

tantly, several viruses target SGs and stress granule components for viral replication (10, 11, 34, 39). Recent studies suggest that SGs and the P body physically interact and that mRNAs may move between the two compartments (1, 6, 22, 28, 30).

miRNAs are a class of small noncoding RNA molecules ~21 to 22 nucleotides (nt) in length. miRNAs usually interact with 3'-untranslated regions (UTRs) of target mRNAs, leading to the downregulation of mRNA expression. Notably, the liver-specific and abundant miR-122 interacts with the 5'-UTR of the HCV RNA genome and facilitates HCV replication (15, 17, 19, 20, 31). Ago2 is at least required for the efficient miR-122 regulation of HCV RNA accumulation and translation (40). However, the molecular mechanism(s) for how DDX6 and miR-122 as well as DDX3 positively regulate HCV replication is not fully understood. Therefore, we investigated the potential role of P-body and stress granule components in HCV replication.

#### MATERIALS AND METHODS

**Cell culture.** 293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). HuH-7-derived RSc cured cells, in which cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) (37) could infect and effectively replicate, were cultured in DMEM with 10% FBS as described previously (3–5, 23).

**Plasmid construction.** To construct pcDNA3-FLAG-DDX6, a DNA fragment encoding DDX6 was amplified from total RNAs derived from RSc cells by reverse transcription (RT)-PCR using KOD-Plus DNA polymerase (Toyobo) and the following pairs of primers: 5'-CGGGATCCAAGATGAGCAGCGCC AGAACAGAGAACCCTGTT-3' (forward) and 5'-CCGCTCGAGTTAAGGT TTCTCATCTTCTACAGGCTCGCT-3' (reverse). The obtained DNA fragments were subcloned into either BamHI-XhoI site of the pcDNA3-FLAG vector (2), and the nucleotide sequences were determined by BigDye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

**RNA interference.** The following small interfering RNAs (siRNAs) were used: human ATXN2/ATX2/ataxin-2 (siGENOME SMRT pool M-011772-01-005), human PABP1/PABPC1 (siGENOME SMRT pool M-019598-01-005), human Lsm1 (siGENOME SMRT pool M-005124-01-005), human Xrn1 (siGENOME SMRT pool M-013754-01-005), human G3BP1 (ON-TARGETplus SMRT pool L-012099-00-005), human PATL1 (siGENOME SMRT pool M-015591-00-005), and siGENOME nontargeting siRNA pool 1 (D-001206-13-05) (Dharmacon, Thermo Fisher Scientific, Waltham, MA), as a control. siRNAs (25 nM final concentration) were transiently transfected into RSc cells (3–5, 23) using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences targeted to DDX6 (DDX6i) as well as the control nontargeting shRNA (shCon) in a lentiviral vector: 5'-GATCC CCGGAGGAACAACTCTGAAGTTCAAGAGACTTCAGAGTTAGTTCCT CCTTTTTGGAAA-3' (sense) and 5'-AGCTTTTCCAAAAGGAGGAACTAA CTCTGAAGTCTCTGAACTTCAGAGTTAGTTCCTCCGGG-3' (antisense) for DDX6i and 5'-GATCCCGAATCCAGAGGTAATCTACTTCAAGAGA GTAGATTACTCTGGATTCTTTTTGGAAA-3' (sense) and 5'-AGCTTTT CAAAAGAATCCAGAGGTAATCTACTCTTGAAGTAGATTACCTC TGGATTCCGGG-3' (antisense) for shCon. The oligonucleotides described above were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER (8), to generate pSUPER-DDX6i and pSUPER-shCon, respectively. To construct pLV-DDX6i and pLV-shCon, the BamHI-Sall fragments of the corresponding pSUPER plasmids were subcloned into the BamHI-Sall site of pRDI292, an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistance marker allowing for the selection of transduced cells (7). pLV-DDX3i, described previously (3), was used.

**Lentiviral vector production.** The vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-based vector system was described previously (27, 44). The lentiviral vector particles were produced by the transient transfection of the second-generation packaging construct pCMV-DR8.91 (27, 44), the VSV-G-

envelope-expressing plasmid pMDG2, as well as pRDI292 into 293FT cells with FuGene6 reagent (Roche Diagnostics, Mannheim, Germany).

**HCV infection experiments.** The supernatants were collected from cell culture-generated HCV-JFH1 (37)-infected RSc cells (3–5, 23) at 5 days postinfection and stored at  $-80^{\circ}\text{C}$  after filtering through a  $0.45\text{-}\mu\text{m}$  filter (Kurabo, Osaka, Japan) until use. For infection experiments with HCV-JFH1, RSc cells ( $1 \times 10^5$  cells/well) were plated onto 6-well plates and cultured for 24 h. We then infected the cells at a multiplicity of infection (MOI) of 1 or 4. The culture supernatants were collected at 24 h postinfection, and the levels of the core protein were determined by an enzyme-linked immunosorbent assay (ELISA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was also isolated from the infected cellular lysates by using an RNeasy minikit (Qiagen, Hilden, Germany) for analysis of intracellular HCV RNA. The infectivity of HCV-JFH1 in the culture supernatants was determined by a focus-forming assay at 48 h postinfection. HCV-JFH1-infected cells were detected by using anti-HCV core (CP-9 and CP-11 mixture).

**Quantitative RT-PCR analysis.** The quantitative RT-PCR analysis of HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously (3–5, 14, 23). We used the following forward and reverse primer sets for the real-time LightCycler PCR: 5'-ATGAGTCATGTGGCAGTGA-3' (forward) and 5'-GCTGGCTGTACTTCTCCAC-3' (reverse) for DDX3, 5'-ATG AGCAGGCCAGAACAGA-3' (forward) and 5'-TTGCTGTGTCTGTGTGC CCC-3' (reverse) for DDX6, 5'-TGACGGGGTCAACCACTG-3' (forward) and 5'-AAGCTGTAGCCGCGCTCGGT-3' (reverse) for  $\beta$ -actin, and 5'-AGA GCCATAGTGGTCTCGGG-3' (forward) and 5'-CTTTCGCAACCAACGC TAC-3' (reverse) for HCV-JFH1.

**Preparation of anti-PATL1 antibody.** The anti-PATL1 antiserum was raised in rabbits using the glutathione *S*-transferase (GST)-fused PATL1 Ct (C-terminal region of PATL1, aa 450 to 770) as an antigen, and immunoglobulins were affinity purified by using the maltose-binding protein (MBP)-fused PATL1 Ct that was immobilized on an *N*-hydroxysuccinimide (NHS) column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

**Preparation of LDs.** Lipid droplets (LDs) were prepared as described previously (26). Cells were pelleted by centrifugation at 1,500 rpm. The pellet was resuspended in hypotonic buffer (50 mM HEPES [pH 7.4], 1 mM EDTA, 2 mM  $\text{MgCl}_2$ ) supplemented with a protease inhibitor cocktail (Nacal Tesque, Kyoto, Japan) and was incubated for 10 min at  $4^{\circ}\text{C}$ . The suspension was homogenized with 30 strokes of a glass Dounce homogenizer using a tight-fitting pestle (Wheaton, Millville, NJ). A 1/10 volume of  $10\times$  isotonic buffer (0.2 M HEPES (pH 7.4), 1.2 M potassium acetate (KoAc), 40 mM magnesium acetate [ $\text{Mg}(\text{oAc})_2$ ], and 50 mM dithiothreitol (DTT)) was added to the homogenate. The nuclei were removed by centrifugation at 2,000 rpm for 10 min at  $4^{\circ}\text{C}$ . The supernatant was collected and centrifuged at  $16,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatant was mixed with an equal volume of 1.04 M sucrose in isotonic buffer (50 mM HEPES, 100 mM KCl, 2 mM  $\text{MgCl}_2$ , and protease inhibitor cocktail). The solution was set in a 13.2-ml Polyallomer centrifuge tube (Beckman Coulter, Brea, CA). One milliliter of isotonic buffer was loaded onto the sucrose mixture. The tube was centrifuged at  $100,000 \times g$  in an SW41Ti rotor (Beckman Coulter) for 1 h at  $4^{\circ}\text{C}$ . After the centrifugation, the LD fraction on the top of the gradient solution was recovered in phosphate-buffered saline (PBS). The collected LD fraction was used for Western blot analysis.

**Western blot analysis.** Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. Supernatants from these lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-DDX3 (catalog no. 54257 [NT] and 5428 [IN]; Anaspec, San Jose, CA), anti-DDX6 (A300-460A; Bethyl Laboratories, Montgomery, TX), anti-adipose differentiation-related protein (ADFP; GTX110204; GeneTex, San Antonio, TX), anti-calnexin (NT; Stressgen, Ann Arbor, MI), anti-HCV core (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan), anti- $\beta$ -actin antibody (A5441; Sigma, St. Louis, MO), anti-ATX2/SCA2 antibody (A302-033A; Bethyl), anti-PABP (sc-32318 [10E10]; Santa Cruz Biotechnology, Santa Cruz, CA), anti-PABP (ab21060; Abcam, Cambridge, United Kingdom), anti-G3BP1 (611126; BD Transduction Laboratories, San Jose, CA), anti-LSM1 (LS-C97364, Life Span Biosciences, Seattle, WA), anti-HSP70 (610607; BD), anti-XRN1 (A300-443A; Bethyl), or anti-PATL1 antibody.

**Immunofluorescence and confocal microscopic analysis.** Cells were fixed in 3.6% formaldehyde in PBS, permeabilized in 0.1% NP-40 in PBS at room temperature, and incubated with anti-DDX3 antibody (54257 [NT] and 5428 [IN]; Anaspec), anti-DDX3X (LS-C64576; Life Span), anti-DDX6 (A300-460A; Bethyl), anti-HCV core (CP-9 and CP-11), anti-ATX2/SCA2 antibody (A302-033A; Bethyl), anti-ataxin-2 (611378; BD), anti-PABP (ab21060; Abcam), anti-G3BP1 (A302-033A; Bethyl), anti-LSM1 (LS-C97364; Life Span), anti-XRN1

(A300-443A; Bethyl), anti-Dcp2 (A302-597A; Bethyl), anti-human Ago2 (011-22033; Wako, Osaka, Japan), or anti-PATL1 antibody at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) for 30 min at 37°C. The cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) at a 1:300 dilution in PBS containing BSA for 30 min at 37°C. Lipid droplets and nuclei were stained with borondipyrromethene (BODIPY) 493/503 (Molecular Probes, Invitrogen) and DAPI (4',6-diamidino-2-phenylindole), respectively, for 15 min at room temperature. Following extensive washing in PBS, the cells were mounted onto slides using a mounting medium of 90% glycerin-10% PBS with 0.01% *p*-phenylenediamine added to reduce fading. Samples were viewed under a confocal laser scanning microscope (LSM510; Zeiss, Jena, Germany).

**Statistical analysis.** A statistical comparison of the infectivities of HCV in the culture supernatants between the knockdown cells and the control cells was performed by using the Student *t* test. *P* values of less than 0.05 were considered statistically significant. All error bars indicate standard deviations.

## RESULTS

**HCV infection hijacks the P-body components.** To investigate the potential role of P-body components in the HCV life cycle, we first examined the alteration of the subcellular localization of DDX3 or DDX6 by HCV-JFH1 infection using confocal laser scanning microscopy as previously described (2), since both DDX3 and DDX6 were identified previously as P-body components (6). For this, we used HuH-7-derived RSc cells, in which cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) (37) can infect and effectively replicate (3, 4, 23). HCV-JFH1-infected RSc cells at 60 h postinfection were stained with anti-HCV core antibody, anti-DDX3, and/or anti-DDX6. Lipid droplets (LDs) and nuclei were stained with BODIPY 493/503 and DAPI (4',6-diamidino-2-phenylindole), respectively. Samples were viewed under a confocal laser scanning microscope. Although we observed that endogenous DDX3 localized in faint cytoplasmic foci in uninfected RSc cells, DDX3 relocalized, formed ringlike structures, and colocalized with the HCV core protein in response to HCV-JFH1 infection (Fig. 1A). On the other hand, endogenous DDX6 was localized in the evident cytoplasmic foci termed P bodies in the uninfected cells (Fig. 1A). DDX6 also relocalized, formed ringlike structures, and colocalized with the core protein in response to HCV-JFH1 infection (Fig. 1A). Although we failed to observe that most of the P bodies of DDX6 perfectly colocalized with DDX3 in uninfected RSc cells (Fig. 1B), we observed a few P bodies of DDX6 colocalized with DDX3 in the uninfected cells (Fig. 1B, arrowheads). Intriguingly, we found that endogenous DDX3 colocalized with endogenous DDX6 in HCV-JFH1-infected cells (Fig. 1B). To further confirm this finding, pHA-DDX3 (41) and pcDNA3-FLAG-DDX6 were cotransfected into 293FT cells. Consequently, we observed that hemagglutinin (HA)-DDX3 colocalized with FLAG-DDX6 in 293FT cells coexpressing HA-DDX3 and FLAG-DDX6 (Fig. 1B), suggesting cross talk of DDX3 with DDX6. Recently, LDs have been found to be involved in an important cytoplasmic organelle for HCV production (26). Indeed, both DDX3 and DDX6 were recruited around LDs in response to HCV infection, while these proteins did not colocalize with LDs in uninfected naïve RSc cells (Fig. 1C). Furthermore, both DDX3 and DDX6 accumulated in the LD fraction of the HCV-JFH1-infected RSc cells; however, we could not detect both proteins in the LD fraction from uninfected control cells (Fig. 1D), suggesting that DDX3 and

DDX6 are recruited around LDs in response to HCV infection.

