NS2 and NS3 and by host proteases including signal peptidase and signal peptide peptidase. Viral structural protein, capsid protein (core) and two envelope proteins (E1 and E2) occupy the N-terminal third of the polyprotein, while nonstructural (NS) proteins located in the remaining region. NS3, NS4A, NS4B, NS5A and NS5B are essentially required for autonomous replication in the replicon cells [5]. NS3 possesses the RNA helicase and protease activities [16,17], and NS4A fulfils anchoring NS3 on the intracellular membrane [18]. NS4B is a membrane protein modelling the ER membrane in order to make it suitable for efficient HCV viral replication [19]. NS5A is a phosphoprotein required for HCV replication [20], because adaptive mutations for efficient RNA replication in the HCV replicon were selectively introduced into the NS5A coding region [21]. NS5B is the active subunit of the replication complex known as an RNA-dependent RNA polymerase [22]. Recent reports suggest that several host proteins attend to the formation of the HCV replication complex [9,10,23,24]. In this review, we summarise the physiological and pathological functions of the host proteins that directly or indirectly participate in the replication of HCV.

IMMUNOPHILINS AND HSP90

The peptide bond *cis/trans* isomerases catalyse the conversion between *cis* and *trans* peptide bonds for correct folding of the protein substrate, including peptidyl prolyl *cis/trans* isomerase (PPIase), such as the families of cyclophilins [25], FK506-binding proteins (FKBP) [26,27] and parvulins [28] and the secondary amide peptide bond *cis/trans* isomerase (APIase) [29]. Cyclophilin and FKBP are classified as immunophilins capable of binding to immunosuppressants cyclosporine and FK506, respectively [30]. The family members do not share a homologous domain with each other, based on their amino acid sequences, substrate specificities and inhibitor sensitivities. Recently, cyclophilin B and FKBP8 were shown to interact with NS5B and NS5A, respectively, and to regulate HCV replication [9,10], suggesting that the immunophilins are promising therapies for chronic hepatitis C (Figure 1).

Cyclophilin B

A study of the host gene related to resistance to retrovirus infection revealed that HIV capsid interacts with cyclophilin A [31], which is incorporated into viral particles, but its precise functions in the viral life cycle have not been elucidated yet. HIV particles lacking cyclophilin A exhibited no abnormality in virus packaging, reverse transcriptase activity or capsid stability [32]. However, in macaque cells, cyclophilin A modulates conformation of gag capsid protein to facilitate the interaction with TRIM5alpha, a potent antiretroviral restriction factor and confers resistance to human

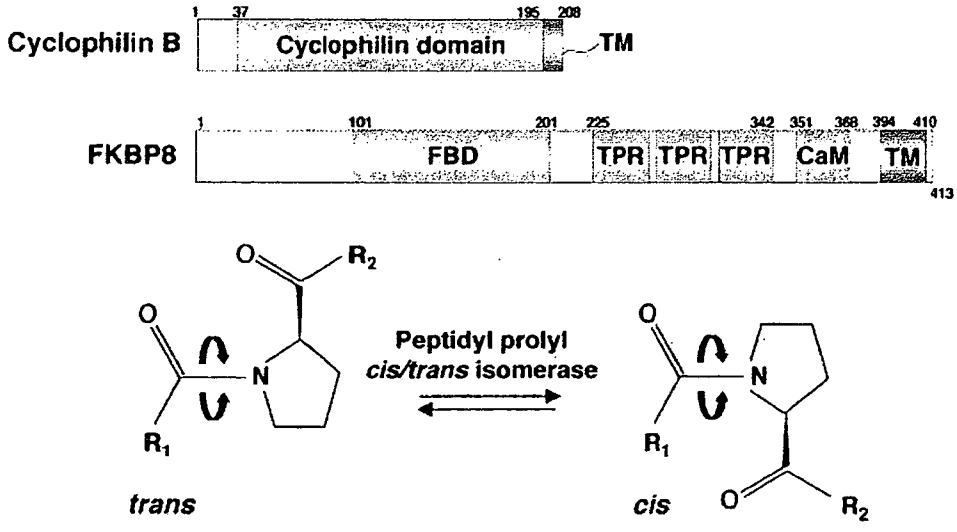

Figure 1. Structures of cyclophilin B and FKBP8. Cyclophilin B possesses a cyclophilin domain and a transmembrane region. FKBP8 has an FK506-binding domain (FBD), three sets of tricopeptide repeats (TPRs), a calmodulin-binding domain (CaM) and a transmembrane region (TM). Both proteins catalyse the conversion between *cis* and *trans* propyl peptide bonds for correct folding of protein substrate

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retrovirus, which participates in the establishment of host range restriction [33,34].

Cyclophilin B, formerly called s-cyclophilin, is identified as a 20 kDa secreted neurotrophic factor for spinal cord cells of chick embryo [35], and it is secreted into human milk and blood [36,37]. Extracellular cyclophilin B enhances the retrotranslocation of prolactin into nucleus [38], is implicated in the presynaptic function by interacting with synaptin I, and impairs the correct folding of prion protein in the presence of cyclosporin A, leading to accumulation in aggresomes [39]. Therefore, cyclophilin B may regulate the correct folding and translocation of host proteins under extracellular and intracellular conditions, although its precise functions are still unknown.

Cyclosporin A and its derivatives capable of inhibiting cyclophilins were shown to inhibit HCV RNA replication and to be effective in the treatment of hepatitis C patients [9,40,41]. Inoue *et al.* [42] reported at the first time that cyclosporin A is effective for the treatment of hepatitis C patients. Cyclosporin derivatives lacking the ability to interact with cyclophilin lost their inhibitory effect on HCV replication [9]. Cyclophilin B was shown to specifically interact with NS5B, the HCV RNA-dependent RNA polymerase, around the ER of the HCV replicon cells and to promote NS5B's association with the viral RNA [9]. Cyclosporin A was shown to disrupt interaction between NS5B and cyclophilin B [9] (Figure 2). Treatment with cyclosporin A and knockdown of cyclophilin B suppressed the replication of HCV, suggesting that cyclophilin B plays an important role in HCV genome replication by enhancing the interaction between NS5B and viral RNA [9].

