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A de-ubiquitinating enzyme USP15 participates in the propagation of hepatitis C virus

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Abstract

De-ubiquitinating enzymes (DUBs) are a large group of proteases that catalyze the release of ubiquitin from their substrates. DUBs involve in many cellular functions such as homeostasis, tumorigenesis and host defense, and anti-cancer drugs targeting to DUBs are now in clinical trials. In this study, I examined the roles of DUBs on the life cycle of hepatitis C virus (HCV). By using RNAi-based screening, I identified an ubiquitin specific protease 15 (USP15) crucial for HCV replication. The reduction of lipid droplets in USP15-knockdown cells was recovered by the overexpression of USP15, suggesting that USP15 is involved in the production of lipid droplets. These data suggest that USP15 participates in the HCV propagation through the regulation of lipid metabolisms in the liver.

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General Introduction

The liver is the largest organ in human body and weights 1KG-1.5 kg in adults. The various functions of the liver are performed by hepatocytes. For example, liver controls glucose concentration in the blood by glycogenesis, glycogenolysis or glycogenesis. The liver also regulates lipid metabolisms through production of cholesterol or triglycerides. Toxic compounds are modified or broken down by the liver.

Because liver is critical for survival and its function is multiple, there are several severe diseases in the liver. About 80% of liver diseases are caused by hepatitis virus infection (Fig. 3B). Human hepatitis viruses consist of 5 different viruses such as shown in Fig. 1 and 2. Before the discovery of hepatitis A virus (HAV) (1, 2) and hepatitis B virus (HBV) (3, 4) during the 1960s and 1970s, patients with viral hepatitis were classified based on epidemiological studies as having either infectious (transmitted person to person by the fecal-oral route) or serum (transmitted by transfusion of blood products) hepatitis (Fig. 1). When diagnostic tests for HAV and HBV infections were developed, HAV was found to be the major cause of infectious hepatitis and HBV was found to be the major cause of serum hepatitis. Hepatitis delta virus (HDV), discovered in 1977, is a defective virus requiring the presence of HBV in order to replicate (5). However, some patients with typical signs and symptoms of viral hepatitis did not have serologic markers of HAV, HBV, or HDV infection and were categorized based on epidemiological characteristics as having either parenterally transmitted non-A, non-B (NANB) hepatitis or enterically transmitted NANB

hepatitis (6). Subsequently, two additional viruses were discovered: hepatitis C virus (HCV) (7) and hepatitis E virus (HEV) (8, 9). HCV is the major cause of parentally transmitted and HEV is the major cause of enterically transmitted NANB hepatitis. In addition, some patients with typical signs and symptoms of acute viral hepatitis do not have serologic markers of any of these types of viral hepatitis and can be classified as having non-ABCDE hepatitis.

HAV belongs to Picornaviridae family (1), has no envelope and possesses a single-stranded RNA as a genome. HAV can be transmitted by fecal oral infection. HAV is resistant to low pH (3.0), but would be inactivated by the treatment with 100°C for 10 minutes or chlorinated disinfective cleanser (10, 11). HAV is acutely infected and is normally excluded within 1-2 months. HBV belongs to Hepadonaviridae family (12), has envelope and a double-stranded partially circular DNA as a genome. HBV is transmitted by blood via perinatal and sexual routes. HBV infection is normally acute infection, however 1% of infected patients cause chronic infection as a carrier. Chronic infection leads to cirrhosis and hepatocellular carcinoma (HCC). HBV infects 240 million people worldwide and a million people in Japan. Reverse transcriptase inhibitors (Lamivudine or Entecavir) can be used for current therapy, however patients have to take them for their life and drug-resistant viruses are emerging. HDV belongs to Deltaviridae family (13). HDV has a single stranded RNA as a genome. HDV is a replication-deficient virus, which needs HBV infection for successful propagation. Therefore, HDV infection can be occurred by co-infection of HBV/HDV or in HBV carriers. HDV infection is mainly seen in South Europe and 1% of HBV carriers are co-infected with HDV in Japan. HEV belongs to Hepeviridae family (14). HEV has a single

stranded RNA as a genome and is transmitted by fecal oral routes. The symptoms of HEV are similar to HAV. Only difference is that the frequency of fulminant hepatitis in HEV infection is about 5 times higher than that in HAV infection.

HCV is a major causative agent of chronic liver diseases including steatosis, cirrhosis and HCC (15). In Japan, liver cancer is the 4th most common cancer (Fig. 3A). About 70% of liver cancers are caused by HCV infection and 20% are HBV infection (Fig. 3B). HCV has several genotypes. Genotype 1b of HCV is major among all genotypes. It occupies 70% in HCV patients (Fig. 3C). Current therapy such as combination of pegylated-interferon (IFN) and ribavirin (RBV) achieved about 50% sustained virological response (SVR) in patients infected with genotype 1b HCV with high viral load (Fig. 3D) (16). As novel potent therapeutics, direct-acting antivirals (DAA) including inhibitors for viral protease and polymerase have been recently developed (17). Clinical trials revealed that DAA treatment achieved SVR in over 80% of chronic hepatitis C patients (18, 19). However, drug-resistant HCV against DAA had been already reported, suggesting that development of novel therapeutics with a low frequency of emergence of breakthrough viruses is needed.

HCV belongs to Flaviviridae family and possesses a positive and single-stranded RNA genome. The viral RNA is translated into a large single polyprotein (about 3000 amino acids) and processed into 10 viral proteins through cleavage by viral-encoded and host proteases (Fig. 4). Core protein is a component of viral capsids and E1 and E2 glycoproteins are envelope glycoproteins. The p7 protein acts as a proton pump for an efficient virus release. Non-structural (NS) 2 and 3 proteins possess protease activity. NS4 is thought to be a scaffold for viral replication complex. NS5A

interacts with various host factors and regulates viral replication. NS5B has an RNA-dependent RNA polymerase activity (20, 21). HCV is trapped by glycosaminoglycans, like heparin and heparan sulfate on cell surface and then transferred to protein receptors. HCV enters via an endocytosis, replicates on the endoplasmic reticulum (ER) membrane, and buds into the ER lumen (Fig. 5).

Molecular mechanisms of HCV pathogenesis

Core protein of HCV is a multifunctional protein, which localizes in many cellular components such as nucleus, ER, lipid droplets (LDs), lipid rafts and mitochondria. Core protein plays roles in apoptosis, autophagy, cell cycles and oncogenesis. Chronic HCV infection leads to steatosis, cirrhosis and hepatocellular carcinoma in the liver (22). On the other hand, HCV infection epidemiologically correlated with extra-hepatic manifestations such as type 2 diabetes, mixed cryoglobulinemia and non-Hodgkin lymphoma (23). Although precise molecular mechanisms of HCV-induced pathogenesis remain unknown (24), the liver-specific HCV core transgenic (CoreTG) mice showed steatosis and hepatocellular carcinoma (25) (Fig. 6). Furthermore, the insulin resistance is occurred in the same mice (26) (Fig. 6). Sterol regulatory element binding transcription factor 1c (SREBP-1c), which positively regulates the production of saturated and monounsaturated fatty acids and triglycerides, is enhanced in CoreTG mice liver (27). It suggests that core protein plays a role in liver diseases and extra-hepatitis manifestations (Fig. 7). Yeast two-hybrid screening revealed HCV core proteins directly interacted to proteasome activator 28 γ

(PA28 γ). PA28 γ regulates cell cycles and protein expression through maintenance of proteasome in nucleus. Although core protein localizes at cytoplasm in CoreTG mice, majority of core protein was detected in nucleus in CoreTG/PA28 γ^{-} mice (28). Surprisingly, CoreTG/PA28 γ^{-} mice did not show any HCV-core induced phenotypes such as insulin resistance, steatosis and HCC even though this mice expresses core protein as same as CoreTG mice (28, 29). CoreTG/PA28 γ^{-} mice show no activation of SREBP-1c compared to CoreTG. These data suggests that degradation of HCV core protein in nucleus through PA28 γ -dependent proteasome might be a key step to develop core-induced diseases (Fig. 7) (30).

Maturation of HCV core protein

Once HCV polyprotein is translated, core protein is firstly cleaved at the position of 191 amino acids by host signal peptidase and then further processed by signal peptide peptidase (SPP) (31, 32). Our laboratory tryied to identify the responsible regions in core protein for processing by SPP (33). Domain II of core protein, which has cluster of hydrophobic amino acid residues, was necessary for processing of core by SPP. Especially substitution of L139, V140 and L144 to alanin (M2 mutant) inhibited SPP cleavage. SPP recognizes the helix-breaking structure in the signal peptide of transmembrane region. Mutation of I176 to alanine and F177 to leucine (M1 mutant) to acquire the α -helix structure in the signal sequence was also impaired SPP cleavage (Fig. 8) (33). As we expected, the maturation of core protein by SPP is needed to localize to detergent-resistant membrane (DRM) (34). The recombinant viruses possessing either M1 or M2 mutation could not

release infectious particles into culture media (34), suggesting that processing of HCV core protein by SPP is essential for propagation of HCV.

HCV infection and receptor candidates

HCV need to bind appropriate host cell surface for an efficient infection. HCV binds to glycosaminoglycan such as heparin or heparan sulfate on cell surface and then binds to specific receptors. Many receptor candidates for HCV had been reported such as, CD81, dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN/L-SIGN), low density lipoprotein receptor (LDL-R), scavenger receptor class B type I (SR-BI), epidermal growth factor receptor (EGFR), claudin 1 (CLDN1), occludin (OCLN), Niemann-Pick C1-like protein 1 (NPC1L1) (Fig. 9A)(35, 36). HCV can internalize into cells derived from human and chimpanzee but not from mice. CD81 was firstly identified to bind to HCV E2 glycoprotein. Human derived CD81 but not of mouse specifically bound to E2 protein, suggesting that CD81 was one of the species-specific receptors for HCV (37). Recently, human OCLN was also identified as a species-specific functional receptor for HCV by using expression-cloning method (38). Transgenic mice expressing human CD81 and human OCLN were susceptible to HCV infection, suggesting that CD81 and OCLD were restrict factors for species-specific infection of HCV (Fig. 9B) (39, 40).

HCV replication and microRNA-122

In 1993, microRNA (miRNA) was firstly identified in *C. elegans* (41). Over 2000 miRNAs have

been registered in the database so far (42). miRNA incorporated into RNA-induced silencing complex (RISC) interacts with a target mRNA via a specific recognition element (Fig. 10A). RISC contains argonaute 2 (Ago2), dicer, and TAR RNA binding protein (TRBP) (43, 44). In human, Ago2 plays a pivotal role in the repression of translation of target genes. It is now commonly believed that miRNAs play important roles in cell homeostasis and abnormality of miRNA expression participates in the development of several diseases including viral diseases. miRNAs encoded by Epstein-Barr virus (EBV) were identified in 2004 and over 200 viral miRNAs have been reported in several DNA viruses, especially in herpesviruses (45). miR-122 is a liver specific miRNA and is the most abundantly expressed in the liver (46, 47, 48). miR-122-deficient mice showed steatosis, inflammation, fibrosis and hepatocellular carcinoma (49, 50), suggesting that miR-122 plays a crucial roles in liver homeostasis. Jopling et al. reported for the first time that the inhibition of miR-122 dramatically decreased HCV RNA replication (51) (Fig. 10B). They identified the miR-122 binding site in the end of the 5' untranslated region (UTR) of HCV RNA. In addition, lack of enhancement of HCV replication by the expression of a mutant miR-122 incapable of binding to the 5'UTR was canceled by the introduction of a complementary mutation in the 5'UTR, suggesting that direct interaction of miR-122 to the 5'UTR is crucial for an enhancement of HCV replication. In subsequent reports, they identified the second adjacent miR-122 binding site in the 5'UTR (52).

Although precise mechanisms of the miR-122 mediated enhancement of HCV replication have not fully elucidated yet, Henke et al. demonstrated that miR-122 might contribute to HCV liver tropism at the level of translation (53). Wilson et al. showed that knockdown of Ago2 in cells HCV RNA autonomously replicating (HCV replicon) and in cells infected with HCV attenuates HCV replication, in addition, knockdown of Ago2 reduced translation of the polymerase defective HCV RNA (54). Shimakami et al. showed that miR-122 stabilizes viral RNA and reduces its decay in

concert with Ago2 and that miR-122-dependent stabilization of HCV RNA was not observed in Ago2 knockout murine embryonic fibroblasts (55). These results suggest that Ago2 is required for an efficient enhancement of both translation and replication of HCV. Furthermore, exogenous expression of miR-122 in non-hepatic cell lines (56) enhanced the efficiency of HCV replication, suggesting that miR-122 plays an important role in an efficient replication of HCV in the liver (Fig. 10C).

Membrane structure for HCV replication

The viruses of Flaviviridae family including HCV utilize ER membrane to form replication machinery (57). Observation of electron microscope showed that HCV infection induced accumulation of folded membrane structures called membranous web (58, 59) (Fig. 11A). This structure contained viral proteins and viral genomes. A membrane structure was observed as a double membrane vesicle (DMV) by HCV infection and HCV RNA replication was occurred in DMV to prevent viral RNA replication from host antiviral responses (59) (Fig. 11B). Host lipids are well known to be essential components in the viral life cycle, including the assembly, budding, and replication of various viruses (60, 61, 62, 63). In the case of HCV, several types of lipids are required for the HCV life cycle. Saturated and monounsaturated fatty acids, but not polyunsaturated fatty acids, enhance HCV RNA replication (64), suggesting that lipid biogenesis is involved in HCV replication. HCV particles bind to lipoprotein receptors for entry (65, 66). It was shown that HCV uses assembly and secretion pathway of very low density lipoprotein (VLDL) for maturation and secretion of viral particles (67, 68). Cholesterol and sphingolipids are required for maturation

and infectivity of HCV, since depletion of cholesterol or down-regulation of sphingomyelin reduces infectivity (69). Accumulation of lipid components in the liver leads to liver steatosis, and is associated with progression to liver fibrosis and HCC as described above.

