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Abstract: Hepatitis C virus (HCV) is a positive-strand enveloped RNA virus and belongs to the Flaviridae family. The heavy health burden associated with the virus infection in humans and the intriguing peculiarities of the interaction between the HCV replication cycle and the hepatocyte host cell have stimulated a flourishing research field. The present review aims at recapitulating the different viral and cellular systems modelling HCV entry and replication, and in particular at gathering the tools available to dissect the HCV entry pathway.

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Hannover, March 14th 2012

Dear Editors,

Thank you very much for your invitation to submit a manuscript to the Methods issue entitled “Methods to study RNA virus molecular biology”. As discussed previously with Pr. Semler, our review, “Entry and replication of recombinant hepatitis C viruses in cell culture”, describes the viral and cellular models for hepatitis C virus entry and replication with a special focus on the main tools available to dissect the HCV entry pathway. We hope the quality of the submitted manuscript reaches your expectations and the requirements of the Methods journal.

Please note that we would like to incorporate in the main text a link for a TCID₅₀ calculator (Excel sheet). This document was obtained from Marco Binder, Heidelberg, who agreed to make it freely accessible on the Heidelberg website. This will however take a few weeks and therefore we planned to add this link in the revised manuscript.

For your convenience, we have listed, in the submission website, a number of internationally renowned experts in the field of HCV cell-culture systems and entry, as potential referees.

Best regards,

Gabrielle Vieyres

Title

Entry and replication of recombinant hepatitis C viruses in cell culture

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Abstract

Hepatitis C virus (HCV) is a positive-strand enveloped RNA virus and belongs to the *Flaviridae* family. The heavy health burden associated with the virus infection in humans and the intriguing peculiarities of the interaction between the HCV replication cycle and the hepatocyte host cell have stimulated a flourishing research field. The present review aims at recapitulating the different viral and cellular systems modelling HCV entry and replication, and in particular at gathering the tools available to dissect the HCV entry pathway.

Keywords

Hepatitis C virus, HCV, cell-culture systems, entry, reporter virus, cell lines, fusion

Entry and replication of recombinant hepatitis C viruses in cell culture

1. Introduction

1.1. Gathering tools to study an elusive virus

The evidence for viral non-A non-B hepatitis dates back to 1975 [1]. The virus responsible for this disease, later called hepatitis C virus (HCV), currently infects chronically around 160 million people worldwide [2]. These are at risk of developing serious liver disease including hepatic steatosis, fibrosis, cirrhosis and ultimately hepatocarcinoma [3]. From the beginning, HCV turned out to be a real challenge for scientists. The molecular identification of the etiologic infectious agent responsible for hepatitis C took 14 years and was finally achieved by Michael Houghton's lab, at Chiron, in 1989 [4]. In addition to that, the difficulty to propagate the virus in cell culture frustrated researchers in the domain for another 16 years, until 2005 [5-7]. However, these roadblocks in HCV research were not fruitless. A panel of tools were generated to circumvent the absence of a cell-culture model sustaining the complete viral life cycle and to study HCV without actually having the virus in a test tube. For instance, the chimpanzee model was established before the identification of the virus [8], and retroviral pseudoparticles [9-11] or replicons [12] were developed before the cell culture model. These surrogate systems allowed to study individual steps of the virus life cycle or to test the first antivirals. Interestingly, the generalization of the cell culture model for HCV did not render these tools obsolete. They remain complementary options in the scientist's toolbox and are still widely used to dissect independent steps of the HCV replication cycle, pathogenesis, host response or as a first screening approach.

The goal of this review is to present the main methods to study the early steps of HCV replication. Due to the large scope of this topic, the review will be principally focused on entry investigation and restricted to cell-culture models. For theoretical and technical details on the replicon system, we invite the readers to consult the following reviews [13, 14]. Moreover, animal models for HCV research are reviewed by Ploss and Rice in another article of this issue.

1.2. Molecular virology of HCV

HCV is small and enveloped. It belongs to the *Flaviviridae* family and the hepacivirus genus [15]. The HCV genome is a short (~9.6 kb) positive-strand RNA organized into three parts: a unique open reading frame (ORF) surrounded by 5' and 3' untranslated regions important for RNA replication and translation. The HCV ORF encodes a polyprotein precursor of around 3,000 amino acids, that is targeted to the endoplasmic reticulum (ER) where it gets processed by cellular and viral proteases into 10 or 11 mature functional proteins, whose key functions are depicted in Figure 1 [16,

17]. Schematically, by analogy with other *Flaviviridae*, these proteins have been assigned to two groups, the structural and non-structural proteins. Note that the detailed composition of the virus particle is not reported yet and therefore that the possible incorporation of some so-called “non-structural” proteins, such as p7 and NS2, in the viral particle is still matter of discussion. Also relevant is the distinction of functional modules within HCV polyprotein (Figure 1). Thus, the minimal replication module has been described in the early stages with the development of HCV replicons, and consists of the NS3 to NS5B proteins, both necessary and sufficient for RNA replication [12]. The entry module contains at least the capsid (Core) and envelope (E1 and E2) proteins. Finally, the assembly module is more difficult to describe since *Flaviviridae* use, in addition to the structural proteins that constitute the bricks for assembly, some of the non-structural proteins to orchestrate this process [18, 19]. The overlap between the different modules illustrates the multifunctionality of HCV proteins, a solution for the challenges associated with a very small genome.

1.3. HCV in the infected patient

Considering its genome structure, HCV may appear very simple. However, the molecular virology of HCV cannot resume its biophysical and biological properties. Its interaction with its host cell indeed leaves a permanent imprint on the virion and influences all the steps of the viral replication cycle. The complexity of the host and of the virus/host interplay makes any attempt to faithfully mimic HCV growth in cell culture extremely challenging.

First of all, consistent with its main clinical manifestations, HCV exhibits a strong tropism for the liver and mainly replicates within hepatocytes. Hepatocytes are very specialized host cells and metabolic specialists [20]. They exhibit a complex and peculiar polarity with several basal and apical poles [21], a feature that is generally not recapitulated by hepatic cell lines. While apical poles of adjacent hepatocytes form a network of bile canaliculi, the basal poles face the liver sinusoids where they secrete various proteins and lipids. In particular, hepatocytes secrete lipoproteins and are the only cells capable to pack and release in the bloodstream lipids within very-low-density lipoproteins (VLDL) [20]. It seems that HCV can hijack this function for its own assembly as it circulates in patient serum in association with lipoproteins [22, 23]. Moreover, host factors crucial for production of VLDL like apolipoprotein E, apolipoprotein B and microsomal triglyceride transfer protein have been found to be important for production and release of infectious HCV particles [24-29]. The association of HCV with lipoproteins influences cell entry with the involvement of several lipoprotein (LDL receptor) [30, 31] and lipid receptors (Scavenger receptor class B type I, SR-BI; Niemann-Pick C1-like 1 cholesterol uptake receptor, NPC1L1) [32, 33]. It might also contribute to protect the virus from the host humoral immune response. It seems moreover that a fraction of patient-derived HCV is attached to immunoglobulins or to molecules of the complement [22]. Finally, the intrinsically high error-rate of HCV RNA polymerase, combined with a high level of viral replication and under

pressure of the host immune response, is responsible for the high virus variability. As a consequence, HCV isolates evolved into 6 major genotypes, further divided into numerous subtypes [34]. These genotypes differ in their response to treatment and disease severity [35, 36]. This adds a level of complexity to HCV research as HCV properties cannot systematically be inferred from the study of a single isolate. Last, clinical isolates are very poorly infectious in cell culture and only a very limited number of strains allow reverse genetics (see Section 2.2). Overall, this complexity of HCV biology and its primary host cell – the hepatocyte – may explain the difficulty to establish cell culture models that would recapitulate at once all aspects of the HCV replication cycle and all HCV properties. As a consequence, a panel of systems have been generated with various benefits and drawbacks. These models gain when being combined to give us the currently best available picture of HCV entry and replication.

2. Theory on the choice of an entry system

2.1. Overview on the viral entry systems

Many systems have been developed to study HCV entry. Serum-derived HCV particles replicate extremely poorly in cell culture, precluding the detection of infectivity and therefore entry studies with traditional assays. Also, until 2005, there was no reverse genetics system for HCV. One requirement to build virus surrogates for entry studies is a basic knowledge of the viral factors involved. HCV only encodes two envelope glycoproteins, E1 and E2 (for a review on E1 and E2 glycoproteins, see [37]), and one capsid protein, Core, which could make the story short. However, the precise structure of these proteins is still unknown and their exact function in assembly and entry is unclear. The picture gets even more complex with the possible role of host factors incorporated into the viral particles. Indeed, virus-associated lipoproteins are now presented at the front line of entry, as possible attachment or entry factors and also potential modulators of virus fusion [23]. Despite these difficulties, related viruses, easier to cultivate, for instance bovine viral diarrhoea virus, have been used as surrogate models [38]. Secondly, recombinant forms of the envelope glycoproteins were used to search for viral receptors [33, 39] but also as probes for antibody screening or neutralization of binding assays [40, 41], to study HCV-induced receptor signalling during entry [42], to delineate E2-receptor interacting surfaces [43, 44], or to draw a theoretical model for E2 structure [45]. Most frequently a soluble form of E2 produced in mammalian or insect cells and capable of inhibiting HCV entry [46] is used. A more elaborated system to study HCV glycoproteins properties is the reconstitution of full-length unmodified E1E2 into liposomes [47]. In addition to that, different viruses can be used as a scaffold for E1E2 glycoproteins, a concept called pseudotyping. The first HCV pseudotypes elaborated were based on modified influenza [48] or vesicular stomatitis virus viruses [49-52]. However, to our knowledge, except for one report [49], only chimeric glycoproteins,

consisting of the ectodomains from E1E2 and both the transmembrane domain and cytoplasmic tail from the heterologous virus, could be incorporated into the pseudovirus envelope. The retroviral HCV pseudoparticle (HCVpp) system was the first described to allow incorporation of non-modified glycoproteins on its surface [9-11]. Reported in the same year by three independent teams, this system overcame the other HCV pseudotypes and is still widely used despite the spread of the cell-cultured HCV (HCVcc) system. More recently, transcomplementation of HCV genomes into infectious particles was described as a new system to study assembly determinants [53] but the transcomplemented HCV particles (HCV_{TCP}) produced are also useful tools to study entry and replication, in absence of virion production. In the next paragraph, we will focus on those three systems which are nowadays the most frequently used to study HCV entry, namely HCVcc which recapitulate the whole HCV replication cycle, but also HCVpp and HCV_{TCP} which allow to focus on a single-round infection (Figure 2).

2.2. The cell-cultured HCV (HCVcc) system and derivatives

HCVcc are authentic HCV particles initially produced by hepatoma cell lines transfected with *in vitro* transcribed HCV RNA derived from the JFH-1 clinical isolate [5-7]. They are infectious in cell culture as well as in chimpanzee and humanized mice [5, 54]. Methods to produce HCVcc have been published elsewhere [55, 56]. Additionally, three other strains, H77-S (genotype 1a), Con1 and NC1 (genotype 1b), were found capable to produce infectious virions in cell culture [57-59]. The H77-S sequence corresponds to the prototype H77 strain with five engineered replication-enhancing mutations [60, 61]. The titers yielded by this virus are however moderate (10-100 times lower than JFH-1 virus, with a maximal titer of 10³ FFU/ml obtained with a particular Huh-7-derived subclone and an extra titer-enhancing mutation [61]). Moreover, the specific infectivity is 400-times lower than that of cell-culture derived JFH-1 [59]. A very recent publication reports the production of small amounts of infectious particles (in the range of 10² FFU/ml) from the NC1 isolate incorporating several replication-enhancing mutations, but without virus propagation [57]. The strategy was different for the establishment of Con1 virus culture, where most replication-enhancing mutations precluded the production of infectious particles *in vivo* and in cell culture [58]. With little or no replication-enhancing mutation, some infectious particles could however be recovered from transfected cells, as evidenced by infection of mice with a humanized liver. Unfortunately, the poor replication efficiency of the non-adapted Con1 genome hindered the detection of infectivity in cell culture and prevented virus titration.

Due to the restricted number of cell-culture infectious HCV clones, production of viruses from different genotypes and strains mostly relies on chimeric genomes incorporating the structural proteins from the heterologous strain but the JFH-1 replication module. Construction of such chimeras and optimization of the junction sites have been investigated and reviewed elsewhere [62-64]. Currently,

chimeras are available with the structural proteins of representatives for all reported HCV genotypes [6, 62, 64-68]. Interestingly, some constructs such as the FL-J6/JFH-1 [6] or the Jc1 [62] chimeras actually spread more efficiently than the original JFH-1 clone, and tend to replace it in HCV research laboratories due to the higher titers reached as compared to the parental JFH-1 strain (up to 1,000 fold increased titer for Jc1). However, the extent of reverse genetics opportunities (for instance specific swapping of the glycoprotein-coding sequences) is limited by the pleiotropic effects that the modifications may have on the RNA structure and therefore replication, on structural protein processing or on virus assembly, sometimes precluding efficient production of infectious particles and entry investigations.

