Vps4 and the ESCRT-III complex are required for the release of infectious hepatitis C virus particles

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The mechanisms by which infectious hepatitis C virus (HCV) particles are assembled and released from infected cells remain poorly characterized. In this regard, many other enveloped viruses, notably human immunodeficiency virus type 1, have been shown to utilize the host vacuolar protein sorting machinery (also known as the endosomal sorting complex required for transport; ESCRT) to traffic through the cell and effect the membrane rearrangements required for the formation of enveloped particles. We postulated that this might also apply to HCV. To test this hypothesis, we established a method of conditional virus-like particle assembly involving trans-complementation of an envelope-deleted JFH-1 genome using plasmid transfection. This system reliably produced virus particles that were infectious and could be enumerated easily by focus-forming assay in Huh7 cells. Following co-transfection with plasmids expressing various dominant-negative forms of either components of the ESCRT-III complex or Vps4 (the AAA ATPase that recycles the ESCRT complexes), a reduction in particle production was seen. No significant effect was observed after co-transfection of dominant-negative ESCRT-I or Alix, an ESCRT associated protein. Dominant-negative Vps4 or ESCRT-III components had no effect on either virus genome replication or the accumulation of intracellular infectious particles. These data were confirmed using cell culture infectious HCV and we conclude that HCV requires late components of the ESCRT pathway for release of infectious virus particles.

INTRODUCTION

Hepatitis C virus (HCV) represents a major global health burden. An estimated 170 million people are chronically infected worldwide and a significant proportion of these will go on to develop end stage liver disease. Current drug treatments are poorly tolerated and are only effective in approximately 50% of individuals. There is, therefore, a pressing need for a greater understanding of the molecular mechanisms involved in HCV infection in order for novel drug targets to be identified.

HCV is the prototype member of the genus Hepacivirus in the family Flaviviridae. It is a single-stranded, positive-sense RNA virus whose genome encodes a single ORF. This is translated in a cap-independent manner from an internal ribosome entry site (IRES) located in the 5’ untranslated region, into a single polyprotein of approximately 3000 aa. The polyprotein is proteolytically cleaved by host and viral proteases to form 10 mature proteins; the structural proteins (core, E1 and E2), a viroporin p7 and the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B).

Although recent advances in the development of tissue culture systems for the study of the HCV life cycle (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) have defined a critical role for lipid droplets in virus assembly (Miyanari et al., 2007), the precise mechanism by which infectious HCV particles are assembled and released remains poorly characterized. In particular, it has not been established how nascent HCV particles acquire a lipid bilayer membrane (lipid droplets are surrounded by a single lipid layer). In this regard, it has been well documented that many enveloped viruses utilize aspects of the host endosomal pathway to acquire a membrane and effect assembly and release following membrane scission. Central to the endosomal pathway is the multivesicular body (MVB), which is generated by inward budding of numerous intraluminal vesicles, a process that is analogous to the mechanism whereby viruses acquire their envelope (for review see McDonald & Martin-Serrano, 2009). The sorting of cargo into MVBs is co-ordinated by several multi-protein complexes collectively termed the endosomal sorting complexes required for transport (ESCRTs). Although the precise workings of the ESCRT machinery remain to be fully characterized, it is generally thought that four ESCRT complexes (0, I, II and III) act sequentially to incorporate ubiquitinated target proteins into the intraluminal vesicle within the MVB (for reviews see Hurley &
Emr, 2006; Teis et al., 2009). When the process is complete, the ESCRT complexes are dissociated from the cytosolic side of the endosomal membrane and recycled by the AAA ATPase, Vps4. In the absence of Vps4 activity, the ESCRT complex cannot function and aberrant endosomes are formed. Numerous enveloped RNA viruses such as human immunodeficiency virus type 1 (HIV-1) (Garrus et al., 2001), Ebola virus (Martin-Serrano et al., 2001) and avian sarcoma virus (AVS) (Pincetic et al., 2008) and, more recently, enveloped DNA viruses including hepatitis B virus (HBV) (Lambert et al., 2007) and herpes simplex virus 1 (HSV-1) (Crump et al., 2007) have been shown to utilize ESCRT complexes during virion morphogenesis. In most cases viruses interact with the ESCRT machinery via late (L) domains, short motifs normally found in capsid or matrix proteins, which act as docking sites for ESCRT components. When mutated, the loss of the L-domain causes defective assembly, whereby separation of host and virus fails during the budding process (Bieniasz, 2006).

