MINIREVIEW

Mechanism of Cell Infection with Hepatitis C Virus (HCV)
– a New Paradigm in Virus-Cell Interaction

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A b s t r a c t

Hepatitis C virus (HCV) is an enveloped, single-stranded RNA virus, belonging to the Flaviviridae family. HCV infection is a major cause of chronic hepatitis worldwide, leading to steatosis, liver cirrhosis and hepatocellular carcinoma. Significant advances in understanding the mechanisms of HCV infection have been made since the development of a cell culture system reproducing the complete HCV cell cycle *in vitro*. HCV represents a new paradigm in interactions between the virus and its target cell, the human hepatocyte, due to the central role of lipoproteins in the HCV life cycle. Very low density lipoproteins are required for virus particle assembly and secretion. Upon the release, the infectious virus circulates in the blood as triglyceride-rich particles and infects cells using lipoprotein-receptor dependent mechanisms. HCV cell entry is a multi-step process: heparan sulphate and/or low-density lipoprotein receptor are cell surface factors mediating an initial virus attachment; subsequent virus interaction with tetraspanin CD81 and the human scavenger receptor SR-BI, the main HCV receptors, triggers virus movement to the tight junctions and its uptake *via* Claudin-1 and occludin. Another originality of HCV is that initiation of productive infection requires dynamic microtubules. Whereas other viruses use kinesin or dynein-dependent transport, HCV exploits mechanisms driven by microtubule polymerization to efficiently infect its target cell, in which virus nucleocapsid protein might play a particular role. An improved of understanding of the cellular events involved in HCV cell entry and transport, leading to the initiation of productive HCV infection, may reveal novel targets for anti-viral interventions.

K e y   w o r d s: Hepatitis C virus, lipoproteins, cellular receptors, mirotubules, virus entry

(Hepatitis C virus (HCV) infection affects about 130 million individuals worldwide, and remains a major cause of chronic liver disease (Shepard *et al.*, 2005). HCV is a unique and potentially lethal human pathogen. In the majority of cases (60–85%), HCV infection progresses to chronic liver disease, which can evolve to hepatic steatosis, cirrhosis and hepatocellular carcinoma (Shepard *et al.*, 2005). In spite of the considerable efforts there is still no vaccine available that can protect against HCV. Current therapy, based on the application of pegylated interferon alpha in combination with ribavirin leads to the clearance of the virus in about 50–80% of cases, depending on the virus genotype and has serious side effects (Keam and Cvetkovic, 2008; Pawlotsky, 2006).

The important feature of the HCV genome is its high degree of genetic variability: 6 major virus genotypes and about 100 subtypes have been identified, which often have distinct geographic distributions (Simmonds *et al.*, 2005). In addition, constant mutations of the viral genome due to a high error rate of the viral polymerase, result in a heterogeneous population of virus quasispecies in infected individuals. HCV is characterized by an extraordinary ability to establish a viral persistence, which it achieves by evading the innate responses of infected hepatocytes and humoral and cellular responses of the adaptive immune system (see for review Gale and Foy, 2005; Rehermann and Nascimbeni, 2005; Meurs and Breiman, 2007).

HCV is an enveloped virus of the *Flaviviridae* family (genus Hepacivirus) with a single-stranded positive sense RNA genome of about 9.6 kb. The virus genome encodes a polyprotein composed of about 3,000 amino acids, which is cleaved by host and viral proteases into three structural proteins (the capsid protein, and two envelope glycoproteins E1 and E2), P7 and several non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The structural proteins form the virus particles, whereas NS3 to NS5 are involved in genome replication (reviewed in Brass *et al.*, 2006;

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Bartenschlager *et al.*, 2004; Penin *et al.*, 2004). Although the detailed structure of HCV virion remains unclear, infectious virus particles are most likely composed of a nucleocapsid bearing the viral RNA, and the lipid envelope into which the E1 and E2 glycoproteins are embedded.

The liver is a major, if not the only human organ that produces HCV. A unique feature of HCV is that both virus RNA replication and virion assembly depend on cholesterol metabolism and fatty acid biosynthetic pathways in the human hepatocyte (Huang *et al.*, 2007; Ye, 2007; Kapadia and Chisari, 2005). As well, HCV infection is known to induce changes in host lipid metabolism, such as to reduce levels of serum lipoproteins upon chronic HCV infection (Petit *et al.*, 2003) and to accumulate lipids in liver parenchymal cells (steatosis) (Barba *et al.*, 1997) (Petit *et al.*, 2003). HCV assembly coincides with the association of virus nucleocapsid protein with lipid droplets, an intracellular organelle that stores neutral lipids, and has been proposed as the site of viral morphogenesis (Boulant *et al.*, 2007; Miyanari *et al.*, 2007). Indeed, the HCV core protein recruits non-structural proteins and replication complexes to lipid-droplet associated membranes, and this recruitment is critical for the production of infectious virus particles (Miyanari *et al.*, 2007) with the help of NS2, NS3, NS5A and P7 (Lanford *et al.*, 2009).