These results suggest that HCV-JFH1 infection disrupts P-body formation. Therefore, we further examined whether or not HCV-JFH1 disrupts the P-body formations of other microRNA effectors, including Ago2; the Sm-like protein Lsm1, which is a subunit of heptameric-ring Lsm1-7, involved in decapping; the 5'-to-3' exonuclease Xrn1; the decapping activator PATL1; and the decapping enzyme DCP2 (6, 21, 30). As expected, HCV-JFH1 disrupted the P-body formations of Ago2, Lsm1, and Xrn1 as well as PATL1 (Fig. 2). Lsm1, Xrn1, or PATL1 relocalized, formed ringlike structures, and colocalized with the HCV core protein in response to HCV-JFH1 infection, whereas they were localized predominantly in P bodies in uninfected RSc cells (Fig. 2). In fact, we observed that DDX6 colocalized with Ago2, a P-body marker (Fig. 2). In contrast, HCV-JFH1 failed to disrupt the P-body formation of DCP2 (Fig. 2). Thus, these results suggest that HCV disrupts P-body formation through the hijacking of P-body components.

**HCV hijacks stress granule components.** Since Nonhoff et al. recently reported that DDX6 interacted with ataxin-2 (ATX2) (28), we examined the potential cross talk among DDX6, ATX2, and HCV. Although ATX2 and G3BP1, a well-known stress granule component (36), were dispersed in the cytoplasm at 37°C, both proteins formed discrete aggregates termed stress granules and colocalized with each other in response to heat shock at 43°C for 45 min, indicating that ATX2 is also stress granule component (Fig. 3A). We did not observe prominent colocalization between DDX6 and ATX2 at 37°C (Fig. 3B). In contrast, we found that DDX6 was recruited, juxtaposed, and partially colocalized with stress granules of ATX2 in response to heat shock at 43°C for 45 min in the uninfected RSc cells (Fig. 3B). Notably, ATX2 was recruited, formed the ring-like structures, and partially colocalized with DDX6 in response to HCV-JFH1 infection even at 37°C (Fig. 3B). Furthermore, we noticed that ATX2 was recruited around LDs in HCV-JFH1-infected cells at 72 h postinfection, while ATX2 did not colocalize with LDs in uninfected cells (Fig. 3C), suggesting the colocalization of ATX2 with the HCV core protein in infected cells. Indeed, ATX2 colocalized with the HCV core protein in HCV-JFH1-infected RSc cells at 37°C (Fig. 3D). Moreover, HCV-JFH1 infection induced the colocalization of the core protein with other stress granule components, G3BP1 or PABP1 as well as ATX2 (Fig. 4 and 5). To further confirm our findings, we examined the time course of the redistribution of DDX6 and G3BP1 after inoculation with HCV-JFH1. Consequently, we still detected the P-body formation of DDX6 and dispersed G3BP1 in the cytoplasm, and we did not observe a colocalization between the HCV core protein and DDX6 at 12 and 24 h postinfection (Fig. 4). In contrast, we found that the P-body formation of DDX6 began to be disrupted at 36 h postinfection (Fig. 4). Consistently, G3BP1 formed stress granules at 36 h postinfection (Fig. 4). We then noticed a ringlike formation of DDX6 or G3BP1 and colocalization with the HCV core protein after 48 h postinfection (Fig. 4), suggesting that the disruption of P-body formation and the hijacking of P-body and stress granule components occur in a late step of HCV infection.

We then examined whether or not HCV-JFH1 infection

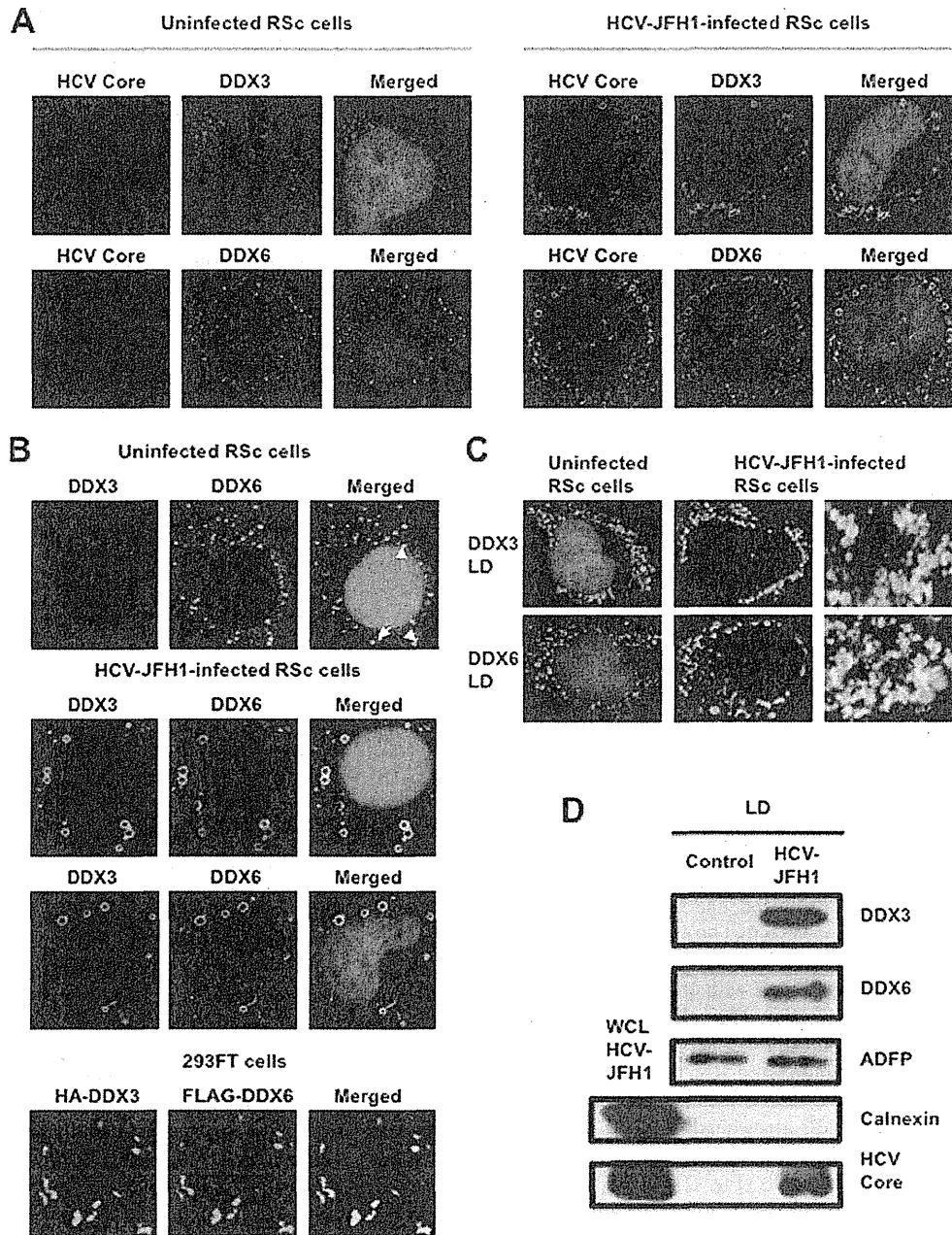


FIG. 1. Dynamic recruitment of DDX3 and DDX6 around lipid droplets (LDs) in response to HCV-JFH1 infection. (A) HCV-JFH1 disrupts the P-body formation of DDX6. Cells were fixed at 60 h postinfection and were then examined by confocal laser scanning microscopy. Cells were stained with anti-HCV core (CP-9 and CP-11 mixture) and either anti-DDX3 (54257 and 54258 mixture) or anti-DDX6 (A300-460A) antibody and then visualized with FITC (DDX3 or DDX6) or Cy3 (core). Images were visualized by using confocal laser scanning microscopy. The two-color overlay images are also exhibited (merged). Colocalization is shown in yellow. (B) HCV-JFH1 recruits DDX3 or DDX6 around LDs. Cells were stained with either anti-DDX3 or anti-DDX6 antibody and were then visualized with Cy3 (red). Lipid droplets and nuclei were stained with BODIPY 493/503 (green) and DAPI (blue), respectively. A high-magnification image is also shown. (C) Colocalization of DDX3 with DDX6. HCV-JFH1-infected RSc cells at 60 h postinfection were stained with anti-DDX3X (LS-C64576) and anti-DDX6 (A300-460A) antibodies. 293FT cells cotransfected with 100 ng of pcDNA3-FLAG-DDX6 and 100 ng of pHA-DDX3 (41) were stained with anti-FLAG-Cy3 and anti-HA-FITC antibodies (Sigma). (D) Association of DDX3 and DDX6 with LDs in response to HCV-JFH1 infection. The LD fraction and whole-cell lysates (WCL) were collected from uninfected RSc cells (control) or HCV-JFH1-infected RSc cells at 5 days postinfection. The results of Western blot analyses of DDX3, DDX6, and the HCV core protein as well as the LD marker ADFP and the endoplasmic reticulum (ER) marker calnexin in the LD fraction are shown.

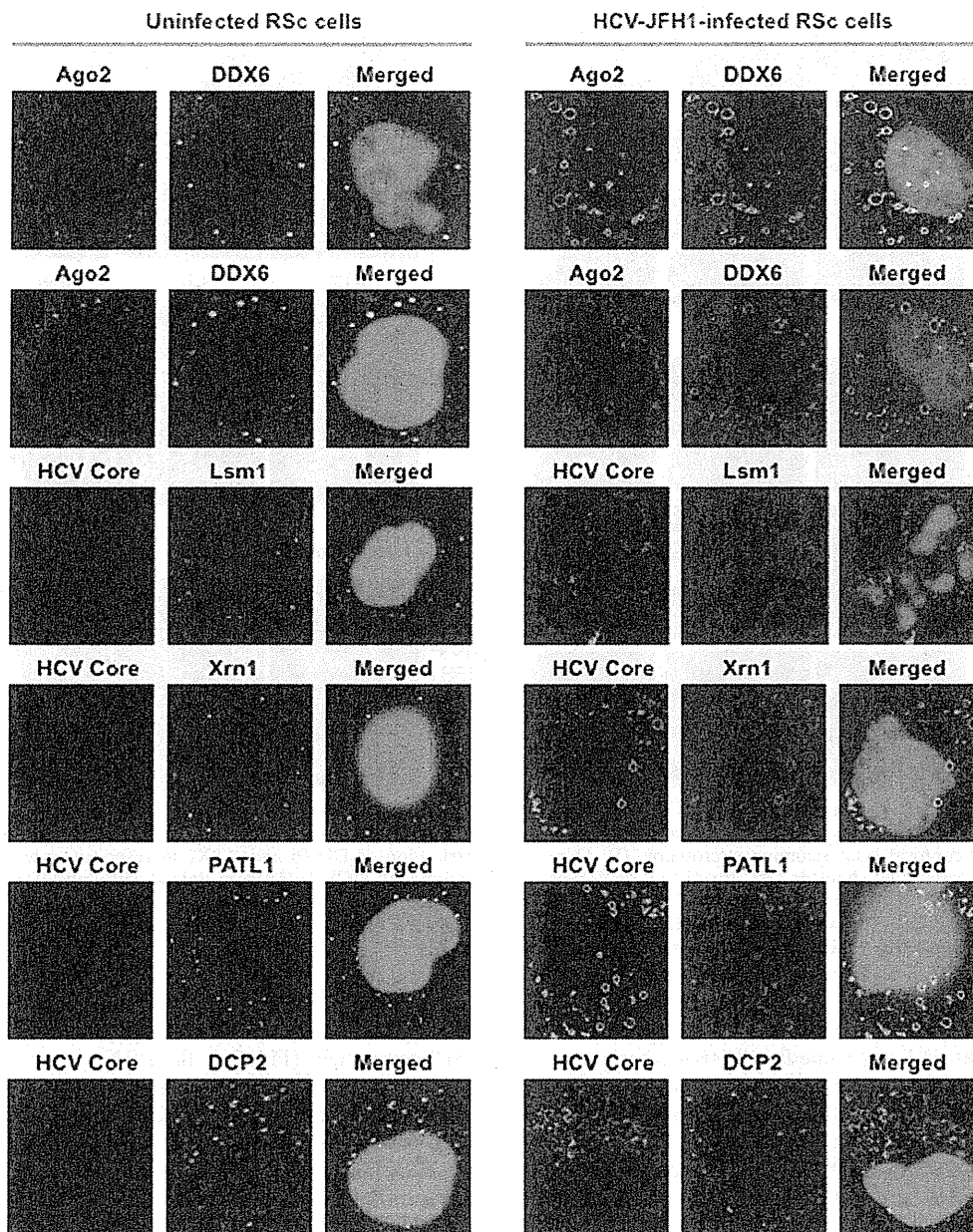


FIG. 2. HCV disrupts the P-body formation of microRNA effectors. Uninfected RSc cells and HCV-JFH1-infected RSc cells at 72 h postinfection were stained with anti-human AGO2 (011-22033) and anti-DDX6 (A300-460A) antibodies. The cells were also stained with anti-HCV core and anti-Lsm1 (LS-C97364), anti-Xrn1 (A300-443A), anti-PATL1, or anti-DCP2 (A302-597A) antibodies and were examined by confocal laser scanning microscopy.

could affect the stress granule formation of G3BP1, ATX2, or PABP1 in response to heat shock or treatment with arsenite. These stress granule components dispersed in the cytoplasm at 37°C, whereas these proteins formed stress granules in response to heat shock at 43°C for 45 min or treatment with 0.5 mM arsenite for 30 min (Fig. 5). In contrast, stress granules were not formed in HCV-JFH1-infected cells at 72 h postinfection in response to heat shock at 43°C for 45 min (Fig. 5), suggesting that HCV-JFH1 infection suppresses stress granule formation in response to heat shock or treatment with arsenite.

Intriguingly, G3BP1, ATX2, or PABP1 still colocalized with the HCV core protein even under the above-described stress conditions (Fig. 5). Furthermore, Western blot analysis of cell lysates of uninfected or HCV-JFH1-infected cells at 72 h postinfection showed similar protein expression levels of ATX2, PABP1, HSP70, DDX3, DDX6, and Lsm1 but not G3BP1 (Fig. 6), suggesting that HCV-JFH1 infection does not affect host mRNA translation.