Three canonical L-domains have been identified in viruses: PT/SAP, YPXL and PPXY, which have been shown to interact with TSG101, Alix and the Nedd4-like E3 ubiquitin ligases, respectively (Demirov & Freed, 2005). More recently, a fourth L-domain (FPIV) was shown to interact with TSG101, Alix and the Nedd4-like E3 ubiquitin ligases, respectively (Demirov & Freed, 2004). More recently, a fourth L-domain (FPIV) was described in a paramyxovirus but there is as yet no information about its binding partner (Schmitt et al., 2005).

Where ESCRT complex function is inhibited, viral particle production is compromised. We postulated that the ESCRT complex may also be involved in the HCV life cycle and that inhibiting ESCRT function would reduce HCV viral particle production. In vitro manipulation of fundamental pathways such as the ESCRT system is extremely toxic to cells and therefore cannot be easily achieved using stable cell lines that constitutively express dominant-negative (DN) ESCRT proteins. We therefore investigated whether a transient trans-complementation method could be used to assess the role of ESCRT components in HCV virus assembly and release. Trans-complementation systems have been developed for a number of positive-strand RNA viruses, such as West Nile (Hanna et al., 2005), Sindbis (Bredenbeek et al., 1993) and Kunjin (Khromykh et al., 1998; Harvey et al., 2004) viruses, but it is only recently that analogous systems have been reported for HCV (Ishii et al., 2008; Steinmann et al., 2008; Adair et al., 2009). In this study, we complemented a JFH-1 derivative lacking the E1 and E2 glycoproteins by transfection of a plasmid expressing dominant-negative (DN) ESCRT proteins. We therefore investigated whether a transient trans-complementation method could be used to assess the role of ESCRT components in HCV virus assembly and release. Trans-complementation systems have been developed for a number of positive-strand RNA viruses, such as West Nile (Hanna et al., 2005), Sindbis (Bredenbeek et al., 1993) and Kunjin (Khromykh et al., 1998; Harvey et al., 2004) viruses, but it is only recently that analogous systems have been reported for HCV (Ishii et al., 2008; Steinmann et al., 2008; Adair et al., 2009). In this study, we complemented a JFH-1 derivative lacking the E1 and E2 glycoproteins by transfection of a plasmid expressing the relevant structural proteins, either in the presence or absence of plasmids expressing DN derivatives of various ESCRT proteins. This system was advantageous in that the effects of ESCRT disruption were limited to virus-producing cells, although we also qualified our findings using fully infectious HCV. We describe the use of this methodology to demonstrate the requirement for a functional ESCRT complex during the release of HCV virus particles.

RESULTS

Provision of the HCV envelope proteins in trans enables efficient virus particle production

We developed a conditional assembly system (JFH-1/D1E2ΔE1E2) based on transfection of Huh7 cells with a JFH-1 derivative containing an internal deletion of 351 aa spanning the envelope glycoproteins. This deletion renders the virus incapable of producing infectious virus particles, yet it retains the ability to undergo productive genome replication in Huh7 cells (Wakita et al., 2005). To restore particle production, E1, E2 and p7 were supplied in trans in the form of a rescue plasmid pCDNA-E1-p7(J4). As subsequent experiments would involve co-transfection of pDNA-E1-p7(J4) with green fluorescent protein (GFP)-tagged ESCRT protein expression constructs, pEGFP was co-transfected as a control for non-specific effects of GFP expression on particle production. Huh7 cells were electroporated with JFH-1/D1E2ΔE1E2 RNA and subsequently transfected with plasmids by lipofection.

