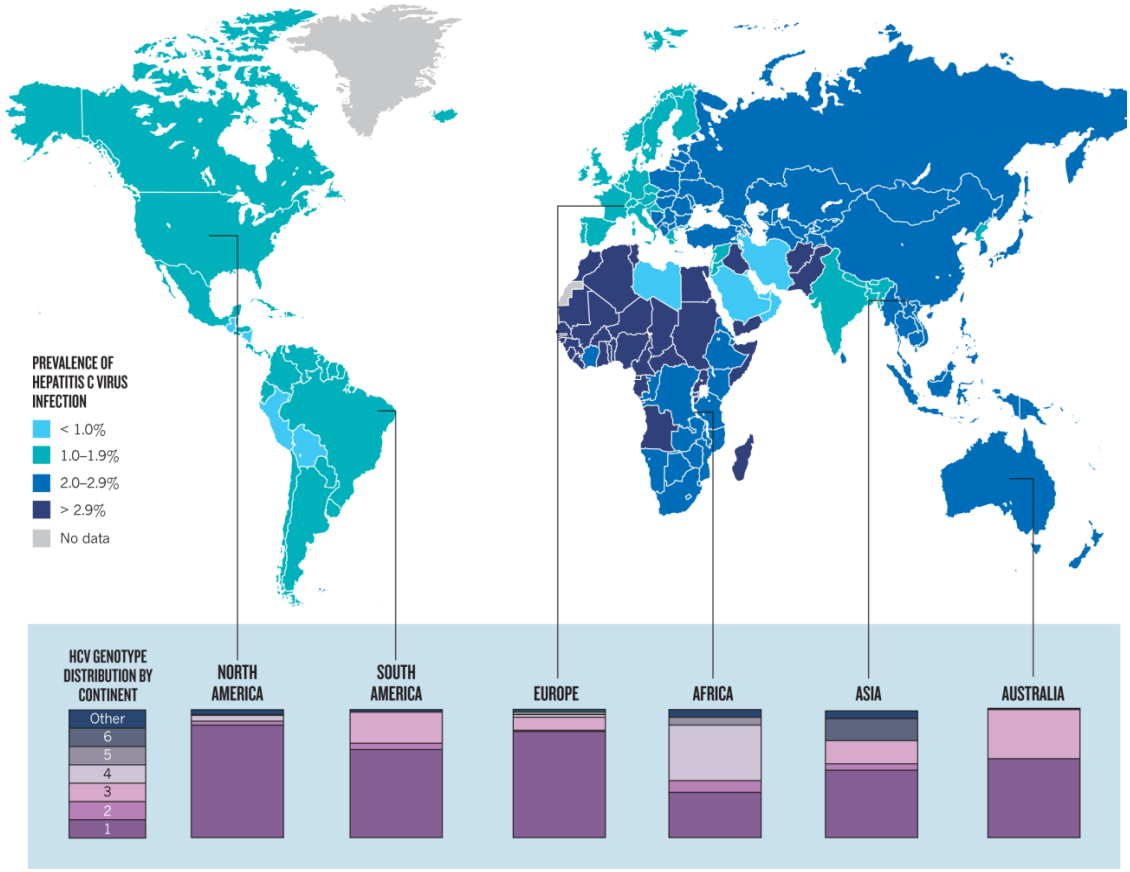

Figure 2. Prevalence of hepatitis C virus infection worldwide. HCV is spread in all continents, with a higher prevalence in north Africa and south Asia. The distribution of the different HCV genotypes is indicated in the lower part. Modified from Gravitz, Nature 2011.

HCV is mainly transmitted through exposure to infected blood. In the developed nations the majority of patients acquired the disease through intravenous drug use or blood transfusion. Other risk factors for HCV infection include high-risk sexual activities or birth to an infected mother. With the current routine tests of blood, HCV transmission via blood transfusion becomes rare, happening in 1 in 500.000 to 2.000.000 transfusions [45]. Moreover, part of the success in HCV prevention has come because the human immunodeficiency virus (HIV) campaigns targeted practices that also decrease HCV transmission. However, in poorer nations the lack of screening of blood donations together with the reuse of medical supplies enhance the viral dissemination [87].

2.3. Natural history of HCV infection

Infection by HCV was unknown for a long time, mainly because the pathogenesis observed was associated to other viruses that also targeted the liver, like hepatitis A and B. It was in the 1970s when scientists realized that another infectious agent was involved in most of the cases of hepatitis that occurred after blood transfusion, naming it non-A, non-B hepatitis. It took almost a decade to isolate this agent and characterize it like a novel hepatitis virus [47].

2.3.1. Acute Hepatitis C

Acute hepatitis C starts after an incubation period that ranges from 2 to 12. The majority of acutely infected patients are asymptomatic and have a clinically non-apparent or mild course, which in turn difficult the diagnostic of the disease [45]. Around 20% of infected patients will clear the virus without any pharmacological help, a proportion that increases up to 50% in teenagers and young adults [87]. Fulminant hepatic failure as a result of HCV acute infection can happen but is not frequent (Figure 3).

2.3.2. Chronic Hepatitis C

The risk of developing a chronic HCV infection is high. Around 80% of infected patients do not clear the virus after 6 months of the onset of acute infection. Many factors affect the rate and the evolution of chronic HCV infection, like the age at time of infection, gender, race and the development of symptoms during acute infection [45]. Once the chronic infection is established the rate of spontaneous clearance is very low. The progression of the infection can lead to structural liver damage, also known as fibrosis, that in 10-15% of individuals with chronic HCV infection will turn into cirrhosis. At this stage the liver becomes fibrotic and scarred, resulting in jaundice and a swollen abdomen, together with an increase of toxins in blood and other serious complications [87]. Unfortunately, the progression to cirrhosis is usually silent, making that some patients discover that they are infected with the virus when they present complications of end-stage liver disease or hepatocellular carcinoma (HCC) [45].

Many studies have estimated the time to develop cirrhosis and HCC after infection starts. The mean time to develop cirrhosis is estimated around 20 years (+/-10 years) and the time to diagnoses HCC is 28 years (+/- 11 years). Once cirrhosis is established, HCC develops at annual rate of 1-4%, causing the death of 5-10% of infected patients every year [35, 199, 220].

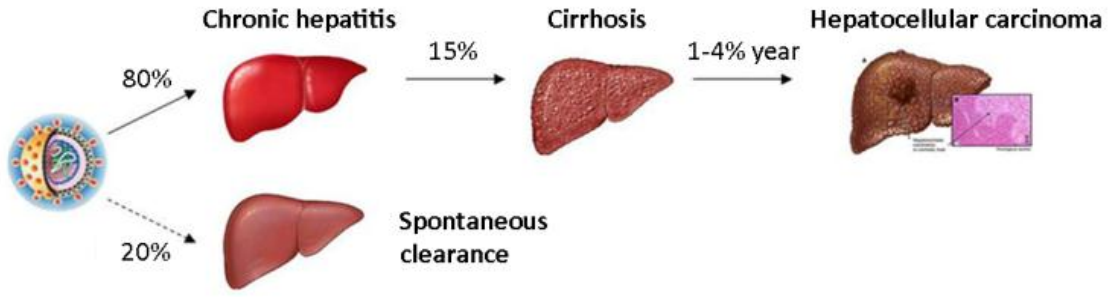

Figure 3. Natural history of hepatitis C infection. Around 80% of HCV-infected patients will develop a chronic hepatitis that in 15% will turn into cirrhosis. HCC will develop at annual rate of 1-4% in cirrhotic patients.

2.4. Viral evolution

RNA viruses are characterized by high levels of genetic heterogeneity, which influences not only the viral evolution but also the course of the pathogenesis. Mutation at the

nucleotide level together with recombination are the main causes of this genetic variation in RNA viruses in general, and HCV in particular [86, 154].

2.4.1. Mutation

As all (+)RNA viruses, the RdRp encoded by HCV lacks a proof reading function. The high level of replication and the high error rate of the RdRp in RNA viruses lead to a mutation rate estimated at 10^{-4} substitutions per site and round of copying [56]. Given that the rate of virus production in an infected individual is around 10^{12} virions per day [162], the production of a highly genetically divers population is assured. This high error rate, which in turn is the primary cause of genetic variation in HCV, causes that the virus circulates as a complex population of distinct but closely related viral variants within the same host named quasiespecies [54].

2.4.2. Recombination

Although recombination events have been reported to be important for the evolution of most RNA viruses, it was believed that HCV mainly evolved through the accumulation of mutations, suggesting that recombination was a rare event *in vivo* and/or the resulting recombinants were not viable [223]. It was in 2002 when the first natural inter-genotypic recombinant strain of HCV (RF1_2k/1b) was identified [106]. Interestingly, this report documented not one but six different isolates, all derived from the same recombination event. Thus, the recombinant strain was circulating in the population. In the last years a number of natural inter-genotypic [20, 52, 57, 94, 105, 117, 118, 124, 125, 152, 153, 164, 214, 222, 239], intra-genotypic [33, 38, 43, 156, 190] and even intra-subtype [155, 200] recombinants of HCV have been identified but much lower recombination events have been described for HCV than for other (+)RNA such as poliovirus. However, it is important to note that the prevalence of recombination in HCV could be underestimated since recombinants are not screened for routine practice. All this together points to recombination as a genetic diversity tool that influences HCV evolution. However, there was no information about the frequency of recombination in HCV, an issue that has been addressed in this PhD work.

Two main recombination mechanisms have been described: the non-replicative and the replicative, mainly differentiated because the first one requires RNA breakage and subsequent ligation while the last one happens under viral genome replication. The most accepted theory for generation of HCV recombinants is the replicative mechanism, also known as template switching mechanism, widely used in recombination for the other (+)RNA viruses. During RNA replication the RdRp switches from copying the donor template RNA to an acceptor template RNA, without releasing the nascent strand [119, 161] (Figure 4). Thus, for recombination to happen between two different HCV strains there are some prerequisites that need to be accomplished. First, the simultaneous infection of the same cell by different viral strains. Second, the simultaneous replication of both viral genomes. Third, the template shift by the RdRp while keeping the reading frame. And fourth, the encapsidation and release of the recombinant genome. In addition, viral recombinants might be difficult to detect since i) the replicative fitness of the recombinant strain could be lower than the one from parental strains [154], and ii) HCV genotyping analyses are based on a single or two small genome regions [139].

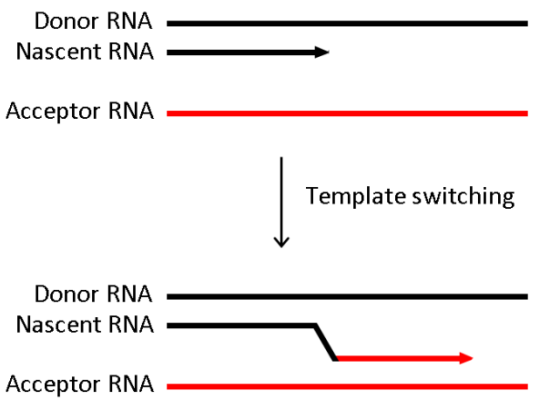

Figure 4. Template switching mechanism. The recombinant RNA results from the jump of the RdRp from the donor RNA to an acceptor RNA.

The discovery of HCV recombinants *in vivo* demonstrates that they have successfully performed each step in the recombination process. Moreover, these recombinants have been under virus selection and have prevailed over parental strains. This indicates that some recombinants can have a greater fitness and, as a result, an important epidemic spreading. Recombination is, from the clinical point of view, a relevant phenomenon because treatment recommendations, as well as the viral response, may differ between genotypes. We are now starting a new therapeutic era against HCV with drugs that specifically target regions of the HCV genome. As happened with other important pathogenic viruses like HIV, natural resistant variants that allow HCV to avoid the effects of these drugs have been already reported [15,

134]. Now that recombination events are proved in HCV, it is no longer necessary that two escape mutations arise simultaneously in the same genome, recombination can provide this antiviral resistance.

2.5. Standard of care of chronic hepatitis C

For almost a decade, the standard of care against chronic hepatitis C consisted of the combined administration of pegylated IFN-α and ribavirin (PEG-IFN/RBV). PEG-IFN stimulates the patient's immune system, and RBV is a general inhibitor of virus replication. This therapy is long, costly and involves multiple severe side effects, like depression and hemolytic anemia, that may result in therapy discontinuation [66]. A treatment response is considered successful when a patient achieves a sustained virological response (SVR) previously defined as having a negative viral load 24 weeks after treatment cessation. Among patients infected with the HCV genotype 1 (HCV-1), the most prevalent worldwide and in Europe [63], only about 40-54% of the cases achieve such a SVR. Moreover, they require higher RBV doses and longer treatment duration (48-72 weeks) in comparison with patients infected with the HCV genotypes 2 or 3 (24 weeks) [66].

To improve the SVR rates in patients infected with HCV-1, different compounds with direct antiviral activity have been developed or are still under development. Phase III of clinical trials combining an HCV protease inhibitor (boceprevir or telaprevir) with PEG-IFN/RBV have been completed and both protease inhibitors have been approved by the Food and Drug Administration and the European Commission. Telaprevir and boceprevir block the NS3/4A protease of HCV, avoiding the cleavage of the viral polyprotein into the viral enzymes and structural proteins. In naïve patients, triple therapies with these compounds improve the SVR rates up to around 70%, even with shorter treatment durations (24-28 weeks) in most of the patients [99, 181, 204]. In previous non-responders to IFN-based treatment, triple therapy also significantly improves SVR rates [13, 241]. These successes led to the modification of previous guidelines and triple therapy has become the new standard of care for the treatment of chronic HCV-1 infections [83], with the consequent 3-fold increase of the present

costs of around 10,000€/year/patient. However, these new protease inhibitors must be given with PEG-IFN/RBV to avoid protease-inhibitor resistance emerging. The continued need for PEG-IFN/RBV is disappointing because not only keep the side effects mentioned above, but also it involves more frequent and severe effects, such as anemia and skin rash, due to the addition of the protease inhibitor. Furthermore, these new drugs can cause pharmacological interactions with drugs metabolized in the liver through cytochrome P450 3A4. Consequently, the current goal is to achieve an efficient PEG-IFN/RBV free treatment. This will require the administration of multiple novel drugs with different modes of action to avoid the appearance of resistant viruses. Currently more than 50 new drugs are in research and development [194, 197]. This includes a second generation of protease inhibitors, agents that block the RdRp or even inhibitors for the NS5A (Figure 5). Besides drugs that target viral proteins, the identification of host factors (HFs) that are required for viral propagation open an extensive area of research. In contrast to the above mentioned drugs, the use of such HFs would be not only HCV genotype independent but given the genetic stability of the host would also make less probable the emerge of resistant strains.

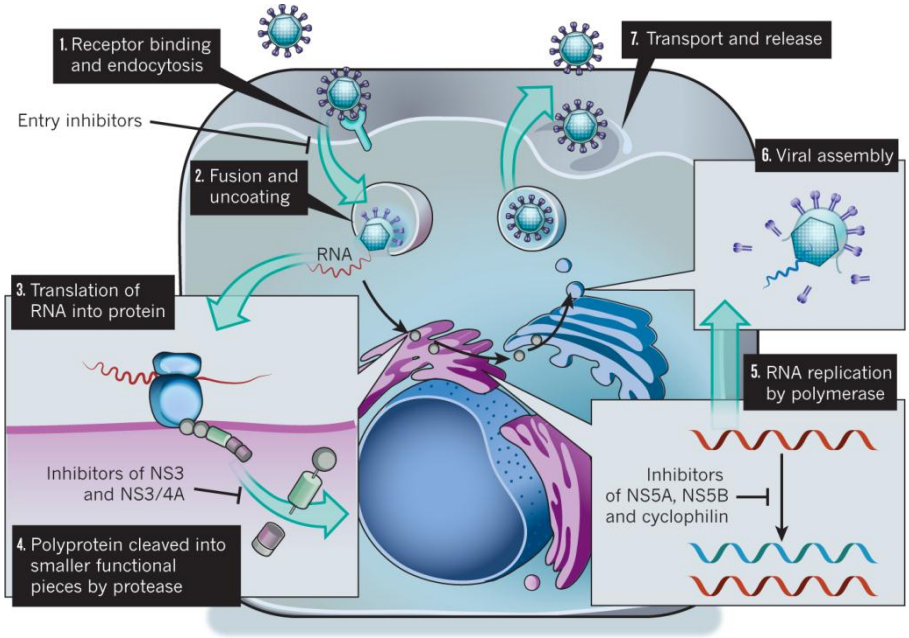

Figure 5. Steps in HCV life cycle susceptible to be targeted by drugs. Multiple drugs targeting different steps of the HCV life cycle are in development or already in clinical trials. The aim of these drugs is to avoid viral propagation by affecting one of the seven key steps in the viral life cycle. Adapted from Schlütter, Nature 2011.