**P-body and stress granule components are required for HCV replication.** Finally, we investigated the potential role of

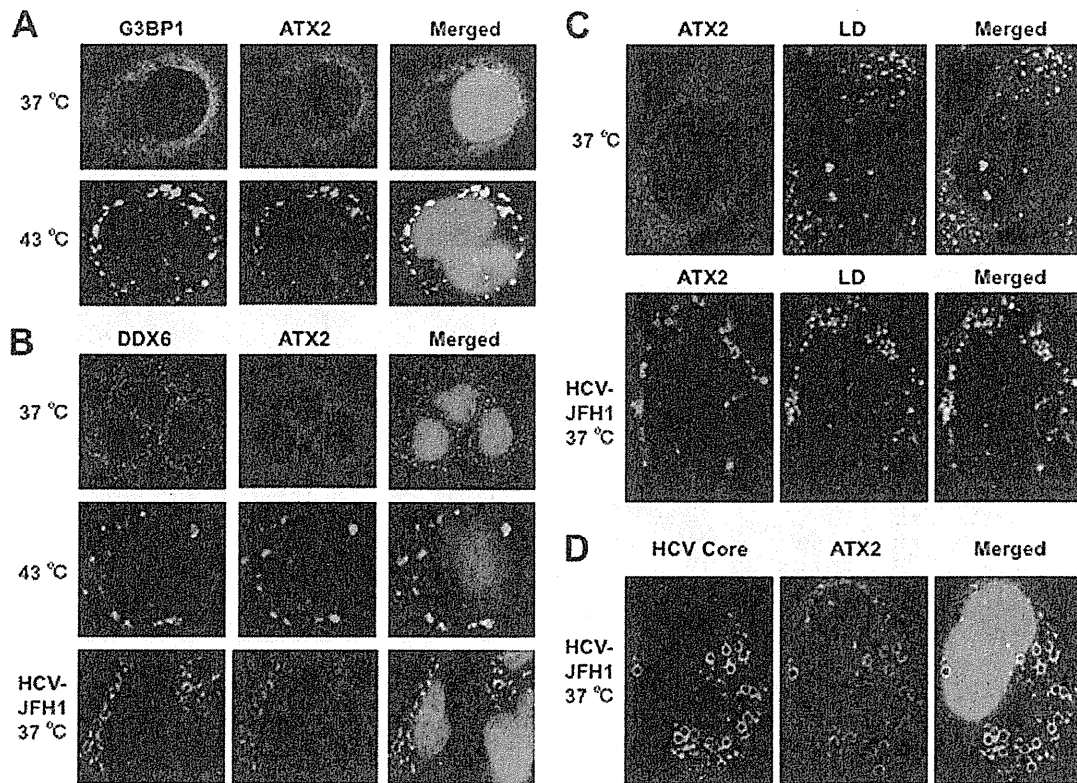


FIG. 3. Dynamic redistribution of ataxin-2 (ATX2) around LDs in response to HCV-JFH1 infection. (A) ATX2 is a stress granule component. RSc cells were incubated at 37°C or 43°C for 45 min. Cells were stained with anti-G3BP1 (A302-033A) and anti-ATX2 (A93520) antibodies and were examined by confocal laser scanning microscopy. (B) Dynamic redistribution of DDX6 and ATX2 in response to heat shock or HCV infection. RSc cells after heat shock at 43°C for 45 min or 72 h after inoculation with HCV-JFH1 were stained with anti-DDX6 and anti-ATX2 (A93520) antibodies. (C) HCV relocates ataxin-2 to LDs. HCV-JFH1-infected RSc cells at 72 h postinfection were stained with anti-ATX2 (A93520) antibody and BODIPY 493/503. (D) ATX2 colocalizes with the HCV core protein. HCV-JFH1-infected RSc cells at 72 h postinfection were stained with anti-ATX2/SCA2 (A301-118A) and anti-HCV core antibodies.

P-body and stress granule components in the HCV life cycle. We first used lentiviral vector-mediated RNA interference to stably knock down DDX6 as well as DDX3 in RSc cells. We used puromycin-resistant pooled cells 10 days after lentiviral transduction in all experiments. Real-time LightCycler RT-PCR analysis of DDX3 or DDX6 demonstrated a very effective knockdown of DDX3 or DDX6 in RSc cells transduced with lentiviral vectors expressing the corresponding shRNAs (Fig. 7A). Importantly, shRNAs did not affect cell viabilities (data not shown). We next examined the levels of HCV core and the infectivity of HCV in the culture supernatants as well as the level of intracellular HCV RNA in these knockdown cells 24 h after HCV-JFH1 infection at an MOI of 4. The results showed that the accumulation of HCV RNA was significantly suppressed in DDX3 or DDX6 knockdown cells (Fig. 7B). In this context, the release of the HCV core protein and the infectivity of HCV in the culture supernatants were also significantly suppressed in these knockdown cells (Fig. 7C and D). This finding suggested that DDX6 is required for HCV replication, like DDX3. To further examine the potential role of other P-body and stress granule components in HCV replication, we used RSc cells transiently transfected with a pool of siRNAs specific for ATX2, PABP1, Lsm1, Xrn1, G3BP1, and PATL1 as well as a pool of control siRNAs (siCon) following HCV-

JFH1 infection. In spite of the very effective knockdown of each component (Fig. 7E), the siRNAs used in these experiments did not affect cell viabilities (data not shown). Consequently, the accumulation of HCV RNA was significantly suppressed in ATX2, PABP1, or Lsm1 knockdown cells (Fig. 7F), indicating that ATX2, PABP1, and Lsm1 are required for HCV replication. In contrast, the level of HCV RNA was not affected in Xrn1 knockdown cells (Fig. 7F), suggesting that Xrn1 is unrelated to HCV replication. Furthermore, we observed a moderate effect of siG3BP1 and siPATL1 on HCV RNA replication (Fig. 7F). Altogether, HCV seems to hijack the P-body and stress granule components around LDs for HCV replication.

## DISCUSSION

So far, the P body and stress granules have been implicated in mRNA translation, RNA silencing, and RNA degradation as well as viral infection (1, 6, 22, 30). Host factors within the P body and stress granules can enhance or limit viral infection, and some viral RNAs and proteins accumulate in the P body and/or stress granules. Indeed, the microRNA effectors DDX6, GW182, Lsm1, and Xrn1 negatively regulate HIV-1 gene expression by preventing the association of viral mRNA

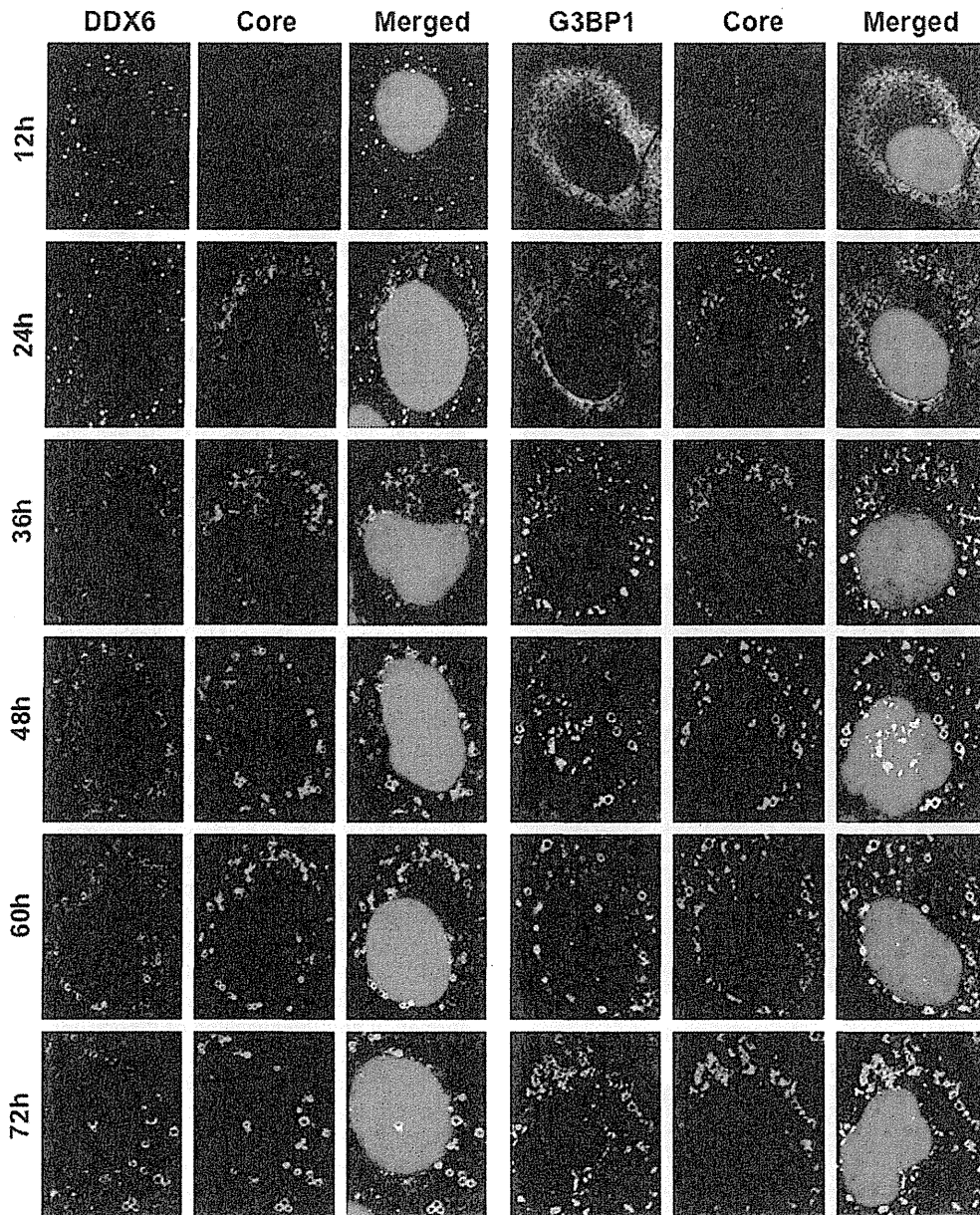


FIG. 4. Dynamic redistribution of DDX6 and G3BP1 in response to HCV-JFH1 infection. RSc cells at the indicated times (hours) after inoculation with HCV-JFH1 were stained with anti-HCV core and either anti-DDX6 (A300-460A) or anti-G3BP1 (A302-033A) antibodies.

with polysomes (9). In contrast, miRNA effectors such as DDX6, Lsm1, PatL1, and Ago2 positively regulate HCV replication (Fig. 7B and F) (16, 31, 33). We have also found that DDX3 and DDX6 are required for HCV RNA replication (3) (Fig. 7B) and that DDX3 colocalized with DDX6 in HCV-JFH1-infected RSc cells (Fig. 1B), suggesting that DDX3 co-modulates the DDX6 function in HCV RNA replication. In this regard, the liver-specific miR-122 interacts with the 5'-UTR of the HCV RNA genome and positively regulates HCV replication (15, 17, 19, 20, 31). Since miRNAs usually interact with DDX6 and Ago2 in miRISC and are involved in RNA silencing, DDX6 and Ago2 may be required for miR-122-

dependent HCV replication. Indeed, quite recently, a study showed that Ago2 is required for miR-122-dependent HCV RNA replication and translation (40). However, little is known regarding how miR-122 and DDX6 positively regulate HCV replication. Accordingly, we have shown that these miRNA effectors, including DDX6, Lsm1, Xrn1, and Ago2, accumulated around LDs and the HCV production factory and colocalized with the HCV core protein in response to HCV infection (Fig. 1 and 2). However, the decapping enzyme DCP2 did not accumulate and colocalize with the core protein (Fig. 2). Consistent with this finding, Scheller et al. reported previously that the depletion of DCP2 by siRNA did not affect HCV

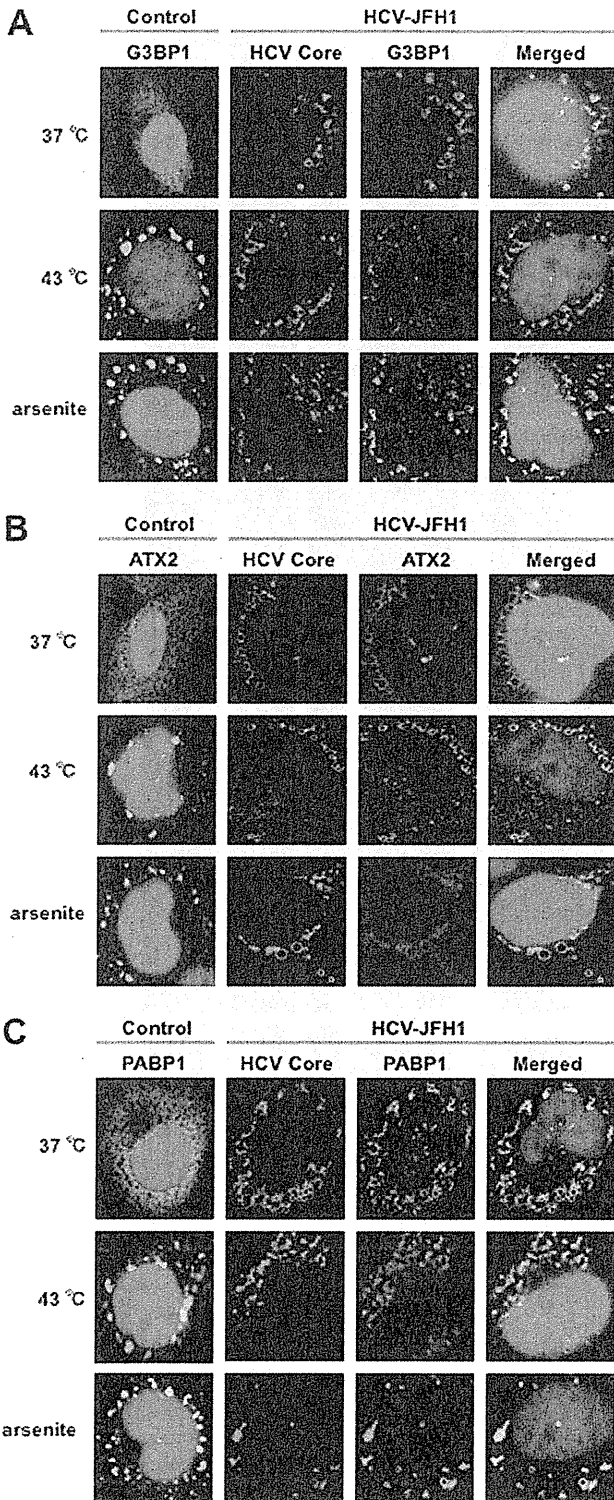


FIG. 5. HCV suppresses stress granule formation in response to heat shock or treatment with arsenite. Naive RSc cells or HCV-JFH1-infected RSc cells at 72 h postinfection were incubated at 37°C or 43°C for 45 min. Cells were also treated with 0.5 mM arsenite for 30 min. Cells were stained with anti-HCV core and anti-G3BP1 (A), anti-ATX2 (B), or anti-PABP1 (ab21060) (C) antibodies and were examined by confocal laser scanning microscopy.