Seventy-two hours after electroporation, protein expression in producer cells was verified by Western blot and virus production in culture supernatants was measured by focus-forming assay (Fig. 1a). In the absence of the pCDNA-E1-p7(J4) rescue plasmid, no virus production was detectable. A titration of plasmid input showed that optimal virus production was obtained when 2 μg pCDNA-E1-p7(J4) was transfected (data not shown), giving a mean titre of 1.1 × 10^6 focus-forming units (f.f.u.) ml^-1 (Fig. 1a); this is equivalent to titres achieved in packaging cell lines (Steinmann et al., 2008). In the context of GFP co-expression, the mean titre was 6.4 × 10^5 f.f.u. ml^-1. Titres achieved via trans-complementation were lower than those seen for full-length JFH-1 (7.5 × 10^5 f.f.u. ml^-1), most likely reflecting the fact that only a fraction of the cell population was transfected with the envelope protein expression plasmid. Nevertheless, these findings confirmed that infectious viruses could be reproducibly generated by trans-complementation and that titres were sufficiently high to discern the effects of manipulating the endosomal pathway in subsequent experiments.

To confirm the activity of various DN ESCRT components, their effects on HIV-1 particle production was verified by co-transfection of both HEK 293T and Huh7 cells with the NL43 proviral clone of HIV-1 alongside ESCRT components (data not shown). As expected, DN Tsg101, charged multivesicular body protein (CHMP) 4 and Vps4 reduced levels of extracellular infectious virus and p24 secretion approximately 10-fold in both producer cell types, whereas wild-type controls or DN Alix (apoptosis-linked gene-2 interacting protein X) did not. It was also important to verify that DN ESCRT components did not interfere with aspects of the virus life cycle distinct from particle production. To this end, Huh7 cells were electroporated with a subgenomic HCV replicon, SGR-Luc-JFH-1 (Targett-Adams & McLauchlan, 2005), prior to transfection with
plasmids expressing the various ESCRT components. RNA replication was assessed by luciferase assay and, as shown in Fig. 1(b), none of the constructs had any significant effect on HCV genome replication.

Inhibition of Vps4 function blocks HCV particle production

Vps4, a member of the AAA ATPase family, is an essential protein for cargo sorting and MVB formation. Vps4 dissociates ESCRT complexes from the MVB membrane and allows them to recycle for further rounds of vesicle formation. A DN ATPase-defective mutant of Vps4, in which the active site glutamic acid has been mutated to glutamine, leads to defective MVB sorting and formation of aberrant endosomes in BHK cells (Bishop & Woodman, 2000) and also in Huh7 cells. Production of infectious particles of HIV-1 (Garrus et al., 2001), HSV-1 (Crump et al., 2007), HBV (Lambert et al., 2007) and ASV (Pincetic et al., 2008) is inhibited upon expression of the Vps4DN protein. The wild-type or DN forms of Vps4, tagged with GFP (Vps4WT–GFP or Vps4DN–GFP) were transfected into Huh7 cells, the distribution of Vps4DN–GFP was distinct from Vps4WT (Fig. 2a), with a concentration in large cytoplasmic punctae consistent with aberrant endosomes. Vps4WT–GFP or Vps4DN–GFP plasmids were then co-transfected with pcDNA-E1-p7(J4) into Huh7 cells containing JFH-1/ΔE1E2 RNA. A control transfection using pcDNA-E1-p7(J4) and pEGFP (GFP) was included to measure particle production in the absence of exogenous Vps4 expression. Virus titre was quantified by focus-forming assay and expressed as a percentage of the GFP control (Fig. 2b). Transfection of Vps4WT–GFP did not significantly alter particle production (98 % of the number of particles produced in the presence of GFP). However, in the presence of Vps4DN–GFP, the extracellular virus titre was reduced approximately 20-fold (5 % of GFP control levels). To ascertain whether inhibition of extracellular particle production was dependent on the level of Vps4DN expression, increasing amounts of Vps4DN–GFP DNA were transfected, from 50 ng to 1 μg, resulting in a proportionate increase in protein expression (Fig. 2c). Even the lowest level of Vps4DN–GFP expression resulted in a greater than fivefold reduction (18 % of GFP control levels), and extracellular particle levels were further diminished with increased Vps4DN–GFP expression, indicating that the DN effect was both potent and dose-dependent. Western blot analysis of lysates from producer cells transfected with the lowest (50 ng) and highest (1 μg) amounts of Vps4DN–GFP DNA confirmed that expression of the HCV proteins was unaffected by Vps4DN–GFP expression (Fig. 2d), consistent
with the observation in Fig. 1(b) that RNA replication was unaffected, and supporting the notion that the effect of Vps4DN was specific to extracellular particle production. These results demonstrate that recycling of ESCRT complex components by Vps4 and a functional endosomal pathway is critical to the production of extracellular HCV particles.