Screening of a genome-wide siRNA library revealed that phosphatidylinositol 4-kinase III alpha (PI4KA) and COPI vesicle coat complex as a human gene associated with HCV replication (70, 71, 72, 73, 74, 75). Phosphatidylinositol 4-phosphate, which is associated with OSBP and CERT (76, 77) as described below, is increased by HCV infection (70, 78, 79, 80). PI4KA is co-localized with NS5A and double stranded RNA in the replication plate form composed of detergent-resistant lipid components, known as a membranous web, and is critical for HCV replication at posttranslational stages in the membranous web (81) (Fig. 11C).

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 (later renamed VAP-A) (82). Furthermore, one homologue and its splicing variant were reported as VAP-B and VAP-C, respectively (83). GST pull-down and immunoprecipitation analyses revealed that NS5A and NS5B interact with human VAP-A/B and that the N-terminal MSP domain and the coiled-coil domain of VAP-A/B are responsible for the binding to NS5B and NS5A, respectively (84, 85). In addition, systematic RNAi screening revealed that 62 target host genes are involved in HCV RNA or proteins including VAP-A/B (86). These findings suggest that VAP-A and -B positively regulate HCV replication by binding to NS5A/B (Fig. 12).

The peptide bond *cis/trans* isomerase converts between *cis* and *trans* peptide bonds leading to correct folding of the protein substrate. Peptidyl prolyl *cis/trans* isomerase (PPIase) includes the families of cyclophilin (87), FK506-binding proteins (FKBP) (88, 89) and parvulins (90), and the secondary amide peptide bond *cis/trans* isomerase (91). Cyclophilin and FKBP are categorized as immunophilins, which are targeted by the immunosuppressants cyclosporin and FK506, respectively (92). Some cyclophilins and FKBP8 were shown to interact with NS5B and/or NS5A and to regulate HCV replication (93, 94, 95, 96, 97), suggesting that immunophilins could lead to promising therapies for chronic hepatitis C.

Interferon therapy and IL28B

IFN is a major agent for HCV therapy. However, 50% of patients infected with genotype 1b with high viral load are resistant to pegylated-IFN/RBV treatment. In 2009, SNPs in IL28B gene were identified as critical SNPs for determination of the efficacy for pegylated-IFN/RBV treatment (98, 99, 100, 101). TT of rs8099917 SNP in IL28B genome was called as a major allele and TG/GG were called minor allele (Fig. 13). Patients possessing major allele were sensitive to pegylated-IFN/RBV treatment.

Introduction

Ubiquitylation is a post-translational modification to regulate protein function in eukaryotes.

Ubiquitin (Ub) is covalently attached to substrate proteins by using E1, E2 and E3 enzymes (102, 103). Firstly, E1 enzyme activates Ub in ATP-dependent manner and transfers to E2 Ub-conjugating enzyme. E3 Ub-ligases recognizes its substrate protein and E2, and activated Ub is transferred to substrate protein. So far, 2 E1 enzymes, 10 E2 enzymes, and hundreds of E3 enzymes were identified. On the other hands, de-ubiquitinases (DUBs) catalyze the opposite reaction to ubiquitylation, which release ubiquitin from the substrates (104). In humans, nearly 100 DUBs are reported and are classified into cysteine proteases and metalloproteases. The cysteine proteases comprise ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), Machado-Josephin domain proteases (MJDs) and ovarian tumour proteases (OTU). The metalloprotease group contains only the Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+) (JAMM) domain proteases. (105). DUBs regulate multi-cellular functions and participate in many diseases including cancer and immune disorders (106, 107). In addition, USP11 inhibits replication of influenza A virus through de-ubiquitination of NP protein (108). However, it remains unclear which DUBs control HCV life cycle.

In this study, I assessed the involvement of DUBs in the HCV propagation. The shRNA based screening revealed that USP15 is an essential host factor in HCV replication. USP15 gene knockout Huh7 cell lines exhibited reduction of lipid droplets formation and this suppression was

cancelled by the overexpression of USP15. Members of the PAT family of proteins, originally named for Perilipin, Adipose differentiation-related protein (ADRP) and Tail Interacting Protein 47, play conserved structural and functional roles on lipid droplets. Among the PAT family proteins, ADRP was ubiquitinated and specifically de-ubiquitinated by USP15, suggesting that USP15 participates in the HCV propagation through the lipid storage in the liver.

Materials and Methods

Plasmids.

cDNAs of USP15 and USP20 were obtained from Dr. Wade Harper (Addgene, Plasmid #23217). USP15/USP20 DNA were amplified by Gflex DNA polymerase (Takara) and cloned into lentiviral transfer vector, FUGW obtained from Dr. David Baltimore (Addgene, Plasmid #14883) together with internal ribosomal entry site (IRES) sequence and puromycin N-acetyl-transferase genes and designated as FUIPW. The sequence was also inserted into pEFLAGPGKpuro by In-Fusion cloning kit (Takara). The cDNAs of Perilipin, ADRP and TIP47 were obtained by PCR by using cDNA derived from Huh7 cells and cloned into pEFOSFPGKpuro. HA-ubiquitin expressing vector was obtained from Dr. Ikuo Shoji (109). The retroviral vectors expressing DUBs shRNA were obtained from (Takara). USP15 DNA was amplified by Gflex DNA polymerase (Takara) and cloned into pFastBac HTb (Lifetechnologies). The pCMV-VSV-G and pCMV-dR8.2 dvpr were obtained from Dr. Robert Weinberg (Addgene #8454 and #8455, respectively). For CRISPR/Cas9 mediated gene targeting, pX330 and pCAGEGxxFP were obtained from Dr. Feng Zhang (Addgene, Plasmid #42230) and Dr. Masahito Ikawa (Addgene, Plasmid #50716), respectively. To target human USP15, the following oligonucleotides, 5'-CACCGCGACTATCGACTAGGTACC-3' and 5'-AAACCGCTCCGGAAAGGGGACACC-3', were annealed and cloned into pX330 and designed as pX330hsUSP15. To target mouse USP15, the following oligonucleotides,

5'-CACCGGTGTCCCCTTCCGGAGCG-3' and 5'-AAACCGCTCCGGAAAGGGGACACC-3', were annealed and cloned into pX330 and designed as pX330mmUSP15. To target human USP20, the following oligonucleotides, 5'-CACCGGCCAGGATGGGGACTCCA-3' and 5'-AAACTGGAGTCCCCATCCTGGCC-3', were annealed and cloned into pX330 and designed as pX330hsUSP20. Genomic DNA derived from Huh7 cells or mouse embryonic fibroblasts (MEFs) were extracted by DirectPCR Lysis Reagents (Viagen Biotech Inc). The genomic DNA of human USP15, mouse USP15 or human USP20 targeting by sgRNA was amplified by PCR, and cloned into pCAG EGxxFP and designed as pCAG EGxxFP hsUSP15, pCAG EGxxFP mmUSP15 and pCAG EGxxFP hsUSP20, respectively. Plasmids pHH21-JFH1-E2p7NS2mt encoding a cDNA of a full-length RNA of JFH1 strain (110). pSGR-JFH1, which encodes a SGR of the JFH1 strain, was provided by Dr. Takaji Wakita. The plasmids used in this study were confirmed by sequencing with an ABI Prism 3130 genetic analyzer (Applied Biosystems).

Cell lines.

HEK293T, immortalized human embryonal kidney cell line, Plat-E, packaging cell lines for generating retrovirus, Huh7 and Huh7.5.1, human hepatocellular carcinoma cell lines, were cultured in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.

Antibodies and reagents.

Antibodies to the following proteins were purchased from indicated manufacturers: anti-USP15 mouse monoclonal antibody (Abcam, ab56900), anti-ubiquitin mouse monoclonal antibody (Cell signaling, clone P4D1), anti-HCV NS5A mouse monoclonal antibody (Austral Biologicals, HCM-131-5), anti- β -actin mouse monoclonal antibody (Sigma, A2228), anti-HA rat monoclonal antibody (Roche, clone 3F10) and horseradish peroxidase (HRP) conjugated anti-FLAG mouse monoclonal antibody (Sigma, clone M2). Agarose conjugated Tandem Ubiquitin Binding Entity (TUBE) and PR-619, a non-selective, reversible inhibitor of DUBs and ubiquitin-like isopeptidases, were purchased from LifeSensors. Polyethylenimine (PEI, Linear, MW 25,000) was obtained from Polysciences inc. For lipid droplets imaging, HCS LipidTOXTM Red neutral lipid stain was obtained Lifetechnologies.

Generation of retro- and lenti-viruses and establishment of stable cell lines.

Retroviruses expressing shRNAs against human DUBs were generated in Plat-E cells. Briefly, 2×10^6 Plat-E cells were seeded on 10 cm dish and incubated at 37°C for 1 day. Five μ g of retroviral transfer vector and 1 μ g of pCMVVSVG were mixed with 500 μ l of Opti-MEM (Lifetechnologies) and 40 μ l of PEI (1mg/ml) and incubated for 15 min. DNA complex was inoculated into seeded Plat-E cells and culture medium was changed at 4 h post-transfection. The culture supernatants collected at 3 days post-transfection were passed through 0.45 μ m filter. In the case of lentivirus, 2×10^6 HEK293T cells were seeded on 10 cm dish and incubated at 37°C for 1 day. Lentiviral transfer vector (FUIPW, 1.5 μ g), 2.5 μ g of pCMV-dR8.2 dvpr and 1 μ g of pCMV-VSV-G were mixed with 500 μ l of Opti-MEM and 40 μ l of PEI and incubated for 15 min. DNA complex was

inoculated into HEK293T cells and culture medium was changed 4 h post-transfection. The culture supernatants 3 days post-transfection were passed through 0.45 μ m filter. For infection of retrovirus/lentivirus into Huh7.5.1 or Huh7 cells, 2×10^5 cells (2 ml) were seeded on 6 well plates and incubated for 1 day. The virus containing culture supernatants (2 ml) and 8 μ l of polybrane (Sigma, 4mg/ml) were inoculated into cells and centrifuged at 2500 rpm for 45 min at 32°C. Cells were selected by puromycin at 2 days post-infection to select stable cell lines.

Preparation of HCV.

HCV derived from the genotype 2a JFH-1 strain mutated in E2, p7 and NS2 as shown (110) was prepared by serial passages in Huh7.5.1 cells. Briefly, 1.5×10^6 of Huh7.5.1 cells were seeded on 10 cm dish and incubated for 1 day. The culture supernatant containing HCV was inoculated at moi of 1.0 and culture medium was changed to fresh medium at 2 h post-infection. Culture supernatants were collected at 4 days post-infection and infectious titers were determined.

RNAi screening.

Huh7.5.1 cells expressing DUB shRNA were seeded on 24 well plates at 3×10^4 cells/well and incubated for 1 day. Cells were infected with HCV at moi of 0.5 and RNAs were extracted at 2 days post-infection by adding Isogen II (Nippon Gene, 500 μ l) following manufacture's protocol. Intracellular HCV RNA was calculated by quantitative RT-PCR.

Quantitative RT-PCR (qPCR).

qPCR for HCV RNA was performed by TaqMan RNA-to-CtTM 1-Step Kit and ViiA7TM real time PCR system (Lifetechnologies). The following primers were used, HCV: 5'-

GAGTGTGCGTGCAGCCTCCA-3' and 5'-CACTCGCAAGCACCCTATCA-3', GAPDH: 5'-TGTAGTTGAGGTCAATGAAGGG-3' and 5'-ACATCGCTCAGACACCATG-3'. The following probes were used, HCV: 5'-6-FAM/CTGCGGAAC/ZEN/CGGTGAGTACAC/-3'IABkFQ, GAPDH: 5'-6-FAM/AAG GTC GGA /ZEN/GTC AAC GGATT GGT C/-3'IABkFQ. HCV RNA was determined by ddCt method by using GAPDH as internal control. The expression of miR-122 was determined by using miR-122-specific RT and PCR primers provided in the TaqMan microRNA assay (Lifetechnologies). U6 small nuclear RNA was used as an internal control. TGF- β targeting genes were quantified by Sybr Green qPCR. RNA was extracted by Isogen II and synthesized cDNA by using a High-Capacity RNA-to-cDNA kit (Lifetechnologies). qPCR was performed by SYBR Select Master Mix following manufacture's protocol. The following primers were used, mouse PAI: 5'-GCCAACAAAGAGCCAATCACA-3' and 5'-AGGCAAGCAAGGGCTGAAG-3', mouse CTGF: 5'-AACTGTGTACGGAGCGTGAC-3' and 5'-GCTGCTTGGAAAGGACTCAC-3', mouse SMAD: 5'-TGGATGGCGTGTGGGTTA-3' and 5'-TGGCGGACTTGATGAAGATG-3', human SMAD: 5'-GGCCGGATCTCAGGCATTC-3' and 5'-GAGTCGGCTAAGGTGATGGG-3',

HCV titration.

Huh7.5.1 were seeded on 24 well plates (3×10^4 cells/well) and incubated for 1 day. The culture supernatants serially diluted by medium were inoculated and incubated for 2 h. The culture supernatants were removed and 1% methylcellulose containing DMEM supplemented with 10%

FBS, 100 U/ml penicillin and 100 µg/ml streptomycin was added and incubated for 2 days. The supernatants were removed, washed once with phosphate buffer saline (PBS) and then incubated with 4% paraformaldehyde (PFA) in PBS for 2 h. Cells were washed by PBS three times, and permeabilized by incubating with 0.2% TritonX-100 containing PBS for 15 min. After washed with PBS three times, cells were incubated with anti-NS5A antibody (1/2000) diluted by 2% FBS/PBS at room temperature for 1 h. After washed with PBS three times, cells were incubated with Alexa Flour (AF) 488 conjugated anti-rabbit antibody (1/2000) diluted by 2% FBS/PBS at room temperature for 1 h. After washed by PBS three times, viral proteins expressing foci were counted under immunofluorescent microscopy (Olympus).

