研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
西口修平	慢性肝炎	山口　徹，北原光夫，福井次矢　編	今日の治療指針	医学書院	東京	2007	373-377.
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研究成果の刊行物・別刷り

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The lipid droplet is an important organelle for hepatitis C virus production

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The lipid droplet (LD) is an organelle that is used for the storage of neutral lipids. It dynamically moves through the cytoplasm, interacting with other organelles, including the endoplasmic reticulum (ER)[1–3]. These interactions are thought to facilitate the transport of lipids and proteins to other organelles. The hepatitis C virus (HCV) is a causative agent of chronic liver diseases[4]. HCV capsid protein (Core) associates with the LD[5], envelope proteins E1 and E2 reside in the ER lumen[6], and the viral replicase is assumed to localize on ER-derived membranes. How and where HCV particles are assembled, however, is poorly understood. Here, we show that the LD is involved in the production of infectious virus particles. We demonstrate that Core recruits nonstructural (NS) proteins and replication complexes to LD-associated membranes, and that this recruitment is critical for producing infectious viruses. Furthermore, virus particles were observed in close proximity to LDs, indicating that some steps of virus assembly take place around LDs. This study reveals a novel function of LDs in the assembly of infectious HCV and provides a new perspective on how viruses usurp cellular functions.

Hepatitis C virus (HCV) has a plus-strand RNA genome that encodes the viral structural proteins Core, E1 and E2, the p7, and the nonstructural (NS) proteins 2, 3, 4A, 4B, 5A and 5B (refs 7, 8). NS proteins are reported to localize on the cytoplasmic side of endoplasmic reticulum (ER) membranes[9]. To elucidate the mechanisms of virus production, we used a HCV strain, JFH1, which can produce infectious viruses[10–12]. We first investigated the subcellular localization of the HCV proteins in cells that had been transfected with JFH1^{E2FL} RNA, in which a part of the hypervariable region 1 of E2 was replaced by the FLAG epitope tag (see Supplementary Information, Fig. S1, S2a–d). Core localized to the lipid droplets (LDs; Fig. 1a), as previously reported[5]. Interestingly, NS proteins were also detected around LDs in 60–90% of JFH1^{E2FL}-replicating cells (Fig. 1a, c). Similar levels of colocalization of LDs with viral proteins were observed in cells that had been transfected with chimeric HCV genomes expressing structural proteins, p7 and part of NS2 of the genotype 1b (Con1) or the genotype 1a (H77) isolate (see Supplementary Information, Fig. S1, S2e)[13]. In contrast, there was no close association between the LDs and NS proteins in cells that had been transfected with JFH1^{dC3} RNA (Fig. 1b, c), which lacked the coding region of Core (Supplementary Information, Fig. S1). NS proteins were diffusely present on the ER, suggesting that NS proteins are translocated from the ER to LDs in JFH1^{E2FL}-replicating cells in a Core-dependent manner. Importantly, there was no association between LDs and PDI, an ER marker protein, indicating that either ER membranes were absent in close proximity to LDs or that PDI was excluded from such membranes (Fig. 1c). These results were supported by western blot analysis of the LD fraction (Fig. 1d). The LD fraction contained ADRP, an LD marker, but not the ER markers Calnexin and Grp78 (data not shown), indicating that there was no ER contamination in the LD fraction. However, the LD fraction from JFH1^{E2FL}-replicating cells contained high levels of viral proteins in contrast to the LD fraction from JFH1^{dC3}-replicating cells (in which HCV proteins were virtually absent (Fig. 1d, LD fraction)), even though the expression levels of the NS proteins in whole-cell extracts were similar (Fig. 1d, whole-cell extract). About 20–45% of the total HCV proteins associated with the LDs in JFH1^{E2FL}-replicating cells (Fig. 1e). Consistent with previous reports that Core enhances the formation of LDs[14], overproduction of LDs was observed in JFH1^{E2FL}-, but not JFH1^{dC3}-replicating cells (Supplementary Information, Fig. S3a–l). Treatment of the cells with oleic acid, which enhanced the formation of LDs, did not affect either HCV protein levels or the recruitment of viral proteins to LDs in JFH1^{dC3}-replicating cells (Supplementary Information, Fig. S3m–p). Thus, the overproduction of LDs is insufficient for the recruitment of HCV proteins to LDs. To examine the ability of Core to recruit NS proteins to LDs, JFH1^{dC3}-replicating cells were transfected with a plasmid-expressing Core (Core^{Wt}) (Fig. 1f, g). NS5A accumulated around LDs (Fig. 1f, arrowheads and panel 2), as did NS3 and NS4AB (Fig. 1g), in cells expressing Core^{Wt}. The translocation of NS proteins to LDs was, however, not observed in JFH1^{dC3}-replicating cells expressing Core$^{PP/AA}$ (Fig. 1g and Supplementary Information, Fig. S2f–h),

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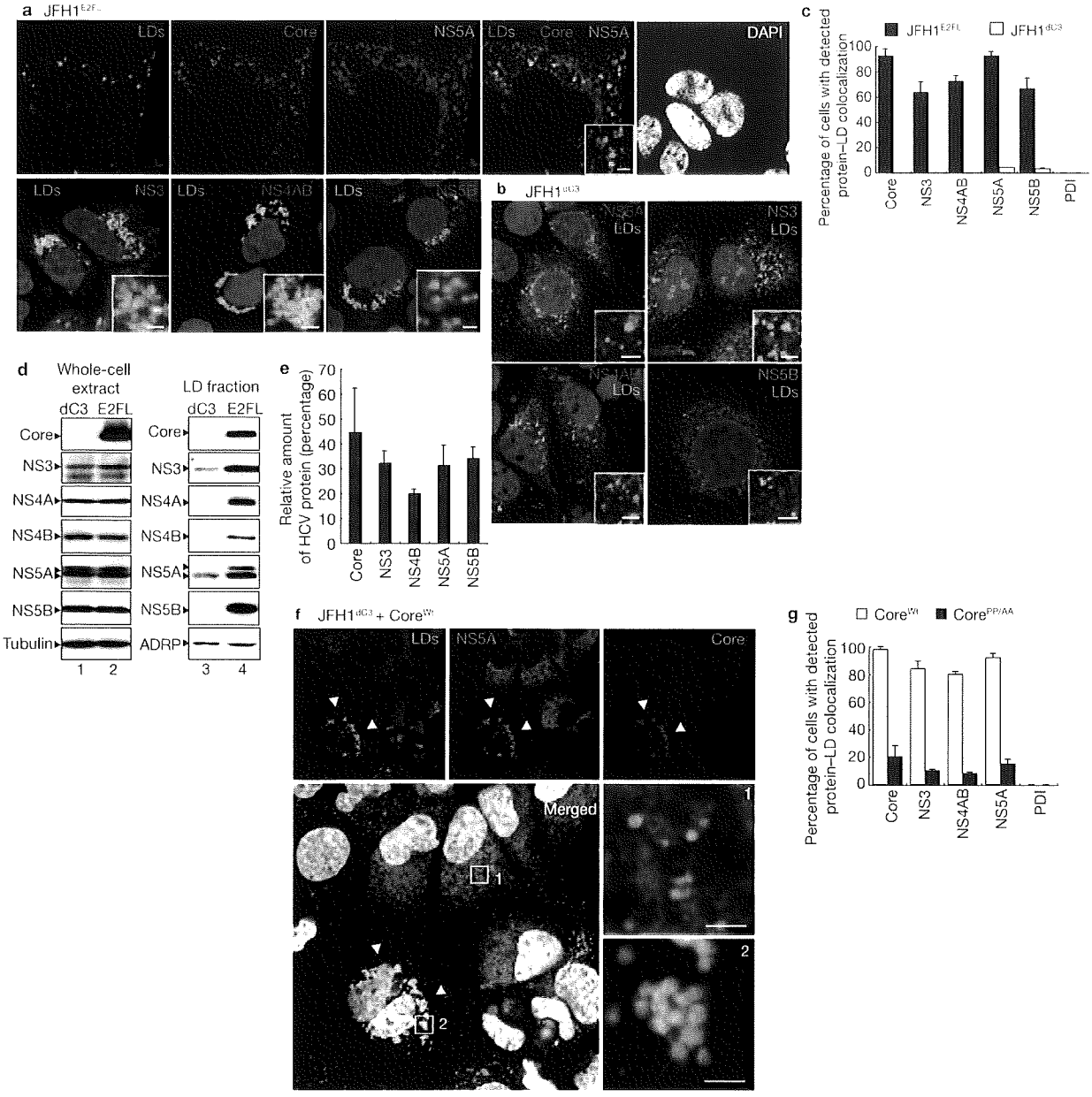