2.6. Host factors as antiviral targets

All steps in the HCV replication cycle depend on host encoded factors [23, 82]. Drugs targeting HFs required for the entry of HCV in the cell, replication or assembly/release of the new viral particles are currently being evaluated [113, 224]. In particular, those drugs targeting HFs involved in replication step have been deeply analyzed. Among them, cyclophilins are of special interest since they are required for all HCV genotypes. Cyclophilins are ubiquitous human cell proteins involved in protein folding. In addition, they have been described as functional regulators of HCV replication through their interaction with some HCV non-structural proteins involved in the replication complex, especially NS5A and NS5B [36, 37, 235]. The Cyclophilin antagonist alisporivir (DEBIO-025) efficiently inhibits HCV replication by blocking the interaction between HCV NS5A and NS5B with cyclophilins [37, 70, 169]. This antagonist is under phase III trial, after showing in phase II that administration of alisporivir together with PEG-IFN/RBV reached a SVR in 76% of the patients compared to 55% in PEG-IFN/RBV treated patients.

Another example of host encoded antiviral target is the microRNA 122 (miRNA122). MiRNA122 is specifically expressed in the liver and stimulates HCV RNA replication through interactions with the 5'NTR [104]. Miravirsen (SPC3649) is a locked nucleic acid complementary to the 5'end of miRNA122 that inhibits miRNA122 function. Recent studies done in chimpanzees demonstrated that miravirsen decreased HCV levels in blood and liver without any significant side effect besides a reduction in serum cholesterol levels [121]. Moreover, this important antiviral effect was shown against HCV genotypes 1-6 *in vitro* [129]. The recent finished phase II study of miravirsen in chronically HCV infected patients has verified the potent antiviral effect also in humans [172].

The increasing understanding of the molecular interactions between HCV and the host cell has resulted in the identification of multiple HFs that are utilized by the virus to multiply. Currently, the challenge is to characterize the action mechanisms by which the identified HFs act on HCV. This will be essential to identify novel promising antiviral targets.

3. HEPATITIS C VIRUS STRUCTURE AND VIRAL REPLICATION

3.1. Viral structure

The HCV genome consists of an RNA molecule with (+)polarity and approximately 9.6kb that contains a long open reading frame (ORF) encoding for a polyprotein precursor of about 3,000 amino acids. The ORF is flanked by 5' and 3' non-translated regions (NTRs) that contain RNA structures essentials for RNA translation and replication. The polyprotein is processed by cellular and viral proteases to generate the individual HCV proteins that will be divided in structural and non-structural proteins. The structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. The non-structural proteins comprise the p7 ion channel, the NS2-NS3 protease, the NS3 serine protease and RNA helicase, the NS4 polypeptide, the NS4B and NS5A proteins and the NS5B RdRp (Figure 6) [151].

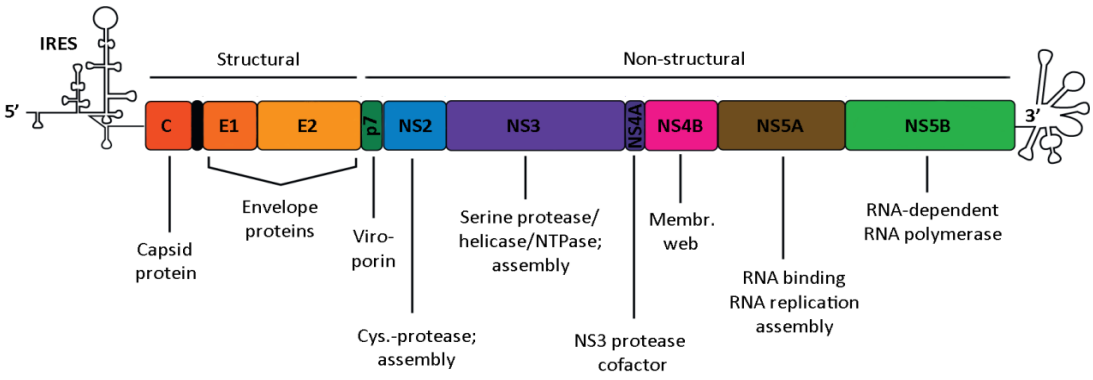

Figure 6. HCV genome organization and viral proteins function. The HCV ORF is flanked by 5' and 3' non-translated regions. HCV codifies for a single polyprotein that is divided into structural and non-structural proteins. For every viral protein the function is indicated. Modified from Bartenschlager *et al*, Trends in Microbiology 2011.

3.1.1. Non-translated regions (NTRs)

The 5'NTR is the most conserved region of the HCV genome [206] and contains an internal ribosome entry site (IRES) required for the cap-independent translation of the viral RNA. It is composed by four highly structured domains, being domain I and II essential for HCV RNA replication [76]. The abundant liver-specific miRNA122 binds to specific sites on the 5'NTR to promote HCV RNA amplification [91, 104]. Although the

miRNA122 specific function remains under debate recent work demonstrated a role in stabilization of the HCV RNA albeit an additional role in promoting translation and replication cannot be excluded [205].

The 3'NTR has a tripartite structure. It is composed by a short variable sequence following the stop codon of the ORF, a poly(U) tract of heterogeneous length, as well as a highly conserved 98 nucleotide sequence (designed as X-tail) [8]. An additional cis-acting RNA element was identified in the C-terminal region of NS5B that forms a long distance RNA-RNA interaction with the X-tail and it is essential for HCV RNA replication [75].

3.1.2. Structural proteins

The core protein is the first structural protein encoded by HCV. It is an RNA-binding protein that forms the viral nucleocapside [58]. The core protein binds viral RNA, regulates HCV RNA translation and interacts with the glycoproteins to generate a complete virion. Besides these functions, it has also been reported to interact with cellular proteins altering host cell functions [144, 187, 217]. Moreover, core has been involved in cell signaling, apoptosis, carcinogenesis and lipid metabolism. Since most of these findings were obtained in the context of an overexpression of core protein, it is yet unclear whether these interactions occur in the course of a normal infection [217].

The E1 and E2 envelope glycoproteins are essential for virus entry. They form a heterodimer that will be exposed in the surface of HCV particles and is therefore the obvious candidate ligand for cellular receptors. Because of this exposure, the envelope glycoproteins are targets of neutralizing antibodies. In addition, they participate in the assembly of the virion [68, 85, 90].

3.1.3. Non-structural proteins

The p7 polypeptide is not required for RNA replication but is fundamental for HCV infection *in vivo* [193]. In addition, it has been shown to form oligomers and to have an ion channel activity [88, 170].

The NS2 protein is a non-glycosylated integral membrane protein. Before cleavage from the polyprotein, NS2 participates in a protease activity responsible for the cleavage at NS2-NS3 junction together with the first 180 residues of NS3 [58]. It is described that this protein is not essential for the formation of the replication complex [22, 133], but is required for the complete replication cycle *in vitro* and *in vivo* [174, 228, 238].

NS3 is a bifunctional protein that carries in the N-terminal a serine-type protease domain and in the C-terminal a helicase/NTPase domain. Despite its proteolytic activity, the NS4A cofactor enhances its cleavage and stabilizes the protease to avoid proteolytic degradation. The NS3-4A resulting protease is responsible for the polyprotein cleavage in the region downstream NS3, generating the components of the viral RNA replication complex [58]. Moreover, the NS3-4A protease also has a role in blocking the ability of the host cell to develop an innate antiviral response [72, 147]. Thus, it is not surprising that this protease had been targeted for the design of specific inhibitors as a novel antiviral treatment strategy [13, 99]. The C-terminal region of NS3 encodes for the RNA helicase DedxH/D-box. Enzymes within this family can unwind double-stranded RNA or single-stranded RNA regions with high secondary structures in an ATP-dependent manner. In the particular case of NS3 it has been observed that monomeric NS3 can bind RNA, although RNA unwinding requires an NS3 dimer [151]. The role that NS3 helicase activity has in the HCV life cycle is not clear, although it has been suggested to cooperate in initiation of RNA replication by unwinding secondary structures, displacing bound proteins that could be interfering with RNA synthesis or even participate in the dissociation of the replicative form [58].

NS4B is a highly hydrophobic non-structural protein detected in association to the endoplasmic reticulum (ER) membranes [96]. It induces intracellular membrane alterations, arguing that the main function of this viral protein is the formation of membranous structures that will support HCV replication [60]. Nevertheless, the structure of the membranous web observed when all HCV proteins are expressed is slightly different from the NS4B-induced membranes, indicating that other viral and/or cellular components participate in this process [8].

NS5A protein can be found in multiple phosphorylated forms, a conserved characteristic in NS5 proteins from hepaciviruses, pestiviruses and flaviviruses [188]. In fact, cell culture adaptive mutations affect residues that are required for hyperphosphorilation, indicating that the phosphorylation state of NS5A is crucial for the correct HCV replication [67]. Moreover, the N-terminal region of NS5A contains an α-helix that anchors NS5A to membranes. This helix has been involved in protein-protein interactions, which in turn are required for the formation of the HCV replication complex [171]. Besides the α-helix, NS5A is composed of three domains. Domain I appears to be involved in RNA binding and domain II has been suggested to inhibit the IFN-induced dsRNA activated protein kinase PKR, while domain III is not well characterized [79, 95].

The NS5B protein is the catalytic center of the HCV replication complex. Its catalytic domain is associated with membranes through a C-terminal transmembrane domain, which is essential for HCV RNA replication in cell culture [150]. The crystal structure of this protein showed the classical shape of a right hand present in other RdRp with palm, finger and thumb domains [27, 127]. It is important to note that the RdRp activity is modulated by the interaction with other viral proteins as well as with cellular proteins, like cyclophilins [17, 227].

The HCV genome has an alternative reading frame that overlaps the sequence of core protein and codifies for a 160 amino acids protein named alternative reading frame protein (ARFP) [25]. Although the ARFP is not required for HCV RNA replication no *in vitro* neither *in vivo* [145], the development of immune response against ARFP in HCV infected patients indicates that this protein is expressed during HCV infection [14]. The function and relevance of ARFP in the HCV life cycle and pathogenesis is not established.

3.2. Viral life cycle

Impressive advances in understanding the HCV life cycle have been achieved over the last years. This progress includes the identification of HFs required for HCV entry into the cell, the definition of the viral multiplication requirements, and the

characterization of cellular compartments necessary for particle assembly and progeny virus production [16].

Attaching and viral entry. HCV infection starts with the virus binding to the hepatocytes, which are the main target cells. Nevertheless, infection of B cells, dendritic cells and other cell types by HCV have been reported [151]. The envelope glycoproteins E1-E2 establish an initial attachment with the glucosaminoglycans (GAGs) and low-density lipoprotein receptors (LDLR) present in the cell surface. After this initial engagement, the particle interacts with the scavenger receptor BI (SR-BI) and the CD81 tetraspanin in the basolateral surface of the hepatocyte to finally move to tight junctions formed by Claudin-1 (CLDN-1) and Occludin (OCLN) (Figure 7). Recently, a potential role for Niemann-Pick-like 1 cholesterol absorption receptor (NPC1L1) and CD229 in HCV entry and attachment has been reported [180]. After the attachment to the surface, HCV enters to the cell by clathrin-mediated endocytosis which appears to happen in a pH-dependent manner, as the low pH facilitates the fusion of viral and endosomal membranes for the subsequent uncoating.

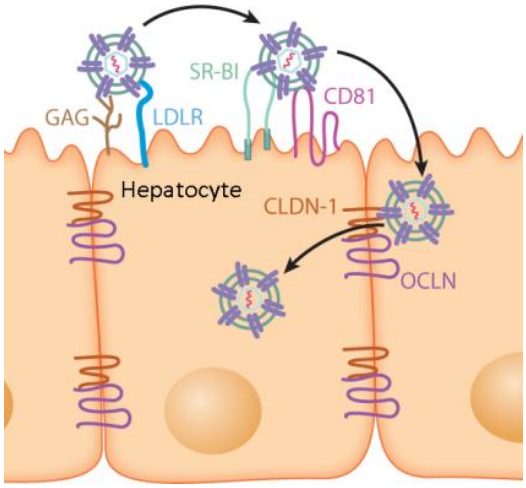

Figure 7. HCV attaching and entry into the hepatocyte. HCV entry is a multi-step process that requires several interactions between viral envelope proteins and cellular receptors. Modified from Rice *et al*, Topics in Antiviral Medicine 2011.

Translation and replication. Like other (+)RNA viruses, the HCV life cycle is characterized by the virion release of a messenger-sense genomic RNA into the cytoplasm. Due to its mRNA nature, the viral genome is used for translation but also as a template for viral replication. Thus, regulation between translation and replication is a key step that has to be highly controlled [2]. Once the viral RNA is released into the cytoplasm, it is directly translated through the IRES located in the 5'NTR. This step takes place in the ER and results in the production of a 3,000 amino acids polyprotein precursor that will be processed by cellular and viral proteases in the 10 structural and non-structural proteins mentioned above [151]. Then, the viral genome will be translationally repressed and recruited for replication in membranous replication

vesicles named membranous web. These structures are formed inside infected cells due to the action of NS4B together with NS5A. It is in these structures where the RdRp will amplify the viral RNA with the support of the replication complex formed by viral NS3, NS4A, NS4B and NS5A proteins and some HFs. Once there, the (+)RNA genome is copied into a negative-strand intermediate, which will be used to synthesize positive-strand RNAs. New generated genomes will go under subsequent rounds of translation, RNA replication or will be packed into virus particles [16].

Viral assembly and exit. The lasts steps of the viral life cycle take place close to cellular organelles named lipid droplets (LDs), where high amounts of core proteins are accumulated. These organelles have and outer phospholipid monolayer derived from ER membranes and are involved in lipid mobility and storage [157]. Although LDs often are localizing close to ER, where they are used as a source for lipids, they dynamically move through the cytoplasm and interact with other organelles. Due to this proximity to ER replication sites, it has been suggested that LDs could facilitate the concentration of core and the other viral components to induce efficient viral assembly [24] (Figure 8).Interestingly, a recent publication suggests that during virus assembly core protein is recruited from the surface of LDs into small transport vesicles, which may contain vial particles or might represent a core-containing intermediate, that will be directed to ER [42]. Following the capsid assembly, new virions bud into the lumen of the ER where E1/E2 glycoproteins and the low-density lipoprotein (LDL) reside [39]. It is important to note that capsid formation is intimately linked to LDL synthesis, which will explain the LDL-like composition of HCV particles and the use of lipoprotein production pathway to exit the cell [16].

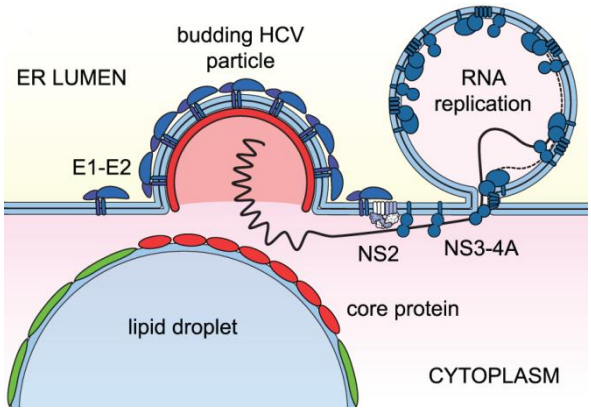

Figure 8. Model of HCV assembly. To complete HCV particle assembly core must be recruited from the surface of LDs and viral RNA from the replication complexes. While this is happening, the new HCV particle buds into the ER. Adapted from Counihan *et al*, Plos Pathogens 2011.