FKBP8

HCV NS5A is an essential component of the viral replication complex, although NS5A's function has not been clarified yet. We screened the human fetal brain and liver libraries using a yeast two-hybrid system that employs HCV NS5A as bait and identified FKBP8 as an NS5A-binding partner [10] (Figure 2). An immunoprecipitation analysis revealed that NS5A bound to FKBP8 but not to FKBP52 or cyclophilin D, all three of which have homology to each other.

FKBP8 belongs to the FKBP family based on sequence similarity, but lacks the amino acid residues essential for either FK506 binding or PPIase activity [43]. Recent biochemical and enzymological studies indicate that FKBP8 has weak PPIase activity and low affinity to FK506 [44,45], suggest-

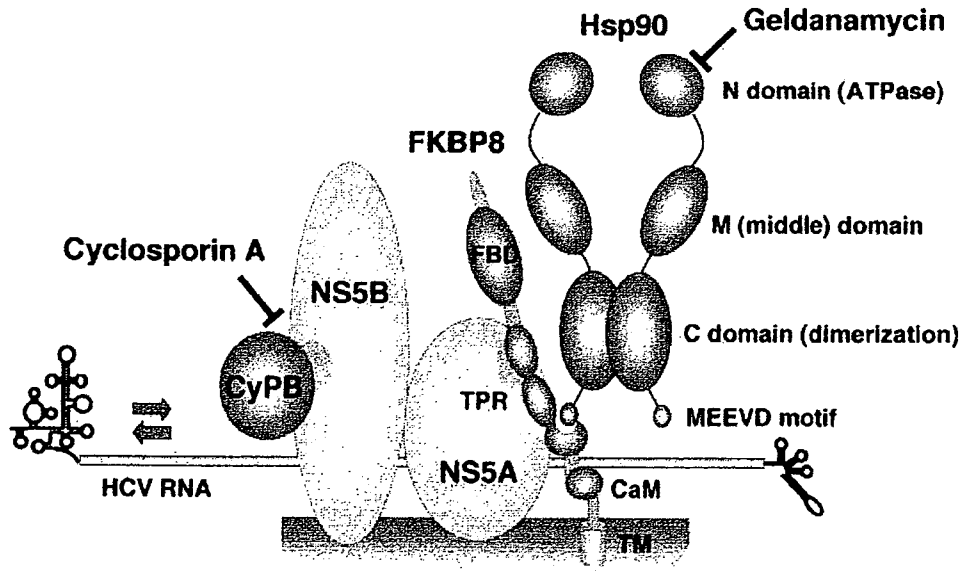

Figure 2. Interaction of HCV NS5A and NS5B proteins with immunophilins and Hsp90. Cyclophilin B interacts with NS5B. FKBP8 interacts with NS5A and Hsp90 through the different regions within TPR domains. Lys^{307} and Arg^{311} of the FKBP8 carboxylate clamp motif are required for binding to the MEEVD motif of Hsp90. Cyclosporin A inhibits interaction between cyclophilin B and NS5B. Geldanamycin is an inhibitor of the ATPase activity of Hsp90

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ing that FK506 is unable to modulate FKBP8 function. Previously, FKBP8 was termed FKBP38 or FKBPr38 (FKBP-related protein 38 kDa) from the deduced molecular weight of 38 kDa based on the fact that the incomplete amino acid sequence was missing the N-terminal part of the authentic FKBP8. The true transcription and translation initiation sites were identified in the upstream of the original start site in the genomic sequences [46]. The FKBP8 splicing variants of 44 and 46 kDa were detected in mouse but not in human, and the 45 kDa of human FKBP8 corresponds to the 44 kDa of murine protein [46].

The physiological function of FKBP8 is largely unknown, but is slightly elucidated from the data of genetically manipulated mice [47]. FKBP8−/− mice exhibit a phenotype similar to that of mutant mice under the excessive activation of the Sonic hedgehog (Shh) protein, a secreted morphogen that regulates the patterning and growth of many tissues in the developing mouse embryo [47]. Human and mouse have three species of hedgehog proteins: Indian hedgehog (Ihh), Desert hedgehog (Dhh) and Shh [48,49]. Ihh and Dhh are predominantly expressed in bone and gonads, respectively, whereas Shh is ubiquitously expressed in many organs such as brain, liver and lungs. Shh is secreted as glycoprotein from the ventral midline of the spinal cord and is involved in the regulation of the genes related to the control of ventral fate in the spinal cord and forebrain [50,51]. Hedgehog protein generally binds to the receptor protein Patched (Ptc) and then inhibits the function of the membrane protein Smoothened (Smo) [52,53]. Smo activates the protein kinase A, which suppresses the transcription factor GLI protein by phosphorylation [54]. Phosphorylated GLI was inactivated by cleavage and acts as a transcriptional repressor against a full length of GLI in hedgehog signalling [54]. Hedgehog protein binds to the receptor Ptc and then inhibits Smo, leading to the accumulation of the full length of the GLI protein [55]. Deficiency in the murine Shh gene or knockouts of the genes required for Shh signal transduction abolished control over morphological formation [51,56]. On the other hand, excessive Shh signalling exhibited the opposite phenotype, including cells that inappropriately adopt ventral identities for dorsal identities [48,57]. FKBP8-deficient mice were reported to exhibit phenotypes similar to those of mice expressing excessive Shh signalling, except that the FKBP8-deficient mice had no abnormalities of the limb pads, bronchial arches or somites [47]. Shh−/− and FKBP8−/− double knockout embryos showed partial rescue of cyclopia and holoprosencephaly, but still showed limb outgrowth defect [47]. These results suggest that Shh signalling in the brain is overlapped with FKBP8-controlled signalling including phosphorylation and protein–protein interaction. Shirane *et al.* [58] suggest that FKBP8 is an inherent phosphatase inhibitor and retains Bcl-2 on mitochondrial membrane to inhibit apoptosis. However, there was no difference between wild-type and FKBP8-deficient mice with respect to apoptosis, suggesting that FKBP8 deficiency does not affect physiological apoptosis. FKBP8 may modulate a phosphatase such as calcineurin to enhance the phosphorylation required for suppression of Shh signalling.