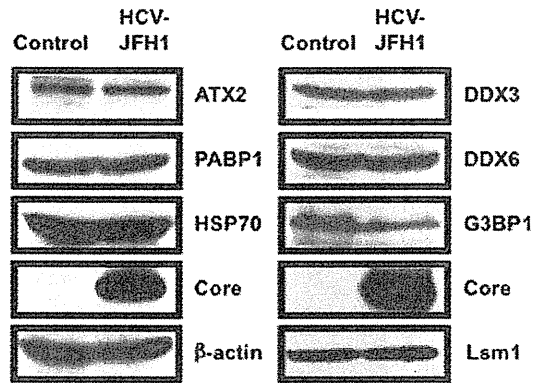


FIG. 6. Host protein expression levels in response to HCV-JFH1 infection. The results of the Western blot analyses of cellular lysates with anti-ATX2/SCA2 antibody (A301-118A), anti-PABP1 (ab21060), anti-HSP70 (610607), anti-HCV core, anti-β-actin, anti-DDX3 (54257 [NT] and 5428 [IN] mixture), anti-DDX6 (A300-460A), anti-G3BP1 (611126), or anti-LSM1 (LS-C97364) antibody in HCV-JFH1-infected RSc cells at 72 h postinfection as well as in naïve RSc cells are shown.

production (33). Since HCV harbors the internal ribosome entry site (IRES) structure in the 5'-UTR of the HCV genome instead of a cap structure, unlike HIV-1, DCP2 may not be recruited on the HCV genome and utilized for HCV replication. Otherwise, DCP2 may determine whether or not DDX6 and miRNAs positively or negatively regulate target mRNA.

Furthermore, we have demonstrated that HCV infection hijacks the P-body and stress granule components around LDs (Fig. 1, 2, 4, and 5). We have found that the P-body formation of DDX6 began to be disrupted at 36 h postinfection (Fig. 4). Consistently, G3BP1 formed stress granules at 36 h postinfection. We then observed the ringlike formation of DDX6 or G3BP1 and colocalization with the HCV core protein after 48 h postinfection, suggesting that the disruption of P-body formation and the hijacking of P-body and stress granule components occur at a late step of HCV infection. Furthermore, HCV infection could suppress stress granule formation in response to heat shock or treatment with arsenite (Fig. 5). In this regard, West Nile virus and dengue virus, of the family *Flaviviridae*, interfere with stress granule formation and P-body assembly through interactions with T cell intracellular antigen 1 (TIA-1)/TIAR (11). Moreover, PABP1 and G3BP1, stress granule components, are known to be common viral targets for the inhibition of host mRNA translation (34, 39). In fact, HIV-1 and poliovirus proteases cleave PABP1 and/or G3BP1 and suppress stress granule formation during viral infection (34, 39). On the other hand, HCV infection transiently induced stress granules at 36 h postinfection (Fig. 4) and did not cleave PABP1 (Fig. 6); however, HCV could suppress stress granule formation in response to heat shock or treatment with arsenite through hijacking their components around LDs, the HCV production factory (Fig. 5). Consistently, Jones et al. showed that HCV transiently induces stress granules of enhanced green fluorescent protein (EGFP)-G3BP at 36 h after infection with the cell culture-generated HCV (HCVcc) reporter virus Jc1FLAG2 (p7-nsGluc2A); however, those authors did not show the recruitment of EGFP-G3BP to LDs (18). Although we do not know the exact reason for this apparent discrepancy,

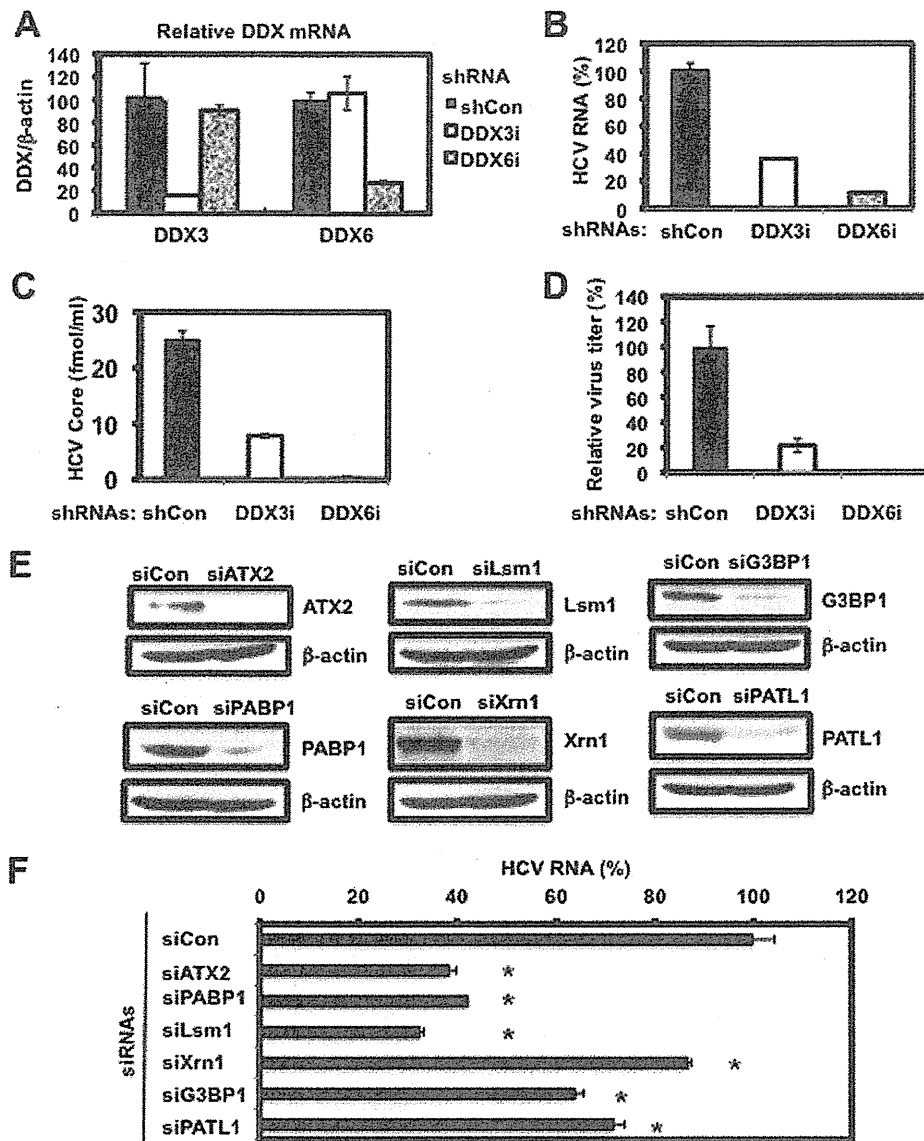


FIG. 7. Requirement of P-body and stress granule components for HCV replication. (A) Inhibition of DDX3 or DDX6 mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for DDX3 or DDX6 was also performed for  $\beta$ -actin mRNA in RSc cells expressing shRNA targeted to DDX3 (DDX3i) or DDX6 (DDX6i) or the control nontargeting shRNA (shCon) in triplicate. Each mRNA level was calculated relative to the level in RSc cells transduced with the control nontargeting lentiviral vector (shCon), which was assigned as 100%. Error bars in this panel and other panels indicate standard deviations. (B) Levels of intracellular genome-length HCV-JFH1 RNA in the cells at 24 h postinfection at an MOI of 4 were monitored by real-time LightCycler RT-PCR. Results from three independent experiments are shown. Each HCV RNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (shCon), which was assigned as 100%. (C) The levels of HCV core in the culture supernatants from the stable knockdown RSc cells 24 h after inoculation of HCV-JFH1 at an MOI of 4 were determined by ELISA. Experiments were done in triplicate, and columns represent the mean core protein levels. (D) The infectivity of HCV in the culture supernatants from stable-knockdown RSc cells 24 h after inoculation of HCV-JFH1 at an MOI of 4 was determined by a focus-forming assay at 24 h postinfection. Experiments were done in triplicate, and each virus titer was calculated relative to the level in RSc cells transduced with a control lentiviral vector (shCon), which was assigned as 100%. (E) Inhibition of ATX2, PABP1, Lsm1, Xrn1, G3BP1, or PATL1 protein expression by 72 h after transient transfection of RSc cells with a pool of control nontargeting siRNA (siCon) or a pool of siRNAs specific for ATX2, PABP1, Lsm1, Xrn1, G3BP1, or PATL1 (25 nM), respectively. The results of Western blot analyses of cellular lysates with anti-ATX2, anti-PABP1, anti-Lsm1, anti-Xrn1, anti-G3BP1, anti-PATL1, or anti- $\beta$ -actin antibody are shown. (F) Levels of intracellular genome-length HCV-JFH1 RNA in the cells at 48 h postinfection at an MOI of 1 were monitored by real-time LightCycler RT-PCR. RSc cells were transiently transfected with a pool of control siRNA (siCon) or a pool of siRNAs specific for ATX2, PABP1, Lsm1, Xrn1, G3BP1, and PATL1 (25 nM). At 48 h after transfection, the cells were inoculated with HCV-JFH1 at an MOI of 1 and incubated for 2 h. The culture medium was then changed and incubated for 22 h. Experiments were done in triplicate, and each HCV RNA level was calculated relative to the level in RSc cells transfected with a control siRNA (siCon), which was assigned as 100%. Asterisks indicate significant differences compared to the control treatment (\*,  $P < 0.01$ ).



several possible explanations can be offered. First, those authors examined the localization of EGFP-G3BP within 48 h postinfection, and we observed it at later times (Fig. 4). Second, they used only EGFP-tagged G3BP instead of endogenous G3BP1. Third, they used a Jc1FLAG2 (p7-nsGlc2A) clone, and an HCV-JFH1 clone could markedly induce the recruitment of the core protein to LDs compared to that of Jc1. Also, Jangra et al. failed to observe the recruitment of DDX6 to LDs at 2 days after infection with HJ3-5 virus (16). Accordingly, we also observed that most of the DDX6 still formed intact P bodies at earlier times (12 h or 24 h postinfection). Importantly, we observed the recruitment of DDX6 to LDs 48 h later (Fig. 4). Furthermore, those authors did not show the ringlike structure formation of the HJ3-5 core protein around LDs, unlike the JFH1 core protein that we used in this study. The interaction of the HCV core protein with DDX6 may explain the recruitment of P-body components to LDs. However, we do not yet know whether the P-body function(s) can be performed on LDs. At least, HCV infection did not affect the translation of several host mRNAs with 5' caps and 3' poly(A) tails despite the disruption of P-body formation at 72 h postinfection (Fig. 6), suggesting that HCV does not affect P-body function and that HCV recruits functional P bodies to LDs.

We need to address the potential role of stress granule components, such as PABP1, in HCV replication/translation, since the HCV genome does not harbor the 3' poly(A) tail. Intriguingly, we have found that the accumulation of HCV RNA was significantly suppressed in PABP1 knockdown RSc cells (Fig. 7F). In this regard, Tingting et al. demonstrated previously that G3BP1 and PABP1 as well as DDX1 were identified as the HCV 3'-UTR RNA-binding proteins by proteomic analysis and that G3BP1 was required for HCV RNA replication (35). Yi et al. also reported that G3BP1 was associated with HCV NS5B and that G3BP1 was required for HCV RNA replication (42). We observed a moderate effect of siG3BP1 on HCV RNA replication (Fig. 7F). In contrast, the accumulation of HCV RNA was significantly suppressed in ATX2 and Lsm1 knockdown cells as well as in PABP1 knockdown cells (Fig. 7F), suggesting that ATX2, Lsm1, and PABP1 are required for HCV replication.

Taking these results together, this study has demonstrated for the first time that HCV hijacks P-body and stress granule components around LDs. This hijacking may regulate HCV RNA replication and translation. Indeed, we have found that the accumulation of genome-length HCV-O (genotype 1b) (14) RNA was markedly suppressed in DDX6 knockdown O cells (data not shown). More importantly, these P-body and stress granule components may be involved in the maintenance of the HCV RNA genome without 5' cap and 3' poly(A) tail structures in the cytoplasm for long periods, since the hijacking of P-body and stress granule components by HCV occurred at later times.

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# The ESCRT System Is Required for Hepatitis C Virus Production

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## Abstract

**Background:** Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for hepatitis C virus (HCV) production. However, the mechanisms of HCV assembly, budding, and release remain poorly understood. Retroviruses and some other enveloped viruses require an endosomal sorting complex required for transport (ESCRT) components and their associated proteins for their budding process.

