



Generation of fluorescent HCV pseudoparticles to study early viral entry events- involvement of Rab1a in HCV entry

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Received: 3 April 2022 / Accepted: 1 June 2022 / Published online: 11 July 2022
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Abstract Understanding the early events in viral biology holds the key to the development of potent preventives. In this study, fluorescent hepatitis C virus pseudoparticles (HCVpp) have been generated where the envelope glycoprotein of Hepatitis C virus (HCV) has an EGFP tag. Using these pseudoparticles, entry assays were conducted where their entry was tracked via confocal microscopy. Using this system, fusion of host and viral membranes is predicted to occur within 15 min of HCV entry. Using cells with a knockdown for Rab1a, HCV trafficking was observed to be altered, indicating a role of Rab1a in HCV trafficking. In conclusion, this study reports the generation and use of fluorescent HCVpp which may be used to understand the early events of viral entry. This system may be adapted for the study of other enveloped viruses as well.

Keywords HCV · Fluorescent pseudoparticles · HCV trafficking · Rab1a

Introduction

A thorough knowledge of viral entry events holds the key to the development of preventives. Details of the very early viral entry events of enveloped viruses undergoing endocytosis, such as timelines of internalisation and fusion, remain scanty. In this work we study the early entry events of one such enveloped virus, Hepatitis C Virus (HCV). HCV infection is considered to be a global health problem, with more than 58 million infected people worldwide [1] for which so far there aren't any vaccination.

HCV, a member of *Flaviviridae* family encodes for three structural and seven non-structural proteins. The enveloped virus exhibits structural glycoproteins E1 and E2 on its surface of which E2 is a better characterized subunit compared to E1. E1E2 are involved in HCV entry by interacting with various cellular receptors. Following cellular attachment, HCV has been reported to enter hepatocytes via clathrin-mediated endocytosis [2] which ultimately leads to fusion with endocytic compartments to cause release of the genome into the cytoplasm [3, 4]. In this endocytosis process, early endocytic sorting and vesicle fission/fusion results in the generation of either recycling endosomes destined for the cell surface or late endosomes that ultimately fuse with lysosomes for degradation [5]. Regulation of such sorting events is not completely understood but is believed to be mediated by the small Rab (Ras-related in brain) GTPases [6, 7]. One such mediator is Rab 1a which is involved in early endocytic sorting for many ligands [8] such as asialoglycoprotein receptor (ASGPR) and Epidermal Growth Factor receptor (EGFR) which have been known to be involved in HCV entry [9, 10].

Details of HCV entry remain elusive and so does an understanding of the timeline of viral entry and membrane

This work is part of a doctoral thesis submitted towards partial fulfilment of a PhD degree from Presidency University, Kolkata, India.

This work has been submitted to bioRxiv (Cold Spring Harbor laboratory) as preprint. doi: <https://doi.org/10.1101/2021.03.11.434898>.

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fusion event. To address this issue, this manuscript describes creation of fluorescent pseudoparticles (HCVpp) for the study of early events of HCV entry. Most of the HCVpps so far used to study HCV entry from different laboratories are GFP/ luciferase reporter based lentiviral particles that have been analyzed via microscopic/ luminescence experiments 72 h of post infection on liver cell lines [11, 12]. To the best of our knowledge, we report here for the first time fluorescent HCVpp that have been used to study the initial 30 min of trafficking events of this virus by fluorescence microscopy.

Materials and methods

Cell culture

All experiments were conducted in either HEK 293T, Huh7 or Rab1a KD Huh7 cells. The Rab1aKD cells were obtained as a kind gift from Dr. Allan Wolkoff, Albert Einstein College of Medicine, Bronx, NY. HEK 293T cells were grown in DMEM media and Huh7 cells in RPMI media, containing L-glutamine (Gibco, ThermoFisher Scientific, USA), supplemented with 10% FBS and 1% penicillin streptomycin. Rab1a KD cells were maintained similar to Huh7 with an additional supplement of 2.0 µg/ml Puromycin.

Plasmid constructs

Plasmid pcDNA-E1E2 containing the full length cDNA of genotype 1a (H77) strain inserted into pcDNA and retroviral structural genes Gag-Pol containing construct pTG5349, were obtained as a kind gift from Prof. Jean Dubuisson, Université Ede Lille, France. GFP reporter construct, pLenti-PKG-GFP was obtained from Dr. Jayanta Roychoudhury, Albert Einstein College of Medicine, New York [13]. EGFP tagged construct was generated in pEGFP-N1 (Clontech).

Antibodies, fluorescent dyes and probes

Rabbit anti-GFP antibody (G1544) was purchased from Sigma Aldrich, USA (G1544). Plasma membrane marker FM 4-64 lipophilic dye (Molecular probes, Invitrogen detection technologies) and lyso-sensor molecular probe LysoTracker (Molecular Probes, Life Technologies) were purchased from Thermo Fisher Scientific, USA (Thermo Fisher Scientific, USA).

Preparation of fluorescence labelled HCV-E1E2

HCV glycoprotein E1E2 from pcDNA-E1E2 was inserted into pEGFP-N1 to prepare a construct expressing E1E2-EGFP fusion protein. For this, E1E2 sequence was amplified via PCR from pcDNA-E1E2 construct using primers (forward 5'CGAAGCTTGCATGGGTTGCTCTTTC 3' and reverse 5' CAGAATTCCCGCCTCCGC 3'), the product was subsequently digested with *HindIII* and *EcoRI* (NEB, USA) and ligated into pEGFP-N1 to create a E1E2-EGFP fusion construct with EGFP at the C-terminal end. Plasmid DNA selected from positive clones was isolated and sequenced to confirm proper insertion of E1E2 to be expressed as a fusion with EGFP.

Transfection of pE1E2-EGFP in HEK 293T cells for cell surface expression of EGFP

HEK 293T cells were seeded on sterile coverslips to achieve 70–80% confluency. pE1E2-EGFP vector was transfected using TurboFect (Thermo Fisher Scientific) reagent following manufacturer's instructions and incubated for 48 h at 37 °C in the presence of 5% CO₂. 48 h later, cells were stained with 5 µg/ml of plasma membrane marker FM 4-64 for 15 min on ice followed by fixation with 4% formaldehyde solution (diluted in 100 mM HBSS buffer, pH 7.6) for 10 min on ice and incubated for another 10 min at room temperature. Overexpression of E1E2-EGFP was checked by confocal microscopy (Leica DMI8) using 63X oil immersion objective. Z-Series images were acquired utilizing FITC and rhodamine filter settings with a multichannel white light source. All the images were taken in 1024 × 1024 format with scanning speed 100 Hz and sequential imaging manner. Microscopic images were quantified by Image J (1.52a) (ImageJ-win-64). Images were pseudocolored and co-localization measured using ImageJ-Fiji image processing software.

Immunoblotting to confirm expression of E1E2-EGFP

To confirm protein expression of pE1E2-EGFP in HEK293T cells, western blot analysis was performed. Approximately 60% confluent HEK293T cells were seeded on 60 × 15 mm dish and pEGFP-E1E2 vector was transfected with TurboFect reagent (Invitrogen) as stated earlier. 48 h post transfection cells were scraped and lysed in 100 µl lysis buffer containing 50 mM Tris, 150 mM NaCl, and 0.1% NP40 supplemented with 100 mM PMSF (Thermo Fisher Scientific) and protease inhibitor (Sigma). Lysed cells were centrifuged at 16,000 g for 20 min and supernatant stored at – 20 °C until use. Total protein

concentration was estimated by the Lowry method [14]. Approximately 30 µg protein was used per well in 12% SDS-PAGE, followed by electro transfer onto PVDF membranes (Millipore, USA). Post transfer immunoblotting was performed using rabbit anti-GFP antibody (1:2000) following standard protocol. The membrane was blocked with 5% milk solution and subsequently incubated overnight at 4 °C with primary antibody. Upon washing, blots were incubated with anti-Rabbit IgG secondary antibody (1:2000 dilution) conjugated to alkaline phosphatase for 1 h at room temperature. Rabbit anti-β-actin was used (1:1000 dilution) as a loading control. Immunoreactive proteins were detected by staining the membrane with NBT and BCIP (Promega Corporation, USA) substrate solution for 5 min. Post staining, the blots were washed in water and dried.

