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Proteomic approaches to analyzing hepatitis C virus biology

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Abstract

Hepatitis C virus (HCV) is a major cause of liver disease worldwide. Acute infection often progresses to chronicity resulting frequently in fibrosis, cirrhosis, and in rare cases, in the development of hepatocellular carcinoma (HCC). Although HCV has proven to be an arduous object of research and has raised important technical challenges, several experimental models have been developed all over the last two decades in order to improve our understanding of the virus life cycle, pathogenesis and virus-host interactions. The recent development of direct acting-agents (DAAs), leading to considerable progress in treatment of patients, represents the direct outcomes of these achievements. Proteomic approaches have been of critical help to shed light on several aspect of the HCV biology such as virion composition, viral replication, and virus assembly and to unveil diagnostic or prognostic markers of HCV-induced liver disease. Here, we review how proteomic approaches have led to improve our understanding of HCV life cycle and liver disease, thus highlighting the relevance of these approaches for studying the complex interactions between other challenging human viral pathogens and their host.

Keywords

Hepatitis C virus; virus-host interactions; virus life cycle; proteomic; mass spectrometry

HCV-host interactions: From transcriptomic to proteomic approaches

The hepatitis C virus (HCV), a member of the *Flaviviridae* family, was first recognized in 1975 as a viral, hepatitis-causing agent distinct from the hepatitis A and B viruses[1]. However, it would take more than a decade to specifically identify HCV as the infectious agent responsible for these observed cases of non-A, non-B hepatitis[2]. The isolation of a cDNA clone in 1989 from the serum of a chimpanzee with non-A, non-B hepatitis paved the way for basic and clinical HCV research. In the 25 years since then, impressive advances have been continuously made in our knowledge about this virus, from the characterization of its life cycle to the resolution of some of its proteins' structure.

The past decade especially has seen numerous breakthroughs in our understanding of HCV biology. Most importantly, the development of promising direct-acting agents (DAAs) against HCV non-structural proteins has revolutionized patient treatments, creating interferon-free, and thus much more tolerable, regimens. The development of ground-

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breaking technologies in the field of transcriptomic, structural, and cell biology as well as the generation of humanized animal models over the last 10 to 15 years has dramatically improved our understanding of HCV-host interactions. Additionally, the development in 2005 of a cell culture system[3–5] where an HCV clone was able to replicate enabled the characterization of the entire HCV life cycle *in vitro*. Since then, transcriptomic analysis and RNA-based approaches have been intensively used to study the dependencies of HCV on the cell host, host responses to infection, and the identification of key host factors involved in the HCV life cycle[6–10]. These studies, combined with the structural resolution of several HCV proteins[11] and the development of HCV-permissive small animal models,[12] have been critical in shedding light on potential therapeutic targets.

However, although the role of transcriptomic analysis in improving our understanding of the regulation of HCV-host interaction is undeniable, these approaches do not give a complete picture of HCV-host interactions. Indeed, mRNA expression does not perfectly reflect protein expression. Additionally, changes in enzymatic activity, protein localization, the assembly/disassembly of protein complexes, and transcriptional modifications are not accounted for by transcriptomic analysis. These changes are likely to have an impact on host-virus interactomes and, ultimately, on disease progression and outcome. Thus, during the last decades, proteomic has emerged as a unique tool to implement complementary approaches to transcriptomic analyses and to allow a more extensive assessment of host-virus interactions. In 2011, the global mapping of a virus-host interactome at a high-throughput resolution in the context of HIV infection symbolizes one of these accomplishments[13]. Proteomic approaches can facilitate evaluating the degree of virally-induced pathogenesis in a host while also providing relevant prognostic markers[14]. Finally, the relevant mapping and analysis of intraviral protein networks and virus-host proteins networks can guide target identification of anti-viral drug discovery against challenging pathogens[1516].

Among the considerable achievements of proteomics in understanding virus biology, HCV likely represents one of the prime examples. Here, we review how proteomic approaches have dramatically expanded our understanding of the HCV life cycle, liver pathogenesis, host-altered metabolism and host immune responses. We also emphasize how these approaches can be applied to study HCV in increasingly more physiological experimental systems.

I. A brief overview of the Hepatitis C Virus

HCV is an enveloped virus of the *Flaviviridae* family, measuring 40–80 nm in diameter with a heterogeneous morphology and no clear form of symmetry[17–19]. For these reasons, a high-resolution model of particle structure is not available, in contrast to the the well-defined particle structures of the related flaviviruses. Similarly, the structure and arrangement of the two envelope glycoproteins, E1 and E2, on the viral membrane are not fully defined.

HCV has a positive-sense, single stranded RNA genome, which encodes for a large polyprotein containing structural and non-structural viral proteins. As with other members of the *Flaviviridae* family, the HCV genome has a long open reading frame (ORF) flanked by two non-coding regions at its 5’ and 3’ ends[20].

The polyprotein encoded by this ORF is around 3000 amino acids and is cleaved co- and post- translationally into 11 structural and non-structural proteins by host or viral proteases. The N-terminal part of the polyprotein contains three structural proteins: core, E1 and E2, followed by seven non-structural proteins (p7, NS2-3-NS4A/B-NS5A/B). An 11th protein, the F protein, is the result of a reading frameshift in the core-encoding sequence. During translation, C/E1, E1/E2, E2/p7 and p7/NS2 junctions are processed by the host signal peptidase, an ER resident-enzyme. NS2 processes its junction with NS3 while the NS3/NS4A serine protease complex processes the remaining junctions to release the non-structural proteins NS4A, NS4B, NS5A and NS5B[20].

The HCV life cycle is a complex, multi-step process (Figure 1) requiring the involvement of a large number of different host proteins. Hepatocytes, the major cell type within the liver, represent the main reservoir of HCV *in vivo.* After attachment to the hepatocyte cell surface through recognition of a large panel of specific receptors and co-factors, HCV is internalized into the hepatocyte via clathrin-mediated endocytosis[8921–25]. Fusion between the viral membrane and the endosomal membrane allows the release of the viral RNA into the cytosol. Viral RNA is then translated and cleaved into its constituent structural and non-structural proteins[20]. Viral replication and particle assembly, which occur in ER-derived membrane structures as observed with many positive-strand RNA viruses, are highly dependent on several host proteins[2627]. The association of structural proteins and newly synthetized HCV genome in ER-derived membranes then allows for the assembly and secretion of new viral particles via the host's very low density lipoprotein (VLDL) secretory pathway[27].