Hsp90

Proteomics analysis reveals that FKBP8 forms a complex with Hsp90 to act as a co-chaperone [10]. Although both NS5A and Hsp90 bound to the TPR domain of FKBP8, interaction between NS5A and FKBP8 did not affect homomultimerisation of FKBP8 or complex formation with Hsp90. The amino acid residues of the carboxylate clump position in the TPR domain of FKBP8 grasp the C-terminal MEEVD motif of Hsp90. Mutations of the residues in the carboxylate clump of FKBP8 suppressed the interaction with Hsp90 but not that with NS5A, suggesting that FKBP8 interacts with NS5A and Hsp90 at different sites within the TPR domain. Knockdown of FKBP8 and treatment with geldanamycin, an ATPase inhibitor of Hsp90, downregulated HCV replication in HCV replicon cells. These data suggest that recruitment of Hsp90 to the replication complex through the interaction between FKBP8 and NS5A is crucial for the replication of HCV (Figure 2). It is also feasible to speculate that NS5A modulates the activity of unidentified phosphatases by the interaction with FKBP8 to facilitate the replication of HCV RNA. Although Hsp90 was shown to be involved in the cleavage between NS2 and NS3 [59], NS2 is not required for the replication of the HCV genome [5].

Hsp90 was suggested to be involved in the enzymatic activity and intracellular localisation of several viral enzymes, including polymerases. Hsp90 was shown to bind to a viral polymerase subunit

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of influenza virus to facilitate the replication complex formation and the nuclear localisation of the viral polymerase subunit [60,61]. The DNA polymerase of herpes simplex virus type 1 required the chaperone activity of Hsp90 for the nuclear localisation of the polymerase [62]. Flock house virus utilises Hsp90 to assemble the complex of the RNA-dependent RNA polymerase on the intracellular membrane [63]. Knockdown and treatment with Hsp90 inhibitor revealed that Hsp90 activity is important for the rapid growth of negative strand RNA viruses [64]. Furthermore, Hsp90 was shown to be required for the activity of the hepatitis B reverse transcriptase [65,66]. Hsp90 generally requires the co-chaperone protein to acquire specificity to the substrate client. Therefore, Hsp90 and co-chaperones are crucial molecules required for the efficient replication of a broad range of viruses and are an ideal target for antivirals with broad spectra. Recently, Hsp90 inhibitors were shown to drastically impair the replication of poliovirus without any emergence of escape mutants [67].

Immunophilins and Hsp90 may be involved in HCV replication through the correct folding of the replication complex required for efficient enzymatic activity. In addition, cyclophilin B may also participate in the translocation of NS5B, as seen in the polymerase subunits of influenza virus, to facilitate binding to viral RNA. Elucidation of the HCV replication complex may lead to the development of new therapeutics for chronic hepatitis C.

VESICLE-ASSOCIATED MEMBRANE PROTEIN-ASSOCIATED PROTEINS

VAPs were originally identified as proteins that bind to vesicle-associated membrane protein (VAMP) in the nematode *Aplysia* and were designated as VAMP-associated protein 33 kDa (VAP-33) [68]. After that, one homologue and its splicing variant were identified as VAP-B and -C, respectively [69], and VAP-33 has been renamed VAP-A. Although VAP-A was suggested to be required for delivery of components into the presynaptic membrane of *Aplysia* ganglion [68,70], in mouse organs both VAP-A and -B localise in the intracellular membrane compartments, including ER, but not in the VAMP [68,71]. In addition, VAP-A, -B and -C are ubiquitously expressed in mammalian organs, such as heart, placenta, lung, liver, skeletal muscle and pancreas [72], suggesting that VAP proteins possess have other functions besides neurotransmitter release [69,70,73].

VAP is a type II membrane protein composed of three functional domains: the N-terminal half of the protein, which is highly homologous with the nematode major sperm protein (MSP); the coiled-coil domain and the transmembrane domain. VAP-A shares 60% identity with VAP-B, while VAP-C is the splicing variant of VAP-B that lacks a transmembrane domain [69]. MSP was identified as one of the major proteins of the nematode sperm [74] and forms a microfilament required for amoeboid motility through the push–pull theory. MSPs form a subfilament by homodimerisation through the Ig-like domain and coiled coil around each other to form a filament. Several filaments are further assembled around each other to make a macrofiber [75,76]. The MSP-like domain was identified in several mammalian, avian, arthropod, plant and fungal proteins but not in protist proteins [77].

VAP-interacting proteins share the FFAT motif represented by the consensus amino acid sequence EFFDAxE as determined by a comparison of oxysterol-binding protein-related proteins (ORPs) [78]. However, both VAMP and tubulin are capable of binding to VAP proteins in an FFAT-independent manner [70,79–81]. In yeast, Opi1p is the transcriptional repressor of the INO1 gene, which encodes an inositol-1-phosphate synthase [72,82]. SCS2p is a yeast homologue of VAP and interacts with Opi1p through the FFAT motif to regulate the expression of the INO1 gene [78]. In mammals, ceramide is transported by the cargo protein CERT from ER to Golgi for the synthesis of sphingomyelin [83,84]. VAP-A and -B could anchor CERT via the FFAT motif to uptake ceramide by CERT in ER [85], suggesting that VAPs serve as anchors for the transporter of ceramide in mammalian cells rather than as a component of neurotransmitter release machinery.

VAP-A and -B were reported to be NS5A-binding host proteins by the screening of the human hepatoma cell line library using NS5A as bait in yeast [23,24]. GST pulldown and immunoprecipitation analyses revealed that NS5A and NS5B interact with human VAP-A and that the N-terminal MSP domain and the coiled-coil domain of VAP-A are responsible for the binding to NS5B and NS5A, respectively [24] (Figure 3). Several host kinases were shown to phosphorylate NS5A,

