Calsyntenin-1 mediates hepatitis C virus replication

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The hepatitis C virus (HCV) RNA genome of 9.6 kb encodes only 10 proteins, and so is highly dependent on host hepatocyte factors to facilitate replication. We aimed to identify host factors involved in the egress of viral particles. By screening the supernatant of HCV-infected Huh7 cells using SILAC-based proteomics, we identified the transmembrane protein calsyntenin-1 as a factor specifically secreted by infected cells. Calsyntenin-1 has previously been shown to mediate transport of endosomes along microtubules in neurons, through interactions with kinesin light chain-1. Here we demonstrate for the first time, we believe, a similar role for calsyntenin-1 in Huh7 cells, mediating intracellular transport of endosomes. In HCV-infected cells we show that calsyntenin-1 contributes to the early stages of the viral replication cycle and the formation of the replication complex. Importantly, we demonstrate in our model that silencing calsyntenin-1 disrupts the viral replication cycle, confirming the reliance of HCV on this protein as a host factor. Characterizing the function of calsyntenin-1 will increase our understanding of the HCV replication cycle and pathogenesis, with potential application to other viruses sharing common pathways.

INTRODUCTION

Hepatitis C virus (HCV) infects an estimated 180 million people worldwide (Gower et al., 2014), and chronic infection is a major cause of cirrhosis, liver failure and hepatocellular carcinoma. Virus entry requires coordinated binding of the viral particle to a number of plasma membrane entry factors, including scavenger receptor B1, the tetraspanin CD81 and the tight junction proteins claudin and occludin (Sabahi, 2009). Entry occurs by clathrin-mediated endocytosis and the endocytosed viral particle is taken up by the early endosome (Blanchard et al., 2006). Acidification of the early endosome is essential for the virus life cycle, facilitating the release of the HCV genome from the viral envelope and capsid (Coller et al., 2009). Once released, the viral RNA is translated at the endoplasmic reticulum (ER), and after production of sufficient non-structural proteins, specifically NS4B and NS5A, the ER undergoes modifications to produce a structure known as the ’membranous web’ (Gosert et al., 2003). The membranous web is a matrix of membranes and characteristic double membrane vesicles, which house the replication complex, shielding it from immune recognition (Romero-Brey et al., 2012; Ferraris et al., 2013). Here, viral RNA is duplicated to form negative-strand RNA, which serves as
RESULTS

Calsyntenin-1 is secreted by HCV-infected cells

We used the JFH1 HCV cell culture model and stable isotope labelling of amino acids in cell culture (SILAC)-based proteomics, coupled with MS, to identify factors secreted by HCV-infected cells. Equal numbers of HCV (JFH1)-infected Huh7 cells and uninfected controls were grown in media labelled with heavy (H) or medium (M) arginine and lysine isotopes. Supernatant was harvested from Huh7 controls (containing medium arginine and lysine) and JFH1-infected cells (containing heavy arginine and lysine isotopes), mixed in a 1:1 ratio, processed and analysed by MS (Fig. 1a). Proteins with higher levels of heavy relative to medium isotopes indicate a higher level of that protein in the supernatant of HCV-infected cells. Ratios of heavy and medium compared with light (endogenous) isotopes show the level of labelling present in the identified protein. H/L or M/L ratios lower than 0.05 are indicative of insufficient labelling and were therefore discarded.

Several proteins were present in higher quantities (at least twofold) in the supernatant of JFH1-infected Huh7 cells, compared with uninfected controls (Table 1). One of these proteins, calsyntenin-1, displayed a striking 43-fold up-regulation in the supernatant of JFH1-infected cells (in addition to a high level of medium and heavy isotope labelling), and was therefore subjected to further analysis. Calsyntenin-1 is a membrane-bound adapter protein with a known affinity for kinesin light chain 1 (KLC1), and is known to play a role in plus-end-directed, kinesin-mediated, microtubule-based transport of vesicular cargo in neurons (Ludwig et al., 2009).

