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H.知的財産権の出願・登録状況

1.特許取得

なし

2.実用新案登録

なし

3.その他

特になし

別紙4　　研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷り

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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

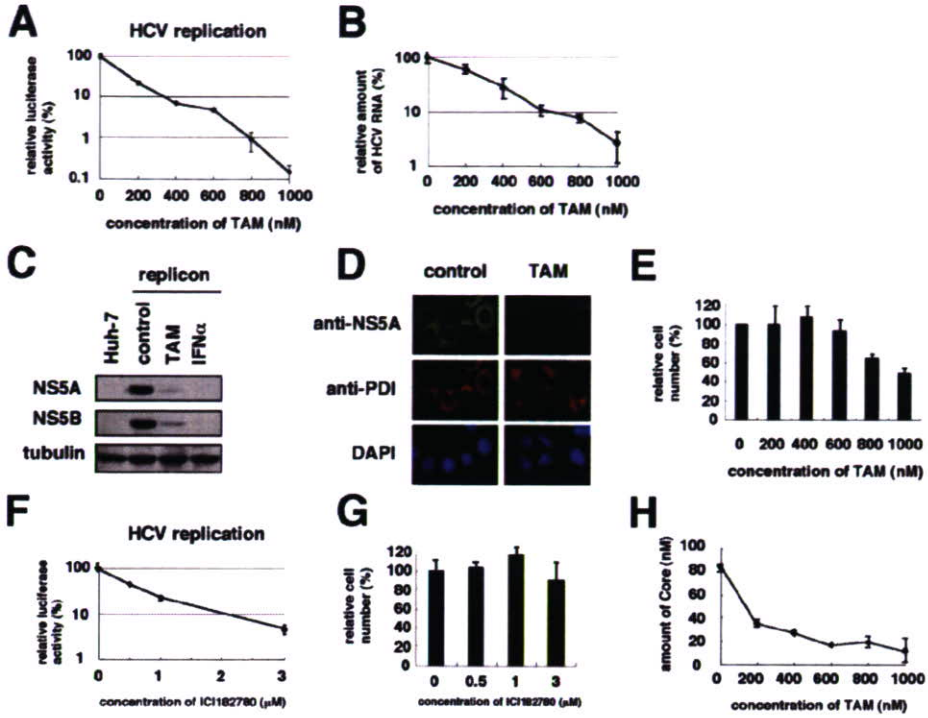

FIGURE 1. **TAM suppressed the replication of the HCV genome.** *A*, luciferase activities were measured using the LucNeo#2 cells, which carried a luciferase-containing replicon RNA, upon treatment with TAM at the indicated doses for 7 days. Relative luciferase activities are plotted against the concentrations of TAM. The data show the means of three independent experiments. The *error bars* are indicated. *B*, HCV RNA was quantified by real time RT-PCR analysis using the lysates from MH-14 cells, harboring the HCV subgenomic replicon, treated with the indicated doses of TAM for 7 days. Relative amounts of HCV RNA are shown. *C*, HCV NS5A and NS5B proteins as well as tubulin as an internal control were detected by immunoblot analysis in the lysates from MH-14 cells (replicon) treated without (control) or with 500 nM TAM or 100 IU/ml interferon-α as a positive control for 7 days and Huh-7 cells. *D*, HCV NS5A and protein-disulfide isomerase (*PDI*) as an internal control were detected by indirect immunofluorescence analysis in the cells treated without (control) or with 500 nM TAM for 7 days. 4′,6-Diamidino-2-phenylindole (*DAPI*) shows a nuclear staining. *E*, cell number was counted after 5 days upon treatment with various concentrations of TAM. Relative cell numbers are shown. *F*, luciferase activities with LucNeo#2 cells treated with various concentrations of ICI182780 were measured as described in *A*. *G*, cell number was counted under treatment with ICI182780 at the indicated concentrations. *H*, core in the culture medium of JFH1 RNA-transfected cells upon treatment with TAM was quantified as described under "Experimental Procedures."

used in this study were mouse normal IgG as a negative control (Zymed Laboratories), anti-NS5B (anti-NS5B#10; a generous gift from Dr. Kohara at the Tokyo Metropolitan Institute of Medical Science), anti-FLAG, and anti-caveolin-2 antibodies.

Fractionation of Cell Extracts—MH-14 cells transfected with the expression plasmid for FLAG-tagged ESRα were fractionated essentially as described previously (25).

HCV Replication Complex Assay—Isolation of HCV RC was done as described previously (16, 21).

In Vitro HCV Infection Experiment—*In vitro* HCV infection was conducted essentially as described (23). Briefly, HCV-infected serum (~2×10^5 copies) was inoculated into HuS-E7/DN24 cells (5×10^4 cells) for 24 h. After washes, cells were cultured in the medium supplemented with 10 μM PD98059 to stimulate HCV translation (27) (*scheme* in Fig. 6*B*). To observe HCV amplification, HCV RNA in the cells was quantified, since HCV RNA was hardly detected significantly in the culture medium (23).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay—The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to examine the cell viability using Cell Proliferation kit II, XTT (Roche Applied Science) according to the manufacturer's protocol.