**Methodology/Principal Findings:** To determine whether or not the ESCRT system is needed for HCV production, we examined the infectivity of HCV or the Core levels in culture supernatants as well as HCV RNA levels in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, expressing short hairpin RNA or siRNA targeted to tumor susceptibility gene 101 (TSG101), apoptosis-linked gene 2 interacting protein X (Alix), Vps4B, charged multivesicular body protein 4b (CHMP4b), or Brox, all of which are components of the ESCRT system. We found that the infectivity of HCV in the supernatants was significantly suppressed in these knockdown cells. Consequently, the release of the HCV Core into the culture supernatants was significantly suppressed in these knockdown cells after HCV-JFH1 infection, while the intracellular infectivity and the RNA replication of HCV-JFH1 were not significantly affected. Furthermore, the HCV Core mostly colocalized with CHMP4b, a component of ESCRT-III. In this context, HCV Core could bind to CHMP4b. Nevertheless, we failed to find the conserved viral late domain motif, which is required for interaction with the ESCRT component, in the HCV-JFH1 Core, suggesting that HCV Core has a novel motif required for HCV production.

**Conclusions/Significance:** These results suggest that the ESCRT system is required for infectious HCV production.

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## Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1]. HCV Core, a highly basic RNA-binding protein, forms a viral capsid and is targeted to lipid droplets [2–6]. The Core is essential for infectious virion production [7]. NS5A, a membrane-associated RNA-binding phosphoprotein, is also involved in the assembly and maturation of infectious HCV particles [8,9]. Intriguingly, NS5A is a key regulator of virion production through the phosphorylation by casein kinase II [9]. Recently, lipid droplets have been found to be

involved in an important cytoplasmic organelle for HCV production [4]. Indeed, NS5A is known to colocalize with the Core on lipid droplets [5], and the interaction between NS5A and the Core is critical for the production of infectious HCV particles [3]. However, the host factor involved in HCV assembly, budding, and release remains poorly understood.

Budding is an essential step in the life cycle of enveloped viruses. Endosomal sorting complex required for transport (ESCRT) components and associated factors, such as tumor susceptibility gene 101 (TSG101, a component of ESCRT-I), charged multivesicular body protein 4b (CHMP4b, a component of ESCRT-III), and apoptosis-linked gene 2 interacting protein X (ALIX, a TSG101- and CHMP4b-binding protein), have been found to be involved in membrane remodeling events that accompany endosomal protein sorting, cytokinesis, and the budding of several enveloped viruses, such as human immunodeficiency virus type 1 (HIV-1) [10–12]. The ESCRT complexes I, II, and III are sequentially, or perhaps concentrically recruited to the endosomal membrane to sequester

cargo proteins and drive vesicularization into the endosome. Finally, ESCRT-III recruits Vps4 (two isoforms, Vps4A and Vps4B), a member of the AAA-family of ATPase that disassembles and thereby terminates and recycles the ESCRT machinery.

Since HCV is also an enveloped RNA virus, we hypothesized that the ESCRT system might be required for HCV production. To test this hypothesis, we examined the release of HCV Core into culture supernatants from cells rendered defective for ESCRT components by RNA interference. The results provide evidence that the ESCRT system is required for HCV production.

## Materials and Methods

### Cell Culture

293FT cells (Invitrogen, Carlsbad, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). The HuH-7-derived cell line, RSc cured cells that cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) [13] could infect and effectively replicate [14–16] and OR6c and OR6 cells harboring the genome-length HCV-O RNA with luciferase as a reporter were cultured in DMEM with 10% FBS as described previously [17,18].

### Plasmid Construction

To construct pcDNA3-FLAG-Alix, a DNA fragment encoding Alix was amplified from total RNAs derived from RSc cells by RT-PCR using the following pairs of primers: Forward 5'-CGGG-ATCCAAGATGGCGACATTCATCTCGGT-3' and reverse 5'-CCGGCGCGCTTACTGCTGTGGATAGTAAG-3'. The obtained DNA fragment was subcloned into *Bam*HI-*Nco*II of pcDNA3-FLAG vector [19], and the nucleotide sequences were determined by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The plasmid of pJRN/3-5B was based on pJFH1 [13] and was constructed as previously described [20].

### RNA synthesis, RNA transfection, and Selection of G418-resistant cells

Plasmid pJRN/3-5B were linearized by *Xba*I and used for the RNA synthesis with the T7 MEGAScript kit (Ambion, Austin, TX). *In vitro* transcribed RNA was transfected into OR6c cells by electroporation [17,18]. The transfected cells were selected in culture medium containing G418 (0.3 mg/ml) for 3 weeks. We referred to them as OR6c/JRN 3-5B cells.

### Immunofluorescence and Confocal Microscopic Analysis

Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS) and permeabilized in 0.1% Nonidet P-40 (NP-40) in PBS at room temperature as previously described [21]. Cells were incubated with anti-HCV Core antibody (CP-9 and CP-11 mixture; Institute of Immunology, Tokyo, Japan), anti-Myc-Tag antibody (PL14; Medical & Biological Laboratories, MBL, Nagoya, Japan), anti-Alix antibody (Covalab, Villeurbanne, France), and/or anti-FLAG polyclonal antibody (Sigma, St. Louis, MO) at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) at 37°C for 30 min. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) or anti-Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch) at a 1:300 dilution in PBS containing BSA at 37°C for 30 min. Lipid droplets and nuclei were stained with BODIPY 493/503 (Molecular Probes, Invitrogen) and DAPI (4',6'-diamidino-2-phenylindole), respectively. Following extensive washing in PBS, cells were mounted on slides using a mounting media of 90%

glycerin/10% PBS with 0.01% *p*-phenylenediamine added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (LSM510; Zeiss, Jena, Germany).

### RNA Interference

The following siRNAs were used: human TSG101 (siGENOME SMARTpool M-003549-01-0005 and 5'-CCUCCAGU-CUUCUCUCGUCUU-3' sense, 5'-GACGAGAGAAGACUG-GAGGUU-3' antisense), human Alix/PDCD6IP (siGENOME SMARTpool M-004233-02-0005), human Vps4B (siGENOME SMARTpool M-013119-02-0005), human CHMP4b (siGENOME SMARTpool M-018075-00-0005), and siGENOME Non-Targeting siRNA Pool#1 (D-001206-13-05) (Dharmacon, Thermo Fisher Scientific, Waltham, MA) as a control. siRNAs (50 nM final concentration) were transiently transfected into either RSc cells [14–16] or OR6 cells [17,18] using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin (sh) RNA-encoding sequences against TSG101, Alix, Vps4B, or CHMP4b in lentiviral vector: TSG101i, 5'-GATCCCC GGAGGAAATGGATCGTGCCTT-CAAGAGAGGCACGATCCATTTCCCTCCTTTTTGGAAA-3' (sense), 5'-AGCTTTTCCAAAAAGGAGGAAATGGATCGTG-CCTCTCTTGAAGGCACGATCCATTTCCCTCCGGG-3' (antisense); Alixi, 5'-GATCCCC GGAGGTGTCCCTGTCTTG-TTCAAGAGACAAGACAGGGAACACCTCCTTTTTGGAA-A-3' (sense), 5'-AGCTTTTCCAAAAAGGAGGTGTTCCTGT-TCTTGTCTCTTGAACAAGACAGGGAACACCTCCGGG-3' (antisense); Vps4Bi, 5'-GATCCCC GGAGAATCTGATGATC-CTGTTCAAGAGACAGGATCATCTCCTTTTTTGGAAA-3' (sense), 5'-AGCTTTTCCAAAAAGGAGAAATCTGATGATCCTGTCTCTTGAACAGGATCATCAGATTCTC-CGGG-3' (antisense); CHMP4bi, 5'-GATCCCC GAGGAG-GACGACGACATGATTCAAGAGATCATGTGCTGCTGCC-TCTCTTTTTGGAAA-3' (sense), 5'-AGCTTTTCCAAAAAGGAGGACGACGACATGATCTCTTGAATCATGTGCTG-TCGTCTCCTCCGGG-3' (antisense); Broxi, 5'-GATCCCCG-GATGACAGTACTAAACCCTTCAAGAGAGGGTTTAGTA-CTGTATCCTTTTTTGGAAA-3' (sense), 5'-AGCTTTTC-CAAAAAGGATGACAGTACTAAACCCTCTCTTGAAGGG-TTTAGTACTGTCTCCTCCGGG-3' (antisense). The oligonucleotides above were annealed and subcloned into the *Bgl*II-*Hind*III site, downstream from an RNA polymerase III promoter of pSUPER [22], to generate pSUPER-TSG101i, pSUPER-Alixi, pSUPER-Vps4Bi, and pSUPER-CHMP4bi, respectively. To construct pLV-TSG101i, pLV-Alixi, pLV-Vps4Bi, and pLV-CHMP4bi, the *Bam*HI-*Sal*I fragments of the corresponding pSUPER plasmids were subcloned into the *Bam*HI-*Sal*I site of pRDI292 [23], an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistant marker allowing for the selection of transduced cells, respectively.

### Lentiviral Vector Production

The vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously [24–26]. The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 [24–26] and the VSV-G-envelope-expressing plasmid pMDG2 as well as pRDI292 into 293FT cells with FuGene6 (Roche Diagnostics, Basel, Switzerland).

### HCV Infection Experiments

The supernatants was collected from cell culture-generated HCV-JFH1 [13]-infected RSc cells [14–16] at 5 days post-

infection and stored at  $-80^{\circ}\text{C}$  after filtering through a  $0.45\ \mu\text{m}$  filter (Kurabo, Osaka, Japan) until use. For infection experiments with HCV-JFH1 virus, RSc cells ( $1 \times 10^5$  cells/well) were plated onto 6-well plates and cultured for 24 hours (hrs). Then, we infected the cells with  $50\ \mu\text{l}$  (equivalent to a multiplicity of infection [MOI] of 0.1) of inoculum. The culture supernatants were collected and the levels of HCV Core were determined by enzyme-linked immunosorbent assay (ELISA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was isolated from the infected cellular lysates using RNeasy mini kit (Qiagen, Hilden, Germany) for quantitative RT-PCR analysis of intracellular HCV RNA. The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 hrs post-infection. The HCV infected cells were detected using anti-HCV Core antibody (CP-9 and CP-11). Intracellular HCV infectivity was determined by a focus-forming assay at 48 hrs post-inoculation of lysates by repeated freeze and thaw cycles (three times).

### Quantitative RT-PCR Analysis

The quantitative RT-PCR analysis for HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously [17,18]. We used the following forward and reverse primer sets for the real-time LightCycler PCR: TSG101, 5'-ATGGCGGTGTCGGAGAGCCA-3' (forward), 5'-AACAGGTTTGAGATCTTTGT-3' (reverse); Alix, 5'-ATGGCGACATTCATCTCGGT-3' (forward), 5'-TACTGGGCTGTCTTCCC-C-3' (reverse); Vps4B, 5'-ATGTCATCCACTTCGCCCAA-3' (forward), 5'-ATACTGCACAGCATGCTGAT-3' (reverse); CHMP4b, 5'-ATGTCGGGTGTTCCGGGAAGCT-3' (forward), 5'-ATCTCTTCCGTGTCGCCGAG-3' (reverse); Brox, 5'-ATGACCCATTGGTTTCATAG-3' (forward), 5'-CCTGGATGACCTCAAGTCAT-3' (reverse);  $\beta$ -actin, 5'-TGACGGGGTCACCACACTG-3' (forward), 5'-AAGCTGTAGCCGCGCTCGGT-3' (reverse); and HCV-JFH1, 5'-AGAGCCATAGTGGTCTGCGG-3' (forward), 5'-CTTTCCGAACCCCAACGCTAC-3' (reverse).

### MTT Assay

Cells ( $5 \times 10^3$  cells/well) were plated onto 96-well plates and cultured for 24, 48 or 72 hrs, then, subjected to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Cell proliferation kit I, Roche). The absorbance was read using a microplate reader (Multiskan FC, Thermo Fisher Scientific) at 550 nm with a reference wavelength of 690 nm.

### Renilla Luciferase (RL) Assay

OR6 cells ( $1.5 \times 10^4$  cells/well) [17,18] were plated onto 24-well plates and cultured for 24 hrs. The cells were transfected with siRNAs (50 nM) using Oligofectamine and incubated for 72 hrs, then, subjected to the RL assay according to the manufacturer's instructions (Promega, Madison, WI). A lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect RL activity.

### Western Blot Analysis

Cells ( $2 \times 10^5$  cells/well) were plated onto 6-well plates and cultured for 24 or 48 hrs. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Supernatants from these lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-

TSG101 antibody (BD Transduction Laboratories, San Jose, CA), anti-Alix antibody, anti-Vps4B antibody (Abnova, Taipei, Taiwan) (A302-078A; Bethyl Laboratories, Montgomery, TX), anti-CHMP4B antibody (sc-82557; Santa Cruz Biotechnology, Santa Cruz, CA), anti-HCV Core antibody, anti- $\beta$ -actin antibody (Sigma), anti-Myc-Tag antibody, anti-FLAG antibody (M2; Sigma), anti-Chk2 antibody (DCS-273; MBL), anti-heat shock protein (HSP) 70 antibody (BD), Living Colors A.v. monoclonal antibody (JL-8; Clontech, Mountain View, CA), anti-HCV NS5A monoclonal antibody (no. 8926; a generous gift from A Takamizawa, The Research Foundation for Microbial Diseases of Osaka University, Japan), or anti-HCV NS5A polyclonal antibody (a generous gift from K Shimotohno, Chiba Institute of Technology, Chiba, Japan).

