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-β-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 OR6c JRN/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.

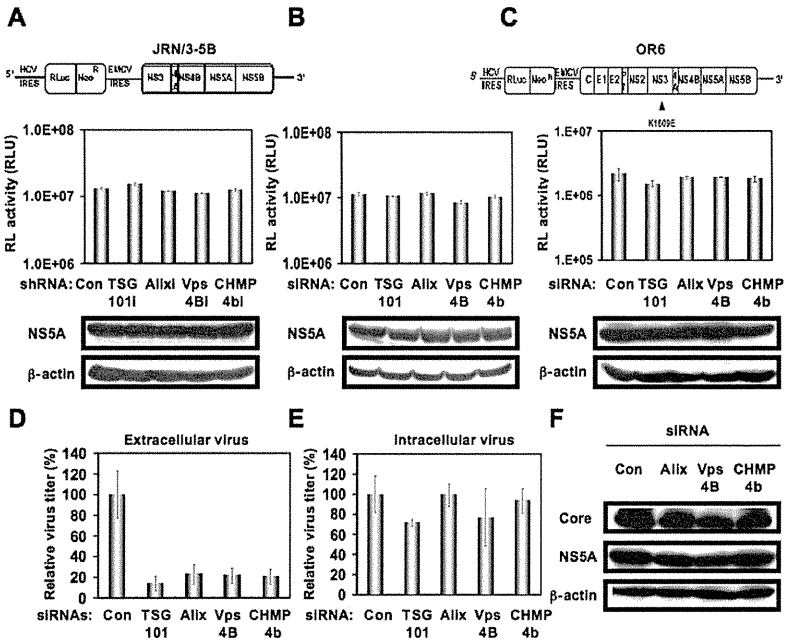

Figure 2. ESCRT system is not required for HCV RNA replication and assembly of intracellular infectious HCV. (A) Schematic gene organization of subgenomic JFH1 (JRN/3-5B) RNA encoding *Renilla* luciferase gene. Renilla luciferase gene (RLuc) is depicted as a box and is expressed as a fusion protein with 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-β-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

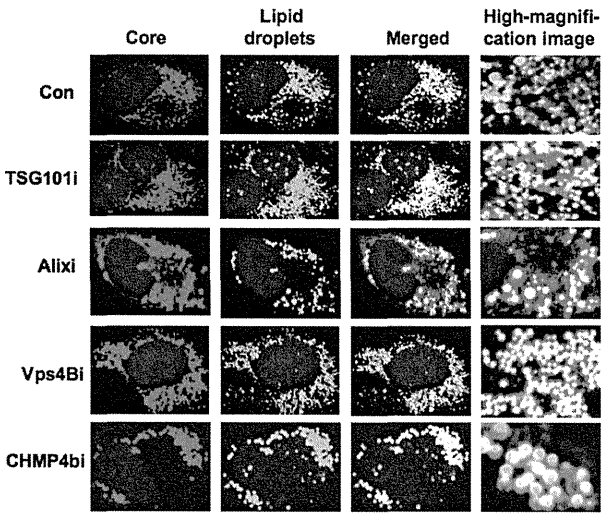

Figure 3. HCV Core is targeted to lipid droplets even in the ESCRT knockdown cells. The RSc cells transduced with a control lentiviral vector (Con), the TSG101 knockdown (TSG101i), the Alix knockdown (Alixi), the Vps4B knockdown (Vps4Bi), or the CHMP4b knockdown (CHMP4bi) cells were infected with HCV-JFH1. 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).
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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 TASG101, 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 PA28γ (11S regulator γ), 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

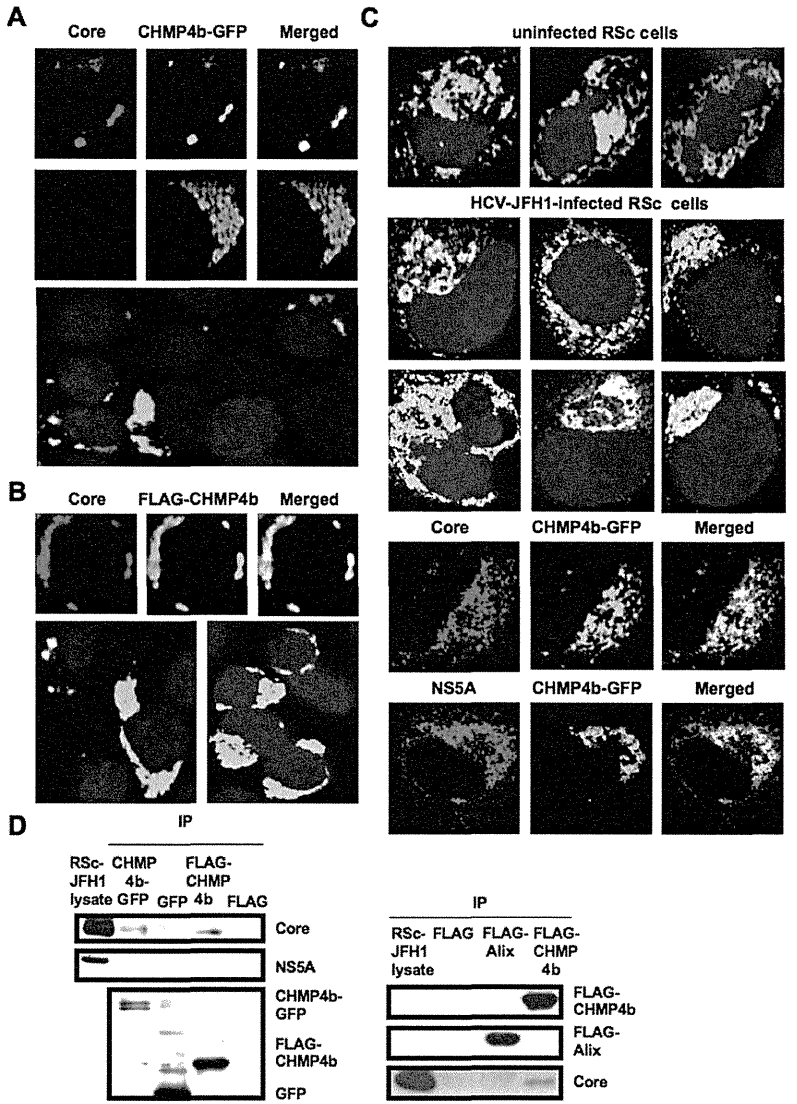

Figure 4. HCV Core interacts with CHMP4b. (A–C) HCV Core colocalizes with CHMP4b. 293FT cells cotransfected with 100 ng of pcDNA3/core (JFH1) and either 100 ng of pCHMP4b-GFP [39] (A) or pFLAG-CHMP4b [39] (B) were examined by confocal laser scanning microscopy. Cells were stained with anti-HCV Core and 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.
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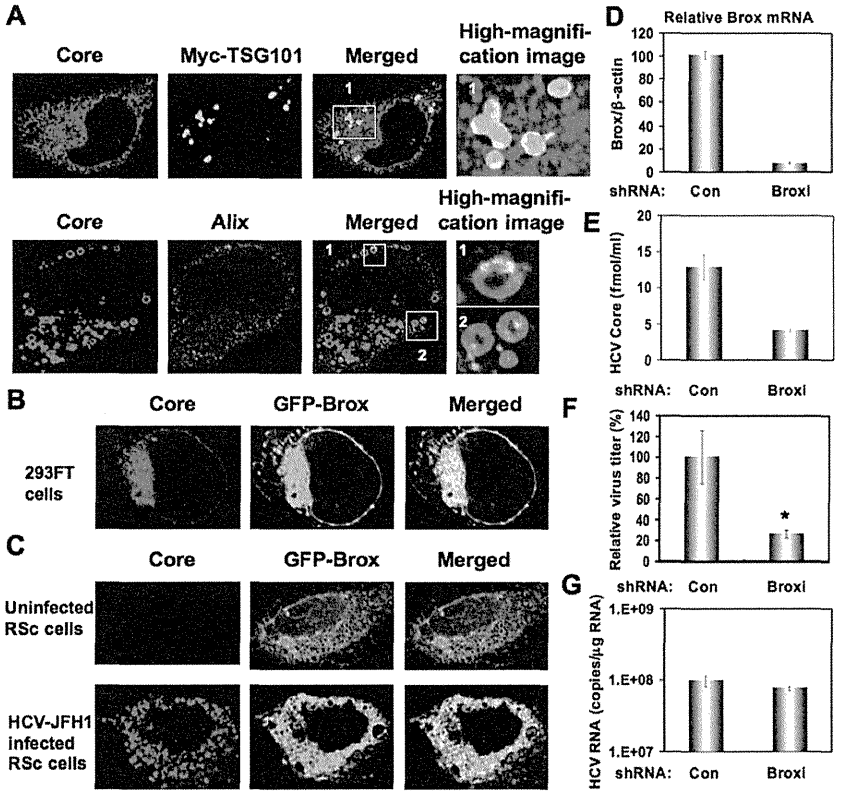

Figure 5. Brox is required for HCV life cycle. (A) Subcellular localization of Myc-tagged TSG101 in HCV-JFH1-infected RSc cells. RSc cells transfected with 100 ng of pBj-Myc-TSG101 [40] were infected with HCV-JFH1. Cells were fixed 60 hrs post-infection and were then examined by confocal laser scanning 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^{WT} [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^{WT} 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 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%. (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 transduced with a control lentiviral vector (Con) which was assigned as 100%. Asterisks indicate significant differences compared to the control treatment. *$P<0.05$; **$P<0.01$. (G) The levels of intracellular genome-length HCV-JFH1 RNA in the cells used in (E) were monitored by real-time LightCycler RT-PCR.
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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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Hepatitis C Virus Core Protein Abrogates the DDX3 Function That Enhances IPS-1-Mediated IFN–Beta Induction

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Abstract

The DEAD box helicase DDX3 assembles IPS-1 (also called Cardif, MAVS, or VISA) in non-infected human cells where minimal amounts of the RIG-I-like receptor (RLR) protein are expressed. DDX3 C-terminal regions directly bind the IPS-1 CARD-like domain as well as the N-terminal hepatitis C virus (HCV) core protein. DDX3 physically binds viral RNA to form IPS-1-containing spots, that are visible by confocal microscopy. HCV polyU/UC induced IPS-1-mediated interferon (IFN)-beta promoter activation, which was augmented by co-transfected DDX3. DDX3 spots localized near the lipid droplets (LDs) where HCV particles were generated. Here, we report that HCV core protein interferes with DDX3-enhanced IPS-1 signaling in HEK293 cells and in hepatocyte Oc cells. Unlike the DEAD box helicases RIG-I and MDA5, DDX3 was constitutively expressed and colocalized with IPS-1 around mitochondria. In hepatocytes (O cells) with the HCV replicon, however, DDX3/IPS-1-enhanced IFN-beta-induction was largely abrogated even when DDX3 was co-expressed. DDX3 spots barely merged with IPS-1, and partly assembled in the HCV core protein located near the LD in O cells, though in some O cells IPS-1 was diminished or disseminated apart from mitochondria. Expression of DDX3 in replicon-negative or core-less replicon-positive cells failed to cause complex formation or LD association. HCV core protein and DDX3 partially colocalized only in replicon-expressing cells. Since the HCV core protein has been reported to promote HCV replication through binding to DDX3, the core protein appears to switch DDX3 from an IFN-inducing mode to an HCV-replication mode. The results enable us to conclude that HCV infection is promoted by modulating the dual function of DDX3.