A further development to the HCVcc system is the incorporation of reporter genes that allow easy and rapid quantification of infectivity [69, 70]. The main reporter viruses are listed in Figure 3. Note that while luciferase viruses allow easy-to-measure quantification of infectivity [63], fluorescent protein-tagged viruses permit infectivity measurement at the single cell level. However, such modified viruses are often not as stable as the original virus in cell culture which might lead to the loss of the reporter gene expression. They also often do not spread efficiently in cell culture [69]. These tools are therefore extremely useful for transient assays but not appropriate to follow the virus spread and evolution in culture along a long time period [63]. For the same reason, it is recommended to harvest reporter virus stocks directly from transfected cells rather than after cell passages (unpublished data). Finally, reporter cell lines that complement the panel of tools to assess HCV infectivity are described in Table 1.

Based on the HCVcc system, it is possible to assay the whole replication cycle of HCV and in particular to design reverse genetics studies but also perturbation studies such as testing potential antivirals or assessing the role of cellular or viral factors in HCV replication cycle with specific chemical or genetic tools. Reporter viruses make such assays adaptable to high-throughput screening, with obvious advantages for antiviral research [76, 126, 129-131]. Figure 4 illustrates a possible assay setup based on a dual reporter system, typically the *Gaussia/Renilla* and firefly luciferases. Note that these two types of luciferases utilize different substrates and therefore their activity can be monitored independently within one sample. In our laboratory [126], we used a permissive hepatoma cell line (Huh-7-Lunet-hCD81) constitutively expressing and secreting the *Gaussia* luciferase, as a marker for cell number and viability along the protocol. These cells can be infected with a firefly luciferase reporter chimeric virus (Luc-Jc1) (Option A) or transfected with the *in vitro*-transcribed corresponding viral RNA (Option B). The firefly luciferase activity in the cell lysate at early time points (typically at 48 hours post-transfection or post-infection, before virus spread can influence the results [62]) reflects the efficiency of the early stages of HCV infection, that is to say translation and replication of the viral genome, but also, in the case of Option A, virus entry. In addition, the cell supernatant can be harvested and used to infect naïve cells. The firefly luciferase activity in these target cells corresponds to the infectivity released by the producer cells and therefore reflects the complete HCV replication

cycle. With this system, it is therefore possible to monitor independently the efficiency of the early steps of HCV replication cycle (by normalizing the intracellular firefly luciferase activity with the secreted *Gaussia* luciferase activity, in the producer cells), as well as the late steps (by normalizing the intracellular firefly luciferase activity in the target cells with this activity in the producer cells). This system proved to be simple, cost-efficient and compatible with high-throughput screens (96- or 384-well dishes). With this strategy, we assessed the antiviral activity of a library of 123 natural compounds [126], 24 and 23 of which had an effect on HCV replication/translation or whole infectious cycle, respectively, while Reiss *et al.*, using different cell line and combinations of reporters, extracted 13 targets important for HCV entry or replication from a pre-selected panel of 178 siRNAs [76]. Typically, this type of assay gives a first indication as to the global efficiency of the replication cycle and distinguishes between its early and late steps. A panel of other tests allows further and closer dissection of the HCV replication cycle and entry in particular. Figure 5 illustrates the HCV entry process and the tools available for its study, whose description will be developed in the following sections of this review.

2.3. Retroviral HCV pseudoparticles (HCVpp): A specific entry system

Retroviral HCVpp are replication-deficient retroviruses harbouring non-modified HCV envelope glycoproteins instead of their own glycoproteins [9-11]. A comprehensive protocol for HCVpp production can be found in the review by Bartosch et Cosset [155]. Briefly, HCVpp are generally produced in human embryonic kidney (HEK) 293T cells co-transfected by three expression vectors (Figure 2). The first vector contains a packaging-competent retroviral minigenome encoding a genetic marker such as the luciferase, green fluorescent protein (GFP) or β -galactosidase. It also includes retroviral sequences necessary for reverse transcription and integration of the reporter gene in the infected cell genome. The second plasmid encodes the retroviral gag-pol precursor that will provide the virus core. Finally, the third plasmid encodes HCV glycoproteins. HCVpp offer several benefits over the HCVcc system. First of all, they can be used in S2 containment facilities, an undeniable advantage in particular for antiviral screens [156]. Secondly, they are flexible tools able to incorporate a collection of patient-derived glycoproteins [157, 158] (a detailed protocol for the generation of such HCVpp can be found in reference [159]), a feature particularly useful to characterize cross-neutralizing antibodies [158, 160]. Thirdly, HCVpp proved to be a relevant model to study the mechanisms and steps of virus entry, with for instance the description of HCV internalization [146, 147], of the cellular signalling cascades activated upon virus entry [141], or to reveal new receptors for the virus [116, 119]. On this last aspect, HCVpp can widen the extent of entry and receptor-complementation assays to cell lines that support low levels of HCV RNA replication, and therefore would not have allowed scoring entry with the HCVcc or HCV_{TCP} (see below) systems. This approach was successfully used to identify Claudin-1 (Cldn1) and Occludin (Ocln) as HCV

coreceptors [116, 119]. However, the predictive value of HCVpp has its limits. Indeed, HCVpp and HCVcc assembly differs largely. Firstly, HCVpp are generally produced in non-liver cells and therefore do not reproduce the virus association with lipoproteins, with a possible impact on antibody-mediated neutralization, entry dependence on the lipid receptors LDL-R, SR-BI and NPC1L1, and eventually fusion. Secondly, HCVpp presumably assemble as retroviruses, in post-Golgi compartments or at the plasma membrane with a rather unspecific envelope protein incorporation. As a consequence, substantial differences between HCVpp and HCVcc were found concerning their viral glycoproteins in term of glycosylation pattern and oligomerization [152, 161-163]. Moreover, the retroviral capsid precludes the use of HCVpp as a model to study uncoating. Altogether, although HCVcc are probably closer to the natural virus, HCVpp are still used as a “pure” system reproducing the entry event of HCV, independent on the downstream steps such as replication and assembly.

2.4. Transcomplemented particles (HCV_{TCP}): an HCV-based entry and replication system

Like HCVpp, HCV_{TCP} [53] are single-round infectious particles. However, they likely comprise the genuine HCV capsid and envelope shells, wrapped around an HCV subgenome. This system can be used to investigate HCV assembly determinants but also in infection assays to study HCV entry and replication independently of virus spread or production [126]. HCV_{TCP} are produced by transfection of an HCV subgenomic replicon (assembly-deficient but replication-competent HCV construct) in a so-called packaging cell line [53]. The packaging or helper cell line provides in *trans* the assembly factors missing in the subgenomic replicon. Typically they constitutively express HCV Core, E1, E2, p7 and NS2 proteins therefore restoring, by transcomplementation, the production of infectious particles containing the subgenomic replicon. With this method, and depending on the replicon used, virus preparations with titers and biophysical properties similar to full-length infectious clones can be obtained, under improved biosafety conditions. This system can furthermore accommodate a variety of reporter genes or labelled viral proteins in the subgenomic replicon, therefore allowing easy infection readouts. Furthermore, the inability of the replicon to produce infectious particles precludes virus spread and new rounds of infection, making infection readouts, even at late time points, independent on virus production. Figure 2 summarizes the properties of these main HCV surrogates. Note that, as mentioned before, the host cell leaves a physical and functional hallmark on the released particles, with potential impact on entry mechanisms. As a consequence, particular care must be taken when choosing not only the target cells of an entry assay but also the host cells used to produce the viral particles.

2.5. Host cells for the investigation of HCV entry

Theoretically, primary human hepatocytes (PHH) would be the *in vitro* system of choice to grow HCV and study HCV entry. However, PHH require stringent culture conditions to keep the differentiation and polarization features reminiscent of the hepatocytes in the liver environment. Moreover, permissivity for HCV infection might vary from one hepatocyte donor to another. For these reasons, robust PHH cultures and mostly their infection with HCV serum particles (HCVsp) or HCVcc seem to remain restricted to a few laboratories. The reader is referred to the reviews by Zhu *et al.* [164] and Pichard-Garcia *et al.* [86] for practical and theoretical details on HCV infection of PHH. Note that, due to their difficult handling and limited availability, PHH are in general used to validate results first obtained in hepatoma cell lines. Indeed, a variety of cell lines are now available to study HCV entry and replication (Table 1). A number of them are derived from the Huh-7 cell line, further selected for better HCV RNA replication (*e.g.* Huh-7 Lunet, Huh-7.5 or Huh-7.5.1 cell lines) or genetically modified to encode a reporter system allowing easy infectivity assay or to permit receptor complementation studies. Importantly, the tools are now available to study individually the 4 main HCV receptors (CD81, SR-BI, Cldn1 and Ocln): cell lines expressing very low levels of one of the receptors and retroviral vectors to rescue the expression of this receptor or one of its homologs have been developed in several laboratories [44, 101, 102, 104, 106, 116, 165] (see Table 1). Thanks to HCVpp, it is also possible to extend these receptor-complementation studies in cell lines that poorly replicate HCV, since the readout of HCVpp infectivity is independent on HCV replication [113, 116, 119].

Nevertheless, two main issues remain as for the relevance of these Huh-7 derived cell lines for HCV entry investigation. First of all, their poor or absent polarization biases HCV entry studies. New models such as the partially polarized and HCV-permissive HepG2 CD81/miR-122 cell line might help tackling this concern [112]. Furthermore, despite some controversy, it is generally accepted that the VLDL secretion is altered in the Huh-7 cell line [28, 166]. A similar defect is observed in the HepG2 cell line, also it can be partially rescued by inhibition of MEK-ERK (mitogen-activated protein kinase kinase - extracellular signal regulated kinase) [166]. As a consequence, HCV particles produced in Huh-7-derived cell lines do not fully resemble the lipovirions circulating in infected patient serum, with possible implications for entry studies. In fact the much higher specific infectivity of HCV re-cultured from infected humanized mice compared to cell culture grown HCV is likely due to different particle properties of viruses grown in these environments [54]. HCV production in mice engrafted with a humanized liver [54], in PHH [91] or development of new cell lines fully competent for VLDL production is necessary to overcome this issue.

2.6. Dissecting HCV entry, one step at a time

2.6.1. Endocytosis and fusion of HCV

A panel of classical tools, developed for the study of cell biology or for other viruses are available to investigate HCV endocytosis, trafficking and fusion [145] (see Figure 5). For HCV, these include chemical and molecular inhibitors of endosomal trafficking and acidification, and of clathrin-dependent endocytosis [78, 146, 147]. Also, virus internalization can be efficiently demonstrated by resistance to protease digestion (proteinase K [146, 167] or trypsin [168-170]). The efficiency of virus adsorption onto the cell surface can be evaluated by detection of the cell-associated viral RNA by reverse transcription and quantitative PCR [168, 171] or with ³⁵S-labelled HCV virions [172]. How the virus triggers a cellular response leading to its internalization and delivery to a compartment favourable for fusion remains incompletely understood and requires systematic studies of the signalling cascades regulated after virus attachment onto the cell surface. Several groups attempted to tackle this issue, by screening libraries of kinases for those that are important for HCV entry [141] or by targeting specific signalling molecules using genetic or pharmaceutical approaches [42, 173].