We confirmed the presence of full-length calsyntenin-1 by Western blot of supernatant from JFH1-infected cells, while levels were undetectable in uninfected controls (Fig. 1b). Such an increase in the levels of an intracellular protein in the supernatant could conceivably be due to increased cell death. Although HCV-infected cells are known to undergo higher rates of apoptosis than uninfected cells, the increased level of calsyntenin-1 in the supernatant of HCV-infected cells is unique among 14 detectable intracellular proteins (Table 1), indicating that this increase is specific to calsyntenin-1 and not a general increase in the extrusion of intracellular protein. In contrast to supernatant, there was a comparatively modest twofold up-regulation of intracellular calsyntenin-1 protein within HCV-infected cells (Fig. 1c).

Calsyntenin-1 knock-down does not decrease secretion of total infectious virus

The striking increase in the levels of calsyntenin-1 in the supernatant of HCV-infected cells indicates that the processing of this protein is altered in response to HCV infection. To identify a potential role of calsyntenin-1 in the HCV replication cycle, we used the Jc1 model of HCV infection in Huh7 cells, owing to the higher levels of infectious virus this model produces compared with JFH1, from which it is derived. We confirmed increased levels of calsyntenin-1 in the supernatant of Jc1-infected cells, similar to those observed for JFH1 (data not shown).

Calsyntenin-1 knock-down in Jc1-infected Huh7 cells was performed using siRNA. After 48 h, quantitative real-time PCR analysis demonstrated >90% knock-down efficiency at the RNA level (Fig. 2a), and Western blot confirmed decreased calsyntenin-1 protein levels (Fig. 2b). After 48 h of calsyntenin-1 knock-down, the supernatant containing siRNA was replaced with fresh medium. This medium was left on the cells for a further 24 h, after which it was harvested and analysed for the presence of infectious HCV virus by an infectivity assay. There was no difference in infectivity between the supernatant from calsyntenin-1-silenced and control cells (Fig. 2c), indicating that this protein is not essential for egress of infectious viral particles.

Calsyntenin-1 is involved in early stages of the HCV replication cycle

In addition to analysing the role of calsyntenin-1 knockdown on viral egress, we examined any potential effects on other steps of the virus replication cycle. To examine its role in the early stages of the replication cycle, calsyntenin-1 was silenced in Huh7 cells by treating with 20 nM siRNA for 48 h,
then cells were infected with cell-culture-generated HCV (Jc1, m.o.i. 0.2). After 48 h, the number of infectious foci was determined by labelling with anti-NS5A antibody (Fig. 3a); intracellular levels of HCV RNA were determined by real-time quantitative PCR (Fig. 3b) and levels of HCV core protein were determined by Western blot (Fig. 3c). Interestingly, calsyntenin-1 knock-down reduced HCV infection, with fewer infectious foci and lower levels of HCV RNA and protein detected 48 h after exposure to infectious virus particles.

Calsyntenin-1 mediates trafficking of early endosomes in hepatoma cells

The decreased rate of virus infection following calsyntenin-1 knock-down may be due to its known molecular functions. In neurons, calsyntenin-1 has been implicated in the trafficking of early endosomes (Ponomareva et al., 2014), but this has not previously been studied in hepatic cells. Early endosomes are required for important stages in the HCV replication cycle, namely the uptake and acidification

Table 1. Proteins identified by SILAC-based MS with at least twofold higher levels in the supernatant of HCV-infected compared with control Huh7 cells (H/M ratio) and sufficient labelling of medium and heavy relative to light (endogenous) isotope (M/L and H/L ratios >0.05)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold up-regulation, H/M</th>
<th>M/L</th>
<th>H/L</th>
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<tr>
<td>Calsyntenin-1</td>
<td>43.5</td>
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<td>109.1</td>
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<tr>
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</tr>
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<td>0.0</td>
<td>0.2</td>
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<tr>
<td>α-Enolase</td>
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<td>0.2</td>
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<td>Cofilin-1</td>
<td>2.7</td>
<td>0.3</td>
<td>0.9</td>
</tr>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>0.2</td>
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<tr>
<td>Ubiquitin</td>
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<td>0.9</td>
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<td>Neureotensin/neuromedin N</td>
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of HCV particles, to release the viral RNA genome (Berger et al., 2009), and the setup of the RNA replication complex (Romero-Brey et al., 2012). Therefore, disruption of early endosomal trafficking via the inhibition of calsyntenin-1 could reduce infection by impairing either of these stages of the HCV replication cycle.