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Introduction

The retinoic acid inducible gene-I (RIG-I) and the melanoma differentiation-associated gene 5 (MDA5) encode cytoplasmic RNA helicases [1–3] that signal the presence of viral RNA through the adaptor, IPS-1/Mitochondrial antiviral signaling protein (MAVS)/Caspase recruitment domain (CARD) adaptor inducing interferon (IFN)-beta (Cardif)/Virus-induced signaling adaptor (VISA) to produce IFN-beta [4–7]. IPS-1 is localized to the mitochondrial outer membrane through its C-terminus [6]. Increasing evidence suggests that the DEAD-box RNA helicase DDX3, which is on the X chromosome, participates in the regulation of type I IFN induction by the RIG-I pathway.

DDX3 acts on the IFN-inducing pathway by a complex mechanism. Early studies reported that DDX3 up-regulates IFN-beta induction by interacting with IKKepsilon [8] or TBK1 [9] in a kinase complex. Both TBK1 and IKKepsilon are IRF-3-activating kinases with NF-kappaB- and IFN-inducible properties. DDX3 has been proposed to bind IKKepsilon, and IKKepsilon is generated after NF-kappaB activation [10]. Yeast two-hybrid studies demonstrated that DDX3 binds IPS-1, and both are constitutively present prior to infection (Fig. 1). Ultimately, DDX3 forms a complex with the DEAD-box RNA helicases RIG-I and MDA5 [11], which are present at only low amounts in resting cells, and are up-regulated during virus infection. Previously we used gene silencing and disruption, to show that the main function of DDX3 is to interact with viral RNA and enhance RIG-I signaling upstream of NAP1/TBK1/IKKepsilon [11]. Hence, DDX3 is involved in multiple pathways of RNA sensing and signaling during viral infection.

DDX3 resides in both the nucleus and the cytoplasm [12], and has been implicated in a variety of processes in gene expression regulation, including transcription, splicing, mRNA export, and translation [13]. A recent report suggested that the N-terminus of hepatitis C virus (HCV) core protein binds the C-terminus of DDX3 (Fig. S1) [14,15], and this interaction is required for HCV replication [16]. Although DDX3 promotes efficient HCV infection by accelerating HCV RNA replication, the processes appear independent of its interaction with the viral core protein [15]. HCV seems to co-opt DDX3, and require DDX3 for replication. In addition, the association between DDX3 and core protein implicates DDX3 in HCV-related hepatocellular carcinoma progression [17]. Therefore, DDX3 could be a novel target for the development of drugs against HCV [18].

A number of reports have demonstrated the formation of the DDX3-core protein complex in the cytoplasm, but the functional relevance of DDX3-core protein interaction is not known. In this report, we show evidence that the HCV core protein participates in suppression of DDX3-augmented IPS-1 signaling for IFN-β induction. Several possible functions of DDX3 are discussed, focusing on its core protein association and IPS-1-regulatory properties.

A

Two representative polyI:C-binding proteins identified by mass-spectrometric analysis

dsRNA-binding protein	ID	Mr (kDa)	polyI:C	polyU	gene name
dsRNA-activated protein kinase	IPI00019463	63 kDa	37	2	PKR
ATP-dependent RNA helicase	IPI00215637	73 kDa	19	12	DDX3

B

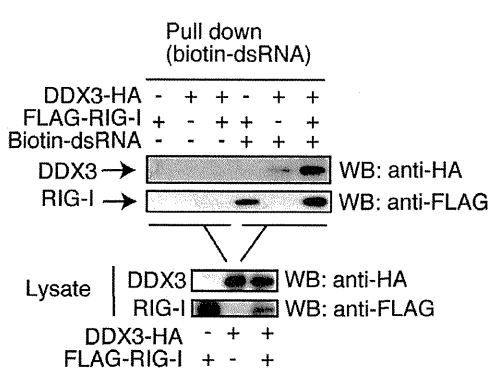

Figure 1. DDX3 is a RNA-binding protein. (A) DDX3 is a polyU- and polyI:C-binding protein. Mass spectrometry analyses indicated that DDX3 binds polyI:C- and polyU-Sepharose, although PKR binds polyI:C but not polyU. The rough data from MASCOT and one representative of six trials are shown. (B) DDX3 binds dsRNA, RIG-I and HCV core protein. Expression vectors for Flag-tagged RIG-I and HA-tagged DDX3 were transfected into HEK293 cells using lipofectamine 2000. Twenty-four hours after the transfection, extract from transfected cells were mixed with biotin-conjugated dsRNA. RNA-protein complex were recovered by pull-down assay using streptavidin-Sepharose. The protein within the pull-down fraction was analyzed by western blotting. The results are representative of two independent experiments.
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Materials and Methods

Cell culture and reagents

HEK293 cells and HEK293FT cells were maintained in Dulbecco's Modified Eagle's low or high glucose medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FCS (Invitrogen) and antibiotics. Huh7.5 cells were maintained in MEM (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FCS. Hepatocyte sublines with HCV replicon (O cells) and without replicon (Oc cells) were established as described previously [19]. O cells with core-less subgenomic replicon (sO cells) were also generated in Dr. Kato's laboratory [16,19]. RIG-I −/− mouse embryonic fibroblasts (MEF) were gifts from Drs. Takeuchi and Akira [1]. Anti-FLAG M2 monoclonal Ab and anti-HA polyclonal Ab were purchased from Sigma. A mitochondria marker (Mitotracker) and Alexa Fluor®-conjugated secondary antibodies were purchased from Molecular probe. Anti-HCV core mAb (C7-50) [20] and anti-human DDX3 pAb were from Affinity BioReagents, Inc and Abcam, Cambridge MA, respectively.

Plasmids

DDX3 cDNA encoding the entire ORF was cloned into pCR-blunt vector using primers, DDX3N F-Xh (CTC GAG CCA CCA TGA GTC ATG TGG CAG TGG AA) and DDX3C R-Ba (GGA TCC GTT ACC CCA CCA GTC AAC CCC) from human lung cDNA library. To make an expression plasmid, HA tag was fused at the C-terminal end of the full length DDX3 (pEF-BOS DDX3-HA). pEF-BOS DDX3 (1-224aa) vector was made by using primers,

DDX3 N-F-Xh and DDX3D1 (GGA TCC GGC ACA AGC CAT CAA GTC TCT TTT C). pEF-BOS DDX3-HA (225-662) was made by using primers, DDX3D2-3 (CTC GAG CCA CCA TGC AAA CAG GGT CTG GAA AAA C) and DDX3C R-Ba. To make pEF-BOS DDX3-HA (225-484) and pEF-BOS DDX3-HA (485-663), the primers, DDX3D2 R-Ba (GGA TCC AAG GGC CTC TTC TCT ATC CCT C) and DDX3D3 F-Xh (CTC GAG CCA CCA TGC ACC AGT TCC GCT CAG GAA AAA G) were used, respectively. HCV core expressing plasmids, pcDNA3.1 HCVO core or JFH1 core, were previously reported by N. Kato (Okayama University Japan) [16]. Another 1b genotype of the core was cloned from a HCV patient in Osaka Medical Center (Osaka) according to the recommendation of the Ethical Committee in Osaka. We obtained written informed consent from each patient for research use of their samples. Reporter and internal control plasmids for reporter gene assay are previously described [21,22].