3.3. Model systems to study HCV

To study the different pathways involved in HCV pathogenesis, as well as to understand the viral life cycle, it is essential to have appropriate model systems. It was almost a decade after the HCV discovery when the molecular cloning of the first HCV genome was described [47]. However, for long HCV could not be replicated in cultured cells. Without a reliable cell culture system, *in vitro* transcripts were inoculated into the livers of chimpanzees, establishing in these animals a productive infection [18]. Major advances had been achieved in the last 10 years to establish the desired cell culture systems.

3.3.1. HCV subgenomic and genomic replicon systems

A major breakthrough in the development of cell-based systems was the development of self-replicating HCV mini-genomes, named replicons [133]. In the initial version they were derived from a genotype 1b HCV isolate in which the structural proteins and some non-structural proteins were replaced by the selectable marker neomycin phosphotransferase (Figure 9). Transfection of the RNA replicon into the human hepatoma cell line Huh7 in the presence of the cytotoxic drug G418 resulted in a low frequency of neomycin-resistant colonies harboring HCV RNA. The accumulation of cell culture adaptive mutations together with the selection of permissive Huh7 cell clones dramatically increased RNA replication levels [18]. After reporting the existence of functional genotype 1b replicons, further research was done to establish replicons for genotypes 1a and 2a. In addition, derivatives that express different reporter genes and even full-length replicons have been constructed [151]. However, the full-length HCV replicons failed in producing infectious particles, suggesting that the adaptive mutations that increase RNA replication are deleterious for particle assembly or release [176].

The main limitation of HCV replicon systems was that only allowed the study of intracellular steps of the viral replication cycle, excluding the assembly and release of HCV infectious particles. Despite this drawback, the use of replicon systems provided the first cell culture model for the screening of antiviral drugs targeting HCV RNA

replication. Moreover it has been exploited to study interactions between viral and host components and to characterize the viral replication complex.

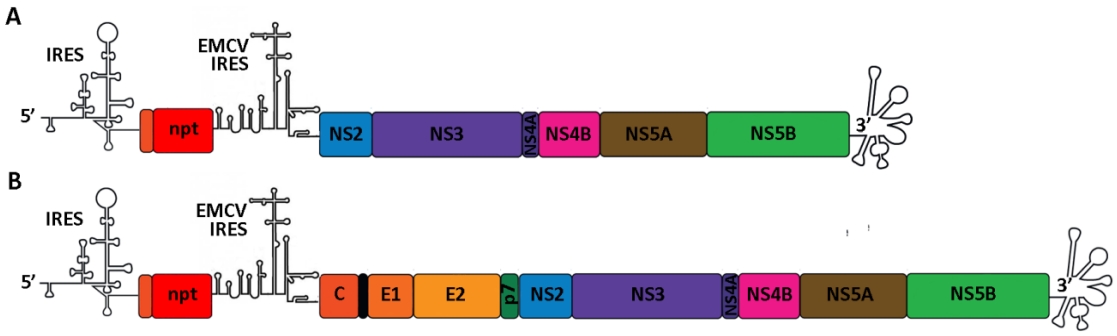

Figure 9. Subgenomic and genomic HCV replicons. (A) In the subgenomic replicon the region encoding for the structural proteins has been replaced by core-neomycin phosphotransferase (npt) fusion protein, followed by the IRES of the encephalomyocarditis virus (EMCV). (B) The HCV genomic replicon also contains the selectable npt gene but codifies for all HCV proteins.

3.3.2. HCVcc

Efficient production of infectious HCV in cell culture was achieved in 2005 with the identification of a genotype 2a isolate that was cloned from a Japanese patient with acute fulminant HCV infection [225]. This isolated, designated as JFH-1 (Japanese fulminant hepatitis 1), can initiate efficient replication in hepatoma cells without needing adaptive mutations. This new HCVcc (cell culture) system produced viral particles that not only infected naïve hepatoma cells, but also chimpanzees and immunodeficient mice with partially human livers [131, 225]. The efficiency of this system was improved with the generation of a chimera in which the region from core to the end of NS2 of JFH-1 was replaced by the analogous coding region of another genotype 2a isolated, the J6, resulting in the chimera Jc1 [175]. These new HCVcc systems solved the gaps of the replicon system, allowing 20 years after the discovery of the virus to reproduce all steps of the HCV life cycle in the laboratory.

3.3.3. Animal models

Development of animal models is fundamental to advance in clinical studies of HCV infection, as well as for the improvement of novel therapeutic and preventive strategies. Unfortunately, the natural species tropism of HCV is limited to humans and

chimpanzees [120]. Chimpanzees can be infected with isolates of the 6 HCV genotypes and have innate and adaptive immune responses similar to those observed in infected humans [31, 32]. However, the high cost, the restricted availability and the ethical concerns make difficult to support these animals as models for HCV research [31]. Since the discovery that tree shrew, close relatives of primates, can be infected with HCV and develop not only a persistent infection but also an evidence of chronic liver disease, they have been proposed as a model for HCV infection [233]. It is worth to notice that the low and variable infection rates and HCV titers achieved in tree shrew experiments have not contributed to the extensive use of these animals [30].

Two approaches have been proposed to establish the long-awaited small animal model. First, HCV needs to be genetically modified in order to infect non-human cells. Some reports described that after adapting the virus to the murine environment, HCV entry and replication can occur [21, 179]. However, it is still unclear which additional viral modifications will be required to complete all HCV life cycle in mice cells. Second, murine models can be humanized either by xenotransplantation of human tissues or by genetic manipulation [179]. Chimeric mice with a HCV-permissive tissue can be obtained through a transplant of human hepatocytes into mice with liver injury and severe immunodeficiency [146]. The urokinase-type plasminogen activator (uPA) transgenic mouse is the most used rodent with this purpose. In this animal, the overexpression of the transgene is hepatotoxic resulting in a mouse with liver damage. Thus, the transplant of non-transgenic human hepatocytes can rescue the phenotype and allows HCV infection. Since those chimeric mice do not have a functional immune system, pathogenesis and immunity studies cannot be approached using this model. Many efforts have been done during last years to obtain a humanized liver model in mice that also harbor a human haematolymphoid system [126]. However, the genetic manipulation of mice to achieve a successful HCV infection is not so obvious. It will require numerous genetic adaptations to humanize the host, mainly due to the limited capacity of murine cells to support HCV life cycle [179]. Although major advances have been achieved in the identification of human HFs required for HCV entry, the list of HFs implicated in HCV replication is still increasing, which in turn hampers the choice of murine factors to be manipulated.

4. HEPATITIS C VIRUS AND HOST FACTORS

4.1. Identification of host factors affecting HCV infection

Major efforts have been carried out during the last years to identify HFs involved in the HCV life cycle. Their identification is essential to understand HCV biology and might also provide novel antiviral targets. To identify such HFs two main strategies have been followed. First, the exhaustive study of specific individual HFs that were chosen based on i) their ability to physically interact with HCV components, such as cyclophilins or miRNA122, or ii) their already described function on other (+)RNA viruses, such as the components of the cellular mRNA decay pathway that were first identified to affect the plant (+)RNA virus brome mosaic virus (BMV) [103, 167, 203]. Second, genome wide-screenings using the RNA interference (RNAi) technology via short-interference RNAs (siRNAs). These are small synthetic dsRNAs, directly delivered by transfection into the cytoplasm of human cells that act through the RNAi pathway to knockdown the complementary targeted cellular or viral mRNAs. As a result, the corresponding encoded protein is depleted. However, silencing conditions need to be carefully optimized and controlled in order to avoid false-negative results due to low transfection efficiency, and false-positive results due to cell toxicity or non-specific binding of the siRNA (off-target effects) [59, 207]. Using this technology, genome-wide RNAi-based approaches have been carried out to promote the identification of HCV co-factors [186, 211, 213]. Among the HFs identified are proteins involved in multiple cellular pathways such as RNA metabolism, intravesicular trafficking, membrane biogenesis, lipid transport or immune response.

4.2. Cellular mRNA decay factors in the HCV life cycle

A key cellular pathway required for the replication of HCV and other (+)RNA viruses is the 5'-3' deadenylation-dependent mRNA decay pathway. It is one of the main pathways of mRNA decay and is highly conserved from yeast to humans. In this pathway, decapping occurs in a two-step model (Figure 10). First, since mRNA translation and mRNA 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. Although this first step is necessary for decapping, it does not inevitably lead to mRNA decay and the mRNA can stably remain in a translationally-repressed state. In a second step of the decapping process, the decapping complex (Dcp1/Dcp2) removes the 5'cap and the mRNA is degraded via the 5'-3'-exonuclease Xrn1 [73].

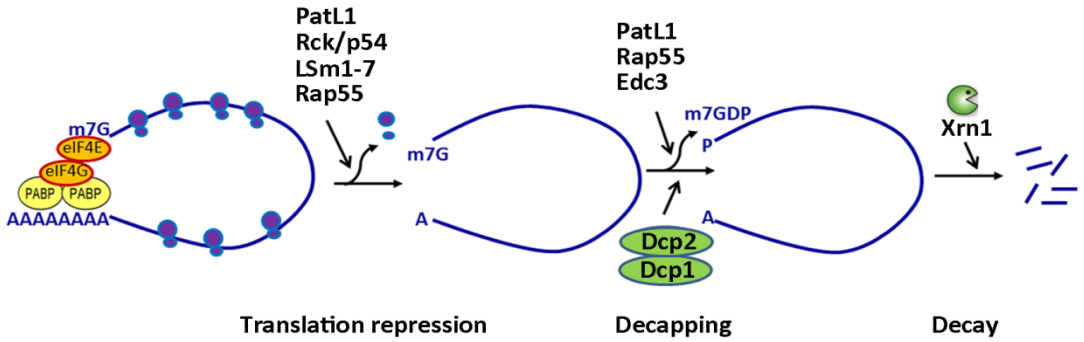

Figure 10. mRNA decapping occurs in two steps. A conserved set of decapping activators repress translation and allow the access of the decapping enzyme to the mRNA to remove the cap. Finally, the decapped mRNA is degradated by Xrn1.

A conserved set of proteins, referred as decapping activators, favor the transition of cellular mRNAs from translation to decapping by accelerating translation repression or by acting directly on the decapping enzyme [50]. The decapping activators DDX6 (also named as Rck/p54), PatL1, LSm1-7 and Rap55 favor decapping by promoting mRNA translation repression. Interestingly, PatL1 and Rap55 seem to have a key role since they promote decapping by both mechanisms. DDX6 is a member of the family DEAD-box helicases that are involved in splicing, ribosome biogenesis, RNA transport, degradation and translation repression, although the exact contribution to most of these processes is mainly unknown. The role of DDX6 in translation repression is suggested to be through its ATPase activity that allows the release of eIF4E from the mRNA, repressing translation at the initiation step and fomenting decapping [73]. Moreover, a recent publication suggests that Dhh1, the homologous in yeast of DDX6, also promotes translation repression by slowing the ribosome movement on mRNA [212]. PatL1 biochemical properties are mostly unknown, but their activity in blocking translation has been proved *in vitro.* Moreover, the different domains of PatL1 interact

with multiple decapping activators such as LSm1-7, DDX6 or Edc3. Due to these multiple interactions, PatL1 has been proposed to act as a scaffold protein [26, 166]. The LSm1-7 complex is composed by seven LSm proteins (LSm1 to LSm7) and forms a ring structure suitable for RNA binding. Curiously, the family of LSm proteins is normally found in hexameric or heptameric complexes *in vivo* and are involved in RNA-related functions. LSm1-7 together with PatL1 associates with the 3' end of mRNAs after deadenylation and have been proposed to promote translation repression by limiting the interaction of translation initiation factors with the mRNA and, in consequence, facilitating the access of the decapping enzyme [48, 218, 219]. However, there are some LSm proteins that do not form hexameric or heptameric complexes. This is the situation of Rap55, which contains an LSm domain and belongs to the LSm14 protein family. It was reported that Rap55 is an RNA-binding component that can repress translation *in vitro* and *in vivo* [215]. Recently, it was described that this repression is mediated by the direct binding of Rap55 to eIF4G and the corresponding inhibition of the joining of the 43S complex [184]. In summary, all proposed mechanisms of action of the decapping activators that promote translation repression are related to effects on the translation initiation step.

Regarding the other set of decapping activators, Dcp1 and Edc3 favor decapping by directly activating the decapping enzyme. Dcp1 is the decapping enzyme subunit that together with Dcp2 forms the decapping complex. Thus, the formation of this complex is essential for the correct removal of the 5'cap structure. The Edc3 protein, like Rap55, contains an LSm domain and belongs to the LSm16 class. Interestingly, Edc3 acts as an scaffold protein that directly interacts, among others, with the decapping enzyme Dcp2 suggesting that it would play a major role in either recruit or activate the decapping complex [51, 221]. Furthermore, recent publications have reported a dual role for PatL1 and Rap55 demonstrating that these two proteins also promote decapping through the activation of the decapping enzyme. Both of them directly interact with Dcp2, and for Rap55 has been proved that stimulates decapping *in vitro* [77, 163]. Recently, our group reported that HCV RNA translation and replication depend on the decapping activators PatL1, LSm1 and DDX6, while the decapping enzyme Dcp2 and the exonuclease Xrn1 do not affect HCV infection. This requirement

was functionally linked to the 5' and 3'NTR regions of the viral genome where the LSm1-7 complex directly interacts [195]. These findings suggest that HCV hijacks the translation repression step that accelerates decapping rather than the whole 5'-3' degradation pathway. Interestingly, some of these decapping activators were described to also influence the replication of other (+)RNA viruses, such as the human dengue virus (DENV) [226], the animal flock house virus (unpublished data, Giménez-Barcons and Díez), the plant BMV [53, 140, 165]and the bacteriophage Qβ [74]. Thus, the dependence of (+)RNA viruses infecting different kingdoms on HFs involved in mRNA decay suggests that they have found a robust solution to promote viral propagation that is evolutionary conserved. The question that arises is why HFs that promote translation repression in mRNA can enhance the translation and replication of HCV and other (+)RNA viruses. While the exact mechanism by which these set of proteins promote HCV translation is not well understood, we can speculate with their role in viral replication (Figure 11). Since all (+)RNA viruses replicate through an mRNA intermediate that is also used for the translation of viral proteins, a key common step to all (+)RNA viruses is the specific recruitment of viral RNA from the cellular translation machinery to the membrane-associated replication complex. As these two steps are antagonistic, and cannot happen at the same time, the exit from translation to replication requires translation repression. For BMV it has been demonstrated that Pat1, LSm1 and Dhh1 promote replication by favoring the recruitment step. Thus, given the role of these proteins in cellular translation repression it is likely that viruses hijack this function. Interestingly, a new role for DDX6 in encapsidation has been determined in DENV, a (+)RNA viral life cycle step that also requires translation repression. The conserved use of these

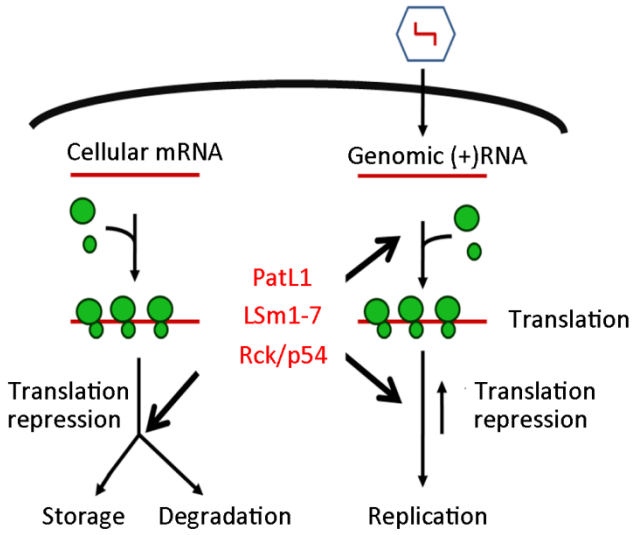

Figure 11. The fate of cellular mRNA and viral genomic (+)RNA has common traits. The same host proteins involved in the shift of cellular mRNA translation to storage or decay also regulate the switch of (+)RNA viral genomes from translation to replication.

proteins by multiple (+)RNA viruses, together with the lack of toxicity in human cells when these HFs are depleted point to PatL1, LSm1 and DDX6 as possible novel targets for novel broad-spectrum antiviral drugs.