RESULTS

Tamoxifen Suppressed HCV Genome Replication—We screened for agents that suppressed HCV genome replication using a HCV subgenomic replicon system (13, 16). Among the compounds tested, we observed that TAM inhibited HCV genome replication. HCV replication activity, monitored by luciferase activity (22), and the amount of HCV RNA were decreased with TAM treatment in a dose-dependent manner (Fig. 1, *A* and *B*). The expression of HCV proteins, NS5A and NS5B, detected by immunoblot (Fig. 1*C*) and indirect immuofluorescence analyses (Fig. 1*D*), also drastically decreased by treatment with TAM. A high concentration of TAM decreased cell proliferation (Fig. 1*E*). However, TAM suppressed HCV replication without any cytotoxicity in another cell line, HuS-E7/DN24 cells (Fig. 6, *C* and *D*). In addition, a pure anti-estrogen compound ICI182780, which had little cytotoxic effect, reduced HCV RNA (Fig. 1, *F* and *G*). Moreover, TAM inhibited the production of core in the culture medium of HCV JFH1-transfected cells, in a recently developed system of the production of infectious HCV particles (Fig. 1*H*) (28–30). The above data indicate that TAM suppresses HCV genome replication.

ESR Was Involved in HCV Genome Replication—Next, we investigated which cellular protein TAM targets to suppress HCV replication. It has been reported that TAM targets 1) ESR (31), 2) P-glycoprotein (32, 33), 3) calmodulin (34), 4) protein kinase C (35, 36), etc. Although other compounds targeting P-glycoprotein, calmodulin, and protein kinase C did not affect HCV replication in our screening (data not shown), ESR was suggested to play a role in HCV replication as shown below.

RNAi-mediated specific knockdown of endogenous ESRα and ESRβ (Fig. 2*A*) reduced HCV RNA in replicon-containing cells to ~20–40% and 60–70%, respectively (Fig. 2*B*). Transient transfection with ESRα and ESRβ expression plasmids, which activated ERE-driven transcription 4–5-fold (Fig. 2*C*), showed that ectopically expressed ESRα augmented HCV replication activity in a dose-dependent manner, whereas ESRβ did not (Fig. 2*D*). ESRα-induced augmentation of the replication was reversed upon TAM treatment (Fig. 2*D*). These results suggested a significant role of ESR, especially ESRα, in HCV

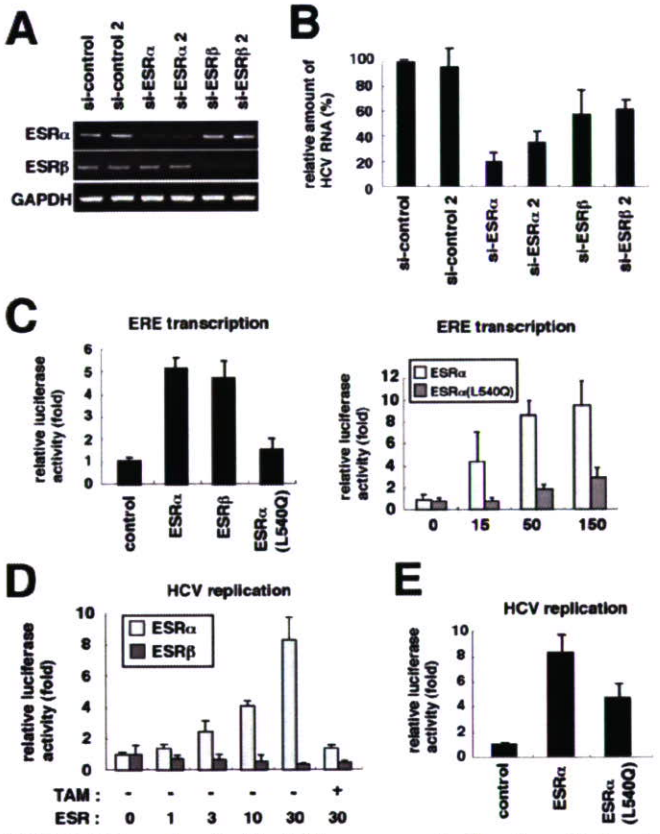

FIGURE 2. **ESR was involved in HCV genome replication.** *A*, specific knockdown of endogenous ESRα and ESRβ. RT-PCR analysis was performed to detect the expression of ESRα, ESRβ, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control in the cells transfected with siRNA recognizing ESRα (si-ESRα, si-ESRα2), ESRβ (si-ESRβ, si-ESRβ2), or randomized siRNA (si-control, si-control2). *B*, HCV RNA was quantified as shown in Fig. 1*B*, using the cells transfected with si-control, si-control2, si-ESRα, si-ESRα2, si-ESRβ, and si-ESRβ2 for 5 days. *C*, the ERE-mediated transcriptional activities were measured by a luciferase assay using the lysates from the cells transfected with pGL3-EREX3-TATA-Luc reporter plasmid together with pcDNA3-ERα (ESRα), pcDNA3-hERβ (ESRβ), pcDNA-ESRα(L540Q), or the empty vector (control) (*left*) or varying amounts (ng) of pcDNA3-ERα (ESRα) or pcDNA-ESRα(L540Q) (*right*) and treated with 100 nM estradiol for 36 h. *D* and *E*, HCV replication activities were examined by quantifying the luciferase activities using cured MH-14 cells transfected with the indicated doses (ng) of ESRα or ESRβ (*D*) or 30 ng of ESRα, ERα(L540Q), or the empty vector (control) (*E*) together with 0.125 μg of LMH14 RNA without or with 1 μM TAM for 4 days.