II. Deciphering the HCV life cycle using Proteomics

1. Virion composition and its interplay with host lipoproteins—One of the most striking features of HCV is its tight interplay with host lipid metabolism. In contrast to all other members of the *Flaviviridae* family, this interplay gives HCV particles *in vivo* a heterogeneous and very low buoyant density. This is due to the distinct lipid composition of HCV particles, which associate with serum triacyglycerol (TG)-rich lipoproteins *in vivo*[28]. Indeed, serum-derived HCV particles have been found to be associated with components of low density lipoproteins (LDL) or very low density lipoproteins (VLDL), such as apolipoprotein B-48 and -100 (apoB-48, apoB-100), apolipoprotein C-I (apoC-I) and apolipoprotein E (apoE)[2930]. Accordingly, HCV particles are routinely qualified as lipoviral particles (LVPs)[2831]. However, the nature of the interactions between lipoproteins and the HCV viral particles, and consequently the architecture of LVPs, is not yet fully understood..

While these gaps in our knowledge do exist, some proteomics studies have made progress in understanding the association between HCV particles and host lipoproteins (Figure 1). In 2011, a study used a replicating HCV cell-culture derived virus (HCVcc) encoding a tagged envelope glycoprotein (E2-FLAG) to allow viral particle purification and protein/lipid composition analysis by mass spectrometry (MS)[32]. HCVcc particles were shown to have very distinct lipid signatures similar to those of VLDLs and LDLs, including apoE, found on viral particles. Recent studies provide evidence that ApoE likely interacts with envelope glycoproteins during late steps of viral assembly, following capsid envelopment but before

viral secretion[3334]. Hence, this observation suggests that viral particles and lipoproteins may not associate as hybrid, single viral particle but rather via transient interactions during viral particle maturation Future proteomic analysis will be needed to analyze the nature of the interaction between E1 and/or E2 with apolipoproteins during virus maturation.

Interestingly, another study aimed to analyze HDL, VLDL, and LDL of HCV obtained from infected patients[35] using 2-D electrophoresis (2DE) and Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry (MALDI–TOF MS). In contrast to healthy donors, there was a decrease in ApoA-I association with LDL in infected donors. It was also shown that Apo-AI is an important player in viral particle production, highlighting the influence of HCV on altering host lipoprotein biogenesis to ensure its replication. ApoB-100 production is also known to be reduced in HCV patients[36]. Using stable isotope labeling by amino acids in cell culture (SILAC), it was demonstrated that ferritin heavy chain (Fth) is an important effector of viral particle production which is responsible for the inhibition of apoB-100 following up-regulation by NS5A[37].This result was consistent with a previous yeast two-hybrid experiment suggesting an interaction between Fth and NS5A[38].

2. Viral Replication

HCV non-structural protein interactions: Due to the tight interplay between the HCV non-structural proteins during viral replication and assembly, proteomics approaches have been used to characterize these interactions (Figure 1), which could also be targeted by anti-viral therapies. In 2000, a group developed a two hybrid screen by transforming yeasts with plasmids carrying a cDNA library encoding for random polypeptides derived from HCV non-structural proteins[39]. Through this strategy, they identified previously known interactions between nonstructural proteins, such as those between NS3 and NS4A, as well as unreported interactions, such as between NS4A and NS2. In 2003, a similar approach was employed to create a map of HCV non-structural protein interactions. GST-pull down assays on *in vitro* translated non-structural HCV proteins, as well as immunoprecipitation assays in adenovirus-transduced Huh-7 cells expressing the HCV non-structural proteins[40], were both utilized. Their results confirmed previously observed interactions and identified several new dimerizations (homo- and hetero-) among the HCV non-structural proteins and also suggested new functions in viral replication for NS2 and NS4B proteins.

Proteome screens to identify host proteins that impact viral replication: In 2005, a study took advantage of a highly permissive cell line expressing a full-length HCV Con1 (genotype 1b) replicon to conduct the first large-scale proteomic analysis of HCV replication (Figure 1). Using multi-dimensional liquid chromatography and MS, the expression of more than 4200 proteins was analyzed[41]. Viral replication-induced changes mostly involved proteins important for lipid metabolism, confirming the tight link suggested by other studies between HCV and lipid host metabolism. Proteome analysis of liver biopsies from HCV-infected patients revealed changes in interferon-regulated proteins and markers of HCV-associated hepatocellular carcinoma, illustrating the potential of proteomic approaches for diagnostic and prognostic applications. Following this work, several studies aimed to apply proteomic approaches to identify other differential protein profiles in hepatoma cell lines expressing or not HCV RNA[4243].Among them, a group identified 13

proteins differentially upregulated within Huh-7 cells expressing full-length HCV Con1 replicon using 2DE and MALDI–TOF MS[43]. Interestingly, several heat shock proteins as well as annexin V were independently identified between different screens, thus highlighting the consistency of these proteomic screens. However, some discrepancies can be found as keratins (Type 1, 8 and 9) were shown to be differentially regulated during HCV replication in one screen, whereas keratin type I was unchanged in another. These discrepancies highlight the importance of determining interaction specificities when conducting proteomic analysis. Indeed, the antibody-mediated capture of specific protein complexes prior to MS may result in non-specific interactions and thus, to inaccurate results. A study recently proposed a novel strategy to capture and purify with an increased specificity targeted protein complexes. This technology, defined as immuno-competitive capture mass spectrometry, allowed to identify 8 novel NS5A host interacting proteins such as the large tumor suppressor homolog 1 and 2, important for NS5A phosphorylation and viral RNA replication[44].

More recently, another study reported the coupling of immunoprecipitation of host-viral protein complexes with MS to identify human proteins interacting specifically with one viral protein among core, NS2, NS3/4A, NS4B, NS5A and NS5B proteins[45]. Following transfection of 293T cells with plasmids encoding individual HCV proteins, 98 proteins involved in a broad panel of functions were identified, including ER proteins, transport proteins and transmembrane proteins. Although 24 of these proteins were previously shown in the literature to interact with HCV proteins, the authors also found, following a genetic shRNA screen, a panel of 11 additional ER proteins involved in HCV RNA replication. Serum from infected patients has proven to be a valuable source material for proteome screens that aim to identify host proteins impacting viral replication. Indeed, a recent study identified eight serum proteins differentially regulated in HCV-infected patients through 2DE and MS[46]. Among them, an *in vitro* assay revealed retinol-binding protein 4 as a novel regulator of HCV replication.