4.3. Processing bodies

Cellular mRNAs associate with proteins in messenger ribonucleoprotein complexes (mRNPs), which composition dictates the fate of mRNAs. In eukaryotic cells it has been described that translationally inactive mRNPs can accumulate into cytoplasmic mRNP granules. Among them, the best-characterized granules are processing bodies (P-bodies), associated with mRNA decay, and stress granules (SGs), that contain mRNA stalled at translation initiation step [62] (Figure 12). P-bodies are discrete and highly dynamic granules present in the cytoplasm of eukaryotic cells under normal growth conditions, although their size and number increase under stress conditions. In contrast, SGs are only present under a stress situation. Although P-bodies and SGs share some components, both granules contain some characteristic markers [62, 84]. The HFs PatL1, LSm1 and DDX6, required for efficient HCV multiplication, localize in P-bodies together with translationally repressed mRNAs [100, 195].

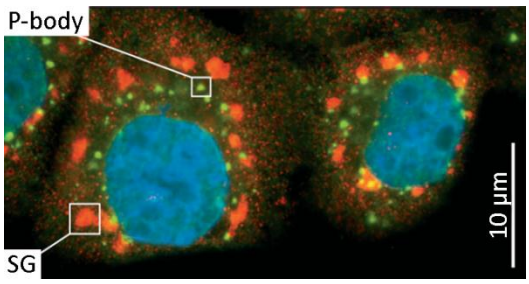

Figure 12. P-bodies and SGs appearance. P-bodies (green) are discrete and smaller than SGs (orange).

4.3.1. P-body composition and formation

P-bodies contain translationally repressed mRNAs together with a conserved core of proteins involved in mRNA decay pathways, including the 5'-3'deadenylation-dependent mRNA decay. These components comprise the decapping complex Dcp1/Dcp2, the exonuclease Xrn1, and the Ccr4/Pop2/Not deadenylase complex. Also factors that participate in the translation repression and decapping activation, such as DDX6, PatL1, LSm1-7, Rap55 and Edc3, are included as characteristic markers of P-bodies [50]. In addition, components of the nonsense-mediated mRNA decay (NMD),

of the AU-rich element (ARE)-mediated mRNA decay and of the miRNA repression pathway have also been described to localize in P-bodies [65, 130]. In contrast to SGs, ribosomal subunits and translation initiation factors have not been detected in these foci, indicating that mRNPs must be free of ribosomes prior to its assembly in P-bodies. Consequently, P-bodies are dispersed by drugs that stabilize polysomes, like cyclohexamide, but their number and size are increased by drugs that avoid ribosome association with mRNA, like puromycin [40, 64, 216]. However, not all P-bodies have the same composition and concentration of components. This leads to a heterogeneous population of foci that hampers its characterization. Despite their well-studied composition, the internal organization of the foci was unclear. A recent publication revealed for the first time an internal bi-compartimentalization of P-bodies, describing the existence of a dense central core where the enzymes Xrn1 and Dcp1 accumulate surrounded by DDX6 [41]. These data suggests that the peripheral area of the foci is dedicated to the anchoring of repressed mRNAs and the core to their decay.

The mechanisms involved in P-body assembly are not well-understood. Once mRNA is free of ribosomes and in complex with P-body components, specific dimerization domains present in these proteins facilitate the aggregation of individual mRNPs into large structures. The available studies that approach the process of P-body assembly have been done in yeast, Drosophila and humans. It is important to note that the literature in the subject is sometimes confusing, as not always is properly indicated from which biological system the results are obtained. Studies in yeast revealed that LSm4, a subunit of the LSm1-7 complex, and the decapping activator Edc3 are fundamental components for P-body assembly. LSm4 contains prion-like glutamine and asparagine (Q/N)-rich regions, the absence of these regions promotes a decrease in P-body size and number [51]. In fact, a study demonstrated that more than 20 yeast P-body components have a higher Q/N content [189]. Edc3 has an homodimerization domain in the C terminal region and it also acts as a scaffold protein through the interaction with many P-body components, such as Dcp2 and Dhh1 (the yeast homolog of DDX6), being essential for P-body formation in yeast [51, 132, 163]. In addition, PatL1 also appears to have a scaffold function, since in yeast and human it has been

demonstrated to interact with many P-body components at the same time that is essential for P-body nucleation [26, 163, 166, 177]. Importantly, not all results observed in yeast are extrapolated to metazoans. For instance, Q/N-rich regions are not present in most metazoan LSm4 proteins. In contrast, although the Edc3 homodimerization domain is conserved in higher eukaryotes [132], Edc3 depletion does not affect P-body assembly, suggesting that additional mechanisms modulate the foci formation in metazoans [64]. Finally, DDX6 was reported to be essential for P-body aggregation in higher eukaryotes, since it is the only P-body component which depletion leads to P-body disruption and even in the presence of arsenite treatment avoid the formation of these granules [148, 201].

P-bodies are highly dynamic structures. Once foci are formed, mRNPs can enter and exit from P-bodies in a reversible way. This dynamic property is well defined, since the number and size of these granules changes throughout the cell cycle, being almost absent in mitotic mammals cells [236]. This reversible localization of mRNAs in P-bodies indicates that mRNAs associated with components from the decay machinery can be degraded, remain in the foci for mRNA storage, or exit the P-body to continue with translation [168]. How the fate of the mRNP is decided is still unclear, although it is probably determined by cellular and environmental conditions. Interestingly, a recent publication in yeast reported that the vast majority of mRNAs that are released from polysomes are degraded and not stored in the cell. In addition, only a limited subgroup of mRNAs has the availability to return to translation [12]. Thus, these findings contradicted the common believe that most translationally repressed mRNAs can move from P-bodies and polysomes. Regarding to the proteins that accumulate in these foci, fluorescence recovery acceptor-photobleaching (FRAP) studies demonstrated that P-body components rapidly cycle in and out of P-bodies [3, 6, 78, 109]. However, different kinetics are observed among P-body components. For instance, Dcp1 rapidly exchanges between P-bodies and the cytosol, whereas Dcp2 is more static. This differential kinetic supports that P-bodies contain a core structure with components that do not rapidly exchange with the cytoplasmic pool.

4.3.2. P-body function

The presence of P-bodies in a wide range of organisms from yeast to humans suggests an essential function for these foci. However, this function remains unclear. Studies in yeast demonstrated that P-bodies are not required for decapping, nor for mRNA stability during stress or for translation repression also under stress [28, 51]. Moreover, mRNA degradation and miRNA mediated translation repression is not affected after P-bodies depletion in metazoans [64, 210]. Thus, P-bodies are not essential for the global control of translation repression and mRNA degradation. However, the formation of these foci might affect the translation and/or degradation of a limited subset of mRNAs or might be important under certain conditions [50].

Currently, there are three possible functions that are being considered. First, P-bodies could be limiting the availability of free decay enzymes in the cytoplasm and preventing inappropriate mRNA degradation. Second, they could be concentrating enzymes and factors to optimize the process. And third, they could be avoiding that repressed mRNPs compete for the translation machinery [62]. The alteration of P-body formation under certain conditions, like a cellular stress or a viral infection, may have consequences *in vivo* beyond viral multiplication. Interestingly, a study in mice suggested that P-body disruption is correlated with and upregulation of global translation and inflammatory processes [242]. Furthermore, the recent work published by Ruggieri *et al.* demonstrated a correlation between modulation in SGs disruption and cell survival under HCV infection conditions [191]. A similar role might be possible for P-body given the intimate link between these two kinds of foci. In summary, unraveling P-bodies function is a fundamental question that remains unanswered, being currently the major challenge in this field.

4.3.3. P-bodies and viruses

Viruses interact with the host machinery affecting translation, localization and degradation of cellular mRNAs. Thus, is not surprising that multiple P-body components are involved in viral life cycles. In addition, it is predicted that those viruses that have multi-functional genomes utilized as messenger RNAs for translation,

as templates for replication and as genomic RNAs for virus assembly, establish higher dependency on such HFs, as they might need them to tightly segregate these steps [19]. In line with this prediction, multiple (+)RNA viruses and retroviruses depend on P-body components for their expansion. During the last years many studies have reported such dependency in HCV [9, 10, 71, 100, 186, 195], poliomyelitis virus [198, 208], DENV [226], HIV-1 [4, 44, 97, 137, 138] and foamy virus (FV) [240]. Importantly, not always the required P-body component had a positive effect in the propagation of the virus. DDX6 is essential for HCV replication, while in HIV-1 negatively regulates viral gene expression by avoiding viral mRNA association with polysomes [44]. In addition, it was believed that HFs could also affect viral RNA recombination, a major driving force in virus evolution [159]. A genome-wide screening in a yeast system infected with the (+)RNA virus tomato bushy stunt virus (TBSV) identified for the first time HFs affecting RNA recombination [203]. Interestingly, many of these HFs participated in the 5'-3' deadenylation-dependent mRNA decay pathway and localized in P-bodies. Among them the exonuclease Xrn1 was found to reduce the frequency of TBSV recombination by degrading RNA recombination intermediates. These results open the exciting possibility that the host can directly influence viral evolution. Currently, it is unknown whether the recombination of other (+)RNA viruses could be influenced by such HFs.

Although many studies have demonstrated the association between P-body components and viral life cycles, the interaction of P-body structures with viruses remained unclear. The first study that approached this question demonstrated that Flaviviruses DENV and West Nile virus (WNV) interfered with P-body formation [61]. More recently, it was shown that P-bodies were also disrupted under poliovirus (PV) infection [55]. Curiously, only for PV has been described that the degradation of P-body components by the viral protease is responsible for such effect. However, other viruses, such as FV, caused an alteration in P-body composition rather than foci disruption [114, 240]. Nevertheless, the implications of P-body alteration for viral life cycles are unknown. When this thesis started there were almost no studies on P-body structures and virus infection, and non for HCV. Although it was known that HCV depends on specific P-body components, it was unexplored whether this dependency

was linked to their localization in P-body foci and whether HCV infection causes changes in P-body formation and composition.

OBJECTIVES

Some P-body components such as the cellular translation repressors PatL1, LSm1 and DDX6 promote the translation and replication of HCV and other (+)RNA viruses. However, whether P-body granules themselves play a role in (+)RNA life cycles remained elusive. In addition, the P-body component Xrn1, an exonuclease, has been shown to influence the evolution rate of the (+)RNA plant virus TBSV by decreasing its recombination frequency. Nevertheless, it was unknown whether this could be extrapolated to other (+)RNA viruses such as HCV. The aim of this thesis has been to further define the interface of P-bodies and HCV biology. This will contribute to a better understanding of the HCV life cycle.

The specific aims of this thesis are:

1. To study whether HCV infection affects the formation of P-body granules.
2. To determine whether P-body granules are required for HCV infection.
3. To establish a selectable HCV replicon-based recombination detection system in cell culture that allows to estimate HCV recombination frequencies.
4. To use the established HCV-replicon-based recombination system to explore the role of P-body components in HCV recombination.

RESULTS

In this section scientific results are presented in two parts, both of them containing research papers and appendices. These appendices include additional data obtained after the publication of the manuscripts during this thesis. This information is relevant for future studies and complements the data presented in the papers.

Part I: Determine the importance of P-body structures for HCV infection and the alterations that the virus causes on these foci.

Publication I: HCV infections alters P-body composition but is independent of P-body granules

Appendices

Part II: Establish a selectable, replicon-based recombination detection system to study HCV recombination events in cell culture and evaluate the possible involvement of P-body components in this mechanism.

Publication II: Hepatitis C virus RNA recombination in cell culture

Appendix

1. Part I: Determine the importance of P-body structures for HCV infection and the alterations that the virus causes on these foci.

1.1. Publication I

HCV infection alters P-body composition but is independent of P-body granules

Gemma Pérez-Vilaró*, Nicoletta Scheller*, Verónica Saludes and Juana Díez

Journal of Virology, Volume 86, Issue 16, 8740-8749, August 2012

Previous work in our lab proved that the P-body components PatL1, LSm1 and DDX6 are required for HCV replication. However, there was no information about the relevance of the localization of these proteins in P-bodies as well as the requirement of P-body formation for HCV infection. By using the HCV virus Jc1, and also a 2a subgenomic replicon, we proved that PatL1, LSm1 and DDX6 do not localize in P-bodies under HCV infection. In addition, the disruption of these granules do not affect the HCV life cycle. I was the main driven force of this work and actively participated in the design, execution and discussion of the experiments.

*These authors contributed equally to this work

1.2. Appendix I

HCV disrupts P-bodies at advanced time of infection

Previously, by using the HCV virus Jc1 we demonstrated that at 96 hours post-infection P-body components PatL1, LSm1 and DDX6 did not localize in these P-body foci. Interestingly, Dcp1 was not affected by the infection, suggesting that HCV alters the localization of HFs required for its propagation. Similar results were obtained with a 2a subgenomic replicon, although the effect on P-body alteration was observed at 48h post-electroporation (Publication I). These results were unexpected since it was widely accepted in the P-body research field that PatL1, LSm1-7 and DDX6 are core proteins required for P-body formation [62, 196]. Thus, their absence in P-bodies should promote P-body disruption. In addition, many studies have reported that P-body components are in dynamic exchange between the cytosol and the foci, being some components more static than others [62]. One possibility is that HCV infection alters this exchange and, as a consequence, the dynamic of P-body disruption could be affected. If so, we reasoned that at advanced time of infection, when HCV infection is chronically established, all components localization might be affected.

To explore this possibility Huh7.5 cells were infected with HCV and then immunostained with antibodies against DDX6 and Dcp1 at 8 days post-infection. P-body number was analyzed using ImageJ particle-counting software as described in Publication I. In contrast to the results obtained at 96 hours post- infection, at 8 days the abundance of P-bodies that contained both DDX6 and Dcp1 were reduced by 55% and 33% relative to that in non-infected cells, respectively. Such alteration did not correlate with higher titers (Figure S1 and S2). A similar result was obtained when the effect of HCV replication was analyzed at advanced times post-electroporation. Huh7.5 cells were electroporated with the HCV replicon, and P-bodies were analyzed at 96 hours post-electroporation. P-bodies were immunostained for DDX6 and Dcp1 and quantified in NS5A-positive cells. In contrast to the results obtained at 48 hours post-electroporation, replicon-containing cells showed a statistically significant decrease of both DDX6- and Dcp1-conatining P-bodies (Figure S3). Together these results strongly suggest that at advanced times of HCV infection the localization of all studied P-body components is affected, although a higher effect is observed for some factors.

These observations, together with the initial alteration of P-body composition (Figure 1 and 7 in Publication I), suggest that HCV modulates P-bodies in a two-step process. First, PatL1, LSm1 and DDX6, P-body components required for HCV replication, decrease their localization in these foci. Since these proteins play a key role in P-body formation, in a second step the low availability in the cytoplasm of free PatL1, LSm1-7 and DDX6 would limit P-body aggregation and the global amount of P-body per cell would be decreased. These findings challenge the current accepted mechanism for P-body formation.