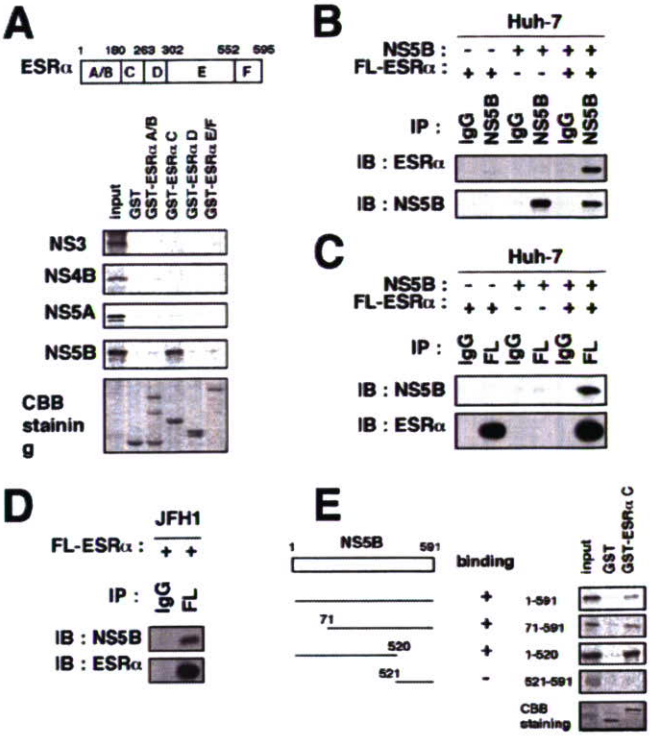

FIGURE 3. **ESRα interacted with HCV NS5B.** *A*, *top*, schematic representation of the primary structure of ESRα. ESRα consists of domains A–F. The amino acid numbers are also shown. *Bottom*, GST pull-down assays were performed using the recombinant proteins of the A/B, C, D, and E/F domain of ESRα fused with GST and *in vitro* translated HCV NS3, NS4B, NS5A, and NS5B protein. *Input*, the one-fifth amount of protein used for the pull-down assay. The Coomassie Brilliant Blue staining pattern of the precipitated fraction is also shown in the *bottom panel*. *B–D*, the lysates from the cells ectopically expressing NS5B (*B* and *C*) or the whole open reading frame of the HCV JFH1 strain (*D*) and/or FLAG-tagged ESRα were immunoprecipitated (*IP*) with anti-NS5B (*B*; NS5B), anti-FLAG antibody (*C* and *D*; FL), or mouse normal IgG as a negative control followed by the detection of ESRα and NS5B by immunoblot analysis (*IB*). *E*, deletion mutants of NS5B were subjected to a GST pull-down assay with GST-fused C domain of ESRα as described in *A*. The *left panel* shows a schematic representation of the full-length and truncated mutants of NS5B. The *numbers* indicate the amino acid numbers in NS5B.

genome replication. ESRα(L540Q), carrying a leucine to glutamine point mutation at aa 540 within the LXXLL motif (aa 536–540) of ESRα (37), had much lower transactivation activity driven from ERE (Fig. 2*C*). However, ESRα(L540Q) stimulated HCV replication activity ~5-fold, although the stimulation was less than that by wild-type ESRα (Fig. 2*E*). Thus, ESRα having lower transactivating capacity could still facilitate HCV replication.

ESRα Interacted with HCV NS5B—Thus, the chemical biology approach revealed the involvement of ESR in HCV genome replication. Then we investigated the molecular mechanism of ESR-induced HCV replication. A binding assay between ESRα and HCV proteins expressed in the HCV subgenomic replicon showed that the C domain of ESRα coprecipitated with NS5B but not NS3, NS4B, and NS5A (Fig. 3*A*). Other ESRα domains, A/B, D, and E/F, did not bind to any HCV proteins. A coimmunoprecipitation assay also indicated the presence of ESRα in the immunoprecipitate by anti-NS5B antibody (Fig. 3, *B* and *D*), and *vice versa* (Fig. 3*C*). Thus, ESRα specifically interacted with NS5B. Deletion analysis indicated that the region of 71–591 and 1–520 but not 521–591 of NS5B coprecipitated with the recombinant C domain of ESRα (Fig. 3*E*). This binding profile is different from that between cyclophilin B and NS5B, which we previously reported (21).

The ESRα-NS5B Interaction Was Important for the Regulation of HCV Genome Replication—To examine whether the interaction between ESRα and NS5B was essential for the ESRα-mediated regulation of HCV replication or not, we searched for a point mutant of ESRα that could not bind to NS5B by alanine-scanning mutation analysis. ESRα mutants, ESRα(255M) and ESRα(258M), in which IRK at aa 255–257 and DRR at aa 258–260 was replaced by TGT and AQT, respectively, had little affinity with NS5B (Fig. 4*A*) but still possessed the ERE-mediated transactivation capacity (Fig. 4*B*). However, both ESRα(255M) and ESRα(258M) caused only weak activations of HCV replication, compared with wild type ESRα (Fig. 4*C*). The data suggest that the interaction of ESRα with NS5B is