Finally, fusion remains one of the dark corners in HCV entry research as models established for other *Flaviviridae* members do not apply to HCV. Several systems have been developed to decipher the mechanisms of HCV fusion independently from the upstream steps of viral entry. They rely on fusion between HCV glycoproteins- and receptors- expressing cells (cell-to-cell fusion assay), or on fusion between virus particles and either the cell surface of permissive cells or artificial liposomes. These different systems are illustrated in Figure 6 and detailed below. In the cell-to-cell fusion assay established by Kobayashi *et al.* (Figure 6A), 293T donor cells expressing a T7-polymerase-dependent GFP and HCV glycoproteins on their surface, are co-cultured with indicator cells that express all HCV receptors (*e.g.* Huh-7 cells) and the T7 polymerase [174]. Fusion between the two cell lines results in the appearance of green syncytia. Variants of this system, using on one hand the luciferase reporter gene under the control of the HIV-1 promoter and the Tat transactivator on the other hand have also been developed [118, 119]. Another experimental setup relies on inducing virus fusion at the cell surface (Figure 6B) [78, 104]. In this case, viral particles are pre-adsorbed onto target cells that have been treated with an inhibitor of endosomal acidification (*e.g.* Bafilomycin A1 or Concanamycin A) so as to prevent the natural HCV entry pathway. Last, fusion can be tested between virus particles and liposomes (Figure 6C) [175, 176]. By labelling one of the protagonists (virions or liposomes) with a fluorescence dye at a self-quenching concentration, it is possible to assess fusion by monitoring fluorescence dequenching and emission. Fusion is quantified as a percentage of the maximal fusion that corresponds to total dequenching of the dye by addition of detergent. This system, as opposed to the two assays described above, is an indicator for hemifusion, as dequenching occurs as soon as the outer hemilayers of virions and liposomes merge. Another liposome-based assay was developed to monitor complete fusion [175]. This “content mixing assay” however depends on a retroviral core and can therefore only be applied to the HCVpp system. It relies on the BlaM (β -lactamase)/CCF2 reporter system, with BlaM being incorporated as a fusion with Vpr in the retroviral capsid while CCF2 is loaded within the liposomes [177]. Upon complete fusion between virions and

liposomes, BlaM cleaves CCF2 thereby resulting in a change of its fluorescence emission wavelength. Finally, a novel fusion assay based on fluorescence dequenching was recently reported [32]. In this setup, fluorescence dequenching of labelled virions is observed upon fusion with the target cells, under normal infection conditions.

Importantly, fusion in all these systems depends on a transient acidification, consistent with HCV entry dependence on endosomal acidification [146, 147]. Yet, HCVcc are pH resistant [78, 178], indicating that a first trigger may be needed to render the glycoprotein fusogenic. Consistent with this, cell-to-cell and cell-surface fusion assays depend on a 37°C incubation period with the target cells prior to the acidic treatment. They also depend on the expression of HCV receptors [174]. In these conditions, up to 10-20% of infectivity can be recovered by virus fusion at the plasma membrane [78, 104], a percentage that might be underestimated considering that some virions might undergo abortive infection through internalization in absence of endosomal acidification. However, it is unclear whether these two systems, as well as the newly reported virus-cell fusion assay [32], really dissociate fusion from the upstream entry events and simplify entry studies. Defining inhibitors that would act on HCV entry without affecting fusion in these setups would consolidate their usefulness. On the opposite, the liposome-based assays are temperature-dependent but receptor-independent [175, 176]. Therefore these systems are simpler than cell-based experiments and more amenable to perform perturbation studies and define the true determinants of fusion. Nevertheless, the fusion scored with these assays is particularly low as compared to what has been obtained with other pH-dependent viruses [179], raising doubts as to whether it really represents the full extent of HCV fusogenicity. Testing HCV fusion with liposomes harbouring individual HCV receptors would be of great interest to better understand the fusion process and requirements.

2.6.2. Dynamics of HCV entry

The large and growing number of entry factors present at different locations of the plasma membrane and involved in HCV entry is reminiscent of the stepwise entry of coxsackie virus B which is transferred from a first set of receptors to tight-junction coreceptors [180]. Several studies attempted to dissect this chronology of receptor interaction and the kinetics and dynamics of virus internalization in the case of HCV. The most intuitive but work-intensive approach is to directly look at virus entry with live-cell microscopy. To this end, Coller *et al.* labelled HCVcc with the DiD or DiI lipophilic dyes and monitored entry of single particles into target cells [154]. The main difficulties to overcome seem to be the low HCV titers and specific infectivity, making the distinction between infectious and non-infectious particles difficult. An indirect approach to assess the entry kinetics is to compare the kinetics of action of entry inhibitors targeting different receptors or steps of entry (*e.g.* neutralizing HCV- or receptor-specific antibodies, inhibitors of endosomal acidification, clathrin-dependent endocytosis or receptor function, etc), using infectivity readouts. Thus, Evans *et al.* suggested that Cldn1 plays a role at a late stage of entry, just before virus fusion, and after CD81 [119], while CD81

and SR-BI might play simultaneous roles in entry [181]. Also, Meertens *et al.* estimated the time lapse between internalization (proteinase K resistance) and fusion (bafilomycin A1 resistance) of HCVpp [146]. One drawback of these studies comes from the different properties of the inhibitors tested (plasma-membrane permeability, affinity for their ligands, etc) that might affect their kinetics of action.

For both approaches detailed above, a pre-requisite is the synchronization of virus entry. This implies blocking entry at a particular stage and suddenly releasing this block. Virus attachment to the plasma membrane is a slow and inefficient process that largely accounts for the asynchronized infections observed in cell culture. Therefore, entry is generally synchronized at the attachment step by incubating the virus particles with the target cells at 4°C, a temperature that allows virus attachment but not its internalization, washing out unbound viruses and shifting the cells to 37°C to promote virus entry. Although improved by spinoculation [182], attachment of virions at 4°C is severely impaired resulting in a significant loss in infectivity [168]. Moreover, cold treatment of the cells, even for minute time periods, and sudden shift to 37°C, trigger cold and hot shock responses [183] including for instance changes in membrane fluidity and in virus receptor localization [184], but also possible artefacts when studying cellular pathways or gene expression. An alternative synchronization method is to couple virions with magnetic particles and pull down the magnetic virions onto the cell surface with a magnet under the cell dish [168]. This way, virions can be massively attached onto the cell surface in no more than 2 minutes, with an efficiency even increased as compared to a standard, non-synchronized, infection. Magnetic adsorption is technically simple, does not appear to disturb virus entry and proved to be useful to study for instance kinetics of antibody neutralization. More recently, virus immobilization on the tissue-culture dish, prior to cell seeding, was described as a new method to transiently submit HCV to various treatments prior to cell infection [185]. This method could possibly also be used to synchronize HCV entry since the virus adsorption time onto the cells is reduced by seeding directly the target cells onto the immobilized viruses. We found that this method resulted in a 10-fold decrease in infectivity as compared to a standard, non-synchronized infection (unpublished data).

2.6.3. Cell-to-cell transmission

In addition to the standard entry route depicted in Figure 5, HCV is able to spread directly from one infected cells to the surrounding cells, without secretion of cell-free virions, resulting in foci formation. Although the quantified contribution of this cell-to-cell transmission and its modalities are still disputed, it can be evidenced in several systems. Usually, cell-to-cell transmission is evaluated between two co-cultured cell populations, one population of producer cells infected beforehand and one population of target cells that can be easily tracked due to a chemical [186], or genetically-encoded fluorescent marker [101, 102]. Infected events in the second populations are considered as indicator of cell-to-cell transmission if the cell-free infectivity is blocked. This last condition can be

achieved by using saturating concentrations of neutralizing antibodies [171, 186, 187], particular virus mutants or target cells [101], or by culturing the cells under an agarose overlay that prevents Brownian motion of cell-free viruses [102, 171]. The dual cell-based reporter system developed by Jones *et al.* [85] (see Table 1) is particularly amenable to investigate cell-to-cell transmission [85, 102, 188] as it allows at the same time distinguishing two different cell populations (producer and target cells) and tracking rare infection events at the single cell level. As an alternative to using two cell populations, counting the number of cells per infection focus after culture under an agarose overlay has been used as an estimate of cell-to-cell transmission efficiency [171, 189].

3. Technologies of HCVcc entry and replication assays

3.1. Preparation, concentration and storage of HCVcc

A number of methodological reviews have been published with detailed protocols on the HCVcc system [55, 56, 63]. In particular, the basic protocols used in our laboratory for HCVcc production and analysis can be found in the following references [14, 63]. Data on HCVcc storage are sparser. Briefly, we found that HCVcc (Jc1 or Luc-Jc1 virus) has a half-life of 6 hours at 37°C, 3.5 days at room temperature and 11 days at 4°C [178]. Other teams reported JFH-1 virus stability at 4°C for up to 4 or 6 weeks [6, 190], a difference that might be imputable to the different virus strains and titers. To our knowledge, HCVcc stability at -80°C over long-term storage has not been documented, but HCVcc stored at -80°C can undergo up to 5 cycles of freeze-thaw without a significant loss of infectivity [6, 190]. Nevertheless, Yi recommends supplementing the medium with 20% foetal calf serum prior to HCVcc freezing [56]. In our laboratory, HCVcc are routinely stored in aliquots at -80°C in standard complete medium (10% fetal calf serum).

Last, concentration of virus stocks has been achieved by different methods including PEG-precipitation [6], centrifugal concentrators [56] or ultracentrifugation [6]. Note that ultracentrifugation, when performed on or through a sucrose cushion, allows a density-dependent partial purification of the virus particles from some medium and cellular contaminants. In our hands, JFH-1 virus particles pelleted by ultracentrifugation through a 20% sucrose cushion and resuspended in PBS could be frozen at -80°C and thawed with no significant loss of infectivity.

3.2. Virus titration methods

Several assays are available to determine HCV titers [191]. Absolute quantification of infectious particles can be obtained by TCID₅₀ (50% tissue culture infectious dose) [6, 191] and FFU (focus forming unit) assays [7, 192] which are both applicable for all infectious HCV constructs and rely on immunolabelling of HCV-infected cells. An alternative is the use of a reporter system, with

either a reporter cell line (see Table 1) or a reporter virus (see Figure 3). Infection can then be quantified as arbitrary units (for instance with the luciferase activity in the cell lysate (*Renilla* or firefly luciferase) or supernatant (*Gaussia* luciferase)) or as a percentage of infected cells with the possibility to extrapolate the absolute number of virus particles (fluorescent reporters visualized by microscopy or fluorescence-activated cell sorting).

The protocol for the TCID₅₀ assay has been described in details elsewhere [191]. A resembling protocol is routinely used in our laboratory, with some modifications in the immunolabelling procedure and TCID₅₀ calculation that are detailed in Section 3.3.1. Briefly, the assay relies on the serial dilution of the virus preparation in a number of replicate wells. The TCID₅₀ titer corresponds to the virus dilution resulting in infection of half of the replicate wells. This assay presents the advantages of a high reproducibility and low margin for subjectivity when observing the plates under the microscope as the wells are counted either positive (one infectious event minimum) or negative (no infected cells), while the infected foci do not need to be counted. It is particularly useful to compare the titers of different virus mutants and to follow virus titers along culture of infected cells as the assay does not depend on virus spread. For the same reason, immunostaining can be performed at different time points post-infection without altering the titer. It is however to be kept in mind that TCID₅₀ assays require a large number of replicates for a reliable titer calculation (typically, a minimum of 6) so that one titration usually requires half a 96-well dish (6 replicates and 8 dilutions). Therefore, TCID₅₀ assays for large experiments require large amounts of staining reagents (antibodies and substrate). Further limitations to this technique to study entry inhibitors are detailed in Section 3.4.

Next, the FFU assay corresponds to the quantification of HCV-infected cell foci [7, 192]. As for the TCID₅₀ assay, cells are infected in replicates (usually a minimum of 3), with a serially-diluted virus preparation, and immunostained (see section 3.3.1). However, rather than counting the positive wells, the investigator counts the number of foci in an appropriate dilution (typically yielding between 20 and 80 foci per well in a 96-well dish, so that the foci are neither overlapping nor too scarce). This assay offers other advantages and drawbacks than the TCID₅₀ assay. First, it is dependent on virus spread. This can be a problem when comparing the titers of different virus mutants that may spread differently so that a second round of infection induces bias in the titration. To overcome this issue, the investigator can stop the infection before virus spread is detectable (the time lapse might differ on the virus strain, but Yi *et al.* reported that the FFU assay remained linear after a 48h infection for JFH-1 or a 96h infection for H77-S [61]) or maintain the cells under an agarose overlay to prevent spread. Note that virus spread is not an issue when titrating HCVpp or HCV_{TCP} that establish single-round infections. Last, the FFU assay is more amenable than the TCID₅₀ assay to perform entry inhibitor assays (see Section 3.4). In this case, cells are infected with a constant virus dose yielding a limited number of foci (typically around 50-80 FFU/well in absence of inhibitor), in presence of different inhibitor concentrations, and the residual foci are counted. One drawback of the method is the small amount of viruses used in the experiment, limiting the measuring window of the assay.

Last, the use of reporter viruses, and in particular the luciferase reporter viruses, is certainly the most convenient and time-saving alternative [63]. The virus does not need to be titered and a single virus dilution (usually the undiluted viral supernatant) can be incubated with various inhibitor concentrations to obtain a dose-response curve. However, this type of assay relies on genetically modified viruses that usually produce lower infectious titers. When performing infectivity assays with different virus mutants, this implies cloning all constructs with the reporter gene. More importantly, mutants that yield low viral titers might become too impaired after incorporation of a reporter gene, precluding their use in infectivity assays. Next, it is important to ensure that the luciferase activity values obtained are in the linear range of the assay and in particular do not reach saturation due to the luminometer properties or to a too high ratio of infected cells. On the opposite, luciferase reporter viruses might yield high signals despite a low number of infection events [69], and therefore, one should be careful that the signals recorded represent a sample of virus that is large enough to be representative. These issues can be tackled by titring beforehand the virus preparations when generating viruses from a new luciferase reporter construct, with the TCID₅₀ or FFU assays. Finally, reporter cell lines offer an interesting alternative to perform quick infectivity assays compatible with any viral strain but often suffer from a relatively low sensitivity [128].