Production of fluorescent HCV pseudoparticles (HCVpp)

HCV pseudoparticles were produced by transfecting constructs encoding pE1E2-EGFP (E1E2 fused to EGFP), retroviral packaging construct pTG5349/gag-pol and a reporter pLenti-PGK-GFP into HEK 293T cells by calcium-phosphate method (Calphos Mammalian Transfection kit, Clontech as per manufacturer's instructions). Cells were seeded on 60 × 15 mm dish a day before transfection to achieve 50–80% confluency. pTG5349/gag-pol (4.2 µg), pLenti-PGK-GFP (4.2 µg) and pE1E2-EGFP (1.4 µg) plasmid DNA were mixed with solution A (32 µl calcium solution and sterile water up to total volume of 260 µl). 260 µl of solution B (260 µl 2X HBS buffer) was then added to solution A mixture and vortexed slowly for few seconds and incubated at room temperature for 15 min. The transfection mixture was added dropwise to HEK 239 T cells and allowed to incubate at 37 °C in 5% CO₂. 3 ml fresh DMEM complete media (with FBS) was added after 16 h post transfection. After 40 h post transfection, HCVpp containing media (approximately 42 ml) was collected, purified via passage through 0.45 µm filter (Millipore, MILLEX-GV), concentrated by Amicon ultra filter (Amicon Ultra Centrifugal Filters, Merck) by centrifugation at 4000xg in a swinging-bucket rotor for 20 min. The fluorescent HCVpp were stored at – 80 °C. Approximately 900 µl HCVpp was obtained from 42 ml of media.

Transduction of Huh7 cells with HCVpp

Confluent Huh7 cells monolayers grown in 100 × 10 mm dish (nuncTM, Thermo Fisher Scientific, China) were transduced with 90 µl HCVpp supplemented with DMEM (910 µl DMEM and 90 µl HCVpp) without FBS and antibiotics on ice for 1 h. Infected dish was incubated for

20 min in 5% CO₂ at 37 °C followed by wash with PBS twice. Post internalization cells were scraped and resuspended in 1 ml PBS on ice, followed by a centrifugation at 500xg for 2 min. Total RNA was isolated using Trizol reagent (Sisco research laboratories, India) with 400 µg Ribonucleoside vanadyl complexes (VRC) from NEB, USA. cDNA was synthesized with 1 µg of RNA, using iScriptTM Reverse Transcription supermix for qRT-PCR kit (BIO-RAD Laboratories, USA) following manufacturer's instructions at PCR conditions (priming at 25 °C for 5 min, reverse transcription at 46 °C × 20 min, inactivation at 95 °C × 1 min at RT (25 °C)). 60 ng of cDNA was amplified by PCR using (20 pmol/ reaction) forward and reverse primer for KGFP (forward 5'TCGACAGTCAGCCGCATCT3' and reverse primer 5'CCGTTGACTCCGACCTTCA3') respectively using 1 µl Taq polymerase (NEB, USA). The PCR amplification was performed as per the following cycle: 95 °C × 5 min, 30 cycles of 95 °C × 30 s, 47 °C × 45 s and 72 °C × 1 min followed by a final extension at 72 °C for 5 min. The PCR amplified product was checked in 1.5% agarose gel electrophoresis.

Trafficking of EGFP labelled HCVpp in cells

To understand HCV trafficking events from 0 to 30 min, approximately 60–70% confluent Huh7 or Rab1a cells were seeded on sterile coverslips placed in a 60 × 15 mm cell culture dish. 50 µl HCVpp diluted in 450 µl of DMEM (without FBS, antibiotics) were added (100 µl mixture each) dropwise on the coverslips placed on ice. After washes with cold Hanks' balanced salt solution without magnesium or calcium (HBSS), HCVpp was allowed to internalize by shifting cells to 37 °C for various times as required in various experiments. Internalization was stopped by shifting the cells to ice, followed by fixation in 4% formaldehyde for 10 min on ice and 10 min at room temperature (RT). For staining with a plasma membrane marker FM 4-64, 5 µg/ml was incubated for 15 min on ice prior to fixation. To study lysosomal colocalisation, lysotracker was added at a concentration of 0.14 µM (diluted in PBS) to both of the cell lines for 30 min at RT prior to shifting cells to ice and incubation with HCVpp. Cells were examined via confocal microscope (Leica DMi8) in 63X oil immersion and images were quantified. z-Series images were acquired via confocal microscope utilizing a multi-channel white light source. All the images were taken in 1024 × 1024 format with scanning speed 100 Hz and sequential imaging manner. Images were pseudocolored and colocalisation measured using ImageJ-Fiji image processing software. Pixel intensities of EGFP or integrated density (IntDen) from all the microscopic images were quantified by Image J (1.52a) (ImageJ-win-64) after

subtraction of background IntDen. IntDen of each of the images for all the time points were normalized with mean IntDen of 0 min and all the replicates (N = 3) were considered.

Statistical analysis

Multiple comparisons were conducted by one-way analysis of variance followed by Bonferonni post hoc analysis. Correlation analysis was performed by linear correlation analysis Pearson’s R (using above threshold values) by Fiji image processing software (ImageJ-win-64). t-score was determined to check the significance of individual Pearson’s R values (above threshold). All statistical analyses were performed using SPSS software (version 17.0; SPSS Inc, Chicago, IL). Difference was considered statistically significant at $p < 0.05$.

Results

Preparation of pE1E2-EGFP construct and its expression in 293T cells

E1E2-EGFP fusion protein was conceptualized in order to generate fluorescent pseudoparticles suitable for microscopy studies. A C-terminal EGFP tagged fusion of E1E2 was created such that ER-signal sequence of the protein remained undisturbed. The E1E2 construct (pcDNA-E1E2) provided by Prof Jean Dubuisson, Université Ede Lille, France consisted 21 amino acids of the HCV core, complete E1E2 (540 amino acids) and 16 amino acids of NS2 sequence has been mentioned in Fig. 1A. As the C-terminal end of E2 is on the cytoplasmic side, an EGFP tag is expected to not significantly affect its ability to interact with the HCV entry receptors. In a study with HA and Myc epitopes tagged at the C terminal end of E1E2 the tags did not alter the topology and functionality of TM domains and were oriented towards cytosolic side [15].

As detailed in material and methods section, E1E2 coding region was amplified and inserted into an EGFP tagged vector pEGFPN1 to create a fusion construct pE1E2-EGFP (sequence shown in Fig. 1A). The fusion protein of E1E2-EGFP has two helical transmembrane domains (TMD) similar to that previously mentioned by Cocquerel et al., 2002 [15]. Among the two domains, the second TM domain towards C terminal of the protein linked glycoprotein E2 and EGFP. Both the TM domains in general consist of ER retention sequence, signal sequence and are involved in heterodimerization of glycoproteins E1 and E2 regions, also present in our constructs [16]. Figure 1B represents our proposed model of E1E2-EGFP where post cleavage of TM domain between E1 and E2,

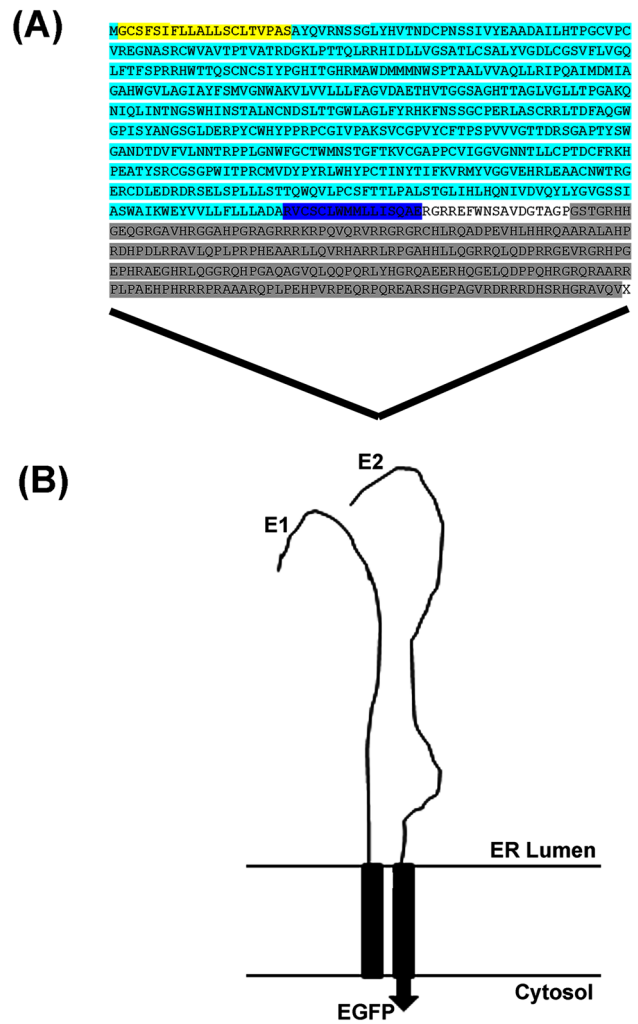


Fig. 1 Sequence and expected orientation of E1E2-EGFP fusion protein. HCV E1E2 was amplified and inserted into EGFPN1 vector to prepare E1E2-EGFP fusion protein. A Protein sequence of E1E2-EGFP with HCV core (Yellow), E1E2 (turquoise), NS1 (Blue) and EGFP sequence (grey). B Schematic representation of expected E1E2-EGFP orientation on the ER/ viral envelope (color figure online)

EGFP tagged with E2 glycoprotein in its C terminal end is oriented towards the cytoplasm or within the pseudoparticle. Details of orientation of E1 and E2 in the ER membrane are well explained in literature [17].