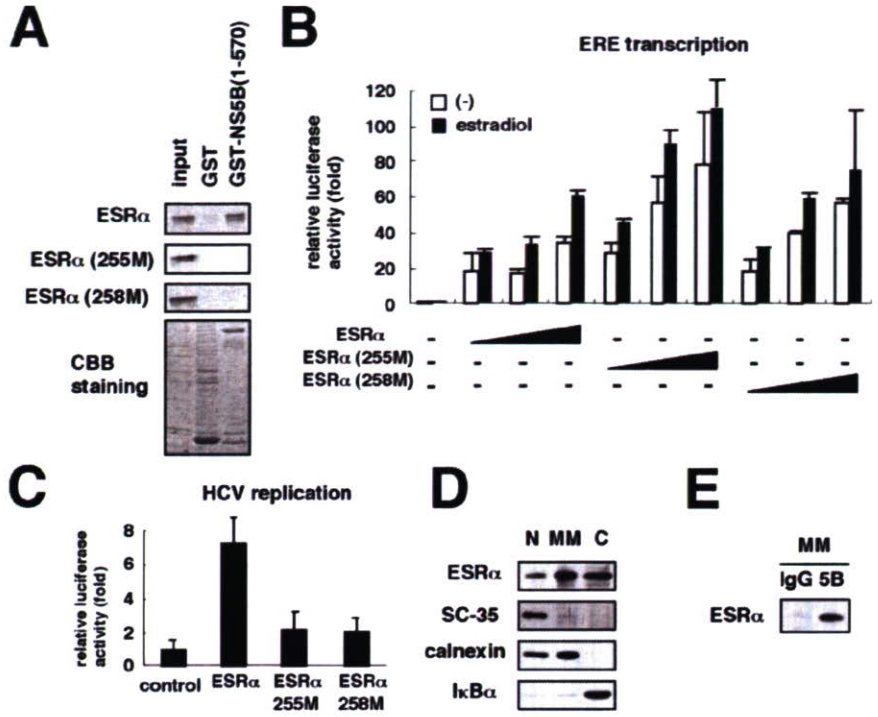

FIGURE 4. **The interaction of NS5B mediated the regulation of HCV genome replication by ESRα.** *A*, GST pull-down assays were performed as described in Fig. 3*A* using the wild type ESRα or point mutant of ESRα, ESRα(255M), and ESRα(258M). *B*, the mutation within ESRα(255M) and ESRα(258M) did not reduce the activation capacity of ERE-mediated transcription. Huh-7 cells were transfected with the expression plasmids for ESRα, ESRα(255M), or ESRα(258M) at doses of 10, 30, and 100 ng each together with pGL3-EREX3-TATA-Luc reporter plasmid and treated without (*white bar*) or with 100 nM estradiol (*black bar*) to quantify the luciferase activity. *C*, HCV replication activities were examined by quantifying the luciferase activities as described in the legend to Fig. 2*D* in the cells upon transfection with the expression plasmids for wild type ESRα, ESRα(255M), or ESRα(258M). *D*, the cells were fractionated into the nucleus (*N*), MM, and cytoplasm (*C*). Each fraction was detected for FLAG-tagged ESRα, SC-35, calnexin, and IκBα, respectively, by immunoblot analysis. Calnexin, an ER marker protein, was detected in the nucleus as well as MM, probably because of the existence of the nuclear membrane in the nuclear fraction. *E*, the MM fraction obtained in *D* was subjected to a coimmunoprecipitation assay using anti-NS5B or IgG followed by immunoblot analysis for the detection for ESRα.

critical for ESRα-mediated regulation of HCV genome replication.

Thus, ESRα interaction with NS5B regulates HCV replication. NS5B is mainly located on the cytoplasmic surface of the ER membrane (21, 38). On the other hand, ESRα as a nuclear hormone receptor is normally distributed in the cytoplasm and translocates into the nucleus upon ligand stimulation. In addition, a part of ESRα localizes on the membrane fraction. In our experiment, NS5B was mainly located around the ER, colocalized with the ER marker, protein-disulfide isomerase (data not shown) (21). Ectopically expressed ESRα showed diffuse distribution in the cells (data not shown). We fractionated cell homogenates and observed that a part of the ESRα resided in the microsomal membrane (MM) fraction (Fig. 4*D*). Moreover, ESRα in the MM fraction was coprecipitated with NS5B (Fig. 4*E*). It suggests the possibility that the interaction between NS5B and ESRα, at least in part of them, occurs on the ER membrane.

ESRα Promoted the Participation of NS5B in the HCV Replication Complex—It was reported that HCV proteins involved in the replication machinery was associated with the lipid raft on the ER and cofractionated with CAV2. A coimmunoprecipitation assay showed that NS5B associated with CAV2 (Fig. 5*A*). In the experiment investigating the role of ESRα in NS5B-CAV2 association, the coprecipitation of NS5B with CAV2 was decreased upon the knocking down of ESRα (Fig. 5*B*). Treatment with TAM abrogated the association of NS5B with CAV2 (Fig. 5*C*), although the total amount of NS5B in the cells is similar in the presence and absence of TAM for 24 h in this experiment (data not shown). Thus, ESRα was suggested to promote the association between NS5B and CAV2. Since a part of CAV2 resided on the lipid raft on the ER (18), ESRα-mediated binding between NS5B and CAV2 was possible to affect the localization of NS5B to the HCV RC. To see the consequential relevance of ESRα on NS5B function, we analyzed the HCV RC by treatment with digitonin/protease as described previously (16). HCV proteins involved in the RC and surrounded by the membrane structure are resistant to the treatment with digitonin followed by protease, whereas those unrelated to the replication outside the RC are digested by the treatment. By using this technique measuring the sensitivity to protease, HCV RC can be distinguished from the ER that is not related to the replication, although the RC and the nucleus cannot be separated. The experimental condition for fractionation was confirmed with the detection with IκBα and calnexin; a cytosolic protein IκBα was washed out following the treatment with digotinin (Fig. 5*D*, *lanes 1* and *2*), and ER protein calnexin, which did not accumulate in the RC, was digested by treatment with digotinin/protease (Fig. 5*D*, *lanes 2–4*). An ER lipid raft component, CAV2, was still detected under the digitonin/protease treatment (the RC-containing fraction) (Fig. 5*D*, *lanes 3* and *4*). Under this condition, a part of NS5B was detected in the digitonin/protease-resistant fraction, as described previously (16) (Fig. 5*D*, *lanes 3* and *4*). However, NS5B in this fraction was decreased upon treatment with TAM (Fig. 5*D*, *lanes 3*, *4*, *7*, and *8*). On the other hand, the amount of NS5A was not significantly changed by TAM treatment. Knocking down of ESRα also disrupted the association of NS5B with the RC-containing fraction (Fig. 5*E*). From the above results, it was suggested that ESRα promoted the participation of NS5B in the RC (also see "Discussion").