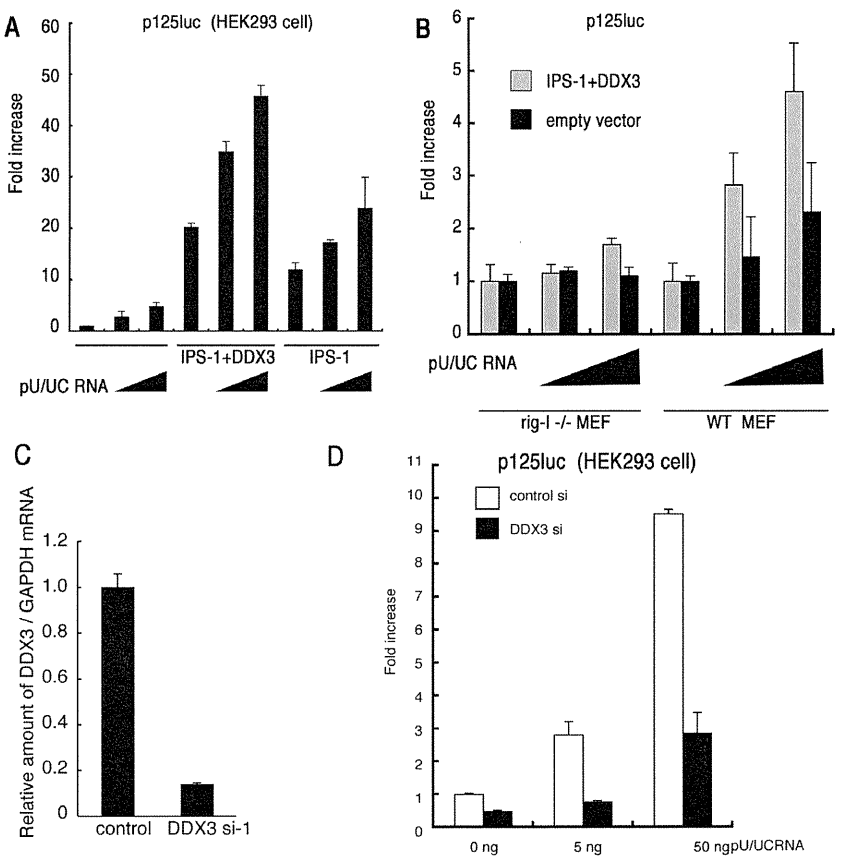

Figure 2. DDX3 is a positive regulator of IPS-1-mediated IFN promoter activation. (A) IFN-β induction by polyU/UC is augmented by DDX3. IPS-1 (100 ng), DDX3 (100 ng) and p125luc reporter (100 ng) plasmids were transfected into HEK293 cells in 24-well plates with or without the HCV 3′ UTR poly U/UC region (PU/UC) RNA (0, 25 or 50 ng/well), synthesized *in vitro* by T7 RNA polymerase. HCV RNA-enhancing activation of IFN-beta promoter was assessed by reporter assay in the presence or absence of the DDX3-IPS-1 complex. (B) RIG-I is essential for the DDX3/IPS-1-mediated IFN-promoter activation. MEF from wild-type and RIG-I −/− mice were transfected with plasmids of IPS-1, DDX3 and p125luc as in panel A, and stimulated with polyU/UC (0, 25 or 50 ng/well). Reporter activity was determined as in panel A. (C) Knockdown of DDX3. Negative control or DDX3 targeting siRNA (20 pmol), DDX3 si-1, was transfected into HEK293 cells, and after 48 hrs, expression of endogenous DDX3 mRNA was examined by real-time RT-PCR. DDX3 si-1-mediated down-regulation of the DDX3 protein was also confirmed by Western blotting (data not shown). (D) DDX3 enhances RIG-I-mediated IFN-beta promoter activation induced by polyU/UC. DDX3 si-1 or control siRNA was transfected into HEK293 cells with reporter plasmids (100 ng). After 48 hrs, cells were stimulated with polyU/UC (5~50 ng/ml) with lipofectamin 2000 reagent for 6 hrs, and activation of the reporter p125luc was measured. The results are representative of at least two independent experiments, each performed in triplicate.
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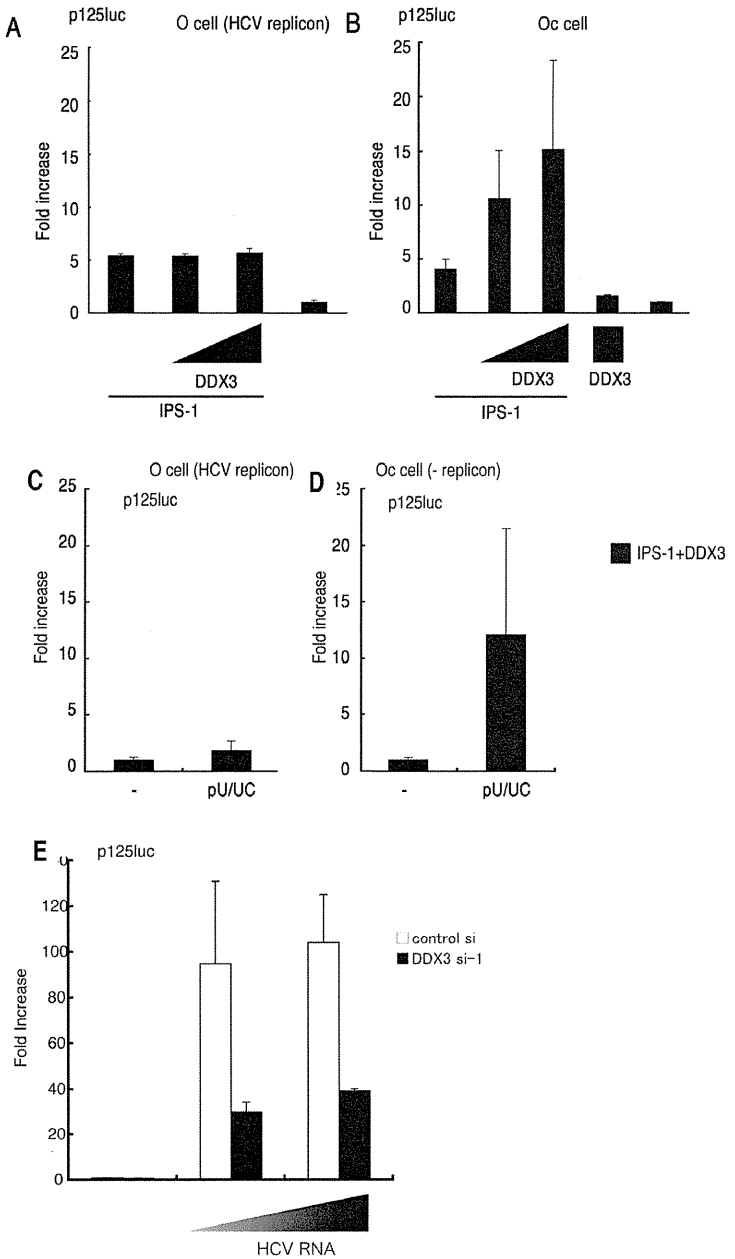

Figure 3. The HCV replicon suppresses IPS-1/DDX3-mediated augmentation of IFN promoter activation. (A,B) O cells with the HCV replicon fail to activate an IFN-β reporter in response to IPS-1/DDX3. O cells contain the full-length HCV replicon, and Oc cells do not [16]. O cells (A) or Oc cells (B) were transfected with IPS-1, DDX3 or p125luc reporter plasmids. At timed intervals (24 hrs), reporter activity was determined as in Fig. 2. (C,D) The HCV replicon suppresses IFN-promoter activation by polyU/UC. O cells and Oc cells expressing IPS-1 and DDX3 were stimulated with polyU/UC. At 48 hrs, reporter activity was determined as in panel A. (E) DDX3 is required for enhanced activation of IFN-beta promoter by O cell HCV 3′UTR. HCV 3′ UTR cDNA was amplified by RT-PCR from RNA extracted from O cells containing full-length HCV replicon. The HCV 3′ UTR RNA was synthesized *in vitro* using T7 RNA polymerase. DDX3 siRNA or control siRNA was transfected into HEK293 cells with the p125luc reporter. After 24 hrs, cells were transfected with HCV RNA, and incubated for 24 hrs. The IFN-beta promoter activation was assessed by luciferase reporter assay. One representative of at least three independent experiments, each performed in triplicate, is shown.
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Preparation of HCV polyU/UC RNA

The HCV genotype 1b polyU/UC RNA (from 9421 to 9480, Accession number: EU867431) [23] was synthesized by T7 RNA polymerase *in vitro*. The template dsDNA sequences were; Forward: TAA TAC GAC TCA CTA TAG GGT TCC CTT TTT TTT CTT TTT TTT TTT TTT TTT TTT TTT TTT CTC CTT TTT TTT TC, Reverse: GAA AAA AAA AGG AGA AAA AAA AAA AAA AAA AAA AAA AAA AGA AAA AAA AAA AGG GAA CCC TAT AGT GAG TCG TAT TA. The synthesized RNA was purified by TRIZOL reagent (Invitrogen). cDNA of HCV 3′ UTR region was amplified from total RNA of O cells using primers HCV-F1 and HCV-R1, and then cloned into pGEM-T easy vector. The primer set sequences were HCV-F1: CTC CAG GTG AGA TCA ATA GG and HCV-R1: CGT GAC TAG GGC TAA GAT GG. RNA was synthesized using T7 and SP6 RNA polymerases. Template DNA was digested by DNase I, and RNA was purified using TRIZOL (Invitrogen) according to manufacturer's instructions.

RNAi

Knockdown of DDX3 was carried out using siRNA, DDX3 siRNA-1: 5′-GAU UCG UAG AAU AGU CGA ACA-3′, siRNA-2: 5′-GGA GUG AUU ACG AUG GCA UUG-3′, siRNA-3: 5′-GCC UCA GAU UCG UAG AAU AGU-3′ and control siRNA: 5′-GGG AAG AUC GGG UUA GAC UUC-3′. 20 pmol of each siRNA was transfected into HEK293 cells in 24-well plate with Lipofectamin 2000 according to manufacture's protocol. Knockdown of DDX3 was confirmed 48 hrs after siRNA transfection. Experiments were repeated twice for confirmation of the results.

Reporter assay

HEK293 cells (4×10^4 cells/well) cultured in 24-well plates were transfected with the expression vectors for IPS-1, DDX3 or empty vector together with the reporter plasmid (100 ng/well) and an internal control vector, phRL-TK (Promega) (2.5 ng/well) using FuGENE (Roche) as described previously [23]. The p-125 luc reporter containing the human IFN-beta promoter region (−125 to +19) was provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). The total amount of DNA (500 ng/well) was kept constant by adding empty vector. After 24 hrs, cells were lysed in lysis buffer (Promega), and the *Firefly* and *Renella* luciferase activities were determined using a dual-luciferase reporter assay kit (Promega). The *Firefly* luciferase activity was normalized by *Renella* luciferase activity and is expressed as the fold stimulation relative to the activity in vector-transfected cells. Experiments were performed three times in duplicate (otherwise indicated in the legends).

PolyI:C or polyU/UC stimulation

PolyI:C was purchased from GE Healthcare company, and solved in milliQ water. For polyI:C treatment, polyI:C was mixed with DEAE-dextran (0.5 mg/ml) (Sigma) in the culture medium, and the cell culture supernatant was replaced with the medium containing polyI:C and DEAE-dextran. Using DEAE-dextran, polyI:C is incorporated into the cytoplasm to activate RIG-I/MDA5.

HCV 3′ UTR poly U/UC region (PU/UC) RNA (0~50 ng/well), which is synthesized *in vitro* by T7 RNA polymerase, transfected into HEK293 cells in 24-well plate by lipofectamin 2000 (Invitrogen) with other plasmids. Cells were allowed to stand for 24~48 hrs and HCV RNA-enhancing activation of IFN-beta promoter was assessed by reporter assay.

Immunoprecipitation (i.p.)

HEK293FT cells were transfected in a 6-well plate with plasmids encoding DDX3, IPS-1, RIG-I or MDA5 as indicated in the figures. 24 hrs after tranfection, the total cell lysate was prepared by lysis buffer (20 mM Tris-HCl [pH 7.5] containing 125 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% NP-40, 30 mM NaF, 5 mM Na_3Vo_4, 20 mM IAA and 2 mM PMSF), and the protein was immunoprecipitated with anti-HA polyclonal (SIGMA) or anti-FLAG M2 monoclonal Ab (SIGMA). The precipitated samples were resolved on SDS-PAGE, blotted onto a nitrocellulose sheet and stained with anti-HA (HA1.1) monoclonal (SIGMA), anti-HA polyclonal or anti-FLAG M2 monoclonal Ab.

Pull-down assay

The pull-down assay was performed according to the method described in Saito T et al. [24]. Briefly, the RNA used for the assay was purchased from JBioS, Co. Ltd (Saitama, Japan). The RNA sequences are (sense strand) AAA CUG AAA GGG AGA AGU GAA AGU G, (antisense strand)CAC UUU CAC UUC UCC CUU UCA GUU U. The biotin is conjugated at U residue at the 3′ end of antisence strand (underlined). Biotinylated double-stranded (ds)RNA were incubated for 1 hr at 25°C with 10 μg of protein from the cytoplasmic fraction of cells that were transfected with Flag-tagged RIG-I and HA-tagged DDX3 expressing vectors. The mixture was transferred into 400 μl of lysis buffer containing 25 μl of streptavidine Sepharose beads, rocked at 4°C for 2 h, collected by centrifugation, washed three times, resuspended in SDS sample buffer.

Proteome analysis of RNA-binding proteins

RNA-binding proteins were identified by affinity chromatography and Mass spectrometry. Briefly, cell lysate was prepared from human HEK293 or Raji cells as will be described elsewhere (Watanabe and Matsumoto, manuscript submitted for publication). The lysate was first applied to polyU-Sepharose and then the pass-through fraction was applied to PolyI:C-Sepharose. The eluted proteins were analyzed on Mass spectrometry using the MASCOT software.