Remarkably, the formation of virus particles and their release from infected cells require their association with VLDL, which is also produced by the human hepatocyte (Huang *et al.*, 2007; Chang *et al.*, 2007; Gastaminza *et al.*, 2008). Consequently, the major population of HCV circulating in the serum is of low density, due to the viral association with β-lipoproteins (André *et al.*, 2002; Agnello *et al.*, 1999; Thomssen *et al.*, 1992). It is generally accepted that light density, infectious HCV in the serum is represented by Lipo-Viro-Particles (LVPs), lipoprotein-like structures composed of triglyceride-rich lipoproteins (TRL) bearing Apolipoprotein B and Apolipoprotein E, viral nucleocapsids, and envelope glycoproteins (Nielsen *et al.*, 2006; André *et al.*, 2002). An intriguing observation is that besides Apolipoprotein B100, which is an integral part of VLDLs, formed, in human liver, as is HCV, LVPs might contain Apolipoprotein B48, produced by enterocytes (Diaz *et al.*, 2006). This would suggest that a part of circulating HCV may originate not only from the liver, but also from the intestine (Diaz *et al.*, 2006).

LVPs are the major and infectious population of serum HCV. However, serum HCV is extremely heterogeneous, with bouoyant density ranging from 1.06 to 1.30 g/ml (see for review André *et al.*, 2005). Only a minor population of the circulating virus could correspond to the canonical "flavivirus-like" particles (Petit *et al.*, 2005). Besides LVPs, other unconven-

tional virus forms have been detected in patient sera: "naked" HCV nucleocapsids, lacking the virus envelope (Maillard *et al.*, 2001) and displaying "FcγR-like" properties (Maillard *et al.*, 2004) and "exosomes" – host cell derived membranous vesicles, containing HCV RNA, envelope proteins, and CD81, the major receptor for the virus (Masciopinto *et al.*, 2004). It remains to be determined whether all these forms of serum HCV are infectious.

A support for the key role of lipoproteins associated to virus particles in HCV infectivity has been provided by experiments *in vivo* and *in vitro*. Indeed, only the very low-density population of serum HCV was found to be infectious in chimpanzees (Bradley *et al.*, 1991) and in tissue culture cells (André *et al.*, 2002; Monazahian *et al.*, 1999). The fact that lipoproteins increase the infectivity of HCV was confirmed by studies in the recently developed *in vitro* HCV replication model, reproducing the complete viral replication cycle in subclones of the human hepatoma cell line Huh-7 (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005). The virus produced *in vitro* (HCV cell culture derived, HCVcc) transmitted infection to naïve hepatoma cells, but also infected chimpanzees and in uPA-SCID mice with human liver grafts (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Lindenbach *et al.*, 2006). However, the JFH1 strain of HCV produced in experimentally infected chimpanzees or uPA SCID-mice with human liver grafts had a higher specific infectivity and lower buoyant density than the same virus strain derived from hepatoma cells (Lindenbach *et al.*, 2006). Thus, the composition of lipoproteins associated with virus particles was different in virus particles produced *in vitro* than *in vivo* and was determinant for virus infectivity.

Currently available data suggest that cellular events leading to HCV cell entry and the establishment of productive infection are a multi-step processes, involving several viral and hepatocyte-specific factors that triggers virus uptake (see for review Burlone and Budkowska, 2009). Strikingly, HCV enters the liver cells via tight junctions using cellular proteins that normally form firm seals between hepatocytes (Evans *et al.*, 2007; Liu *et al.*, 2008). The liver tight junctions separate the canalicular domain of the hepatocyte involved in bile secretion from the luminal domain of the cell that is in contact with the blood. This structure maintains cell polarity by forming a kind of intramembrane, regulating the diffusion of various molecules (Shin *et al.*, 2006). The human picornavirus group-Coxackieviruses, also exploit the tight junction molecules for cell entry. Indeed, their binding to the cell receptor triggers virus movement to the tight junctions and virus uptake (Coyne *et al.*, 2007).