3.3. Protocols for virus titration

This section provides standard protocols to titrate a virus stock using the three main infectivity assays introduced above (TCID₅₀, FFU and luciferase assays). In all cases, 10⁴ Huh-7.5 target cells are seeded per well in 96-well dishes 24h before infection. Note that for the luciferase assay, experiments can easily be scaled up to larger wells. Target cells are inoculated for 3 to 4 h at 37°C prior to supernatant removal and exchange with complete medium (FFU and luciferase assays). In the case of the TCID₅₀ assay, medium change is usually not required. The infection volume for the TCID₅₀ assay is 200 µl/well (to facilitate 1/10 serial dilutions), and 50 µl/well for the FFU and luciferase assays. We typically use 6 replicates for the TCID₅₀ assay, and between 3 and 6 replicates for the FFU and luciferase assays.

In general, in the TCID₅₀ and FFU assays, our virus preparations are titrated using dilutions covering the 1/10-1/10⁸ range (8 serial 10-fold dilutions). For low-titer virus preparations or when comparing samples with subtle titer differences, a more narrow range of dilutions may be advantageous (for instance 3-fold dilutions). On the contrary, the luciferase assays do not require sample dilutions, unless the titer is such that the linear range of the assay is overpassed (see section 3.2).

After inoculation, cells are cultivated for 40h for the FFU assay and for 72h for the TCID₅₀ and luciferase assays, before cell fixation (TCID₅₀ and FFU assays, see section 3.3.2) or lysis (*Renilla* / firefly luciferase assay, see section 3.3.3).

3.3.1. Immunostaining protocol for TCID₅₀ and FFU assays

The staining procedure used for our FFU and TCID₅₀ assays is derived from the protocol first described by Lindenbach *et al.* [6, 191], but simplified and adapted to our home-made substrate. It is detailed below.

- a. Gently wash the cells once in PBS and fix in cold methanol (50 µl/well, equilibrated beforehand at -20°C) for 20 mn at -20°C. The methanol fixation also allows permeabilization of the plasma membrane. Discard methanol waste according to the chemical safety rules of your institution.
- b. Gently wash the cells twice in PBS. Note that we observe a good staining efficiency irrespective of whether the washing steps (in steps b, d, e and f) are performed by submerging the plates in a large bechar of PBS or by using a multichannel pipette. In all cases, the PBS is discarded by gently shaking it off from the plate over a sink. After the last wash of a series, the plates are blotted upside down on paper tissues to remove as much PBS as possible.
- c. Incubate 45 mn at RT (or overnight at 4°C), under gentle agitation, with the primary antibody (30 µl/well). Routinely, we use the mouse monoclonal anti-NS5A 9E10 antibody, generously provided by C.M. Rice (Rockefeller university) [6], at 0.5 µg/ml in PBS. Note that if an antibody-mediated neutralization experiment has been performed with a mouse antibody, it is advised to stain the cells with an antibody from another species so as to avoid detection of the neutralizing antibody. In many cases however, we did not observed a significant background after neutralization and detection with mouse antibodies, maybe because of a short half-life time of the neutralizing antibody and of the high concentration of the primary staining antibody.
- d. Gently wash the cells three times in PBS as above.
- e. Incubate 1h at RT, under gentle agitation, with the peroxidase-conjugated secondary antibody (30 µl/well, anti-mouse-HRP, Sigma A4416), diluted 1/200 in PBS.
- f. Gently wash the cells three times in PBS as above. Add the home-made substrate reagent (30 µl/well), prepared extemporaneously (see below). Incubate for 10 mn or up to 1 h at RT under gentle agitation, regularly inspecting the plates under a microscope until the desired staining intensity is reached. Positive cells will show a typical strong cytoplasmic red-brown staining pattern, with unstained nuclei. Discard the substrate according to the chemical safety rules of your institution, and replace it with water to stop the reaction. Plates can be stored at 4°C for several weeks.
- g. For the FFU assay, count the number of foci per well in a dilution yielding between 20 and 80 FFU/well. For the TCID₅₀ assay, count the number of positive (at least one FFU) and negative (no FFU) wells per dilution. Several statistical methods can be used for the TCID₅₀ calculation [191]. We routinely use the method established by Spearman and Kärber [193, 194]. A pre-

filled Excel sheet for the TCID₅₀ calculation can be freely accessed at [\[link, to be added in the revised manuscript\]](#) (courtesy of Marco Binder, Heidelberg).

Carbazole-based substrate reagent:

Solution A (37.5 mM sodium acetate, 15 mM acetic acid):

75 ml of 0.5M sodium acetate solution (Roth 6779.1)

30 ml of 0.5M acetic acid solution (Roth 37385) (prepared under the fume hood, by pipetting first the water and then the acid)

H₂O up to 1L

Solution B (Handle under the fume hood):

Dissolve 1.6 g 3-amino-9-ethyl-carbazole (Sigma A5754) in 500ml nn-dimethylformamid (Fluka 40255).

Both stock solutions A and B can be stored at 4°C for several months.

Working solution (handle with eyes and skin protected)

Extemporaneously, mix 5 volumes of solution A with 1.5 volume of solution B and 0.02 volume of H₂O₂ (Roth 8070.1). The resulting solution is yellowish. Filter at 0.45 µm (the solution becomes transparent) and use fresh.

3.3.2. Renilla luciferase activity assay

For the luciferase assays, we and others also developed protocols using home-made substrates [63, 76, 126]. In particular, a detailed protocol for the firefly luciferase assay has been published elsewhere [63]. Here, we explain the procedure for the *Renilla* luciferase assay. Note that this protocol is directly applicable to the *Gaussia* luciferase assay, with the modifications indicated in step a.

- a. Gently wash the cells once in PBS and lyse them in passive lysis buffer (35 µl/well, Promega E1941) for 5 mn at RT. We routinely freeze the plates at -20°C until luciferase assay, with no loss in sensitivity. Note that in the case of the *Gaussia* luciferase system, luciferase activity is generally assessed in the cell culture supernatant. In consequence, 45 µl of cell culture supernatant are harvested, inactivated for 5 mn at RT by addition of 5 µl of 10% Triton X-100 (prepared in PBS) for 5 mn at RT (final concentration of 0.1% TritonX-100), and kept at 4°C until luciferase assay.
- b. Before the assay, thaw and equilibrate the samples at room temperature and transfer 20 µl of the lysates (*Renilla* or *Gaussia* luciferase) in white 96-well dishes (Berthold, reference 23300).
- c. Prepare the substrate solution extemporaneously as described below and protect from the light. Note that the half-life time of the substrate is limited and therefore we strongly recommend having positive (no inhibitor) and negative (no infection) controls on each 96-well plate read, as the values obtained might decrease along time when reading several plates.

- d. Read the luciferase activity in a plate luminometer (Berthold Centro XS³ LB960 luminometer, Freiburg, Germany), with automatic injection of 60 µl substrate per well, and measurement over a 0.1s time period (after 1 s shaking, and 5 s delay). Adaptation of this protocol to larger wells is possible. In this case, we use a tube luminometer (Lumat LB 9507 apparatus, Berthold Technologies, Bad Wildbad, Germany), which allows us to measure the luciferase activity from a larger lysate volume. Also be aware that the measurement parameters should be established for every luminometer as the number of arbitrary relative light units measured might widely differ between instruments.

Substrate solution for Renilla or Gaussia luciferase activity assay:

Stock solution of Coelenterazin:

Dissolve 1mg Coelenterazin (P.J.K., Kleinblittersdorf, Germany, reference 102171) in 2.36 ml methanol. Store at -80°C for several months.

Working solution:

Dilute the stock solution 1/2000 in PBS, freshly before use. Keep in the dark.

3. 4. Application to entry inhibitor assays

The assays described above are particularly useful in entry studies. In particular, they can be used to compare the infectivity of different virus mutants, or to test new molecules for their potential to block virus entry. In the first case, the TCID₅₀ is the technique of choice. Indeed, this assay is highly robust, does not require the addition of a reporter gene to the viral genome and does not depend on virus spread. However, in the second case (testing of entry blockers), FFU and luciferase assays are more appropriate. Indeed, they do not require virus dilution for the titer determination. The target cells are inoculated with a unique dose of infectious particles (virus dilution that gives around 50-80 FFU/well for the FFU assay or the undiluted virus stock for the luciferase assay) previously mixed with varying concentrations of the molecule of interest. The infectivity is measured as the residual FFU/well or luciferase activity. On the opposite, with the TCID₅₀ assay, for every inhibitor concentration, one needs to titrate the [virus-inhibitor] mix, with a minimum of 6 replicates and a number of serial dilutions, rapidly leading to a large number of titration plates. But more importantly, titrating the [virus-inhibitor] mix results in the dilution of the tested molecule alongside the virus. There are then several possible scenarios. First, if the inhibitor acts on the virus particle (*e.g.* antibody targeting the viral particle or virucidal agent), its effect will be evidenced by TCID₅₀, as long as the [virus-inhibitor] interaction or the virucidal effect is not reversible upon dilution. However, if the inhibitor acts on the target cells (*e.g.* antibody targeting a virus receptor), the TCID₅₀ assay will not be appropriate to quantify the inhibitory effect. Indeed, as, for a given inhibitor concentration, the [virus-inhibitor] mixture will be diluted, virus and inhibitor dilutions will have opposite effects on the

infection that will distort the TCID₅₀ titer of the sample. FFU and luciferase assays are also more convenient for inhibitor kinetics studies (inhibitor added at different time points before, during or after inoculation).

4. Conclusion: New challenges and hot topics on HCV entry

As pointed out, a persisting hurdle in studying HCV is the complexity of the host/virus system. It is still difficult to obtain, in cell culture, virus particles and cells that resume the properties of patient hepatocytes and circulating virions. However, the HCVcc system and its growing compatibility with PHH cultures or small animal models might refine our current picture on the virion and its entry process. Although entry research has principally – and successfully – focused on the search for cellular receptors, several mechanistical aspects of HCV entry are still poorly understood. First of all, an entry model in polarized cells, that could integrate the involvement of the different HCV receptors, is still missing. The newly elaborated HepG2-derived cell lines [112] or particular PHH culture conditions might help filling this gap. Secondly, the signalling cascades triggered by HCV interaction with its receptors are only partially uncovered. In particular, the signals for internalization or the link between receptor binding and assembly of clathrin-coated pits are unclear. The HCVpp model is an interesting tool to dissect these signals independently of replication-induced signalling. Next, an in-depth basic knowledge on HCV virion and glycoprotein structures is sorely needed to understand how HCV overcomes its pH resistance and becomes able to fuse with the cell membrane. The always improved performance of the HCVcc system in term of virus titer and purification methods [195] might constitute a step further towards this goal. Finally, genome uncoating has been neglected until now but might get insight from the developing field of HCV assembly research and from the generation of new tools that will facilitate the investigation of HCV entry by live cell microscopy.

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Figure captions

Figure 1: HCV polyprotein

Structural proteins (Core, E1 and E2) are coloured whereas non structural (NS) proteins are depicted in white. F designates a protein translated from an alternative reading frame in Core coding sequence; its expression in an infection system is however still disputed.

Figure 2: Main cell-culture systems to study HCV entry

The three plasmids used to generate HCVpp contain the following sequences: 1) packaging-competent retroviral minigenome encoding a reporter gene (Rep.), surrounded by the retroviral long-terminal repeats (black boxes); 2) retroviral gag-pol precursor; and 3) HCV glycoproteins. For further details, please refer to [155]. For further details on the HCV_{TCP} and HCV_{cc} systems, please see the main text.

Figure 3: Main reporter viruses

The first 3 schemas represent the parental WT viruses used to create most of the reporter viruses. White rectangles correspond to JFH-1 (genotype 2a) sequences whereas grey rectangles correspond to sequences from the J6 (genotype 2a) isolate. Sequences derived from isolates that do not belong to the 2a genotype are shown with hatched rectangles (Con1 or see note 2). Note that, in the absence of a systematic study comparing the efficiency of these reporter genomes, we gathered the infectivity data from the published literature as reported by the cited authors. Due to different experimental conditions used in these studies, the values indicated (“fold infectivity reduction”) should therefore be taken as a simple indication and not used to compare the efficiencies of different reporter genomes.