Confocal analysis

HCV replicon-positive (O) or –negative (Oc) cells were plated onto cover glass in a 24-well plate. In the following day, cells were transfected with indicated plasmids using Fugene HD (Roch). The

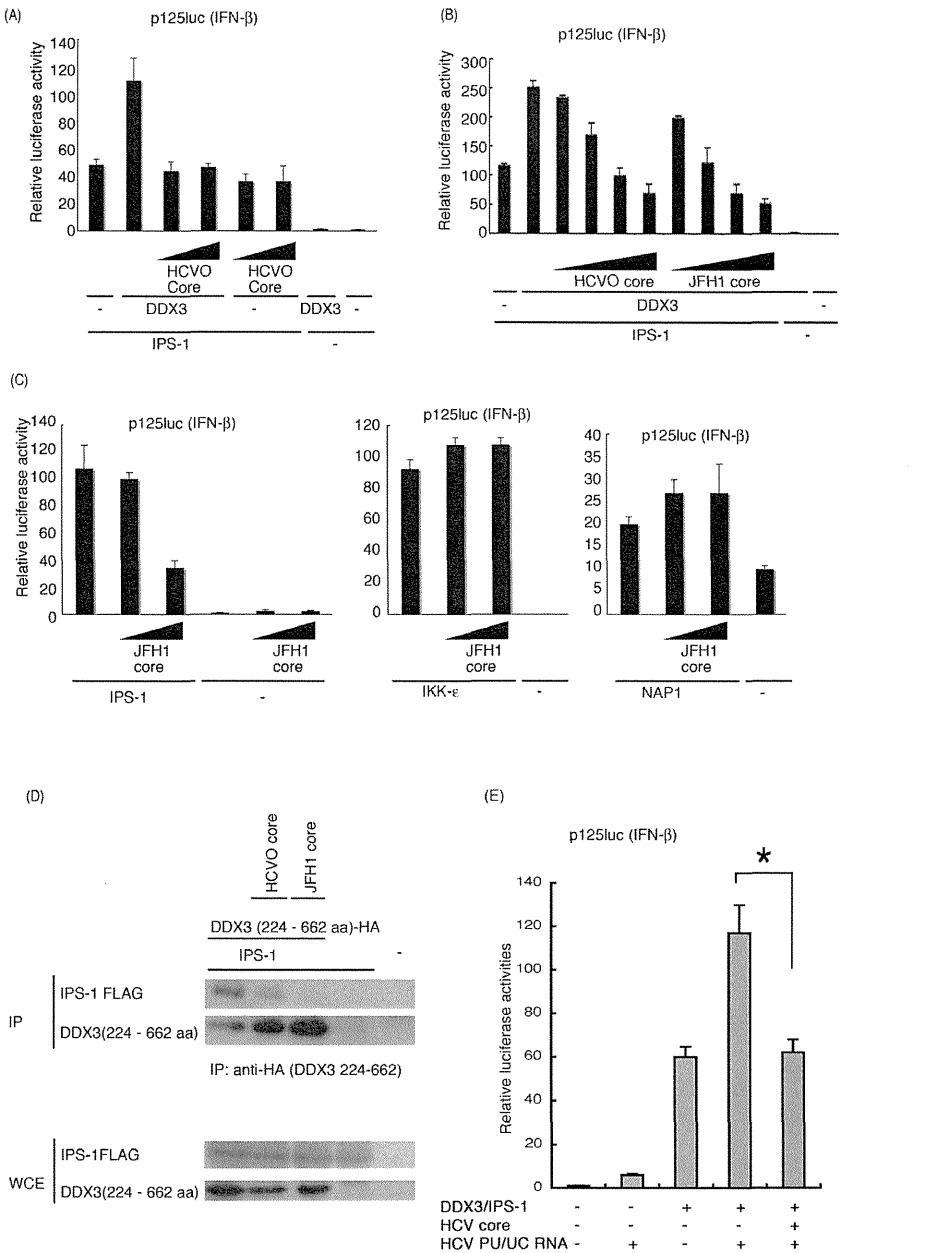

Figure 4. HCV core protein inhibits DDX3 promotion of IPS-1-mediated IFN-beta induction. (A) Expression plasmids for IPS-1 (100 ng), DDX3 (200 ng) and/or HCVO core (50 or 100 ng) were transfected into HEK293 cells in 24-well plates with reporter plasmids, and reporter activity was examined. (B) Expression plasmids for IPS-1 (100 ng), DDX3 (100 ng), and/or HCVO or JFH1 core (10, 25, 50 or 100 ng) were transfected into HEK293 cells, and reporter gene expression was analyzed. (C) IPS-1-, IKKepsilon- or NAP1-expressing plasmids were transfected into HEK293 cells with HCV JFH1 core-expressing plasmids (25 or 100 ng), for reporter gene analysis. (D) Plasmids for expression of FLAG-tagged IPS-1 (400 ng), HA-tagged DDX3 partial fragment (400 ng) and HCVO or JFH1 core (400 ng) were transfected into HEK293FT cells. 24 hrs later cells were lyzed and the lysate was incubated with anti-HA Ab for immunoprecipitation. The DDX3 (224-662)-bound IPS-1 was blotted onto a sheet and probed with anti-Flag Ab. Whole cell lysate was also stained with anti-tag Abs. (E) IPS-1 (100 ng), DDX3 (100 ng), JFH1 core (50 ng) and/or p125 luc reporter (100 ng) plasmids were transfected with HEK293 cells, with HCV 3′UTR poly-U/UC (PU/UC) RNA (25 ng), synthesized *in vitro*. Cell lysates were prepared after 24 hrs, and luciferase activities measured. One representative of at least three independent experiments is shown except for panel D, which is a representative of two sets of the experiments.
doi:10.1371/journal.pone.0014258.g004

amount of DNA was kept constant by adding empty vector. After 24 hrs, cells were fixed with 3% of paraformaldehyde in PBS for 30 minutes, and then permeabilized with PBS containing 0.2% of Triton X-100 for 15 min. Permeabilized cells were blocked with PBS containing 1% BSA, and were labeled with anti-Flag M2 mAb (Sigma) or anti-HA pAb (Sigma) in 1% BSA/PBS for 1 hr at room temperature [25]. In some cases, endogenous proteins were directly stained with anti-core (C7-50) mAb (Affinity BioReagents, Inc) or anti-DDX3 pAbs (Abcam, Cambridge MA). The cells were then washed with 1% BSA/PBS and treated for 30 min at room temperature with Alexa-conjugated antibodies (Molecular Probes). Thereafter, micro-cover glass was mounted onto slide glass using PBS containing 2.3% DABCO and 50% of glycerol. The stained cells were visualized at ×60 magnification under a FLUOVIEW (Olympus, Tokyo, Japan).

Results

DDX3 binds RNA species

We have performed proteome analyses of RNA-binding fractions in human dendritic cell lysate eluted from polyU and polyI:C Sepharose. 127 cytoplasmic proteins were reproducibly identified as polyI:C-binding proteins (Watanabe and Matsumoto, unpublished data). Four of them are DEAD/H box helicases. In this setting, we found DDX3 is a RNA-binding protein (Fig. 1A). DDX3 in cell lysate bound both polyU and polyI:C, while the control PKR bound only to polyI:C.

Using biotinylayed dsRNA, RNA-binding properties of DDX3 and RIG-I were tested by pull-down assay. DDX3 or RIG-I protein was co-precipitated with dsRNA in HEK293 cells expressing either alone of DDX3 or RIG-I (Fig. 1B). Strikingly, higher amounts of DDX3 and RIG-I were precipitated with dsRNA in cells expressing both proteins (Fig. 1B). This, taken together with previous results [11,14,16], indicates that DDX3 assembles in some RNA, RIG-I, IPS-1 and HCV core protein in its C-terminal domain (Fig. S1).

PolyU/UC but not replicon enhances IFN-β induction via IPS-1/DDX3

A polyU/UC sequence is present in the 3′-region of the HCV genome, and serves as a ligand for RIG-I in IPS-1 pathway activation [23]. We produced the polyU/UC RNA and tested its IFN-beta-inducing activity in the presence or absence of DDX3 and IPS-1 (Fig. 2A). HCV polyU/UC promoted IPS-1-mediated IFN-beta induction, and this was further enhanced by forced expression of DDX3/IPS-1 (Fig. 2A). Similar results were obtained with wild-type mouse embryonic fibroblasts (MEF) (Fig. 2B). We also investigated whether DDX3 enhanced IPS-1-mediated IFN-β promoter activation in a RIG-I −/− MEF background (Fig. 2B). In IPS-1/DDX3-expressing MEF cells, polyU/UC IFN-induction was almost totally abrogated by the lack of RIG-I, suggesting that the trace RIG-I protein in the IPS-1 complex is required for DDX3 enhancement of the polyU/UC-mediated IFN response.

DDX3 mRNA (Fig. 2C) and protein [11] were depleted in HEK293 cells by gene silencing with si-1 siRNA, so this was used for DDX3 loss-of-function analysis. Control or DDX3-silinced cells were transfected with increasing amounts of polyU/UC and IFN-beta promoter activation was determined by luciferase assay. DDX3 loss-of-function resulted in a decrease of promoter activation by intrinsic polyU/UC (Fig. 2D). The result was confirmed with cells over-expressing RIG-I and exogenous polyI:C stimulation. HEK293 cells were transfected with a plasmid for the expression of RIG-I and stimulated with polyI:C, an activator of the IPS-1 pathway (Fig. S2A). IFN-beta reporter activation was suppressed in si-1-treated cells that expressed RIG-I, since polyI:C lots often contain short size duplexes that can activate RIG-I [26]. In addition, DDX3 augmented the IFN-beta response in cells expressing MDA5/IPS-1 (Fig. S2B). Thus, DDX3 was also crucial for IPS-1-mediated IFN-beta promoter activation.

We next determined whether the HCV replicon triggers IPS-1/DDX3 IFN promoter activation, using human hepatocyte lines with the HCV replicon (O cells) or without it (Oc cells). In O cells with the HCV replicon, IPS-1/DDX3 expression showed minimal enhancement of IFN-beta promoter activation (Fig. 3A), while in control Oc cells with no replicon, DDX3 facilitated IFN-beta promoter activation (Fig. 3B). Similarily, an augmented IFN promoter response to polyU/UC was observed in control Oc cells, but not in O cells (Figs. 3C and 3D). HCV RNA was prepared from O cells, and its ability to activate the IFN-beta reporter was tested in HEK293 cells (Fig. 3E). The HCV RNA of O cells had a high potency to induce reporter activation, and this activity was largely abrogated by si-1 siRNA treatment. Therefore, DDX3 augments IPS-1-mediated IFN-beta promoter activation in hepatocyte O cells, and HCV RNA, presumably the 3′UTR, participates in this induction. However, no IFN-beta reporter activation was detected in O cells which harbor HCV replicon. Therefore, an unidentified viral factor appeared to participate in suppressing virus RNA-mediated IFN-beta induction, which occurred in O cells overexpressing DDX3/IPS-1.

HCV core protein inhibits IPS-1 signaling through DDX3

What HCV proteins participate in IFN-beta induction was tested in a pilot study using protein expression analysis. We found that expression of HCV core protein as well as NS3/4A led to suppression of IFN-beta reporter activity in Oc cells (data not shown). The HCV core protein physically binds DDX3 [14,16], and co-localizes with DDX3 in the cytoplasm of HeLa cells transfected with HCV core protein [14]. Furthermore, we showed that DDX3 binds IPS-1, which resides on the mitochondrial outer membrane, and assembles into RNA-sensing receptors. Since some populations of the HCV core protein localize on the mitochondrial outer membrane [27], we tested if HCV core