***In vitro* transcription, RNA transfection, and colony formation.**

The plasmid pSGR-JFH1 was linearized with XbaI and transcribed *in vitro* by using a MEGAscript T7 kit (Life Technologies) according to the manufacturer's protocol. The *in vitro* transcribed RNA (10 µg) was electroporated into Huh7 cells or its relative cells at 10^7 cells/0.4ml under conditions of 210 V and 960 µF using a Gene Pulser apparatus (Bio-Rad) and plated on DMEM containing 10 % FCS. The medium was replaced with fresh DMEM containing 10 % FCS and 1 mg/ml G418 at 24 h post-electroporation. Colonies were visualized by staining with Giemsa (Merck). at 3 weeks post-electroporation.

Subcellular localization of USP15.

Huh7.5.1 cells cultured on glass slides for 1 day were fixed with 4% PFA in PBS for 2 h. Cells were washed by PBS three times and permeabilized by incubating with 0.2% TritonX-100 in PBS

for 15 min. After washing with PBS three times, cells were incubated with anti-USP15 antibody (1/1000) diluted by 2% FBS in PBS at room temperature for 1 h. After washing with PBS three times, cells were incubated with Alexa Flour (AF) 488 conjugated anti-mouse antibody (1/2000) and HCS LipidTOXTM Red neutral lipid stain diluted by 2% FBS in PBS at room temperature for 1 h. The stained cells were covered with Prolong Gold AntiFade Reagent with DAPI (Lifetechnologies) and observed with FluorView FV1000 confocal microscopy (Olympus).

Immunoblotting.

Cell lysates were prepared by adding lysis buffer consisted of 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 1% glycerol and protease inhibitor cocktail tablets (Roche Molecular Biochemicals), incubation for 30 min at 4°C, and centrifugation at 14,000 x g for 15 min at 4°C. The supernatants were incubated at 95°C for 5 min. Proteins were resolved by SDS-PAGE (Novex gels, Invitrogen), transferred onto nitrocellulose membranes. These membranes were blocked with Tris-buffered saline containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 0.05% Tween 20 and 5% skim milk, incubated with primary antibody at room temperature for 1 h, and then with HRP-conjugated secondary antibody at room temperature for 1 h. The immune complexes were visualized with Super Signal West Femto substrate (Pierce) and detected by an LAS-3000 image analyzer system (Fujifilm).

Generation of USP15 knockout Huh7 cells.

Huh7 cells were transfected with pX330 hsUSP15 and pCAG EGxxFP hsUSP15 by PEI. GFP-positive cells were sorted by FACS AriaTM at 7 days post-transfection and incubated to form

single colonies. Genomic DNA extracted from each grown cell clones and their mutations were confirmed by sequencing. The lack of protein expression was confirmed by immunoblotting.

***In vitro* ubiquitinating assay.**

HEK293T cells transfected with expression plasmids encoding FLAG-taggedUSP15, HA-tagged ubiquitin and OSF-tagged Perilipin, ADRP or TIP-47 were lysed at 2 days post-transfection, and the lysates were incubated with Strept-Tactin beads (IBA) for 1 h. After washing with lysis buffer 5 times, beads were incubated with 1x sample buffer at 95°C for 5 min. The protein samples were subjected to immunoblotting.

HCV pseudovirues.

HCV pseudovirus was generated as described elsewhere (111). Briefly, 1×10^6 cells of 293T cells were seeded on 10cm dish and incubated for overnight. 2 μ g of pCAG C60E1/E2 (genotype 1b, strain con1), which expressed HCV E1 and E2 proteins or pCAG VSVG was transfected by PEI. After 6 hours, culture medium was changed, and incubated further for 24 hours. VSV Δ G/Luciferase *G was infected in transfected 293T cells at moi=1 and adsorbed by incubated for 1 hour at 37°C. After washing 293T cells three times by warmed PBS(-), 5 mL of fresh culture media was added on infected cells and incubated for 24 hours. Culture supernatants were collected and filtered by 0.45 μ m filter. 3×10^4 cells of Huh7 or its relatives were seeded on 24 well plates and incubated overnight. 50 μ L of viral containing culture media was added on seeded cells and incubated for 1 hour. Cells were washed by warmed PBS and incubated for 24 hours. Luciferase activity was determined by Steady-Glo luciferase assay system (Promega).

Generation of USP15 knockout mice.

USP15 knockout mice were generated by collaboration with NPO for Biotechnology Research

Development in Research Institute for Microbial Diseases. Briefly, B6D2F1 female mice were superovulated and mated with B6D2F1 males, and fertilized eggs were collected from the oviduct. The pronuclear stage eggs were injected with pX330mmUSP15. The eggs were cultivated in kSOM overnight then transferred into the oviducts of pseudopregnant ICR females (112). The tails of F0 mice were used USP15 genotyping by using primer sets of 5'-ATTTGGTACAGACCTGCCGG-3' and 5'-TCGGAATAATGGGGAACTTGGG-3'. Mice mutated in USP15 genome were crossed with wild type mice and mice possessing 223 bp deletion in USP15 sgRNA targeting genome were further crossed with wild type 5 times.

Preparation USP15^{+/−} MEFs.

USP15^{+/−} female and male mice were crossed and pregnant female mice were killed in E13.5 and obtained embryos. The embryos were extracted liver and brain and then filtered 100 µm cell strainers. Filtered cells were seeded on a collagen coated T25 flask per each embryo. After a few days, confluent MEFs were passaged all cells into T75 flask. Then they passed into 3 bottles of T75 flask. Finally, all MEFs (Passage 3) were kept in -80°C until use for experiments. In some cases, MEFs were immortalized by a lentivirus carrying the simian virus 40 (SV40) large T antigen.

VSV infection and ELISA.

3x10⁴ cells of MEFs were seeded on 24 well plates. After 24 hours, moi=5 of VSV was infected for 1 hours. Infected cells were washed by warmed PBS and incubated further. Culture supernatants were collected in each 4 hours and kept in -80°C. The concentration of IFN-β was determined by ELISA (R&D system).

TGF-β stimulation.

3x10⁴ cells of MEFs or Huh7 cells were seeded on 24 well plates. After 24 hours, 1ng/mL of

TGF- β was added and cells were collected in indicated time points.

Production of recombinant USP15 by baculovirus expression system.

DH10Bac E-coli competent cells were transformed by pFastBac HTb hsUSP15. LacZ negative colony were cultured and extracted Bacmid DNA. Sf-9 cells were maintained in SF900II supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin. 5×10^5 cells of Sf-9 cells were seeded on 6 well plates and Bacmid DNA (5 μ L of miniprep products) were transfected into Sf9 cells by mixing with 5 μ L of X-tremeGENE HP DNA transfection reagent (Roche) and 500 μ L of antibiotics-free SF900II. After 4 days, culture supernatants were collected as P1 virus. Transfected cells were lysed and confirmed USP15 expression by western blotting. 500 μ L of P1 virus was used to expand virus for a stock virus. 1×10^6 cells in 10 cm dish were infected with recombinant baculovirus expressing 6xHis-USP15 at moi=1. Infected cells were collected after 3 days infection. Cells were lysed by lysis buffer consisted of 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 1% glycerol, 10mM Imidazole and protease inhibitor cocktail tablets (Roche Molecular Biochemicals), incubation for 30 min at 4°C, and centrifugation at 14,000 x g for 15 min at 4°C. Supernatants were added by Ni beads and incubated at 4°C for 1 hour. Beads were washed by lysis buffer three times and then eluted by lysis buffer containing 0.25M Imidazole. Eluted proteins were exchanged buffer to PBS by Slide-A-Lyzer Dialysis Cassettes (Pierce).

In vitro DUBs enzymatic activity assay.

Recombinant USP15 (rUSP15) was diluted to 400nM by using assay buffer (50mM Tris-HCl (pH 8.0), 0.05% CHAPS, 10mM DTT). A DUB substrate, DiUb48-5 (Lifesensors) is a diubiquitin, which is K48-linked ubiquitin molecule. DiUb48-5 was also diluted to 400nM by using assay buffer. In 96-well black assay plates, rUSP15 and DiUb48 were mixed and performed a kinetic read to measure fluorescent of TAMRA for 1 hour.

Statistical analysis.

The data for statistical analyses are average of three independent experiments. Results were expressed as means \pm standard deviation. The significance of differences in the means was determined by Student's *t* test.

Results

HCV infection suppresses ubiquitination of cellular proteins.

Ubiquitylation is a post-translational system to control numerous cellular processes including protein degradation, signal transduction, endocytosis and gene expression. To examine the effect of HCV infection on the ubiquitination of cellular proteins, total ubiquitinated proteins in HCV infected Huh7.5.1 cells were purified by agarose-conjugated tandem ubiquitin binding entity (TUBE) (Fig. 14A). Ubiquitinated proteins in cells infected with HCV were decreased in accord with the increase of NS5 expression, suggesting that ubiquitinated proteins in cells infected with HCV were degraded by proteasome or ubiquitin was removed by DUBs. To examine the involvement of DUBs on the HCV replication, Huh7.5.1 cells infected with HCV were treated with a cell-permeable and broad-spectrum DUB inhibitor, PR-619 (113), at 24 h post-infection. Intracellular HCV RNA was significantly reduced by the treatment with PR-619 at a concentration exhibiting no cell toxicity (Fig. 14B). These data suggest that DUB activity is required for HCV replication.

RNAi screening to identify DUBs participate in HCV replication.

The human genome encodes approximately 100 DUBs. DNA microarray data suggested that majority of DUBs is expressed in Huh7.5.1 cells (Fig. 15A). Next, I set up a screening system to identify DUBs involved in the regulation of HCV replication. Stable knockdown Huh7.5.1 cell lines established by infection with retroviruses expressing shRNA against 65 different DUBs were

infected with HCV and intracellular HCV RNA were determined by qPCR at 2 days post-infection.

Screening data suggested that expression of shRNA against USP15 inhibited HCV replication in the same level with that against PI4KA reported to be involved in HCV replication (Fig. 15B).

Interestingly, amounts of mRNA of USP15 were increased in HCV infected cells (Fig. 15C).

Therefore, I focused on USP15 on the replication of HCV in this study.

USP15 specifically participates in the propagation of HCV.

To clarify the roles of USP15 in the replication of HCV in more detail, I established 2 independent USP15 deficient Huh7 cell clones (#8 and #22) by using CRISPR/Cas9 system (114, 115) (Fig. 16).

Lack of USP15 expression was confirmed by immunoblot analyses (Fig. 17A). These USP15

knockout Huh7 cell lines exhibit similar growth curve and expression of miR-122, a critical

microRNA for HCV replication, with parental Huh7 cells (Figs. 17B and 17C). Next, to determine

the effect of knockout of USP15 on the HCV replication, subgenomic HCV RNA replicon of JFH1

strain was electroporated into parent and USP15 knockout Huh7 cells and cultivated for 2 weeks in

the presence of G418 to form drug-resistant colonies. Numbers of colony in USP15 knockout cells

were significantly reduced than those in parental Huh7 cells (Fig. 18A), suggesting that USP15

plays crucial roles in HCV replication. To further examine the roles of USP15 on HCV propagation,

intracellular RNA and infectious titers in parental and USP15 knockout Huh7 cells at 4 days

post-infection were determined by qPCR and plaque assay, respectively. Both intracellular viral

RNA and infectious titers in the culture supernatants were drastically reduced in USP15 knockout

cells in compared with parent Huh7 cells (Figs. 18B and 18C). To examine the specificity of the

participation of USP15 in the viral replication, parental and USP15 knockout Huh7 cells were infected with Japanese encephalitis virus (JEV). Intracellular JEV RNA and infectious titers in the culture supernatants were comparable between parental and USP15 knockout Huh7 cells (Figs. 18D and 18E). I further examined the effect of USP15 knockout on the replication of hepatitis B virus (HBV). A plasmid encoding genotype C of HBV was transfected into parental and USP15 knockout Huh7 cells and intracellular HBV DNA was quantified by qPCR at 3 days post-transfection. No significant difference between parental and USP15 knockout Huh7 cells was observed (Fig. 18F). Collectively, these data suggest that USP15 specifically participates in the propagation of HCV.

USP15 participates in the lipid droplet formation.

To examine the subcellular localization of USP15, Huh7 cells were examined by immunofluorescence observation by using anti-USP15 antibody. USP15 was detected as dot-like structure and co-localized with lipid droplets in parental Huh7 cells (Fig. 19A upper). Upon infection with HCV, co-localization of USP15 with large sizes of lipid droplets in compared with mock-infected cells were detected (Fig. 19A lower). Because lipid droplets were suggested to be important for HCV assembly (116), I hypothesized that USP15 participates in the regulation of biogenesis or function of lipid droplets. FACS analyses revealed that USP15 knockout Huh7 cell lines contain smaller amounts of lipid droplets than parental cells (Fig. 19B). In USP15 knockout Huh7 cells, amounts of lipid droplets were severely reduced and overexpression of USP15 in the USP15 knockout cells induced formation of larger size and number of lipid droplets (Fig. 19C).

These results suggest that USP15 participates in formation of lipid droplets.

ADRP is a specific target for USP15.