Several studies have also focused on the identification of host proteins impacting viral replication through binding to the HCV viral RNA. Using a sequence-specific biotinylated peptide nucleic acid for the specific capture of the HCV RNA, a group reported the identification of 83 host factors bound to the HCV genome, which either positively or negatively impacted viral replication and translation in HCV replicon cell lines following siRNA silencing[47]. Mir-122 is a liver-specific micro-RNA required for HCV replication which binds to the stem-loop I (SLI) of the 5’ untranslated region (UTR) of the HCV viral RNA[48]. Using a human proteome microarray, a group identified the heterogeneous nuclear ribonucleoprotein K (hnRNP K) as a strong interacting factor of the SLI 5’UTR and as an important host determinant for viral replication[49]. Interestingly, a previous screen also identified another hnRNP, the hnRNP H1, as interacting with HCV core in infected cells following relocalisation of nuclear hnRNP H1 into the cytoplasm[50]. Several challenges still remain to be addressed when elucidating the HCV viral RNA replication process. Among them, the question regarding the role(s) of the NS3 protein, along with the potential interplays between the RNA cis-acting replication elements and the viral and host proteins, can be of potential interest for further interactome analysis[26].

Detecting changes in enzyme catalytic activities: Beyond the ability to analyze protein presence or abundance within infected cells, proteomic approaches can also be used to characterize changes in the catalytic activity of enzymes within infected cells. Indeed, a 2009 study applied a method called activity-based protein profiling (ABPP), which combines the use of protease probes mimicking different protein substrates with 2DE and MS, to characterize the activity of protease families during HCV replication (Figure 1) by using Huh-7 cells expressing a subgenomic replicon[51]. Using this method, several protease classes with altered catalytic activity during HCV replication were identified, highlighting the relevance of such methods to find novel host dependencies and potential anti-viral targets.

Proteome screens within sub-cellular compartments: Because of the tight interplay between HCV and host lipid metabolism, several screens have been performed on lipid-related cellular compartments or structures to identify active sites of HCV RNA replication and to unveil specific changes in lipid metabolism induced by viral RNA replication[52–56] (Figure 1). A study notably reported a proteomic screen on lipid droplets isolated from cells expressing HCV core protein[53]. Use of 1DE/MALDI-TOF MS or direct nano-flow liquid chromatography–MS/MS unveiled the involvement of core protein in lipid droplet biogenesis. Moreover, the detection of proteins involved in RNA metabolism within lipid droplets of core-expressing cells suggested a close link between core protein, lipid droplets and RNA replication.

Viral RNA replication is thought to occur within detergent resistant membrane (DRM) structures in HCV-infected cells. In 2006, a proteomic screen performed on DRM structures isolated from Huh-7 cells expressing a full-length genotype 1b replicon was reported[54]. Although the authors identified 39 proteins differentially expressed by using 2DE with MS, they were able to identify 150 proteins by combining one-dimensional electrophoresis (1DE) separation, mass spectrometry and SILAC. By using a siRNA screen, it was demonstrated that HCV replication depends on several proteins, such as the small GTPases Cdc42 and RhoA or syntaxin-7. Furthermore, it was shown that up-regulation of several proteins involved in HCV replication was not due to an increase in gene expression but to a relocalization of these proteins into lipid rafts, thus highlighting the relevance of performing proteome analysis on particular sub-cellular compartments. Consistently, another screen on DRM structures isolated from HCV subgenomic replicon-expressing cells uncovered a Ras-GTPase-activating protein binding protein, G3BP1, to be important for HCV replication through association with HCV NS5B[55]. G3BP1 was also identified in another screen as able to bind to the 5’ untranslated region of the negative strand of the HCV RNA, along with the polyadenylate-binding protein 1 and the nucleolysin TIA-1[57]. Consistently with the study mentioned above, G3BP1 siRNA-mediated downregulation impaired RNA replication.

However, a study recently focused its attention on the dominant type of detergent-resistant membranous compartments within HCV-infected cells, the double-membrane vesicles (DMVs). In this work, authors generated HCV replicon expressing a hemagglutinin-tagged form of NS4B protein, thus allowing them to purify NS4B associated membranes[56]. Interestingly, most of these membranes were DMVs and their isolation permitted the identification of critical host proteins for viral replication. These findings are consistent with

the idea that DMVs likely represent the pro-active sites of viral replication within HCV-infected cells[26].

Detecting post-translation modifications impacting viral replication: A considerable benefit of proteomic approaches – in contrast to transcriptomic approaches – is its ability to identify post-translation modifications (PTM) in viral or host proteins that can impact virus replication (Figure 1). For instance, a study from 2013 analyzed NS5A phosphorylation sites upon HCV replication using cells expressing a subgenomic replicon[58]. Following NS5A immunoprecipitation, SDS-PAGE gel separation and MS, a specific phosphorylation site within NS5A was found to act as a negative regulator of RNA replication. However, the precise role of NS5A, as well as the role(s) of its distinct phospho-isoforms during RNA replication still remain to be precisely defined[26] and can thus be of interest for further proteomic analysis.

3. Virus entry& Assembly

Host entry factors and envelope glycoproteins: Although a high number of proteomic studies focus on host dependencies related to HCV replication, some studies have also used proteomic approaches to identify host determinants involved in HCV entry and assembly (Figure 1). HCV entry is a complex, multi-step process involving several receptors and entry co-factors, allowing the viral particle to fuse with host cell endosomal membranes. EGFR-mediated signaling has previously been shown to mediate the formation of CD81-claudin-1 (CLDN1) receptor complexes, which are critical for HCV entry[59]. However, the mechanism linking EGFR signaling to the formation of CD81-CLDN1 was unknown. A study in 2013 used SILAC, co-immunoprecipitation assays and MS to identify an association between the GTPase HRas, located downstream of the EGFR signaling pathway, and CD81 and CLDN1[60]. In combination with several other *in vitro* assays, a novel mechanism was uncovered, mediating the formation of receptor complexes during HCV entry. Alternatively, proteomics computational models have also been applied to generate structural models for the HCV E1 envelope glycoprotein, whose structure still remains unknown. In 2003, a study suggested that HCV E1 may be the HCV fusion protein based on its homology with other class II fusion proteins,[61] even though HCV E2 was thought for several years to be the putative HCV fusion protein[62]. Interestingly, the recent structural resolution of a large fragment of the E2 ectodomain[63–64] has challenged the ability of E2 to be a fusion protein and now supports a model where E1 would be the putative HCV fusion protein.