### Immunoprecipitation Analysis

Cells were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1 mM PMSF, and protease inhibitor cocktail containing  $104\ \mu\text{M}$  4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 80 nM aprotinin, 2.1  $\mu\text{M}$  leupeptin, 3.6  $\mu\text{M}$  bestatin, 1.5  $\mu\text{M}$  pepstatin A, and 1.4  $\mu\text{M}$  E-64 (Sigma). Lysates were pre-cleaned with 30  $\mu\text{l}$  of protein-G-Sepharose (GE Healthcare Bio-Sciences). Pre-cleaned supernatants were incubated with 5  $\mu\text{l}$  of Living Colors A.v. monoclonal antibody or anti-FLAG antibody at  $4^{\circ}\text{C}$  for 1 hr. Following absorption of the precipitates on 30  $\mu\text{l}$  of protein-G-Sepharose resin for 1 hr, the resin was washed four times with 700  $\mu\text{l}$  lysis buffer. Proteins were eluted by boiling the resin for 5 min in  $1 \times$  Laemmli sample buffer. The proteins were then subjected to SDS-PAGE, followed by immunoblotting analysis using either anti-FLAG antibody, Living Colors A.v. monoclonal antibody or anti-HCV Core antibody.

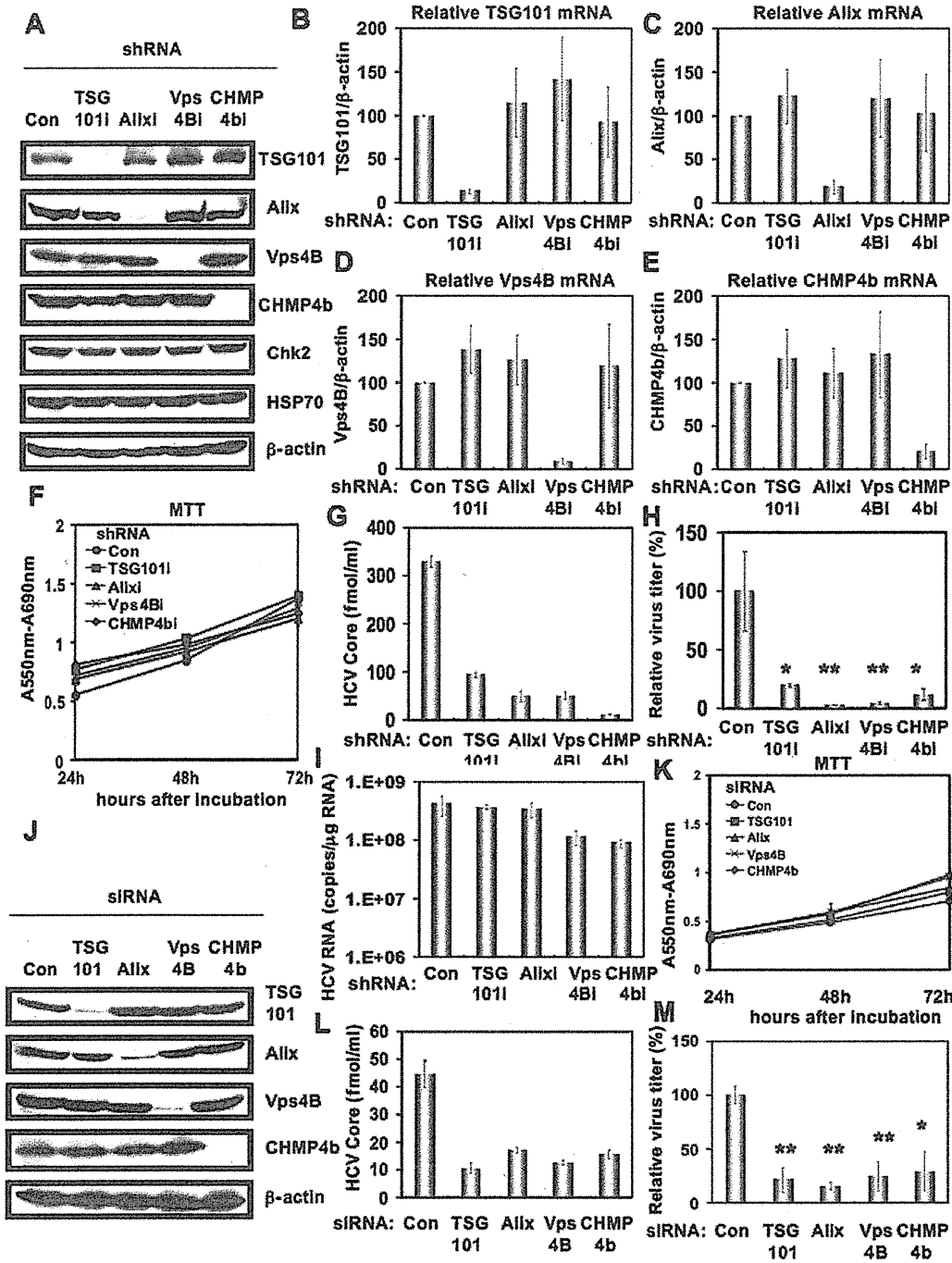
### Statistical Analysis

Statistical comparison of the infectivity of HCV in the culture supernatants between the knockdown cells and the control cells was performed using the Student's *t*-test. *P* values of less than 0.05 were considered statistically significant. All error bars indicate standard deviation.

## Results

### The ESCRT system is required for HCV production

To investigate the potential role(s) of the ESCRT system in the HCV life cycle, we first used lentiviral vector-mediated RNA interference to stably knockdown the ESCRT components, including TSG101, Alix, Vps4B, or CHMP4b in HuH-7-derived RSc cured cells that cell-culture-generated HCVcc (HCV-JFH1, genotype 2a) [13] could infect and effectively replicate [14–16]. We used puromycin-resistant pooled cells 10 days after the lentiviral transduction in all experiments. Western blot and real-time LightCycler RT-PCR analyses for TSG101, Alix, Vps4B, or CHMP4b demonstrated a very effective knockdown of each ESCRT component in RSc cells transduced with lentiviral vectors expressing the corresponding shRNAs (Fig. 1A–E). Importantly, we noticed that the depletion of ESCRT components did not affect the levels of several cellular proteins, including HSP70, Chk2, and  $\beta$ -actin (Fig. 1A). To test the cell toxicity of each shRNA, we examined colorimetric MTT assay. In this context, we demonstrated that the shRNAs did not affect the cell viabilities (Fig. 1F). We next examined the levels of HCV Core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in the TSG101, Alix, Vps4B, or CHMP4b stable knockdown RSc cells 97 h after HCV-JFH1 infection at an MOI of 0.1. The results showed that the release of HCV Core into the culture supernatants



**Figure 1. ESCRT components are required for the infectious HCV production.** (A) Inhibition of TSG101, Alix, Vps4B, or CHMP4b protein expression by shRNA-producing lentiviral vectors. The results of the Western blot analysis of cellular lysates with anti-TSG101, anti-Alix, anti-Vps4B, anti-CHMP4b, anti-Chk2, anti-HSP70, or anti-β-actin antibody in RSc cells expressing shRNA targeted to TSG101 (TSG101i), Alix (Alixi), Vps4B (Vps4Bi), or CHMP4b (CHMP4bi) as well as in RSc cells transduced with a control lentiviral vector (Con) are shown. Real-time LightCycler RT-PCR for TSG101 (B), Alix (C), Vps4B (D), or CHMP4b mRNA (E) was performed as well as for β-actin mRNA in triplicate. Each mRNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (Con) which was assigned as 100%. Error bars in this panel and other figures indicate standard deviations. (F) MTT assay of each knockdown RSc cells at the indicated time. (G) The levels of HCV Core in the culture supernatants from the stable knockdown RSc cells 97 h after inoculation of HCV-JFH1 at an MOI of 0.1 were determined by ELISA. Experiments were done in triplicate and columns represent the mean Core protein levels. (H) The infectivity of HCV in the culture supernatants from the stable knockdown RSc cells 97 hrs after inoculation of HCV-JFH1 at an MOI of 0.1 was determined by a focus-forming assay at 48 hrs post-infection. Experiments were done in triplicate and each virus titer was calculated relative to the level in RSc cells transduced with a control lentiviral vector (Con) which was assigned as 100%. Asterisks indicate significant differences compared to the control treatment. \**P*<0.05; \*\**P*<0.01. (I) The level of intracellular genome-length HCV-JFH1 RNA in the cells at 97 hrs post-infection was monitored by real-time LightCycler RT-PCR. Results

from three independent experiments are shown. (J) Inhibition of TSG101, Alix, Vps4B, or CHMP4b protein expression by 72 hrs after transient transfection of RSc cells with a pool of control siRNA (Con) or a pool of siRNA specific for Alix, Vps4B, or CHMP4b (50 nM). The results of the Western blot analysis of cellular lysates with anti-TSG101, anti-Alix, anti-Vps4B, anti-CHMP4b, or anti- $\beta$ -actin antibody is shown. (K) MTT assay of each knockdown RSc cells at the indicated time. (L) The levels of HCV Core in the culture supernatants were determined by ELISA 24 hrs after inoculation of HCV-JFH1. RSc cells were transiently transfected with a pool of control siRNA (Con) or a pool of siRNA specific for TSG101, Alix, Vps4B, or CHMP4b (50 nM). At 48 hrs after transfection, the cells were inoculated with HCV-JFH1 at an MOI of 5 and incubated for 2 hrs. Then, culture medium was changed and incubated for 22 hrs. Experiments were done in triplicate and each Core level was calculated relative to the level in the culture supernatants from the control cells and indicated below. (M) The infectivity of HCV in the culture supernatants from the transient knockdown RSc cells 24 hrs after inoculation of HCV-JFH1 at an MOI of 5 was determined by a focus-forming assay at 48 hrs post-infection. Experiments were done in triplicate and each virus titer was calculated relative to the level in RSc cells transfected with a control siRNA (Con) which was assigned as 100%. Asterisks indicate significant differences compared to the control treatment. \* $P$ <0.05; \*\* $P$ <0.01.

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was significantly suppressed in these knockdown cells after HCV-JFH1 infection (Fig. 1G). Importantly, the infectivity of HCV in the culture supernatants was also significantly suppressed in these knockdown cells (Fig. 1H), while the RNA replication of HCV-JFH1 was not affected in the TSG101 or Alix knockdown cells and was somewhat decreased in the Vps4B and CHMP4b knockdown cells (Fig. 1I). This suggested that the ESCRT system is associated with infectious HCV production. To further confirm whether or not the ESCRT system is involved in HCV production, we analyzed the single-round HCV replication. For this, we used RSc cells transiently transfected with a pool of siRNAs specific for TSG101, Alix, Vps4B, or CHMP4b as well as a pool of control siRNAs (Con) following HCV infection. In spite of very effective knockdown of each ESCRT component (Fig. 1J), we demonstrated that the siRNAs did not affect the cell viabilities by MTT assay (Fig. 1K). Consistent with our finding using the stable knockdown cells, we observed that the release of HCV Core or the infectivity of HCV into the culture supernatants was significantly suppressed in these transient knockdown cells 24 hrs after HCV-JFH1 infection (Fig. 1L and 1M). Furthermore, we examined the effect of siRNA specific for TSG101, Alix, Vps4B, or CHMP4b in HCV RNA replication using the subgenomic JFH1 replicon, JRN/3-5B, encoding *Renilla* luciferase gene for monitoring the HCV RNA replication in HuH-7-derived OR6cJRN/3-5B cells (Fig. 2A and 2B) or an OR6 assay system, which was developed as a luciferase reporter assay system for monitoring genome-length HCV RNA replication (HCV-O, genotype 1b) in HuH-7-derived OR6 cells (Fig. 2C) [17,18]. The results showed that these siRNAs could not affect HCV RNA replication as well as the levels of intracellular NS5A proteins (Fig. 2A–C). Although we have demonstrated that the ESCRT system is required for production of extracellular infectious HCV particles, it is not clear whether or not these findings are associated with the assembly of intracellular infectious particles. To test this point, infectivity of intracellular infectious particles was analyzed following lysis of HCV-JFH1-infected knockdown cells by repetitive freeze and thaw. Consequently, we did not observe any significant effects of siRNAs on the accumulation of intracellular infectious HCV-JFH1, while the accumulation of extracellular HCV was significantly suppressed in these knockdown cells (Fig. 2D and 2E), indicating that inhibition of the ESCRT system does not block the accumulation of intracellular infectious HCV particles. Furthermore, Western blot analysis of cell lysates demonstrated that the level of intracellular HCV Core and NS5A was not affected in these knockdown cells 72 hrs post-infection (Fig. 2F). Thus, we conclude that the ESCRT system is not required for the assembly of infectious particles but the ESCRT system is required for late step of HCV production.