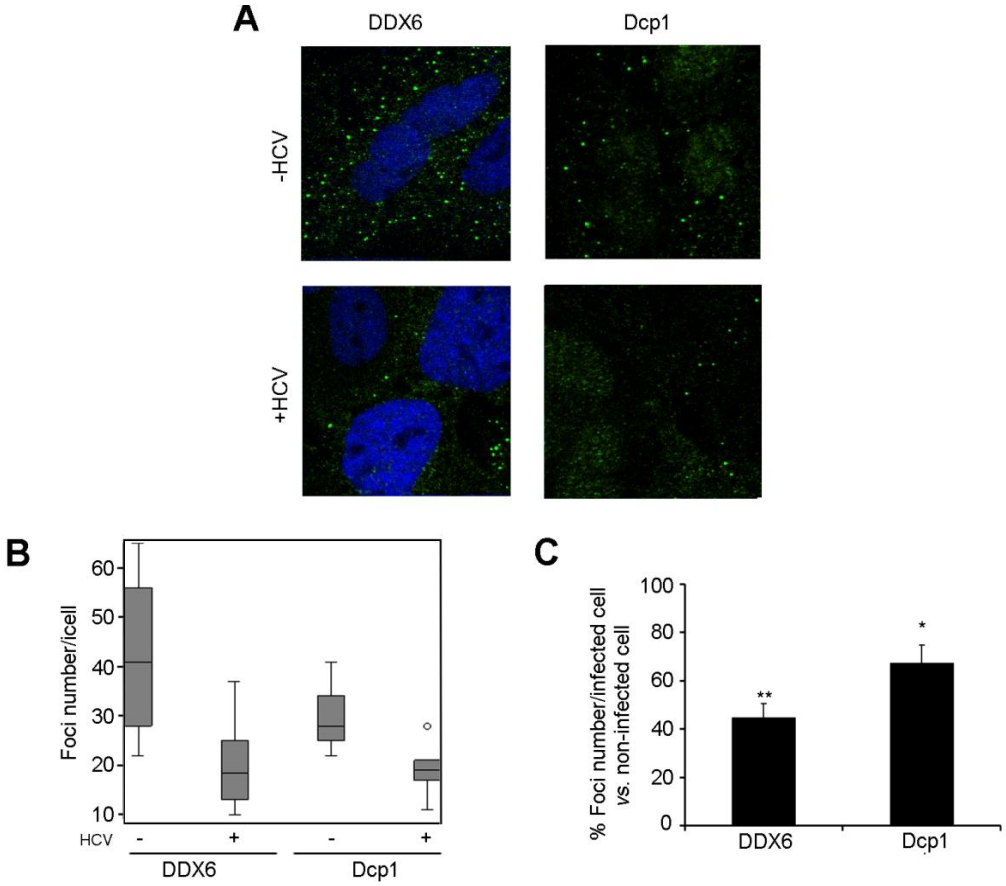

Figure S1. HCV disrupts P-bodies at advanced time of infection. (A) Huh7.5 cells were infected for 8 days with HCV and immunostained with antibodies for DDX6 and Dcp1 (green). Nuclei were visualized using TROPRO-3 (blue). (B) P-body number was quantified using at least 100 cells. Graphed are box plots showing medians, upper and lower quartiles, and outliers. (C) Results for the number of foci containing DDX6 or Dcp1 in HCV-infected cells were also plotted as percentages relative to those in non-infected cells. Error bars indicate the standard error of the mean (*P< 0.01; **P<0.005).

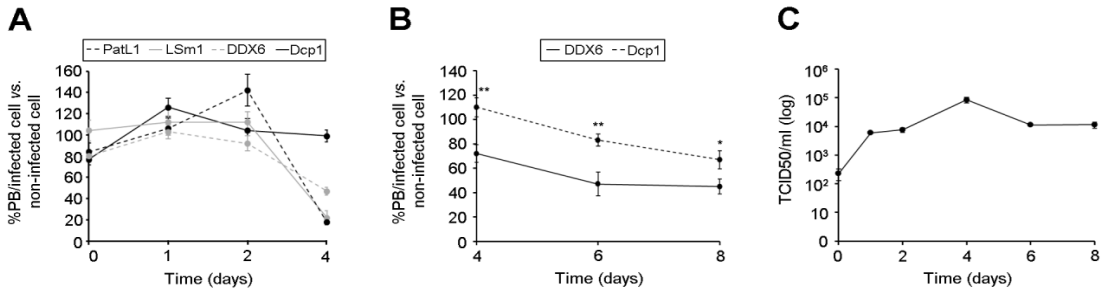

Figure S2. Kinetic of P-body abundance during HCV infection. (A) Huh7.5 cells were infected with HCV and at different times post-infection immunostained with antibodies to quantify DDX6-, LSm1-, PatL1- and Dcp1-containing P-bodies in at least 100 cells. Given is the relative abundance of DDX6-, LSm1- and PatL1- versus Dcp1- containing P-bodies in HCV infected related non-infected cells during the first 96 h post-infection. (B) DDX6-, as a representative example of LSm1- and PatL1, together with Dcp1- containing P-bodies at advanced times of infection. (C) Kinetic of HCVcc infectivity in the supernatant of HCV-infected cells in a representative experiment during the first 8 days of infection. Error bars indicate the standard error of the mean.

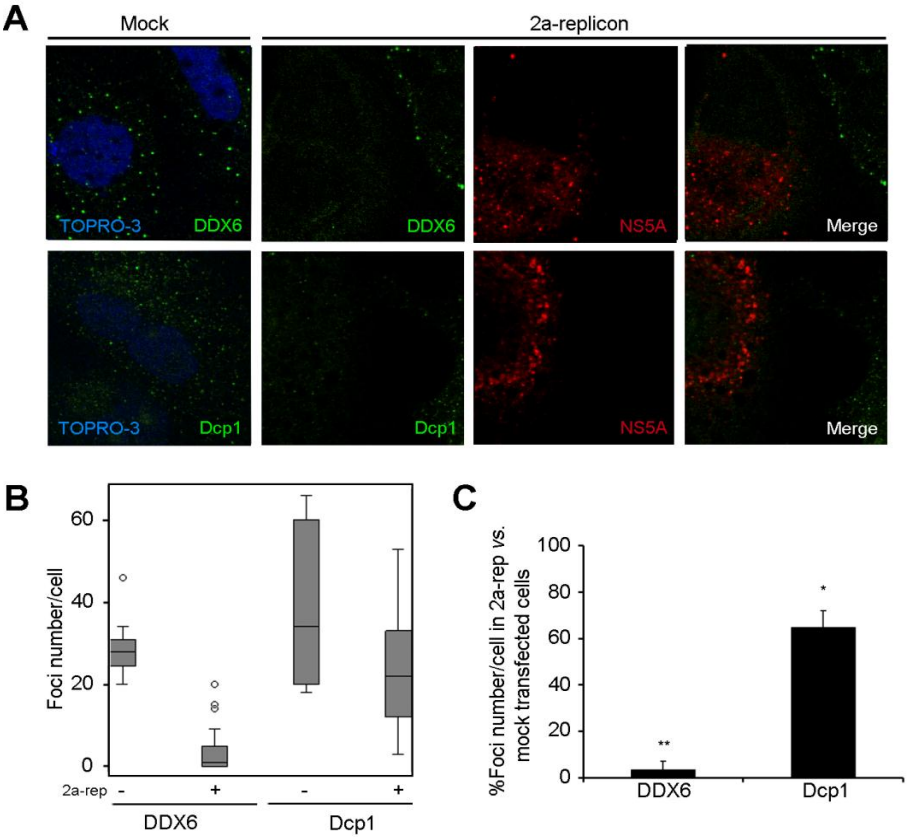

Figure S3. HCV RNA translation and/or replication disrupt P-bodies at advanced time of infection. (A) Huh7.5 cells were electroporated with a subgenomic 2a replicon RNA and coimmunostained after 96 hours to detect NS5A and DDX6 or Dcp1. (B) P-body number was quantified using at least 50 cells. Graphed are box plots showing medians, upper and lower quartiles, and outliers. (C) Results for the number of foci containing DDX6 or Dcp1 in replicon-transfected NS5A-positive cells were also plotted as percentages relative to those in mock-transfectd cells. Error bars indicate the standard error of the mean (*P< 0.05; **P<0.005).

1.3. Appendix II

HCV infection alters SGs composition

SGs are another kind of mRNP granules dynamically linked to P-bodies. The mRNAs in SGs are stalled in the translation initiation step together with translation initiation factors and ribosomal subunits. Among these factors, the RNA-binding proteins TIA-1 and TIAR contain QN-rich domains that promote SGs assembly [110]. SGs are solely detected under stress conditions. Importantly, the phosphorylation of eIF2α by the kinases PKR, PERK, GCN2 or HRI is one of the most characterized pathways of SG induction [108, 110, 142]. However, alternative pathways such as inhibition of eIF4A RNA helicase or viral infection have been described to promote SGs formation [49, 115, 141]. The observation that SGs localize close in proximity to P-bodies [62, 84], together with the fact that both granules share components, have suggested that SGs are an intermediate step between active translation that take place in free polysomes and mRNA degradation that happens in P-bodies. A growing body of evidence indicates that viruses manipulate SGs in order to control the stress that the host suffers when a viral infection occurs [19, 231]. In addition, since HCV alters P-body granules and P-bodies are intimately linked to SGs, it is likely that HCV infection could also influence the formation/composition of SGs.

To better understand the relation between HCV and SGs we infected Huh7.5 cells with HCV. At 96 hours post-infection, when more than 90% of cells were infected, cells were immunostained with antibodies against the SGs markers TIA-1 and the translation initiation factor eIF3. The percentage of cells showing SGs was analyzed. Under HCV infection eIF3 but not TIA-1 localized in punctual foci compatible with SGs. To characterize whether HCV was altering SGs composition by changing the localization pattern of some components or by inhibiting their expression, we performed a Western blot. The expression levels of both SGs markers were unchanged under HCV infection (Figure S4). Although additional experiments are missing, these results suggest that HCV infection alters SGs composition.

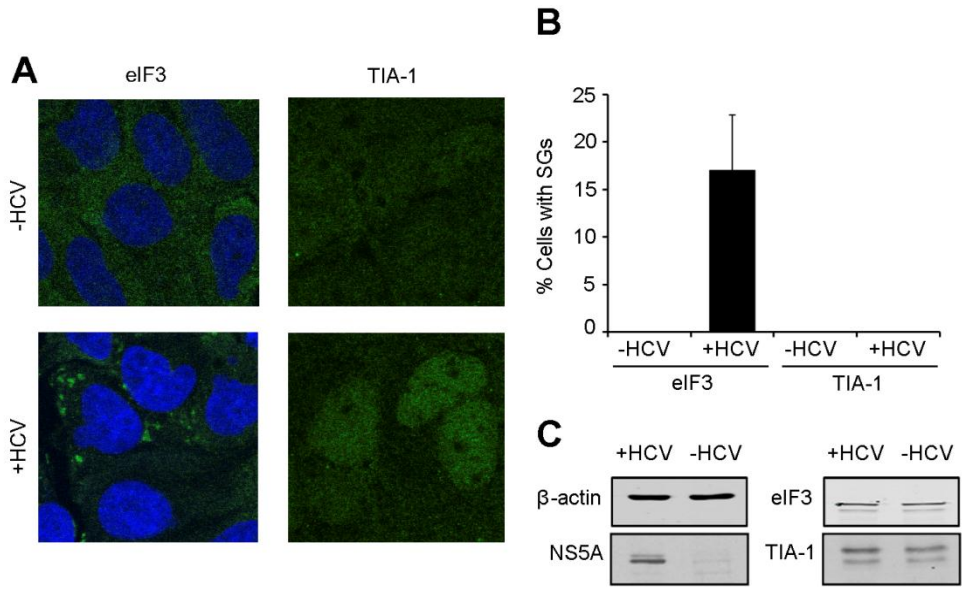

Figure S4. HCV infection alters SGs composition without degrading SGs components. (A) Huh7.5 cells were infected for 96 hours with HCV and immunostained with antibodies for eIF3 and TIA-1 (green). Nuclei were visualized using TROPRO-3 (blue). Notice that TIA-1 has not been stained with TOPRO-3 to no interfere with the signal of the antibody in the nucleus. (B) Percentage of cells showing SGs was evaluated in at least 100 cells. Graphed are medians and error bars indicate the standard error of the mean. (C) Western blot of SGs components at 96 hours post-infection. NS5A and β-actin are shown as controls for infection and protein loading, respectively.

2. Part II: Establish a selectable, replicon-based recombination detection system to study HCV recombination events in cell culture and evaluate the possible involvement of P-body components in this mechanism.

2.1. Publication II

Hepatitis C virus RNA recombination in cell culture

Jochen Reiter*, Gemma Pérez-Vilaró*, Nicoletta Scheller*, Leonardo-Bruno Mina, Juana Díez and Andreas Meyerhans

Journal of Hepatology, Volume 55, Issue 4, pages 777-783, October 2011

Recombination in RNA viruses is a widely described phenomenon. Although the first HCV recombinant *in vivo* was reported 10 years ago, the recombinant frequency for this virus was still no determined. With the new generation of drugs and the increasing number of detected HCV recombinant strains, a system in which to study the impact of recombination was needed. Here we set up a selectable, replicon-based recombination detection system in cell culture that allowed us to determine a recombinant frequency of $4x10^{-8}$ per nucleotide in HCV genotype 1. Except for the generation of mutated plasmids, I was fully involved in the design, execution and discussion of the results described in this article.

*These authors contributed equally to this work

Supplemental Material

Supplemental Literature:

[1] Lohmann V, Hoffmann S, Herian U, Penin F, Bartenschlager R. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. J Virol 2003;77: 3007-3019.

[2] Tellinghuisen TL, Lindenbach BD. Reverse transcription PCR-based sequence analysis of hepatitis C virus replicon RNA. Methods Mol Biol 2009;510: 165-175.

Reiter J, Perez-Vilaro G, Scheller N, Mina LB, Diez J, Meyerhans A. Hepatitis C virus RNA recombination in cell culture. Supplemental material. J Hepatol. 2011 Oct;55(4):777-783.

2.2. Appendix III

The 5'-3' exonuclease Xrn1 does not affect the recombination rate of HCV

Major advances have been done over the last years to characterize the factors influencing recombination rates in (+)RNA viruses. Genome wide screenings and global proteomics approaches with the system TBSV-yeast reported 38 HFs affecting the recombination of this (+)RNA virus. Interestingly, some of these HFs were identified as key members of the 5'-3' deadenylation-dependent mRNA decay pathway or factors influencing this pathway [158]. Among them, the exonuclease Xrn1 had the major effect and was reported to increase viral RNA recombinants 50-fold when it was absent [203]. In Xrn1-depleted cells abundant 5´truncated TBSV RNAs were found, suggesting that these RNAs might serve as recombination substrates promoting viral RNA recombination [46]. With the now available selectable, replicon-based recombination detection system in cell culture (Publication II), it is possible to study the influence of HFs in the recombinant frequency of HCV.

First we focused on the effect of Xrn1 protein. Huh7 cells were electroporated with a shRNA against Xrn1 (shXrn1) that specifically silence the endogenous Xrn1 protein, or with a non-targeting shRNA (shirr). At the time of maximum silencing, cells were electroporated with the corresponding combination of neomycin-selectable, subgenomic replicons. In these replicons several mutations were introduced that allow only the survival of those cells in which recombination between two different mutated replicons raised and restored the wt phenotype. We used a replicon with the mutation at the 5' end of the neomycin phosphotransferase gene (5'm) and the one with the mutated active site of the viral polymerase (Δ5B). This combination allows the longest cross-over length, thus increases the possibility to have a recombination event. After the co-transfection of 5'm+Δ5B replicons or the single transfection with the wt replicon, cells were grown for 3 weeks under the presence of G418. In this system the presence of colonies after 3 weeks of selection indicates at least one recombination event. If Xrn1 affects HCV recombination its depletion is expected to increase the number of HCV recombinants when compared to non-depleted cells. However, the

recombination frequency determined for shXrn1 cells was similar than in shIrr cells (Figure S5). In Table S1 are displayed the colonies counted under each condition.

The role of Xrn1 in TBSV recombination has been associated with its capacity to degrade TBSV RNA. Thus, in the absence of Xrn1, TBSV RNA fragments might accumulate in the cytoplasm and could be recruited for their use as templates for viral replication. Our preliminary results suggest that this is not the case for HCV. Currently it is unknown whether Xrn1 plays a key role in HCV RNA degradation. Although our results would not support a role for the 5'-3' deadenylation-dependent mRNA decay pathway in HCV recombination, they do not exclude that other HFs could influence recombination rates

Table S1. Total number of G418-resistant cell colonies after co-transfection of mutated/wt HCV replicon RNA.