HCV assembly, particle production and budding: Virus particle production and release is a multi-step process involving nucleocapsid formation, envelopment of the viral particle, maturation and release through the VLDL secretory pathway[27]. However, the mechanism of HCV assembly is still not fully understood. This mechanism is thought to involve a broad panel of host cellular proteins, notably those involved in host lipid metabolism and lipid droplet biogenesis. Thus, proteomic approaches can be useful tools to decrypt the host dependencies of virus assembly and improve our understanding of this complex process (Figure 1). A good example is a study from 2012 where an interaction was identified between a tyrosine motif within the HCV core protein and AP2M1, a subunit of clathrin adaptor protein complex 2 (AP-2), using microfluidics affinity assays[65]. Microfluidics

affinity assays consist in the capture of molecular complexes via mechanical trapping of molecular interactions, and allow a high-fidelity analysis of weak and transient protein-protein interactions with very limited off-target effects[66]. This interaction was shown to mediate the recruitment of AP2M1 to lipid droplets, which is critical for core colocalization with E2 and efficient viral particle assembly.

Proteomic methods can also be used to identify novel host substrates of viral proteases that can influence viral particle production. SILAC, SDS-PAGE electrophoresis and MS were used to identify novel cellular substrates of NS3-4A protease through the use of NS3-4A inducible-expressing cell lines[67]. GPx8 was found to be a novel NS3-4A substrate *in vitro* and in infected-patient biopsies, highlighting its role as a proviral factor for viral particle production. Yeast two-hybrid systems have also been shown to be useful to identify host determinants of viral particles production[6869]. By using such system, interaction between the proteasome activator PA28γ and HCV core was demonstrated both in cell culture and in the liver of HCV core transgenic mice[69]. This interaction with PA28γ likely regulates the stability of core protein, thus enhancing virus particle production and promoting HCC. A more recent study used a high-throughput Western array, an assay able to analyze expression changes and PTMs among a broad panel of signaling proteins, to identify proteomic changes within liver samples of core-transgenic mice with or without PA28γ expression[70]. VTI1A, a vesicular transport associated factor, was subsequently identified as a novel player in HCV budding, whose expression is regulated by the interplay between core protein and PA28γ.

4. Innate host immune responses against HCV—During HCV infection, hepatocytes respond to viral invasion by secreting type 1 interferons (IFN-α and IFN-β) which play a key role in the first steps of host anti-viral responses against infection. IFNs induce the expression of several interferon-stimulated genes (ISGs) that restrict viral replication and also trigger the stimulation of a future HCV-specific adaptive immune response[71]. In the past, genome-wide RNAi screens were traditionally used to identify upregulated effectors of anti-viral responses against HCV[710]. However, proteomic approaches are powerful tools for studying the interactions between viral proteins and the host innate immune responses (Figure 1). Several host effectors of these responses are dependent on PTMs, undergone changes in their intrinsic activities, relocalized to particular sub-cellular compartments and assemble/disassemble into protein complexes[72] –all these processes remaining not detectable by transcriptomic analysis. In 2008, a proteome-wide view of 1134 interactions between HCV and human proteins was made by using data mining and two-yeast hybrid system. The results of this study suggested that HCV core is a major instigator of the Jak-STAT and TGFβ pathways[73]. Another study, using affinity purification and MS, analyzed 70 viral open reading frames (viORFs) from 30 different viruses, identifying 579 host proteins targeted by the viORFs, One such host protein ubiquitin-specific peptidase 19 (USP19), a protein involved in the unfolded protein response and in cell proliferation, was found to interact with NS5A[74]. It was shown that the NS5A-USP19 interaction induces USP19 relocalization to HCV replication compartments, thus inhibiting cell proliferation and promoting viral persistence through perturbation of cell metabolism. In addition, another proteome screen found that HCV NS3/4A protease is able to interact with

transport proteins involved in nucleocytoplasmic transport and can block STAT1 nuclear accumulation, thus highlighting a novel mechanism for immune evasion[45].

III. Applying proteomics to the study of HCV in more physiologically relevant systems

The development of replicon systems—Proteomics approaches have emerged as useful tools to improve our understanding of viral replication. However, for a long time, interactome studies were not possible due to the lack of cell culture systems for HCV, as serum-derived HCV particles poorly replicated in hepatoma cell lines and in primary human hepatocytes[75]. In 1999, Huh7 cell lines were generated that constitutively expressed a subgenomic HCV RNA derived from a cDNA isolated and cloned from the liver of a chronically infected patient with a genotype 1b strain[76]. This HCV RNA replicon was called "subgenomic" as only the sequence encoding the HCV non-structural proteins (from NS2 or NS3 to NS5B) was cloned. This allowed for HCV replication to be studied in hepatoma cell lines, as it was observed that HCV RNA replication did not require the presence of structural proteins as for several other *Flaviviridae*[7778]. This major breakthrough, along with i) the development of a full-length HCV replicon[79], ii) the identification of adaptive mutations within non-structural proteins that enhanced replication[80] and iii) the generation of highly permissive cell lines,[79] thus paved the way for the characterization of HCV replication and large-scale proteome analysis in Huh7.5 cell lines expressing a full-length HCV replicon (see above; Viral Replication). However, as this model only focuses on replication, the other stages of the HCV life could not be characterized (Figure 2).