**Tsg101 and Alix are not required for efficient HCV particle production**

The first virus shown to utilize ESCRT complex components during assembly, HIV-1, enters the endosomal pathway via interactions between late domains found in the Gag structural protein and either the ESCRT-I component Tsg101 (Garrus et al., 2001) or the ESCRT-I/III associated protein, Alix (Demirov & Freed, 2004). Accordingly, HIV-1 viral particle production is significantly reduced when Tsg101 is disrupted, whilst disrupting Alix function can be compensated for by Tsg101 interactions (McDonald & Martin-Serrano, 2009).

Although sequence analysis failed to identify any canonical late domains in any HCV proteins (data not shown), we investigated whether HCV similarly recruited the ESCRT machinery via interaction with Tsg101 or Alix. To this end,
a DN mutant of Tsg101 tagged with GFP (TSG101DN–GFP) that blocks ESCRT recruitment via PT/SAP motifs, was used to assess the importance of Tsg101 for HCV particle production. Consistent with the lack of canonical L domains, expression of TSG101DN–GFP had no significant effect on particle production (Fig. 3a, b). Although a modest reduction was seen in the presence of TSG101DN–GFP (64–73 % of GFP control levels), no dose-dependent relationship with expression levels was observed, meaning that the low level of disruption is likely due to non-specific effects associated with overexpression.

Alix acts as a bridge between ESCRT-I and ESCRT-III (Hurley & Emr, 2006). Three expression constructs of Alix were obtained: AlixDN(Y)–YFP lacks the first half of the Bro1 domain, which is the binding site for the ESCRT-III component CHMP4; AlixDN(Bro)–GFP is the wild-type Bro1 domain in isolation and so will bind CHMP4 but not to YPXL motifs (and therefore may or may not be dominant-negative); and AlixDN(V)–GFP is the wild-type V domain in isolation and so will bind to YPXL motifs but is unable to interact with the remainder of the ESCRT machinery, and therefore inhibits any YPXL motifs from interacting with wild-type Alix (Munshi et al., 2007). A wild-type Alix plasmid (AlixWT) was transfected as a further control. As seen for Tsg101, however, expression of neither the wild-type nor the DN forms of Alix showed any significant effect upon particle production (Fig. 3c, d). These results suggest that recruitment of the ESCRT complexes via PT/SAP or YPXL interaction with Tsg101 or Alix, respectively, are not required for HCV particle production.

Inhibition of ESCRT-III function blocks HCV particle production

ESCRT-III comprises numerous CHMPs – 2A, 2B, 3, 4A, 4B, 4C and 6 – as well as the associated proteins CHMP1A, 1B, 5 and 7, which act in concert to mediate the formation of the intraluminal vesicles in the MVB. The CHMPs are also known to be involved in membrane scission activities alongside Vps4 and can facilitate scission in the absence of ESCRT-I and -II (Wollert et al., 2009).

To generate DN versions of the CHMPs (CHMP-DN) they were cloned as yellow fluorescent protein (YFP) fusion proteins such that the YFP moiety was C-terminal (Pawliczek & Crump, 2009), this prevents the C-terminus-mediated interaction of the CHMPs with Vps4 (Obita et al., 2007; Stuchell-Brereton et al., 2007) and therefore perturbs ESCRT complex recycling. These constructs were then introduced into the trans-complementation system, as described above, to determine if they were able to inhibit HCV particle production. As shown in Fig. 4(a), each of the CHMP-DN constructs dramatically reduced extracellular particle production (to between 1 and 15 % of GFP control levels). To assess whether this effect was dose-dependent, increasing amounts of the construct...
expressing the most effective inhibitor of HCV particle production, CHMP4B–YFP, were transfected into Huh7 cells containing JFH-1/ΔE1E2 RNA (Fig. 4b). This experiment confirmed that expression of CHMP4B–YFP reduced extracellular particle production in a dose-dependent fashion. Western blot analysis of lysates from cells transfected with either low (50 ng) or high (1 μg) amounts of CHMP-DN constructs confirmed that expression of the HCV proteins was not affected by CHMP-DN expression (Fig. 4c). We conclude that production of extracellular HCV particles requires the action of the ESCRT-III complex.