Figure 1 Core recruits NS proteins to LDs. (**a**) Huh-7 cells transfected with JFH1^{E2FL} RNA were labelled with antibodies against Core (red), NS5A (blue), NS3 (red), NS4AB (red) or NS5B (red). Lipid droplets (LDs) and nuclei were stained with BODYPI 493/503 (green) and DAPI (white in upper panel, blue in lower panels), respectively. Insets are high magnification images of areas in the respective panel. (**b**) JFH1^{dC3} replicon-bearing cells were labelled with DAPI (blue), BODIPY 493/503 (green) and indicated antibodies (red). The insets are high magnifications of the corresponding panel. (**c**) Percentages of JFH1^{E2FL}- or JFH1^{dC3}-bearing cells in which hepatitis C virus (HCV) proteins or PDI colocalize with LDs ($n > 200$). (**d**) Western blot analysis of HCV proteins and marker proteins in whole-cell extracts and the LD fractions from cells transfected with JFH1^{E2FL} (E2FL) or JFH1^{dC3} (dC3) RNA. (**e**) HCV proteins were quantified by using western blotting data of the purified LD fraction and whole-cell extracts of JFH1^{E2FL}-replicating cells. Results are shown as relative amounts of HCV proteins co-fractionated with LDs. This results correspond well with results obtained by quantitative immunofluorescence staining (data not shown). (**f**) Trans-complementation with Core^{Wt} relocates NS proteins to LDs. JFH1^{dC3} replicon-bearing cells were transfected with pcDNA3–Core^{Wt} and labelled with BODIPY 493/503 (green), DAPI (white) and antibodies against NS5A (red) and Core (blue). Arrowheads indicate Core^{Wt}-expressing cells. Higher-magnification images of area 1 and area 2 are shown in panels 1 and 2, respectively. Scale bars, 2 μm. (**g**) The percentages of cells in which HCV proteins colocalize with LDs in the presence of Core^{Wt} or Core$^{P7/AA}$ ($n > 200$). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.

a variant of Core containing two alanine substitutions at amino-acid positions 138 and 143 that fails to associate with LDs[17]. These results show that LD-associated Core recruits NS proteins from the ER to LDs.

Next, we investigated whether Core also recruited HCV RNA to LDs. *In situ* hybridization analysis showed that in more than 80% of JFH1^{E2FL}-replicating cells, both plus- and minus-strand RNAs were diffusely

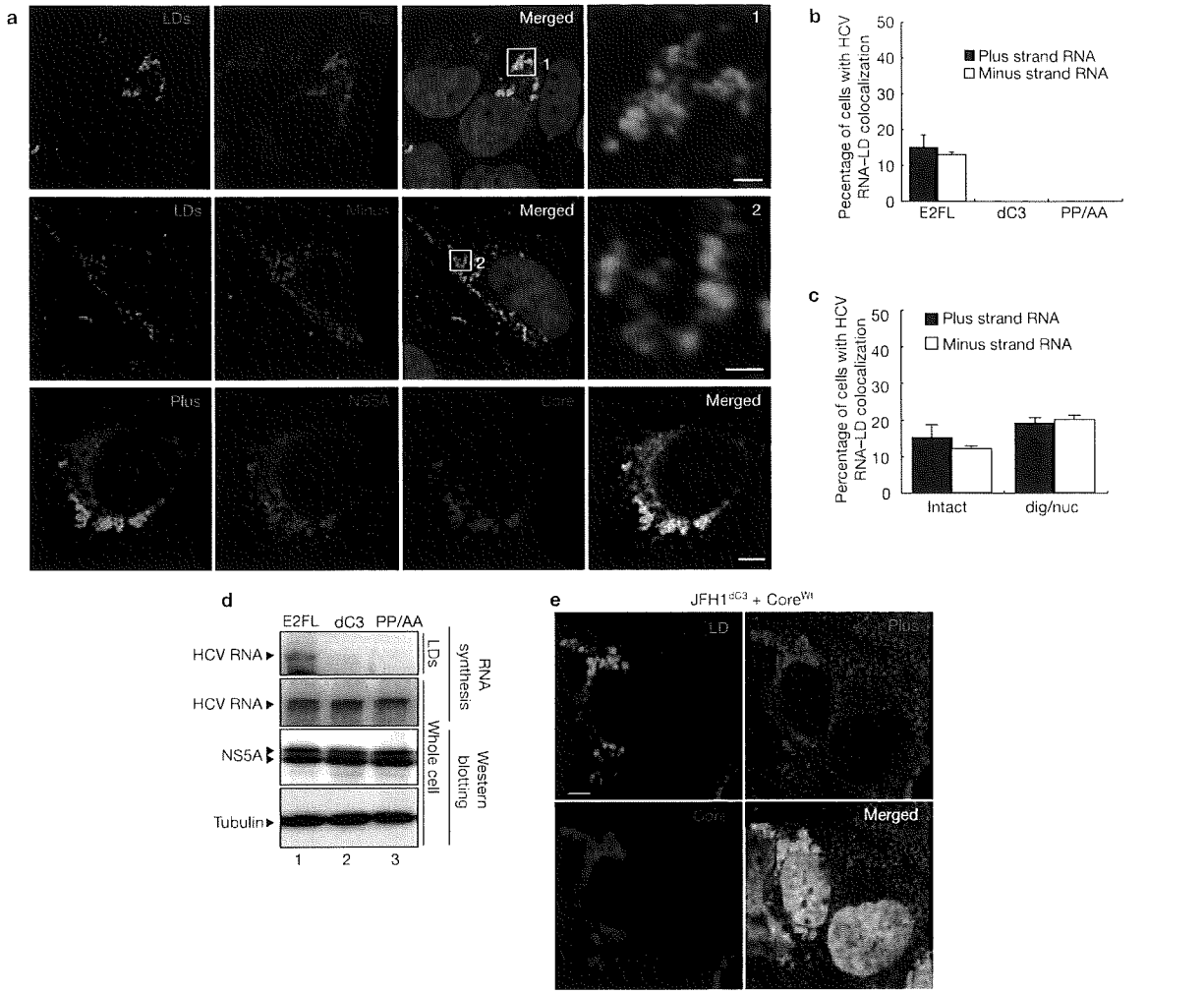

Figure 2 Core-dependent recruitment of active HCV replication complexes to the LD. (**a**) Huh-7 cells transfected with JFH1^{E2FL} RNA were analysed by *in situ* hybridization with strand-specific probes (plus or minus). The cells were labelled to simultaneously visualize lipid droplets (LDs), NS5A and Core (lower panels). Higher-magnification images of area 1 and area 2 are shown in the upper and middle right panels 1 and 2, respectively. Scale bars: 2 μm (panels 1, 2); 10 μm (lower right panel). (**b**) The percentages of JFH1^{E2FL}-, JFH1^{dC3}- and JFH1$^{PP/AA}$-expressing cells positive for overlapping signals for LDs and plus- or minus-strand hepatitis C virus (HCV) RNA ($n > 200$). (**c**) Intact or digitonin and nuclease-treated (dig/nuc) JFH1^{E2FL} replicon-bearing cells were analysed by *in situ* hybridization. The percentages of cells with overlapping signals for LD and plus- or minus-strand HCV RNA are shown ($n > 200$). (**d**) RNA-synthesizing activity in the LD fractions purified from cells transfected with JFH1^{E2FL}, JFH1^{dC3} or JFH1$^{PP/AA}$ RNA (top panel). As a control, HCV RNA synthesis activity in digitonin-permeabilized cells was analysed (second panel from the top). HCV protein levels represented by NS5A are shown, together with the level of tubulin (bottom two panels). (**e**) Localization of plus-strand HCV RNA and Core in JFH1^{dC3} replicon-bearing cells transfected with pcDNA3–Core^{Wt} (Scale bar, 10 μm). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.