The generation of HCV pseudo-particles—In 2003, the generation of the first HCV infection system gave way to major advances in our understanding of the HCV entry process. For the first time, this system allowed for the generation of HCV-like particles with functional HCV envelope glycoproteins incorporated onto retroviral or lentiviral core[81–83]. These HCV-like particles, called "HCV pseudoparticles" (HCVpp), were able to infect hepatoma cell lines and primary human hepatocytes *in vitro* by recapitulating HCV viral entry[8184]. HCVpp are produced by transfection into HEK293t cells of three expression vectors encoding i) E1E2 HCV envelope, ii) a retro- or lenti-viral core and iii) a packaging-competent retrovirus genome containing a marker gene such as Green Fluorescent Protein (GFP) or Luciferase[81]. These marker genes allowed HCV infection to be monitored and quantified via flow cytometry or luminometery. However, the HCVpp model harbors some important caveats, as it does not recapitulate the complex association between HCV particles and lipoproteins and does not allow for the study of the complete HCV life cycle (Figure 2). Thus, the HCVpp model offers limited opportunities for proteomic studies, as it is restricted to analysis of interactions between HCV envelope glycoproteins and cell host factors.

The HCVcc and HCVpc system—In 2005, cell culture-derived HCV (HCVcc) was developed using a full-length RNA genome derived from the JFH-1 (Japanese Fulminant Hepatitis 1) replicon[85]. Following transfection into highly permissive Huh7-derived cell clones, JFH-1 was able to efficiently replicate in cell culture without any adaptive mutations, leading to the production of infectious viral particles both *in vitro* and *in vivo*[3–5]. These particles had a specific tropism for hepatocytes and were associated with lipoprotein components, thus presenting the opportunity to study the early steps of virus attachment

(which involves lipoproteins), assembly and egress (Figure 2). HCVcc particles were found to have a lipid composition similar to VLDL and LDL[32] and consistently associated with lipoprotein components apoC-I and apoE, as determined by immunoprecipitation assays[86–89]. Over the years, the HCVcc system has been improved by selection for adaptive mutations that improve viral production[9091], the development of Huh7-derived highly permissive cell line clones[3 5], the construction of JFH-1 chimeric genomes[392] and novel full-length HCV clones from other genotypes[9394]. The few studies that have used proteomic approaches with the HCVcc system have been dedicated to studying the impact of the HCV life cycle on oxidative stress, cellular homeostasis or on metabolism pathways such as glycolysis and lipogenesis[9596]. However, despite its innovative features, the HCVcc system has been under-utilized by proteomic analysis in comparison to the replicon systems, especially regarding the characterization of viral assembly and egress. This could possibly be due to concerns about the use of Huh7-derived cell lines as HCVcc producer cells. Huh7-derived cell lines have some defects in their lipid metabolism, likely affecting the association of viral particles with lipoprotein components and thus virus assembly. Indeed, infection of chimeric mice or chimpanzees demonstrated that the buoyant densities of HCVcc particles are lower in animal models than in Huh7-derived cell culture system[97]. Moreover, the degree of HCVcc association with apoB remains unclear[19329899]. Other concerns center around particular features of Huh7-derived cell lines, such as their deregulated gene expression,, defective immune response and abnormal proliferation[100101]. Altogether, the use of Huh7 as HCVcc producer cells proved an obstacle in performing relevant proteome screens to decipher viral assembly and association with lipoproteins. A response to these concerns is the long term culture of primary human hepatocytes (PHH) in which HCVcc can replicate at an efficient level. These cultures present all the cellular marker of human hepatocytes and were able to mimic liver architecture *in vitro*[99102103]. HCV particles produced in PHH, or HCVpc (Figure 2), have a lower buoyant density than HCVcc particles that is more consistent with the buoyant density of serum-derived HCV particles. Interestingly, HCVpc particles from the VLDL fraction were highly associated with ApoB, whereas ApoB was undetectable in the HCVcc VLDL fraction, confirming that Huh7-derived cell clones are unable to secrete authentic VLDL[99]. Proteomic analysis of PHH infected with HCV remains challenging as the frequency of infected cells is usually low and differences in protein expression may not become apparent in pools of naïve, bystander and HCV-containing hepatocytes.

Proteomic screens using this system, combined with transcriptomic screens, could likely improve our understanding of HCV assembly and viral particle production. More recently, another HCV cell culture system using hepatocyte-like cells derived from human induced pluripotent stem cells (iPSCs) has been developed[104]. These cells support the entire HCV life cycle and may also represent a valuable model for proteomic analysis as unlimited quantities of cells from defined genetic backgrounds can be generated, improving interexperimental reproducibility.

HCV humanized mice as a new step for HCV-related proteomic screens—Due to the ethical and financial limitations restricting the use of HCV research in naturally-susceptible animal models such as chimpanzee and tree-shrew (*Tupaia belangeri*), small and

cost-effective animal models for HCV research are critically needed. While a popular model for biomedical research of other pathogens, rodents do not support HCV infection[105]. This restriction does not occur at the level of viral assembly or egress but rather during entry and replication[106]. Thus, in order to render rodents susceptible to HCV infection, they must be humanized, either through the engraftment of human liver cells (simultaneously with or without human immune cells) or via genetic humanization. During the past decade, several human liver chimeric mice have been developed and proven relevant to the study of HCV infection (Figure 2), such as the immunodeficient uPA-SCID[107–110] or the *Fah*$^{-/-}$ *Rag1 or 2*$^{-/-}$ *IL2R*γ^{NULL} models[107111–113]. These models harbor either a genetic defect *(Fah−/−)* or a hepatotoxic transgene (uPA), which enables the selective destruction of murine hepatocytes and promotes engraftment of human hepatocytes. Infection of these mice with HCVcc or serum-derived HCV particles were productive[110111114] and can even be neutralized by anti-receptor antibodies[115–117] or with DAAs[118]. However, these mouse models lack a functional immune system, hampering assessment of virus-host interactions.

To counteract these limitations, mice can be co-transplanted with both adult human hepatocytes and CD34+ hematopoietic stem cells to allow the development of chimeric mice with a humanized liver and immune system. The AFC8-huHSC/Hep mice have been shown to support infection with serum-derived HCV particles and to establish a specific T cell response against HCV. However, no B cell response or viremia could be detected[119]. Other similar models have been developed such as the double humanization of NOD-background mice[120]. However, no studies have characterized HCV infection within these mice so far. In addition to xenografted mice, genetically humanized mice can be used to study HCV infection *in vivo*. It was demonstrated that adenoviral expression of the HCV entry factors CD81 and occludin (OCLN) facilitates HCV uptake into hepatocytes of Rosa26-Fluc mice. However, in this model a combination of murine innate and adaptive immune responses limited HCV replication, so *de novo* viral particle production and release were not observed[105]. To overcome this restriction, transgenic mice were created expressing human CD81, SCARB1, CLDN1 and OCLN on genetic backgrounds with impairments in antiviral immunity. Mice lacking *STAT1* allowed persistent, low-level replication of HCV, production of *de novo* infectious viral particles and the development of B- and T-cell specific responses[121], thus making these mice the first opportunity to study HCV life cycle in an immune system context *in vivo* (Figure 2). The model is currently being refined to increase the robustness of infection. ICR-background mice have also been humanized with human CD81 and OCLN. Following infection with HCV, sustained viremia was observed for 12 months as well as hepatopathologic manifestations of infection[122]. However, viral loads in serum were lower than in humanized Rosa26-Fluc mice and no anti-B cell and T-cell responses were reported.