¹ Fold titer reduction as compared to the parental virus. dpt = days post-transfection; dpi = days post-infection; ~ comparable titers; > higher than; < lower than; n.t. = not tested

² x = 1a(H77), 1a(TN), 1b(J4), 2a(J6) [J6/JFH-1 chimera], 2b(J8), 3a(S52), 4a(ED43), 5a(SA13), 6a(HK6a), or 7a(QC69). These constructs allow the generation of reporter viruses from all 7 HCV genotypes.

Figure 4: Whole HCV replication cycle assay

For details on the assay, please see the main text and associated references.

Figure 5: HCV entry into hepatocytes and relevant tools to dissect the individual steps of the entry process

Theoretical model for HCV entry into a non-polarized cell. Note that some chemicals listed may have broader effects than the ones depicted on the figure. The references are cited as examples of studies using the described tools but are not exhaustive. Arrows indicate stimulatory, while bars denote

inhibitory activities of respective treatments on HCV cell entry. Abs, antibodies; BLTs, block lipid transport drugs (BLT-2, 3, 4).

Figure 6: Principle of HCV fusion assays

A. Fusion at the surface of Bafilomycin A1-treated cells; B. Cell-to-cell fusion assay; C. Liposome-based fusion assays. See the main text for the explanations. Note that a novel HCV fusion assay between fluorochrome-labelled virions and target cells has also recently been reported [32]. It corresponds to the 2nd setup described in panel C but with permissive cells rather than liposomes.

Table 1: Main cell systems for the study of HCV entry and replication

Name	Origin	Characteristics and usefulness for HCV research	Ref.
PHH and related (Low and heterogeneous permissivity for HCVcc entry and replication. Rare reports of infection with HCVsp. Autofluorescence hinders immunofluorescence assays [85] (Haid and Pietschmann, personal communication). Reviewed by [86]).			
Fetal PHH	Long-lived as compared to adult PHH [87-90]. Structures looking like bile canaliculi [87]. Inhibition of innate immune response promotes HCVcc productive infection and spread [87]		[87] [88]
Adult PHH	Retain differentiation for up to 2 weeks. Permissive for HCVcc infection, with virus production reaching up to 10 ² -10 ⁵ FFU/ml [91]. However, the low number of successful reports underscores the difficulty to work with this system.		[91]
Micropatterned PHH cultures	Adult PHH seeded on collagen spots and surrounded by mouse fibroblast "feeder" cells [89, 92]	Polarized cells, stronger and more stable differentiation than PHH monocultures [92]. Replication only detectable at the single-cell level. Inhibition of innate immune response promotes HCVcc productive infection [87]	[85, 89, 93]
Induced hepatocyte-like cells	Obtained by induction of pluripotent stem cells	Ressemble fetal human hepatocytes. Permissive for the whole HCVcc replication cycle.	[94]
Huh-7 and derived cell lines			
Huh-7	Well-differentiated hepatocellular carcinoma from a 57-year-old male Japanese patient.	Rapid growth, poor differentiation and poor or absent polarization [21]. Non clonal population with important differences between laboratories strains [95, 96]. Permissive to HCV infection and production	[97]
Huh-7.5	Replicon-expressing Huh-7 cell clone cured with interferon- α . RIG-I mutation ^a .	Highly permissive for HCV RNA replication	[98]
DMSO-differentiated Huh-7.5	DMSO treatment for 6 days minimum, culture on collagen-coated plastic	Growth arrested, partially differentiated cells, stable for up to 200 days in culture. Permissive to HCV infection and production	[99, 100]
Huh7.5-268, Huh7.5-732	Retrov. T. ^b of a CD81-specific shRNA	Refractory to HCV entry. Receptor complementation assays	[101]
Huh-7.5/shSR-BIkd	Retrov. T. ^b of a doxycycline-inducible SR-BI-specific shRNA	Low permissivity to HCV entry. Receptor complementation assays	[44]
Huh-7.5/OCLN ^{low}	Retrov. T. ^b of an Ocln-specific shRNA, isolated clone	Very low permissivity to HCV entry. Receptor complementation assays	[102]
Huh-7.5.1	Replicon-expressing Huh7.5 cells cured with interferon- γ	Highly permissive for HCV RNA replication	[7]
Huh-7 Lunet	Replicon-expressing Huh-7 cell clone cured with a selective drug	Heterogenous and low CD81 expression. Highly permissive for HCV RNA replication. Low permissivity to HCV entry [84]	[103]
Huh-7 Lunet N	Huh-7 Lunet subclone with a particularly low CD81 expression	Very low CD81 expression. Refractory to HCV entry. Receptor complementation assays	[101, 104]
Lunet N hCD81	Retrov. T. ^b of human CD81 in Huh-7 Lunet N cells	Permissive to HCV infection and production	[104]
Lunet N mCD81	Retrov. T. ^b of mouse CD81 in Huh-7 Lunet N cells	Low permissivity to HCV entry	[104]
S29	Huh-7 subclone with a particularly low CD81 expression	Refractory to HCV entry. Receptor complementation assays	[105]
Other human hepatoma cell lines			
HuH6	Hepatoblastoma from a 1-year-old male Japanese patient	Low endogenous Cldn1 expression. Refractory to genotype 2a HCV entry. Receptor complementation assays	[106- 108]
HuH6-hCldn1	Lentiviral transduction of human Cldn1	Permissive to HCV infection and production	[106]
HepG2	Well-differentiated hepatocellular carcinoma from a 15-year-old male Caucasian Argentinean patient	Partially differentiated and polarized. Presence of bile canaliculi between adjacent cells [21]. Refractory to HCVcc entry [6, 109]. Poor HCV RNA replication	[110]
HepG2 CD81	Retrov. T. ^b of human CD81 in HepG2 cells	Permissive to HCVcc infection and production [6]	[111]
HepG2 miR-122	Retrov. T. ^b of a miR-122 encoding construct in HepG2 cells	Increased HCV RNA replication	[112]
HepG2 CD81/miR-122	Retrov. T. ^b of human CD81 in HepG2 miR-122 cells	Increased permissivity to HCV infection	[112]
Non-human cell lines			
BRLA3	Rat hepatoma cell line	No detectable SR-BI expression. Receptor complementation assays	[113]
Murine cell lines (e.g. NIH3T3, AML12, Hepa1-6, Hep56.1D, MMH1-1, etc)	NIH3T3: mouse embryonic fibroblast cell line; AML12, MMH1-1: mouse hepatocyte-derived cell lines; Hep56.1D, Hepa1-6: mouse hepatoma cell lines	AML12 are differentiated, with bile canaliculi between adjacent cells [21]. Only allow replication of subgenomic HCV replicons when selected (Hepa1-6, AML12, MMH1-1) [114, 115]. Not permissive for HCV entry. Receptor complementation assays [116] and test of mouse receptor-adapted viruses [104]. Assembly defect restored by ApoE expression (Hep56.1D, AML12, MHH1-1) [117].	
CHO	Chinese hamster ovary cell line	Binding assays with recombinant HCV glycoproteins [33], HCVpp [118] or HCVcc [119] after ectopic expression of HCV receptors	[120]

Non-liver cell lines (No VLDL production)			
293-T	Human embryonal kidney cell line	Used for HCVpp production. Refractory to HCV entry and poor HCV RNA replication [119]. No Cldn1 expression. Receptor complementation assays [119].	[121]
293-T Cldn1	Retrov. T. ^b of human Cldn1	Low permissivity to HCV entry [119]	[106, 116, 119]
HeLa	Human cervix adenocarcinoma cell line	No expression of Cldn-1 and Ocln [116]. Refractory to HCV infection, low permissivity for HCV RNA replication [122]	[123, 124]
Caco-2	Human colon adenocarcinoma cell line	Polarized cells but with a simple polarity (single apical and basal poles). Permissive for HCV infection	[125]
Indicator cell lines			
Huh7-Lunet-hCD81-Gluc	Retrov. T. ^b of GLuc in Huh7-Lunet-hCD81 cells	<i>Gaussia</i> luciferase expression and secretion for non-invasive cell viability assay	[126]
Huh7.5 FLuc	Retrov. T. ^b of FLuc in Huh-7.5 cells	Firefly luciferase expression for cell viability assay	[76]
Huh-7-GFP and Huh7-derived cell lines-GFP	Retrov. T. ^b of EGFP in Huh-7 or Huh-7-derived cell lines	Constitutive EGFP expression. Allows tracking one cell population to follow cell-to-cell transfer	[101]
Infection reporter cell lines: Expression of a reporter protein containing the NS3/4A protease target recognition sequence, phenotype change upon HCV infection and reporter cleavage. Adapted to all genotypes without virus modification			
Huh7-J20	Retrov. T. ^b of the EGFP-SEAP ^c fusion in Huh-7 cells ³	Release of secreted alkaline phosphatase upon infection. Non-invasive infectivity assay	[127]
n4mBid	Retrov. T. ^b of mBid ^d into the Huh7.5 cell line	Cell apoptosis upon infection (cleavage of mBid). Amenable to the identification of HCV restriction factors	[128, 129]
Huh-7.5/EGFP-IPS	Retrov. T. ^b of EGFP-IPS ^e	Mitochondrial to cytosolic (Huh-7.5/EGFP-IPS) or nuclear (Huh-7.5/RFP-NLS-IPS) translocation of tagged IPS upon infection.	[85, 93]
Huh-7.5/RFP-NLS-IPS	Retrov. T. ^b of RFP-NLS-IPS ^{e,f}	Non-invasive and single-cell infectivity assay. Compatible with live-cell microscopy. System also applicable to PHH and to poorly infectious patient-derived isolates [89]. Dual reporter can be used to detect simultaneously infection events in two cell populations, application to cell-to-cell transmission assays [85].	[85, 89, 93]

^a RIG-I = retinoic acid-inducible gene I; the RIG-I mutation in the Huh-7.5 cell line impairs virus-induced IFN- β synthesis

^b Retrov. T. = Retroviral transduction

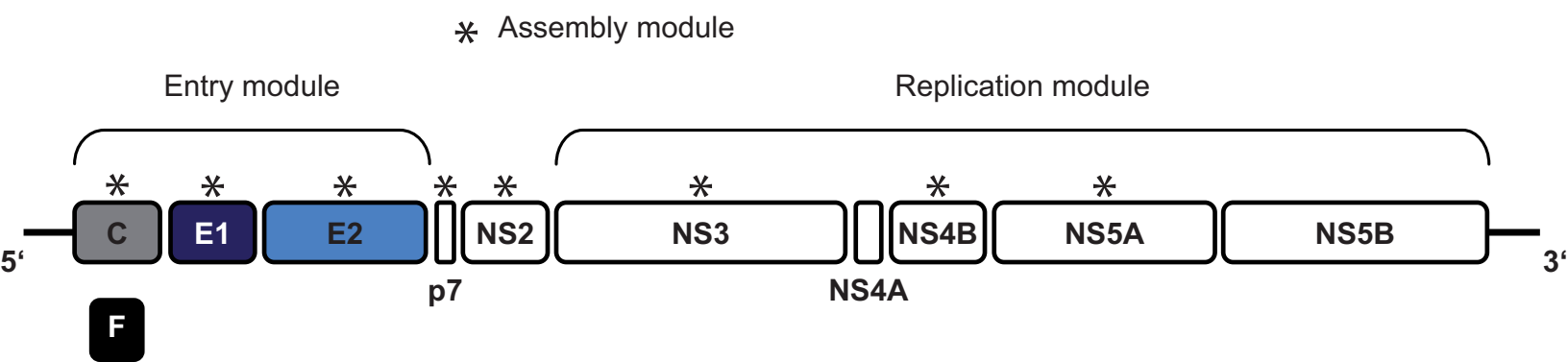
^c SEAP = secreted alkaline phosphatase; fused to the EGFP protein via the HCV NS3/4A protease recognition cleavage

^d mBid = modified Bid; contains the HCV NS3/4A protease recognition cleavage sequence instead of its own cleavage sequence

^e IPS = Interferon- β promoter stimulatory protein-1; mitochondrially-tethered protein cleaved by NS3/4A

^f NLS = nuclear localization signal

Figure 1 Colour



HCVpp

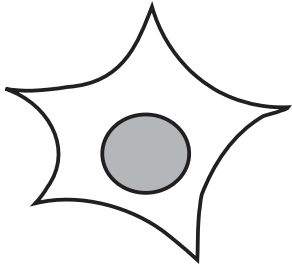
HCV_{TCP}

HCVcc

Virus production

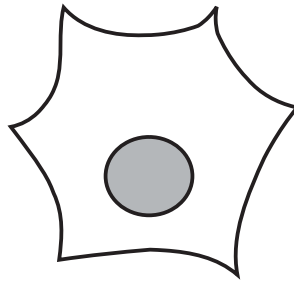
Transfection of three plasmids:

- 1) — CMV — **Rep.** —
- 2) — CMV — gag-pol —
- 3) — CMV — E1 E2 —



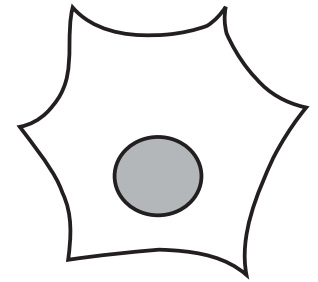
HEK 293T

Transfection of *in vitro* transcribed HCV subgenomic replicon RNA (+/- reporter gene)



Huh-7.5 [CE1] [E2p7NS2]
packaging cell line

Full-length *in vitro* transcribed HCV RNA (+/- reporter gene)



Huh-7 cell line or derived
(see Table 1)

Virus particles

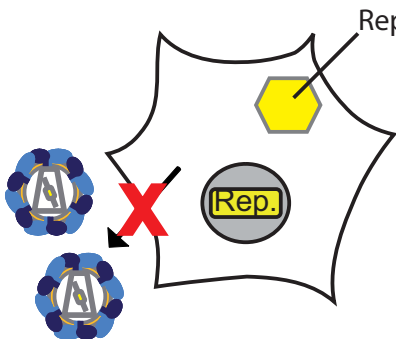
Retroviral scaffold
No lipoprotein association



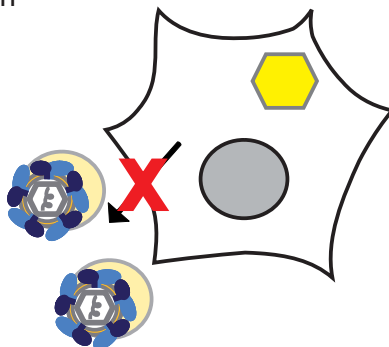
HCV scaffold
Lipoprotein association



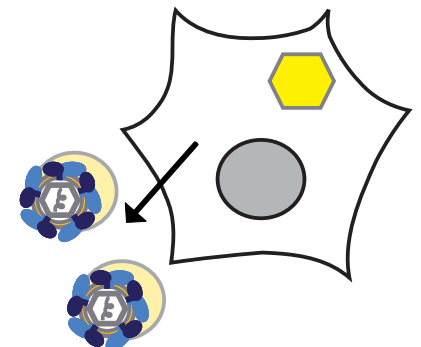
Infection of target cells (e.g. Huh-7.5)



- Single-round infection
- Integration of the reporter gene in the host cell genome
- Mimics HCV entry but depends on retroviral uncoating and gene expression



- Single-round infection
- Reproduces HCV entry and replication



- Whole HCV replication cycle
- Persistent infection

Figure 3 Colour

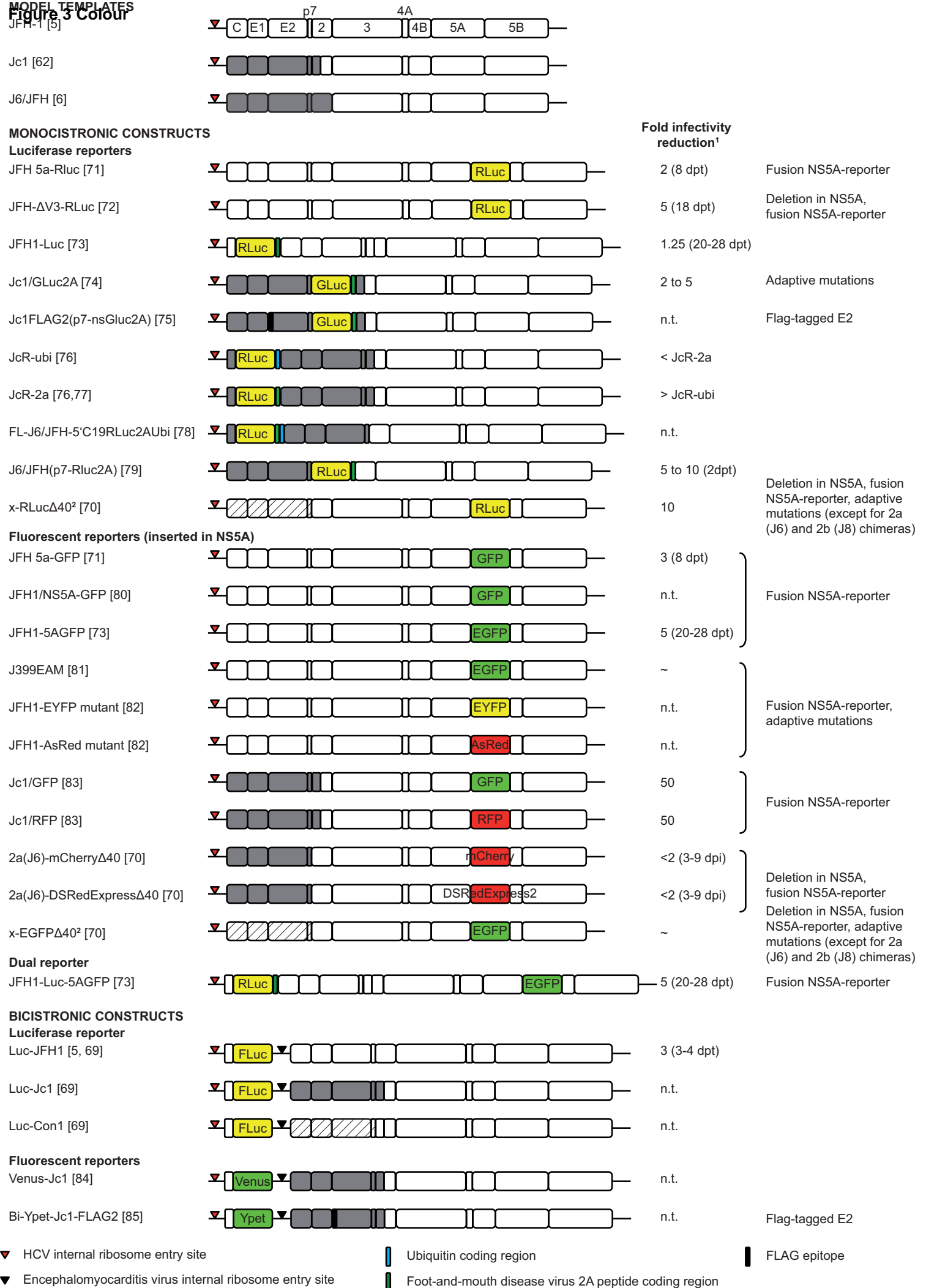
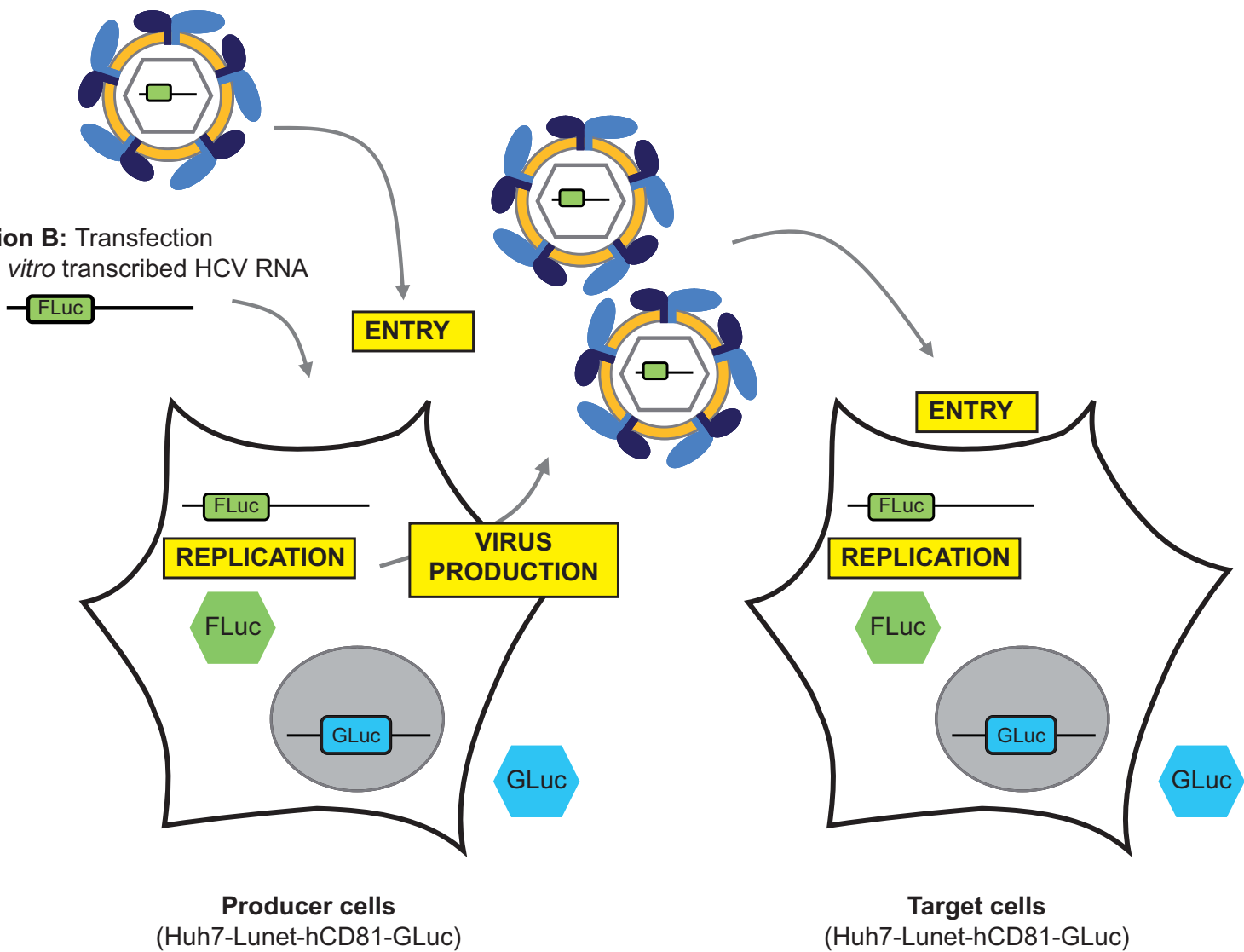


Figure 4 Colour

Option A: HCVcc infection

Option B: Transfection of *in vitro* transcribed HCV RNA



GLuc activity (secreted) = Cell viability
FLuc activity (intracellular) = HCV RNA replication
[+ Entry (Option A)]

GLuc activity (secreted) = Cell viability
FLuc activity (intracellular) = Whole HCV replication cycle in the producer cells