To investigate whether early endosomal trafficking is dependent on calsyntenin-1 in Huh7 cells, we used a combination of live cell and immunofluorescent imaging to analyse early endosomal distribution and movement. Plasmids coding for Rab5a-EGFP or Calsyntenin-1-DsRed fusion proteins (Rab5a being a marker for early endosomes) were transfected into Huh7 cells. Immunofluorescence microscopy showed co-localization of Rab5a and calsyntenin-1 (Fig. 4a), consistent with a role for calsyntenin-1 in early endosomal trafficking.

Colocalisation of calsyntenin-1 and Rab5a suggested a role for calsyntenin-1 in trafficking of early endosomes in Huh7 cells, as previously shown in neurons. To investigate this, we knocked down calsyntenin-1 using siRNA, then transfected cells with the Rab5a-EGFP plasmid. Immunofluorescence microscopy showed a more condensed distribution of early endosomes following calsyntenin-1 knockdown, covering a smaller percentage of the overall cytoplasm, compared with scrambled siRNA controls (Fig. 4b–e). This suggests a functional defect in plus-end directed transport of early endosomes towards the cell periphery.

Early endosomes are dynamic subcellular organelles mediating multiple aspects of intracellular trafficking. To look for functional effects of calsyntenin-1 knock-down on endosomal trafficking in real time, we performed live cell imaging of cells transfected with Rab5a-EGFP. In Huh7 cells treated with calsyntenin-1 siRNA, the mean velocity of early endosomes (Rab5a-EGFP) was reduced, both in the perinuclear region and at the periphery (Fig. 4f, g). Consistent with lower mean velocities, in cells depleted for calsyntenin-1, early endosomes moved over a smaller mean distance during a fixed time period than control cells (data not shown).

To determine whether silencing calsyntenin-1 affects the function of early endosomes in Huh7 cells, we compared the distribution of transferrin receptor in calsyntenin-1-silenced cells with that of scrambled control cells. Usually, the transferrin receptor is internalized into the cytoplasm, then recycled through early endosomes back to the plasma membrane (Herbst et al., 1994). Consistent with this, in control cells treated with scrambled RNA there was clear co-localization between the transferrin receptor and early endosomes (Fig. 5a). However, in cells treated with siRNA against calsyntenin-1, there was markedly decreased co-localization between the transferrin receptor and early endosomes (Fig. 5b), suggesting a deficit in early endosomal trafficking and function.

Altogether, these data suggest that calsyntenin-1 mediates plus-end-directed trafficking of early endosomes by KLC1.
towards the cell periphery. In cells deficient in calsyntenin-1, the condensed distribution of early endosomes and their reduced motility could make it less likely for early endosomes to 'encounter' an endocytosed viral particle, and also inhibit formation of the replication complex, both steps being essential for the replication cycle of HCV.

Calsyntenin-1 does not mediate binding or entry of HCV into hepatocytes

The effect of calsyntenin-1 on HCV entry was further evaluated by using a HCV pseudoparticle (HCVpp) assay, as previously described (Drummer et al., 2003). HCVpp entry involves binding, endocytosis and uptake into early endosomes. We observed that calsyntenin-1 knock-down did not inhibit HCV pseudoparticle entry, as luciferase reporter activity was unchanged in calsyntenin-1-silenced cells, compared with controls (Fig. 6). Thus there was no effect on HCV entry per se.