Due to their ability to produce HCV particles similar to patient-derived particles and to partially recapitulate HCV-host interactions in an integrative context, humanized mice represent a promising portal for further relevant, cost-effective proteomic studies *in vivo* on different primary materials such as immune cell subsets, mice-derived serum and infected-liver tissues. In the future, the development of relevant double humanized mice model, along with the development of mice models that harbor a more functional human immune system,

could provide a unique platform to uncover novel aspect of HCV-host interactions (Figure 2).

IV. Clinical proteomics: From fundamental knowledge to bedside care

Proteomics as a diagnostic tool—Beyond the use of proteomic approaches as a way to decipher the HCV life cycle, proteomics can also be a valuable tool for clinical diagnostics. Indeed, by infecting the liver, HCV infection induces several metabolic disorders which produce specific signatures on several cellular pathways involved in glycolysis, lipogenesis and cellular stress. These signatures, which may reflect viral-induced pathogenesis and liver disease, represent interesting targets for proteomics, thus allowing for the development of new tools for patient diagnosis and treatment strategies. Several studies have reported the use of proteomics to identify and characterize HCV-induced metabolic changes. In 2007, proteomic alterations reflecting liver disease progression were first reported. Through stable isotope labeling and accurate mass and time (AMT) tag approach, 210 proteins within HCV-infected liver tissues were identified whose expression levels correlated with fibrosis[123]. It was shown that impairment of specific mitochondrial processes, including fatty acid oxidation and oxidative stress, reflects fibrosis progression. Three years later, the same group used LC-MS (liquid chromatography-mass spectrometry) to perform a new proteomic and lipidomic screen of HCVcc-infected Huh-7.5 cells. They showed that temporal perturbation in glycolysis pathways, the metabolism of several lipid species and lipotoxic ceramide species are signatures of viral replication, cellular stress and direct cytopathic effects[95]. They also found specific metabolic signatures reflecting high replication rate and cellular stress and confirmed the role of specific mitochondrial processes as key markers of HCV-induced metabolic disorders and disease progression. Thus, mitochondrial proteins appear as attractive markers of HCV-induced metabolic disorders.

HCV core has been previously shown to be implicated in HCV-induced oxidative stress[124] and some proteomic screens have focused on identifying core-interacting host partners within hepatocytes[125]. Consequently, some screens aimed to uncover specific interactions between HCV core and mitochondrial proteins. Through the use of 2DE and MS, a screen identified prohibitin as up-regulated in full-length HCV replicon cells and in the liver of core-expressing transgenic mice. Authors proposed that core destabilizes the interaction between prohibitin and the mitochondrial DNA-encoded subunits of cytochrome c oxidase (COX), which may favor oxidative stress[126].

Very recently, a HCV-host proteins interaction screen within hepatoma cells unveiled an interaction between the mitochondrial protein within bgcn homolog (WIBG) and HCV core through combination of affinity purification/mass spectrometry with RNAi knockdown. This interaction was shown to negatively impact the nonsense-mediated mRNA decay pathway, thus leading to an accumulation of damaging transcripts within infected cells[127]. Studies have also reported the identification through 2DE of several low-abundance serum proteins (such as several complement proteins and some apolipoproteins) to be correlated with liver fibrosis[128129]. Finally, as described above, the quantification of apolipoproteins A-I or B-100 within serum and their association with lipoproteins can also be a relevant marker of HCV infection[3537].

Proteomics as a prognostic tool—Similar to its ability to characterize disease progression, proteomics can also be applied to the detection of markers reflecting disease progression or treatment outcome. Using LC-MS on a cohort of chronically infected patients who did or did not develop sustainable virological response (SVR) following pegylated-IFN and Ribavirin treatment – as well as a reference cohort carrying a polymorphism predictive of SVR (IL28B)[130]– a serum-based protein signature able to predict treatment response was identified in correlation with patient IL28B polymorphism, viral genotype and stage of liver fibrosis[131]. Similarly, surface-enhanced laser desorption ionization TOF MS (an ionization method allowing the study of protein mixtures through MS) was also used to identify a temporal protein signature predicting SVR in infected patients treated with pegylated-IFN/Ribavirin[132]. Another study using 2DE-MS identified 21 proteins, involved in IFN-mediated antiviral immunity, stress response and energy metabolism, as highly predictive markers of SVR in patients[133]. Interestingly, some studies also identified PTMs associated with treatment outcome[133134], highlighting PTMs as a putative prognostic marker. For example, differential phosphorylation state of six distinct proteins (such as AKT or JAK1) was found to correlate with SVR in patients[134]. Beyond responsiveness to treatment, several proteomic analyses have also been conducted to identify predictive markers for the development of severe liver injuries in HCV-infected patients such as liver fibrosis or HCC. Likewise, molecular signatures have been defined with proteomic approaches to predict early progression to fibrosis in HCV-infected patients. Analysis of HCV-infected patient biopsies at different times following liver transplantation revealed 250 proteins differentially regulated in patients displaying a rapid progression to fibrosis. Authors highlighted a link between elevated level of oxidative stress-associated metabolites in serum and development of severe liver injury[135]. Finally, studies have also identified prognostic markers of HCC. MS profiling of plasma from cirrhotic and HCC patients found osteopontin was upregulated in HCC patients[136]. Another study, based on the finding that Ku86 protein (a multi-function nuclear protein) is upregulated within HCC tissues, showed that the level of anti-Ku86 antibodies is upregulated within HCC patients, suggesting these antibodies may be a prognostic marker of HCC[137]. In conclusion, when combined with other markers (e.g. viral genotype, IL28B polymorphism, etc.), proteomics represents a unique and valuable approach to efficiently predict disease and treatment outcome during HCV infection.