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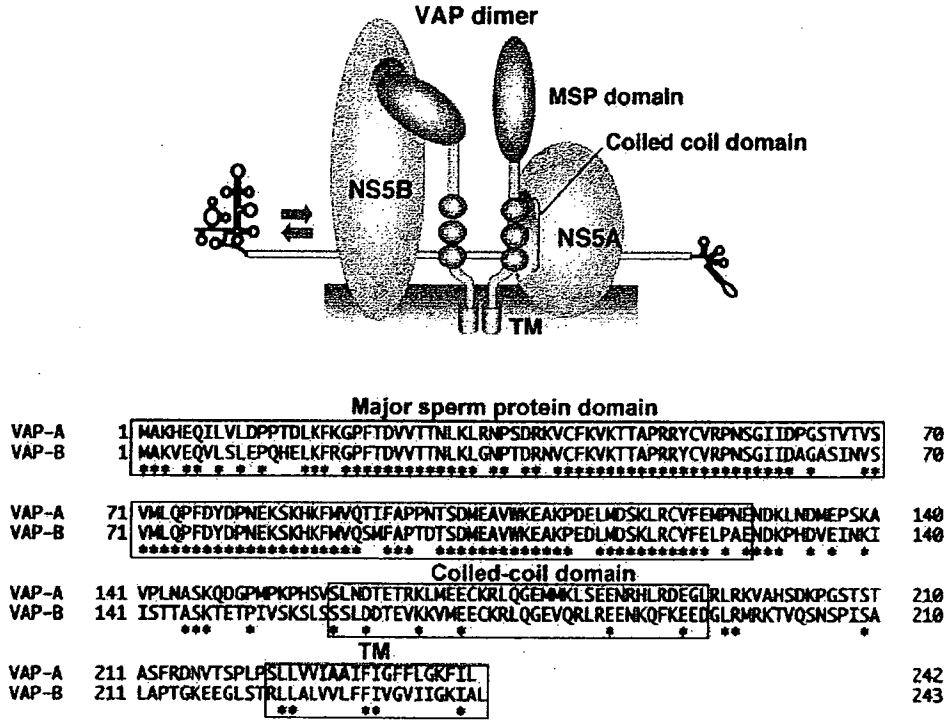

Figure 3. Interaction between HCV NS5A protein and VAPs. VAP-A and VAP-B make homo- and hetero-dimers with each other. The VAP dimer interacts with NS5A and NS5B through the coiled-coil domain and the MSP domain, respectively. VAP-A and VAP-B share 62.9 and 84.9% homology in total and in the MSP domain, respectively

and the hyperphosphorylation of NS5A abrogates the interaction with human VAP-A, which leads to the downregulation of HCV replication [20,86–88]. Adaptive mutation for an efficient replication of HCV RNA in the Huh7 cell line was associated with hypophosphorylation of NS5A, which enhances binding to VAP-A [20]. NS5A of HCV genotype 1a H77 strain was shown to be hyperphosphorylated in both yeast and replicon cells, and no interaction with VAP-A was detected in yeast, suggesting that hyperphosphorylation of NS5A may suppress HCV RNA replication through by counteracting binding to VAP-A [20]. However, we have demonstrated that NS5A of genotype 1a H77 strain is capable of binding not only to VAP-A but also to VAP-B at levels similar to that of genotype 1b in mammalian cells [23].

Several reports suggest that HCV replication takes place on the detergent-resistant membrane fraction [6,89,90]. NS4B is predominantly associated with a lipid-raft-like detergent-resistant fraction, and both NS5A and NS5B are co-localised in the similar fraction in the presence of NS4B [89]. VAP-A was also localised in the detergent-resistant fraction, suggesting that it plays an important role in HCV replication, because the dominant negative mutant of VAP-A suppressed the replication of HCV RNA [89]. VAP-B forms a homodimer and heterodimer with VAP-A, and knockdown of VAP-A or VAP-B led to a substantial suppression of HCV replication [23,91], suggesting that heterodimerisation of VAPs could regulate HCV replication (Figure 3). The host proteins possessing the FFAT motif are related to biosynthesis and translocation of lipid [81], whereas NS5A and NS5B do not have the typical FFAT motif. Although replication of HCV RNA did not affect lipid biosynthesis, lipid components are required to form the HCV replication complex as described below. VAPs might be involved in the transport of lipid components to the HCV replication complex through the interaction with NS5A and NS5B, resulting in the upregulation of HCV replication. VAP-B was shown to interact with Nir2 protein through the FFAT motif and to remodel the ER structure [92]. It can therefore be speculated that VAPs are asso-

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ciated with remodelling of the HCV replication complex in the ER membrane through interaction with Nir2 protein.

HOST PROTEINS MODIFIED BY LIPID AND INVOLVED IN LIPID BIOSYNTHESIS

Lipid components are required for the assembly, budding and replication of several viruses [93–97]. Increases in saturated and monounsaturated fatty acids enhance HCV RNA replication, in contrast to suppression by polyunsaturated fatty acids [98], suggesting that enzymes associated with lipid biosynthesis are also involved in HCV replication. SREBP-1c regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase and stearoyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides [99]. Expression of HCV core protein induces the production of lipid droplets composed mainly of triglyceride [100]. Our recent study suggests that SREBP-1c was upregulated in the liver of transgenic mice expressing HCV core protein through the LXRalpha/RXRalpha-dependent pathway, which leads to the development of fatty liver [101]. The upregulation of SREBP-1c in the core transgenic mice was required for expression of PA28gamma, an HCV core-binding host protein involved in the activation of nuclear proteasome activity. Saturated or monounsaturated fatty acid may be utilised for the formation of HCV replication complex with cholesterol and sphingolipid [98]. A lipophilic long-chain compound derived from microbial metabolites, an inhibitor of sphingolipid biosynthesis, was shown to inhibit HCV replication [6]. The HCV replication complex is shown to be localised in the lipid raft including sphingolipid [89,90,102]. Therefore, compounds disrupting sphingolipid biosynthesis may inhibit the replication of HCV through the modification of the lipid raft (Figure 4).