ESRα Could Serve as a Molecular Target of Anti-HCV Agents—Finally, we assessed the possibility that the association of ESRα with NS5B could serve as a target of anti-HCV agents. By introducing a decoy peptide against ESRα-NS5B interaction, consisting of the C domain of ESRα into replicon-bearing cells,

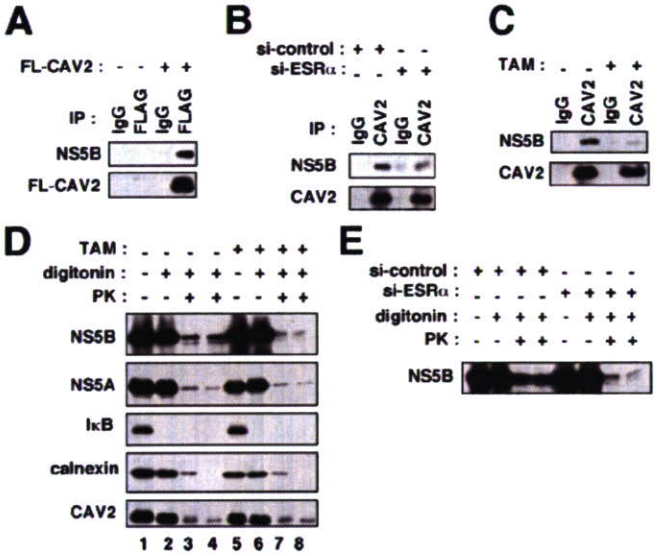

FIGURE 5. **ESRα promoted the participation of NS5B in HCV RC.** *A–C*, a coimmunoprecipitation assay (*IP*) was performed with anti-FLAG (*A*), anti-CAV2 (*B* and *C*) antibody, or mouse normal IgG from the lysates of the cells transfected without or with FLAG-tagged CAV2 (*A*), transfected with si-control or si-ESRα (*B*), or treated without or with 1 μM TAM (*C*). NS5B (*top*) and CAV2 (*bottom*) were detected by immunoblot analysis. *D*, detection of the amount of NS5B in the digitonin/protease-resistant fraction. MH-14 cells were treated without (*lanes 1–4*) or with 1 μM TAM (*lanes 5–8*) for 24 h. Cells were then treated without (*lanes 1* and *5*) or with digitonin (*lanes 2–4* and *6–8*), followed by digestion with proteinase K (0 μg/ml for *lanes 2* and *6*, 0.3 μg/ml for *lanes 3* and *7*, and 1 μg/ml for *lanes 4* and *8*). NS5B, NS5A, IκBα, calnexin, and CAV2 were detected by immunoblot analysis. *E*, HCV RC was isolated as described in *D* using the cells transfected with si-control or si-ESRα, and NS5B was detected. A similar result was obtained by using si-ESRα2.

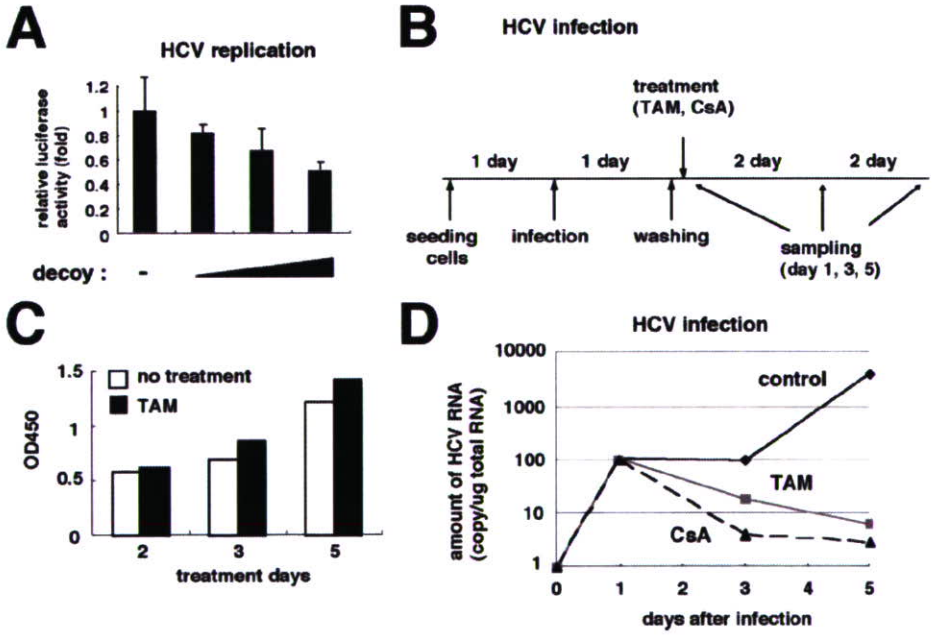

FIGURE 6. **ESRα could serve as a molecular target for anti-HCV agents.** *A*, HCV replication activity was measured by quantifying the luciferase activity as described in the legend to Fig. 2*D* in the cells overexpressing a decoy peptide consisting of the C domain of ESRα. *B*, experimental scheme of *in vitro* HCV infection experiment. After seeding the HuS-E7/DN24 cells, HCV-positive serum was inoculated for 24 h. After extensive washes, the cells were cultured with the medium supplemented without (control) or with 1 μM TAM or 3 μg/ml cyclosporin A. HCV genome RNA was quantified along with the time course (days 1, 3, and 5 postinoculation) by real time RT-PCR analysis. *C*, the treatment with 1 μM TAM did not show any cytotoxic effect on HuS-E7/DN24 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed as described under "Experimental Procedures" to examine the viability of the cells at days 2, 3, and 5 postinoculation. *D*, HCV genome RNA was quantified as described in *B* and plotted against the time course.