To check whether the E1E2-EGFP construct was successfully prepared, protein expression of EGFP was analyzed after its transfection into HEK 293T cells. 48 h of post transfection EGFP expression was analyzed by microscopic analysis as well as by immunoblotting. EGFP expression was observed to be in vesicular structures throughout the cytoplasm (Fig. 2A). Quantitation of the pixel intensity (IntDen) revealed significantly ($p < 0.005$) higher fluorescence intensity signal compared to mock transfected cells (Fig. 2B). EGFP expression was also

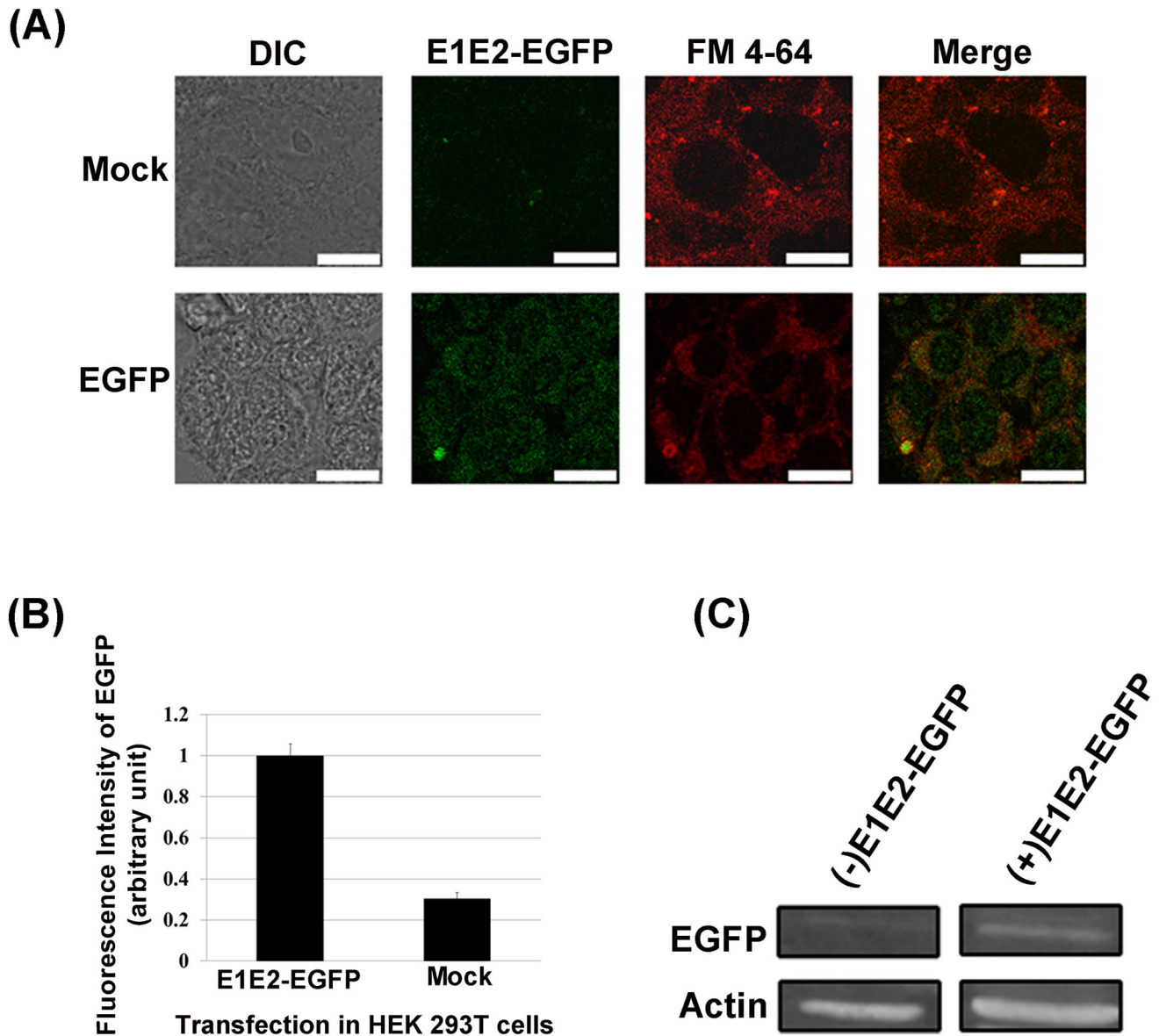


Fig. 2 E1E2-EGFP fusion construct expression in cells. **A** Representative z-plane confocal images of fixed HEK 293T cells where expression of EGFP (green) in HEK 293T cells after 48 h of transfection with pE1E2-EGFP fusion construct was observed. Cells were co-stained with FM4-64 (red) and the merged images depicted.

Scale bars = 10 μ m. **B** Graphical representation of fluorescence intensities of EGFP expression in transfected (E1 E2-EGFP, N = 124) and Mock (N = 68) in 293T cells **C** Immunoblot for GFP and Actin (control) indicating expression of EGFP in E1E2-EGFP transfected cell lysate (color figure online)

observed after 48 h of post transfection by immunoblotting analysis (Fig. 2C).

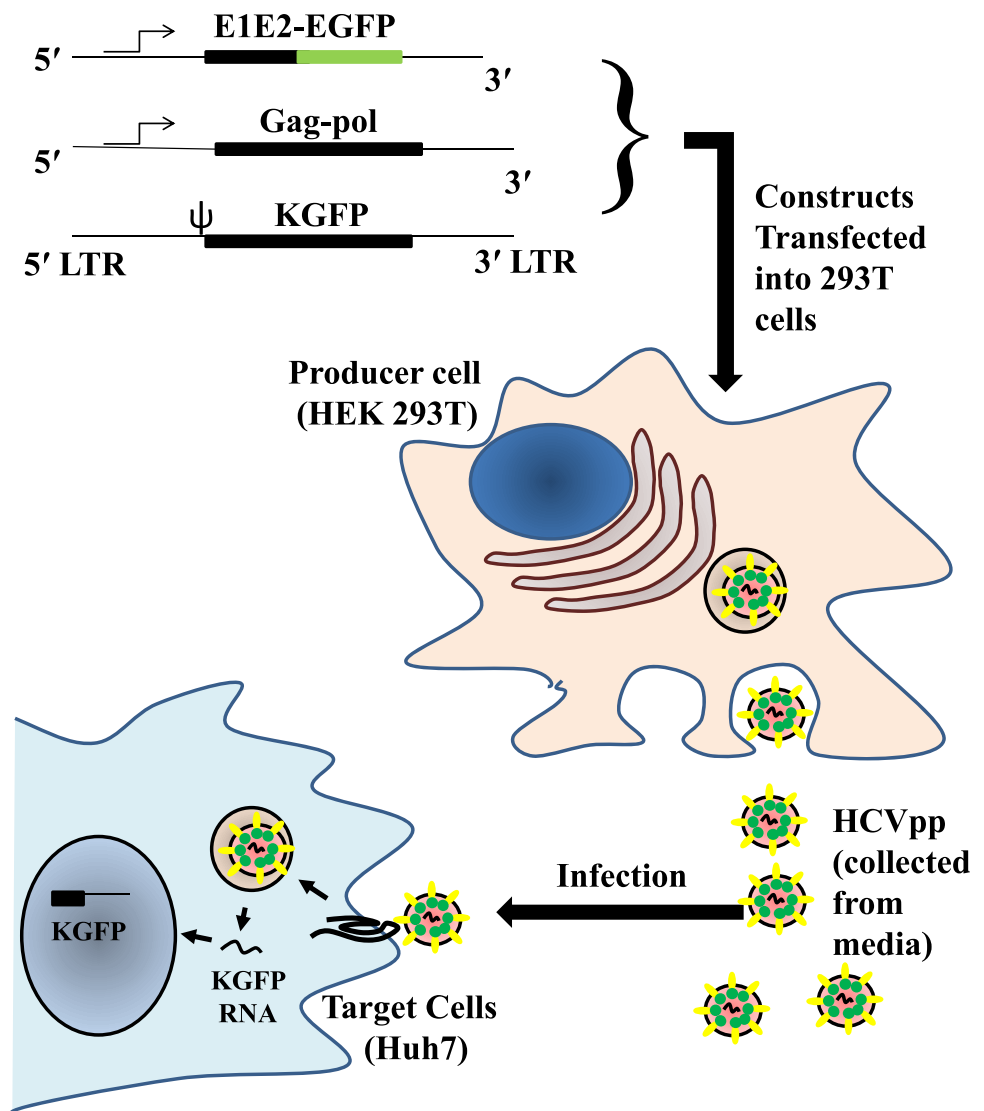
Preparation of fluorescent pseudoparticles

Lentiviral pseudoparticles (HCVpp) were prepared as depicted schematically in Fig. 3 and detailed in the methods section. Transfection of the pE1E2-EGFP, a Gag/Pol packaging construct (pTG5349/gag-pol) and a KGFP containing reporter construct (pLenti-PGK-GFP) into HEK 293T cells yielded fluorescent pseudoparticles from the

media. To check for successful production of HCVpp in HEK 293T cells, harvested HCVpp were allowed to internalize for 20 min in Huh7 cells. EGFP expression was observed under confocal microscope (Fig. 4A). Fluorescence was observed throughout the cytoplasm of Huh7 cells in punctate structures. Fluorescent images were compared with directional interference contrast (DIC) images of same field of cells and approximately 70% transduction efficiency was observed.