The PAT family proteins interact with intracellular lipid droplets and regulate biogenesis of lipid droplets (117). The PAT family consists of 5 proteins in human, Perilipin, ADRP, TIP47, S3-12 and OXPAD. OSF-tagged Perilipin, ADRP and TIP47 were co-expressed with HA-tagged ubiquitin in the presence or absence of FLAG-tagged USP15 in 293T cells and cell lysates were incubated with Strept-Tactin beads to purify OSF-tagged proteins. Purified proteins were subjected to immunoblot analysis by using anti-HA antibody to assess ubiquitination of the OSF-tagged proteins. ADRP but not Perilipin and TIP47 was clearly ubiquitinated in 293T cells and its ubiquitination were reduced by the expression of USP15 (Fig. 20A). To further examine the specific cleavage of ubiquitin from ADRP by USP15, OSF-tagged ADRP, HA-tagged ubiquitin and wild type or catalytically inactivated mutant of FLAG-tagged USP15 were expressed in 293T cells and ubiquitination status of ADRP was determined by immunoblotting. Overexpression of wild type but not of mutant of USP15 suppressed ubiquitination of ADRP in a dose dependent manner (Fig. 20B). Finally, I examined the interaction of ADRP with USP15 by immunoprecipitation analysis. OSF-tagged ADRP interacted with both wild type and mutant FLAG-tagged USP15, suggesting that ADRP is a specific substrate for USP15 (Fig. 20C).

HCV infection efficiency is equivalent in Huh7 and USP15 knockout cells.

There is still possibility that HCV replication was inhibited in USP15 deficient Huh7 cells due to impairment of entry in USP15 deficient cells. To examine this possibility, Pseudotype v (Vesicular

stomatitis virus bearing HCV envelope proteins in place of VSV G protein (VSV Δ G HCV) was infected to Huh7 cells and USP15 deficient Huh7 cells. Efficiency of infection was determined by measurement of luciferase activity. Expression of luciferase in cells infected with VSV Δ G HCV was comparable to those infected with a control virus bearing VSV G protein (VSV Δ G G) (Fig. 21), suggesting that the reason of inhibited HCV replication in USP15 deficient Huh7 cells was not due to impairment of infection steps.

Physiological significance of USP15 *in vivo*.

To examine the physiological importance of USP15, I collaborated with NPO for Biotechnology Research Development in Research Institute for Microbial Diseases and generated USP15 deficient mice by using CRISPR/Cas9 (Fig. 22A). One of offspring mice, which had 223 bp deletion of USP15 genome (Fig. 22B), was obtained. To avoid the risk of off-target mutation in CRISPR/Cas9 system, mutated mice were crossed with wild type mice 5 times (Fig. 22A). Primary mouse embryonic fibroblasts (MEFs) were prepared and confirmed the loss of USP15 protein by western blot (Fig. 22C). USP15 $^{-/-}$ mice were viable and followed in Mendelian rules (Fig. 22D). However, the body weight of USP15 $^{-/-}$ mice was smaller than that of USP15 $^{+/+}$ and USP15 $^{+/+}$ mice until 2 month olds (Fig. 23). These data suggested that loss of USP15 affects on mice growth in unknown mechanisms.

Effects of USP15 on innate immune responses.

Pauli et al. reported that USP15 had a critical regulator of the TRIM25- and RIG-I-mediated innate immune response (118). They showed siRNA-mediated knockdown of USP15 suppressed IFN- β

production in HEK 293T cells and VSV infection was enhanced in USP15 knockdown HEK293T cells. They used siRNA-mediated gene knockdown strategy, therefore, I examined the effect of USP15 on innate immune response by using USP15^{-/-} MEFs. VSV was infected to USP15^{+/+}, USP15^{-/-}, TBK1^{+/+} and TBK1^{-/-} MEFs at moi of 5 and culture supernatants were collected in indicated time points. USP15^{-/-} MEFs secreted IFN- β same levels of USP15^{+/+} MEFs. On the other hands, TBK1^{-/-} MEFs did not secreted IFN- β , suggesting that USP15 did not affect on IFN- β production by VSV infection (Fig. 24A). Furthermore, VSV titers in USP15^{+/+}, USP15^{-/-} MEFs did not changed (Fig. 24B). These data suggested that USP15 did not play a role in RIG-I mediated innate immune response.

Effects of USP15 on TGF- β signaling.

Recently, USP15 had been reported to be critical for regulation of target promoters by SMAD, which is a transcriptional factor regulated by TGF- β signaling pathway (119, 120). Therefore, I next investigated the effect of USP15 on TGF- β signaling by using USP15^{-/-} cells. In MEFs, TGF- β targeting transcriptional factors such as PAI (Fig. 25A), CTGF (Fig. 25B) and SMAD (Fig. 25C) were no significant difference between in USP15^{+/+} or in USP15^{-/-} MEFs. However, SMAD activation through TGF- β stimulation was seen in Huh7 cells but not in USP15 deficient Huh7 cells (Fig. 25D). These data suggested that the roles of USP15 in TGF- β stimulation were cell-type specific manner.

Establishment of high-throughput screening (HTS) system for development of USP15 inhibitors.

My data suggest that USP15 plays a role for HCV replication and lipid droplets formation. Therefore, USP15 inhibitors may be useful to control viral loads in HCV patients and metabolic diseases. So, I produced recombinant USP15 (rUSP15) in insect cells by using baculovirus expression system. Large amounts of rUSP15 were obtained by baculovirus system (Fig. 26A). DiUb48, the C-terminus of wild type ubiquitin is conjugated via an isopeptide bond to lysine 48 (K48) of a second ubiquitin molecule with the resultant diubiquitin forming an internally quenched fluorescent FRET pair (IQF). Each ubiquitin is labeled with a single molecule of either a fluorescent reporter (TAMRA). Once DiUb48 is cleaved by rUSP15, the intensity of TAMRA is increased following incubation (Fig. 26B). Adding rUSP15 enhanced the intensity of TAMRA fluorescent in time-dependent manner (Fig. 26C). On the other hand, DiUb48 alone did not enhance intensity (data not shown). These data suggested that DiUb48 was specifically cleaved by rUSP15 *in vitro*. This system may be useful system to develop USP15-specific inhibitors by HTS system.

USP20 regulates not only HCV replication but also JEV replication.

My shRNA screening data showed USP20 was also affected on HCV replication (Fig. 15B). So, I also investigated the role of USP20 by establishment of USP20 deficient Huh7 cells (Fig. 27A). To examine the roles of USP20 on HCV propagation, intracellular RNA and infectious titers in parental, USP20 knockout Huh7 cells and USP20 knockout Huh7 cells expressing exogenously Flag-USP20 were determined at 4 days post-infection. Both intracellular viral RNA and infectious titers in the culture supernatants were drastically reduced in USP20 knockout cells in compared with parent Huh7 cells (Figs. 27B and 27C). Interestingly, exogenously overexpression of USP20

in USP20 knockout Huh7 cells rescued HCV RNA and infectious titer (Figs. 27B and 27C). To examine the effect of the participation of USP20 in JEV infection, parental and USP20 knockout Huh7 cells were infected with JEV. Intracellular JEV RNA and infectious titers in the culture supernatants in USP20 knockout Huh7 cells were reduced compared to parental Huh7 cells (Figs. 27D and 27E). These data suggested that USP20 played roles not only in HCV infection but also JEV infection.

Discussion

HCV utilizes several cellular machineries to achieve an efficient propagation. For example, Hsp90-mediated protein folding system is required for an efficient HCV replication. NS5A interacts with FKBP8 and human butyrate-induced transcript 1 (hB-ind1) to recruit Hsp90 to viral replication complex (97, 121). To form viral replication complexes in the ER membrane, HCV recruits phosphatidylinositol 4 phosphates (PI4P) to ER from Golgi (70, 78, 79, 80). HCV NS5A and NS5B interact with vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtype A (VAP-A) and B (VAP-B) (85), which transfer PI4P to ER membrane through their FFAT motif (122). Furthermore, lipid droplets and apolipoproteins are necessary for efficient viral releases (123). Therefore, I hypothesized that other unknown host machineries should be involved in HCV propagation.

DUBs regulate many cellular processes. For example, A20 regulates NF κ B signaling pathway through its DUB and E3 ligase activity (124). USP9x controls ubiquitination of Mcl-1, which is a member of Bcl-2 protein family and regulates cell survival through DUB enzyme (125). The tumor suppressor cylindromatosis (CYLD) inhibits NF κ B and mitogen-activated protein kinase (MAPK) through deubiquitination of NEMO, TRAF2, TRAF6 and TAK1. Liver specific CYLD deficiency leads to liver fibrosis, inflammation and hepatocellular carcinoma (126). USP33 and USP20 modulate post-endocytic trafficking of the asthma β 2 adrenergic receptor (β 2AR) (127). AMSH regulates ESCRT proteins stability to control receptor trafficking (128). USP10 regulates p53

localization and stability (129). These reports indicate that the functions of DUBs cover almost all events in live cells. Although human genes encodes only less than 100 DUBs, identification of DUBs essential for HCV replication may discover new host machineries to be utilized by HCV or novel functions of DUBs through cellular components known to be used by HCV.

I established 65 different Huh7 cells stably expressing each of shDUBs. Although I did not check knockdown efficiency in all cells, I found that Huh7 cells expressing shRNA against USP15 significantly inhibited HCV replication. USP15 had been reported to regulate in TGF- β signaling through stabilization of SMADs (119, 120). USP15 also regulates Nrf2, a master regulator of antioxidant response, through de-ubiquitination of Keap1 (130). Furthermore, USP15 stabilizes MDM2 in T cells, an E3 ubiquitin ligase to be essential for p53 stability and function (131). However, physiological roles of USP15 in the liver remain unknown.

In this study, I identified USP15 as a critical host factor for HCV life cycle. Although it is still needed to clarify how USP15 contributes to HCV life cycle, USP15 deficient cells clearly showed the reduction of the number of lipid droplets. Lipid droplet is a cytosolic lipid storage organelle playing central roles in energy and lipid metabolism. Lipid droplet is consisted of neutral lipids such as phospholipids, triacylglycerides and their intermediates. The surface of lipid droplet binds to several regulatory proteins. Among them, Perilipin and ADRP are located on the surface of lipid droplets. My *in vitro* data suggested that USP15 localizes on lipid droplets. Furthermore, ADRP but not Perilipin was an ubiquitinated proteins and USP15 specifically interacts with ADRP and de-ubiquitinates ADRP through its DUB activity. These data suggest that USP15 regulates

formation of lipid droplets in the liver.

Once HCV core proteins are synthesized in ER, some fraction of core proteins localize on lipid droplets. The localization of core proteins on lipid droplets is required for virus assembly and release. Lipid droplets also recruit non-structural proteins such as NS5A and lipid droplet-associated membrane including viral replication complexes (132). HCV infection was reported to accumulate lipid droplets around nucleus (133). Upregulation of USP15 expression in cells upon infection with HCV suggests that HCV utilizes USP15 in the formation of lipid droplets. Further studies are needed to clarify how USP15 regulates lipid metabolism in the liver. Further analysis of USP15 knockout mice will be clarified more detail roles of USP15 in the liver.

My data suggests that USP15 plays important roles in HCV replication through controls of lipid droplets. Therefore, USP15 inhibitors may impair HCV replication in patients and control metabolic diseases. So, USP15 targeting compounds are attractive in clinics. In vitro HTS system is needed for development of USP15 inhibitors. By using baculovirus expressing system, large amounts of rUSP15 were obtained. The rUSP15 and DiUb48 system will be useful and valuable tools to identify USP15 inhibitors. This rUSP15 may also be useful for determinant of three-dimensional structure by generating crystals.

I also found that USP20 was involved in HCV replication. USP20 was demonstrated to regulate recycling and resensitization of β 2 adrenergic receptor (134). USP20 targets TRAF6 to regulate NF- κ B signaling (135). USP20 also deubiquitinates and stabilizes hypoxia-inducible factor (HIF)-1 α (136), which is a sequence-specific DNA-binding transcriptional complex with HIF-1 β , HIF1

regulates genes involved in angiogenesis, glucose metabolism, cell proliferation and invasion/metastasis. Although I need to clarify how USP20 regulates HCV/JEV replication, USP20 may be a good candidate for not only HCV but also JEV therapy.

Figure 1

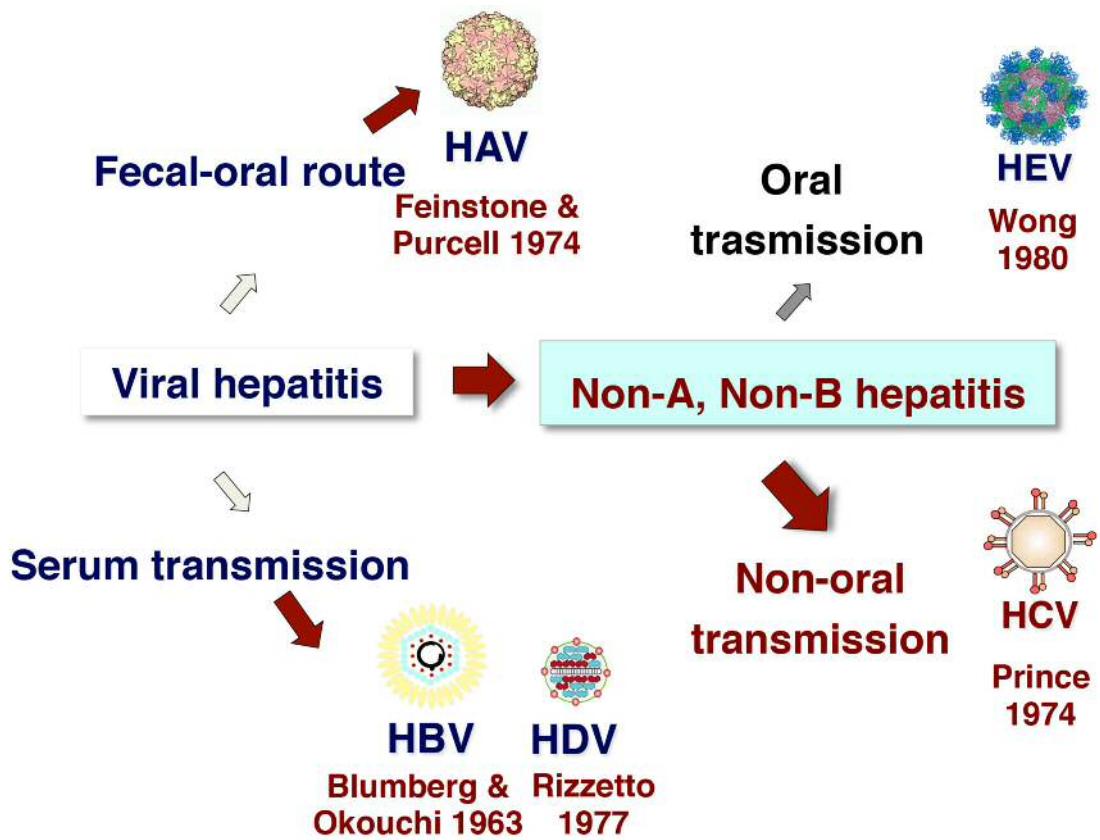


Figure 2

Hepatitis	Hepatitis A	Hepatitis B	Hepatitis C	Hepatitis D	Hepatitis E
Hepatitis virus	HAV	HBV	HCV	HDV	HEV
Family	<i>Picornaviridae</i>	<i>Hepadnaviridae</i>	<i>Flaviviridae</i>	<i>Deltaviridae</i>	<i>Hepeviridae</i>
Genus	<i>Hepadovirus</i>	<i>Orthohepadnavirus</i>	<i>Hepacivirus</i>	<i>Deltavirus</i>	<i>Hepevirus</i>
Viral genome	ssRNA	dDNA	ssRNA	ssRNA	ssRNA
Source	Fecal-Blood	Blood	Blood	Blood	Fecal-Blood
Fulminant infection	+	+	+	+	+
Chronic infection	-	+	+	+	-
<i>Cell culture</i>	+	-	+/-	-	+
Vaccine	+	+	-	-	-