Calsyntenin-1 is involved in the trafficking of HCV replication complexes

When Huh7 cells were silenced for calsyntenin-1 then infected with virus, we observed a relatively greater reduction in the amount of intracellular HCV RNA than in the number of infectious events (Fig. 3a, b). This observation, in addition to the finding that silencing calsyntenin-1 had no effect on HCVpp uptake, led us to hypothesize that calsyntenin-1 plays a role in the replication of viral RNA. Indeed, endosomes have been proposed to contribute to the HCV replication complex (Eyre et al., 2014) and replication complexes themselves are aligned with microtubules and require a functional microtubule network to replicate viral RNA (Lai et al., 2008). To test this hypothesis, we used a JFH1-derived HCV subgenomic replicon (genotype 2a) containing a luciferase reporter (SGR-luc) to measure RNA replication (Targett-Adams & McLauchlan, 2005). Huh7 cells were treated with siRNA against calsyntenin-1, or scrambled RNA as a control, then electroporated with RNA encoding the SGR-luc. This system allowed us to focus on...
viral replication, as it bypasses all the steps of viral entry and virus particle uptake into early endosomes, with replicon RNA being delivered directly into the cell for protein translation and subsequent setup of the replication complex.

Consistent with our hypothesis, the levels of HCV viral RNA in calsyntenin-1 siRNA-treated cells began to increase at 16 h, compared with 12 h for the cells treated with scrambled control siRNA, demonstrating a delay in the establishment of HCV RNA replication (Fig. 7a). In calsyntenin-1 siRNA-treated cells, HCV RNA levels were significantly reduced at all time points after 12 h. When cells were transfected with replication-defective control SGR-luciferase constructs carrying the GND mutation in the polymerase, no difference was observed between calsyntenin-1- and control-siRNA-treated cells, as both failed to establish replication as expected (data not shown). To investigate the mechanism whereby calsyntenin-1 influences HCV RNA replication, HCV-infected Huh7 cells were immunolabelled for both the essential replication complex protein NS5A and calsyntenin-1 and examined by fluorescence microscopy. There was clear co-localization of NS5A with calsyntenin-1 (Fig. 7b), suggesting a role for calsyntenin-1 in the function of the HCV replication complex.

An HCV replicon containing an NS5A-GFP fusion (SGR-NS5A-GFP) (Moradpour et al., 2004) was used to image the localization of NS5A in real time. This cell line was transfected with a Calsyntenin-1-DsRed plasmid and 24 h later analysed by live cell fluorescence microscopy. Replication complexes and calsyntenin-1 were shown to co-localize (Fig. 7c), confirming our previous observations. This further suggests that replication complexes require calsyntenin-1 for normal function. Next, we measured the velocities of mobile replication complexes and showed that their mean velocity was reduced after silencing calsyntenin-1 by siRNA (Fig. 7d).

Although NS5A is an integral component of HCV replication complexes, NS5A is also found elsewhere in HCV-infected cells, including the ER (Boulant et al., 2008). Therefore, to confirm that the motile structures we observed were indeed replication complexes, we labelled for dsRNA. HCV replication complexes generate dsRNA as a replicative intermediate, which can be labelled with anti-
Fig. 7. Calsyntenin-1 is involved in motility of the HCV replication complex. (a) HCV subgenomic replicon-luciferase assay of calsyntenin-1- and control-siRNA-treated cells. Uninfected control cells silenced with CLSTN1 siRNA have a lower production of HCV RNA when electroporated with RNA encoding an HCV subgenomic replicon with luciferase reporter (SGR-luciferase). (For all time points after 12 h, $P<0.05$.) (b) Immunolabelling of calsyntenin-1 (green) and HCV NS5A (red) and merged image. (c) Live cell image showing expression of fluorescent SGR-NS5A-GFP (green) and Calsyntenin-1-DsRed (red), and merged image. Scale bars in (b) and (c), 15 µm. (d) Real-time microscopy measuring the velocity of replication complex (labelled with NS5a-GFP) trafficking shows a deficit in cells treated with CLSTN1 siRNA compared with cells treated with scrambled control siRNA ($^*P<0.05$). (e, f) Proximity ligation assay showing signal obtained using antibodies against dsRNA and calsyntenin-1 (e) and calsyntenin-1 only control (f). Scale bar in (e) and (f) 15 µm. Error bars in (a) and (d) indicate ±SEM.
dsRNA antibodies, appearing as discrete foci within replication complexes, surrounded by NS5A protein (Boulant et al., 2008).