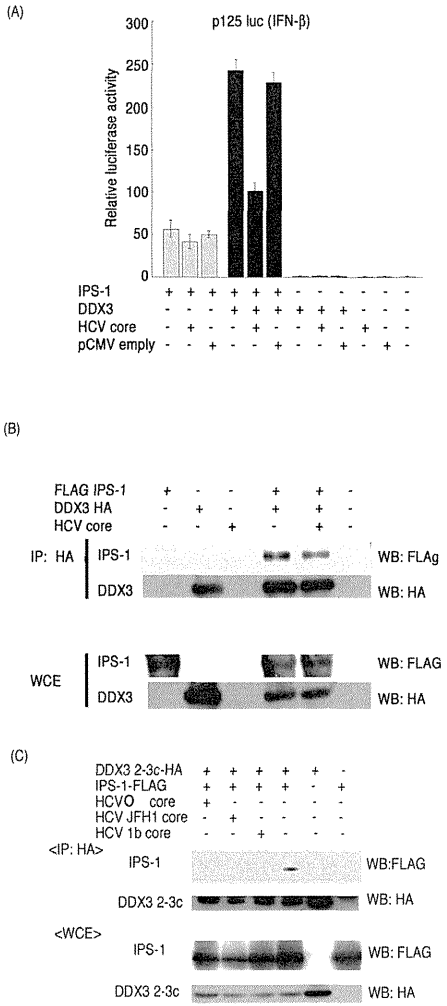

Figure 5. Properties of a 1b-type core protein in the IPS-1 pathway. (A) A core protein derived from an HCV patient suppressed DDX3-mediated activation of IPS-1 signaling. The 1b-type core protein was cloned into the pCMV vector from a patient with hepatitis C. IPS-1 (100 ng), DDX3 (100 ng) and HCV core (100 ng) expression vectors were transfected into HEK293 cells with a reporter plasmid (p125luc), for analysis as in Figure 4. (B) The core protein reduced interaction between full-length DDX3 and IPS-1. The plasmids encoding core protein (400 ng), DDX3-HA (400 ng) and FLAG-IPS-1 (400 ng) were transfected into HEK293FT cells. After 24 hrs, cell lysates were prepared and immunoprecipitation was carried out using anti-HA (DDX3-HA). (C) The core protein blocked interaction between the C-terminal fragment of DDX3 and IPS-1. The C-terminal region of DDX3 (199–662 aa) called DDX3 2-3c, IPS-1, HCV (O) and JFH1 or 1b core expression plasmids were transfected into HEK293FT cells. After 24 hrs, cell lysates were prepared and immunoprecipitation was carried out with anti-HA (DDX3 2–3c). Immunoprecipitates were analyzed by SDS-PAGE and Western blotting with anti-HA or FLAG antibodies. The results are representative of two independent experiments.
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protein affects IPS-1 signaling by binding to DDX3. The cDNAs for HCV core proteins, genotype 1b (HCVO) and 2a (JFH1) [16], were co-transfected into HEK293 together with IPS-1, DDX3, and reporter plasmids, and core protein interference with IPS-1/DDX3-mediated IFN-beta promoter activation was examined. We found that the core proteins of HCVO and JFH1 suppressed IPS-1/DDX3-augmented IFN-beta-induction in a dose-dependent manner (Fig. 4A and 4B). Without DDX3 transfection, core protein had no effect on IPS-1-mediated IFN-beta promoter activation (Fig. 4A). JFH1 core slightly more efficiently inhibited IPS-1/DDX3-augmented IFN-beta-induction than HCVO core (Fig. 4B).

Although some endogenous DDX3 was present in the cytoplasm without DDX3 transfection, only IPS-1 transfection permitted minimal induction of IFN-beta. It is notable that high doses of the HCV JFH1 core protein was needed to inhibit the IPS-1-mediated IFN-beta-induction signal (Fig. 4C, left panel). Since the imaging profile of DDX3 is not always monotonous in human cells, its distribution may be biased in the cytoplasm, which may reason that only a high dose of HCV core involves preoccupied DDX3 protein to inhibit the IPS-1 pathway. This is consistent with earlier reports on an NS3-independent mechanism to block IFN induction using HCV-infected Huh 7 cells [28].

IPS-1 transduces a RNA replication signal to result in IFN-beta output using downstream proteins, such as NAP1 and IKKeysilon. If the HCV core protein interferes with IPS-1 function through DDX3, the core should not inhibit over-expressed downstream molecules. As predicted, HCV core protein did not suppress the IKKepsilon- or NAP1-mediated IFN-beta-inducing signal (Fig. 4C, center and right panels). Hence, the core protein blocks the action of endogenous DDX3 and overexpressed IPS-1 to facilitate minimal IFN-beta promoter activation, and this IFN-beta blocking function of core does not target IKKepsilon or NAP1 (Fig. 4C). An upstream molecule of IKKepsilon and NAP1 is predicted to be the target of the HCV core protein, which is in line with the fact that the HCV core protein interacts with DDX3 [14,16].

To further confirm this model, we examined whether the HCV core protein inhibits the physical interaction between IPS-1 and DDX3. Full length IPS-1 and the C-terminal fragment of DDX3, which binds to the IPS-1 CARD-like region, were transfected into HEK293 cells, with or without the HCV core protein, and the DDX3 fragment was immunoprecipitated. Expression of HCV core proteins strongly inhibited interaction between the DDX3 C-terminal fragment and IPS-1 (Fig. 4D). JFH1 core appeared to show greater inhibition to DDX3-IPS-1 interaction than HCVO. We then examined this IFN-beta blocking function of JFH1 core in a similar cell condition plus polyU/UC. DDX3/IPS-1-enhanced p125luc reporter activity in cells stimulated with polyU/UC (Fig. 4E) was decreased in cells expressing HCV core. The results suggest that the role of the core in HCV-infected cells is to remove DDX3 from IPS-1, and facilitate its interaction with HCV replication complex (Fig. S1).

PolyU/UC HCV RNA activates the IFN-beta promoter (Fig. 2A), and this activity was inhibited by expression of the HCV core protein (Fig. 4E). PolyI:C/RIG-I-mediated IFN-β promoter activation was similarly suppressed by the core protein

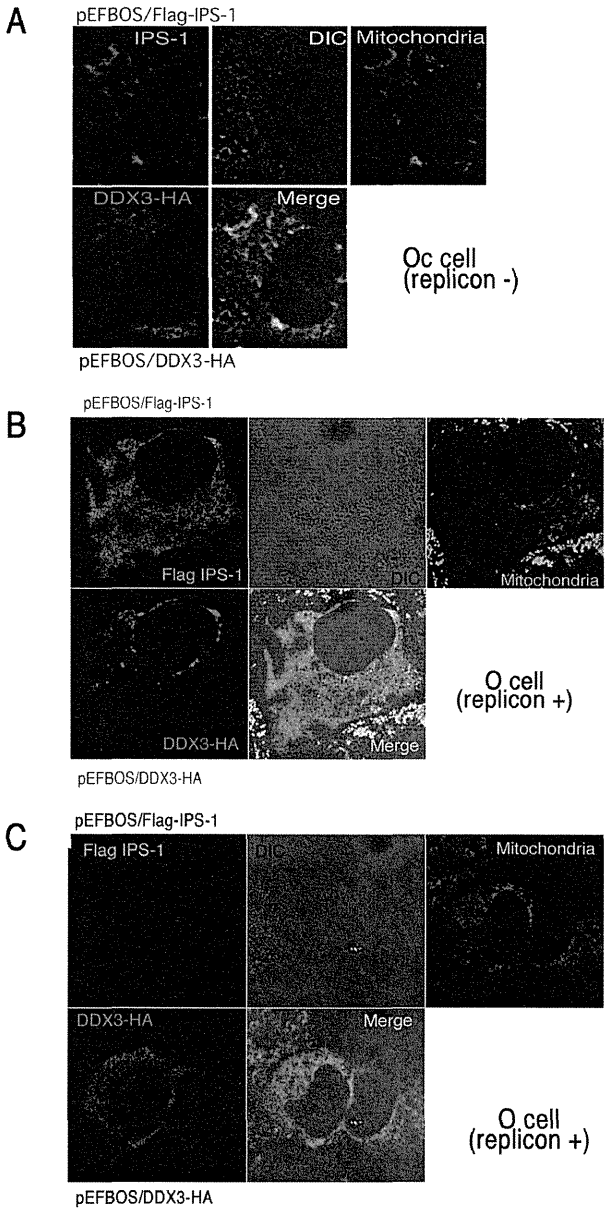

Figure 6. Distribution of DDX3 and IPS-1. (A) DDX3 colocalizes with IPS-1 on the mitochondria in Oc cells. HA-tagged DDX3 and FLAG-tagged IPS-1 were co-transfected into Oc cells. After 24 hrs, cells were fixed with formaldehyde and stained with anti-HA polyclonal and FLAG monoclonal Abs. Alexa488 (DDX3-HA) or Alexa633 antibody was used for second antibody. Mitochondria were stained with Mitotracker Red. Similar IPS-1-DDX3 merging profiles were observed in Huh7.5.1 cells (Fig. S3). (B,C) O cells with the HCV replicon poorly formed the DDX3-IPS-1 complex. Plasmids carrying IPS-1 (100 ng) or DDX3 (150 or 300 ng) were transfected into O (HCV replicon +) as in Oc cells (no replicon, panel A). After 24 hrs, localization of IPS-1 and DDX3 was examined by confocal microscopy. Two representatives which differ from the conventional profile (as in panel A) are shown. Similar sets of experiments were performed four times to confirm the results.
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(Fig. S2A). MDA5-dependent IFN-beta promoter activation was also suppressed by the core expression (Fig. S2B). The inhibitory effect of the core protein on DDX3-IPS-1 interaction was further confirmed using an 1b core isoform isolated from a patient. This HCV core protein also reduced interaction as well as IPS-1-mediated IFN-beta promoter activation (Fig. 5A). The blocking effect was relatively weak in cells expressing IPS-1 and full-length DDX3 (Fig. 5B). We presume that this is because there are multiple binding sites for IPS-1 in the DDX3 whole molecule [11]. For binding assay, we used DDX3 2-3c (across a.a. 199~662, longer than 224~662) instead of the whole DDX3. In fact, DDX3(199-662)-IPS-1 interaction was blocked by the additional expression of core protein (HCVO, JFH1 or 1b core) in Fig. 5C. Ultimately, HCV core protein suppresses IPS-1 signaling by blocking the interaction between the C-terminal region of DDX3 and the CARD-like region of IPS-1, and this inhibition apparently causes the disruption of the active RIG-I/DDX3/IPS-1 complex that efficiently induces IFN-beta production signaling.

Localization of DDX3 and HCV core protein in O cells

We attempted to confirm this finding by tag-expressed proteins and imaging analysis. In Huh7.5 cells IPS-1 colocalized with DDX3 around the mitochondria (Fig. S3), and so did in the hepatocyte lines Oc cells with no HCV replicon (Fig. 6A). In Oc and Huh7.5.1 cells with no HCV replicon, abnormal distribution of IPS-1 was barely observed (Fig. 6A, Fig. S3). In O cells expressing DDX3 and IPS-1, by contrast, two distinct profiles of IPS-1 were observed in addition to the Fig. 6A pattern of IPS-1: diminution or spreading of the IPS-1 protein over mitochondria (Fig. 6B,C). IPS-1 may be degraded by NS3/4A in some replicon-expressing O cells as reported previously [5,28]. We counted number of cells having the pattern represented by Fig. 6 panel B and those similar to Fig. 6 panel C, and in most cases the latter patterns were predominant.

What happens in the O cells with replicon when the core protein is expressed was next tested. Using O and Oc cells, we tested the localization of the core protein and DDX3 in comparison with IFN-inducing properties (Fig. 3). In O cells with full-length HCV replicon, DDX3 was localized proximal to the lipid droplets (LD) (Fig. 7A top panel) around which HCV particles assembled [29]. HCV core protein and DDX3 were partly colocalized in the HCV replicon-expressing cells (Fig. 7A center panel). The results were confirmed with HCV replicon-expressing O cells where endogenous core and DDX3 were stained (Fig. 7B upper panel). Partial merging between core and DDX3 was reproduced in this case, too. In contrast, sO cells, which possess a subgenomic replicon lacking the cording region of the core protein, showed no merging profile of DDX3 and LD (Fig. 7A bottom panel). Likewise, Oc cells barely formed assembly consisting of LD (where the core assembles) and overexpressed DDX3 (Fig. 7A bottom panel) or endogenous DDX3 (Fig. 7B lower panel). O cells expressing DDX3 tended to form large spots compared to Oc cells (with no replicon) and sO cells (core-less replicon) with DDX3.