Figure 3

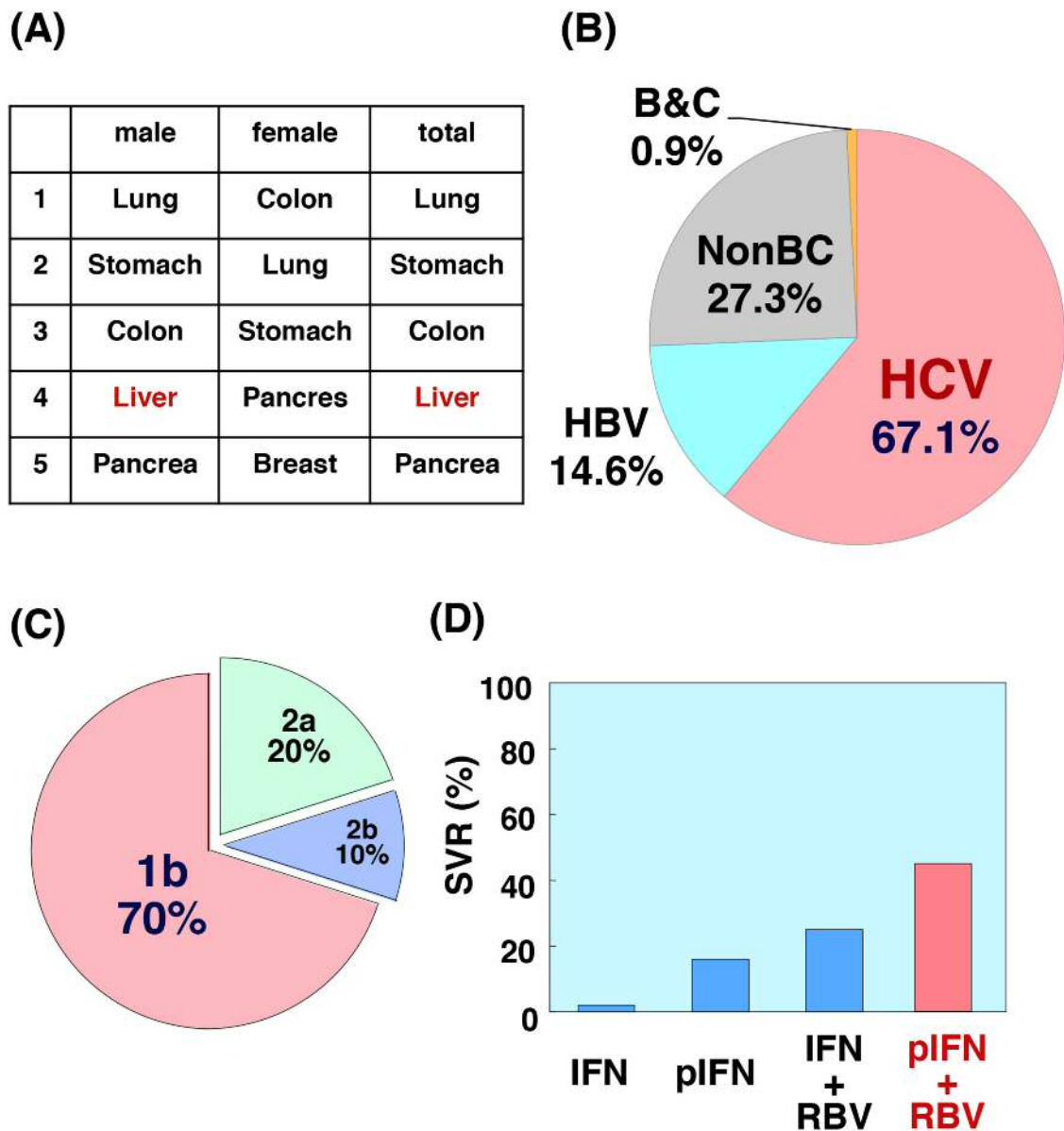


Figure 4

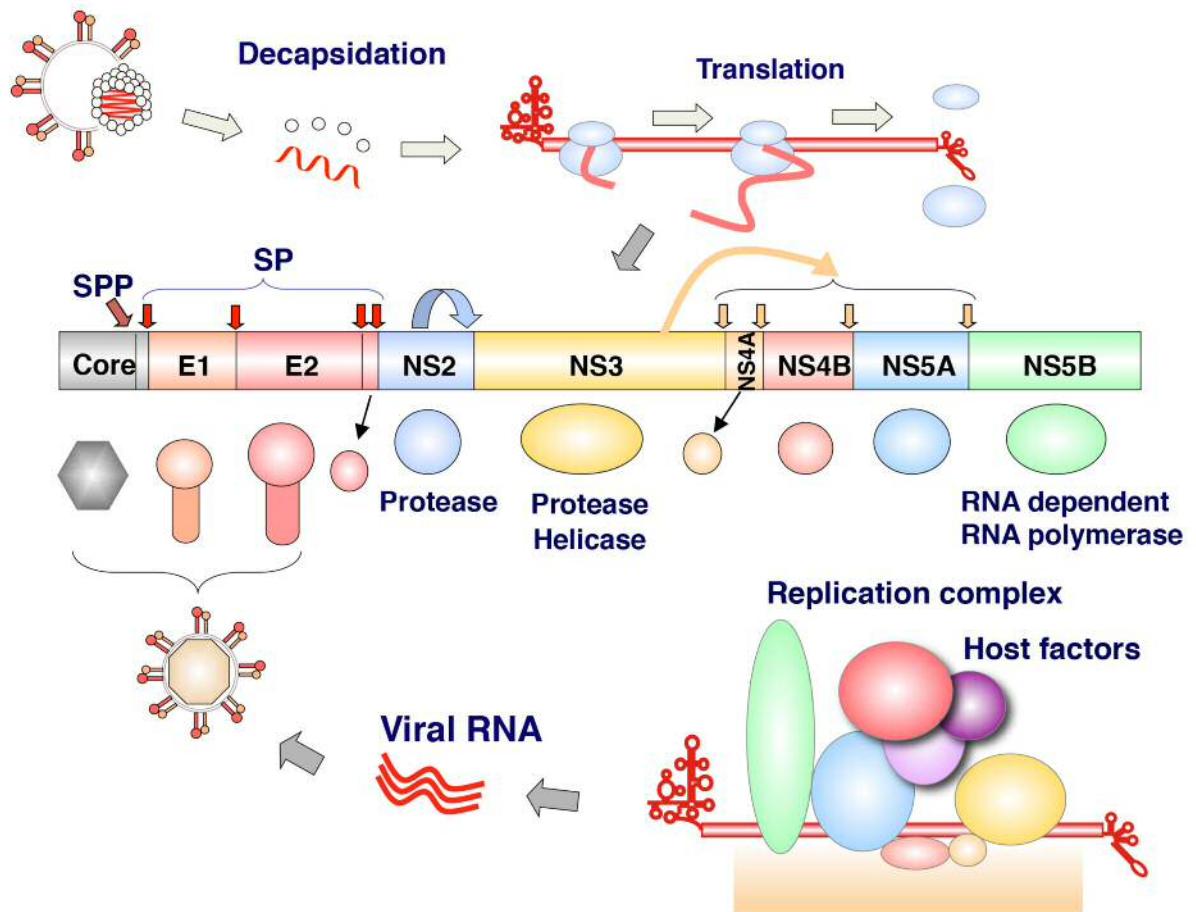


Figure 5

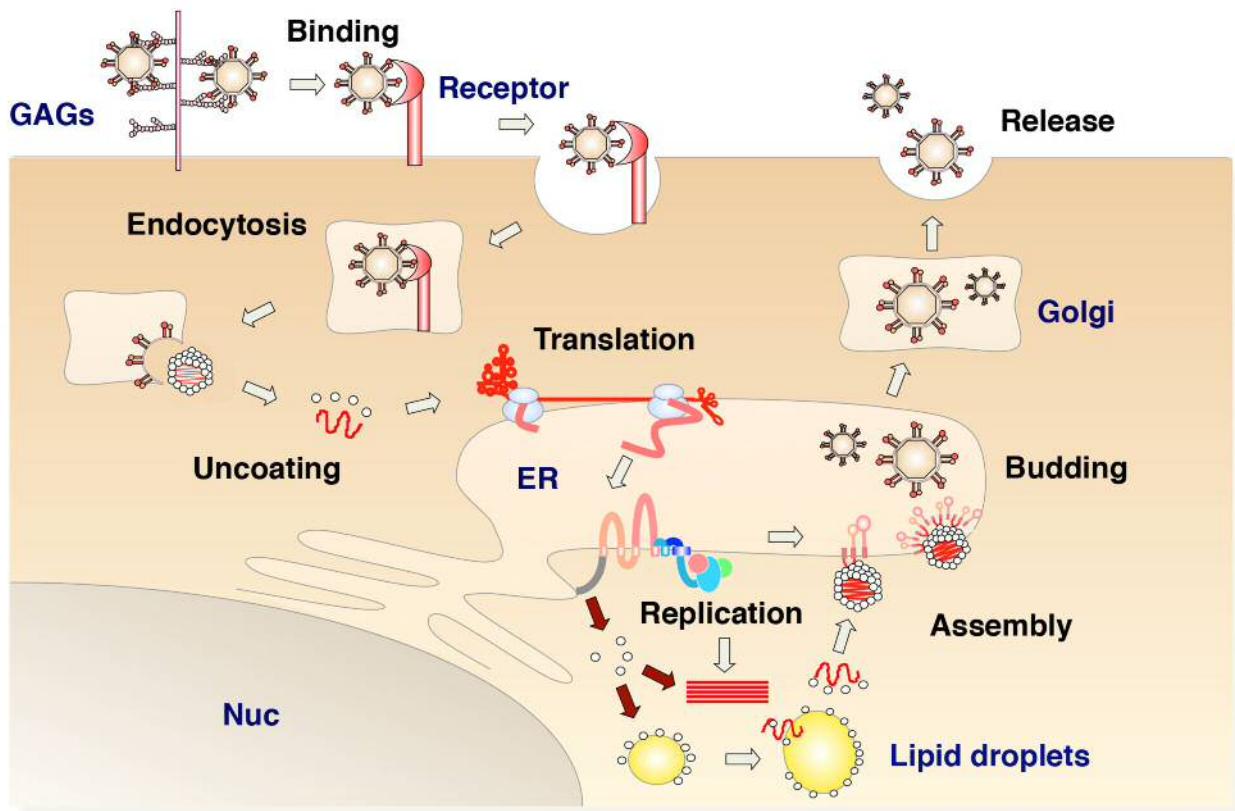


Figure 6