We used proximity ligation assay (PLA), to explore the proximities of calsyntenin-1 and the HCV replication complex. PLA involves labelling cells with antibodies against two targets of interest (in this case viral dsRNA and calsyntenin-1), followed by the addition of secondary antibodies with attached probes. Connector oligos are then hybridized and if within 40 nm, these oligos will form a complete DNA circle, which facilitates rolling circle amplification, which produces a distinct signal upon addition of a fluorescent probe. We observed a strong signal when PLA was carried out using antibodies against calsyntenin-1 and dsRNA (Fig. 7e), confirming close association between calsyntenin-1 and HCV replication complexes.

DISCUSSION

In this study we identified the transmembrane, kinesin light chain-1 (KLC1) adaptor protein calsyntenin-1 as a factor secreted by Huh7 hepatoma cells in response to HCV infection. Our data demonstrate that calsyntenin-1 is involved in the establishment and motility of HCV replication complexes after virus entry. Calsyntenin-1 is known to play a role in plus-end-directed, kinesin-mediated, microtubule-based transport of vesicular cargo in neurons (Ludwig et al., 2009). Consistent with our identification of calsyntenin-1 in the secretome, previous studies have shown that calsyntenin-1 resides in the membrane of secretory vesicles found in body fluids (Rindler et al., 2008). We found that calsyntenin-1 and KLC1 co-localize in Huh7 cells using immunolabelling and PLA (Fig. S1, available in the online Supplementary Material), consistent with the studies carried out in neurons.

Entry of HCV is achieved through clathrin-mediated endocytosis, after which viral particles are taken up by early endosomes. For this to occur, the early endosomes need to be at the periphery of the cell, ready to take up newly entered viral particles. Our data show that in calsyntenin-1 silenced cells early endosomes are more condensed around the nucleus and occupy a smaller proportion of the cytoplasm, owing to a defect in trafficking. This is consistent with the functional role of calsyntenin-1 as an adaptor for KLC1, which is a plus-end-directed, microtubule-associated motor. A functional defect in early endosome function was confirmed by co-localization studies with the transferrin receptor. After silencing calsyntenin-1 there was decreased co-localization between early endosomes and the transferrin receptor, indicating that internalized receptors were not being taken up by early endosomes. However, in HCV uptake studies, no effect on HCVpp entry was observed, indicating that the reduction in HCV infection seen after calsyntenin-1 knock-down involves disruption of the virus replication cycle at stages after virus entry.

After incoming virus is taken up by early endosomes, acidification induces release of the HCV RNA genome into the cytoplasm. The single ORF is translated to form a polyprotein, which is cleaved by viral and host proteases (Hijikata et al., 1993). After threshold levels of specific non-structural proteins (namely NS4B and NS5A) have been produced, they induce the formation of the membranous web (Berger et al., 2009), which houses the HCV replication complex (Romero-Brey et al., 2012; Gosert et al., 2003). This is the location of NS5B, the viral polymerase that replicates the HCV RNA genome, which is either translated into more viral proteins or packaged into nascent viral capsids and exported.

Microtubules are well documented to be involved in trafficking of the replication complex, as treatment with the microtubule stabilizer colchicine inhibits movement and function of the replication complex (Lai et al., 2008). Studies have also demonstrated that the location of HCV RNA replication is important, as it needs to be in close proximity to either the site of protein translation or virus packaging (Vogt et al., 2013). Thus, the dynamic nature of the replication complex is of great importance to the viral replication cycle. Our data show a reduction in the velocity of replication complex trafficking after silencing calsyntenin-1, as well as decreased amounts of HCV RNA. This is consistent with the role of calsyntenin-1 as an adaptor protein, and reinforces the importance of replication complex trafficking for HCV replication. The role of calsyntenin-1 in mediating microtubule-based transport could also have implications for facilitating cell-to-cell spread of HCV, which may partially explain the relatively greater reduction in HCV RNA than focus-forming units after calsyntenin-1 silencing. However, analysis of cell to cell spread was beyond the scope of this study.