For further confirmation, in a parallel study, presence of KGFP RNA in E1E2 tagged HCVpp was detected from

Fig. 3 Production of EGFP-expressing HCV pseudoparticles (HCVpp). Human embryonic kidney cells (HEK 293T) were transfected with three expression vectors; E1E2-GFP, Gag/Pol packaging construct and pLenti-PGK-GFP, KGFP reporter construct. Fluorescent pseudoparticles (expressing fluorescent E1E2 due to presence of tag) were extracted from the media of 293T cells and used for infection/entry studies in Huh7 cells and visualised via confocal microscope. Entry of pseudoparticles can be assayed by estimation of KGFP RNA within the target Huh7 cells



20 min post transduced Huh7 cells by RT-PCR which indicated successful production and transduction of HCVpp (Fig. 4B).

HCVpp trafficking in Huh7

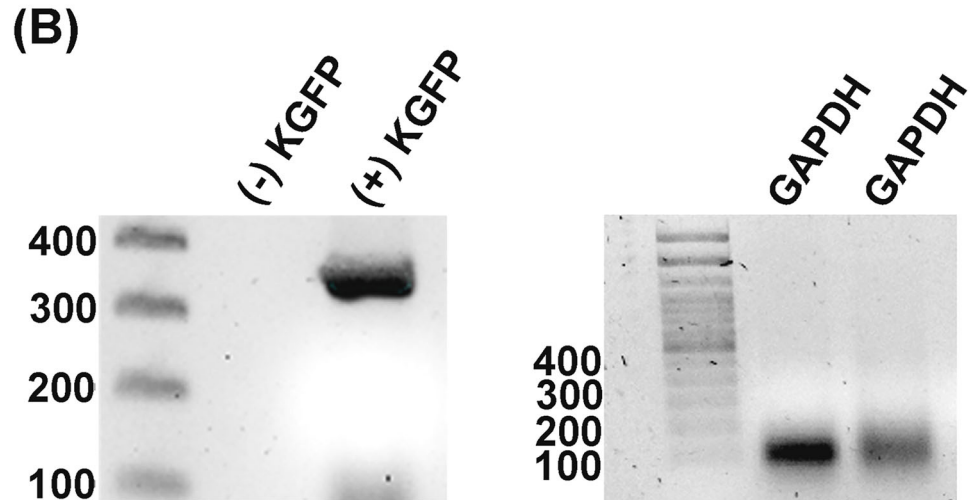
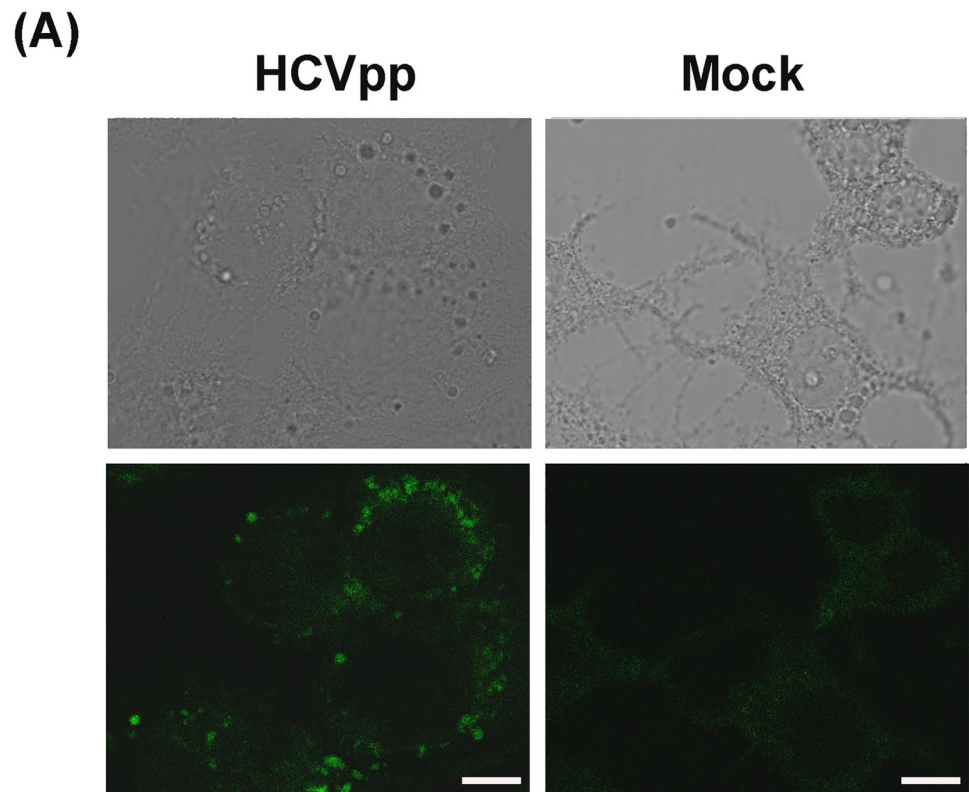
To study HCVpp trafficking, we conducted a time lapse experiment of HCVpp entry in Huh7 cells. As previously characterized, usual endocytic processing in Huh7 results in delivery of ligands to lysosomes within 30 min [8, 18]. Hence we developed entry assays to track the entry of fluorescent HCVpp in the initial 30 min itself. Figure 5A shows representative images of Huh7 cells that were fixed 0, 5, 10, 15 and 30 min after HCVpp internalization.

Measurement of pixel intensities of EGFP tagged HCVpp in Huh7 is depicted in Fig. 5B. It was observed that while pixel intensity remained constant between 0 and 5 min, it rose to a peak level by about 15 min (N = 556).

This is possibly due to coalescing of vesicles due to endocytic processing and fusion-fission events. Thereafter the pixel intensity dropped between 15 and 30 min (N = 906) of internalization possibly due to degradation or dissipation of E1E2-EGFP.

To further study the trafficking details, the cells were stained with a plasma membrane dye FM 4-64. Figure 5A shows representative individual and merged images which indicate colocalization between HCVpp and FM 4-64 at various time points. FM 4-64 is a plasma membrane marker that is expected to label the cellular membrane and not be internalised while on ice, a condition where all endocytosis is halted. As seen in the images and quantitative data of colocalization between HCVpp and FM 4-64, no colocalization between FM 4-64 and EGFP was observed at early time points. HCVpp is expected to be at the plasma membrane or cellular surface at 0 min, but lack of colocalization with FM 4-64 can be explained by a uniform