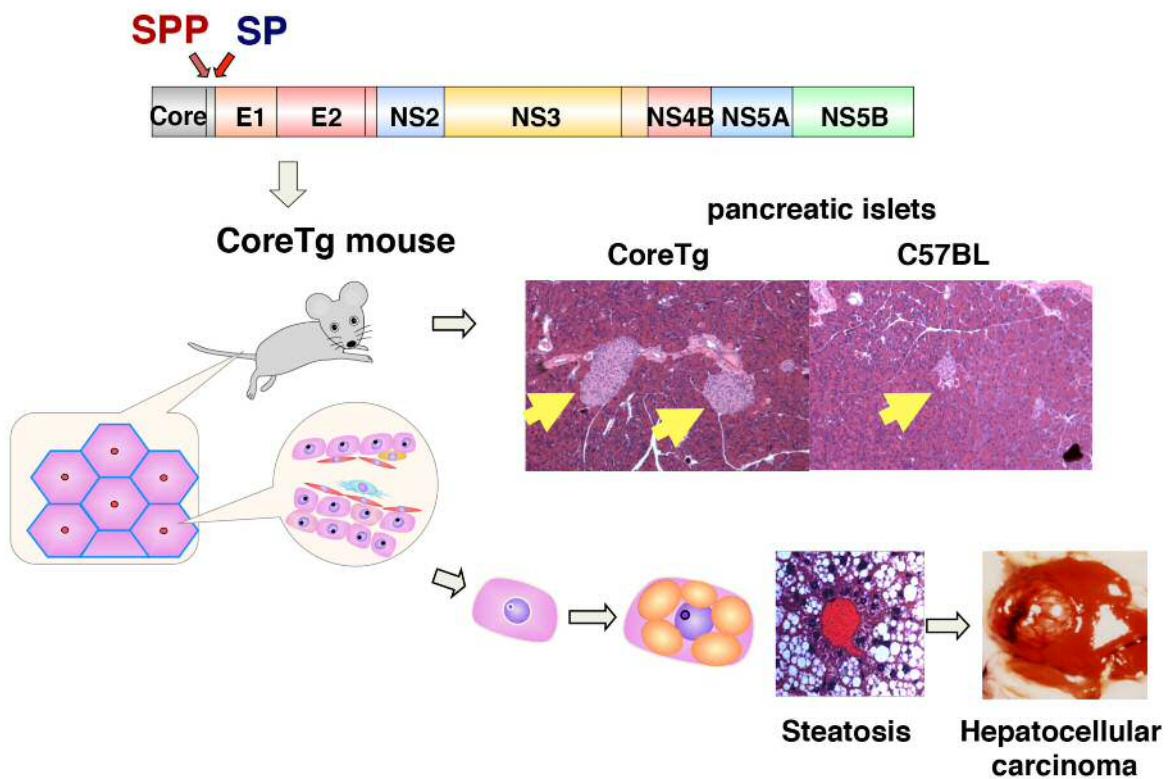


Figure 7

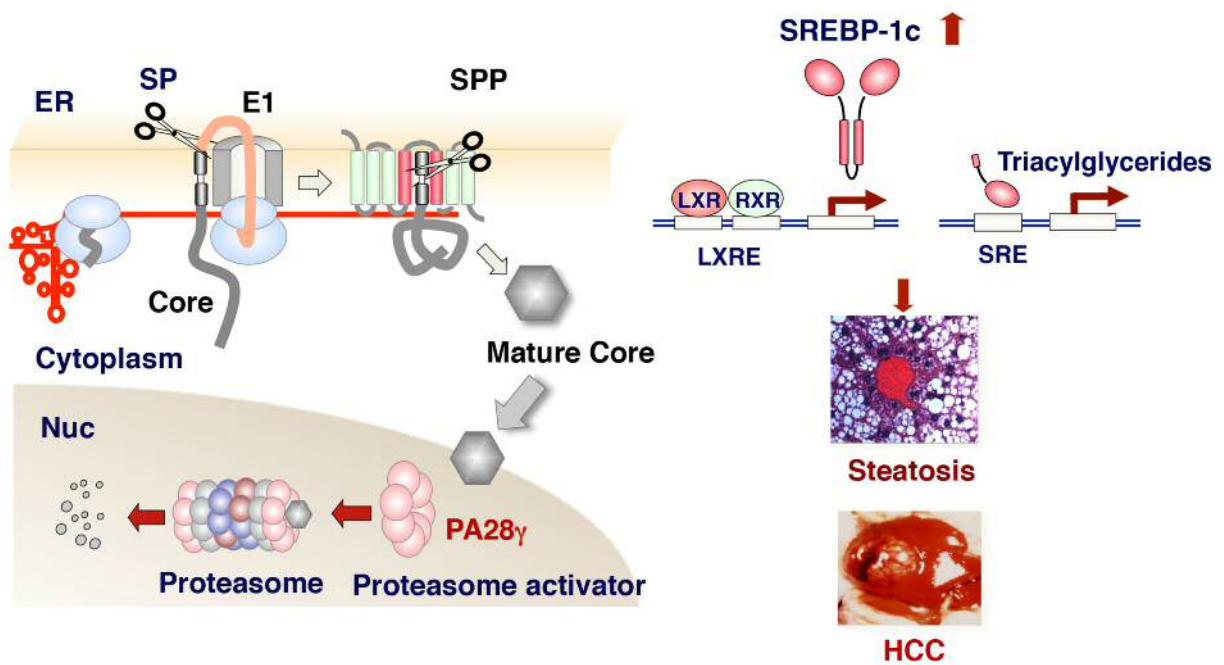


Figure 8

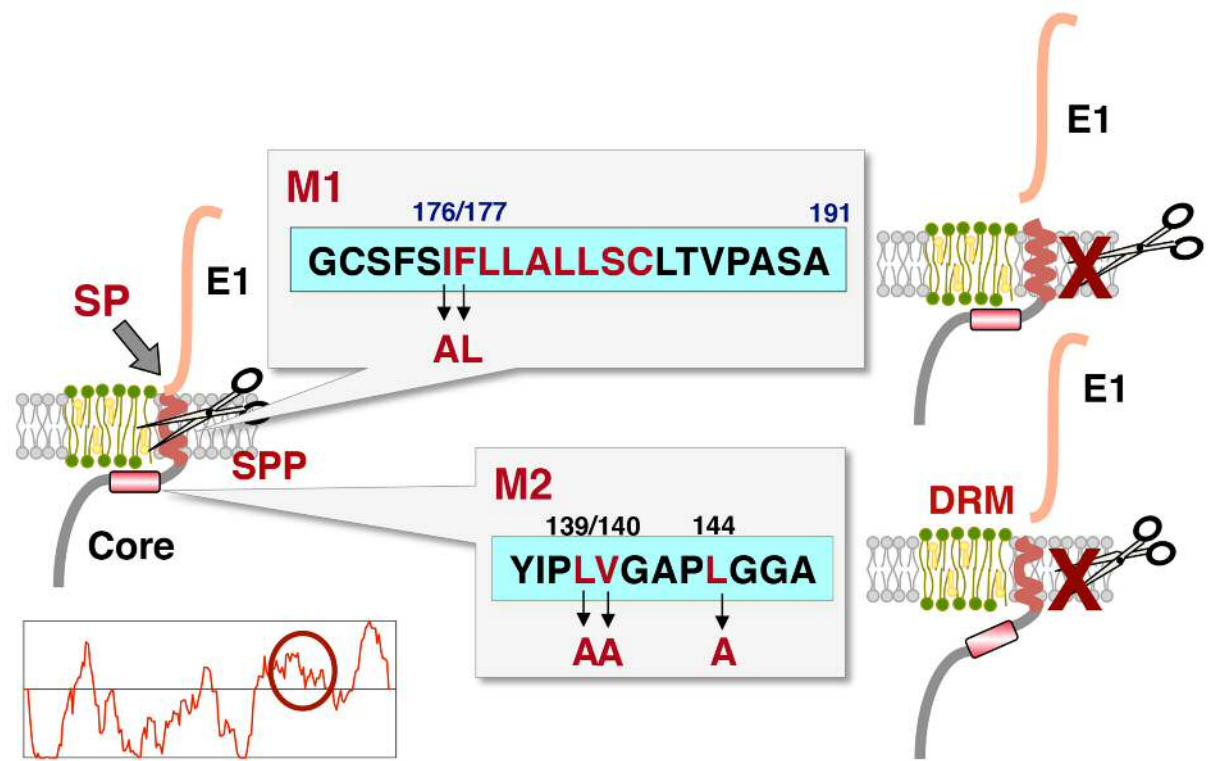
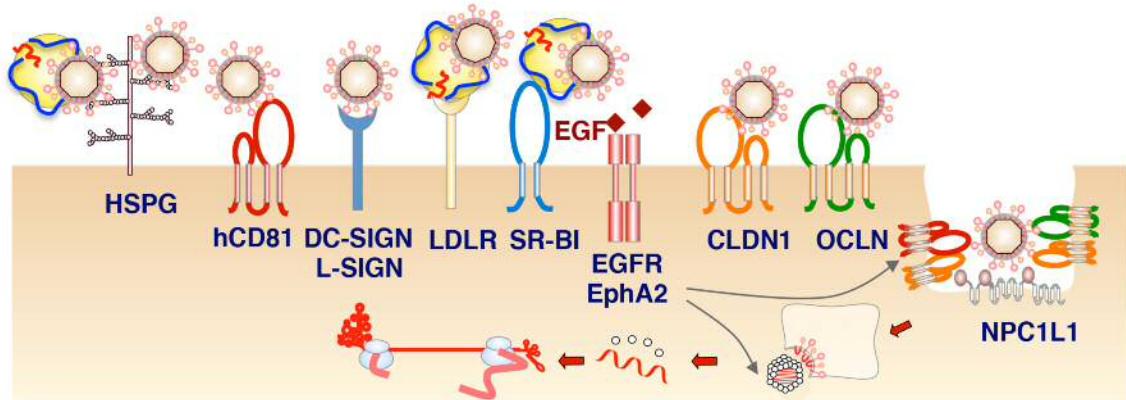


Figure 9

(A)



(B)