HCV replication was also disrupted with an inhibitor of geranylgeranyl transferase I but not with that of farnesyl transferase [103], suggesting that geranylgeranylation of viral or host protein regulates HCV replication efficiency [103]. Geranylgeranylate is an intermediate of the mevalonate pathway and is attached to various cellular proteins for anchoring to plasma or intracellular membrane [99]. Wang *et al.* [104] reported that geranylgeranylated FBL2 is required for the efficient replication of HCV genomic RNA. FBL2 had been identified as a structural homologue of Skp2, which interacts with Skp1 for S-phase entry and conserves the structural motif of F-box for Skp1 binding [105]. The immunoprecipitation analysis revealed that NS5A interacts with FBL2 [104]. The F-box motif is located in the N-terminus of FBL2, followed by 11 leucine-rich repeats [105]

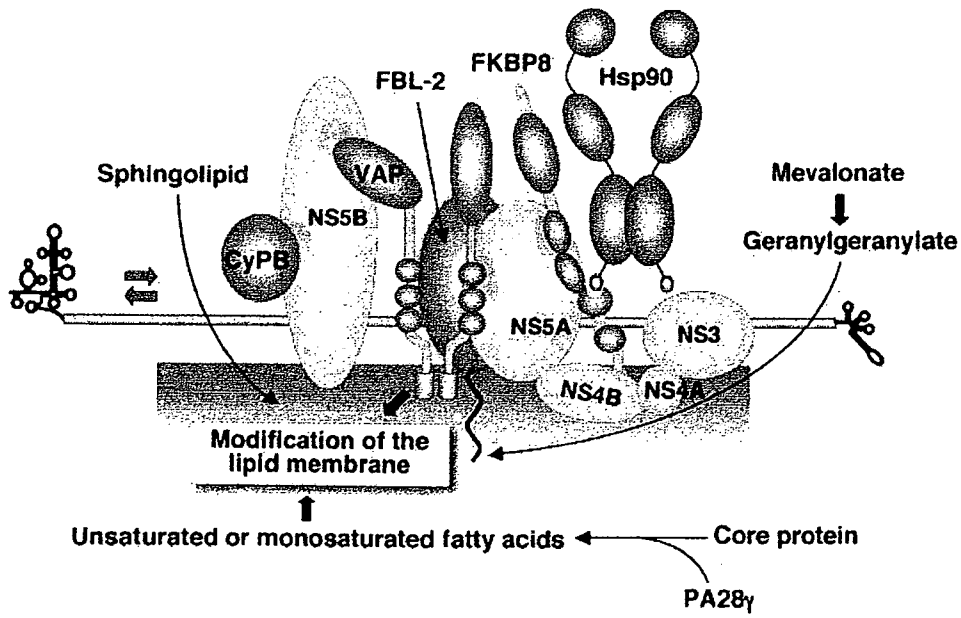

Figure 4. Putative model of HCV replication complex composed of viral and host proteins

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and the CAAX motif (CVIL), which is suggested to be modified by geranylgeranylation [104]. FBL2 lacking the CAAX motif was not modified by geranylgeranylation and lost the interaction with NS5A [104]. An F-box-truncated FBL2 mutant suppressed the replication of HCV as a dominant negative, whereas a mutant in the residues responsible for geranylgeranylation exhibited no suppressive effect [104]. The geranylgeranylated FBL2 is required for the replication of HCV but not for that of West Nile virus [104]. Furthermore, knockdown of FBL2 in the replicon cells induced suppression of HCV replication but not in cells expressing an siRNA-resistant FBL2 [104]. The F-box motif is generally essential for the formation of the ubiquitin ligase complex [105], suggesting that FBL2 regulates the ubiquitination of host or viral proteins through the interaction with NS5A. Another possibility is that FBL2 may retain the viral replication complex by interacting with NS5A (Figure 4).

CONCLUSION

The host machineries of lipid biosynthesis, protein folding and anchoring in the intracellular compartment may cooperate with HCV proteins to facilitate the replication of the viral genome. In addition, translation of the viral genome is also expected to utilise the host proteins to generate viral proteins. Other host factors such as cellular RNA helicase p68 and nucleolin were also reported to be involved in HCV RNA replication [106,107]. The primary concern of chronic hepatitis C is the development of hepatocellular carcinoma through liver steatosis and fibrosis. HCV proteins could potentiate the production of reactive oxygen species, which may activate STAT3 leading to carcinogenesis [101,108–111]. Among HCV proteins, only the core protein was shown to be involved in the induction of carcinogenesis [112–114]. Data on the replication of HCV cooperating with host proteins have been accumulated by using RNA replicon and cell culture systems. Further studies on the host proteins involved in viral replication and carcinogenesis are needed for the development of therapeutic measures for chronic hepatitis C.

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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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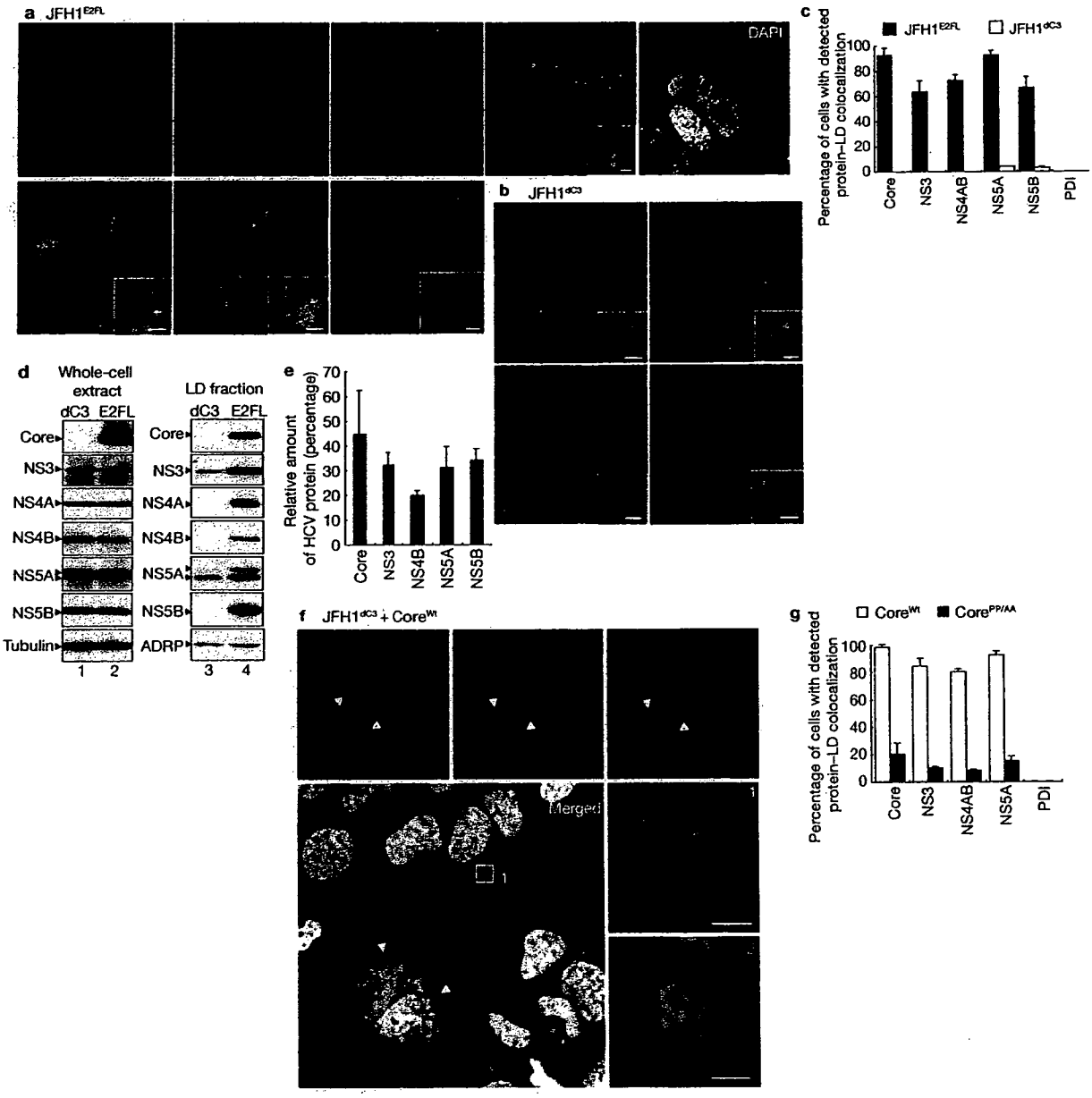