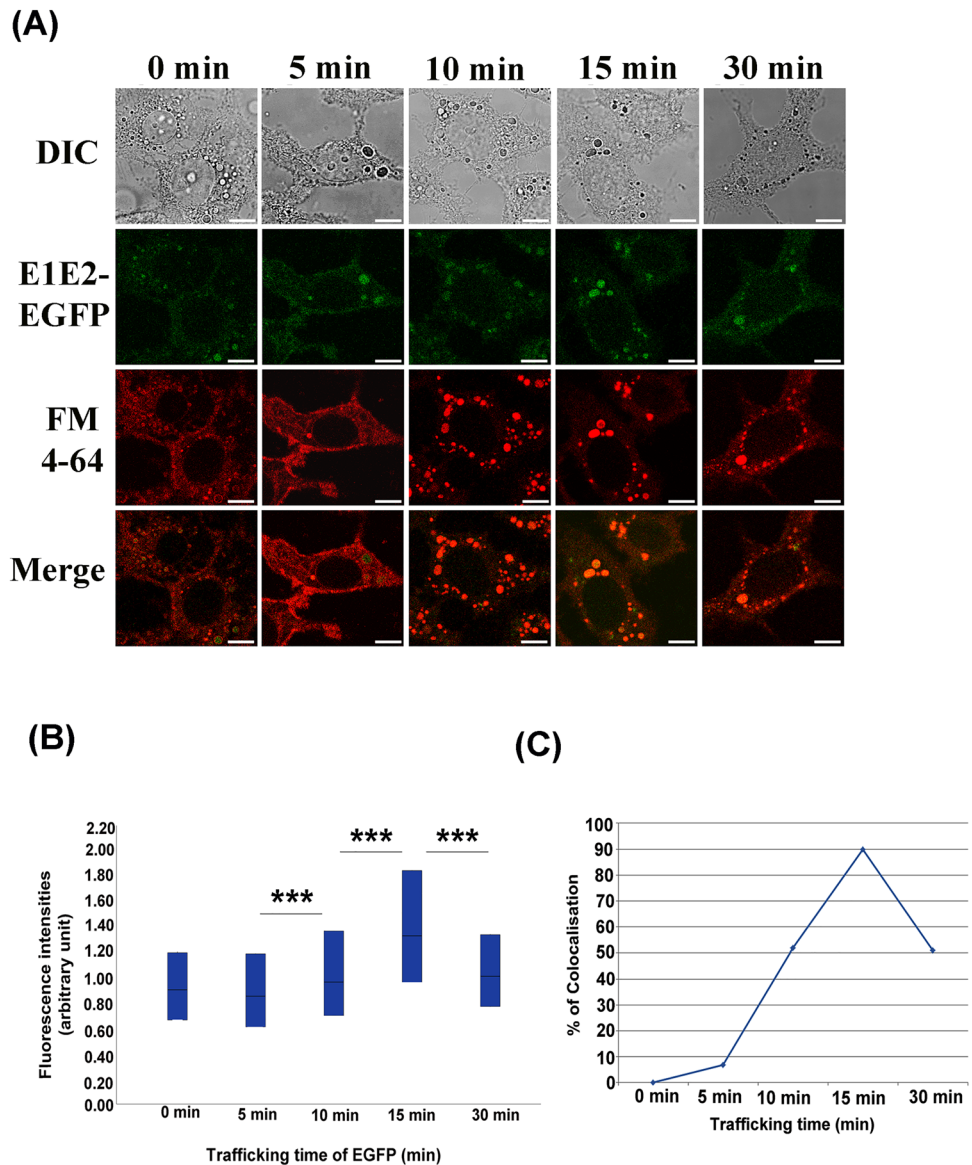
Fig. 4 Entry assay for HCVpp in Huh7 cell line. **A** Expression of EGFP in HCVpp infected Huh7 cells via confocal microscopy. **B** RT-PCR amplification of KGFP from total RNA obtained from Huh7 transduced with HCVpp, separated in 1.5% agarose gel. GAPDH was run as control



distribution of HCVpp on the cell surface resulting in low fluorescent intensity of EGFP that was below the detection limits of the microscope. The EGFP signals rose sharply to a peak value at 15 min ($N = 556$) that was also the time when maximum pixel intensity of HCVpp was being observed (Fig. 5B). As FM 4-64 is a plasma membrane dye that is not internalized, a high colocalization at 15 min ($N = 60$) is indicative of trafficking of E1E2-EGFP to the plasma membrane (Fig. 5C). This would indicate that viral

and host membrane fusion has been completed by 15 min of internalization and hence the E1E2-EGFP has trafficked back to the cell surface via recycling endosomes. However by 30 min a drop in colocalization as well as E1E2-EGFP pixel intensity is perhaps indicative of either dissipation of E1E2-EGFP or its degradation. To cross check degradation in lysosomes, we used a lysosome marker LysoTracker and conducted similar type of time lapse experiments. Interestingly we did not find any colocalization even at 30 min

Fig. 5 HCV pseudoparticles infection and trafficking in Huh7 cells. Representative z-plane confocal images of fixed Huh7 cells. **A** Images of HCVpp transduced in Huh7 cells, fixed at indicated times of post internalisation and analysed via confocal microscope. Scale bars = 10 μ m. **B** Integrated density (IntDen) of EGFP from each of the individual infected Huh7 cells are represented in a box plot profile. Each box represents the median, first and third quartile. ** represents significant difference ($p < 0.05$) and *** represents significant difference ($p < 0.001$) as tested by ANOVA followed by Bonferonni post hoc analysis. For Huh7 (0 min), N = 1748; for 5 min, N = 1018; for 10 min, N = 768; for 15 min, N = 556; for 30 min, N = 906. **C** Graphical representation of positive colocalization percentage (%) between EGFP and FM 4-64 observed at indicated time points. N (0 min) = 67, N (5 min) = 46, N (10 min) = 33, N (15 min) = 60 and N (30 min) = 76. Total number of IntDen measurements for images throughout a z-stack for all replicates of a time point were counted as N. t-score was determined to check the significance of Pearson's R (above threshold) values



of internalization in Huh7 (Fig. 6). This finding indicates that E1E2 does not reach late endosome or are not degraded in lysosome after 30 min of internalization. Hence it is possible that the E1E2 dissipated or was degraded via some other mechanism.

Rab1a is important for HCVpp trafficking

In order to study the role of cellular proteins in HCV entry using these fluorescent pseudoparticles, the importance of Rab proteins in HCV trafficking was studied. A number of Rab proteins have already been implicated in HCV life cycle [19]. In particular, Rab 5 and Rab13 have already been established to be affecting HCV entry [20, 21]. In this study, we investigated the role of another Rab protein, Rab1a. Rab1a, apart from its role in ER-Golgi trafficking

has also been previously characterized to be important in early endocytic sorting events as well as trafficking of early endosomes by recruiting kinesin motor Kifc2 thereby enabling microtubule based movement [8]. Moreover, Rab1a has also been characterized to be important in the trafficking of EGF and ASOR [18], receptors of which have been implicated in HCV entry [22]. Hence, we decided to investigate the role of Rab1a in HCV trafficking by using a Huh7 based Rab1a KD cell line where Rab1a is knocked down by stably expressing shRNA against Rab1a. Experiments previously described for Huh7 were performed in Rab1a KD cell line and compared to the events occurring in the parental Huh7 cells. Observation of trafficking and measurement of pixel intensity (Fig. 7), revealed increased pixel intensity of EGFP at all time points compared to Huh7 cells. This was similar to that

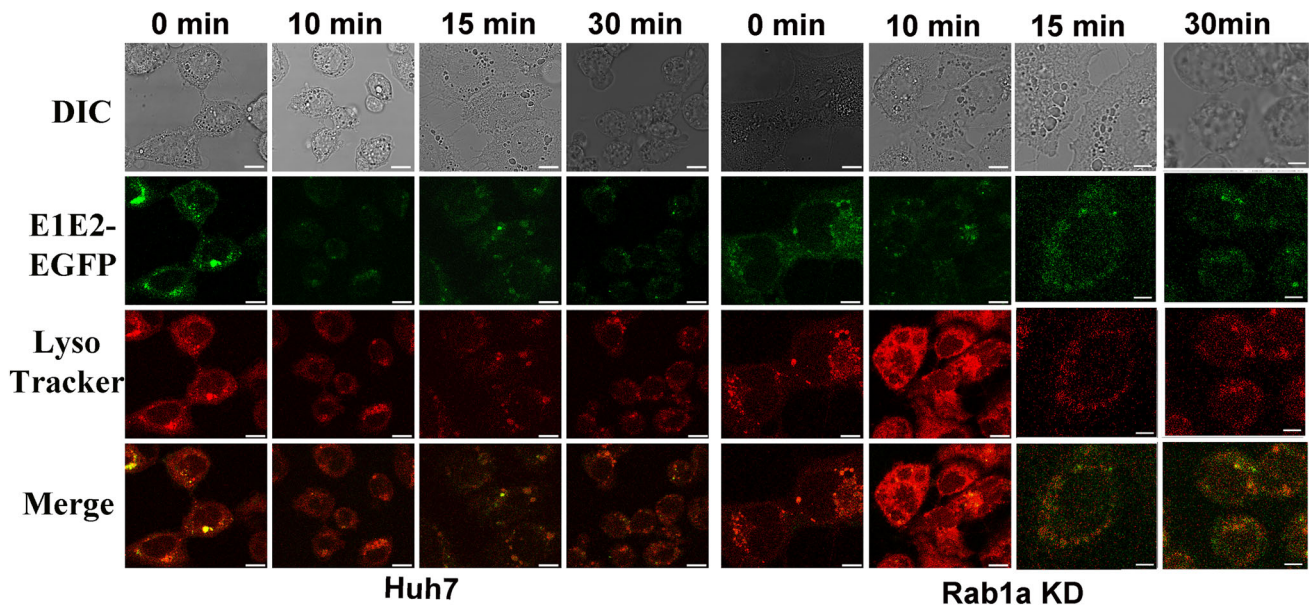


Fig. 6 HCVpp fails to reach lysosomes. Representative z-plane confocal images of fixed Huh7 and Rab1a KD cells represent time lapse trafficking of HCVpp in Rab1a KD and Huh7 cell line. HCVpp infected Huh7 and Rab1a KD cells were co-stained with lysosome marker, LysoTracker (depicted in red). Colocalisation of HCVpp (green) and LysoTracker were assayed. Total numbers of cells for