The main hepatocyte receptors mediating HCV cell entry are: Tetraspanin CD81 (Pileri *et al.*, 1998),

the human scavenger receptor SR-BI (Scarselli *et al.*, 2002), and tight junction molecules Claudin-1 (Evans *et al.*, 2007) and occludin (Liu *et al.*, 2008, Ploss *et al.*, 2009). In addition, cell surface glycosaminoglycans such as heparan sulphate (Barth *et al.*, 2003) and/or low-density lipoprotein receptor (LDL-R) (Agnello *et al.*, 1999) are potential accessory factors that may play a role in initial virus attachment.

Virus cell entry is probably initiated by the interaction between HCV-associated lipoproteins (mainly VLDL) with lipoprotein receptors: LDL-R and/or SR-BI and/or cell surface heparan sulphate proteoglycans (GAGs) (Fig. 1). HCV subsequently interacts with CD81, a "post-entry" receptor, which forms a complex with SB-BI. CD-81 plays a fundamental role in HCV infectivity as it triggers signalling cascades essential for virus entry and further downstream events (Brazzoli *et al.*, 2008). The virus is then transferred to the tight junction proteins Claudin-1 (however virus can also use either Claudin-6 or Claudin-9 (Zheng *et al.*, 2007)), and the more recently discovered occludin (Ploss *et al.*, 2009; Liu *et al.*, 2008) which provides "the final entry key" for HCV cellular uptake (Pietschmann, 2009). Indeed, human occludin in combination with other receptors CD-81, SR-BI and Claudin1 renders mouse cells permissive for virus entry (Ploss *et al.*, 2009). Although all other HCV receptor molecules that mediate HCV infection are ubiquitous, and none is exclusive to liver cells, only human hepatocytes might express all the cellular factors required for virus penetration and the accomplishment of a complete replication cycle.

Studies carried out using virus pseudotypes (HCVpp) that harbour the E1 and E2 envelope glycoproteins integrated into the lipid envelope on lentiviral cores (Bartosch *et al.*, 2003) and the more recently developed cell culture-produced virus (HCVcc model), (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005) permitted it to be established that like other flaviviruses, HCV enters the cell by clathrin-mediated endocytosis, with the delivery of the viral nucleocapsid in early endosomes (Blanchard *et al.*, 2006; Koutsoudakis *et al.*, 2006; Meertens *et al.*, 2006). E1 and E2 envelope glycoproteins mediate pH-dependent fusion with limiting membranes of early endosomes (Lavillette *et al.*, 2007) which triggers nucleocapsid release into the cell cytoplasm and permits the virus to escape the lipoprotein degradation pathway.

Recent data from our group suggest that authentic serum-derived HCV might directly recognise SR-BI at the cell surface and thus enter the cell via SR-BI (Maillard *et al.*, 2006). Moreover, we showed that the virus interacts with the receptor, not by viral envelope glycoproteins but via ApoB-containing lipoproteins associated with virus particles. Indeed, the virus-receptor interaction was not inhibited by a large panel of polyvalent antibodies directed against the HCV envelope, while anti-β-lipoprotein antibodies and VLDL, one of the natural ligands of the receptor, efficiently blocked virus uptake (Maillard *et al.*, 2006). These data could explain the co-existence of lipoprotein-associated virus with potentially neutralizing antibodies, in patient's sera, the latter being not able to control infection (Haberstroh *et al.*, 2008).

Natural ligands of SB-BI, VLDL (Maillard *et al.*, 2006) and oxydated LDL (von Hahn *et al.*, 2006), are inhibitors of HCV cell entry, whereas HDL facilitates infection, probably activating SR-BI or modifying the structure of lipid membranes (Bartosch *et al.*, 2005; Voisset *et al.*, 2005). Natural components of VLDL such as apolipoproteins ApoC1, (Dreux *et al.*, 2007; Meunier *et al.*, 2005), ApoB (Huang *et al.*, 2007) or ApoE (Chang *et al.*, 2007) represent efficient regulators of HCV infectivity. In addition, ApoC3, a known inhibitor of lipoprotein receptors (LDL-R and LRP) has recently been identified as a plasma biomarker associated with the resolution of acute HCV infection (Molina *et al.*, 2008).

Another evidence for the impact of lipoproteins on virus cellular uptake was brought by the findings showing that lipoprotein lipase (LPL) a key enzyme in lipoprotein metabolism which targets TRL-rich lipoproteins to the liver and mediates their hepatic uptake could also introduce HCV into hepatic cells (Andréo *et al.*, 2007). Since the enzyme had an inhibitory effect on HCV infection, in HCVcc model, it can be hypothesised that LPL may either affect the lipoprotein composition of virus particles or mediate an alternative pathway of virus cell entry leading to abortive infection, and as such, could act as a natural modulator of HCV infectivity (Andréo *et al.*, 2007). Apart from lipoproteins and their protein components other host molecules could interfere with virus infectivity acting on virus cell entry. EWI-2wint, a cellular partner and natural inhibitor of CD81, regulates the virus interaction with its main receptor, and thus could influence virus cell entry and infection (Rocha-Perugini *et al.*, 2008).