CD81	SR-B1	CLDN-1	OCLN	Infectivity
H	H	H	H	○
H	H	H	M	✗
H	H	M	H	○
H	M	H	H	○
M	H	H	H	✗
H	M	M	H	○
M	M	M	M	✗



Figure 10

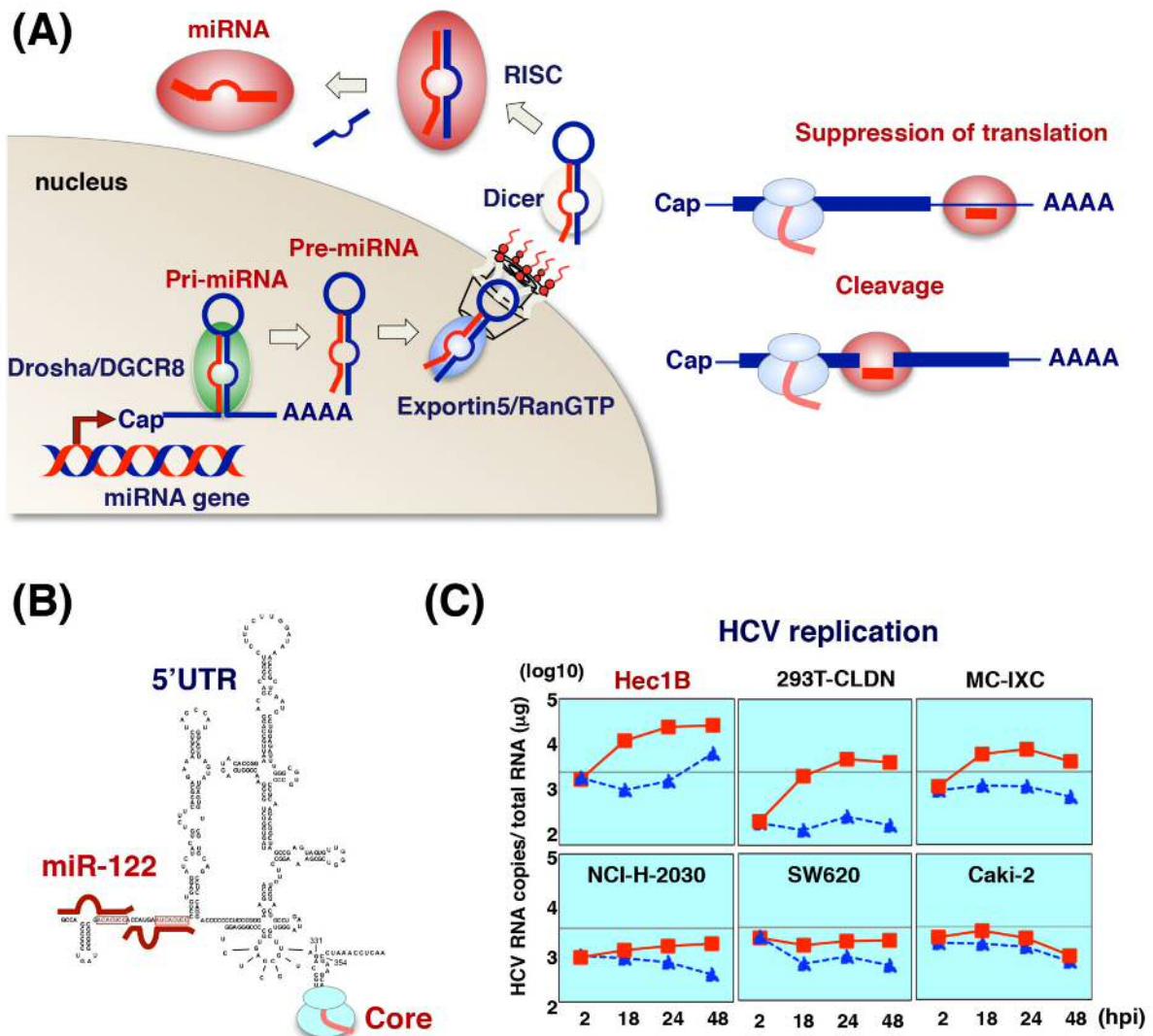


Figure 11

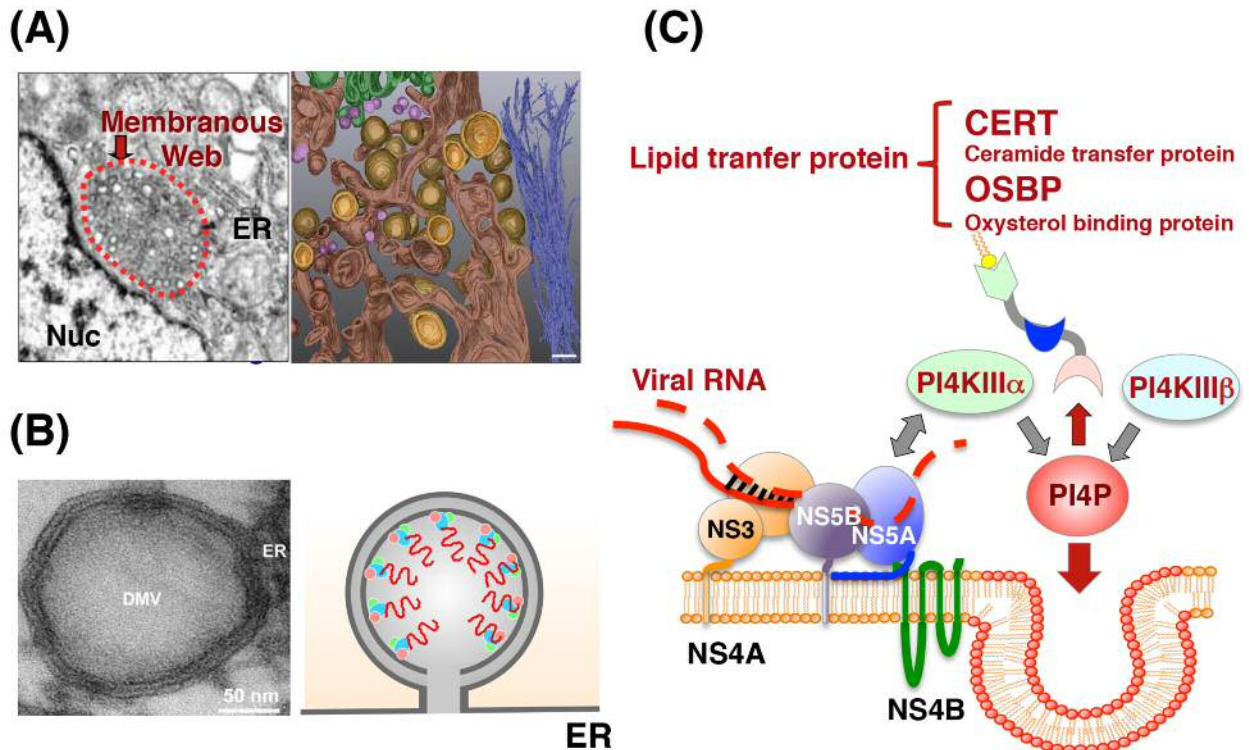


Figure 12

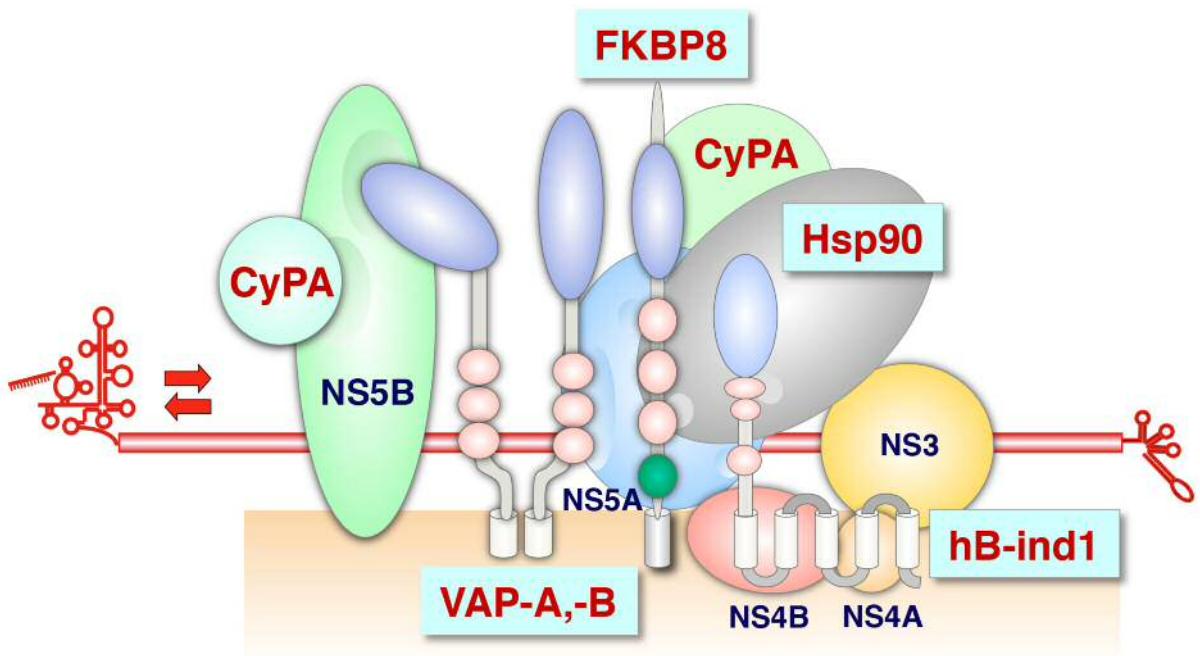


Figure 13

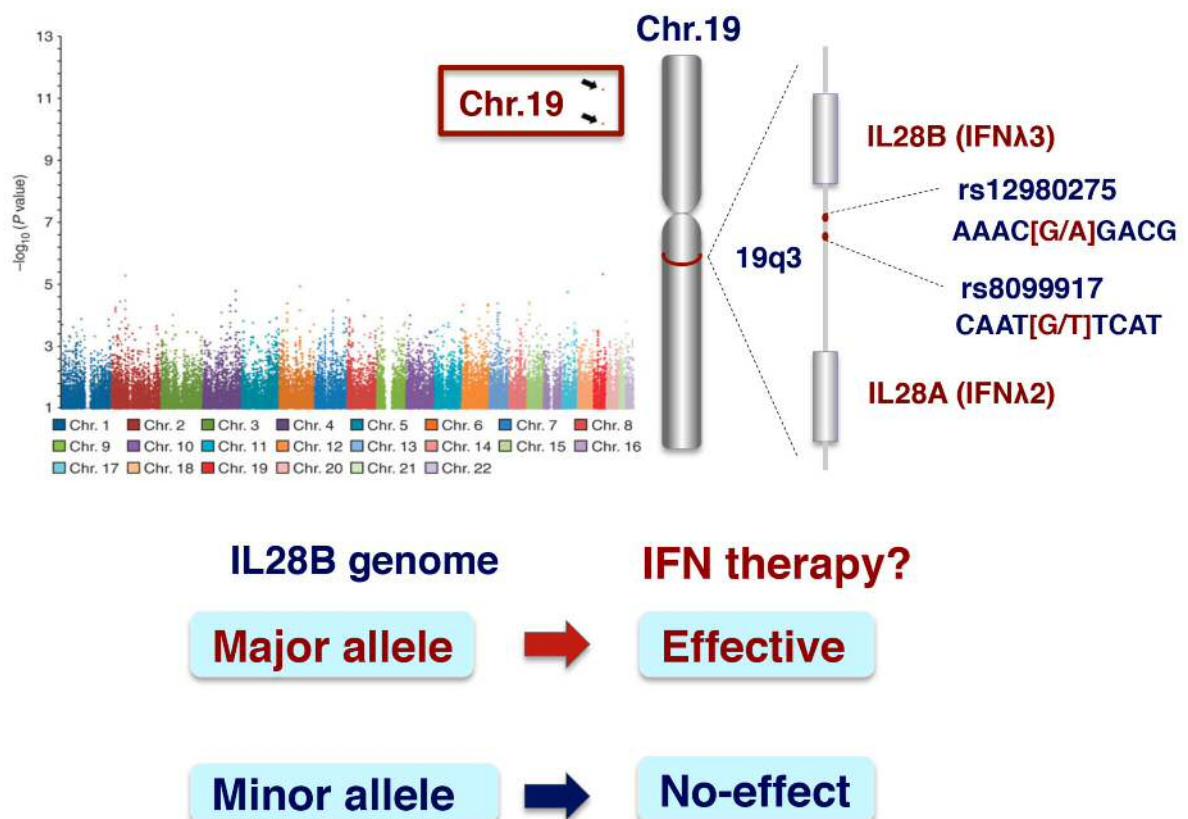