each time points are counted as N. For Huh7 cell lines, N (0 min) = 50, N (5 min) = 45, N (10 min) = 45, N (15 min) = 50 and N (30 min) = 55; and for Rab1a KD, N (0 min) = 50, N (5 min) = 37, N (10 min) = 40, N (15 min) = 55 and N (30 min) = 56 (color figure online)

observed for trafficking of other ligands in Rab1a KD cells lines and is indicative of delayed trafficking leading to accumulation of ligand (Fig. 7B). Maximum pixel intensity was observed at 15 min (N = 425) and stayed almost constant till 30 min (N = 555). Furthermore, colocalization with lipophilic dye FM 4-64 revealed that while in Huh7 co-localisation with HCVpp reduced between 15 and 30 min, there was no such reduction observed in Rab1aKD cells (Fig. 7C). As Rab1a is known to slow down endocytic trafficking at early time points [8], between 5 and 15 min, we conclude that Rab1a has an important role to play in the endocytic trafficking of HCV where sustained colocalisation with FM 4-64 could indicate trafficking of E1E2-EGFP into a slower recycling route as has been previously studied in the case of transferrin trafficking in the absence of Rab1a [8]. To cross check for an association of E1E2-EGFP with lysosomes in the Rab1a KD cells, we co-stained with lysoTracker and did not observe any colocalization (Fig. 6).

Discussion

In this study we have described the preparation of EGFP tagged HCVpp which have been used in microscopy assays on fixed cells to study the very early events of HCV entry. These HCVpp can possibly be used for live cell imaging as well, which in our preliminary studies proved to be

successful (data not shown). To the best of our knowledge, this is the first description of fluorescent pseudoparticles which have been used in trafficking assays to determine the very early events of viral entry. In all previous experiments using HCVpp, fluorescent expression is only visible or analysed 48–72 h of post transduction. In this study, this is first description of EGFP tagged HCVpp where viral glycoprotein is fluorescent and hence can be visualised directly without having to wait for expression of a reporter.

As with all fusion proteins, orientation of EGFP and its possible interference in E1E2 dynamics in the E1E2-EGFP fusion protein is a pertinent question. As previously described, evidence is available in literature where similar constructs with Myc and HA tags had the tagged portion present in the cytoplasm [23]. Furthermore, previous reports suggested that GFP is pH sensitive and the low pH in the secretory pathway during endocytosis decreases fluorescence activity [24, 25]. In this study we did not find any alteration or degradation of fluorescent activity after 20 min of cellular internalisation which is only possible if the EGFP is oriented within the HCVpp and hence not exposed to the low pH of the endosome during entry. Such orientation supports the cytoplasmic presence of EGFP during synthesis at the ER. Such an orientation could not be involved in any steric hindrance to the receptor-ectodomain interactions happening at the surface of the HCVpp and the host cell.

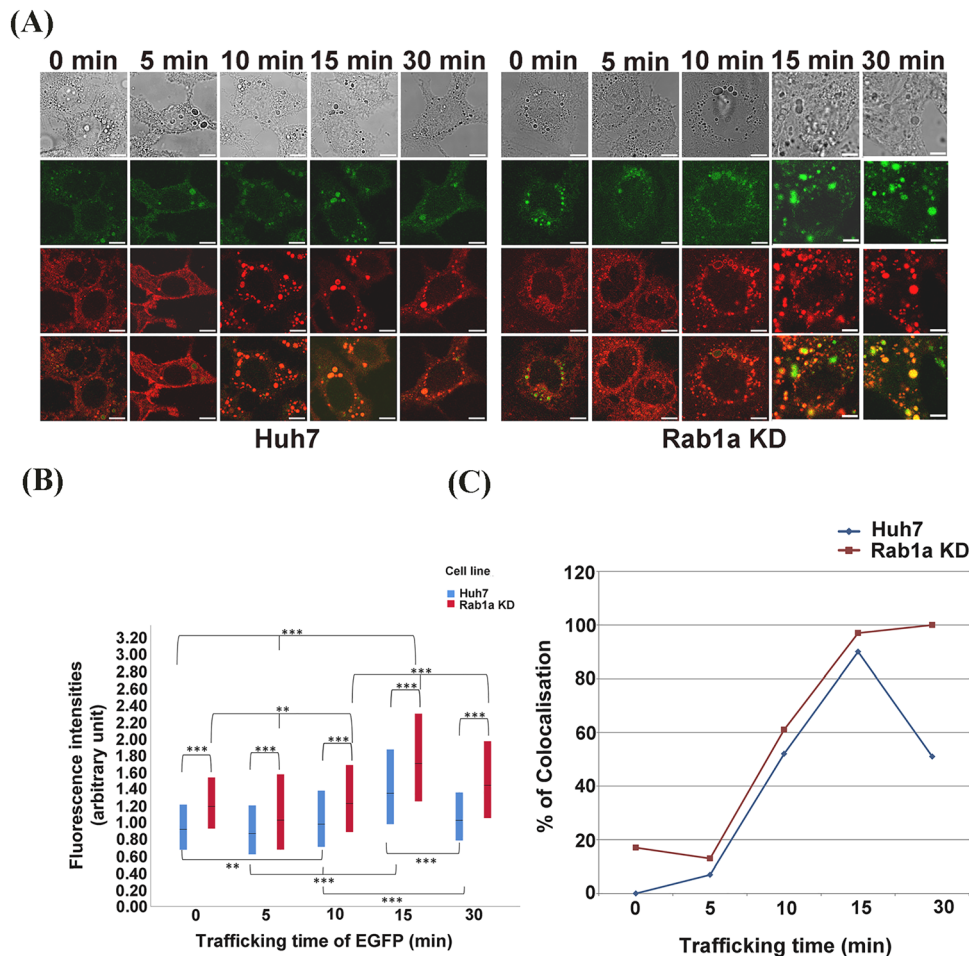


Fig. 7 HCV pseudoparticles infection and trafficking in Rab1a KD cells. Representative z-plane confocal images of fixed Rab1a KD Huh7 cells represent time lapse trafficking of HCVpp in Rab1a KD cell line. **A** Representative images of E1E2 GFP (green) trafficking in Huh7 (left) and Rab1a KD (right) cells for indicated time points. Cells were co-stained with FM 4-64 indicated in red. Top panel DIC images, second panel HCVpp trafficking depicted in green. Third panel: FM4-64 staining and bottom panel depict merged images of HCVpp and FM4-64. Scale Bars = 10 μ m. **B** Integrated density (IntDen) of EGFP from each of the individual infected cells (Huh7 and Rab1aKD) are represented in a box plot profile. Each box represents median, first and third quartile. ** represents significant difference ($p < 0.05$) and *** represents significant difference ($p < 0.001$) as tested by ANOVA. For Huh7 (0 min), N = 1748; for

5 min, N = 1018; for 10 min, N = 768; for 15 min, N = 556; for 30 min, N = 906 and for Rab1a KD (0 min), N = 504; for 5 min, N = 286; for 10 min N = 468; for 15 min, N = 425; for 30 min N = 555. **C** Graphical representation of positive colocalization percentage (%) between GFP and FM 4-64 are observed at indicated time points. For Huh7 cell lines, N (0 min) = 67, N (5 min) = 46, N (10 min) = 33, N (15 min) = 60 and N (30 min) = 76; and for Rab1a KD, N (0 min) = 30, N (5 min) = 47, N (10 min) = 28, N (15 min) = 74 and N (30 min) = 30. Total number of IntDen measurements for images throughout a z-stack for all replicates of a time point were counted as N. One way ANOVA followed by Bonferonni post hoc analysis was performed for statistical analysis. t-score was determined to check the significance of Pearson's R (above threshold) values (color figure online)

In this study we report HCV entry events between 0 and 30 min (Fig. 8). This time span has been selected as most of the published literature suggest that endocytic entry mechanisms of ligands occur within a time span of 30 min [26]. Visualisation of viral entry is difficult for many technical reasons, as all viral particles may not have similar virulence. In our study, analysis of KGFP RNA from Huh7 soon after transduction represents successful delivery of the KGFP RNA genome which can only be possible if pseudoparticles have been able to gain entry into the Huh7 cells. Any non-specific signal or unattached pseudoparticle

would be removed by washes prior to shifting of the cells to 37 $^{\circ}$ C. Hence by analysis of KGFP RNA in Huh7 cells and visualisation of EGFP fluorescence we are confident about the successful preparation of fluorescent HCVpp.