Figure 5 Colour

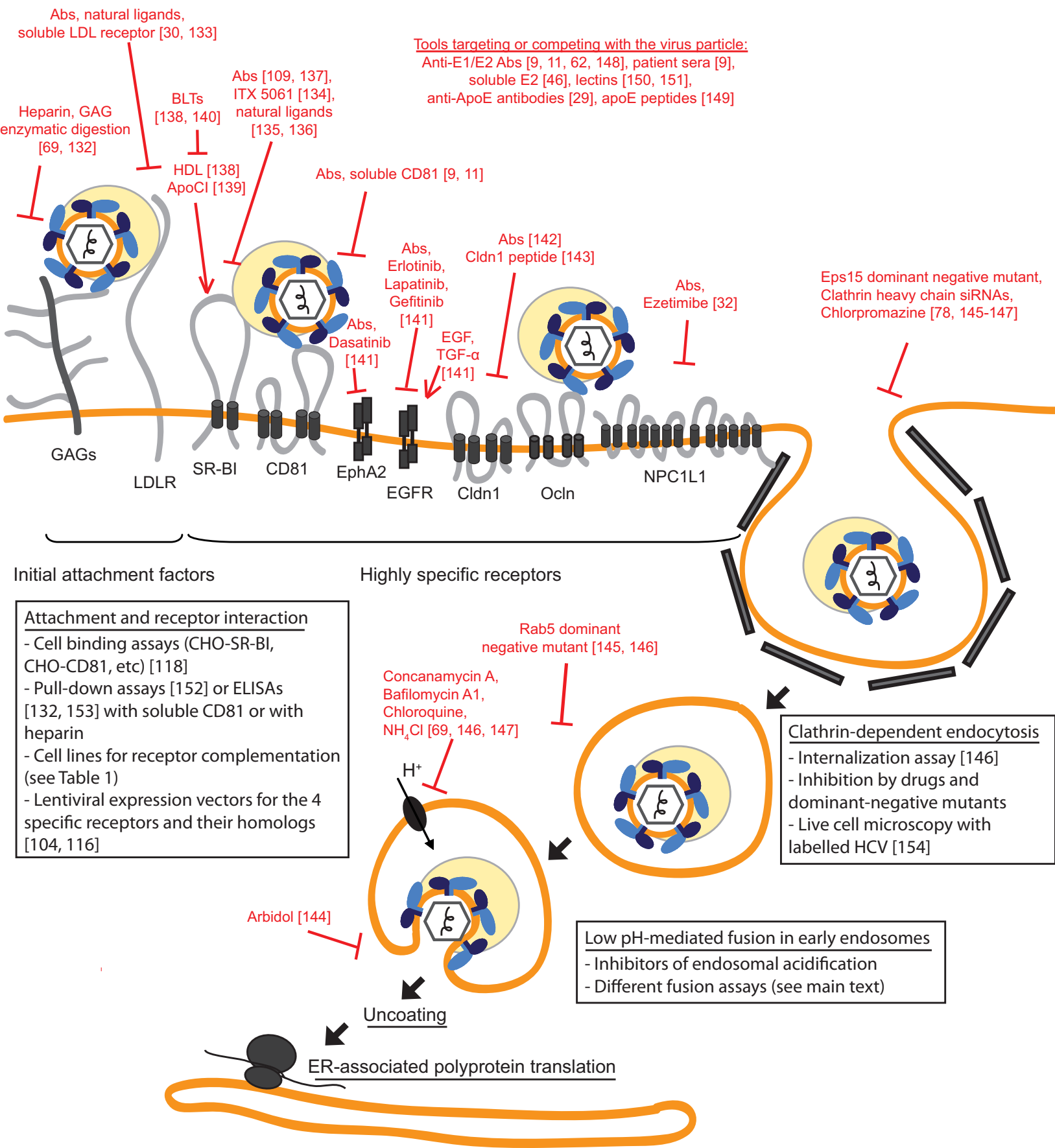


Figure 6 Colour

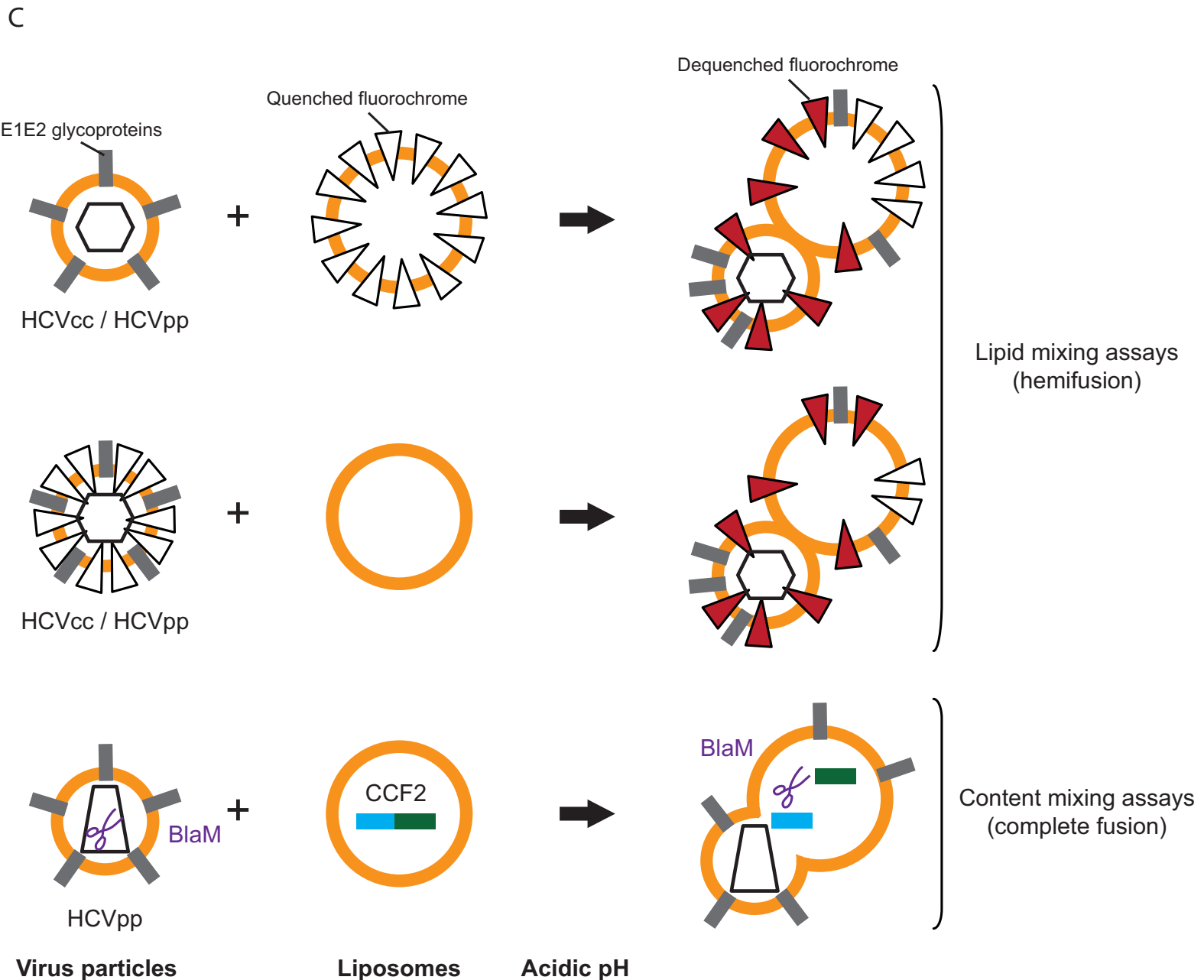
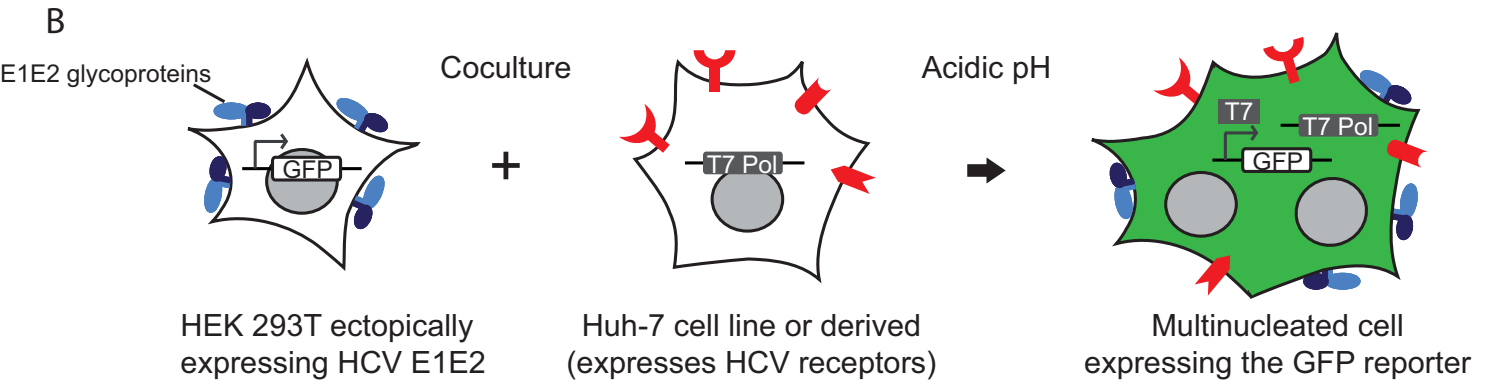
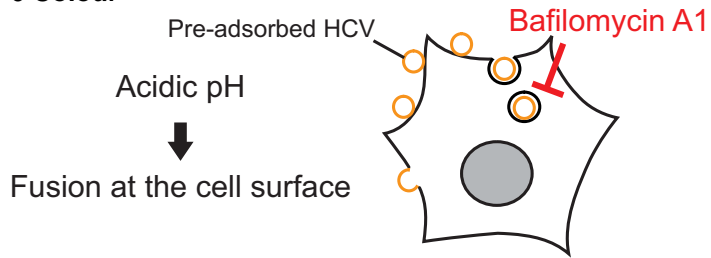
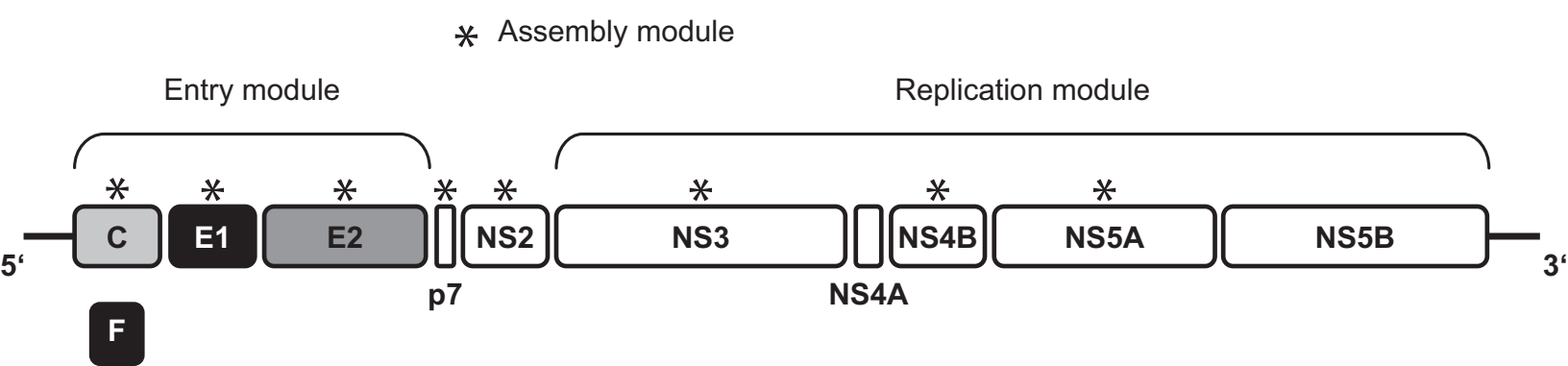


Figure 1 Black & White



HCVpp

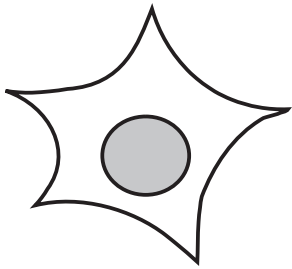
HCV_{TCP}

HCVcc

Virus production

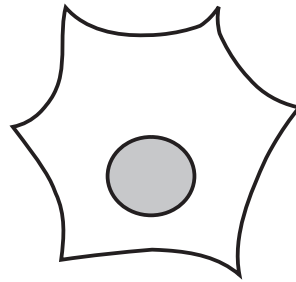
Transfection of three plasmids:

- 1) — CMV — **Rep.** —
- 2) — CMV — **gag-pol** —
- 3) — CMV — **E1 E2** —



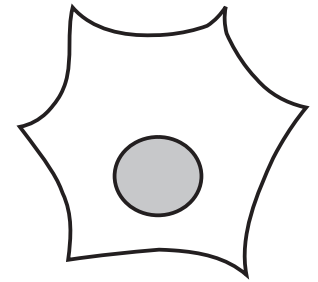
HEK 293T

Transfection of *in vitro* transcribed HCV subgenomic replicon RNA (+/- reporter gene)



Huh-7.5 [CE1] [E2p7NS2]
packaging cell line

Full-length *in vitro* transcribed HCV RNA (+/- reporter gene)



Huh-7 cell line or derived
(see Table 1)

Virus particles

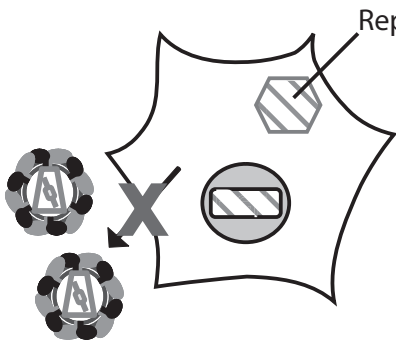
Retroviral scaffold
No lipoprotein association