Overexpressed DDX3 allowed the Oc cells to induce IPS-1-mediated IFN-beta promoter activation (Fig. 3B), while this failed to happen in O cells having HCV replicon (Fig. 3A). Ultimately, overexpressed IPS-1 did not facilitate efficient merging with DDX3 in O cells with replicon (Fig. 6B,C) compared to Oc cells or Huh7.5 cells with no replicon (Fig. 6A, Fig. S3). The results on the functional and immnoprecipitation analyses, together with the imaging profiles, infer that the IPS-1-enhancing function of DDX3 should be blocked by both NS3/4A-mediated IPS-1 degradation and the HCV core which translocates DDX3 from the IPS-1 complex to the proximity of LD in HCV replicon-expressing cells.

Discussion

We investigated the effect of the HCV core protein on the cytosolic DDX3 that forms a complex with IPS-1 to enhance the RIG-I-mediated RNA-sensing pathway. We demonstrated that the core protein removes DDX3 from the IFN-β-inducing complex, leading to suppression of IFN-β induction. DDX3 is functionally complex, since its protective role against viruses may be modulated by the synthesis of viral proteins. DDX3 acts on multiple steps in the IFN-inducing pathway [30]. In addition, DDX3 interacts with the HCV core protein in HCV-infected cells and promotes viral replication [16]. This alternative function is accelerated by the HCV core protein, resulting in augmented HCV propagation [14,16]. More recently, Patal et al., reported that interaction of DDX3 with core protein is not critical for the support of viral replication by DDX3, although DDX3 and core protein colocalize with lipid droplet [15]. If this is the case, what function is revealed by the interaction between DDX3 and HCV core protein remain unsettled. At least, HCV replication is not blocked by this molecular interaction [15].

It remains unclear in Fig. 4C why higher doses of JFH1 core protein are required to inhibit enhancement of IPS-1 signaling by endogenous DDX3 than by exogenously overexpressed DDX3. One possibility is that endogenous DDX3 is preoccupied in a molecular complex other than the IPS-1 pathway since DDX3 is involved in almost every step of RNA metabolism and its localization affects its functional profile [18,30].

Together with these findings, the results presented here suggest that the HCV core inactivates IPS-1 in a mode different from NS3/4A [5,31]. The core protein may switch DDX3 from an antiviral mode to an HCV propagation mode. The core protein localizes to the N-terminus of the HCV translation product, and is generated in infected cells before NS3/4A proteolytically liberates non-structural proteins and inactivates IPS-1. Our results on how the HCV core protein interferes with the interaction between DDX3 and IPS-1 add several possibilities to notions about the HCV function on the IFN-beta-inducing pathway [18].

DDX3 appears to be a prime target for viral manipulation, since at least three different viruses, including HCV [14], Hepatitis B virus [32], and poxviruses [8], encode proteins that interact with DDX3 and modulate its function. These viruses seem to co-opt DDX3, and also require it for replication. The viruses are all oncogenic, and may confer oncogenic properties to DDX3.

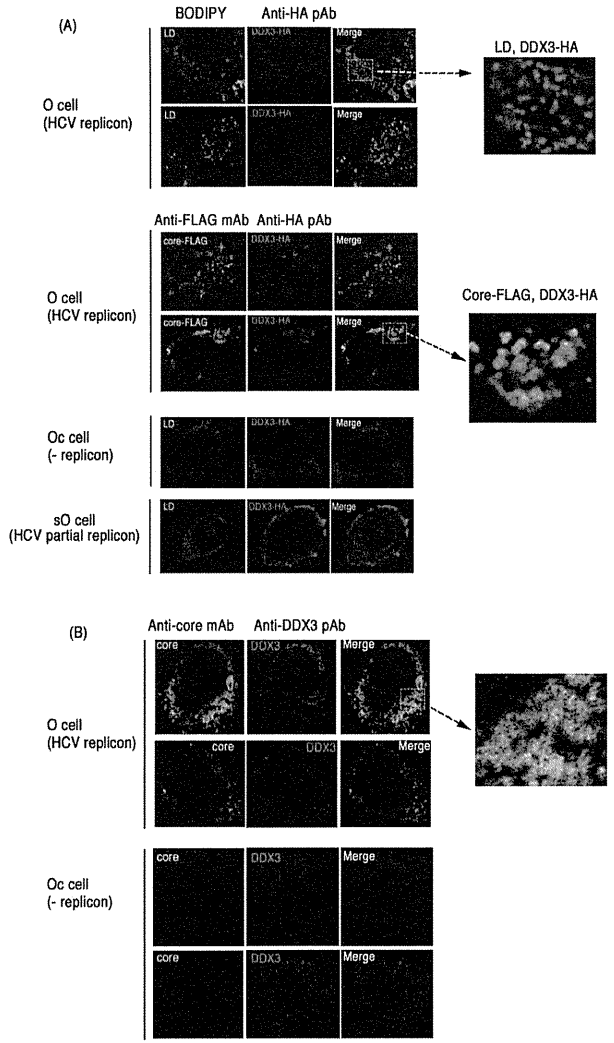

Figure 7. Partial association of endogenous and overexpressed DDX3 with HCV core protein in hepatocyte lines. (A) O cells with the HCV replicon form DDX3-containing speckles in the cytoplasm. O cells contain full-length HCV replicon, and Oc cells do not [16]. O cells were transfected with a plasmid expressing HA-tagged DDX3 (top panel). In other experiments, O cells were transfected with plasmids expressing HA-tagged DDX3 and FLAG-tagged HCV core protein (center panel). After 24 hrs, cells were stained with anti-HA or FLAG antibodies. Proteins were visualized with Alexa488 or 564 second antibodies and the LD was stained with BODIPY493/503. In the bottom panel, Oc cells (no replicon) and sO cells with the core-less subgenomic replicon [16] were transfected with a plasmid expressing HA-tagged DDX3. After 24 hrs, cells were stained with anti-HA antibodies. LD was stained with BODIPY493/503. (B) Endogenous DDX3-HCV core association in O cells. O or Oc cells were cultured to amplify the HCV replicon. Cells were stained with anti-core mAb and anti-DDX3pAb and secondary antibodies. Similar sets of experiments were performed three times to confirm the results.
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DDX3 is also involved in human immunodeficiency virus RNA translocation [33]. The DDX3 gene is conserved among eukaryotes, and includes the budding yeast homolog, Ded1 [34]. The Ded1 helicase is essential for initiation of host mRNA translation, and human DDX3 complements the lethality of Ded1 null yeast [14,35]. Another function of DDX3 is to bind viral RNA to modulate RNA replication and translocation. Constitutive expression of the HCV core or other DDX3-binding proteins may impede IFN induction and promote cell cycle progression. These reports are consistent with the implication of DDX3 in various steps of RNA metabolism in cells that contain both host and viral RNAs.

A continuing question is the physiological role of the molecular complex of DDX3 and IPS-1 during replication of HCV in hepatocytes. HCV proteins generated in host hepatocytes usually induce an HCV-permissive state in patients, for example in the IFN-inducing pathways. NS3/4A protease induces rapid degradation of IPS-1 [5,31] and TICAM-1/TRIF [36]. NS5A interferes with the MyD88 function [37]. Viral replication ultimately blocks the STAT1-mediated IFN-amplification pathway [38]. PKR may be an additional factor by which HCV controls type I IFN production [39]. Our results add to our knowledge of the mechanism of how HCV circumvents IFN induction in host cells: HCV core protein suppresses the initial step of IFN-beta induction by interfering with DDX3-IPS-1 association. Indeed, the core protein functions as the earliest IFN suppressor, since it is generated first in HCV-infected cells, and rapidly couples with DDX3 to retract it from the IPS-1 complex, resulting in localization of DDX3 near the LD (Fig. 7). It is HCV that hijacks this protein for establishing infection. Although gene disruption of DDX3 makes mice lethal, this issue will be further tested using IPS-1 −/− hepatocytes expressing human CD81 and occludin [40], in which HCV replication would proceed.

DDX3 primarily is an accelerating factor for antiviral response through IPS-1-binding. Many host proteins other than DDX3 may positively regulate HCV replication in hepatocytes in association with the IPS-1 pathway. In this context, we know LGP2 [41] and STING [42] act as positive regulators in virus infection. Peroxisomes serve as signaling platforms for recruiting IPS-1 with a different signalosome than mitochondria [43]. It appears rational that HCV harbors strategies to circumvent these positive regulators in the relevant steps of the IFN-inducing pathway.

Imaging studies suggest that the complex of IPS-1 involving the membrane of mitochondrial/peroxisomes differ from that free from the membrane. Although IPS-1 is liberated from the membrane by NS3/4A having largely intact cytosolic domain, it loses the IFN-inducing function [5,31]. Our results could offer the possibility that the clipped-out form of IPS-1 immediately fails to form the conventional complex for IRF-3 activation any more [44] or is easily degraded further to be inactive (Fig. 6C). Indeed, there are a number of mitochondria-specific molecules which assemble with IPS-1 [45]. Formation of the molecular complex on the mitochondria rather than simple association between IPS-1 and DDX3 may be critical for the DDX3 function.

Evidence is accumulating that HCV checks many steps in the IFN-inducing pathway throughout the early and late infection stages, and suppresses IFN production by multiple means. Disruption of IPS-1 function by both NS3/4A and core protein may be crucial in HCV-infected Huh7.5 cells, even though the cells harbor dysfunctional RIG-I [46]. Type I IFN suppresses tumors by causing expression of p53 and other tumor-suppressing agents [47]. These unique features of the HCV core protein require further confirmation, and should be minded in investigation of HCV persistency, chronic infection and progression to cirrhosis and carcinoma.

Supporting Information

Figure S1 The IPS-1 complex. IPS-1 and HCV core bind C-terminal regions of DDX3. DDX3 captures dsRNA at the C-terminal domain. This figure is constructed from [11], [14] and [16].
Found at: doi:10.1371/journal.pone.0014258.s001 (0.41 MB TIF)

Figure S2 DDX3 enhances RIG-I-mediated IFN-β promoter activation induced by polyI:C. (A) DDX3 si-1 or control siRNA was transfected into HEK293 cells with reporter plasmids and RIG-I-expression plasmid or control plasmid (100 ng). After 48 hrs, cells were stimulated with polyI:C (20 μg/ml) with dextran for 4 hrs, and activation of the reporter p125luc was measured. (B) MDA5 (25 ng), IPS-1 (100 ng), DDX3 (100 ng), JFH1 core (50 ng) and/or p125 luc reporter (100 ng) plasmids were transfected with HEK293 cells. Cell lysates were prepared after 24 hrs, and luciferase activities measured. The results are representative of two independent experiments, each performed in triplicate.
Found at: doi:10.1371/journal.pone.0014258.s002 (0.17 MB TIF)

Figure S3 DDX3 colocalizes with IPS-1 on the mitochondria in Huh7.5.1 cells. HA-tagged DDX3 and FLAG-tagged IPS-1 were co-transfected into Huh7.5.1 cells. After 24 hrs, cells were fixed with formaldehyde and stained with anti-HA polyclonal and FLAG monoclonal Abs. Alexa488 (DDX3-HA) or Alexa633 antibody was used for second antibody. Mitochondria were stained with Mitotracker Red. A representative result from three independent experiments is shown.
Found at: doi:10.1371/journal.pone.0014258.s003 (0.92 MB TIF)

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Author Contributions

Conceived and designed the experiments: HO MM TS. Performed the experiments: HO MM. Analyzed the data: HO MM KS TS. Contributed reagents/materials/analysis tools: MI AW OT SA NK KS. Wrote the paper: HO TS.