located in the perinuclear region (see Supplementary Information, Fig. S4a). More importantly, in about 20% of these cells, plus- and minus-strand RNAs accumulated around LDs (Fig. 2a, upper and middle panels; 2b) and colocalized with HCV proteins such as Core and NS5A (Fig. 2a, lower panels). No association between HCV RNA and LDs was detected in JFH1^{dC3}- or JFH1$^{PP/AA}$-replicating cells (Fig. 2b). Northern blot analysis revealed that 4.8% and 5.4% of total plus- and minus-strand HCV RNA, respectively, were detected in purified LD fractions of JFH^{E2FL}-replicating cells (data not shown). Induction of LD formation with oleic acid did not affect HCV RNA accumulation around LDs (data not shown). These results provide strong evidence that Core recruits HCV RNA as well as NS proteins to LDs.

The HCV replication complex is compartmentalized by lipid bilayer membranes[16–18]. Therefore, HCV RNA in the complex is resistant to nuclease treatment in digitonin-permeabilized cells[17] (Supplementary Information, Fig. S4b–d). *In situ* hybridization analysis did not reveal a significant difference in the number of cells containing LD-associated HCV RNA before and after nuclease treatment (Fig. 2c), indicating that HCV RNA around LDs is part of the replication complex. An RNA synthesis assay showed that the purified LD fraction from JFH1^{E2FL}-, but not JFH1^{dC3}- or JFH1$^{PP/AA}$-replicating cells, possessed HCV RNA synthesis activity, even though the expression levels of viral proteins and RNA-synthesizing activities in total cell lysates were similar (Fig. 2d). Moreover, the addition of Core^{Wt} rescued the localization of plus- and minus-strand

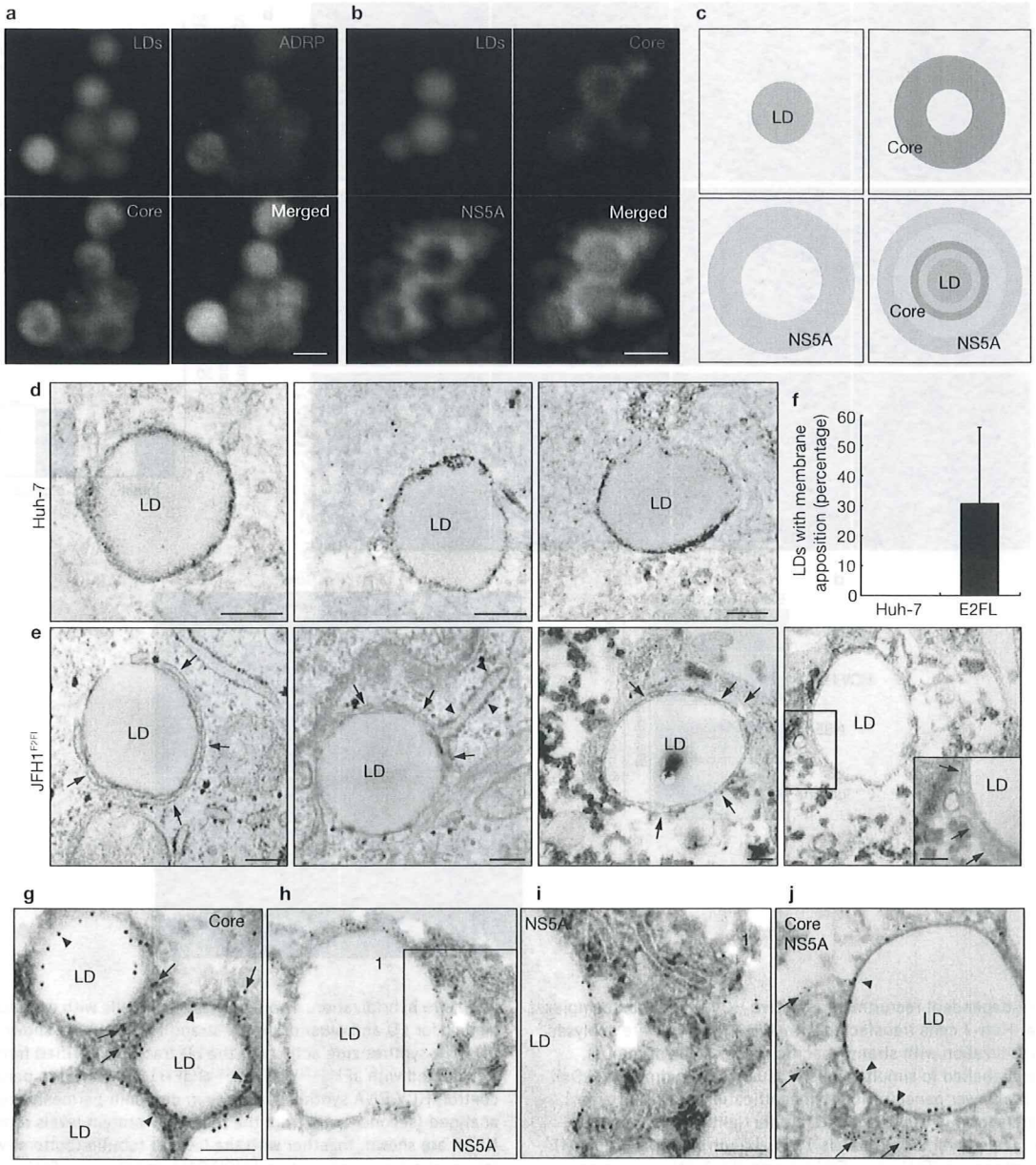

Figure 3 Spatial distribution of Core and NS5A relative to the LD. (**a**, **b**) The localizations of Core, NS5A and ADRP around the lipid droplets (LDs) in JFH1^{E2FL} replicon-bearing cells were analysed using immunofluorescence microscopy. Scale bars, 1 μm. (**c**) Typical images of the localization of LDs, Core, NS5A and merged images are shown with the relative scale of each image. (**d**, **e**) Transmission electron micrographs of LDs in naïve Huh-7 cells and JFH1^{E2FL}-expressing cells. Arrows and arrowheads indicate LD-associated membranes and rough ER membranes, respectively. (**f**) Frequency of LDs with close appositions of membrane cisternae. About 100 Huh-7 cells or JFH1^{E2FL}-expressing cells, respectively, were chosen randomly. LDs with apposed membrane cisternae, as exemplified in panel **e**, were counted as positive. The LDs judged as positive were divided by the total number of LDs. (**g**–**j**) Immunoelectron micrographs of LDs labelled with antibodies against Core (**g**), NS5A (**h**, **i**) or both (**j**) are shown. Panel **i** is a higher magnification of area 1 in panel **h**. In panel **j**, Core and NS5A are labelled with 15 nm and 10 nm gold particles, respectively. Scale bars, 200 nm. All error bars are derived from s.d.

HCV RNA around LDs in JFH^{dC3}-replicating cells (Fig. 2e and data not shown). Both plus- and minus-strand RNA associated with LDs were nuclease resistant (data not shown). These results demonstrate that Core recruits biologically active replication complexes to LDs.