Inhibition of ESCRT function does not block the accumulation of intracellular infectious HCV particles

Although our data demonstrate clearly that DN Vps4 or CHMPs were able block the production of extracellular infectious HCV particles, it was not clear whether this block occurred at the stage of assembly of intracellular infectious particles or during their release to the extracellular environment. To test this, intracellular infectious particles were quantified following lysis of transfected cells by repetitive freeze–thaw (Hughes et al., 2009). As shown in Fig. 5(a), none of the DN ESCRT components had any significant effect on the accumulation of intracellular infectious particles. Appropriate expression of the ESCRT components and HCV viral proteins was demonstrated by Western blot (Fig. 5b). We conclude that ESCRT function is not required for the assembly of intracellular infectious HCV particles, but plays a role in the release of these particles from infected cells.

Release of full-length infectious HCV is dependent on Vps4 and ESCRT-III function

As discussed above, we used a trans-complementation system to facilitate the analysis of the role of the ESCRT complex in HCV particle assembly. We considered it...
important to verify that the results obtained were replicated in the context of the full-length, tissue-culture-infected virus. To test this, we utilized the J6/JFH-1 chimeric virus (Jc1), as in our hands this gave significantly higher titres than JFH-1 (Griffin et al., 2008). Huh7 cells were infected with Jc1 and subsequently transfected with the appropriate ESCRT constructs as described above. Titres of both intra- and extracellular virus were determined at 96 h post-infection (p.i.). As shown in Fig. 6(a), TSG101DN and AlixDN expression resulted in a modest but significant reduction in released virus consistent with the non-specific effect observed in the context of the trans-complementation assay (Fig. 3). Furthermore, as observed in the trans-complementation assay (Figs 2 and 4), both CHMP4BDN and Vps4DN expression resulted in a fivefold reduction in released virus (Fig. 6a). Additionally, none of the ESCRT constructs had any significant effect on the intracellular virus titres (Fig. 6b), further confirming that ESCRT function is required for release of infectious HCV particles, but not for their assembly.

**DISCUSSION**

Our data clearly demonstrate that the release of HCV particles requires late stages in the ESCRT pathway, as DN mutants of either ESCRT-III complex components (CHMPs) or the AAA ATPase Vps4 inhibited production of extracellular infectious virus particles. In contrast with these results, we found no evidence for a role of either ESCRT-I or Alix in the HCV life cycle. These results are similar to recent findings for HSV-1, which has also been shown to be dependent on ESCRT-III and Vps4, but not ESCRT-I or Alix (Pawliczek & Crump, 2009). The observation that DN mutants of all the individual CHMPs tested had a dramatic effect on particle production correlates with the observation that an ordered assembly of the CHMP proteins in ESCRT-III is required for appropriate MVB sorting (Teis et al., 2008).

One explanation of these data might be that DN CHMPs or Vps4 have a general cytotoxic effect by disrupting normal endosome formation. However, the observation that transient expression of the DN Vps4 mutant did not affect production of either influenza virus (Bruce et al., 2009) or respiratory syncytial virus (Utley et al., 2008), whereas it had a marked effect on production of HSV-1 particles (Crump et al., 2007), argues against this explanation. The latter observations suggest that expression of this protein, although undoubtedly detrimental to cell function, does not result in gross perturbation of endosome formation to the extent that all enveloped virus production is impaired.

How might HCV interact with the ESCRT system? For other enveloped viruses, the direct interactions of viral proteins with components of the ESCRT machinery via L-domains has been well characterized (for review see McDonald & Martin-Serrano, 2009). Consistent with the
lack of a requirement for Tsg101 or Alix in HCV particle production, sequence analysis of HCV proteins did not reveal the presence of any canonical L-domains. However, the lack of identifiable L-domains does not necessarily mean that there are no potential associations with ESCRT proteins, as it is clear that the interactions between viruses and the ESCRT system are varied and complex. For example, viruses containing the PPxY motif are able to recruit Nedd4L and subsequently co-opt the ESCRT machinery in an as yet uncharacterized manner. Furthermore, despite not containing the PPxY motif, HIV-1 Gag protein deficient in Tsg101- and Alix-binding L-domains, can be rescued by overexpression of Nedd4L (Chung et al., 2008; Usami et al., 2008), highlighting the interchangeability and flexibility inherent in L-domains. Thus, it is feasible that HCV gains entry to the ESCRT machinery by an as yet uncharacterized interaction, in a manner that is Tsg101- and Alix-independent. In this regard, it should be noted that two HCV proteins with roles in virus assembly – core and NS2 – are ubiquitinated (Shirakura et al., 2007; Suzuki et al., 2001; Welbourn et al., 2009), providing possible mechanisms by which HCV proteins might be recruited to the ESCRT complex.