Concluding remarks

Several mechanisms regulating protein expression and activity are not detectable using transcriptomic analysis, such as change in enzymatic activity, PTMs, assembly of protein complexes, and sub-cellular relocalization processes. However, these mechanisms may be critically important during viral infection by modulating cell host metabolism, triggering innate immune responses, and impacting disease progression or treatment outcome. Thus, proteomic approaches appear as valuable tools to decipher the interaction between a virus and its host and improve patient care and treatments. Here, we have shown that HCV constitutes a relevant proof of principle on how proteomic approaches can first, improve our understanding of virus biology and second, highlight the advantages and limitations of a given experimental system. Humanized mouse models present an opportunity for further

quantitative and temporal proteomic screens *in vivo*, which may uncover a new dimension of HCV-host interactions.

Beyond HCV, little is still known about the biology and host-virus interaction of other members of the *Flaviviridae* family, such as the flaviviruses Dengue Virus, Yellow Fever Virus and West Nile Virus. More importantly, no therapies are currently available against these viruses, which represent major health concerns worldwide. The current knowledge accumulated over the past decades on HCV-host proteins interactions represent a precious source of information for uncovering important host determinants of flavivirus infection, as suggested by interactomes overlaps between HCV and Dengue Virus[68].

Importantly, the recent development of novel proteomic methods such as the affinity capture of tagged viral proteins to analyze particular cellular compartments[56], the immuno-competitive capture of viral proteins to reduce off-target effects[44] or the analysis of viral RNA-bound host proteins through the specific capture of viral RNA[47], can represent valuable approaches to comprehensively decrypt the different stages of flavivirus' life cycle.

Alternatively, the recent application of proteomic approaches for quantifying proteome evolution during a virus infection *in vitro* (quantitative temporal viromics)[138], as well as the development of the ratiometrix apex tagging[139] which allows the proteome of previously inaccessible sub-cellular compartments to be examined, may also represent new opportunities to refine our understanding of HCV infection.

In the near future, taking advantage of the proteomic achievements in virology and of the continuous technological improvements of these methods will be crucial to pave the way toward a better characterization of the biology of challenging human tropic viruses, as well as for implementing effective anti-viral strategies against them.

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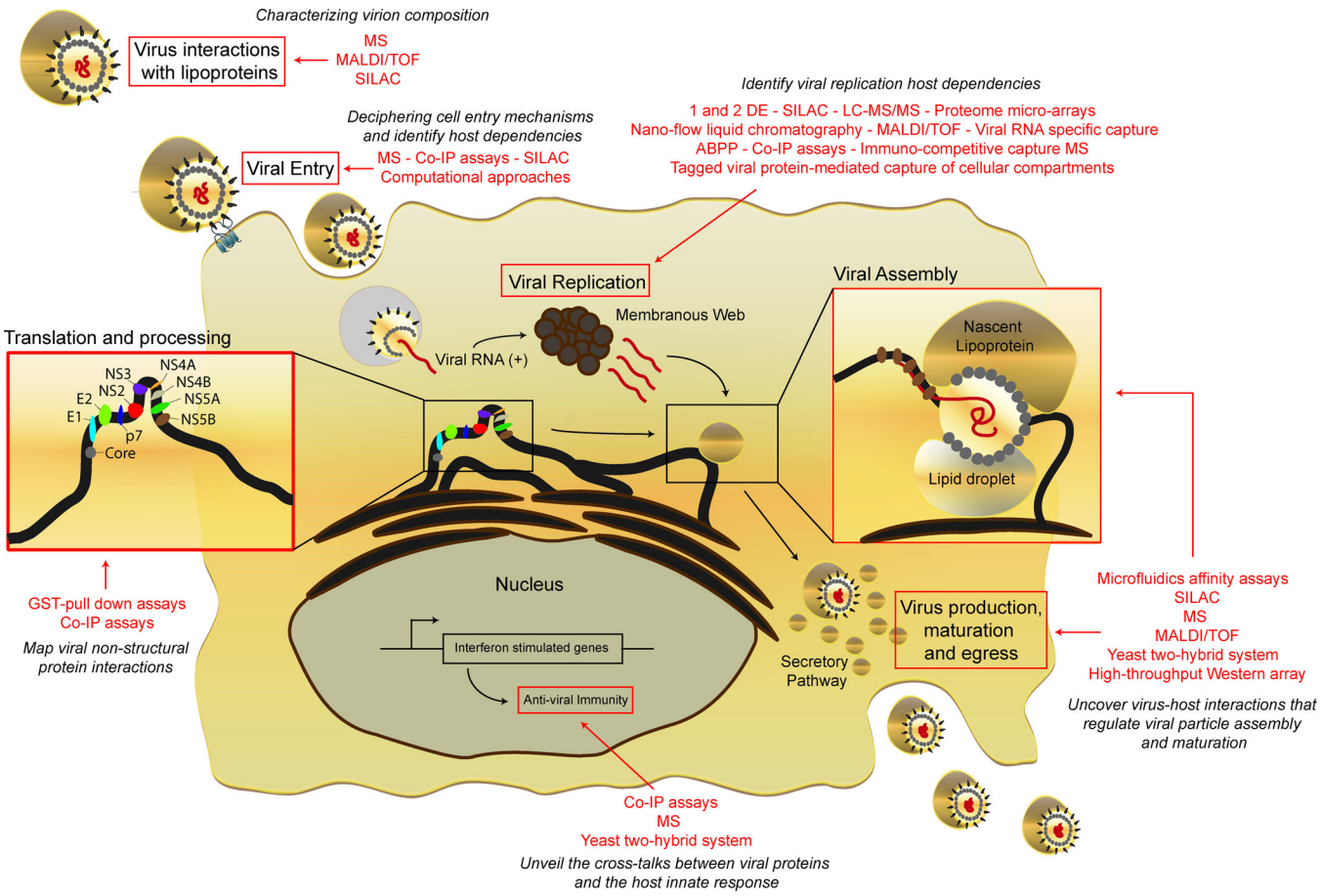