The LD is surrounded by a phospholipid monolayer[19], whereas HCV replication complexes are likely to be surrounded by lipid bilayer membranes[16,17]. Therefore, the replication complexes might not be directly associated with the membranes of LDs. To characterize the colocalization of LDs, viral proteins and replication complexes more precisely, we analysed the localization of NS5A with high-resolution immunofluorescence microscopy. Core was completely colocalized with ADRP, residing on the surface of LDs[20] (Fig. 3a), thus indicating that Core also directly associates with the surface of LDs. More importantly, NS5A mainly localized around the Core-positive area, resulting in a doughnut-shaped signal with a diameter slightly

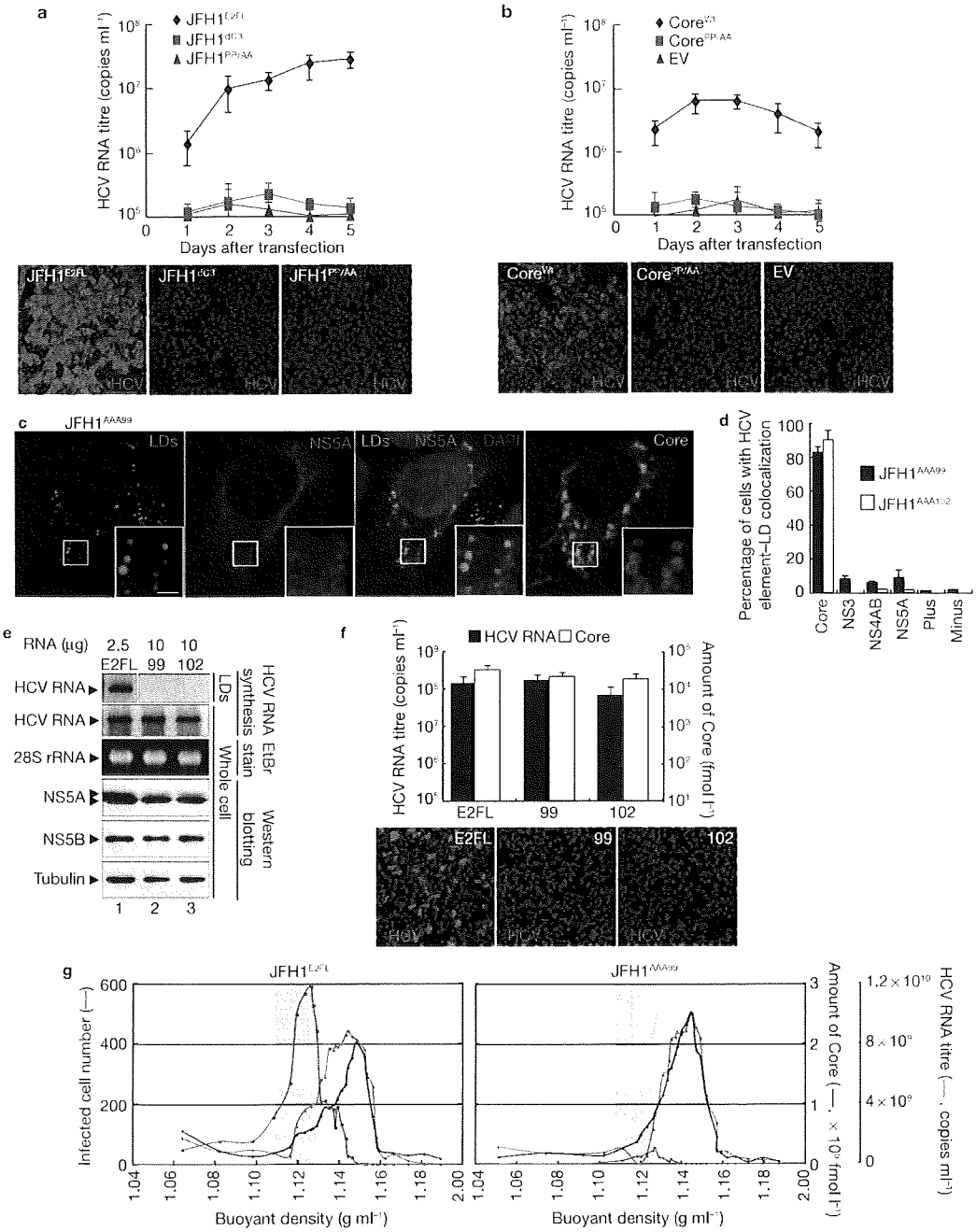

Figure 4 LD associations of Core and NS proteins are necessary for the production of infectious HCV particles. (**a**) The culture medium from JFH1^{E2FL}-, JFH1^{dC3}- or JFH1$^{PP/AA}$-replicating cells was collected at the indicated time points and the titre of hepatitis C virus (HCV) RNA was measured by real-time RT-PCR (upper panel, $n = 3$). The culture medium was added to naïve Huh7.5 cells and, 24 h after inoculation, and cells were labelled with anti-HCV antibodies (lower panels, red). (**b**) JFH1^{dC3} replicon-bearing cells were transfected with pcDNA3 (EV), pcDNA3–Core^{WT} (Core^{WT}) or pcDNA3–Core$^{PP/AA}$ (Core$^{PP/AA}$). The level of HCV RNA and the infectivity of the culture medium were examined as described above ($n = 3$). (**c**) Subcellular localization of NS5A and Core in cells expressing JFH1^{AAA99}. The insets are high magnifications of the area of the corresponding panel. Scale bar, 2 μm. (**d**) Percentages of cells in which the signals for given HCV proteins, and plus- and minus-strand HCV RNA, overlapped with those for LDs ($n > 200$). (**e**) Different amounts of JFH1^{E2FL} (E2FL), JFH1^{AAA99} (99) or JFH1^{AAA102} (102) RNAs, respectively, were transfected into the same number of Huh-7 cells. HCV RNA synthesis activity in purified LD fractions (LD) and whole-cell lysates (whole cell) was analysed (HCV RNA synthesis). 28S rRNA was used as a control. Western blot analysis of NS5A, NS5B and tubulin in cells is also shown. All the RNA samples in the top panel were run on the same gel. (**f**) Analysis of HCV released from cells expressing JFH1^{E2FL}, JFH1^{AAA99} or JFH1^{AAA102}. HCV RNA titres (black bars) and amounts of Core (white bars) accumulated in the culture medium at 5 d after RNA transfection were measured (upper panel, $n = 3$). Infectivity of the culture medium for naïve Huh-7.5 cells was analysed as described above (lower panels). (**g**) Concentrated culture medium from JFH1^{E2FL}- and JFH1^{AAA99}-replicating cells was fractionated using 20–50% sucrose density-gradient centrifugation at 100,000 *g* for 16 h. For each fraction, the amounts of Core (black line), HCV RNA (blue line) and infectivity (represented by infected cell numbers in a well; red line) are plotted against the buoyant density (*x*-axis) ($n = 3$). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.