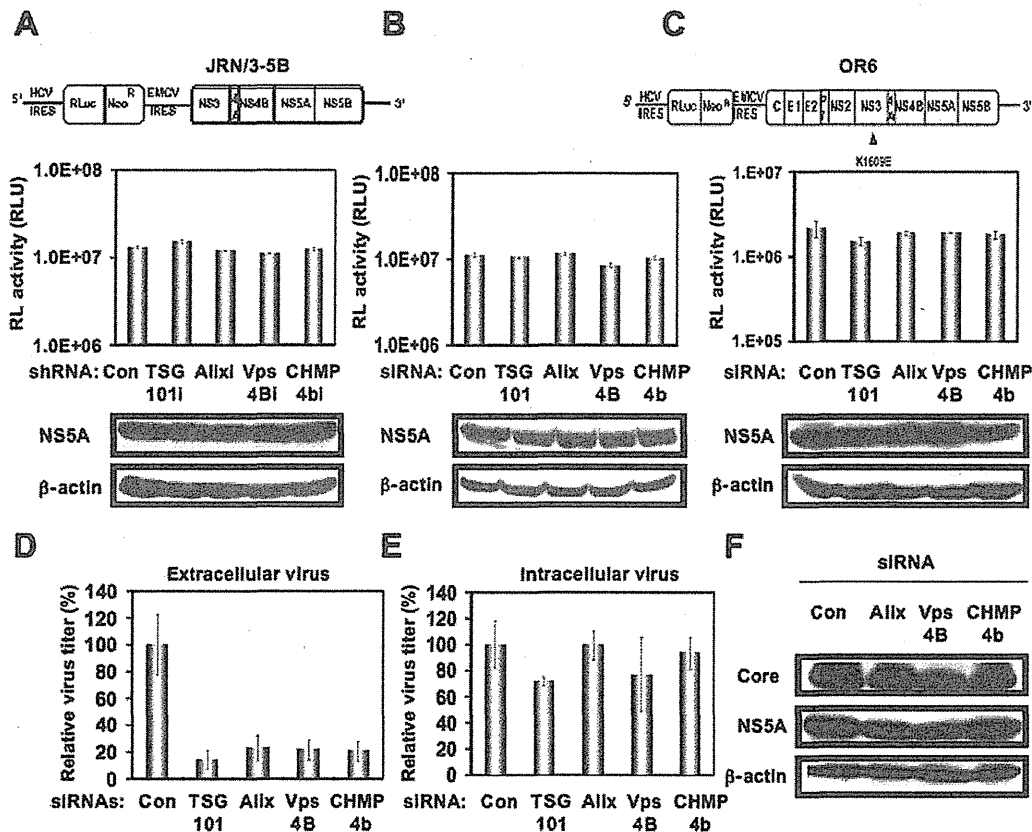
#### HCV Core can target into lipid droplets in the ESCRT knockdown cells

Since lipid droplets have been shown to be involved in an important cytoplasmic organelle for HCV production [4], we

performed immunofluorescence and confocal microscopic analyses to determine whether or not HCV Core misses localization into lipid droplets in the ESCRT knockdown cells. We found that the Core was targeted into lipid droplets even in TSG101 knockdown, Alix knockdown, Vps4B knockdown, or CHMP4b knockdown RSc cells as well as in the control RSc cells after HCV infection (Fig. 3). This suggests that the ESCRT system plays a role in the late step after the Core is targeted into lipid droplets in the HCV life cycle.

#### HCV Core interacts with CHMP4b

To determine whether or not HCV Core can interact with ESCRT component(s), we examined their subcellular localization by confocal laser scanning microscopy. Consequently, the Core mostly colocalized with CHMP4b-green fluorescent protein (GFP) or FLAG-tagged CHMP4b in the perinuclear region of 293FT cells coexpressing them (Fig. 4A and 4B), while the CHMP4b-GFP alone was slightly diffused in the cytoplasm (Fig. 4A), indicating the recruitment of CHMP4b in the Core-expressing area. Importantly, we observed similar partial colocalization in HCV-JFH1-infected RSc cells expressing CHMP4b-GFP (Fig. 4C), whereas the CHMP4b-GFP alone was diffused in the cytoplasm in the uninfected RSc cells (Fig. 4C), suggesting the interaction of HCV Core with CHMP4b. Unfortunately, we failed to observe endogenous CHMP4b using several commercially available anti-CHMP4b antibody (data not shown). Consistent with a previous report that interaction between HCV Core and NS5A is critical for HCV production [3], we found the partial colocalization of NS5A with CHMP4b-GFP as well as the colocalization of Core with CHMP4b-GFP in HCV-JFH1-infected RSc cells (Fig. 4C). Then, we examined whether or not HCV Core can bind to CHMP4b by immunoprecipitation analysis. 293FT cells transfected with 4 mg of pCHMP4b-GFP, pEGFP C3 (Clontech), pcDNA3-FLAG [19], pcDNA3-FLAG-Alix or pFLAG-CHMP4b and RSc cells 5 days after inoculation of HCV-JFH1 at an MOI of 4 were lysed and performed immunoprecipitation of lysate mixtures of HCV-JFH1-infected RSc cells and 293FT cells expressing CHMP4b-GFP, GFP alone, FLAG-CHMP4b or FLAG-epitope alone with anti-FLAG or anti-GFP antibody. Consequently, we observed that the Core but not the NS5A could bind to FLAG-CHMP4b (Fig. 4D). However, the Core was not immunoprecipitated with anti-FLAG antibody using the lysate mixtures of HCV-JFH1-infected RSc cell lysates and 293FT cells expressing FLAG-epitope alone or FLAG-Alix (Fig. 4D). Furthermore, the Core was coimmunoprecipitated with CHMP4b-GFP but not GFP when lysate mixtures of HCV-JFH1-infected RSc cells and 293FT cells expressing CHMP4b-GFP or GFP alone were used (Fig. 4D). In contrast, we failed to observe the marked colocalization of HCV-JFH1 Core with Myc-tagged TSG101 in HCV-JFH1-infected RSc cells expressing Myc-TSG101 or endogenous Alix in HCV-JFH1-infected RSc cells (Fig. 5A). Thus, we concluded that the HCV Core was associated with CHMP4b.



**Figure 2. ESCRT system is not required for HCV RNA replication and assembly of intracellular infectious HCV.** (A) Schematic gene organization of subgenomic JFH1 (JRN/3-5B) RNA encoding *Renilla* luciferase gene. *Renilla* luciferase gene (RLuc) is depicted as a box and is expressed as a fusion protein with Neo. The HCV RNA replication level in each ESCRT knockdown OR6c JRN/3-5B cells by lentiviral vector-mediated RNA interference (shRNA) was monitored by RL assay. The RL activity (RLU) is shown. The results shown are means from three independent experiments. (B) 72 hrs after the transfection of OR6c JRN/3-5B polyclonal cells with each of the siRNA (50 nM), the HCV RNA replication level was monitored by RL assay as described in (A). (C) Schematic gene organization of genome-length HCV-O RNA encoding *Renilla* luciferase gene. The position of an adaptive mutation, K1609E, is indicated by a triangle. 72 hrs after the transfection of OR6 cells with each of the siRNA (50 nM), the HCV RNA replication level was monitored by RL assay as described in (A). (D) The infectivity of HCV in the culture supernatants from the transient knockdown RSc cells 24 hrs after inoculation of HCV-JFH1 at an MOI of 2 was determined by a focus-forming assay at 48 hrs post-infection. Experiments were done in triplicate and each virus titer was calculated relative to the level in RSc cells transfected with a control siRNA (Con) which was assigned as 100%. (E) Intracellular HCV infectivity was determined by a focus-forming assay at 48 hrs post-inoculation of lysates by repeated freeze and thaw cycles as described in (D). (F) RSc cells were transiently transfected with a pool of control siRNA (Con) or a pool of siRNA specific for Alix, Vps4B, or CHMP4b (50 nM). At 24 hrs after the transfection, the cells were inoculated with HCV-JFH1 at an MOI of 0.2 and incubated for 48 hrs. Then, culture medium was changed and incubated for 24 hours. Western blotting of cell lysates 72 hrs post-infection with anti- $\beta$ -actin, anti-HCV NS5A, or anti-HCV Core antibody is shown.  
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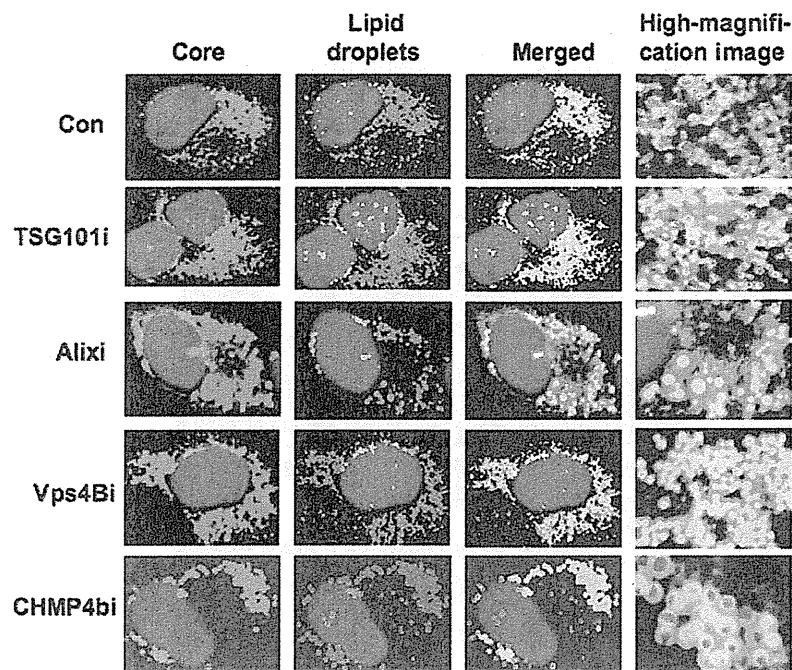
Finally, we examined the subcellular localization of HCV Core and Brox, a novel farnesylated Bro1 domain-containing protein, since Brox was recently identified as a CHMP4-binding protein [27]. In this context, the Core partially colocalized with GFP-Brox in 293FT cells coexpressing of HCV Core and GFP-Brox (Fig. 5B). Importantly, we observed similar partial colocalization in HCV-JFH1-infected RSc cells expressing GFP-Brox (Fig. 5C). On the other hand, the CHMP4b-GFP alone was diffused in the cytoplasm of uninfected RSc cells (Fig. 5C). To examine the potential role of Brox in HCV life cycle, we established the Brox knockdown RSc cells by lentiviral vector expressing shRNA targeted to Brox (Fig. 5D). Consequently, we found that the release of HCV Core or the infectivity of HCV into the culture supernatants was significantly suppressed in the Brox knockdown cells 4 days after HCV-JFH1 infection (Fig. 5E and 5F), while the RNA replication of HCV-JFH1 was marginally affected in the

knockdown cells (Fig. 5G) in spite of the very effective knockdown of Brox mRNA (Fig. 5D), suggesting that Brox is also required for the infectious HCV production.

**Discussion**

In this study, we have demonstrated that the ESCRT system is required for infectious HCV production, and that HCV Core but not NS5A binds to CHMP4b, a component of ESCRT-III. Although RNA replication of HCV-JFH1 was not affected in the TSG101 knockdown or the Alix knockdown cells, the infectivity of HCV in the culture supernatants was significantly suppressed in these knockdown cells after HCV-JFH1 infection (Fig. 1G and 1H). Furthermore, siRNA targeted to TSG101, Alix, Vps4B, or CHMP4b significantly suppressed HCV Core level or the infectivity of HCV in the culture supernatants (Fig. 1L, 1M, and





**Figure 3. HCV Core is targeted to lipid droplets even in the ESCRT knockdown cells.** The RSc cells transduced with a control lentiviral vector (Con), the TSG101 knockdown (TSG101i), the Alix knockdown (Alixi), the Vps4B knockdown (Vps4Bi), or the CHMP4b knockdown (CHMP4bi) cells were infected with HCV-JFH1. Cells were fixed 60 hrs post-infection and were then examined by confocal laser scanning microscopy. Cells were stained with anti-HCV Core (CP-9 and CP-11 mixture) and were then visualized with Cy3 (red). Lipid droplets and nuclei were stained with BODIPY 493/503 (green) and DAPI (blue), respectively. Images were visualized using confocal laser scanning microscopy. Colocalization is shown in yellow (Merged). doi:10.1371/journal.pone.0014517.g003

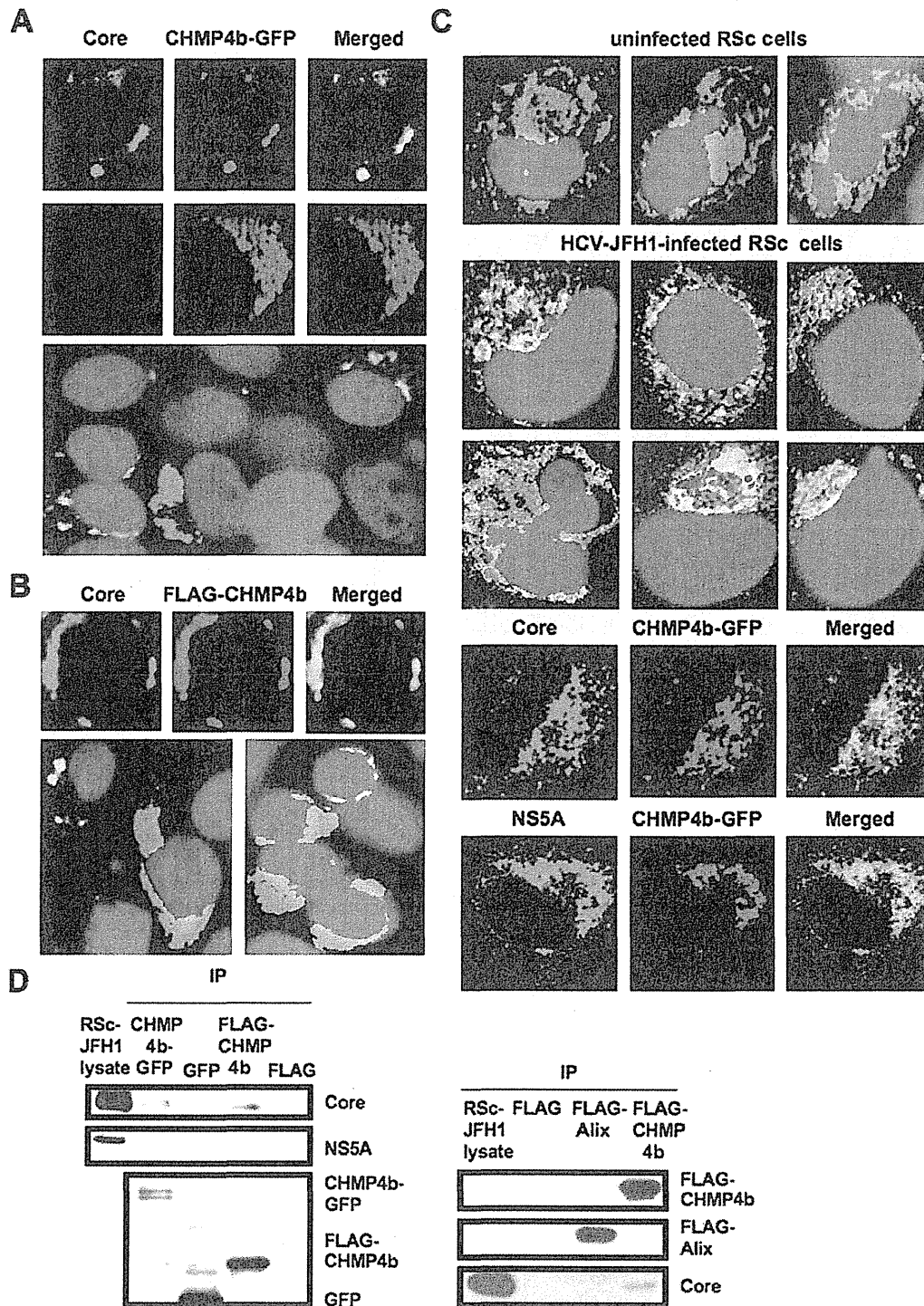
2D), while the siRNA did not affect intracellular HCV Core level (Fig. 2E and 2F). Accordingly, we noticed some discrepancy that shRNA targeted to Vps4B or CHMP4b somewhat decreased intracellular HCV RNA replication (Fig. 1I), whereas siRNA targeted to TSG101, Alix, Vps4B or CHMP4b did not affect the RNA replication of subgenomic replicon of JFH1 (Fig. 2A and 2B). Furthermore, siRNAs targeted to TSG101, Alix, Vps4B, or CHMP4b did not affect HCV-O (genotype 1b) RNA replication using the OR6 assay system [17,18] (Fig. 2C), indicating that the ESCRT system is unrelated to the HCV RNA replication of genotype 1b. Thus, we suggested that the ESCRT system is not significant for HCV RNA replication. Within the family *Flaviviridae*, NS3 of the Japanese encephalitis virus (JEV) is also known to interact with TSG101 and microtubules, suggesting their potential roles in JEV assembly [28]. Accordingly, HCV NS3 has been involved in HCV particle assembly and infectivity [29,30]. However, whether HCV NS3 or another HCV protein binds to TSG101 remains to be investigated. At least, we did not observe the interaction between HCV NS5A and CHMP4b (Fig. 4D), Alix (Fig. 4D), TSG101, or Vps4B (data not shown).