HCV replication activity was reduced in a dose-dependent manner (Fig. 6*A*). To further observe the significance of ESRα in a physiological condition, we performed an *in vitro* infection experiment using serum from an HCV-infected patient as a nascent virus inoculum and nonneoplastic human hepatocytes as highly infection-permissive cells (Fig. 6*B*). Treatment with 1 μM TAM did not show a cytotoxic effect on these cells in any time course examined (Fig. 6*C*). However, treatment with TAM as well as cyclosporin A as a positive control inhibited the multiplication of viral genome RNA in the cells along with the time course (Fig. 6*D*). Thus, ESRα could serve as a potent molecular target of anti-HCV agents.

DISCUSSION

In general, viruses take advantage of host cell factors for their replication. So far, some factors have been shown to relevantly regulate HCV replication, including hVAP33 (39, 40), FBL2 (41), and cyclophilin B (21). Among these, FBL2 and cyclophilin B were identified by a chemical biological approach; FBL2 from the observation of an anti-HCV activity of lovastatin and an inhibitor of geranylgeranyl transferase (41–43); cyclophilin B from the inhibitory effect of cyclosporin A on HCV replication (20, 21). In this study, we found a suppressive capacity of TAM to HCV genome replication. Through further examination using TAM, we revealed ESRα as a host cell factor regulating HCV replication and suggested its regulation mechanism.

Currently, it is proposed that HCV RC that replicates the HCV genome is formed on the intracellular membrane, including the ER membrane (14–17). It was also reported that HCV genome replication was associated with the lipid raft on the intracellular membrane (18). Most HCV proteins are not related to the RC, whereas only a minor portion of HCV proteins take part in the RC to drive the viral replication (16). It has remained widely unknown, however, how HCV proteins are regulated to participate in the RC. It was reported that hVAP-33 binds to NS5A and NS5B, and this protein is related to the amount of NS5B in the lipid raft (40). hVAP-33 was speculated to recruit NS5B to the lipid raft, although its molecular mechanism has not been analyzed. This study suggested the interaction between ESRα and NS5B in the ER fraction, although we did not show the existence of ESRα in the RC, since the RC and the nucleus cannot be separated in the digitonin/protease treatment experiment. ESRα promoted the interaction of NS5B with CAV2. Previous papers reported that ESRα bound to CAV1 and CAV2 (6). From these observations, ESRα is

likely to function as a bridging factor that connects NS5B to CAV2, although we cannot fully neglect the possibility that ESRα augments NS5B-CAV2 binding via another function, such as transcriptional activity. Because CAV2 resided on the lipid raft of the intracellular membrane (18), this action of ESRα may recruit NS5B to the lipid raft and the HCV RC. In fact, ESRα promoted the participation of NS5B in the HCV RC. Thus, ESRα is suggested to escort NS5B to the HCV RC, although it is also possible that ESRα augments the number of the RC itself. However, ESRα at least augments the amount of NS5B involved in HCV replication machinery to stimulate the replication. It was reported that the membrane-associated ESRα served as a platform where signalsomes, including receptor tyrosine kinase, nonreceptor tyrosine kinase Src, and G proteins, assembled and activated downstream signaling pathways (44–46). HCV may also take advantage of such platform characteristics of ESRα to form the RC for their efficient replication. Although the mechanisms of the nuclear receptor function of ESRα have been extensively elucidated, the functions of membrane-associated ESRα have not been widely characterized so far. This study suggested a novel physiological relevance of membrane-associated ESRα as a regulator of the viral replication.

Until now, there are no clinical studies that report a direct interaction of TAM treatment with HCV replication in patients infected with HCV. Given our results, examinations on the effect of TAM or other anti-estrogen drugs may be one of the useful approaches to develop a new anti-HCV strategy. On the other hand, we disclosed the mechanism of ESR-mediated regulation of HCV genome replication. Screening for compounds that inhibit this mechanism expectedly led to novel types of anti-HCV agents. Further analyses on ESR are needed to develop anti-HCV therapeutics as well as reveal the regulation mechanism of HCV replication.