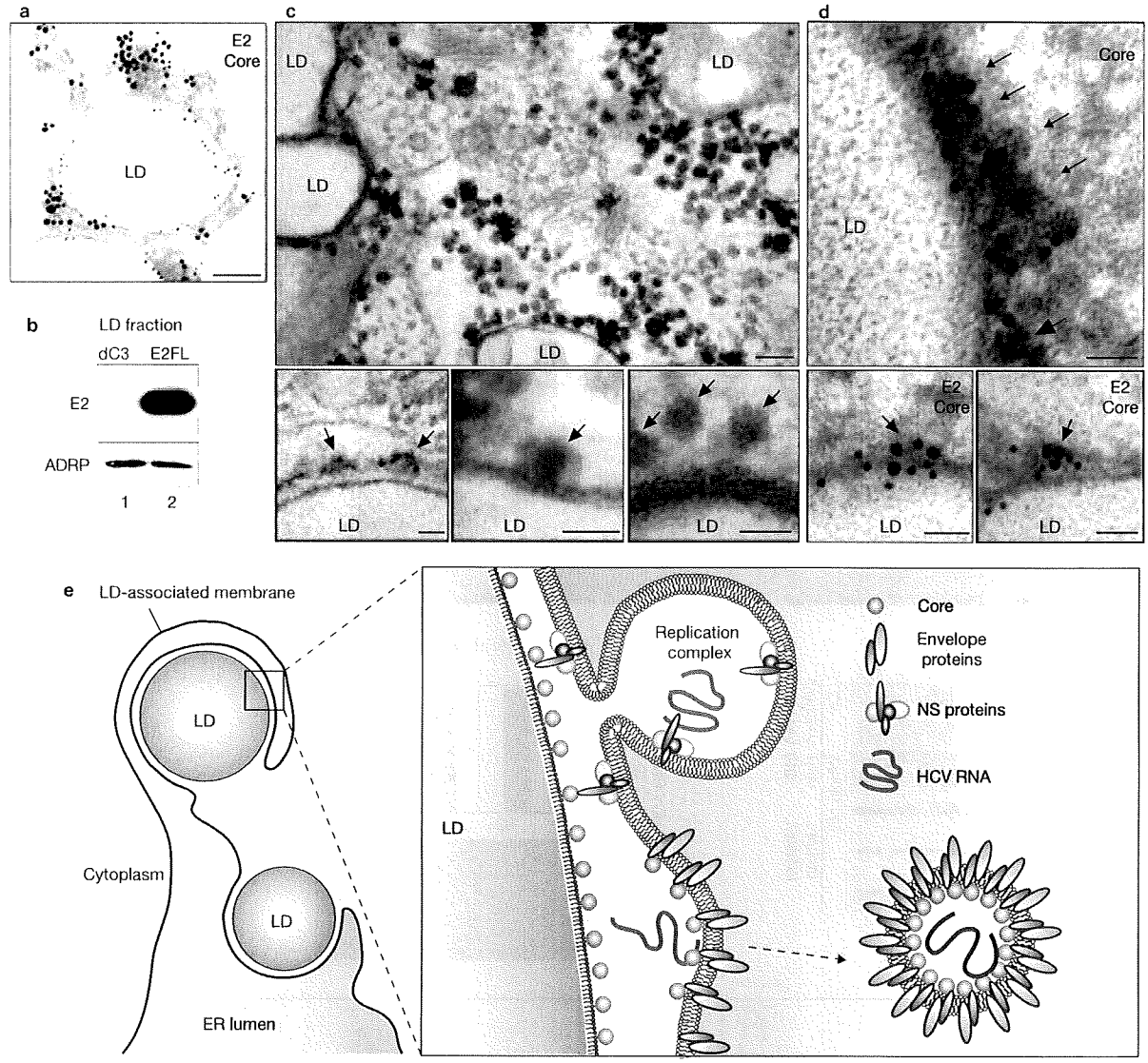

Figure 5 Virus-assembly takes place around the LDs. (**a**) Immunoelectron microscopic detection of E2 and Core in JFH1^{E2FL}-replicating cells. E2 and Core are labelled with 15 nm and 10 nm gold particles, respectively. (**b**) Western blot analysis of the lipid droplet (LD) fraction from JFH1^{E2FL} and JFH1^{dC3} replicon-bearing cells with anti-E2 and anti-ADRP antibodies. (**c**) Transmission electron micrographs of JFH1^{E2FL}-replicating cells. Arrows indicate virus-like particles. (**d**) Immunoelectron micrographs of LDs labelled with antibodies against Core (10 nm) and E2 (15 nm) are shown. Arrows show Core in electron-dense granules. Scale bar: **a** and upper panel of **c**: 100 nm; in **d** and lower panels of **c**: 50 nm. (**e**) A model for the production of infectious hepatitis C virus (HCV). Core mainly localizes on the monolayer membrane that surrounds the LD. HCV induces the apposition of the LD to the endoplasmic reticulum (ER)-derived bilayer membranes (LD-associated membrane). Core recruits NS proteins, as well as replication complexes, to the LD-associated membrane. NS proteins around the LD can then participate in infectious virus production. E2 also localizes around the LD. Through these associations, virion assembly proceeds in this local environment. Uncropped images of gels are shown in Supplementary Information Fig. S6.

larger than that of Core (Fig. 3b). The LD-proximal NS5A signal partially overlapped with the Core signal (Fig. 3b, c, grey). This concentric staining pattern was also observed with the other NS proteins (Supplementary Information, Fig. S5a), indicating that NS proteins associate with Core on the surface of LDs. Electron microscopic analysis only rarely revealed a close association of LDs with other organelles in naïve Huh-7 cells (Fig. 3d, f). However, in the case of JFH1^{E2FL}-replicating cells, about 30% of the LDs were in close proximity to membrane cisternae (Fig. 3e, arrows; 3f), arguing for a HCV-induced membrane rearrangement around LDs. Core was mainly located on the periphery of LDs, and occasionally signals were observed in more distal areas of the LDs (Fig. 3g, arrowheads and arrows, respectively). Although some NS5A signals were observed on the surface of the LD, the majority of NS5A signals were detected more distal of LDs (Fig. 3h, i). Furthermore, we often observed membrane cisternae as white lines in the same area as NS5A signals (Fig. 3i, arrows). When the same section was labelled with anti-Core and anti-NS5A antibodies, Core was detected on the surface of the LDs, whereas NS5A was mainly observed in the peripheral area of the LDs (Fig. 3j, arrowheads and arrows, respectively). In summary, these results show that Core recruits NS proteins, as well as HCV replication complexes, to the LD-associated membranes.

The above results prompted us to ask whether Core–LD colocalization is important for the production of infectious virus particles. JFH1^{E2FL}-replicating cells released virions into the culture medium and these viruses were highly infectious for naïve Huh-7.5 cells[11,21], although culture medium from JFH1$^{PP/AA}$- or JFH1^{dC3}-replicating cells did not contain significant levels of HCV RNA and infectious virus (Fig. 4a). However, following trans-complementation with Core^{WT}, a high titre of HCV RNA and infectious virus could be rescued from JFH1^{dC3}-replicating cells (Fig. 4b; and see Supplementary Information, Fig. S5b, c). In contrast, the production of infectious viruses was not rescued by trans-complementation with Core$^{PP/AA}$ (Fig. 4b). RNA-binding properties and oligomerization of Core^{WT} and Core$^{PP/AA}$, which are both necessary for virus assembly, were similar (Supplementary Information, Fig. S5d; ref. 22), arguing that the primary defect of this mutant in preventing infectious virus production is the inability to associate with LDs.

To investigate the contribution of NS proteins around LDs to infectious virus production, we used variants of NS5A, which were not recruited to LDs even in the presence of Core. We assumed that NS5A was crucial for recruiting other NS proteins to LDs, because the level of NS5A recruited to LDs via Core was higher than the levels of the other recruited NS proteins (Fig. 1c, JFH1^{E2FL}). Using alanine-scanning mutagenesis within the NS5A coding region of JFH1^{E2FL}, we generated two mutants, JFH1^{AAA99} and JFH1^{AAA102}, in which the amino-acid sequence APK (aa 99–101 of NS5A) or PPT (aa 102–104 of NS5A) was replaced by AAA (Supplementary Information, Fig. S1). In JFH1^{AAA99}- and JFH1^{AAA102}-replicating cells, NS5A was rarely detected around LDs, whereas Core was still localized to LDs (Fig. 4c, d). Importantly, these mutations impaired not only the NS5A association with LDs, but also the recruitment of other NS proteins and viral RNAs to LDs (Fig. 4d). These results indicate that NS5A is a key protein that recruits replication complexes to LDs. Importantly, HCV RNA synthesis activity in the LD fractions from these mutant JFH1-replicating cells was also severely impaired (Fig. 4e), corroborating the lack of association of HCV replication complexes with LDs.