	Total number of colonies	
	5'm+Δ5B	wt
shirr	2,4	8800
shXrn1	1	5000

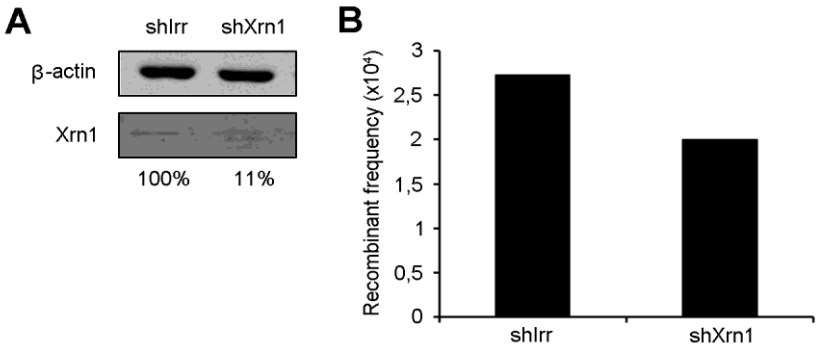

Figure S5. Silencing of Xrn1 does not affect recombinant frequency of HCV. Huh7 cells were electroporated with shRNA targeting Xrn1 (shXrn1) or non-targeting shRNA (shirr). At maximum time of silencing cells were electroporated with the mutated or wt replicons and grew under selection for 3 weeks. (A) Immunoblot analysis of Xrn1 and β-actin at maximum time of silencing. Percentages indicate the expression level of Xrn1. (B) Recombinant frequency for shirr and shXrn1-electroporated cells was calculated by relating the amount of colonies in 5'm+Δ5B plates to wt plates.

DISCUSSION

P-body components are intimately linked with viral propagation. However, little is known about the interface of P-bodies granules with important human pathogens like HCV. In this work we demonstrated for the first time that HCV infection modulates P-body formation first by limiting the localization in these foci of components required for its propagation, and finally by promoting P-body disruption. In addition, we demonstrated that HCV does not require P-body granules for its life cycle and that it also alters the composition of other well characterized mRNPs granules named SGs. Finally, by setting up a system to detect HCV recombination events in cell culture we provided the first estimation of HCV recombination frequency. In addition, we explored the possible involvement of HFs in HCV recombination by testing the effect of Xrn1, a P-body component shown to affect the (+)RNA virus TBSV recombination rates, in the established system.

HCV alters P-body formation in a two-step process

Like other (+)RNA viruses, HCV depends on the decapping activators PatL1, LSm1-7 and DDX6 to translate and replicate its viral RNA [10, 100, 195]. These proteins localize in P-bodies but also present a diffuse cytoplasmic localization. This is due to the rapid cycle of P-body components in and out of these foci. In the present study we demonstrated that HCV infection alters the localization of PatL1, LSm1 and DDX6 in P-bodies while Dcp1 still concentrates in these foci. As HCV does not affect the expression levels of any P-body component analyzed, the virus specifically interferes with the localization of these three decapping activators in P-bodies. Moreover, the remaining Dcp1 granules co-localized with GW182, another P-body component, indicating that these foci are P-bodies and not vestigial remains of Dcp1 protein. This unexpected result argues against the common believe that depletion of PatL1, LSm1-7 and DDX6 inevitably leads to P-body disruption in mammalian cells. PatL1 interacts with multiple P-body components via different domains [26, 166]. These interactions are proposed to promote the formation of protein-protein complexes that will result in P-body aggregates. Given that the domain responsible for the localization of PatL1 in P-bodies also interacts with the LSm1-7 complex [26], and LSm1-7 binds to mRNA it is likely that both components, PatL1 and LSm1-7, are essential for promoting mRNPs

aggregations. In agreement with this, RNAi mediated knockdown of LSm1 or PatL1 resulted in a dramatic reduction of P-bodies number [6, 177, 196]. Finally, the helicase DDX6 has also been reported to have a central role in P-body assembly as it depletion favor P-body disruption, even under stress conditions [148, 201]. Interestingly, at very advanced times of infection HCV also altered the localization of Dcp1 in P-bodies, strongly suggesting that the depletion of PatL1, LSm1-7 and DDX6 from these granules is the initial step that leads to foci disruption. The question that arises is whether this two-step process, that initially implies an alteration in composition that leads to granule disruption, is characteristic of HCV-infected cells or could be also detected in non-infected cells under silencing conditions of some P-body components.

Two possible explanations can be proposed for the fact that depletion of PatL1, LSm1-7 and DDX6 does not lead immediately to P-body disruption in HCV infected cells. First, the widely accepted properties of P-body disassembly derived from experiments in non-infected cells might not be completely accurate. To analyze effects on P-body disassembly most reports determine the influence of one P-body component depletion on P-body integrity by analyzing the localization of only two or three P-body markers. It is possible that such depletion would affect the localization of some interacting-partners but would not lead to a total P-body disruption. To clarify this point a higher number of P-body markers should be analyzed. Second, HCV infection could affect the requirements for P-body disruption, changing the rules when the virus is present. Then, under these circumstances the depletion of PatL1, LSm1-7 and DDX6 from P-bodies would not lead to P-body disruption. Recently, it has been described that P-bodies have an internal organization based in two different compartments: a central core where an external area is anchored [41]. This might explain the differential exit kinetic of P-body components during P-body disassembly observed in our experiments. It is likely that the internal localization of decay enzymes such as Dcp1 and Xrn1, in contrast to the peripheral localization of DDX6, and probably of PatL1 and LSm1-7, affect the kinetic exchange between cytosolic pools and foci. In fact, many FRAP studies approached this issue and already revealed that P-body components have differential kinetics [3, 64, 107, 109]. For instance, GW182 and Dcp2 are mainly immobile while Dcp1 exhibits an exchange with the cytoplasmic pool that depends on

the size of the P-body, being faster in smaller ones [109]. There is no information yet about the kinetic exchange of PatL1, LSm1 or DDX6.

In agreement with our results, while our work was in progress Ariumi *et al.* observed that under HCV infection PatL1, LSm1, DDX6 and Xrn1 localize outside P-bodies but not Dcp2 [10]. Since Xrn1 is a protein not required for HCV propagation [10, 195] and it localizes in the central core of P-bodies [41] one could speculate that once PatL1, LSm1-7 and DDX6 are hijacked from P-bodies by HCV, the disassembly of the structure and the exit of the different components will depend on the kinetic exchange with the cytoplasmic pool of the remaining components. While studies analyzing the exchange of Dcp1 with the cytoplasm have been addressed [109], such studies lack for Xrn1. Thus, one could hypothesize that Xrn1 would be faster exchanged than Dcp1.

With the current available data it is not possible to determine whether the observed HCV-induced P-body disassembly kinetic could also be reproduced in non-infected cells under conditions in which some fundamental P-body marker is depleted. However, a recent publication shows that in a fibrosarcoma cell line Dcp1 localizes in discrete foci under DDX6-silencing conditions, which would support this hypothesis [240].

How HCV induces P-body alterations?

The altered localization of PatL1, LSm1-7 and DDX6 in P-bodies under HCV infection can be linked to changes in the dynamic equilibrium of mRNA and protein components established between P-bodies and the cytoplasmic pool. A simple interpretation would be that HCV alters this equilibrium by sequestering PatL1, LSm1-7 and DDX6 in the cytosol, which thereby decreases the amount of available P-body proteins. This in turn would result in a depletion of PatL1, LSm1-7 and DDX6 from P-bodies (Figure 1). Two main observations support this idea. First, P-body disruption did not affect HCV propagation, suggesting that HCV can utilize P-body components that are diffuse in the cytosol. Second, detection of changes in P-body composition required a certain threshold of HCV components and was reversed when HCV infection levels were decreased. Thus, the equilibrium between P-bodies and their components can be shifted in both directions. In such a case, HCV infection would induce a complete P-

body disruption under certain circumstances, such as a very high replication rates or a sustained chronic infection level. In agreement with this idea, at very advanced time of infection we observed that HCV alters the localization in P-bodies of Dcp1, a P-body component localized in the core part of the foci. This alteration strongly suggests a complete P-body disruption. Immunofluorescence analysis in HCV-infected cells showed that PatL1, LSm1 and DDX6 did not relocate to specific sites. Only in a low-percentage of infected cells LSm1 co-localized with core in the ER. This indicates that i) only a small fraction of PatL1, LSm1-7 and DDX6 interacts with HCV components, ii) a very dynamic P-body-HCV components interactions occur, or iii) there is no direct interaction between P-body and HCV components. This last possibility seems unlikely since *in vitro* binding assays demonstrated that LSm1-7 establishes direct interactions with essential translation/replication regulatory sequences in the 5' and 3'NTR of the HCV genome [195]. Additionally, in HCV-infected cells DDX6 has been shown to interact with the viral genome and HCV core protein by coimmunoprecipitation assays [100].

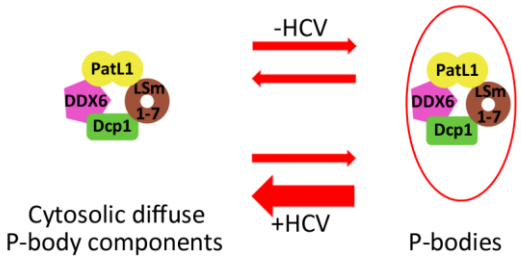

Figure 1. Model of P-body disruption under HCV infection. In non-infected cells P-body components are in a dynamic equilibrium between the cytosolic pool and the foci structures. However, under HCV infection some P-body components would be sequestered by de virus or undergo different modifications altering this equilibrium. This would result in an alteration of multiple interactions between P-body components, and P-body formation would be hampered. The global result would be an increase in the cytosolic pool of P-body components.

A growing body of evidence indicates that post-translational modifications influence the assembly of P-bodies in non-infected cells [29, 92, 123]. Specific phosphorylations of three P-body components have been shown to determine not only their localization in P-bodies but also their function [122, 185, 192]. Thus, an exciting alternative explanation to our observation would be that HCV induces such post-translational modifications in some P-body components. This would increase their diffuse cytosolic concentration making them available for the virus.

A recent publication in yeast revealed that Pat1 can be phosphorylated by protein kinase A (PKA). Once Pat1 is phosphorylated several protein-protein interactions

between Pat1 and other P-body components are altered resulting in a decrease in Pat1-, Dhh1-, Edc3- and Dcp2- containing foci in yeast. Importantly, the phosphorylation status of Pat1, and in turn P-body formation, correlates with cell survival. When PKA phosphorylates Pat1, yeast cells cannot enter in stationary phase, without this resting state cells fail to survive [185]. Although parallel studies in humans are missing, HCV infection has been related to an increase of cAMP levels which in turn results in the activation of PKA and the subsequent phosphorylation of cell substrates [69]. Similar to Pat1, the human Dcp1 can be phosphorylated. In this case, phosphorylation occurs via the c-Jun N-terminal kinase (JNK) and it decreases the number and size of Dcp1-, Edc4- and Xrn1-containing P-bodies. However, no effect in the decapping activity of Dcp1 was detected [192]. Interestingly, under a situation of chronic hepatitis or HCC it has been demonstrated that the JNK pathway is activated [136], suggesting once more that the viral infection could lead to the modification of P-body components. Finally, the decapping activator Edc3 can be phosphorylated by protein kinase B (PKB). Under this situation a higher number of P-bodies containing Edc3 are detected. Moreover, the phosphorylation status of Edc3 creates alternative complexes, increasing its interaction with PABP when is not phosphorylated. Thus, the non-phosphorylated Edc3 not only do not localize in P-bodies but also associates preferentially to actively translated mRNAs [122]. Curiously, the HCV E2 protein downregulates PKB-phosphorylation, so one could speculate that under this situation Edc3 no longer will localize in P-bodies. Although further studies are required, for instance to verify that the human PatL1 also can be phosphorylated by PKA, the idea that the virus creates rapid and reversible post-translational modifications that will push the equilibrium of P-body components to the cytoplasmic pool is an interesting working hypothesis that we would like to explore (Figure 1).

HCV translation and/or replication are sufficient to promote P-body alterations

Since PatL1, LSm1-7 and DDX6 are required for HCV translation and replication we wondered whether these steps of the viral life cycle were enough to alter P-body formation. By using a subgenomic 2a replicon, that only allows translation and

replication of HCV RNA, we observed the same results as with the infectious virus. Thus, translation and replication events are sufficient to induce P-body alterations. In contrast to our results, Ariumi *et al.* determined that PatL1, LSm1-7 and DDX6 were hijacked to LDs where they co-localize with HCV core protein. Thus, they reasoned that P-body alterations were related to late steps of HCV life cycle [10]. We did not observe any of these components localizing with core in LDs, neither at early or at advanced times of HCV infection. Only in few infected cells LSm1 co-localized with core in the ER. Likewise, a recent publication reported that DDX6 co-immunoprecipitates with HCV core, but the authors failed to demonstrate co-localization between core and DDX6, suggesting that only a small portion of core protein complexes with DDX6 [100]. These conflicting results are most probably related to the two different HCV strains used, Jc1 in our studies and JFH1 in Ariumi *et al*. It is well described that the core protein of Jc1 mainly localizes in ER while JFH1 core protein localizes in LDs. Moreover, this core localization correlates with viral infectivity. The main interpretation is that JFH1 assembles virus with lower efficiency than Jc1, thus, more core protein from JFH1 is localized in LDs waiting for participating in the assembly process [24, 39].

In any case the localization of PatL1, LSm1-7 and DDX6 with core in LDs in the JFH1 strain does not directly imply a role in encapsidation. The DEAD-box RNA helicase DDX3, which also localize in P-bodies and is required for HCV replication, has been described to interact with JFH1 core protein in LDs. However, a recent publication discards that DDX3 mediates its function in HCV life cycle through core interaction [7]. Interestingly, encapsidation of (+)RNA genomes, like replication, also requires translation repression [2]. Thus, HFs from the 5'-3' deadenylation-dependent mRNA decay pathway might influence this step of the (+)RNA viral life cycle . At least for Flavivirus dengue virus (DENV) and the retrovirus foamy virus (FV) DDX6 has been shown to be required for efficient encapsidation [240].

P-bodies alterations by other viruses

The modulation of P-body formation by HCV infection described in this thesis is one of the few reports that addresses the interface of viruses with these granules. The first

study that approached this question demonstrated that the Flaviviruses (DENV) and West Nile virus (WNV) disrupt P-bodies. In these studies only the Dcp1 marker was analyzed. They observed that Dcp1 changes the localization from discrete foci to the cytosolic pool under infection [61]. More recently, it was shown that poliovirus (PV) infection disrupts P-bodies by a combination of viral-modulable degradation of the deadenylase Pan3 and lower expression of key P-body components such as Xrn1 and Dcp1 [55]. In our studies we observed that HCV at advanced times after infection decreases by 40% the number of P-bodies while for DENV, WNV and PV no foci or a dramatic reduction were detected. Given that the formation of P-bodies correlates with the amount of mRNAs repressed in translation, it is much more likely that a proper balance between translating and non-translating mRNAs is required for cell survival especially under critical conditions such as viral infection. The maintenance of this equilibrium is predicted to be very important for viruses that chronically infect cells, such as HCV. On the contrary, viruses like DENV, WNV or PV that promote acute infections, would not need to keep long this homeostasis. Interestingly, for the retrovirus FV it was only reported to induce alterations in the composition of P-bodies rather than foci disruption. While DDX6, a HF required for FV infectivity, partially relocated with viral capsid proteins, other P-body components like Dcp1, Dcp2, Ago2 and GW182 remained in P-bodies after the viral infection [240].

It is important to note that, with the exception of our study, all other studies addressing the interaction between P-bodies and viruses only analyze one time-point or do not perform quantitative analysis after infection. So, it cannot be eliminated that the 2 step-process of P-body disassembly in response to HCV infection could be also happening in other viral infections. It is possible that the rapid replication kinetics of for example WNV, DENV and PV made difficult the identification of the time point when some components still localized in P-bodies. Oppositely, in FV and in the Ariumi *et al.* publication on HCV the scenario may be the other way round, since they do not observe P-body disruption and this may be linked to the analysis of only early time-points after infection. Further analyses in these (+)RNA viruses and retroviruses are required to clarify whether the kinetics of P-body alteration/disruption follow the two-

step process observed in HCV. This could confirm a conserved strategy of P-body disruption by these viruses.