What might be the role of the ESCRT machinery in HCV virus production? It is generally thought that the role of the ESCRT machinery in enveloped virus production is limited to the processes of membrane deformation and scission – key events in both virus budding and production of intraluminal MVB vesicles. However, our data clearly show that ESCRT perturbation does not block assembly of intracellular infectious virus. We propose instead, that the ESCRT system is involved in either trafficking of complete nascent HCV particles through (and out of) the cell, or possibly the scission event whereby infectious particles are released from cellular membranes.

One explanation for our findings is the observation made several years ago that Vps4DN-expressing cells demonstrated impaired cholesterol trafficking (Bishop & Woodman, 2000), suggesting that defects in the ESCRT system might disrupt normal cellular lipid metabolism. Lipids are known to play a vital role in HCV particle production; a number of HCV proteins are known to associate with lipid droplets, notably core (Boulant et al., 2006) and NS5A (Miyanari et al., 2007), and these organelles play a key, albeit uncharacterized, role in virus production (Miyanari et al., 2007; Huang et al., 2007; Ye, 2007). It is possible therefore that ESCRT function might be required to transport nascent virus particles from the site of assembly (assumed to be proximal to lipid droplets) to the cell surface.

In conclusion, this study is the first to show a requirement for the ESCRT machinery in the HCV life cycle. Further investigation of the mechanisms of this interaction will help clarify the role of the ESCRT complex in HCV assembly release and could pave the way for future therapeutic interventions.

**METHODS**

**Cell culture and cell lines.** Huh7 cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids,
20 mM HEPES-KOH (pH 7.4) and 1% penicillin/streptomycin (complete DMEM).

**Plasmid construction and transfection.** pcDNA-E1-p7(J4) containing the coding sequence for E1-E2-p7 of the genotype J4 infectious clone, pJ4CV6LS (Yanagi et al., 1998), has been described previously (Griffin et al., 2005). Generation of the CHMP and Alix DN constructs was described recently by Pawliczek & Crump (2009). pcDNA3-myc-ALIX (referred to throughout as AlixWT) was provided by Harald Stenmark (University of Oslo). Wild-type (WT) and dominant-negative (DN) GFP-tagged Vps4 plasmids (Vps4WT–GFP and Vps4DN–GFP) were obtained from Philip Woodman (University of Manchester) (Bishop & Woodman, 2000). The DN Tsg101 plasmid pCR3.1-Tsg101(1-157)–GFP (TSG101DN–GFP) and the DN Alix pCR3.1-Alix–YFP (AlixDN–YFP) were obtained from Juan Martin-Serrano (Kings College, London) (Martin-Serrano et al., 2003).

**In vitro transcription and electroporation of RNA.** T7 in vitro transcripts were made from linearized DNA templates of the JFH1 subgenomic replicon (SGR-luc-JFH1) (Targett-Adams & McLauchlan, 2005) or the non-replicating GND mutant (Wakita et al., 2005), using an Ambion T7 megascript kit. JFH-1/ΔE1E2 and Jc1 were not linearized as they contain a T7 terminator sequence. To electroporate Huh7 cells with RNA, subconfluent cells were trypsinised, washed with diethyl pyrocarbonate (DEPC)-treated PBS, then resuspended at 10⁶ cells ml⁻¹ in DEPC-treated PBS. Cells (8 × 10⁶; 400 μl) were mixed with 10 μg RNA in a 4 mm cuvette, then pulsed in a Bio-Rad Gene Pulser II at 975 μF, 270 V. Cells were resuspended in 12 ml DMEM, then 2 ml of this was added to each well of a six-well plate.