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Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes

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Abbreviations; DHCR24, 24-dehydrocholesterol reductase; HCV, hepatitis C virus; MoAb, monoclonal antibody; HCC, Hepatocellular carcinoma; HBV, Hepatitis B virus

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Key words: hepatitis C virus; replication; DHCR24; U18666A

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Abstract

Background & Aims: The role of 24-dehydrocholesterol reductase (DHCR24) in hepatitis C virus infection (HCV) was characterized, because DHCR24 is a cholesterol biosynthetic enzyme and cholesterol is a major component of lipid rafts, which is reported to play an important role in HCV replication. Therefore, we examined the potential of DHCR24 as a target for novel HCV therapeutic agents.

Methods: We examined DHCR24 expression in human hepatocytes in both the livers of HCV-infected patients and those of chimeric mice with human hepatocytes. We targeted DHCR24 with siRNA and U18666A which is an inhibitor of both DHCR24 and cholesterol synthesis. We measured the level of HCV replication in these HCV replicon cell lines and HCV infected cells. U18666A was administrated into chimeric mice with humanized liver, and anti-viral effects were assessed.

Results: Expression of DHCR24 was induced by HCV infection in human hepatocytes *in vitro*, and in human hepatocytes of chimeric mouse liver. Silencing of DHCR24 by siRNA decreased HCV replication in replicon cell lines and HCV JFH-1 strain-infected cells. Treatment with U18666A, suppressed HCV replication in the replicon cell lines. Moreover, to evaluate the anti-viral effect of U18666A *in vivo*, we administrated U18666A with or without pegylated interferon to chimeric mice and observed an inhibitory effect of U18666A on HCV infection and a synergistic effect with interferon.

Conclusions: DHCR24 is an essential host factor which is augmented its expression by HCV infection, and plays a significant role in HCV replication. DHCR24 may serve as a novel anti-HCV drug target.

Introduction

Extensive epidemiological studies have identified multiple risk factors for hepatocellular carcinoma HCC, including chronic infection with hepatitis C virus (HCV) and hepatitis B virus (HBV), and cirrhosis due to non-viral etiologies, such as alcohol abuse and aflatoxin B1 exposure [1,2]. Of these factors, HCV appears to be the dominant causative factor for HCC in many developed countries. The World Health Organization estimates that 170 million people worldwide are infected with HCV and are therefore at risk of developing liver cirrhosis and HCC [3]. The combination of pegylated interferon-α (PEG-IFN-α) and ribavirin is currently the standard treatment regimen for patients with chronic HCV infection. However, viral clearance is achieved in only 40 to 60% of patients and depends on the HCV genotype with which the patient is infected [4].

We previously established the RzM6 cell line, a HepG2 cell line in which the full-length HCV genome (*HCR6-Rz*) can be conditionally expressed under control of the Cre/*lox*P system and is precisely self-trimmed at the 5' and 3' termini by ribozyme sequences [5]. Anchorage-independent growth of these cells accelerates after 44 days of continuous passaging, during which the cdk-Rb-E2F pathway is activated [5]. In a previous study, we developed monoclonal antibodies (MoAbs) against cell surface antigens on HCV-expressing cells that had been passaged for over 44 days [6]. One of the targets of these MoAbs was *24-dehydrocholesterol reductase* (DHCR24 is also called 3-β-hydroxysterol-Δ-24-reductase, seladin-1, desmosterol delta-24-reductase), a molecule that is frequently overexpressed in the hepatocytes of HCV-infected patients.

DHCR24 confers resistance to apoptosis in neuronal cells [7]. It also regulates the cellular response to oxidative stress by binding to the amino terminus of p53, thereby displacing mouse double minute 2 homolog isoform MDM2 (Homo Sapiens) (MDM2) from p53 and inducing the accumulation of p53 in human embryonic fibroblasts [8].

DHCR24 is a cholesterol biosynthetic enzyme that is also called desmosterol reductase [9,10]. Cholesterol is a major component of lipid rafts, which are reported to play an important role in HCV replication [11]. Therefore, we characterized the role of DHCR24 in HCV replication and evaluated its potential as a target for novel HCV therapeutic agents. We also examined the synergistic antiviral effect of U18666A which is an inhibitor of both DHCR24 [12] and cholesterol synthesis [13] with IFN-α in the treatment of HCV.

Materials and methods

Cells and Plasmids

Cell culture methods of the HuH-7 [14], HepG2 [15], Hybridoma and myeloma PAI cells, RzM6 cells [5], and the HCV subgenomic replicon cells lines FLR3-1 [genotype 1b, strain Con-1; [16]], R6FLR-N [genotype 1b, strain N; [17]], and Rep JFH Luc3-13 [genotype 2a, strain JFH-1[18]] were utilized to evaluate HCV replication [19] were described in *Supplementary data.*

The DHCR24 cDNA was synthesized and amplified by PCR using Phusion *Taq* polymerase (Finnzymes) and cloned into the pcDNA3.1 vector (Invitrogen) or lentivirus vector, as described previously [6].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis

The detailed procedures are described in the *Supplementary data* and [20].

Immunohistochemistry and Western blot analysis.

The detailed procedures are described in the *Online Supplementary data.*

The antibodies used in this experiment were: anti-Core, anti-NS3, anti-NS4B, anti-NS5B [5], and anti-NS5A (kindly provided by Dr. Matsuura, Osaka University), and anti-actin (Sigma).

Inhibition of DHCR24 by siRNA

We synthesized two siRNAs that was directed against human DHCR24 mRNA: siDHCR24-417 and siDHCR24-1024. The target sequence of siDHCR24-417 was

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5'-GUACAAGAAGACACACAAATT-3', while that of siDHCR24-1024 was 5'-GAGAACUAUCUGAAGACAATT-3'. Additionally, we used siRNAs targeted against the HCV genome (siE-R7 and siE-R5) [17,21]. The siCONTROL Non-Targeting siRNA #3 (Dharmacon RNA Technologies) was used as the negative control siRNA. The chemically synthesized siRNAs were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (Invitrogen) by reverse-transfection. Cells were characterized 72 hours after transfection.

Inhibition of viral replication by U18666A

U18666A (Calbiochem) was utilized to treat HCV replicon cells at a concentration of 62.5 to 1,000 nM and chimeric mice at a concentration of 10 mg/kg (i.p.).

To determine whether cholesterol can reverse the U18666A treatment by the addition of cholesterol, we performed the experiments using HCV replicon cells (4×10^3/well in a 96-well white plate, SUMILON). Culture medium was replaced after the cells had spread (at 24 hours), and LDL (Calbiochem) was added to reach a final cholesterol concentration of 50 μg/ml. After a 24 hours-incubation, U18666A (62.5, 125, 250, 500, and 1,000 nM) was added to each well, and the cells were incubated for an additional 48 hours. HCV replication activity was measured by the luciferase assay, and cell viability was measured by the WST-8 cell counting kit according to the manufacturer's instructions (Dojindo Laboratories). Cholesterol measurements are described in the *Online Supplementary data.*

Inhibition assay of HCV replication in replicon cells and persistent infected cells

For evaluation of the anti-HCV replication effect of the inhibitor U18666A in replicon cells and HCV persistently infected cells were described in the *Online Supplementary data.*

Real-time detection (RTD)-PCR

Total RNA was purified from JFH-K4 cells that had been treated with siRNA or U18666A by the acid guanidium-phenol-chloroform method. HCV RNA was quantified by RTD-PCR as previously described [22].

HCV infection of chimeric mice with humanized liver and mRNA quantification by RTD-PCR

We used chimeric mice that were created by transplanting human primary hepatocytes into severe combined immunodeficient mice carrying a urokinase plasminogen activator transgene [23,24] that was controlled by the albumin promoter. These hepatocytes had been infected with plasma from a HCV-positive patient HCR6 (genotype 1b) [19]. The HCV 1b RNA level reached 2.9–18.0 × 10^6 copies/ml in mouse sera after 1–2 months of infection. HCV RNA in the mouse serum or total RNA from liver tissue from humanized chimeric mice with/without HCV infection was extracted using the acid guanidium-phenol-chloroform method. HCV RNA and DHCR24 mRNA levels were quantified by RTD-PCR [22]. The primers and probes for HCV were prepared as previously described [22], and the primers and probes for DHCR24 were prepared using TaqMan^R Gene Expression assays (Applied Biosystems) according to the manufacturer's instructions. PEG-IFN α-2a (Chugai) was administered subcutaneously at a concentration of 30 μg/kg, at day 1, 4, 8, and 11 (the amount administered to the

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chimeric mice was 20-fold amounts of PEG-IFN relative to that used in humans), and U18666A was administered intraperitoneally at a concentration of 10 mg/kg, every day for 2 weeks (Fig. 6A). The protocols for the animal experiments were approved by the local ethics committee.

Human serum albumin in the blood of humanized chimeric mice was measured using a commercially available kit according to the manufacturer's instructions (Alb-II kit; Eiken Chemical).

Results

Identification of DHCR24

We inoculated mice (BALB/c) with RzM6 cells that expressed HCV protein and had been cultured for over 44 days (denoted as RzM6-LC cells); mice were inoculated at least 7 times over a 2-week period. Then we fused the splenocytes from the mice that had been immunized with RzM6-LC cells to myeloma cells to establish hybridomas. Characterization of the culture supernatant from more than 1,000 hybridoma cells by ELISA (data not shown) revealed that one MoAb clone (2-152a) recognized a molecule of approximately 60 kDa in various cells (Fig. S1A and S1B). This molecule was more highly expressed in RzM6-LC cells (Fig. S1A), HeLa cells, and HCC cell lines (HepG2, HuH-7, Hep3B, and PLC/PRF/5) than in HEK293 cells and several normal liver cell lines (NKNT, TTNT, and WRL68) (Fig. S1B). To further characterize this molecule, we performed matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and obtained seven peptide sequences (Fig. S1C, underlined). These peptide sequences suggested that the molecule that was recognized by the 2-152a antibody was DHCR24. We constructed a lentivirus expression vector containing myc-tagged DHCR24 (DHCR24-myc) and transduced it into HepG2 cells. By western blot analysis with 2-152a and anti-Myc antibody, we then confirmed that DHCR24 was expressed in the transfected cells (Fig. S1D). We found that the 2-152a antibody specifically recognized DHCR24.

HCV infection in vivo *induces persistent overexpression of DHCR24*

We next examined whether HCV infection could induce DHCR24 expression in human hepatocytes. DHCR24 was overexpressed more frequently in liver tissues from

HCV-positive patients than in tissues from HBV- and HCV-negative (NBNC) patients (Fig. 1A, Table S1). The liver tissue from HCV-positive patients was stained more strongly for DHCR24 expression than the liver tissue from NBNC patients (Fig. 1B). We inoculated chimeric mice [19,23,25] with HCV ($10^{6.2}$ copies/ml) that had been isolated from the plasma of HCV-infected patients (patient R6, HCV genotype 1b). The serum concentration of human albumin (Fig. S2A) in the chimeric mice after transplantation of hepatocytes indicated that human hepatocytes had engrafted in the mouse livers. At 30 days after transplantation, the mice were infected with HCV, and HCV RNA titers were analyzed both before and after inoculation (Fig. S2B). The average amount of HCV RNA that was present in the serum of the infected chimeric mice at 28 days post-infection was 1.1×10^7 copies/ml (Fig. 1C, Fig. S2B). The DHCR24 mRNA levels in the livers of the chimeric mice were also quantified at 28 days post-infection by real-time detection (RTD)-PCR [22]. The results revealed that there was a significant increase in DHCR24 expression as measured by mRNA levels in HCV infected chimeric mice (Fig. 1D). Next, we examined the extent to which translation of DHCR24 occurred in the chimeric mice (Fig. 1E), and higher DHCR24 protein levels were present in hepatocytes from HCV-infected mice (No.192-8 and 192-9) than in those of uninfected mice (No.164-5 and 172-9). These findings indicate that expression of DHCR24 is significantly up-regulated by HCV infection in human hepatocytes.