HCV does not require P-body formation for its propagation

P-body components are involved in the life cycle of multiple viruses, but the relevance of P-body granules *per se* for these viruses is confusing. Many publications state that P-bodies are required for viral infections, however such studies prove the requirement of P-body components but not of the entire P-body granule [19]. Thus, the significance of P-body aggregation for viral propagation was an unresolved issue. We have proved, by depleting the core P-body component Rap55, that P-bodies are disrupted without influencing the production of HCV. This is the first report demonstrating that HCV does not require P-body formation to complete its viral life cycle. In other words, neither PatL1, nor LSm1-7 or DDX6 have to localize in P-bodies to be used by HCV. Currently, the relevance of P-body formation in non-infected cells is also of uncertain meaning. Many efforts have been done over the last years to clarify this fundamental question with the only outcome that global mRNA degradation and translation repression do not depend on such structures [51, 64, 209, 210]. However, under certain stress conditions, like viral infections, the relevance of these foci could have important implications *in vivo*. For instance, the formation of P-bodies has been reported to be essential in yeast for the long-term survival of stationary phase cells [185]. Moreover, another publication reported the use of P-bodies as a source of mRNPs and HFs for inflammatory processes. They observed a reduced number of P-bodies in swollen bronchial epithelial cells in mice which related with a decrease of mRNA decay and upregulation of translation [242]. It is likely that under chronic HCV infection, where inflammatory events occur in the liver, the disruption of P-bodies could contribute to upregulate global translation, including the viral mRNAs. The implications of P-body disruption in the context of an infected individual is an interesting issue that needs to be addressed to better understand the putative physiological consequences.

HCV infection alters SGs composition by avoiding TIA-1 localization in these foci

Eukaryotic cells contain other mRNPs granules named SGs. They are formed under stress conditions and contain non-translating-pools of mRNA stalled in translation initiation together with translation initiation machinery and RNA binding proteins [231]. Although SGs are often associated with P-bodies and both share some components, the viral modulation of SGs has been more exhaustively studied [135, 231]. The regulation of SGs by viruses is expected, since viruses manipulate host gene expression activating antiviral responses in the host, such as stress responses that lead to global translation repression. If viral mRNAs are repressed in SGs the viral infection cannot succeed, then viruses manipulate SGs to promote viral and cell survival during the infection course [191, 231]. Viruses have been grouped in two classes depending on the interaction established with SGs [231]. First, there are some viruses, such as mammalian orthoreoviruses, Semiliki Forest virus, PV and cricket paralysis virus that initially induce SGs formation but later during the infection they repress these granules [114, 143, 178, 183, 229, 230]. Interestingly, these repressions have been associated to SGs disruption or manipulation of the composition in the granule. A second group of viruses, including rotaviruses, influenza A virus, WNV, DENV or HIV-1, do not induce SGs formation at any point during the infection. This granules inhibition would allow that viral and some cellular proteins are translated and, as result, viral expansion would be ensured [1, 61, 112, 149].

In the present work we did a preliminary research to characterize the effect of HCV on SGs formation and determine in which group it is classified. We observed that early after the infection HCV can induce SGs in a low percentage of cells, where TIA-1 and eIF3, two SGs markers, co-localize. As infection developed, eIF3 still forms SGs but HCV selectively alters the localization of TIA-1 from these granules. Importantly, since both SGs markers presented the same expression levels as in non-infected cells such alteration is not related to a lower protein expression. While this work was in progress Ariumi *et al*. also reported the ability of HCV to initially induce SGs formation, but SGs disappear at 72 hours post-infection [10]. Since we were using different SGs markers, this makes us wonder whether the eIF3-granules that we observed are SGs with an

altered composition, or just vestigial remains of eIF3 protein, like reported with PV [230].

While this thesis was being written two relevant publications have addressed the relation between HCV and SGs. In both studies SGs formation was followed in the presence of HCV infection, but also under the addition of IFN to increase stress responses. This IFN addition was essential for these studies, as hepatoma cell lines fail to generate IFN and this might affect SGs formation [111]. Although, both publications observed an oscillation in SGs formation under viral infection they presented conflicting results. Ruggieri *et al.* co-related the formation of SGs with the ability of establishing chronic infections [191]. They monitored in single-cells the formation of SGs during 72 hours post-infection using TIA-1 as a marker. Excitingly, they observed a dynamic oscillation of SGs formation concomitantly with phases of active and stalled translation. However, the extent of virus replication did not affect the frequency at which SGs assembled and disassembled. Thus, the authors argued that SGs oscillations are essential for cell survival, as a permanent situation of translation repression lead to cell death. Finally, the silencing of SGs components demonstrated that G3BP1 and TIAR restrict HCV replication [191]. In contrast, the second publication by Garaigorta *et al.* reported that G3BP1, TIA-1 and TIAR are required for early and late steps of HCV life cycle. Moreover, they showed an inverse correlation between the presence of SGs and the induction of IFN-stimulated proteins. Thus, SGs would promote HCV infection in two ways i) co-opting SGs components for its expansion and ii) downregulating the translation of IFN-stimulated genes. In addition, they demonstrated, like we have shown with P-bodies, that the role of SGs components in HCV propagation is independent of their aggregation in these granules [80]. Further experiments are needed to solve this conflicting data.

Detection of HCV recombination in cell culture by using a new selectable replicon-based recombination system

(+)RNA viruses harbor the ability of rapidly adapt to new environments via the accumulation of point mutations and recombination events. The first HCV recombinant

was detected 10 years ago [106], marking a breakthrough in the field of HCV evolution, and raised in multiple questions, some of them still unsolved. For instance, HCV recombination rate remained unknown.

To better understand HCV recombination events we set up a neomycin-selectable, HCV replicon-based recombination detection system in cell culture. The HCV replicon was mutated to create neomycin-sensitive or replication defective replicons. Thus, only in those cells in which recombination between two different mutated replicons has occurred, the drug-resistant and replicative wt phenotype is restored and the production of neomycin allows the survival of cells under antibiotic selection. This was the first system that allowed the study of HCV recombination in cell culture and allowed to estimate for first time an HCV recombinant frequency of $4x10^{-8}$ per nucleotide. Although it occurs at low frequency when compared with other viruses like HIV, PV and BMV, whose recombinant frequency ranges around 10^{-6} [81, 101, 102, 128, 232], the recent implementation of drugs that target viral proteins may increase the fitness of the recombinant strains. Thus, the selection of HCV recombinants may become more common. Moreover, by combining the mutated replicons with replicons without adaptive mutations, that strongly influence the replication capacity in cell culture, we created a big panel of combinations with different recombination cross-over range. This demonstrated that recombinant frequencies linearly increased with the selectable cross-over length. Thus, no hotspots were detected through the HCV genome indicating that the recombinant breakpoint can be placed along the viral genome.

However, the mentioned system has some limitations. One of the most important restrictions is that we are not selecting single recombination events, we are detecting cells where at least one recombinant replicon is generated. Thus, it is possible that within a single cell more than one recombination episode is happening and the recombinant frequency would be underestimated. In addition, the detection of cells in which recombination has succeeded requires a 3-weeks selection treatment to ensure that cells without wt replicons are dead. This long waiting time could be an important obstacle to implement the system for screening approaches.

The P-body component Xrn1 does not influence HCV recombination rates

A major finding in viral RNA recombination was the demonstration that HFs could affect the rate of viral recombination. Genome wide screenings using yeast as a model host provided the first evidence of multiple yeast genes affecting TBSV recombination. Such studies identified that the depletion of five host genes increased the number of viral recombinants [203]. Four of them are involved in RNA degradation pathways. Among them, the depletion of Xrn1, the exonuclease of the 5'-3' deadenylation-dependent mRNA decay pathway, was found to increase 50-fold the accumulation of recombinant strains. Given that the other 3 genes are predicted and/or known to influence Xrn1 activity, it is likely that TBSV recombination is affected by cellular degradation pathways. Further studies in yeast demonstrated that under Xrn1 depletion more 5'-truncated TBSV RNAs were generated. Moreover, Xrn1 has the ability to degrade 5' truncated TBSV RNAs *in vitro* [46]. All these data together suggests that the influence of Xrn1 in TBSV recombination is through the degradation of viral RNAs that are used as recombination substrates. Additional genome wide screenings increased the number of yeast genes affecting TBSV recombination up to 38, being the big majority of these genes involved in RNA metabolism [202].

Within the established recombination system we were able to address whether the Xrn1 protein plays a similar role in HCV recombination. Our preliminary results do not support a conserved use of the P-body component Xrn1 and, in consequence, of the 5'-3' deadenylation-dependent mRNA decay pathway in the recombination of (+)RNA viruses. Given that for HCV is unclear how viral RNA is degraded by the host, it might be possible that other degradation pathway, different from the 5'-3' deadenylation-dependent mRNA decay pathway might be involved in promoting recombination. This might justify why the depletion of the main 5'-3' exonuclease does not increase HCV recombinants. Alternatively, other HFs could influence HCV recombination through the modification of the activity of the RdRp, then affecting the frequency of the template switching. Interestingly, it has been described for TBSV that the deletion of the yeast Pmrp1, an ion pump that controls Ca^{2+}/Mn^{2+} influx into the Golgi from the cytosol, increases 160-fold the recombination of TBSV [98]. When Pmrp1 is not present the

Mn^{2+} accumulates in the cytosol and the viral polymerase uses it instead of Mg^{2+}. *In vitro* studies with RdRp of many RNA viruses demonstrated that high Mn^{2+} makes the polymerase action less specific for templates [5, 11, 89, 182, 237], promoting the template switching mechanism. The established HCV recombination system will be a useful tool to elucidate the influence of these putative HFs in the evolution of HCV. However, the experimental approach would be improved by using stable-silenced cell lines instead of transient siRNA-mediated silencing. This would allow a more homogeneous and predictable silencing among cells.

CONCLUSIONS

From the results presented in this thesis, the following conclusions can be drawn:

HCV infection alters P-body composition but is independent of P-body granules

- HCV infection induces changes in P-body composition by specifically altering the localization pattern of PatL1, LSm1-7 and DDX6.
- Treatment with a polymerase inhibitor that partially reduces HCV replication restores P-body composition, suggesting that there is an HCV production threshold at which P-body composition is altered.
- A subgenomic HCV replicon that only supports translation and replication of HCV RNA also alters P-body composition, indicating that these early steps of the viral life cycle are sufficient to selectively alter the localization pattern of P-body components.
- The translation repressor and P-body component Rap55 is essential for P-body formation but is not required for HCV replication.
- Disruption of P-bodies by Rap55 depletion does not affect HCV production, indicating that the localization of PatL1, LSm1-7 and DDX6 in P-bodies granules is not necessary for their function on HCV life cycle.
- At very advanced times post-infection/post-electroporation HCV disrupts P-bodies.
- HCV infection alters the composition of SGs by limiting the localization of TIA-1 marker in these granules without affecting eIF3. Such alteration is not related to protein degradation by the virus.

Hepatitis C virus RNA recombination in cell culture

- A selectable, replicon-based recombination detection system has been established in hepatoma cell lines to observe and quantify recombination events during HCV replication.

- This system enabled the first quantitative estimation for the recombination of HCV in cell culture: the mean recombinant frequency is calculated to be $4x10^{-8}$ per nucleotide or $4x10^{-4}$ per HCV genotype 1b genome.
- The recombination frequency is low when compared to other (+)RNA viruses and dependent on the available cross-over range without major recombination hotspots.
- Depletion of the P-body component, the exonuclease Xrn1, does not affect the HCV recombination frequency.

APPENDICES

1. ABBREVIATIONS

ARFP	Alternative reading frame protein
BMV	Brome mosaic virus
DENV	Dengue virus
ER	Endoplasmic reticulum
FV	Foamy virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCV-1	Hepatitis C virus genotype 1
HF	Host Factor
HIV	Human immunodeficiency virus
IFN	Interferon
IRES	Internal ribosome entry site
JFH-1	Japanese fulminant hepatitis 1
LD	Lipid droplet
LDL	Low-density lipoprotein
miRNA	microRNA
mRNP	Messenger ribonucleoprotein
NTR	Non-translated region
ORF	Open reading frame
P-bodies	Processing bodies
PV	Polio virus
RBV	Ribavirin
RdRp	RNA-dependent RNA polymerase

RNAi	RNA interference
SG	Stress granule
SVR	Sustained virological response
TBSV	Tomato bushy stunt virus
WNV	West Nile virus
(+)RNA	Positive-strand RNA

2. OTHER PUBLICATIONS DURING THIS THESIS

Host factors in viral life cycles

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Abstract

Viruses are obligate intracellular parasites that rely on the host cell for expansion. With the development of global analyses techniques like transcriptomics, proteomics and siRNA library screening of complete cellular gene sets, a large range of host cell factors have been discovered that either support or restrict virus growth. Here we summarize some of the recent findings and focus our discussion on the hepatitis C virus and the human immunodeficiency virus, two major pathogens that threat global health. The identification of cellular proteins affecting multiple viruses points to the existence of central regulation nodes that might be exploited for both, a quantitative description of host-virus interactions within single infected cells and the development of novel, broad-spectrum antiviral drugs.

Viruses are simple infectious agents that totally depend on living cells for expansion. They are formed by a genomic nucleic acid, either DNA or RNA, and a protein shell that surrounds it. Some viruses also present an external bilipid layer that they hijack from the host when exiting the cell. Since viral genomes have a limited coding capacity, they depend on cellular functions. In fact, all fundamental cellular processes, such as for example the transcription and the translation machineries, are parasitized by viruses. It is worthy to note that, in spite of their simplicity, viruses are far of being simplistic organisms. On the contrary, they have evolved very sophisticated and elegant mechanisms to hijack the cellular functions and persist in a living cell. One challenge for the twenty-one century is precisely to unravel this intimate host-virus relationship for gaining efficient control over virus infections and to use viruses as tools for health benefit.

Viruses are extremely diverse not only in size, shape, and genetic organization but also in their replication strategies. Despite this great diversity, there are five common steps that all viruses need for expansion (Figure 1). First, they attach to the host cell through specific interactions between components in the surface of the cell and the virus. This step is critical in determining the virus host range. Second, once attached, viruses enter into the host cell and loose many or all of the proteins contained within the virus particle in a process named uncoating. Third, now viruses are ready to amplify their genomes. In this step, they must coordinate two important events, the production of viral proteins and the replication of viral genomes. The strategies used in this step are greatly influenced by the nature of the viral genome that may be DNA or RNA, either single or double-stranded. A major difference between DNA and RNA viruses is that the latter replicate by viral polymerases that have such a high mutation rate that is unlikely that two copies of the same RNA have exactly the same nucleotide sequence. This has paramount consequences in viral evolution, pathogenesis, resistance to antiviral therapies and vaccine development (see review in this issue by Domingo and Perales). Fourth, when sufficient numbers of new genomes and proteins are produced within the host cell, they form new viruses in a process called assembly. And last, once new viruses are formed the viral progeny exit the cell, either by lysing it or by budding off into the extracellular compartment. Please note that to avoid confusion, in this review we will name the complete viral life cycle as the expansion cycle and the term replication will be used only for the specific replication step.

The expansion cycle completely depend on cellular factors. The identification of such host factors is of major importance since it provides fundamental insights into virus biology and may also suggests candidates targets for novel and efficient antiviral therapies. This review will focus in the identification and use of host factors within the cell that either promote or restrict viral expansion. In particular, we will emphasize the findings of cellular factors affecting human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV) life cycles. Both viruses are major human pathogens that chronically infect around 30 (www.unaids.org/globalreport/Global_report.htm) and 170 (www.who.int/mediacentre/factsheets/fs164/en/index.html) million people worldwide, respectively. Currently, there are no vaccines available. A curative antiviral treatment has not yet been discovered for HIV. Nevertheless, the new therapies have successfully slowed the progression of the disease and prevent the emergence of opportunistic infections. In contrast, curative therapies do exist for HCV treatment. The current triple therapy that includes interferon, ribavirin and novel drugs that target the HCV protease eliminate the virus in 70% of infected patients. However, multiple side effects have been associated with this treatment (34, 64, 73). Furthermore, the RNA nature of their genomes prompts the rapid generation of mutations and consequently resistance to antiviral treatments directed against viral proteins. Thus, there is an urgent need for novel therapeutic interventions. In this context, the development of drugs that target cellular proteins required for cellular expansion are of great interest since, given the genetic stability of the host, they are less likely to select virus escape mutants. In addition, as viruses from the same viral group share some fundamental properties in their replication steps, they are predicted to use common cellular pathways and thus it might be possible to develop broad-spectrum antiviral treatments.