Although we have identified an important role for calsyntenin-1 in the HCV replication cycle, we have no definitive answer for why it is present in the supernatant of HCV-infected cells. Based on Western blot analysis, it appears to be in its full-length form, ruling out the possibility that it is cleaved off the plasma membrane. Calsyntenin-1 has been shown to be present in secretory vesicles from the pituitary gland (Rindler et al., 2008). This suggests that full-length calsyntenin-1 is present in a population of secretory vesicles specific to HCV-infected cells. A subset of HCV RNA genomes has been shown to be associated with exosomes (Cosset & Dreux, 2014; Bukong et al., 2014; Ramakrishnaiah et al., 2013), raising the possibility that calsyntenin-1 is associated with such a population of secretory vesicles. We have preliminary data showing that exosome fractions from the supernatant of HCV-infected cells silenced for calsyntenin-1 are less infectious than those from control infected cells (Fig. S2). However, detailed analysis of exosomes is technically challenging, owing to the heterogeneous nature of this vesicular population, making isolation of pure exosome populations extremely difficult to confirm. Owing to these restraints, a thorough characterization of
exosome-mediated infectivity needs to be confirmed in future studies.

The involvement of the host cell factor calsyntenin-1 in the establishment of the HCV replication cycle is testament to its importance for replication of the virus. The setup and trafficking of replication complexes depend on microtubule-based transport, which in turn is dependent on the membrane adaptor qualities of calsyntenin-1. This study demonstrates the importance of early endosomes and their transport in facilitating HCV replication, since silencing the expression of the adaptor protein calsyntenin-1 results in disruptions to the viral replication cycle. Further insights into the roles of early endosomes in the replication cycle of HCV and other related viruses could provide novel targets for small-molecule inhibitors, paving the way for new classes of antiviral drugs.

**METHODS**

**SILAC and MS.** Growth medium was low glucose Dulbecco’s modified Eagle’s medium (DMEM) deficient in leucine, arginine, lysine and phenol red (Sigma). Glucose was adjusted to 4.5 g L\(^{-1}\), leucine was added to a concentration of 0.105 g L\(^{-1}\), and phenol red was added to 0.0159 g L\(^{-1}\). Arginine and lysine were added at 25 % of the normal concentration in DMEM to avoid conversion of excess isotopes into proline (O’Quinn et al., 2002). Arginine isotopes were added back at a concentration of 0.021 g L\(^{-1}\); lysine isotopes were added back at a concentration of 0.0365 g L\(^{-1}\). After addition of all ingredients, medium was passed through a 0.20 µm filter. Medium containing ‘heavy’ isotope was added to JFH1-infected HuH7 cells and ‘medium’ isotope medium was added to uninfected control cells. Cells were incubated with labelled medium for 48 h, after which supernatant was collected, and centrifuged at 1500 g for 5 min to remove dead cells; then protein was precipitated with trichloroacetic acid.

Peptide extraction and MS were performed as previously described (Hocking et al., 2010). Briefly, protein precipitate was resuspended in Laemmli buffer and run on a 10 % polyacrylamide gel. Lanes were sliced into 12 equal-sized portions and peptides were extracted for MS analysis. Samples were loaded onto a Waters Ultima tandem mass spectrometer. Peak picking and database searching were performed using the Mascot Distiller software package, version 2.3.1.0. MDRO 2.3.1.0 (Matrix Sciences), The SwissProt database on Mascot server 2.2 was used for protein identification. The presence of a signal peptide in detected proteins was determined using SignalP 3.0 (Blagoev et al., 2003). ‘Medium’ and ‘heavy’ isotopes are expressed here in relation to ‘light’ (endogenous amino acid) isotopes. Ratios of isotope to light isotope below 0.05 were discounted as ‘no label incorporation’. Keratin peptides were in all cases ignored.

**Plasmid constructs and live cell imaging.** Full-length calsyntenin-1 was amplified from a human brain cDNA library and then cloned into a pDsRed-monomer-C1 vector (Clontech). The forward and reverse primers used were 5’-tttctgagggcggtacctagc-3’ and 5’-aaaaaggtacgtcactagc-3’ respectively. The early endosomal marker Rab5a was amplified from HuH7 cell-derived cDNA and cloned into a pEGFP-N1 vector (Origene), using forward primer 5’-tttgctgagggctagc-3’ and reverse primer 5’-aaaaaggtacgtcactagc-3’.