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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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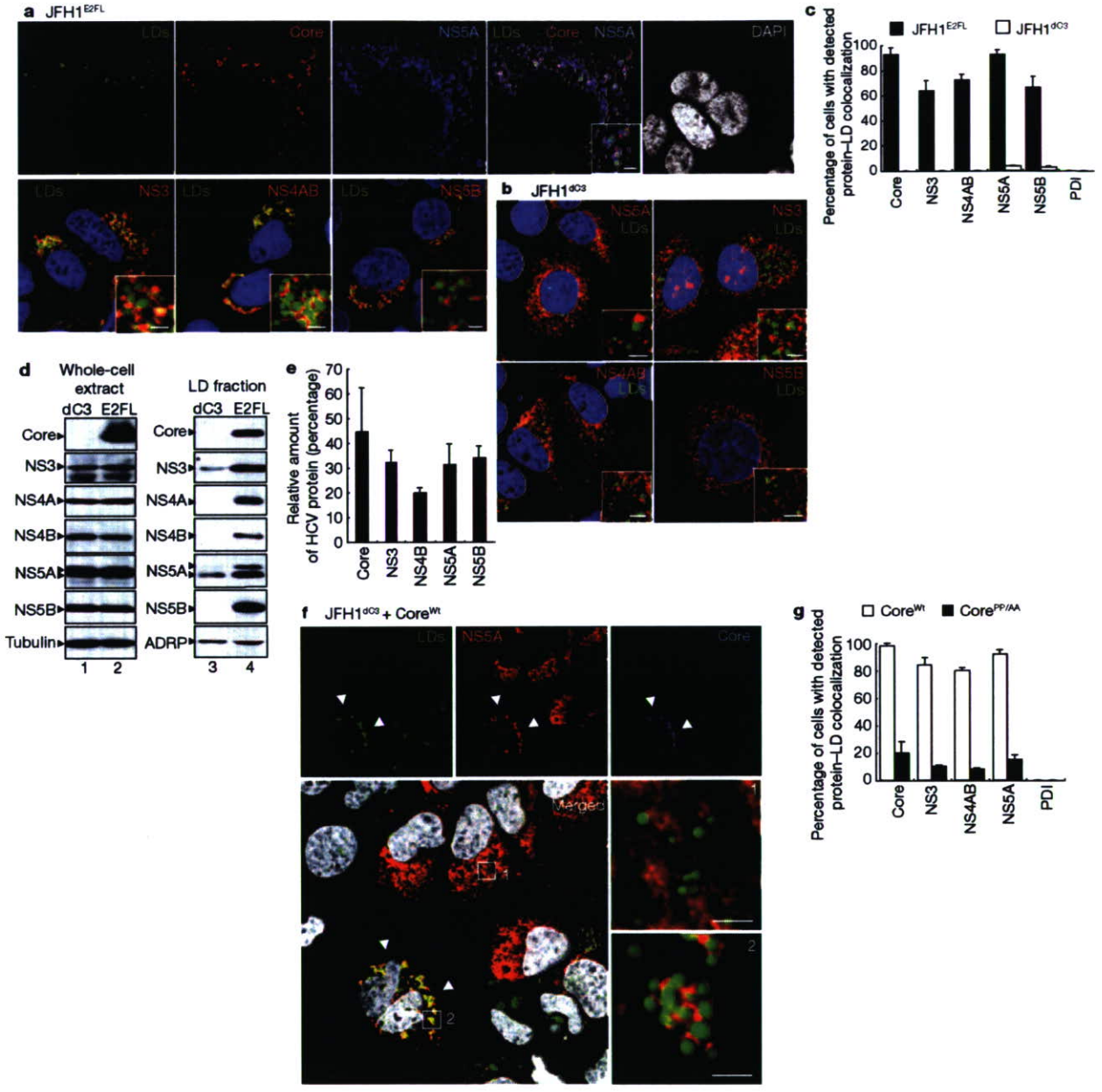

Figure 1 Core recruits NS proteins to LDs. (**a**) Huh-7 cells transfected with JFH1^{E2FL} RNA were labelled with antibodies against Core (red), NS5A (blue), NS3 (red), NS4AB (red) or NS5B (red). Lipid droplets (LDs) and nuclei were stained with BODYPI 493/503 (green) and DAPI (white in upper panel,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