Identification of host proteins involved in viral expansion.

Due to the complexity of host genomes and host-virus interactions it is a difficult task to identify host factors that affect virus expansion. However, while our current knowledge in this field is still very limited, this situation is rapidly changing due to the implementation of recent global approaches that allow high-throughput analyses. These include genome-wide functional assays to study the effect of host factors depletion on the viral life cycle and global proteomic approaches, such as mass spectrometry and microarrays analysis, to detect direct interactions between host factors and viral proteins or genomes. Since host factors playing a critical role in viral expansion often interact

with viral proteins or genomes, the combination of functional and interactome high-throughput analyses together with computational meta-analysis will result in a big step forward in the understanding of the host-virus interface. Importantly, the results obtained at a global scale would need to be later studied in an individualized way to precisely define the mechanism of action of the identified host factors.

Use of yeast systems to identify host factors affecting viral expansion.

The development of systems which allow the life cycle of higher eukaryotic viruses in yeast was a milestone in virus research and enabled the use of versatile yeast tools for unraveling viral replication mechanisms. In fact, the first functional global identification of host factors affecting virus expansion was performed in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) (42). The use of *S. cerevisiae* as a model organism for these studies has major advantages. In contrast to the big genomes of plants and animals, yeast is built out of only 6000 genes from which over 60% have an assigned function. Furthermore, 40% of yeast genes share conserved amino acid sequences with at least one known or predicted human protein, and all the fundamental cellular processes are conserved between yeast and human cells (10, 60). In addition, the commercial accessibility of powerful tools and platforms allows large-scale functional analyses in yeast. These comprise (i) a gene deletion mutant collection covering around 85% of all yeast genes in which each strain contains a deletion of one non-essential gene (83), (ii) a downregulable essential-gene library that allows the study of essential genes (52), and (iii) gene libraries with fluorescent or affinity tags that facilitate interactome analyses (24, 25, 31).

Whereas the first higher eukaryotic virus able to replicate in yeast was the plant brome mosaic virus (BMV) (35), the list rapidly expanded to other plant viruses but also to viruses that infect insects, mammals and humans (4, 57, 59, 65-67, 88). The BMV-yeast system harbors additional advantages as steps of the viral life cycle can be dissected and studied separately making it possible to identify host factors affecting different steps. This is important because effects on viral progeny production could be due to alterations of each previous expansion step. Effects on the replication step of the BMV life cycle were analyzed in the ~5000 strains that comprise the non-essential yeast deletion collection. From this systematic genome-wide screening, around 100 genes were found to affect BMV replication (42). A similar study was performed for another plant virus, the tomato bushy stunt virus (TBSV). Interestingly, around 100 genes were also

identified in this case (58). Surprisingly, against all predictions only a low number of genes were found to be common in both studies. This was suggested to reflect differences in the viral replication strategies or in the experimental screening set-ups. To extend these studies to essential genes, additional genome-wide screenings were performed in the yeast/BMV and yeast/TBSV systems using an essential-gene library that covers 80% of the essential genes and in which each essential gene can be specifically turned off. With this approach 24 and 30 genes that affect the replication of BMV and TBSV were identified, respectively (23, 37). Taken together, these studies uncovered previously unconsidered cellular pathways, such as mRNA turnover, stress response, ribosome biosynthesis and the ubiquitin pathway of protein degradation, that either enhanced or inhibited viral replication.

Importantly, as fundamental cellular processes are highly conserved between yeast and human, host factors identified using the yeast model system might be transferred to clinically important human viruses. This principle has been already proven for the cellular LSm1, Pat1 and Dhh1 yeast proteins, referred to as LSm1, PatL1 and Rck/p54 (also named DDX6) in humans, respectively. These proteins were identified in the yeast system to be essential for BMV replication (3, 14, 51). Subsequent studies in human cells demonstrated that the same set of proteins were required for the replication of the human pathogen HCV in human hepatocytes (36, 71). Despite this functional conservation, studies in higher eukaryotic systems are essential to validate the yeast-derived data and to explore further interactions, as not every plant or mammal protein has a functional counterpart in yeast.

Identification of host factors affecting viral expansion in human cells.

RNA interference (RNAi) is widely used in human cells as a screening tool to identify cellular factors implicated in viral expansion. The RNAi pathway silences gene expression by inducing enzymatic degradation of targeted mRNAs (20). In cell culture, RNAi is achieved by delivering small interfering RNAs (siRNAs). These are small synthetic dsRNAs, directly delivered by transfection into the cytoplasm of human cells, that act through the RNAi pathway to knockdown the complementary targeted mRNA. As a result, the corresponding encoded protein will be depleted. However, the achieved depletion is transitory due to the dilution of the intracellular siRNA concentration by cell division and degradation (11). To increase RNAi persistence, plasmids have been designed that are introduced into the nucleus and integrate into the cell genome. These

plasmids express a perfectly complementary dsRNA (short hairpin RNA, shRNA) that is subsequently processed to siRNA by cellular enzymes producing a long-term silencing effect (79, 84). It is important to note that silencing conditions need to be carefully optimized and controlled in order to avoid false-negative results due to low transfection efficiency, and false-positive results due to cell toxicity or non-specific binding of the siRNA/shRNA (off-target effects) (16, 75). Thus, a subsequent functional validation of the host factors showing a potential impact on the viral life cycle should be performed. Moreover, in contrast to the yeast/virus system in which the gene of interest is deleted from the yeast genome and thus its expression completely depleted, RNAi-mediated silencing in human cells does not achieve a complete depletion due to technical limitations. As a consequence, identification by siRNA analysis of key factors that are very abundant in the cell might be difficult, since it is predicted that large depletion levels would be required to make these factors limiting in the cell and thus to observe an effect on viral life cycles.

Recent advances in genomic technologies and RNAi methodologies have allowed the development of high-throughput techniques for genome-wide RNAi-based screenings. These screenings are based on the transfection of genome-wide siRNA or shRNA libraries usually containing pools of siRNAs or shRNAs, respectively. Using this technology, genome-wide RNAi-based approaches have been carried out in order to identify the complete set of cellular factors affecting the life cycle of important human pathogenic viruses, such as HIV-1 and HCV. Four major genome-wide RNAi-based screens have been published in the context of HIV-1 infection. Three of these screenings were based on the transfection of genome-wide siRNA libraries (8, 40, 89), and led to the identification of around 800 cellular factors affecting HIV-1 infection. While some of them were described previously, the majority were novel host factors not implicated in the HIV-1 life cycle before. When the results obtained from these studies were compared later by Bushman and colleagues in a meta-analysis, the overlapping results among the identified genes after pair-wise screening comparisons was surprisingly small (<7%) (9). These differences were attributed to the use of diverse experimental conditions in terms of viral strains, cell lines, siRNA concentrations, siRNA libraries and transfection methodologies. Despite these controversies, subsequently functional analyses showed a superior overlap in terms of biological pathways. More recently, another genome-wide RNAi screening was performed based on the transfection of a shRNA library (85). Again, although little overlap was reported

on candidate genes in comparison with the previous siRNA screenings, several cellular pathways overlapped between siRNA and shRNA analyses.

Regarding HCV, two major siRNA-based genome-wide screens have been performed to study cellular factors involved in its life cycle. By transfecting siRNA pools, Tai and colleagues identified 96 host genes implicated in HCV replication (78). An alternative approach that allowed to screen for cellular genes involved in early and late HCV expansion steps identified a total of 262 host genes, 44 of these were involved in late steps of viral infection (44). By performing a bioinformatic meta-analysis to compare the obtained results with other screens, the authors revealed a strong statistical enrichment for several host cell pathways and complexes, as well as multiple direct interactions between the functionally defined data and comprehensive proteomic studies. Interestingly they also identified 10 host genes, such as for example DDX3, SPCS3 or Rap9p40/REBEK, that are needed by HCV and HIV-1 and thus may represent novel targets that could be exploited in instances of co-infection. In fact, a second generation of DDX3 inhibitors already demonstrates *in vitro* the potential of this HF as an anti-HIV target (49).

In summary, the yeast system is a fast and excellent alternative to achieve hints of putative human host factors involved in viral life cycles. However, further studies in human cells are required. In spite of the technical limitations associated with genome-wide RNAi screens, which include the possibility of false negative or positive hits, these screens are providing priceless information that in combination with global proteomics and computational meta-analyses are identifying key host proteins and pathways that promote or restrict viral life cycles. In addition, recent advances in genomics also open the option of comparing genomes among patients identifying differences that might be responsible for disease severity or progression, as well as for treatment response. One would predict that these global approaches will allow in the near future the drawing of a detailed road map of viral life cycles in infected cells.

Identification of host non-coding RNAs involved in viral expansion.

All the above described studies aiming to identify host factors affecting viral life cycles were focused in the identification of cellular proteins. However, the recent discovery of non-coding RNAs (ncRNAs) uncovered an additional level of gene regulation that is essential for cell function and is predicted to have profound implications in viral life cycles. Although these implications still remain to be fully explored, very active

research has been already done for one class of ncRNAs, the microRNAs (miRNAs). MiRNAs are small non-coding RNAs that under normal cellular conditions negatively regulate gene expression by translational repression and/or mRNA degradation (43). However, in viral infections miRNAs can modulate viral replication and infection either negatively or positively by direct interaction with the viral genome. Clear examples are those miRNAs which have been previously described to directly influence the HCV life cycle. For example, the liver-specific miR-122 facilitates HCV replication (38) and translation (27), while others like miR-196 represses HCV expression (29). These findings have also important clinical implications. In fact, a novel antagonist targeting the miR-122 has shown potent antiviral effects in chronic HCV-infected patients in early clinical trials (63).

All these findings were obtained analyzing a limited set of miRNAs. An ever-expanding list of high-throughput analyses are being performed to globally identify miRNAs that modulate viral life cycles. Most of the screenings are made using microarrays and aim to identify those miRNAs showing a deregulated expression after viral infection. Those miRNAs showing a potential role on viral life cycles should be then validated by testing the effect of their depletion or overexpression in viral expansion. Several global miRNA analyses have been carried out in the setting of HIV-1 and HCV infections, both in cell culture and *in vivo*. From these, key studies that also include functional validations of the obtained results have identified multiple miRNAs, miRNA families and miRNA-mRNA regulatory modules that play a role in HIV-1 (30, 54, 77, 80) and HCV (7, 33, 47, 61) life cycles. Given the growing interest in this exciting field of research, it is expected that this list will rapidly grow and will include other species of ncRNAs that still remain to be explored.

Conserved use of host factors in viral life cycles: universal host factors?

Viruses are classified into seven groups on the basis of different strategies for storing and replicating their genomes through RNA and/or DNA intermediates. Despite major differences, three of these groups, the positive-strand RNA viruses, the double-stranded RNA viruses and the retroviruses, share two fundamental common features in their replication process. First, they all replicate their genomes through an RNA intermediate that also functions as a mRNA. Second, this mRNA is captured into protected compartments, membrane invaginations in positive-strand RNA viruses and subviral complexes in dsRNA and retrovirus, where replication takes place and competing

processes such as translation are excluded (1). The emergence of these common underlying principles suggests a common evolutionary origin and has practical implications since these shared features might provide novel targets for broad-spectrum strategies of virus control. The development of antivirals that target multiple viruses within these groups is of great clinical interest since it would allow to simplify and improve the treatment of co-infected patients, an important issue for instance in HCV (a positive-strand RNA virus) and HIV-1 (a retrovirus) co-infections. Importantly, it would also allow having a first line of defense against new emerging viruses since most of them belong to the positive-strand RNA group.

One example of cellular proteins affecting multiple viruses within these groups are the components of the cellular processing bodies (P-bodies) and stress granules (SGs). A growing number of viruses including the positive-strand RNA viruses HCV (5, 6, 21, 36, 41, 68, 70, 71, 86), poliomyelitis virus (72, 76), dengue virus (DENV) (81), west nile virus (18, 45); and the retroviruses HIV-1 (2, 32, 48, 50) and foamy virus (87) depend on P-bodies and SGs components for their expansion. P-bodies and SGs are cytoplasmatic granules highly conserved from yeast to human (19, 26). P-bodies contain translationally repressed mRNAs together with proteins from the mRNA decay machinery and, in humans, from the miRNA machinery as well. SGs also contain translationally repressed mRNAs, however they are stalled in the process of translation initiation, together with translation initiation factors and ribosomal subunits. Both types of granules are highly dynamic but while P-bodies are observed under normal growth conditions in higher eukaryotic cells, SGs are formed in response to conditions that result in translational repression, including different types of environmental stresses. Interestingly, many viruses have been shown to modulate the number and composition of P-bodies and SGs (6, 15, 17, 39, 62, 82).

Specific examples of components of these granules with a wide effect on viruses are the proteins Rck/p54 and PatL1, and the LSm1-7 heptameric complex. All these components accumulate in P-bodies and function as translation repressors on cellular mRNAs. This function seems to be hijacked by positive-strand RNA viruses and retroviruses to promote their expansion by assisting the transfer of the viral genomes from translation to the steps of replication or encapsidation (14, 51, 71, 87), processes that require translation repression. The remarkably common use of Rck/p54, PatL1 or LSm1-7 by at least one retrovirus, the foamy virus (87), and by different positive-strand RNA viruses of different kingdoms, including the human HCV (6, 36, 71) and DENV

(81), the plant BMV (14, 51, 55, 56) and the phage Qβ (22) point out to the existence of central regulation nodes that might be exploited for both, improving the quantitative description of host-virus interactions within single infected cells and the development of novel, broad-spectrum antiviral drugs.

Conclusions and perspectives

Host factors play a pivotal role in all aspects of viral life cycles within infected cells. With the omics-technologies of systems biology, the last years have seen a boost of knowledge in this area. However, to fully understand virus-cell interactions, a quantitative and dynamic description of the interacting components with the help of mathematical models would be most valuable. Until today, most modeling attempts have concentrated on viral dynamics in infected individuals and did not consider specific viral features within single infected cells but rather assumed constant virus production rates. The rapidly increasing knowledge on host-virus cell interactions should now be considered and may substantially extend the few modeling attempts on the single cell level that have been performed (12, 13, 28, 46, 53, 69, 74). With the inclusion of key regulatory steps in the viral life cycles that are mediated by host components under the conditions of infected cells, the new models may improve our understanding of virus-induced pathogenic processes and help to develop novel treatment regimens.

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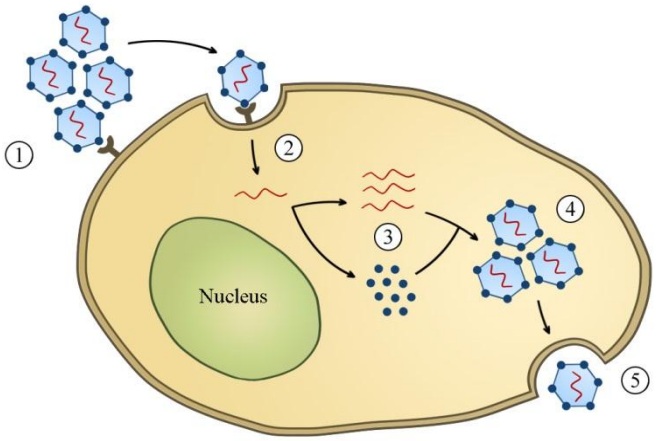
Figure 1. General steps in viral life cycles. All viruses infect cells through a first attachment step to the cell surface (1). Once internalized, the virus releases the viral genome into the cytoplasm (2), allowing the translation of viral proteins and the replication of the genome (3). This then generates new progeny (4) that after maturation will exit the cell to infect new cells (5).



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“¿Pies? Para qué los quiero, si tengo alas para volar”

Frida Kahlo