The study describes the trafficking of fluorescent HCVpp in Huh7 cells and predicts an approximate fusion timing. After interaction with a series of cellular receptors, the virus is internalised into hepatocytes via clathrin mediated endocytosis. Trafficking of HCV-receptor complex promotes viral endocytosis and fusion between cellular membrane and HCV glycoproteins. This is the final

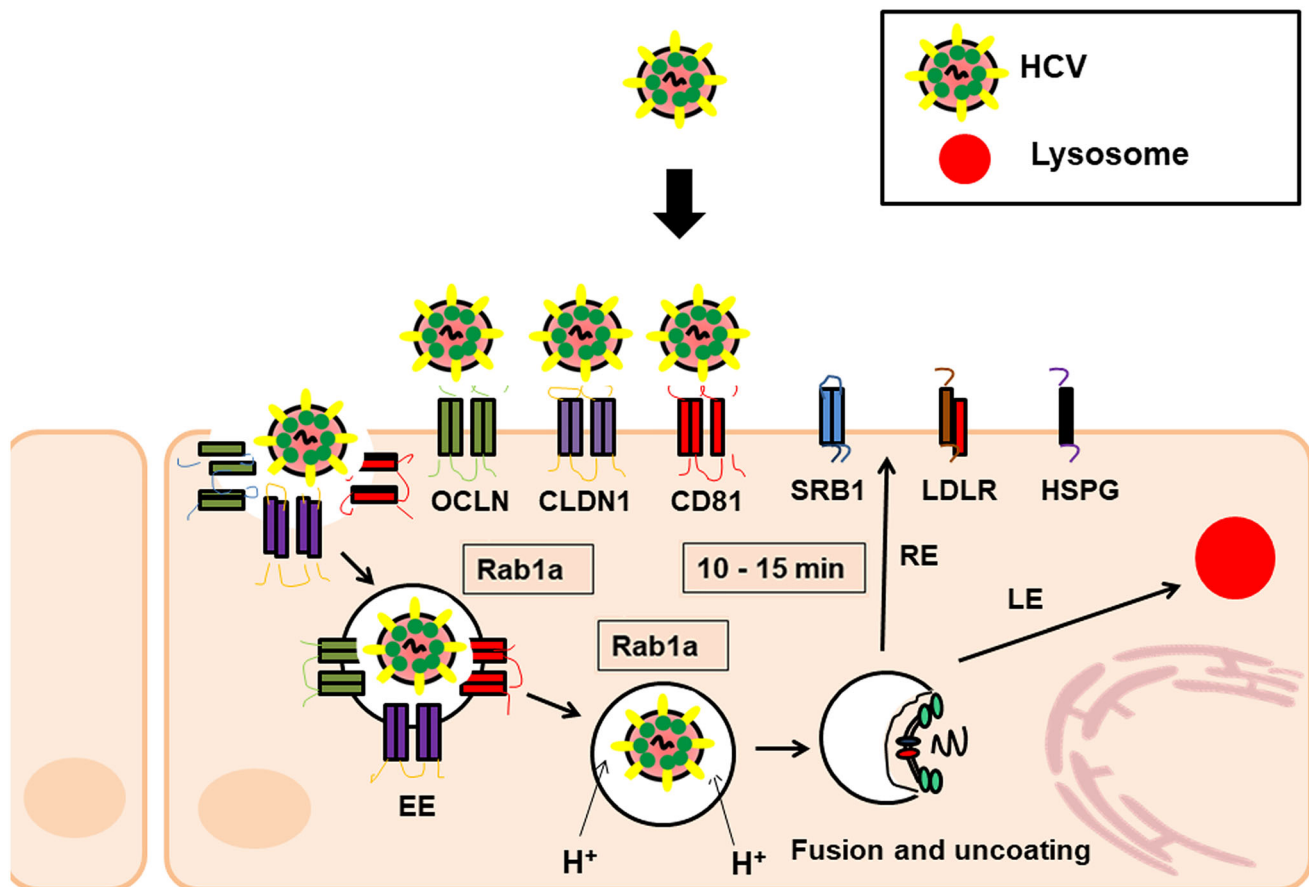


Fig. 8 HCV trafficking in hepatocytes. HCVpp enters cells via interaction with several receptors like CD81. Upon internalization, the virus is endocytosed and undergoes fusion with the endocytic membrane between 5 and 15 min of internalization. Thereafter the

surface glycoprotein travels to the plasma membrane after which it is perhaps degraded. Rab1a has a role to play during the early entry processes, perhaps in trafficking

step before the HCV RNA gets released in cytosol to start viral replication and protein synthesis [27]. Since we observed maximum colocalisation with plasma membrane marker after 15 min of internalisation, we propose that by this time fusion is completed and the fluorescent glycoprotein, still attached to the membrane travels back to the plasma membrane via a recycling route (Fig. 8). Maximum fluorescence is also observed at 15 min due to coalescing of the recycling vesicles at the plasma membrane. More experiments however will help to prove our hypothesis. Nevertheless, this study helps to understand the early entry and fusion events of the virus.

We also report for the first time a role of the Rab GTPase in early trafficking events of this virus. In HCV trafficking scanty information is available for Rab5 and Rab13 [20, 21]. Role of Rab1a in HCV endocytosis has not been studied so far; although it is associated with both early and late endocytic vesicles for many ligands. Rab1a KD cell line exhibited slower processing and degradation of different ligands, such as asialoorsomuroid (ASOR), Transferrin and Epidermal Growth Factor that suggest a

defect in endosomal sorting [8]. Rab1a is also important in life cycle of many viruses including Human Immunodeficiency Virus (HIV) for its ER-Golgi trafficking and Herpes Simplex Virus (HSV-1) to maintain Golgi function [28, 29]. In case of HCV, Rab1a is directly involved in viral replication and promotes lipid droplets formation on ER membrane [30, 31]. Rab1a has also been involved in viral entry of other viruses. It has been reported that Rab1a is rapidly recruited to cell membrane within 30 min in Vaccinia Virus (VACC) infected cells, indicating a possible role in Vaccinia entry [32].

Using EGFP tagged HCVpp, this study describes the probable role of the Rab1a GTPase in HCV cellular trafficking for the first time. HCV follows clathrin mediated endocytosis and uses EGF receptor and ASGPR for its hepatocellular entry, receptors known to be endocytosed with the help of Rab1a. In Rab1a KD cells, an enhanced fluorescence intensity of HCVpp was observed at all time points in comparison to the parental Huh7 cells. This was indicative of a slower/delayed trafficking where vesicles were perhaps coalescing. Colocalisation of HCVpp with

plasma membrane marker FM 4-64 was also enhanced at all time points in contrast to parental cells. This can perhaps be explained by the possibility that in the absence of Rab1a, the vesicles perhaps don't internalise well and remain close to the plasma membrane (Fig. 8). Whether this affects fusion of the viral and host membranes or not remains to be investigated. Expression of EGFP was high even after 30 min indicating that cellular vesicles returning to the cellular surface perhaps remained at the cell surface and are possibly not further processed. Altogether, these observations conclude Rab1a plays an important role in HCV trafficking.

In conclusion, this study explains understanding of HCV biology and describes the preparation of HCVpp which can be used for microscopic assays of viral entry. To the best of our knowledge this is the first preparation of fluorescent HCVpp, used in hepatocellular trafficking assays to determine the very early events of HCV entry. Moreover, this system and these trafficking assays can also be used to understand the entry of any pseudotyped virus including SARS CoV-2. Such assays have the potential to be a powerful tool to screen and understand the mechanistics of any preventive of viral entry. EGFP tagged HCVpp can be used to understand viral entry, rate of transmission or most importantly early trafficking details of any virus by simple RT-PCR, FACS analysis or confocal microscopy. Furthermore, such knowledge perhaps helps to understand the time line of the viral replication cycle and predict a prognosis. With respect to HCV biology, this system is expected to help develop more specific inhibitors of host-HCV fusion complex in future and develop potent therapeutics or preventives for HCV which may be used during blood transfusion and liver transplantation.

Acknowledgements The authors acknowledges funding from Department of Biotechnology-BioCARE Project (BT/Bio-CARE/07/10139/2013-14) awarded to AM and DBT-BUILDER and DST-FIST awarded to Presidency University. The authors would also like to acknowledge funding from Presidency University- Faculty Research Professional Development Fund. The authors also wish to acknowledge contributions of Sayantan Adhikary, Pratik Das, Debisukti Das and Ritisri Mondal to this work.