Coverglass chambers (Lab-Tek II) were used for live cell imaging involving pEGFP-Rab5a, pDsRed-CLSTN1 and pSGR-Luc-GFP-JFH1. Plasmid transfection was performed using FuGENE (Promega), according to the manufacturer’s protocol. Briefly, DNA plasmid and FuGENE were mixed with Opti-MEM medium (Gibco BRL) at a ratio of 1:3. After 15 min at room temperature, the transfection mixture (30–50 µl) was added to each well and cells were incubated under normal culture conditions (37 °C, 5 % CO\(_2\)) for 24 h. Live cells were imaged on a Delta-Vision microscope (Applied Precision) at varying time points, and velocities (nm s\(^{-1}\)) of early endosomes and replication complexes were measured using inbuilt DeltaVision software according to the manufacturer’s instructions.

**Antibodies, immunocytology and microscopy.** Mouse monoclonal antibodies against KLC1 (ab23586), EEA1 (ab70521) and Rab11A (ab170134) were purchased from Abcam. J2 antibody for dsRNA (J2–1406) was purchased from Sciomics. Rabbit polyclonal antibody against HCV core (308) and sheep polyclonal antibody against HCV NSSA were gifts from Dr John McLauchlan (University of Glasgow, UK) and Professor Mark Harris (University of Leeds, UK), respectively. Antibodies against calsyntenin-1 (3176–1), and transferrin receptor (13–6800) were purchased from Epitomics and Invitrogen, respectively. For fixed cell immunolabelling, cells were grown on glass coverslips and fixed with 4 % paraformaldehyde, then blocked with 10 % FBS for 1 h. Incubation with respective antibodies, diluted 1:100 in 10 % FCS, was performed overnight at 4 °C. Appropriate secondary antibodies conjugated with Alexa fluor tags (Invitrogen) were used to visualize cells using a DeltaVision microscope (DV) (Applied Precision) and 60× objective. Proximity ligation assays were performed, using reagents from Olink Bioscience according to the manufacturer’s instructions.

**Image analysis.** Images were opened in ImageJ, and separated into three channels; thresholds were adjusted and areas of interest selected. The ImageJ plugin ‘measure’ was used to analyse the required area of cytoplasm. The area occupied by early endosomes was determined by outlining the region that contains the bulk (i.e. >90 %) of the organelles by visual inspection of images. The area of the nucleus was removed both from the area of the cell occupied by early endosomes and from the area of the whole cell (to give the area for the cytoplasm). Early endosome redistribution was measured as the ratio between the area occupied by early endosomes and the area of the cytoplasm. The relative redistribution was calculated considering the area of the cytoplasm as 100 %. The percentage of total cell area containing early endosomes was calculated using the following formula: percentage area = (b–c)/(a–c) × 100, where a is the area of total cytoplasm in the cell, c is the area of nucleus and b is the area of cytoplasm showing early endosomes (Boulant et al., 2008). To measure velocities of early endosomes, HuH7 cells were silenced for CLSTN-1 with siRNA, transfected with pEGFP-Rab5A (early endosome marker) for 24 h, and examined by live cell imaging, and velocities of at least 10 random early endosomes per cell (total 10 cells counted) were measured using inbuilt DeltaVision software according to the manufacturer’s instructions.

**In vitro transcription, electroporation and infection assays.** In vitro-transcribed HCV RNA was generated from Xbal-linearized subgenomic replicons, i.e. pSGR-luc or pSGR-luc/GND (Dr John McLauchlan, University of Glasgow) (Targett-Adams & McLauchlan, 2005), and MluI-linearized plasmid pJc1 (Professor Ralf Bartenschlager, University Heidelberg) using the T7 RiboMAX Express RNAi System (Promega). Purified viral RNA (10 µg) was electroporated into HuH7 cells at 0.34 kV, 974 µl. Infection of HuH7 cells was quantified by immuno-labelling with anti-NSSA antibody and was consistently found to be 100 %.