Efficient enveloped virus release requires *cis*-acting viral late domains (L-domains), including P(T/S)AP, YPXnL (where X is any amino acid), and PPXY amino acid L-domain motif that are found in the structural proteins of other enveloped viruses [10,11]. Unlike the case with these enveloped viruses, we failed to find the three types of conserved viral L-domain motif in the HCV-JFH1 Core (data not shown). Nevertheless, we observed that the Core bound to CHMP4b, whereas NS5A did not (Fig. 4D), suggesting that the Core has a novel motif required for the HCV production. In this regard, Blanchard *et al.* reported that the aspartic acid at position 111 in the Core is crucial for virus assembly [31].

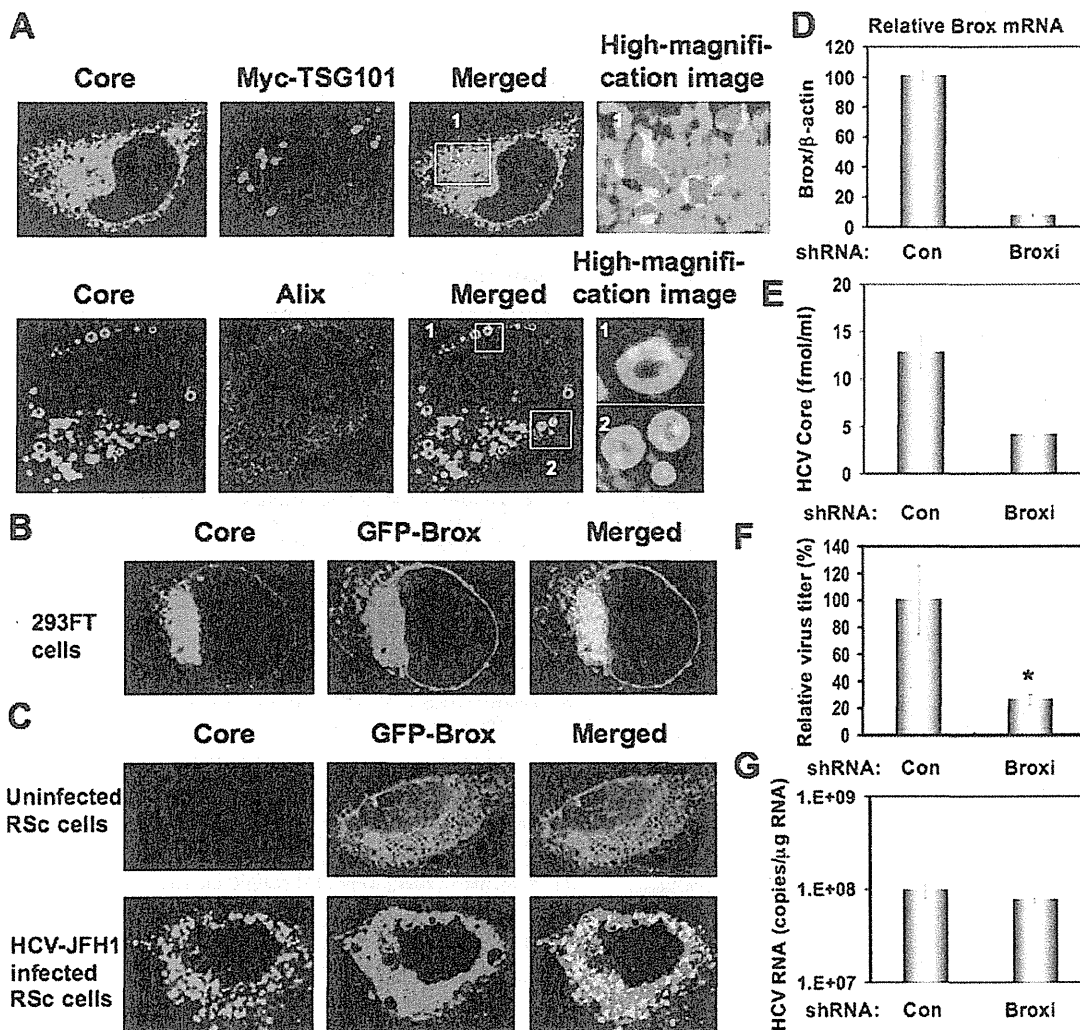
Interestingly, the conversion of the aspartic acid into alanine at amino acid 111 (PTDP to PTAP), which creates the PTAP L-domain motif, enhanced the release of HCV Core in the cell culture medium (a 2.5-fold increase) compared with the wild-type Core [31]. In contrast, Klein *et al.* demonstrated that the D111A mutation in the Core had no effect on HCV capsid assembly [32]. Furthermore, Murray *et al.* found that serine 99, a putative phosphorylation site, in the Core was essential for infectious virion production [7]. In any event, the Core motif that is needed for interaction with the ESCRT components remains to be identified.

Ubiquitin modification of viral protein has been implicated in virion egress as well as in protein turnover. Indeed, the ubiquitin/proteasome system is required for the retrovirus budding machinery, since proteasome inhibition interferes with retroviral Gag polyprotein processing, release, and maturation. The ubiquitin modification of the HIV-1 p6 domain of Gag enhances TSG101 binding [12]. In the case of HCV Core, proteasomal degradation of the Core is mediated by two distinct mechanisms [33–36]. E6AP E3 ubiquitin ligase mediates ubiquitylation and degradation of the Core [34]. In contrast, proteasome activator PA28 $\gamma$  (11S regulator  $\gamma$ ), an HCV Core-binding protein, is involved in the ubiquitin-independent degradation of the Core and HCV propagation [33,35,36]. However, little is known whether or not the ubiquitin modification of the Core might be involved in HCV egress like other enveloped viruses.

Finally, the identification of the site of viral particle assembly and budding is an intriguing issue. In case of HCV, at present, it is very difficult to visualize the HCV budding site in the infected cells by an electron microscopy. However, recent studies suggested that lipid droplets are an important cytoplasmic organelle for HCV production [4]. In this regard, Shavinskaya *et al.* demonstrated that



**Figure 4. HCV Core interacts with CHMP4b.** (A–C) HCV Core colocalizes with CHMP4b. 293FT cells cotransfected with 100 ng of pcDNA3/core (JFH1) and either 100 ng of pCHMP4b-GFP [39] (A) or pFLAG-CHMP4b [39] (B) were examined by confocal laser scanning microscopy. Cells were stained with anti-HCV Core and anti-FLAG polyclonal antibody and were then visualized with FITC (FLAG-CHMP4b) or Cy3 (Core). Images were visualized using confocal laser scanning microscopy. The right panels exhibit the two-color overlay images (Merged). Colocalization is shown in yellow. (C) The Core or NS5A partially colocalizes with CHMP4b in HCV-JFH1-infected RSc cells. RSc cells transfected with 100 ng of pCHMP4b-GFP were infected with HCV-JFH1. Cells were fixed 60 hrs post-infection and were then examined by confocal laser scanning microscopy as shown in panel (A). (D) HCV Core binds to CHMP4b. 293FT cells transfected with 4 μg of pCHMP4b-GFP, pEGFP C3 (Clontech), pcDNA3-FLAG, pcDNA3-FLAG-Alix or pFLAG-CHMP4b and RSc cells 5 days after inoculation of HCV-JFH1 at an MOI of 4 were lysed. The mixtures of these lysates were immunoprecipitated with either anti-FLAG or Living Colors A.v. monoclonal antibody (anti-GFP antibody), followed by immunoblot analysis using anti-HCV Core, anti-HCV NS5A, anti-FLAG, and/or Living Colors A.v. monoclonal antibody. doi:10.1371/journal.pone.0014517.g004



**Figure 5. Brox is required for HCV life cycle.** (A) Subcellular localization of Myc-tagged TSG101 in HCV-JFH1-infected RSc cells. RSc cells transfected with 100 ng of pBj-Myc-TSG101 [40] were infected with HCV-JFH1. Cells were fixed 60 hrs post-infection and were then examined by confocal laser scanning microscopy as shown in Fig. 3. High magnification image of area 1 is shown. Subcellular localization of endogenous Alix in HCV-JFH1-infected RSc cells 60 hrs post-infection. Cells were stained with anti-Alix and anti-HCV Core antibodies and were examined by confocal laser scanning microscopy. High magnification images of area 1 and area 2 are shown. (B) HCV Core partially colocalizes with Brox. 293FT cells cotransfected with 100 ng of pcDNA3/core (JFH1) and 100 ng of pmGFP-Brox<sup>WT</sup> [27] were examined by confocal laser scanning microscopy. (C) The HCV Core partially colocalizes with Brox in HCV-JFH1-infected RSc cells. RSc cells transfected with 100 ng of pmGFP-Brox<sup>WT</sup> were infected with HCV-JFH1. Cells were fixed 60 hrs post-infection and were then examined by confocal laser scanning microscopy. (D) Inhibition of Brox mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for Brox was performed as well as for  $\beta$ -actin mRNA in triplicate. Each mRNA level was calculated relative to the level in RSc cells transfected with a control lentiviral vector (Con) which was assigned as 100%. (E) The levels of HCV Core in the culture supernatants from the Brox knockdown RSc cells (Broxi) 72 hrs after inoculation of HCV-JFH1 were determined by ELISA. (F) The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 hrs post-infection. Experiments were done in triplicate and each virus titer was calculated relative to the level in RSc cells transfected with a control lentiviral vector (Con) which was assigned as 100%. Asterisks indicate significant differences compared to the control treatment. \* $P$ <0.05; \*\* $P$ <0.01. (G) The levels of intracellular genome-length HCV-JFH1 RNA in the cells used in (E) were monitored by real-time LightCycler RT-PCR. doi:10.1371/journal.pone.0014517.g005

the lipid droplet-binding domain of the Core is a major determinant for efficient virus assembly [6]. The HCV Core induces lipid droplet redistribution in a microtubule- and dynein-dependent manner, since disrupting the microtubule network reduced the HCV titer, implicating transport networks in virus assembly and release [2]. Furthermore, Sandrin *et al.* reported that HCV envelope proteins localized to ESCRT-associated multivesicular body (MVB) [37]. Quite recently, Corless *et al.* have

demonstrated that Vps4B and the ESCRT-III complex are required for HCV production [38]. Consistent with our findings, their dominant-negative forms of Vps4B and CHMP4B clearly suppressed HCV production. However, their dominant-negative forms of TSG101 and Alix failed to suppress the HCV production and they suggested that both TSG101 and Alix are unrelated to the HCV production. In contrast, we have demonstrated both TSG101 and Alix are also required for the infectious HCV

production by using shRNAs and siRNAs (Fig. 1). We may partly explain such discrepancy due to the difference of the methodology in our study utilized shRNAs and siRNAs instead of dominant-negative forms of TSG101 and Alix. Importantly, they demonstrated that the dominant-negative forms of Vps4B and CHMP4b did not affect the HCV RNA replication and the accumulation of intracellular infectious particles, suggesting that Vps4B and CHMP4b are unrelated to HCV RNA replication. Indeed, we have demonstrated that the ESCRT system is not required for the assembly of intracellular infectious HCV particles (Fig. 2E). Notably, we have demonstrated for the first time that HCV Core associated with CHMP4b (Fig. 4). Accordingly, we have also demonstrated that Brox, a CHMP4b binding protein, is required for HCV production (Fig. 5D–G). Taking together the past and present findings, we propose that the ESCRT system is involved in

infectious HCV production after the HCV assembly that occurs on lipid droplets.

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## Author Contributions

Conceived and designed the experiments: YA NK. Performed the experiments: YA MK. Analyzed the data: YA. Contributed reagents/materials/analysis tools: MM MI HD TW. Wrote the paper: YA NK.

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