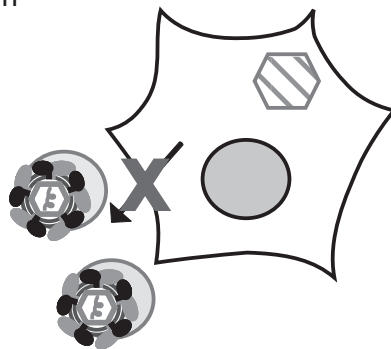
HCV scaffold
Lipoprotein association



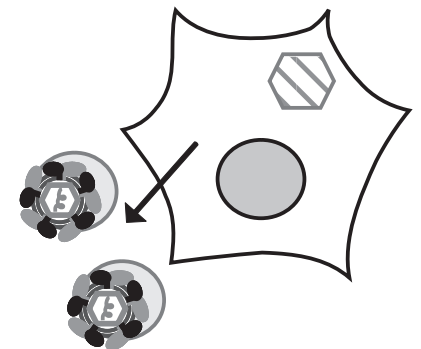
Infection of target cells (e.g. Huh-7.5)



- Single-round infection
- Integration of the reporter gene in the host cell genome
- Mimics HCV entry but depends on retroviral uncoating and gene expression



- Single-round infection
- Reproduces HCV entry and replication



- Whole HCV replication cycle
- Persistent infection

DEFINITION OF TEMPLATES

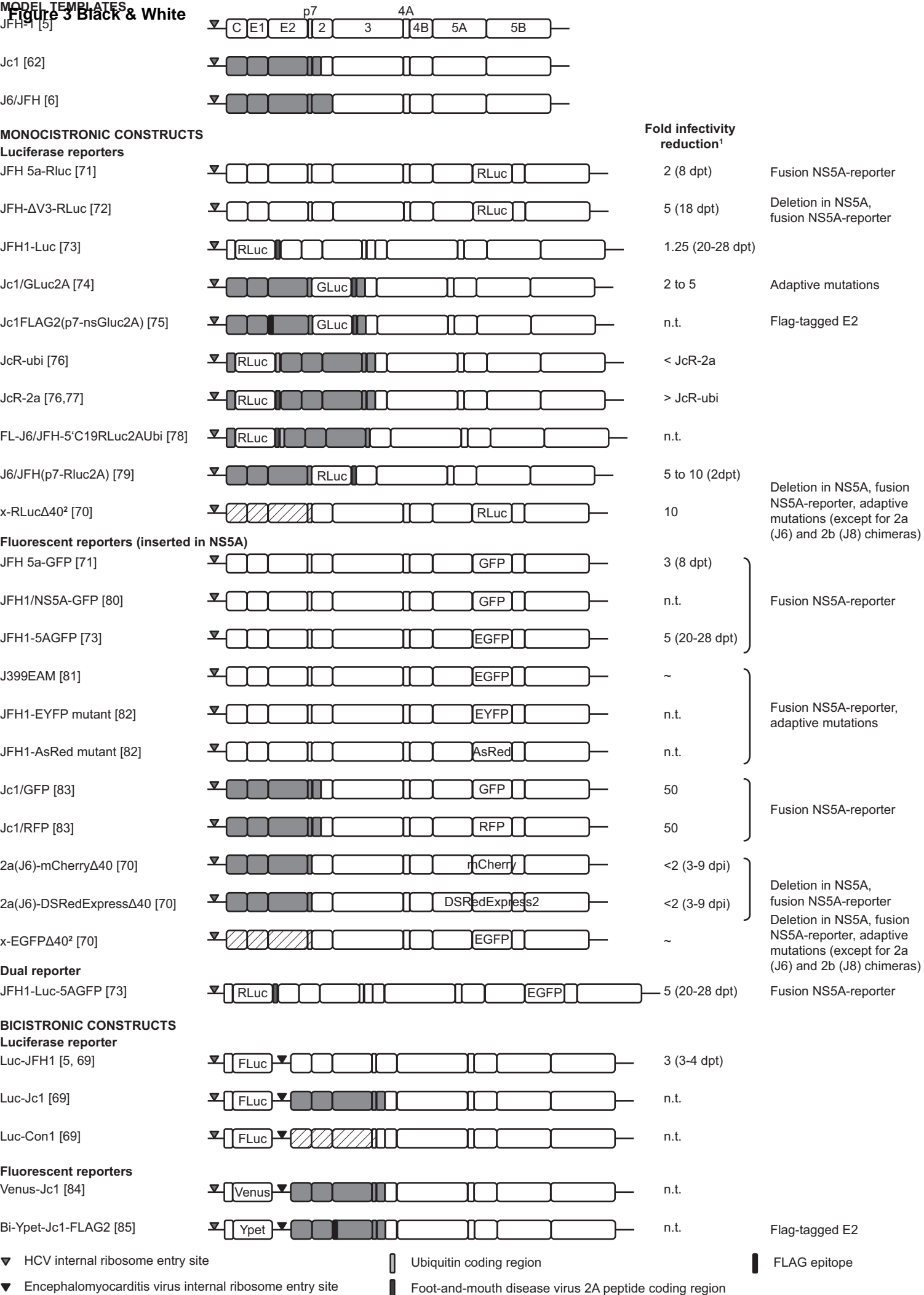
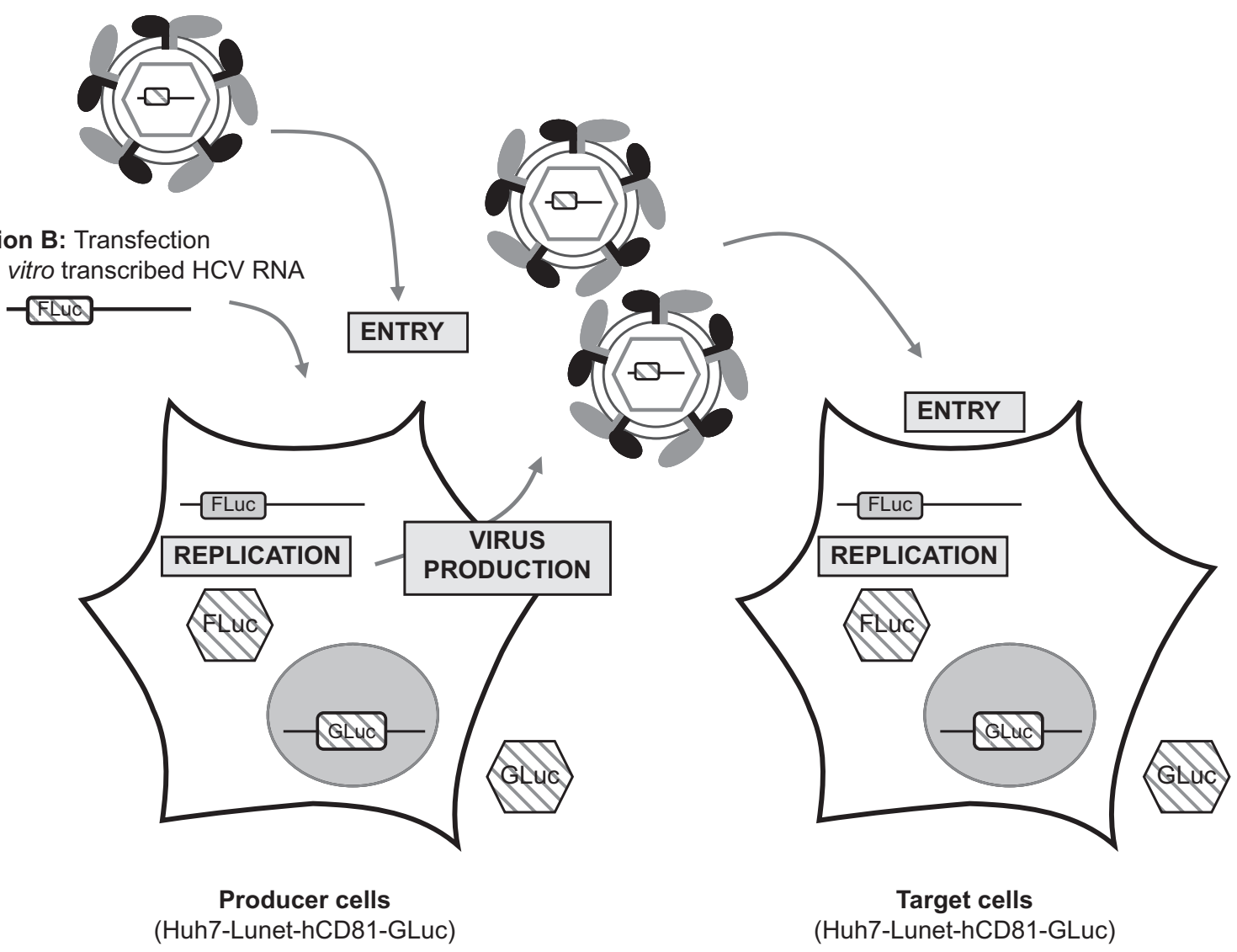


Figure 4 Black & White

Option A: HCVcc infection

Option B: Transfection of *in vitro* transcribed HCV RNA



GLuc activity (secreted) = Cell viability
FLuc activity (intracellular) = HCV RNA replication
[+ Entry (Option A)]

GLuc activity (secreted) = Cell viability
FLuc activity (intracellular) = Whole HCV replication cycle in the producer cells

Figure 5 Black & White

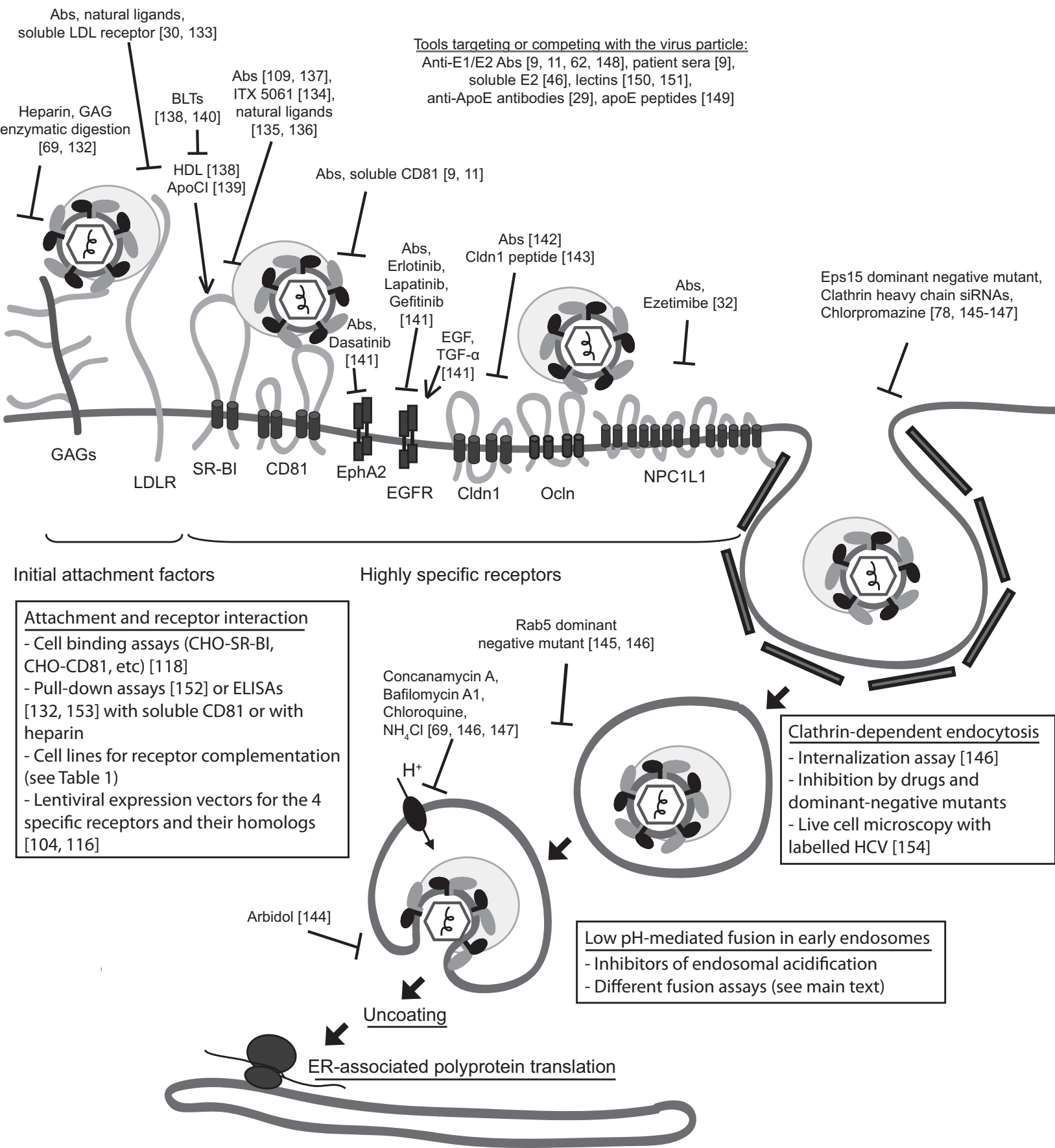


Figure 6 Black & White