Role of DHCR24 in HCV replication

Since augmentation of DHCR24 expression was observed by HCV infection in humanized chimeric mice, we next examined whether DHCR24 was involved in HCV

replication or not. We transfected siRNA into HCV replicon cell lines FLR3-1 (Figs. 2A, B) and R6FLR-N (Figs. 2C, D). Treatment with either two different DHCR24 siRNA molecules (siDHCR24-417 or -1024) decreased HCV replication in a dose-dependent manner (Figs. 2A, C) but did not appear to have a significant effect on cell viability (Figs. 2B, D). Western blot analysis using HCV subgenomic replicon cell lines confirmed these findings (Figs. 2E, F). We also transfected the DHCR24 siRNAs into HCV JFH-1 strain [18]-infected HuH7/K4 cell lines and found that the siRNAs inhibited HCV protein expression by western blot analysis (Figs. 2G, H). These results indicate that DHCR24 may play a role in HCV replication.

The expression level of DHCR24 is linked to intracellular cholesterol levels

Human DHCR24 is involved in cholesterol biosynthesis [10]. It participates in multiple steps of cholesterol synthesis from lanosterol [26] (Fig. 3A). To examine the effect of cholesterol on the DHCR24 expression level in HuH-7 cells, we added cholesterol to cultured cells and determined the DHCR24 expression level (Fig. 3B). Expression level of DHCR24 in HuH-7 cells were decreased approximately 50% by addition of cholesterol compared to that of untreated control (Fig. 3B). On the other hand, that of DHCR24 in HepG2 cells was increased to 2.5 fold by depletion of cholesterol using methyl-β-cyclodextrin (M β CD) (Fig. 3C).

These results indicate that the expression of DHCR24 in a cell correlates with the cholesterol level in that cell. Furthermore, silencing DHCR24 reduced the cholesterol level in cells compared to control cells (Fig. 3D), suggesting that DHCR24 is essential for cholesterol synthesis.

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Effect of U18666A on HCV replication in vitro

We further examined the role that DHCR24 plays in HCV replication by treating cells with U18666A. Treatment of U18666A (62.5, 125, 250, 500, and 1,000 nM) to HCV replicon cells (FLR3-1) decreased HCV replication in a dose-dependent manner by luciferase assay (Fig. 4A) and western blot analysis (Fig. 4B). Notably, DHCR24 protein appeared as doublet bands in the absence of U18666A, but the lower band shifted to the upper band after treatment with U18666A (Fig. 4B). U18666A also suppressed HCV replication in other replicon cell lines (R6FLR-N and Rep JFH Luc 3-13; Figs. 4C, D). Treatment with U18666A (<250 nM) suppressed viral replication without producing significant cytotoxicity. We also examined the effect of 7-dehydrocholesterol reductase (DHCR7) (Fig. 3A) on HCV replication using the specific inhibitor BD1008 [26]. Treatment with BD1008 also suppressed HCV replication, but a much higher concentration was required to suppress replication than was needed in the U18666A assays (Fig. 4E); the concentration also greatly exceeded the intrinsic IC_{50} value for inhibition of σ-receptor binding (47 ± 2 nM) [27]. Therefore, DHCR24 may play a more significant role than DHCR7 in HCV replication. We next evaluated the compensatory effect that the addition of cholesterol had on cells treated with U18666A (Figs. 4F, G) by examining low density lipoprotein (LDL)-replaceable dissolved cholesterol levels as described in the Materials and methods. Treatment with cholesterol led to partial restoration of HCV replication (Fig. 4F). These results suggest that U18666A suppresses HCV replication by depleting cellular cholesterol stores.

Next, we characterized the effect that U18666A had on HCV JFH-1 infection. Adding of U18666A (62.5, 125, 250, and 500 nM) to HCV JFH-1-infected cell lines for 72 h, reductions of NS5B protein level were observed in cells treated more than 500 nM

of U18666A (Figs. 5A and 5B). Additionally, the HCV RNA copy number in infected cells was suppressed by addition of 250 or 500 nM of U18666A (Fig. 5C). Examination of the cytotoxicity that U18666A (62.5-500 nM) had on infected cells revealed that it had little effect on cell viability (Fig. 5D). These results demonstrate that inhibition of DHCR24 by U18666A suppresses viral replication in HCV replicon cells and HCV-infected cells.

Evaluation of the anti-HCV effect of U18666A in vivo

To examine the effect of U18666A on HCV infection *in vivo*, we administered U18666A to HCV-infected chimeric mice with humanized liver. The mice were infected with HCV via inoculation of patient serum HCR6 five weeks after transplantation of human hepatocytes. U18666A (10 mg/kg) and PEG-IFN-α (30 μg /kg) were then administrated to these mice for 2 weeks (Fig. 6A). HCV RNA quantity (Fig. 6B) and serum human albumin levels (Fig. 6C) were measured in the mice after -1, 4 and 14 days of HCV infection. Treatment with U18666A alone significantly decreased HCV RNA levels in the serum (from 1×10^8 to 3×10^5 copies/ml) after 2 weeks, and its suppressive effect was more pronounced than that of PEG-IFN-α alone (8×10^5 copies/ml; Fig. 6B). Moreover, co-administration of U18666A and PEG-IFN-α synergistically (combination index<1) enhanced the anti-viral effect of PEG-IFN-α (5×10^4 copies/ml). Treatment with these drugs did not significantly affect the serum human albumin concentrations in treated mice (Fig. 6C).

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Discussion

The results in this study revealed that DHCR24, an enzyme that participates in cholesterol synthesis (last step; Fig.3A), also plays a significant role in HCV replication. To our knowledge, this is the first report that this molecule is involved in HCV infection. The mevalonate pathway of cholesterol synthesis pathway (starting from acetyl Co-A) has previously been reported to be involved in HCV replication [28]. The present findings are the first evidence that overexpression of one of the enzymes down stream of the mevalonate pathway, i.e., DHCR24, can be induced by HCV infection. In a previous study, 3-hydroxy 3-methyl-glutaryl Co-A (HMG-CoA) reductase was found to be inhibited by lovastatin, subsequently resulting in suppression of HCV replication [28]. The product of the mevalonate pathway that is required for HCV replication is reported to be a geranyl geranyl lipid [29]. Many lipids are crucial to the viral life cycle, and inhibitors of the cholesterol/fatty acid biosynthetic pathway inhibit viral replication, maturation, and secretion [30,31]. We found that inhibition of DHCR24 downregulated HCV replication. DHCR24 catalyzes the reduction of the delta-24 bond of the sterol intermediate and works further downstream of farnesyl pyrophosphate (Fig. 3A) and, therefore, may not influence geranyl-geranylation. Thus, our findings indicate the existence of regulatory pathway of HCV replication by cholesterol synthesis and trafficking through DHCR24 rather than by protein geranyl-geranylation. DHCR24 deficiency reduces the cholesterol level and disorganizes cholesterol-rich detergent-resistant membrane domains (DRMs) in mouse brains [32]. Additionally, the HCV replication complex has been detected in the DRM fraction [11]. Therefore, a deficiency in DRM, induced by silencing DHCR24, may suppress HCV replication.

We demonstrated that addition of cholesterol to HCV-infected hepatocytes treated

with U18666A, led to partial recovery of HCV replication, which suggests that cholesterol may be an important factor in HCV replication. U18666A impairs the intracellular biosynthesis and transport of cholesterol and inhibits the action of membrane-bound enzymes, including DHCR24, during sterol synthesis [33]. Moreover, the DHCR7 inhibitor BD1008 also suppresses HCV replication. Thus, the findings in this study further substantiate the fact that cholesterol plays an important role in HCV replication and infection.

Although monotherapy with statins is reportedly insufficient to induce anti-viral activity in HCV-infected patients [34], a synergistic action between statins and IFN has been observed [35]. The effect of the statin is thought to be mainly mediated by the depletion of geranyl geranyl lipids. It is important to note that higher doses of statins may increase the risk of myopathy, liver dysfunction, and cardiovascular events [36]. Moreover, the EC_{50} values of the statins that are associated with a reduction in HCV replication are reported to be 0.45-2.16 μM, while the IC_{50} of U18666A was estimated to be 125 nM in the present study. Therefore, U18666A may serve as a novel anti-HCV drug that could be utilized with IFN as a combined therapeutic regimen.

In summary, we demonstrated that the expression of DHCR24 is induced by infection with HCV and that DHCR24 is an essential host factor that is required for HCV replication. HCV may increase cholesterol synthesis in cells via the action of a host regulatory factor, such as DHCR24, that is correlated with cholesterol synthesis and is also directly involved in replication. Genome-wide analysis of the host response to HCV infection revealed the upregulation of genes related to lipid metabolism [37]. DHCR24 expression was found to be upregulated in the cDNA microarray analysis of chronic hepatitis C cases [38]. Future studies are needed to examine the detailed

mechanism by which HCV infection augments DHCR24 expression in hepatocytes.

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Figure Legends

Fig 1. HCV induces DHCR24 overexpression *in vitro* and *in vivo*.

(A) Expression of DHCR24 in non-cancerous regions of the livers of HCV-infected (+) and NBNC-HCC patients. Lysates (25 μg/lane) of non-cancerous liver tissues from HCC patients were analyzed by western blot analysis using MoAb 2-152a. The patient numbers (Table S1) are indicated at the top of the blot. (B) Immunohistochemical staining (left) of HCV-infected non-cancerous tissues derived from an HCC patient using the monoclonal antibody 2-152a (Alexa488), anti-TO-PRO-3, or a merge (600X magnification) (upper). Tissues from an NBNC patient stained with the monoclonal antibody 2-152a (Alexa488) as well as TO-PRO-3 (640X magnification). (C) The amount of HCV RNA that was present in the HCV-R6 (genotype 1b)-infected chimeric mice with humanized liver was quantified using RTD-PCR. The results of HCV uninfected (n=4) and infected (n=7) were indicated. (D) The amount of DHCR24 mRNA present in total RNA isolates of in HCV-R6 (genotype 1b)-infected chimeric mice with humanized liver was quantified using RTD-PCR. **P*<0.05 (Mann-Whitney test). The results of HCV uninfected (n=4) and infected (n=7) were indicated. (E) DHCR24 protein was detected by western blot analysis using MoAb 2-152a as a probe, and quantitated by LAS3000. They are normalized with actin and ratio was indicated.

Fig. 2. Effect of knockdown of DHCR24 on HCV replication

(A-D) Effect of knockdown of DHCR24 on HCV replication in HCV replicon cells (FLR3-1 and R6FLR-N) at 72 hours after the anti-DHCR24 siRNAs (417 and 1024), siRNAs against HCV (siE-R7 for FLR3-1 and JFH-1; siE-R5 for R6-FLR-N), or non-target control siRNAs were transfected into HCV replicon cells. Replication