Figure 1 Core recruits NS proteins to LDs. **(a)** Huh-7 cells transfected with JFH1^{E2FL} RNA were labelled with antibodies against Core (red), NS5A (blue), NS3 (red), NS4AB (red) or NS5B (red). Lipid droplets (LDs) and nuclei were stained with BODYPI 493/503 (green) and DAPI (white in upper panel, 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 $^{PP/AA}$ ($n > 200$). 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[15]. 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

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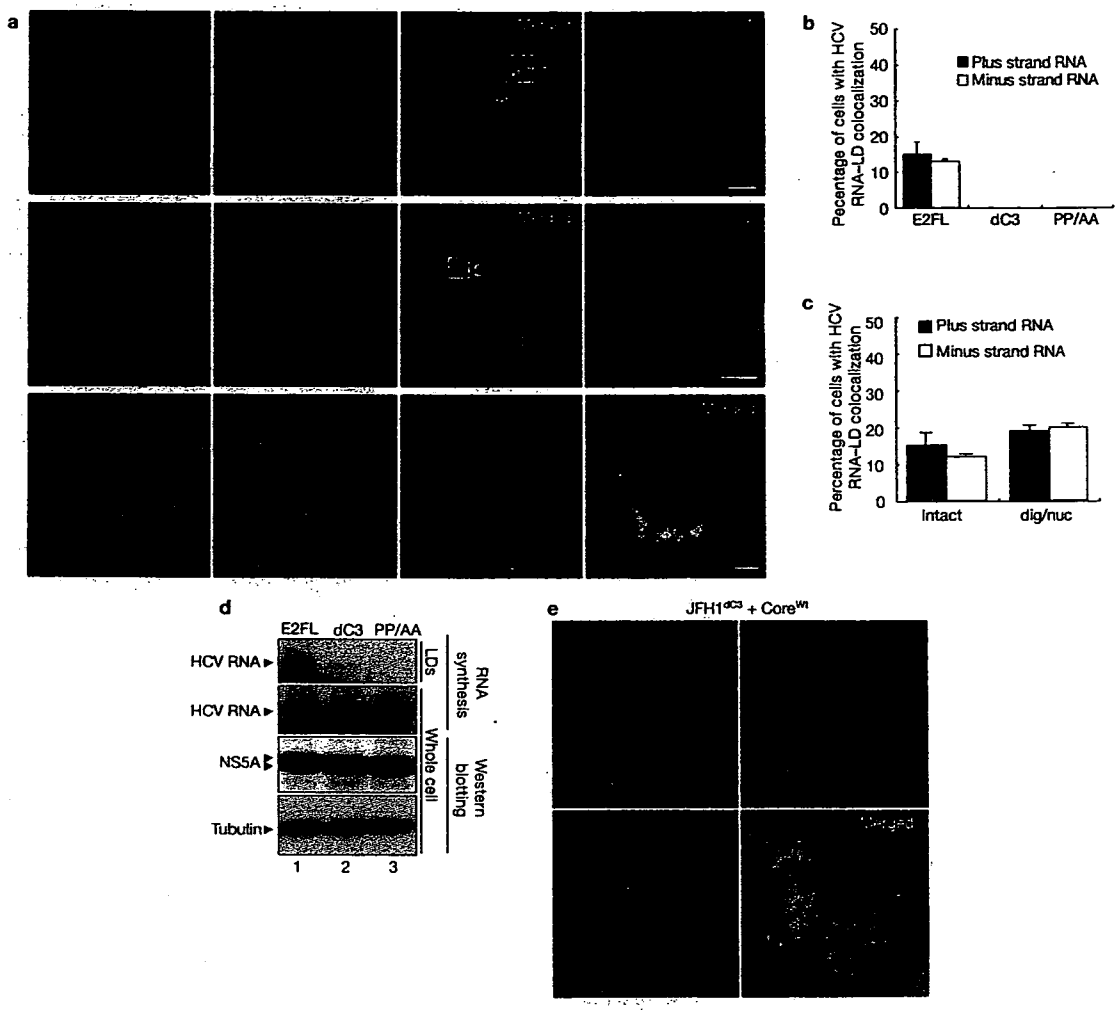

Figure 2 Core-dependent recruitment of active HCV replication complexes to the LD. (**a**) Huh-7 cells transfected with JFH1^{E2FL} RNA were analysed by *in situ* hybridization with strand-specific probes (plus or minus). The cells were labelled to simultaneously visualize lipid droplets (LDs), NS5A 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