To investigate the infectious virus production of these NS5A mutants, we prepared cells expressing similar levels of HCV proteins and RNA by adjusting the amount of transfected HCV RNA (Fig. 4e). This was necessary, because replication activities of these mutants were lower compared with JFH1^{E2FL}. Under these conditions, the amounts of Core and HCV RNA that were released into the culture medium from cells transfected with the mutants were comparable to JFH^{E2FL} (Fig. 4f, upper graph). However, infectivity titres of the mutants were severely reduced (Fig. 4f, lower panels). In sucrose density-gradient centrifugation of culture medium from JFH1^{E2FL}-bearing cells, two types of HCV particles were detected: low-density particles (about 1.12 g ml^{-1}) with high infectivity (Fig. 4g, green area of JFH1^{E2FL}), and high-density particles (about 1.15 g ml^{-1}) without infectivity (yellow area). This result indicates that only a minor portion of released HCV particles is infectious, whereas the majority of released particles lack infectivity. In contrast, cells bearing the JFH1^{AAA99} mutant almost exclusively released non-infectious particles of around 1.15 g ml^{-1}, whereas infectious particles were barely detectable (Fig. 4g, JFH1^{AAA99}). Taken together, these results provide convincing evidence that the association of NS proteins and replication complexes around LDs is critical for producing infectious viruses, whereas production of non-infectious viruses seems to follow a different pathway.

The results described so far imply that some step(s) of HCV assembly take place around LDs. To explore this possibility, we analysed the distribution of the major envelope protein E2 around the LD. Electron microscopic analysis revealed that, in about 90% of JFH1^{E2FL}-replicating cells, E2 was localized in the peripheral area of the LDs (Fig. 5a, large grains). This labelling pattern was similar to the one observed for NS5A (Fig. 3j), indicating that E2 also localizes on the LD-associated membranes. Western blot analysis of the LD fraction supported this conclusion, because the LD fraction that was purified from JFH1^{E2FL}-replicating cells, but not from JFH1^{dC3}-replicating cells, contained E2 (Fig. 5b). Furthermore, spherical virus-like particles with an average diameter of about 50 nm were observed around LDs in JFH1^{E2FL}-replicating cells (Fig. 5c, upper panel). These particles were never observed in naïve Huh-7 cells. A more refined analysis indicates that these particles are closely associated with membranes in close proximity to LDs (Fig. 5c, lower panels, arrows). Finally, these particles around the LDs reacted with Core- and E2-specific antibodies, arguing that the particles represent true HCV virions (Fig. 5d). These results suggest that infectious HCV particles are generated from the LD-associated membranous environment.

In this study, we have demonstrated that Core recruits NS proteins, HCV RNAs and the replication complex to LD-associated membranes. Mutations of Core and NS5A (Fig. 4), which failed to associate with LDs, impaired the production of infectious virus. We note that the mutant Core retains the ability to interact with RNA (Supplementary Information, Fig. S5b) and to assemble into nucleocapside[22]. Similarly, the NS5A mutant still supports viral genome replication and the formation of capsids or virus-like particles, arguing that the introduced mutations in Core and NS5A do not affect overall protein folding, stability or function (Fig. 4). Taken together, the data show that the association of HCV proteins with LDs is important for the production of infectious viral particles (Fig. 5e).

Our results also indicate that NS proteins around the LDs participate in the assembly of infectious virus particles. In one scenario, NS proteins may indirectly contribute to the different steps of virus production — for example, by establishing the microenvironment around the LDs that is required for infectious virus production. Alternatively, NS proteins around the LDs may directly participate in virus production — for example, as components of the replication complex that provide the RNA genome to the assembling nucleocapsid.

In support of the role of LDs in virus formation, we observed that colocalization of HCV protein with LDs was low in cases of the chimera Jc1, supporting up to 1,000-fold higher infectivity titres compared with JFH1 (ref. 13). In a Jc1-infected cell, only about 20% of LDs demonstrated detectable colocalization with Core, but this value increased to 80% in the case of a Jc1 mutant lacking most of the envelope glycoprotein genes and thus being unable to produce infectious virus particles (data not shown). This inverse correlation between the efficiency of virus production and Core protein accumulation on LDs indicates that rapid assembly and virus release results in the rapid liberation of HCV proteins from the LDs.

Steatosis and abnormal lipid metabolism caused by chronic HCV infection may be linked to enhanced LD formation[14]. In fact, the overproduction of LDs is induced by Core (Supplementary Information, Fig. S3) and HCV also induces membrane rearrangements around LDs (Fig. 3d–f). Our findings suggest that excessive Core-dependent formation of LDs

and membrane rearrangements are required to supply the necessary microenvironment for virus production. NS proteins and HCV RNA seem to be translocated from the ER to the LD-associated membranes. Interestingly, the LD-associated membranes were occasionally found in continuity with ribosome-studded rough ER (Fig. 3e, arrowheads). Thus, at least parts of the LD-associated membranes are likely to be derived from ER membranes. ER marker proteins, however, were not detected in the LD fraction, suggesting that the LD-associated membrane is characteristically distinct from that of ER membranes.

To our knowledge, this is the first report showing that LDs are required for the formation of infectious virus particles. The fact that capsid protein of the hepatitis G virus also localizes to LDs[15] indicates that LDs might be important for the production of other viruses as well. Our findings demonstrate a novel function of LDs, provide an important step towards elucidating the mechanism of HCV virion production and open new avenues for novel antiviral intervention. □

METHODS

Antibodies. The antibodies used for immunoblotting and immunolabelling were specific for Core (#32-1 and RR8); E2 (AP-33 (ref. 23); 3/11, CBH5 and Flag M2 (Sigma-Aldrich, St Louis, MO); NS3 (R212)[17]; NS4A and 4B (PR12); NS5A (NS5ACL1); NS5B (NS5B-6 and JFH1-1)[24]; ADRP (Progen Biotechnik, Heidelberg, Germany); tubulin (Oncogene Research Products, MA, USA); Grp78 (StressGen, Victoria, Canada); PDI (StressGen); and Calnexin-NT (StressGen). Antibodies specific for Core (#32-1 and RR8), NS3 (R212) and NS4AB (PR12) were gifts from Dr Kohara (The Tokyo Metropolitan Institute of Medical Science, Japan). Anti-E2 antibody (AP-33) was provided by Dr Patel (MRC Virology Unit, UK). Anti-NS5B (NS5B-6) antibody was kindly provided by Dr Fukuya (Osaka University, Japan). Rabbit polyclonal antibodies specific for NS5A were raised against a bacterially expressed GST–NS5A (1–406 aa) fusion protein. In the case of the HCV chimeras Con1/C3 and H77/C3, immunofluorescence analyses were performed by using the following antibodies: Core (C7/50)[5], a JFH1 NS3-specific rabbit polyclonal antiserum; NS4B (#86)[25]; and NS5A (Austral Biologicals, San Ramon, CA).

Indirect immunofluorescence analysis. Indirect immunofluorescence analysis was performed essentially as described previously[17], with slight modifications. Cells transfected with JFH1 RNA were seeded onto a collagen-coated Labtech II 8-well chamber (Nunc, NY, USA). The coating with collagen was performed using rat-tail collagen type I (BD Bioscience, Palo Alto, CA) according to manufacturer's instructions. Three days after seeding, the cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4) and fixed with fixation solution (4% paraformaldehyde and 0.15 M sodium cacodylate at pH 7.4) for 15 min at room temperature. After washing with PBS, the cells were permeabilized with 0.05% Triton X-100 in PBS for 15 min at room temperature. For the precise localization of the proteins, the cells were permeabilized with 50 μg ml^{-1} of digitonin in PBS for 5 min at room temperature[26]. After incubating the cells with blocking solution (10% fetal bovine serum and 5% bovine serum albumin (BSA) in PBS) for 30 min, the cells were incubated with the primary antibodies. The fluorescent secondary antibodies were Alexa 568- or Alexa 647-conjugated anti-mouse or anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA). Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI). LDs were visualized with BODIPY 493/503 (Invitrogen). Analyses of JFH1 were performed on a Leica SP2 confocal microscope (Leica, Heidelberg, Germany). Analysis of the Con1/C3 and the H77/C3 chimeras was performed in the same way, except that imaging was performed on a Nikon C1 confocal microscope (Nikon, Tokyo, Japan).