Author contribution CB: Investigation, Formal analysis, Writing-original draft. AM: Conceptualization, Methodology, Writing-Review and Editing, Supervision, Funding acquisition.

Declarations

Conflict of interest The authors declare that there are no conflict of interest.

Ethical approval This work did not involve the use of animal or humans.

References

1. WHO. Hepatitis C. WHO fact sheet. <https://www.who.int/news-room/fact-sheets/detail/hepatitis-c> (2021)
2. Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C, Rouillé Y, Rouille Y, Rouillé Y. Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol Am Soc Microbiol.* 2006;80:6964–72. <https://doi.org/10.1128/JVI.00024-06>.
3. Bartosch B, Dubuisson J, Cosset F-L. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med.* 2003;197:633–42. <http://www.ncbi.nlm.nih.gov/pubmed/12615904>.
4. Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA. Hepatitis C virus glycoproteins mediate PH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci USA.* 2003. <https://doi.org/10.1073/pnas.0832180100>.
5. Willingham MC, Pastan IH. Transit of Epidermal Growth Factor through Coated Pits of the Golgi System. *J Cell Biol.* 1982;94:207–212. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6288737.
6. Bananis E, Murray JW, Stockert RJ, Satir P, Wolkoff AW. Microtubule and motor-dependent endocytic vesicle sorting in vitro. *J Cell Biol.* 2000;151:179–186. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11018063.
7. Murray JW, Sarkar S, Wolkoff AW. Single vesicle analysis of endocytic fission on microtubules in vitro. *Traffic* 2008;9:833–847. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18284582.
8. Mukhopadhyay A, Quiroz JAA, Wolkoff AWW. Rab1a regulates sorting of early endocytic vesicles. *Am J Physiol Gastrointest Liver Physiol.* 2014;306:G412–24. <https://doi.org/10.1152/ajpgi.00118.2013>.
9. Saunier B, Triyatni M, Ulianich L, Maruvada P, Yen P, Kohn LD. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. *J Virol.* 2003;77:546–59. <https://doi.org/10.1128/jvi.77.1.546-559.2003>.
10. Diao J, Pantua H, Ngu H, Komuves L, Diehl L, Schaefer G, Kapadia SB. Hepatitis C virus induces epidermal growth factor receptor activation via CD81 binding for viral internalization and entry. *J Virol Am Soc Microbiol.* 2012;86:10935–49. <https://doi.org/10.1128/JVI.00750-12>.
11. Bartosch B, Cosset FL. Studying HCV cell entry with HCV pseudoparticles (HCVpp). *Methods Mol Biol.* 2009. https://doi.org/10.1007/978-1-59745-394-3_21.
12. Voisset C, Callens N, Blanchard E, Op De Beeck A, Dubuisson J, Vu-Dac N. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. *J Biol Chem.* 2005;280:7793–9. <https://doi.org/10.1074/jbc.M411600200>.
13. Follenzi Antonia NL HIV-based vectors. preparation and use - PubMed. *Methods Mol Med.* 2002;69:259–274. <https://pubmed.ncbi.nlm.nih.gov/11987783/>.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein Measurement with the Folin Phenol Reagent. *J Biol Chem.* 1951;193:265–275. <http://www.ncbi.nlm.nih.gov/pubmed/14907713>.
15. Cocquerel L, Op de Beeck A, Lambot M, Roussel J, Delgrange D, Pillez A, Wychowski C, Penin F, Dubuisson J. Topological changes in the transmembrane domains of hepatitis C virus envelope glycoproteins. *EMBO J Eur Mol Biol Organ.* 2002;21:2893–902. <https://doi.org/10.1093/emboj/cdf295>.

16. Op De Beeck A, Montserret R, Duvet S, Cocquerel L, Cacan R, Barberot B, Maire L, Penin M, Dubuisson FJ. The transmembrane domains of hepatitis C virus envelope glycoproteins E1 and E2 play a major role in heterodimerization. *J Biol Chem*. 2000. <https://doi.org/10.1074/jbc.M003003200>.
17. Lavie M, Goffard A, Dubuisson J. HCV glycoproteins: assembly of a functional E1–E2 heterodimer. *Hepat C Viruses Genomes Mol Biol Horiz Biosci*. 2006 <https://www.ncbi.nlm.nih.gov/books/NBK1628/>.
18. Mukhopadhyay A, Nieves E, Che F-YY, Wang J, Jin L, Murray JWW, Gordon K, Angeletti RHH, Wolkoff AWW. Proteomic analysis of endocytic vesicles: Rab1a regulates motility of early endocytic vesicles. *J Cell Sci*. 2011;124:765–75. <https://doi.org/10.1242/jcs.079020>.
19. Elgner F, Hildt E, Bender D. Relevance of Rab proteins for the life cycle of hepatitis C virus. *Front Cell Dev Biol*. 2018;6:166. <https://doi.org/10.3389/FCCELL.2018.00166/BIBTEX>.
20. Takeda M, Ikeda M, Satoh S, Dansako H, Wakita T, Kato N. Rab13 Is Involved in the Entry Step of Hepatitis C Virus Infection. *Acta Med Okayama*. 2016;70:111–118. https://ousar.lib.okayama-u.ac.jp/files/public/5/54190/20160528123706251413/70_2_111.pdf.
21. Farquhar MJ, Hu K, Harris HJ, Davis C, Brimacombe CL, Fletcher SJ, Baumert TF, Rappoport JZ, Balfe P, McKeating JA. Hepatitis C virus induces CD81 and claudin-1 endocytosis. *J Virol Am Soc Microbiol*. 2012;86:4305–16. https://doi.org/10.1128/JVI.06996-11/SUPPL_FILE/MOVIES2.AVI.
22. Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J, Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Dofföl M, Raffelsberger W, Poch O, McKeating JA, Brino L, Baumert TF. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat Med*. 2011. <https://doi.org/10.1038/nm.2341>.
23. Carrère-Kremer S, Montpellier-Pala C, Cocquerel L, Wychowski C, Penin F, Dubuisson J. Subcellular localization and topology of the P7 polypeptide of hepatitis C virus. *J Virol Am Soc Microbiol*. 2002;76:3720–30. <https://doi.org/10.1128/jvi.76.8.3720-3730.2002>.
24. Patterson GH, Knobel SM, Sharif WD, Kain SR, Piston DW. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys J*. 1997. [https://doi.org/10.1016/S0006-3495\(97\)78307-3](https://doi.org/10.1016/S0006-3495(97)78307-3).
25. Ward WW. Properties of the coelenterate green-fluorescent proteins. *Biolumin Chemilumin*. 1981. <https://doi.org/10.1016/b978-0-12-208820-9.50035-5>.
26. Nicola AV, Straus SE. Cellular and viral requirements for rapid endocytic entry of herpes simplex virus. *J Virol*. 2004. <https://doi.org/10.1128/jvi.78.14.7508-7517.2004>.
27. Harrison SC. Viral membrane fusion. *Nat Struct Mol Biol*. 2008;15:690–8. <https://doi.org/10.1038/nsmb.1456>.
28. Nachmias D, Sklan EH, Ehrlich M, Bacharach E. Human immunodeficiency virus type 1 envelope proteins traffic toward virion assembly sites via a TBC1D20/Rab1-regulated pathway. *Retrovirology*. 2012. <https://doi.org/10.1186/1742-4690-9-7>.
29. Zenner HL, Yoshimura S-I, Barr FA, Crump CM. Analysis of Rab GTPase-activating proteins indicates that Rab1a/b and Rab43 are important for herpes simplex virus 1 secondary envelopment. *J Virol*. 2011. <https://doi.org/10.1128/jvi.00500-11>.
30. Nevo-Yassaf I, Yaffe Y, Asher M, Ravid O, Eizenberg S, Henis YI, Nahmias Y, Hirschberg K, Sklan EH. Role for TBC1D20 and Rab1 in hepatitis C virus replication via interaction with lipid droplet-bound nonstructural protein 5A. *J Virol*. 2012. <https://doi.org/10.1128/jvi.00496-12>.
31. Sklan EH, Serrano RL, Einav S, Pfeffer SR, Lambright DG, Glenn JS. TBC1D20 is a Rab1 GTPase-activating protein that mediates hepatitis C virus replication. *J Biol Chem*. 2007. <https://doi.org/10.1074/jbc.M705221200>.
32. Schroeder N, Chung C-S, Chen C-H, Liao C-L, Chang W. The lipid raft-associated protein CD98 is required for vaccinia virus endocytosis. *J Virol*. 2012. <https://doi.org/10.1128/jvi.06610-11>.

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