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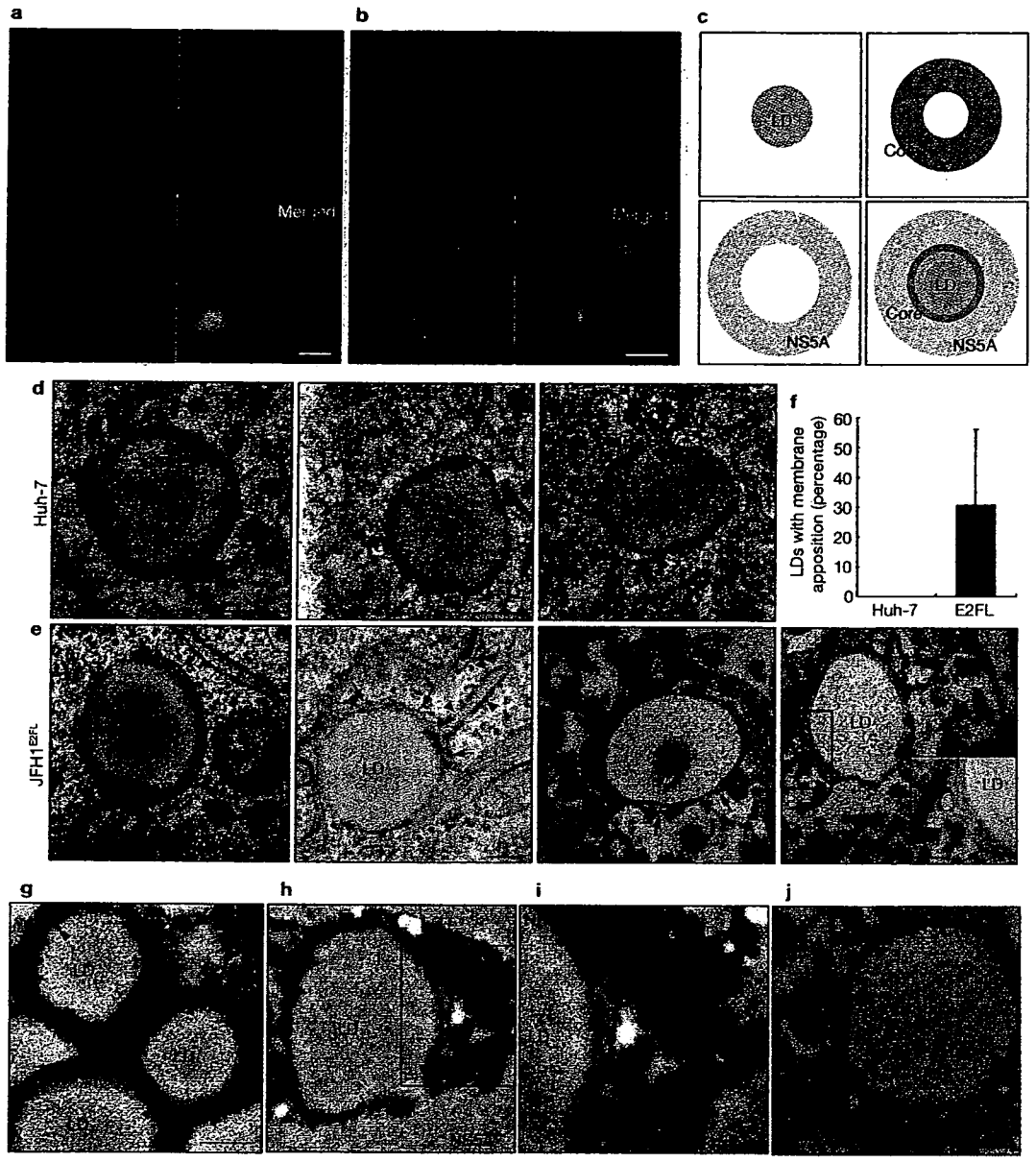

Figure 3 Spatial distribution of Core and NS5A relative to the LD. (**a**, **b**) The localizations of Core, NS5A and ADRP around the lipid droplets (LDs) in JFH1^{E2FL} replicon-bearing cells were analysed using immunofluorescence microscopy. Scale bars, 1 μm. (**c**) Typical images of the localization 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

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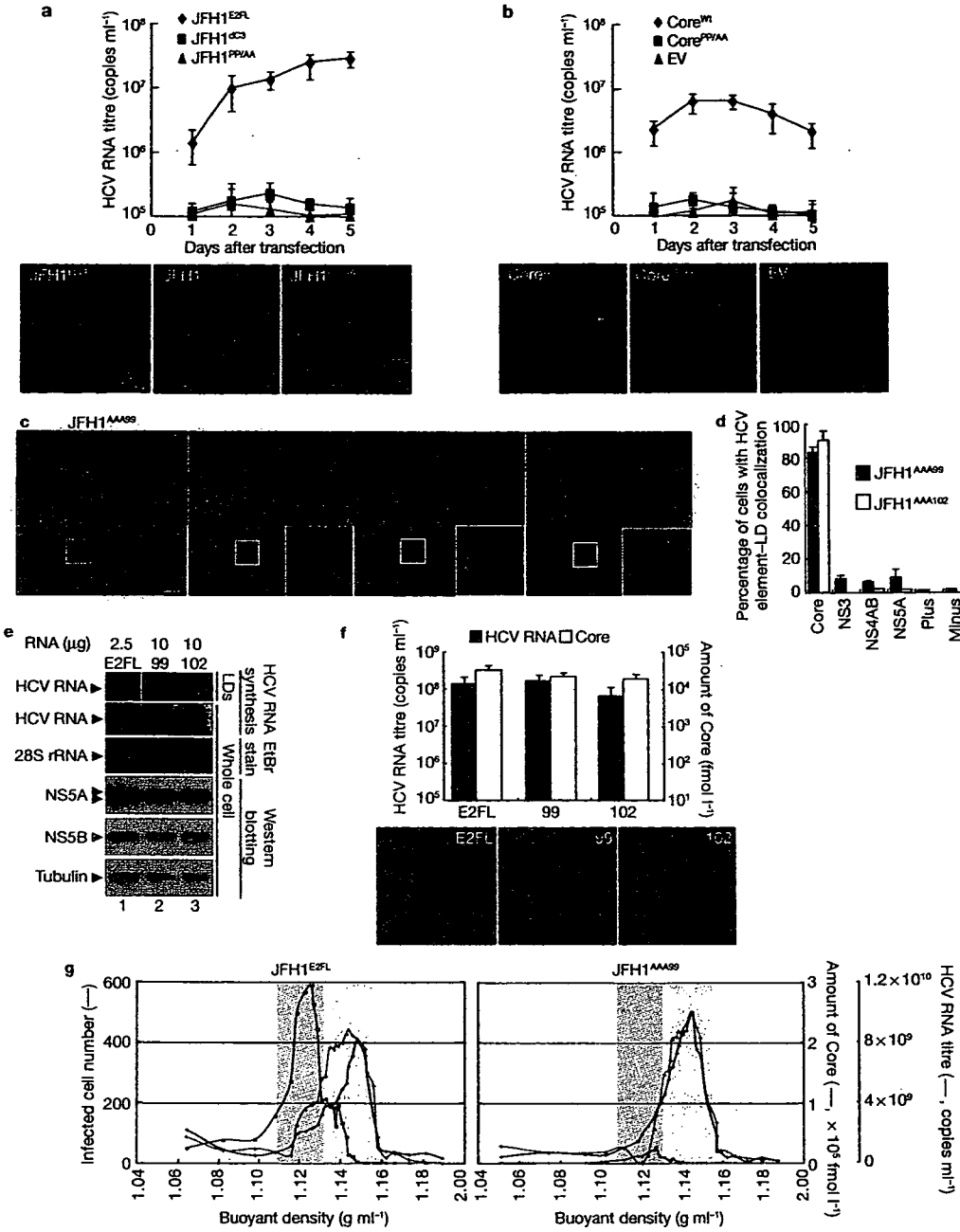