Electron microscopy. For conventional electron microscopy, cells cultured in plastic Petri dishes were processed *in situ*. The cells were fixed in 2.5% glutaraldehyde and 0.1 M sodium phosphate (pH 7.4), and then in OsO_4 and 0.1 M sodium phosphate (pH 7.4). The cells were then dehydrated in a graded ethanol series and embedded in an epoxy resin. Ultrathin sections were cut perpendicular to the base of the dish. For immuno-electron microscopy, cells were detached from the dish with a cell scraper after fixation in 4% paraformaldehyde, 0.1% glutaraldehyde and 0.1 M sodium phosphate (pH 7.4) for 24 h, and washed in 0.1 M lysine, 0.1 M sodium phosphate (pH 7.4) and 0.15 M sodium chloride. After dehydrating the cells in a graded series of cold ethanol, they were embedded in Lowicryl K4M at −20 °C. Ultrathin sections were labelled with primary antibodies and colloidal gold particles (15 nm) conjugated to anti-mouse IgG or anti-rabbit IgG antibodies. For double labelling, colloidal gold particles with different diameters (10 nm and 15 nm) conjugated to anti-mouse IgG or anti-rabbit antibodies were used. Samples were observed after staining with uranyl acetate and lead citrate with a JEM 1010 electron microscope at the accelerating voltage of 80 kV. Anti-Core (#32-1 and RR88), anti-NS5A (NS5ACL1) and anti-E2 (Flag M2) antibodies were used.

Preparation of the lipid droplets. Cells at a confluency of ~80% on a dish with a diameter of 14 cm were scraped in PBS. The cells were pelleted by centrifugation at 1,500 rpm. The pellet was resuspended in 500 μl of hypotonic buffer (50 mM HEPES, 1 mM EDTA and 2 mM $MgCl_2$ at pH 7.4) supplemented with protease inhibitors (Roche Diagnostics, Basel, Switzerland) and was incubated for 10 min at 4 °C. The suspension was homogenized with 30 strokes of a glass Dounce homogenizer using a tight-fitting pestle. Then, 50 μl of 10× sucrose buffer (0.2 M HEPES, 1.2 M KoAc, 40 mM $Mg(oAc)_2$ and 50 mM DTT at pH 7.4) was added to the homogenate. The nuclei were removed by centrifugation at 2,000 rpm for 10 min at 4 °C. The supernatant was collected and centrifuged at 16,000 *g* for 10 min at 4 °C. The supernatant (S16) was mixed with an equal volume of 1.04 M sucrose in isotonic buffer (50 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$ and protease inhibitors). The solution was set at the bottom of 2.2-ml ultracentrifuge tube (Hitachi Koki, Tokyo, Japan). One milliliter of isotonic buffer was loaded onto the sucrose mixture. The tube was centrifuged at 100,000 *g* in an S55S rotor (Hitachi Koki) for 30 min at 4 °C. After the centrifugation, the LD fraction on the top of the gradient solution was recovered in isotonic buffer. The suspension was mixed with 1.04 M sucrose and centrifuged again at 100,000 *g*, as described above, to eliminate possible contamination with other organelles. The collected LD fraction was used for western blotting or the HCV RNA synthesis assay.

HCV RNA synthesis assay. An assay of HCV RNA synthesis using digitonin-permeabilized cells was performed as described previously[17]. For RNA synthesis assays using the LD fraction, the LD fraction collected by sucrose-gradient sedimentation was suspended in buffer B, which contained 2 mM manganese (II) chloride, 1 mg ml^{-1} acetylated BSA (Nacalai Tesque, Kyoto, Japan), 5 mM phosphocreatine (Sigma), 20 units/ml creatine phosphokinase (Sigma), 50 μg ml^{-1} actinomycin D, 500 μM ATP, 500 μM CTP, 500 μM GTP (Roche Diagnostics) and 1.85 MBq of [α-^{32}P] UTP (GE Healthcare, Little Chalfont, UK), and incubated at 27 °C for 4 h. The reaction products were analysed by gel electrophoresis followed by autoradiography.

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

Y.M. and K.S. planned experiments and analyses. Y.M. was responsible for experiments for Figs 1, 2, 3a–c, 4a–e and 5b. K.A., N.U., electron microscopy; T.H., Fig. 1e; M.Z., R.B., Fig. S2e; and K.S. and K.W., Fig. 4f–g. T.W. provided JFH1 strain. Y.M. and K.S. wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Anti-hepatitis C Virus Activity of Tamoxifen Reveals the Functional Association of Estrogen Receptor with Viral RNA Polymerase NS5B*

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Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. HCV genome replication occurs in the replication complex (RC) around the endoplasmic reticulum membrane. However, the mechanisms regulating the HCV RC remain widely unknown. Here, we used a chemical biology approach to show that estrogen receptor (ESR) is functionally associated with HCV replication. We found that tamoxifen suppressed HCV genome replication. Part of ESRα resided on the endoplasmic reticulum membranes and interacted with HCV RNA polymerase NS5B. RNA interference-mediated knockdown of endogenous ESRα reduced HCV replication. Mechanistic analysis suggested that ESRα promoted NS5B association with the RC and that tamoxifen abrogated NS5B-RC association. Thus, ESRα regulated the presence of NS5B in the RC and stimulated HCV replication. Moreover, the ability of ESRα to regulate NS5B was suggested to serve as a potential novel target for anti-HCV therapeutics.

Estrogen receptor (ESR)[2] belongs to the steroid hormone receptor family of the nuclear receptor superfamily (1). ESR consists of two subtypes, ESRα and ESRβ. As a primary physiological function, ESR is involved in the transcription for downstream genes in response to stimulation by the ligand, estradiol. In the normal state, ESR is mainly located in the cytoplasm and nucleus. Upon binding of the ligand, ESR dimerizes and translocates into the nucleus, where it binds to the ESR-responsive elements (ERE) in the DNA promoter of downstream genes and drives transcription. In addition to this classical genomic action, a portion of ESR is located on the membrane, such as the plasma membrane, and involved in the nongenomic function of triggering signal transduction pathways, such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C (2–4). Although the molecular basis of ESR membrane retention is not fully understood, one mechanism involves a membrane protein, caveolin (CAV); ESRα interacted with CAV, and this interaction facilitated ESRα localization to the membrane (5, 6). It was also reported that ESRα localizes to the lipid rafts on the plasma membrane (7). The lipid rafts are microdomains of the membrane that form platforms enriched in cholesterol and glycosphingolipids. However, the characteristics and relevance of membrane-associated ESR have not been fully disclosed. Here, we report the novel role of ESRα in the regulation of viral replication.

Hepatitis C virus (HCV), a causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, constitutes a serious health problem worldwide (8). HCV has a positive strand RNA genome that produces at least 10 functional viral proteins: core, envelope 1, envelope 2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (9, 10). NS5B is an RNA-dependent RNA polymerase, which plays a central role in viral genome replication (11, 12). HCV genome replication can be evaluated using a HCV subgenomic replicon system, which Lohmann *et al.* (13) first established. In this system, cells carry an HCV subgenome RNA encoding NS3 to NS5B. Using this system, it has been proposed that HCV genome replication occurs in the replication complex (RC), which contains the viral genome RNA and HCV NS proteins. The RC forms on the surface of the intracellular membranes, including the endoplasmic reticulum (ER) membrane, and is surrounded by a membrane structure (14–17). It also has been reported that HCV genome replication associates with the lipid rafts on these intracellular membranes, such as the ER membrane (14, 18). These lipid rafts accumulate CAV2, and HCV proteins involved in viral genome replication cofractionate with CAV2 (18). However, it is largely unknown how the RC is formed and under what mechanism the HCV proteins participate in the RC.