After virus cell entry, HCV-RNA replication takes place in membrane-associated replication complexes designated as membranous webs (Gosert *et al.*, 2003). HCV replication complexes are subjected to intracellular transport, and their formation is closely linked to the dynamic organisation of the endoplasmic reticulum, actin filaments and the microtubule network (Jones *et al.*, 2007; Lai *et al.*, 2008; Wolk *et al.*, 2008). However, how the virus is trafficked from the virion attachment at the cell surface to the delivery of the viral genome to its replication site remains unclear.

Indeed, HCV continues to teach us about the mechanisms which viruses can use to infect cells. In a recent study, we provided evidence that intact and

dynamic microtubules play a key role in the early steps of the virus cycle, leading to the establishment of productive HCV infection (Roohvand *et al.*, 2009). Using virus pseudotypes (HCVpp), we demonstrated that the first steps of virus internalisation from attachment at the cell surface until fusion of the viral envelope within an endosomal compartment required microtubule-dependent transport. Studies carried out on the HCVcc JFH1 replication model using drugs affecting the main cytoskeleton components showed that post-fusion steps of the virus cycle, involving nucleocapsid release and early transport, also required intact and dynamic microtubules or polymerisation-dependent "treadmilling" mechanisms (Roohvand *et al.*, 2009). We also discovered a unique property of the HCV core protein: its capacity to directly bind tubulin and to enhance microtubule polymerisation in vitro (Roohvand *et al.*, 2009). It is conceivable that HCV core could also directly interact with tubulin *in vivo* or integrate into the microtubule lattice to exploit their assembly dynamics and/or "treadmilling" mechanisms (Rodionov and Borisy, 1997) and to enable transport of the virus nucleocapsid in infected cells. Indeed, in our studies the virus core protein co-localised with microtubules *in vivo* and *in vitro* as evidenced by confocal and electron microscopy, respectively, (Roohvand *et al.*, 2009). Moreover, other studies evidenced that HCV also takes advantage of microtubules for virus egress: microtubules are essential for viral morphogenesis and the secretion of progeny virus from infected cells (Boulant *et al.*, 2008).

Conclusions

HCV represents a new paradigm in virus host-pathogen interactions due to the central role of lipoproteins in the virus life-cycle: the requirement of VLDL for virus particle assembly and the secretion, association of circulating virus with lipoproteins, a lipoprotein-receptor dependent mechanism of infection, as well as the virus impact on the hepatic lipid metabolism. Besides the dependence of the virus cell cycle on the lipoprotein metabolism, another originality of HCV is that the initiation of a productive infection requires dynamic microtubules. Indeed, although many viruses use the microtubule network for virus transport at various steps of their life cycle; most viruses use kinesin or dynein-dependent transport, or interact with microtubule-associated proteins (Radtke *et al.*, 2006). Our observations suggest that to efficiently infect its target cell HCV exploits mechanisms driven by microtubule polymerization, in which virus nucleocapsid protein might play particular role.

Significant advances in the understanding of the mechanisms leading to productive infection have been made due to the development of cell culture systems *in vitro* that reproduce the complete HCV cell cycle in hepatoma cells. This progress mainly concerns the molecular biology of the virus; especially genome replication, viral polyprotein processing and studies of HCV cell entry. Nevertheless, the structure of infectious virus particle, as well as cellular processes leading to virion assembly and release from infected cells, are the least understood aspects of the HCV life cycle.

An important handicap in HCV studies remains the very limited number of virus isolates that are capable of undergoing an entire viral cycle in cultured hepatoma cells (HCVcc model). These cells have also several characteristics that differ them from human primary hepatocytes, especially concerning synthesis of lipoproteins (Bukh and Purcell, 2006). Further advances in our knowledge of the mechanisms leading to cell infection would help to propose new therapeutic approaches targeting HCV cell entry, such as cyclic synthetic peptides (Lanford *et al.*, 2009) or lectins, such as Cyanovirin-N, (Helle *et al.*, 2006), and/or drugs interfering with microtubule-dependent transport, in addition to the currently developed virus protease-helicase or replication inhibitors (Lanford *et al.*, 2009).