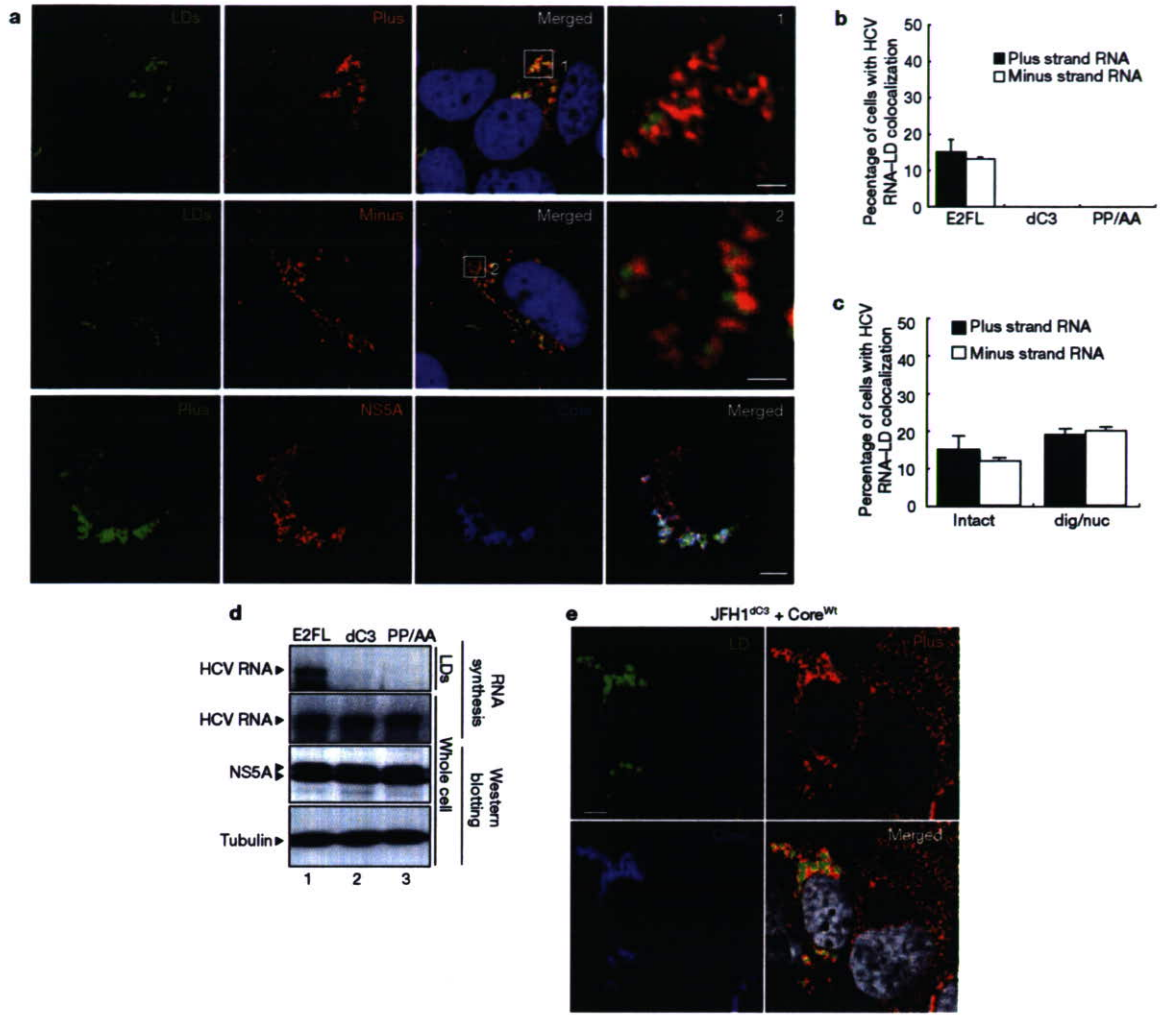

Figure 2 Core-dependent recruitment of active HCV replication complexes to the LD. (**a**) Huh-7 cells transfected with JFH1^{E2FL} RNA were analysed by *in situ* hybridization with strand-specific probes (plus or minus). The cells were labelled to simultaneously visualize lipid droplets (LDs), NS5A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

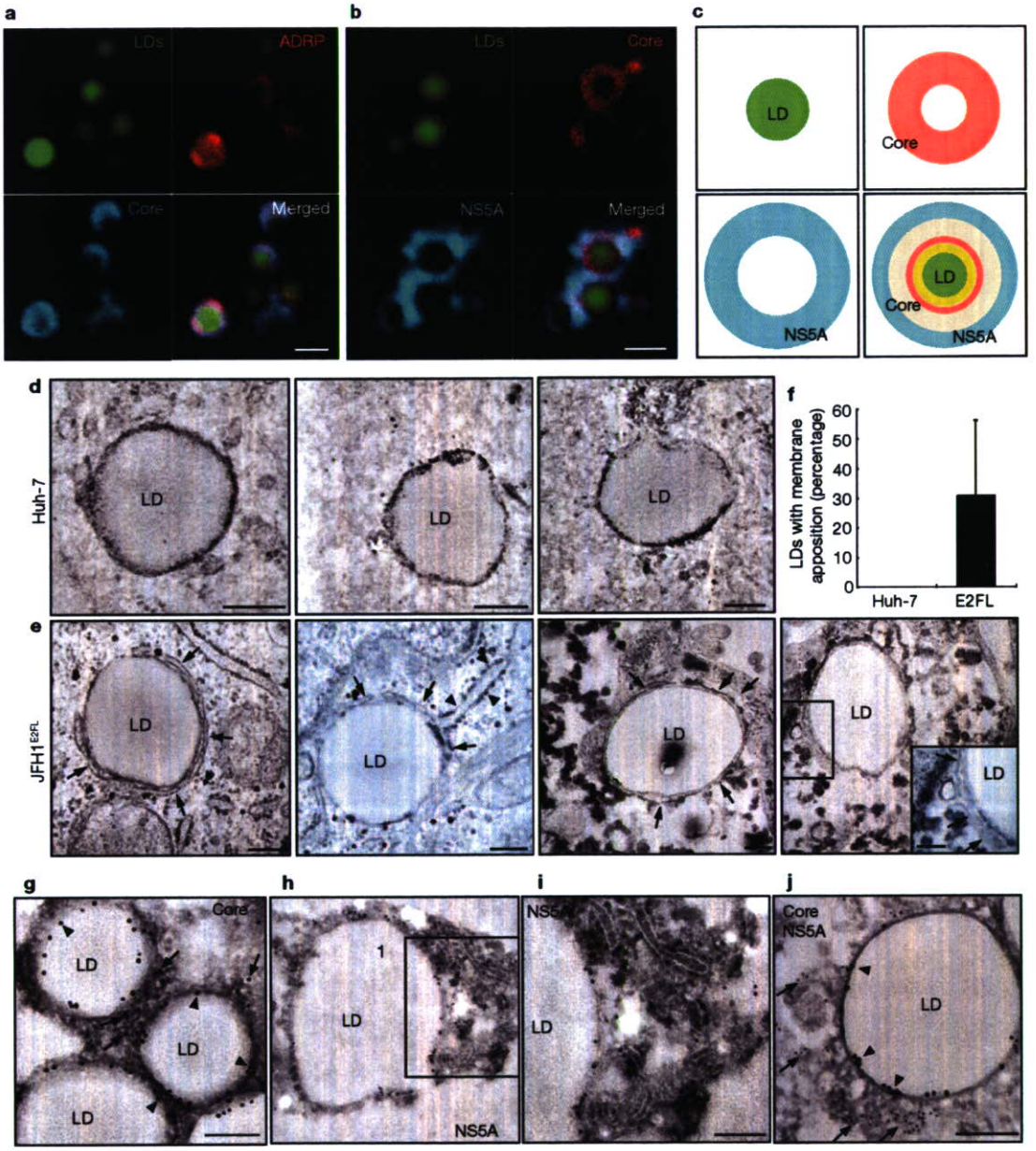

Figure 3 Spatial distribution of Core and NS5A relative to the LD. (**a**, **b**) The localizations of Core, NS5A and ADRP around the lipid droplets (LDs) in JFH1^{E2FL} replicon-bearing cells were analysed using immunofluorescence microscopy. Scale bars, 1 μm. (**c**) Typical images of the localization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