Figure 1. Proteomic approaches to decipher HCV life cycle

The HCV life cycle steps targeted by proteomic approaches are presented. Following virus entry, viral RNA is released into the host cytosol and translated as a large polyprotein (polyprotein translation). Non-structural proteins processing (polyprotein processing) then allow the formation of viral replication complexes (membranous web) and production of new viral RNA copies (viral replication). Viral particles are then assembled into ER-derived membranes through tight interactions with lipid metabolism (viral assembly) before viral particle maturation and particle budding (virus production maturation and egress) out of the cells via the secretory pathway. Red arrows link the different HCV life cycle steps (red rectangles or squares) to the proteomic tools (written in red) used to decipher these steps. RNA translation and viral assembly steps are enlarged. The fundamental purpose for using proteomic approaches for each step of the HCV life cycle is mentioned in italic. In addition, proteomic approaches used to decrypt virus association with lipoproteins, as well as to characterize virus cross-talks with host innate immune responses are also presented. MS, Mass spectrometry; MALDI/TOF, Matrix-assisted laser desorption /ionization time of flight mass spectrometry; SILAC, Stable isotope labeling by amino acids in cell culture; Co-Ip, Co-immunoprecipitation; 1/2DE, 1 or 2 dimensional electrophoresis; ABPP, Activity-based protein profiling; LC, Liquid chromatography.

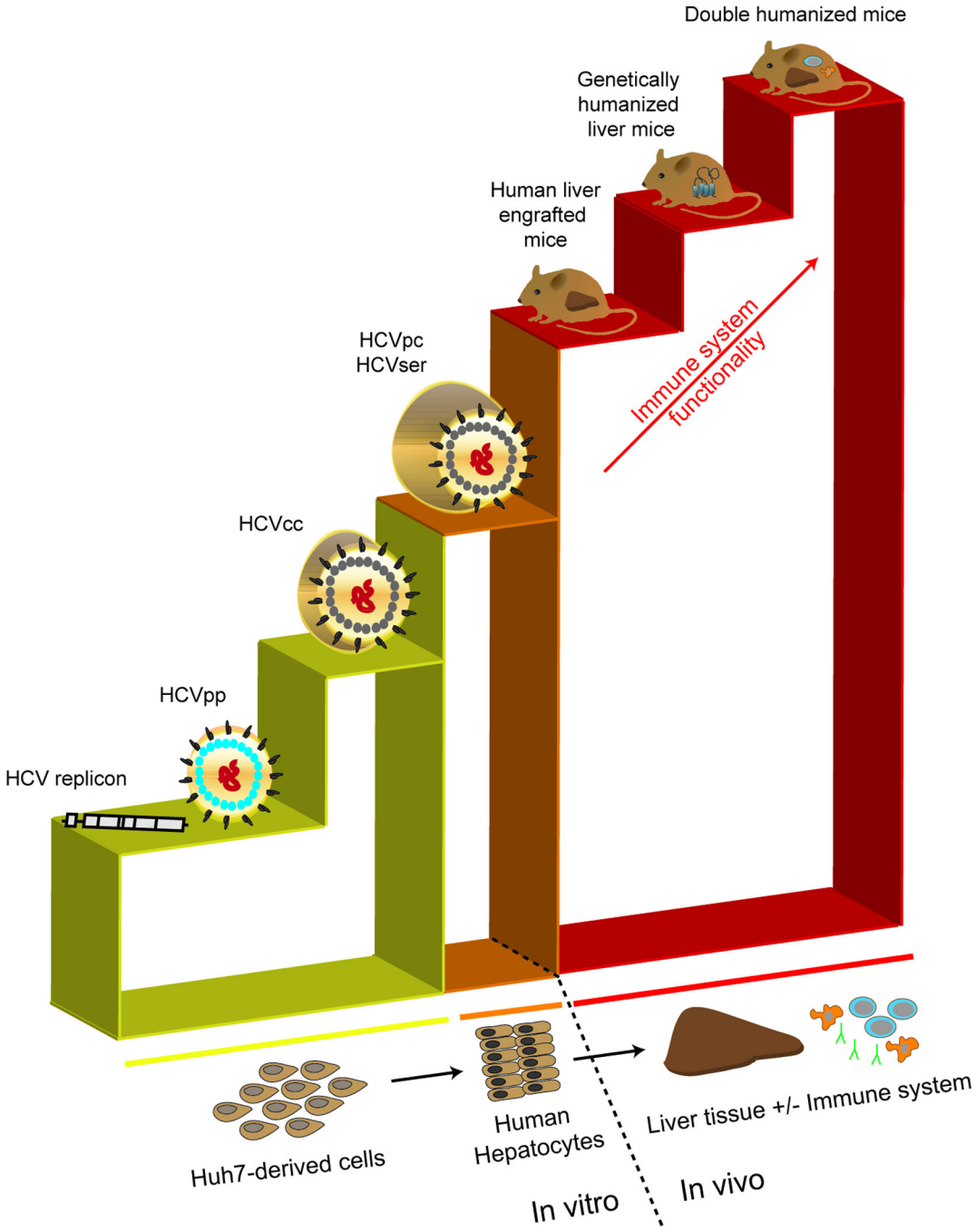

Figure 2. Increasing complexity of HCV experimental models

A three dimensional stair symbolizes the increase in complexity of the different HCV experimental systems currently used. In front of the stair are shown the major cells or tissue(s) associated with each experimental system. The yellow steps represent in vitro system using Huh7-derived cells for the study of specific steps of the HCV life cycle (viral replication, replicon; virus entry, HCVpp). The orange steps represent the in vitro systems using human primary hepatocytes (PHH) or hepatocyte-like cells derived from iPSCs infected by HCVcc particles (then named HCVpc after passage in human hepatocytes) or by

serum-derived HCV particles (HCVser). The red steps represent the current and future in vivo models used to study HCV infection. The first model relies on the use of immunodeficient mice engrafted with human hepatocytes (Human liver engrafted mice) although the second model relies on the use of mice expressing human HCV host factors but harboring defect in their host innate immune response (Genetically humanized liver mice). The final model (still under development) represents HCV-permissive, fully immunocompetent mice able to develop persistent infection similar to human (Double humanized mice). HCVpp, HCV pseudoparticle; HCVcc, HCV cell culture; HCVpc, HCV primary cell culture; HCVser, serum-derived HCV particle.