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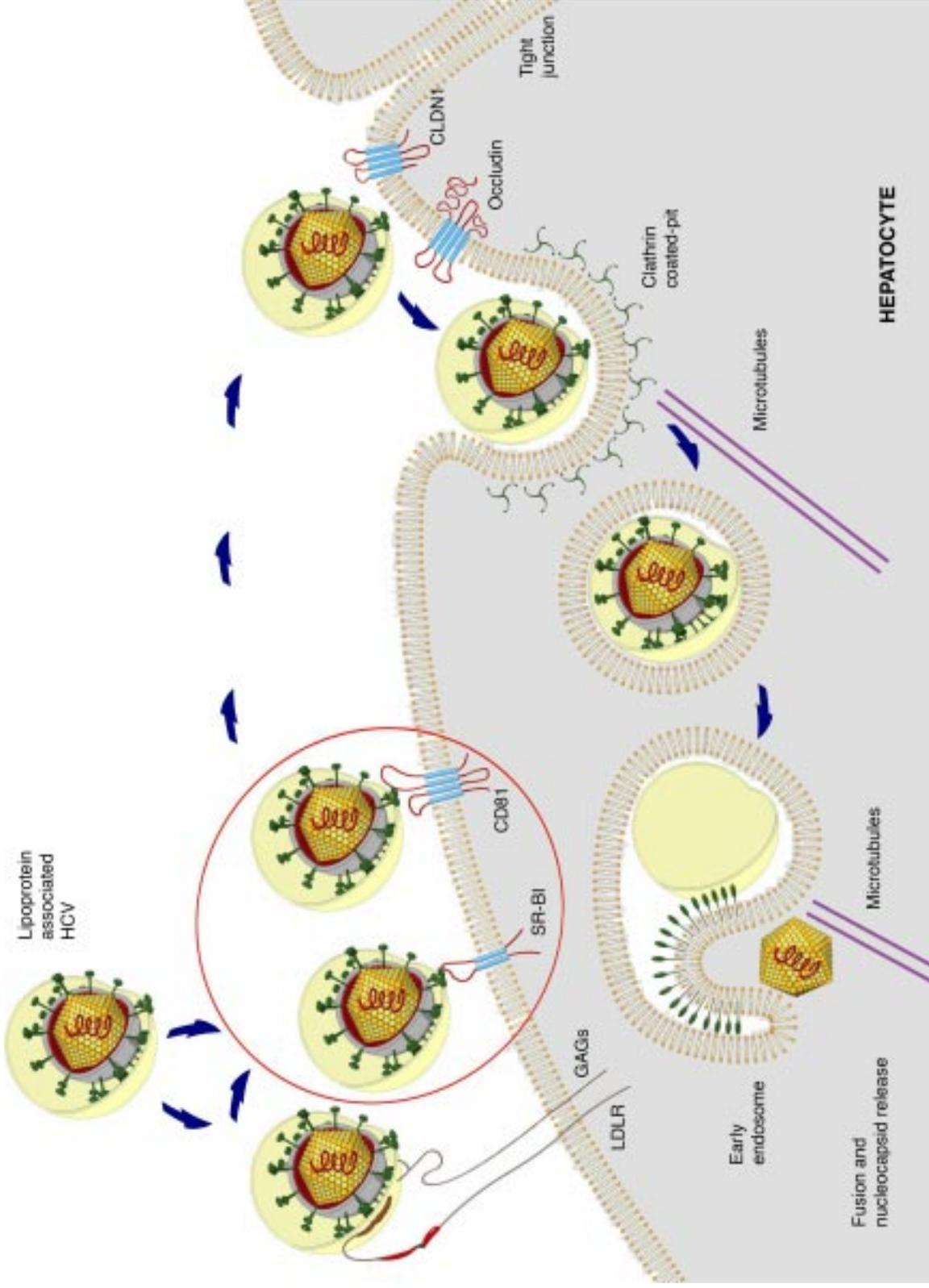
Fig. 1. Mechanisms of HCV cell entry.

HCV circulates in a patient's serum in association with Apo-B containing lipoproteins (mainly VLDL). Initial attachment of the virus to the hepatocyte surface involves virus-associated lipoproteins, which bind to LDL-receptor and/or heparan sulfate proteoglycans. HCV might also directly bind to SR-BI. HCV interacts with SR-BI and CD-81 receptor complex, and this interaction triggers signalling events required for virus entry and the initiation of infection. HCV is subsequently transferred to tight junctions where it binds to CLDN1 and occludin. The virus enters the cell from the tight junctions via clathrin-mediated endocytosis followed by fusion in the endosomal compartment mediated by HCV envelope glycoproteins and release of the virus nucleocapsid. HCV cell entry and early transport require the dynamic microtubule network.