**Trans-complementation.** Ten hours after electroporation of JFH-1/ΔE1E2 RNA, medium was removed and producer cells were washed with complete DMEM. Cells were then transfected with the appropriate plasmids by lipofection using Lipofectin (according to the manufacturer’s instructions). Overall DNA input was kept constant by supplementing with an appropriate quantity of pcDNA3.1 vector. Transfection mixes were removed after 12 h and cells were incubated at 37 °C in complete DMEM for a further 50 h. For quantification of extracellular virus particle production, cell culture supernatant was removed, clarified by centrifugation, then infectivity was determined by focus-forming assay as described by Griffin et al. (2008). To determine intracellular virus particle production, cells were resuspended in 50 μl PBS and lysed by repetitive freeze–thaw action (five times); lysates were clarified by centrifugation at 2800 g in a microcentrifuge for 5 min, and infectivity was determined by focus-forming assay.

**Quantification of particle production using the infectious Jc1 isolate.** Concentrated virus inoculum was prepared by electroporation of cells with Jc1 RNA and collection of the media from repeated passages for 2 weeks. Virus was concentrated by centrifugation of supernatants at 150,000 g through a 20% sucrose cushion; this was resuspended in PBS and titrated by focus-forming assay. To infect Huh7 cells with concentrated Jc1, 5 × 10⁵ cells were seeded in a six-well plate and left to settle overnight. Jc1 (at an m.o.i. of 0.5 in complete DMEM) was added, and removed at 10 h. Cells were then transfected with plasmids by lipofection. Transfection mixes were removed after 12 h and cells were incubated at 37 °C in complete DMEM for a further 74 h. Extracellular and intracellular virus infectious particle production was quantified as described above.

**Transient HCV replication assays using luciferase reporter genomes.** Ten hours after electroporation of in vitro-transcribed RNA, medium was removed and cells were washed with complete DMEM. Cells were then transfected with the appropriate plasmids by lipofection. Transfection mixes were removed after 12 h and cells were incubated at 37 °C in complete DMEM for a further 26 h. Cells were washed in PBS, lysed in 200 μl passive lysis buffer (Promega) and briefly centrifuged to pellet insoluble material. A luciferase assay was performed using a luminometer (BMG Labtech) and the appropriate reagent (Promega). Assays were performed in duplicate.

**Western blot analysis.** For protein analysis, cells were washed twice with PBS, lysed in 40 μl lytic buffer (50 mM Tris/HCl (pH 8.0), 140 mM NaCl, 0.1% SDS, 0.5% NP-40, supplemented with EDTA-free complete protease inhibitor cocktail (Roche), then mixed with 15 μl 4 × Laemmli buffer. Lysates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore) using a semi-dry blotting apparatus (Bio-Rad). After blocking with 5% dried skimmed milk in TBS-T [150 mM NaCl, 50 mM Tris/HCl (pH 7.5), 0.1% Tween-20], primary and secondary antibodies (diluted in 5% dried skimmed milk in TBS-T) were added sequentially with TBS-T washing after each step. Blots were visualized using chemiluminescence and autoradiography.

**Indirect immunofluorescence.** In addition to focus-forming assays, immunofluorescence (IF) was also used to verify expression of the ESCRT and HCV proteins. Cells were fixed in 4% (v/v) paraformaldehyde/PBS for 20 min at room temperature, washed in PBS, then permeabilized with 0.2% Triton X-100 (v/v) in PBS at room temperature for 10 min. Primary antibody (diluted in 10% v/v; FCS/PBS) was applied directly and detected using appropriate Alexa-fluor-conjugated secondary antibodies (Invitrogen Molecule Probes) diluted in 10% FCS in PBS at 1/500.

**Antibodies.** Proteins were detected using the primary antibodies x-E2 (AP33; courtesy of Genetech and Arvind Patel, MRC Virology Unit, Glasgow) at 1:1000 (Western blot) or 1:500 (IF), x-core (#308; gift from John McLauchlan, MRC Virology unit, Glasgow) at 1:3000, polyclonal x-NS5A (Macdonald et al., 2003) at 1:10000 (Western blot) or 1:4000 (IF), x-GAPDH-6C5 (Abcam) at 1:30000 and x-GFP at 1:10000 (gift from Frank von Kuppeveld, Radboud University, Netherlands).

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