A chemical biology approach is a useful method to analyze the molecular mechanism of viral life cycles as well as cellular physiological processes (19). We employed forward chemical genetics in which we analyzed HCV replication activity as a phenotypic indicator of a cell-based assay to screen chemical

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[2] The abbreviations used are: ESR, estrogen receptor; HCV, hepatitis C virus; RC, replication complex; ER, endoplasmic reticulum; TAM, tamoxifen; ERE, ESR-responsive element(s); CAV, caveolin; NS, nonstructural protein; MM, microsomal membrane; siRNA, small interfering RNA; si-ESR, small interfering ESR; GST, glutathione *S*-transferase; aa, amino acid(s); RT, reverse transcription; NS3, NS4A, NS4B, NS5A, and NS5B, nonstructural protein 3, 4A, 4B, 5A, and 5B, respectively.

compounds that inhibited HCV replication. Using this system, we previously identified an immunosuppressant, cyclosporin A, as an anti-HCV compound (20). We also reported that cyclophilin B regulated the RNA binding activity of NS5B (21). In the current study, this chemical screening approach linked ESRα to HCV replication. We showed that tamoxifen (TAM) suppressed HCV genome replication. Using TAM as a bioprobe, we found that ESRα interacted with NS5B and regulated the participation of NS5B in the RC.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Huh-7 and cured MH-14 cells (21) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acid (Invitrogen), and kanamycin (Meiji). MH-14 cells, carrying HCV subgenomic replicon (16), and LucNeo#2 cells, carrying luciferase-containing subgenomic replicon (22), were cultured in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Hus-E7/DN24 cells, a human hepatocyte cell line established by immortalization with HPV E6E7 and hTERT from human primary hepatocytes and introduction with a dominant negative form of interferon regulatory factor-7 (23), were cultured with Dulbecco's modified Eagle's medium with 20 mM Hepes (Invitrogen), 15 g/ml L-proline, 0.25 g/ml insulin (Sigma), 50 nM dexamethasone (Sigma), 44 mM $NaHCO_3$, 10 mM nicotinamide, 5 ng/ml epidermal growth factor, 0.1 mM Asc-2P, 100 IU/ml penicillin G (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 5% fetal bovine serum, 1% Dulbecco's modified Eagle's medium, and 2 UG/ml Fungizone (Invitrogen) (24). Plasmid transfection was performed with FuGENE 6 transfection reagent (Roche Applied Science), as described previously (25). RNA transfection was achieved using DMrie-C transfection reagent (Invitrogen), as described previously (21). siRNA was transfected by using siLentFect (Bio-Rad) (21).

Plasmid Construction—pCMV-FL-ESRα, encoding the whole open reading frame of ESRα fused with a FLAG tag, was generated by inserting the PCR product using 5′-GTTGAATTCATGACCATGACCCTCCAC-3′ and 5′-GTTGATCTCGAGTCAGACTGTGGCAGGGAAAC-3′ as primer set and human lymphocyte cDNA library (Clontech) as a template into the EcoRI-XhoI site of pCMV-FLAG vector (21). pCAG-HA-NS5B, encoding the NS5B protein fused with a hemagglutinin tag, was made by subcloning the PCR product with 5′-GTTGCGGCCGCTATGTCAATGTCCTACTCA-3′ and 5′-GTTCTCGAGTCACCGGTTGGGGAGCAGGTA-3′ as primers and pMH14 as a template into NotI-XhoI digestion of PCAG-HA vector (21). Expression plasmids for HCV NS3, NS4B, NS5A, and NS5B (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, and pcDNA-NS5B, respectively) were described in Ref. 21. pGEX-ESRα A/B, C, D, and E/F, expressing the fusion protein of the domain A/B, C, D, and E/F of ESRα with GST, were prepared by the insertion of the PCR product with pCMV-FL-ESRα as a template and appropriate primers into the EcoRI-XhoI site of pGEX-6P1 vector (Clontech). The expression plasmids for the point mutants of ESRα, ESRα(L540Q), ESRα(255M), and ESRα(258M), of which Leu at aa 540, IRK at aa 255–257, and DRR at aa 258–260 were replaced by Gln, TGT, and ANT, respectively, was generated by oligonucleotide-directed mutagenesis. pCMV-FL-CAV2, encoding FLAG-tagged CAV2, was prepared by inserting the PCR product amplified with 5′-GTTGTCGACTATGGGGCTGGAGAC-3′ and 5′-GTTAAGCTTTCAATCCTGGCTC-3′ as primers and human liver cDNA library (Clontech) as a template into the SalI-HindIII site of pCMV-FLAG vector (21). The mammalian expression vector for the C domain of ESRα was generated by replacing the EcoRI-XhoI digestion of pCMV-FLAG vector (21) by that of pGEX-ESRα C. pLMH14 was described previously (26). pGL3-EREX3-TATA-Luc, pcDNA3-ERα, pcDNA3-hERβ were kindly provided by Dr. Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo). JFH1 expression plasmid was provided by Dr. Wakita (National Institute of Infectious Diseases).

Luciferase Assay—A luciferase assay monitoring HCV replication activity was performed as described previously (22, 26). In Fig. 1, *A* and *F*, we used LucNeo#2 cells, stably carrying luciferase-containing subgenomic replicon (22). In Figs. 2 (*D* and *E*), 4*C*, and 6*A*, we transiently transduced luciferase-containing replicon LMH14 RNA together with each expression plasmid into cured MH-14 cells (26). A luciferase assay detecting the transcriptional activity driven from the ERE was performed as described previously (25).

Real Time RT-PCR Analysis—Real time RT-PCR analysis was performed as previously described (20).

Immunoblot Analysis—Immunoblot analysis was performed as previously described (25). The antibodies used in this study are anti-NS5A (kindly provided by Dr. Takamizawa (Osaka University)), anti-NS5B (anti-NS5B#14; a generous gift from Dr. Kohara (Tokyo Metropolitan Institute of Medical Science)), anti-NS5B (NS5B#6; a kind gift from Dr. Fukuya (Osaka University)), anti-tubulin (Oncogene), anti-FLAG (Sigma), anti-IκBα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-calnexin (StressGen), and anti-caveolin-2 antibodies (BD Biosciences Pharmingen).

Indirect Immunofluorescence Analysis—Indirect immunofluorescence analysis was performed as described previously (25). The antibodies used were anti-NS5A and anti-protein-disulfide isomerase antibodies (StressGen).

siRNA—siRNA duplexes (5′-GUGUGCAAUGACUAUGCUUCA-3′ for si-ESRα and 5′-CGCAUCGGGAUAUCACUAUGG-3′ for si-ESRβ) were synthesized (Proligo). A randomized siRNA, si-control, was purchased from Dharmacon (nonspecific control duplex IX).

Enzyme-linked Immunosorbent Assay—HCV core was quantified in the culture medium of the cells transfected with JFH1 RNA (29) with an enzyme-linked immunosorbent assay according to the manufacturer's protocol (HCV antigen enzyme-linked immunosorbent assay test; Ortho-Clinical Diagnostics).

RT-PCR Analysis—RT-PCR analysis was performed as described (20) by using the following primer sets: 5′-CCTACTACCTGGAGAACG-3′ and 5′-GCTGGACACATATAGTCG-3′ for the detection of ESRα and 5′-AGCCATGACATTCTATAGC-3′ and 5′-CCACTTCGTAACACTTCC-3′ for ESRβ.

GST Pull-down Assay—The GST pull-down assay was conducted as described previously (25).

Immunoprecipitation Analysis—Immunoprecipitation analysis was performed as described previously (25). The antibodies