Figure 4 LD associations of Core and NS proteins are necessary for the production of infectious HCV particles. (**a**) The culture medium from JFH1^{E2FL}-, JFH1^{dC3}- or JFH1$^{PP/AA}$-replicating cells was collected at the indicated time points and the titre of hepatitis C virus (HCV) RNA was measured 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.

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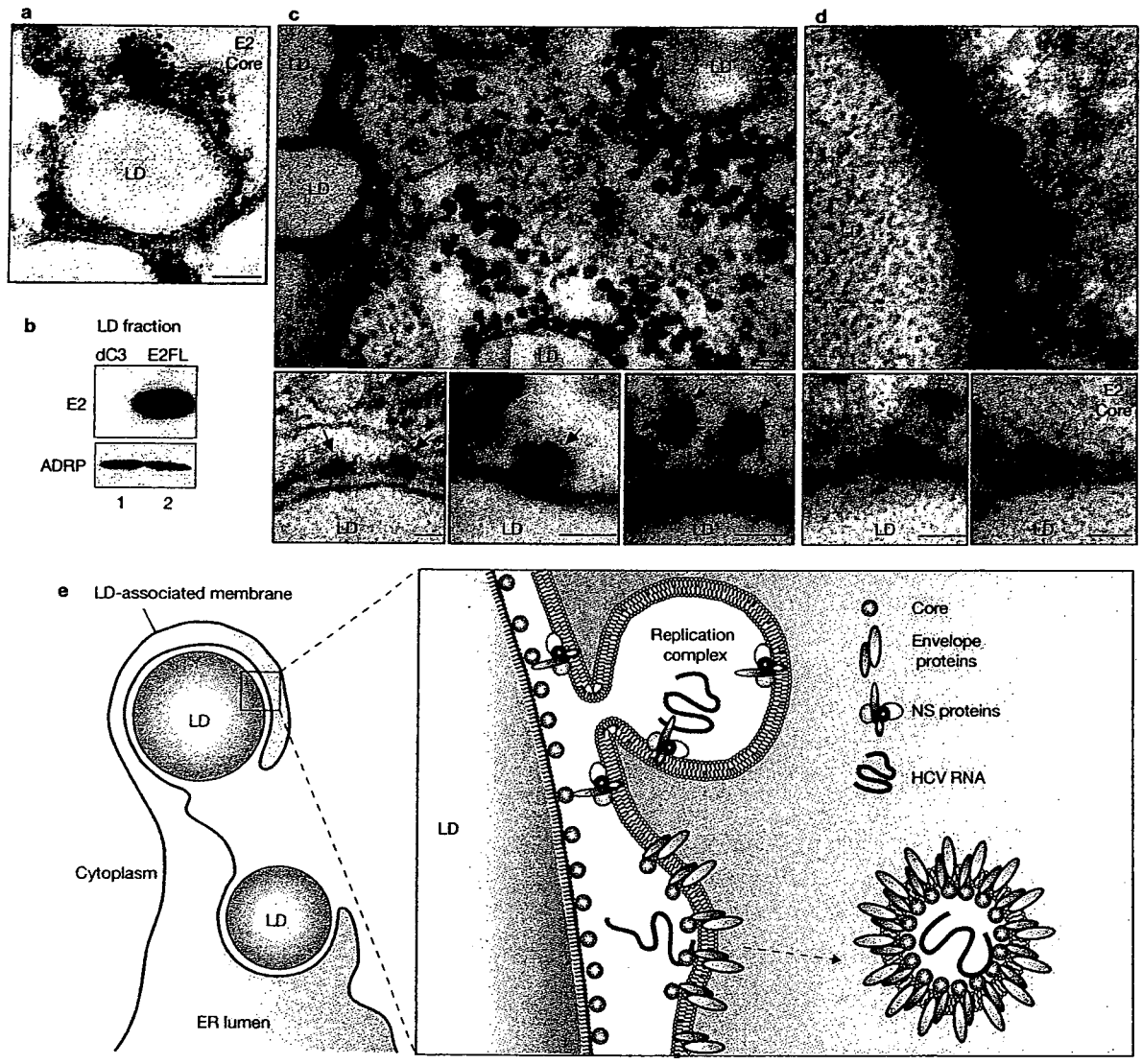

Figure 5 Virus-assembly takes place around the LDs. (**a**) Immunoelectron microscopic detection of E2 and Core in JFH1^{E2FL}-replicating cells. E2 and Core are labelled with 15 nm and 10 nm gold particles, respectively. (**b**) Western blot analysis of the lipid droplet (LD) fraction from JFH1^{E2FL} 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.

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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

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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.

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