HCV infection assays (TCID\(_{50}\)) were performed as described previously (Shahidi et al., 2014). Briefly, HuH7 cells were infected with 1:5 serial dilutions of HCV supernatant for 72 h and immuno-labelled with NSSA antibody, and wells were analysed for infectious foci. The TCID\(_{50}\)s from each dilution were calculated using the following formula: percentage area = (b–c)/(a–c) × 100, where a is the area of total cytoplasm in the cell, c is the area of nucleus and b is the area of cytoplasm showing early endosomes (Boulant et al., 2008). To measure velocities of early endosomes, HuH7 cells were silenced for CLSTN-1 with siRNA, transfected with pEGFP-Rab5A (early endosome marker) for 24 h, and examined by live cell imaging, and velocities of at least 10 random early endosomes per cell (total 10 cells counted) were measured using inbuilt DeltaVision software according to the manufacturer’s instructions.
siRNA silencing, RNA extraction and real-time PCR. Huh7 cells were silenced for CLSTN-1 with siRNA (OriGene), using Lipofectamine RNAiMAX (Invitrogen) for 48 h, according to manufacturer’s instructions. Total RNA was extracted from cells using the QiAgen RNeasy mini kit according to the manufacturer’s instructions. Total RNA (500 ng) was reverse transcribed using the Superscript III RT kit (Invitrogen) as per the manufacturer’s protocol. Real-time quantitative PCR was carried out using SYBR Green (Applied Biosystems) and TaqMan protocols on a Corbett 6000 Rotor-Gene. mRNA levels were normalized to 18S ribosomal RNA and analysed using Rotor-Gene 6000 Corbett software. Calycyntenin-1 primers used for qPCR were: forward primer 5’-cagattgagtgccgg-3’ and reverse primer 5’-gtagataggctcctgctcgag3’.

Protein extraction from supernatant. Trichloroacetic acid (TCA) protein precipitation was used for extracting protein from supernatants. Briefly, TCA was added to a concentration of 10% (v/v) and chilled at −20°C for 1 h, centrifuged at 15 000 x g at 4°C on a bench top centrifuge, and the protein pellet was washed with acetone and air dried.

Quantitation of luciferase activity. Transient HCV RNA replication assays were performed using the pSGR-luc subgenomic replicon, which contains a luciferase reporter (Targett-Adams & McLauchlan, 2005). A replication-defective replicon containing a GND mutation in the polymerase gene was used as a negative control (Targett-Adams & McLauchlan, 2005). Luciferase activity was quantified using the Luciferase Assay System (Promega) in transfected cells according to the manufacturer’s instructions. In brief, cells were harvested at different time points post-transfection and lysed in 100 µl of lysis buffer for each well. Clear supernatant (20 µl) was mixed with 100 µl luciferase reagent and emitted light was measured using a Wallac Victor 1420 Multilabel counter microplate reader.

Concentration of Jc1 virus and sucrose density gradient analysis. Viral supernatants were concentrated using polyethylene glycol 8000 as previously described (Lindenbach et al., 2005). Concentrated virus was overlaid on a gradient of sucrose, prepared from 80 to 10% (w/v) sucrose, and centrifuged at 200 000 x g for 16 h using an SW-41 rotor and Beckman Optima XL-100K ultracentrifuge. Low-density fractions (1–8) were collected and quantified for viral RNA by real-time PCR and for infectivity by TCID50 assay (Ito et al., 2001).

HCvp assay. The effect of calycyntenin-1 on HCV entry was evaluated using the H77c pp [HCV genotype 1a (H77c)-derived pseudoparticles] assay, as described previously (Drummer et al., 2003). These HIV-1 derived pseudoparticles are coated in full-length HCV E1 and E2 glycoproteins from an HCV genotype 1a strain (H77), and contain a firefly luciferase reporter (Drummer et al., 2003). For our experiment, Huh7 cells were seeded at 30 000 cells well−1 in a 48-well plate for 24 h then silenced for calycyntenin-1 using siRNA. After 24 h, silenced cells were washed and H77c-derived pseudoparticles, or empty pseudoparticles were added. After 3 days, cells were lysed and measured for relative luciferase units using the Luciferase Assay System (Promega) as described above.

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REFERENCES