Figure 14

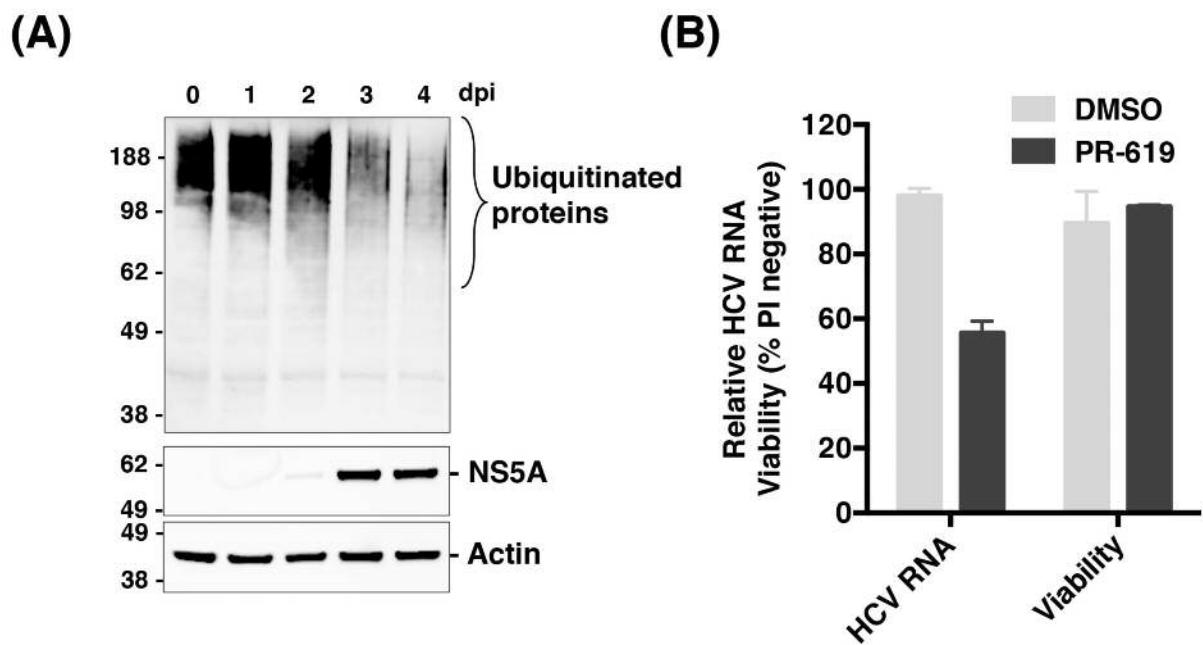


Figure 15

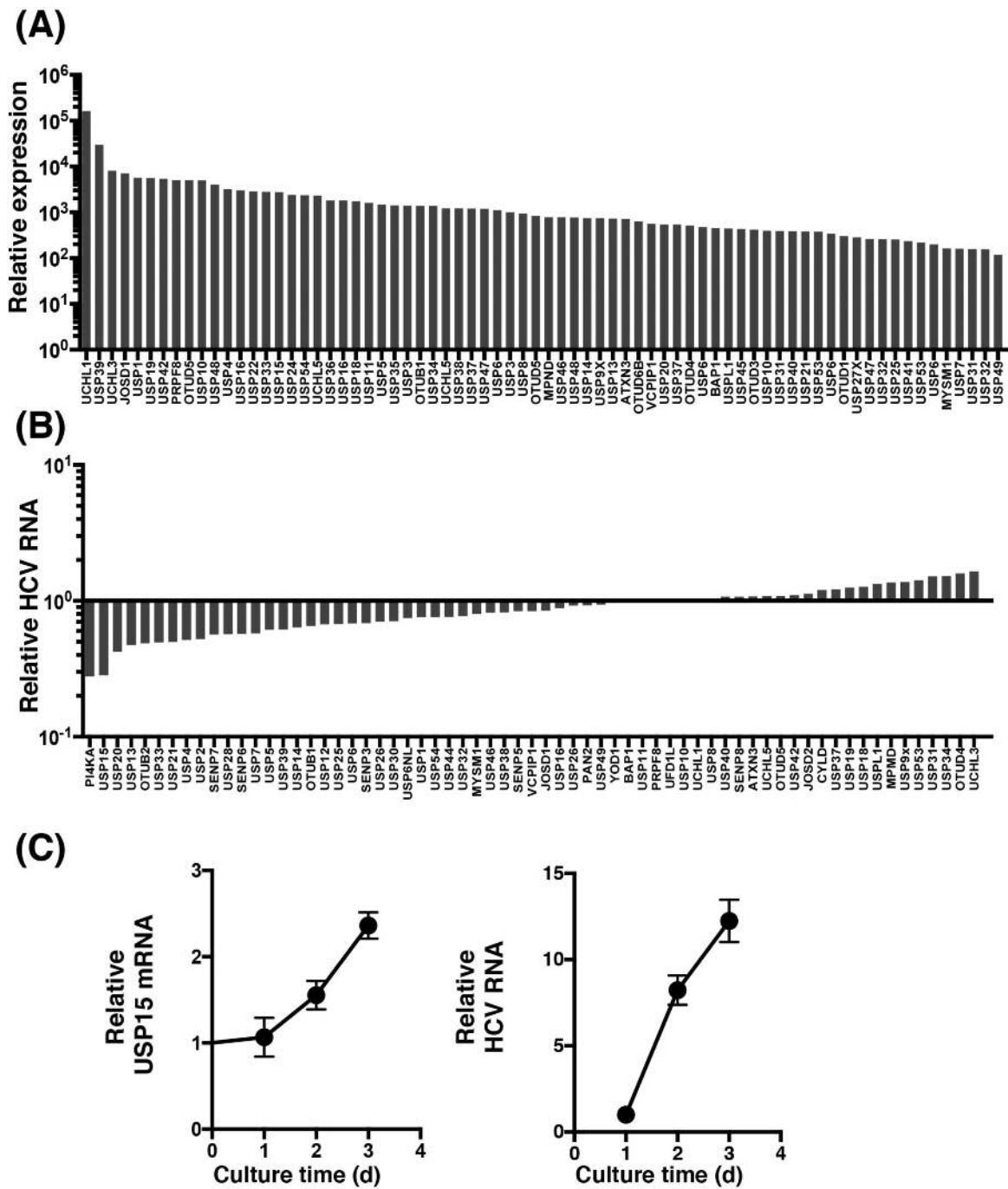


Figure 16

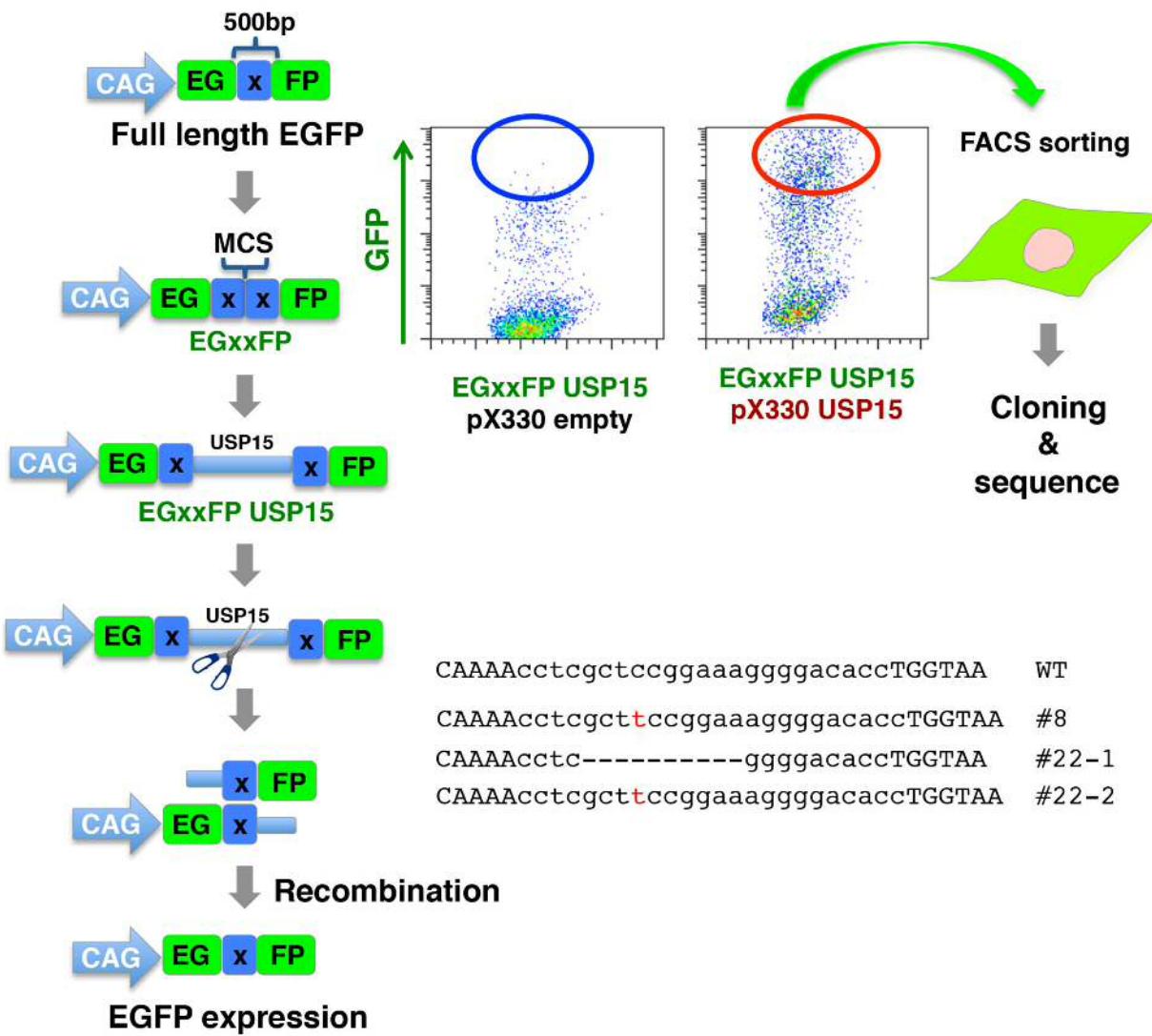


Figure 17

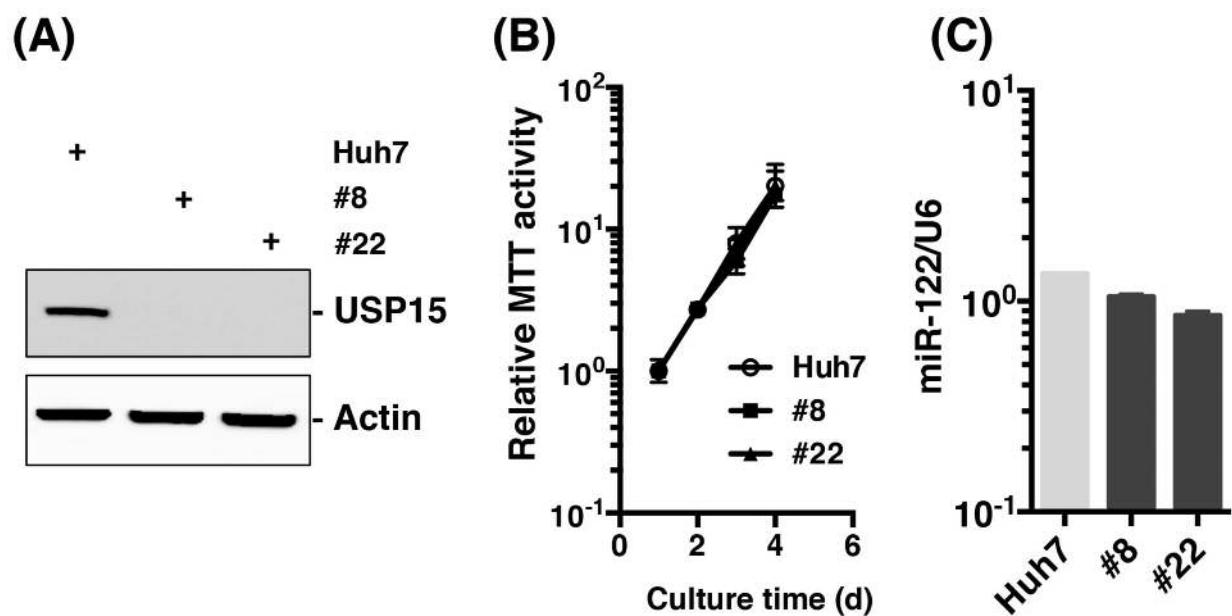


Figure 18

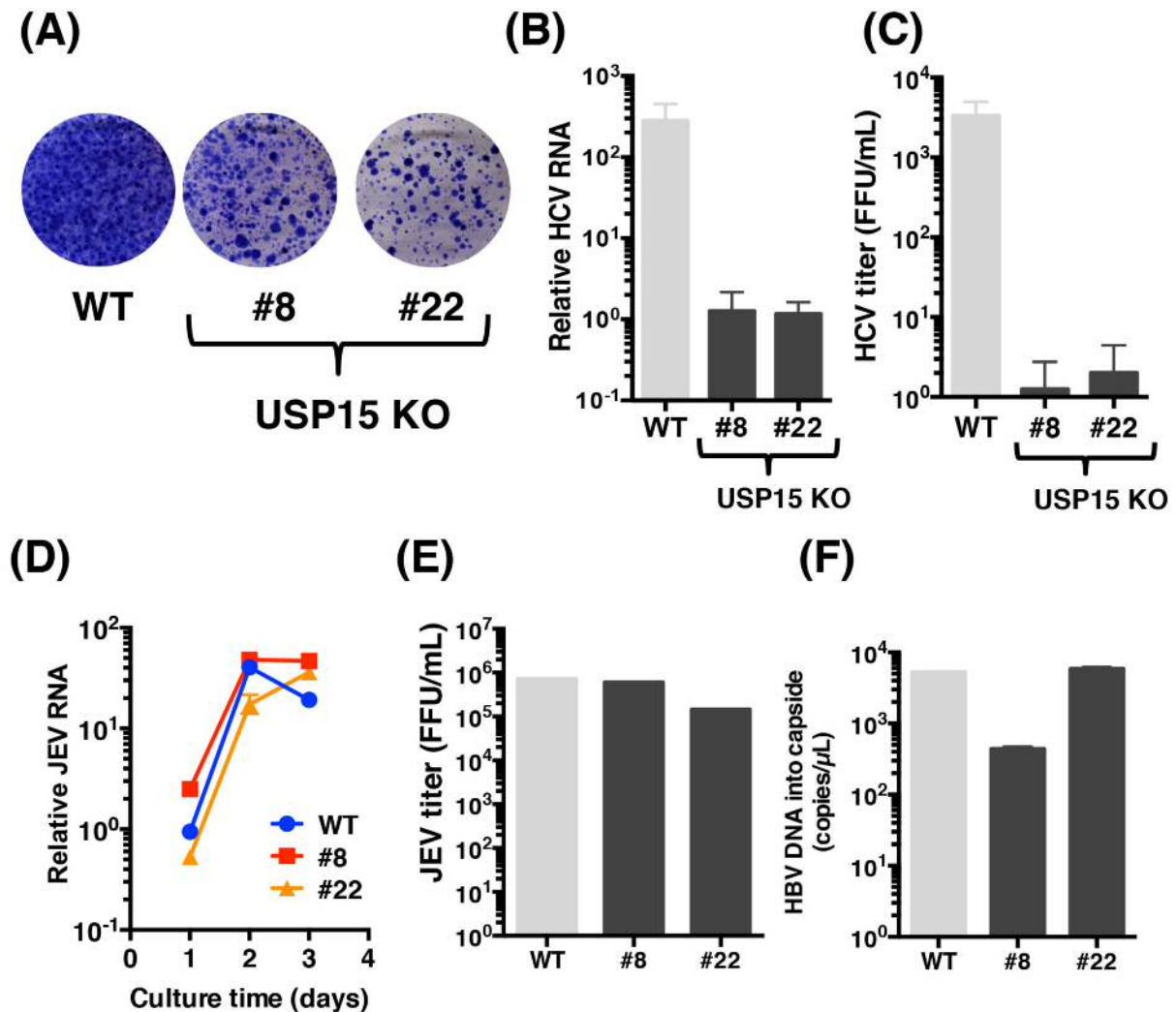


Figure 19

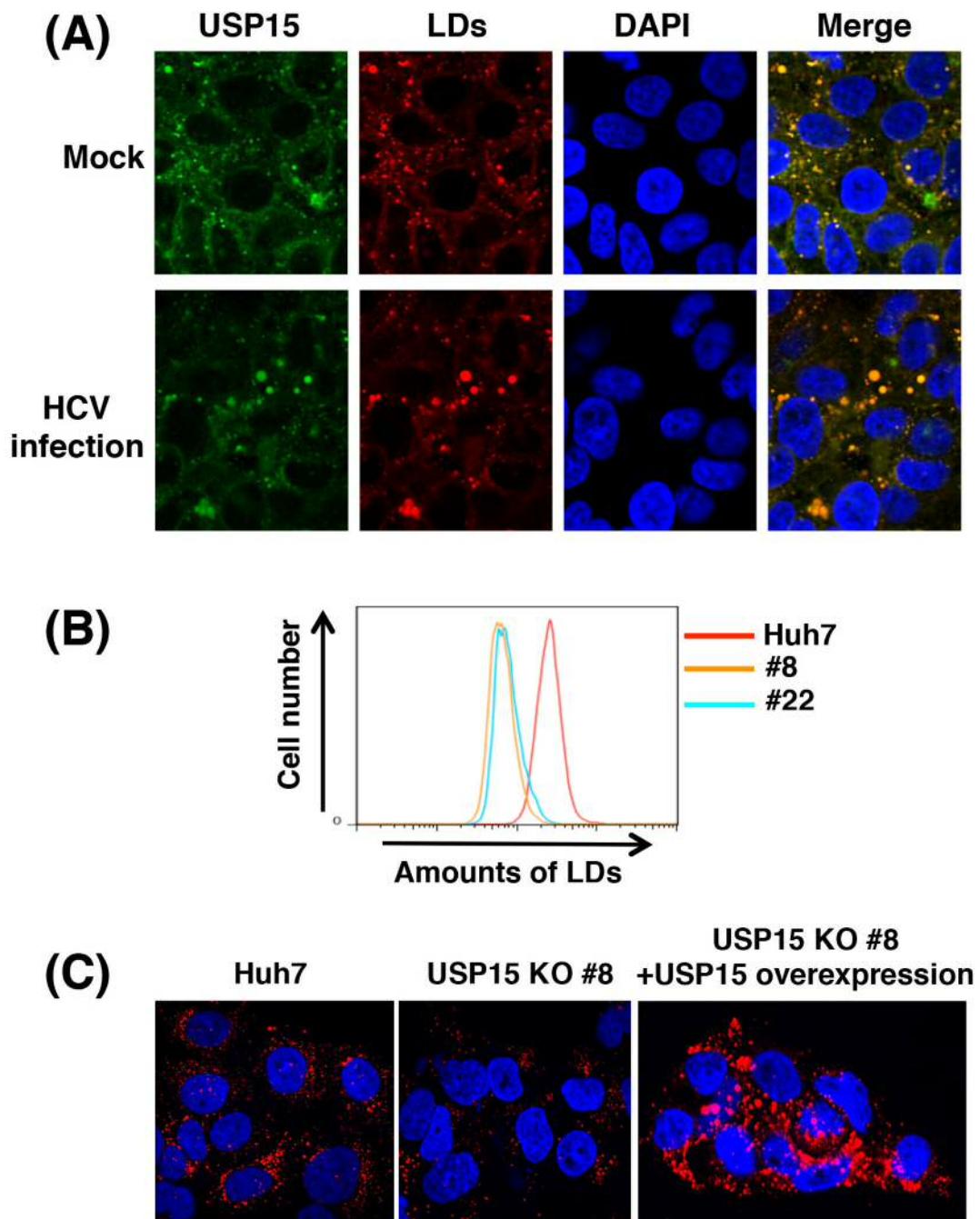


Figure 20

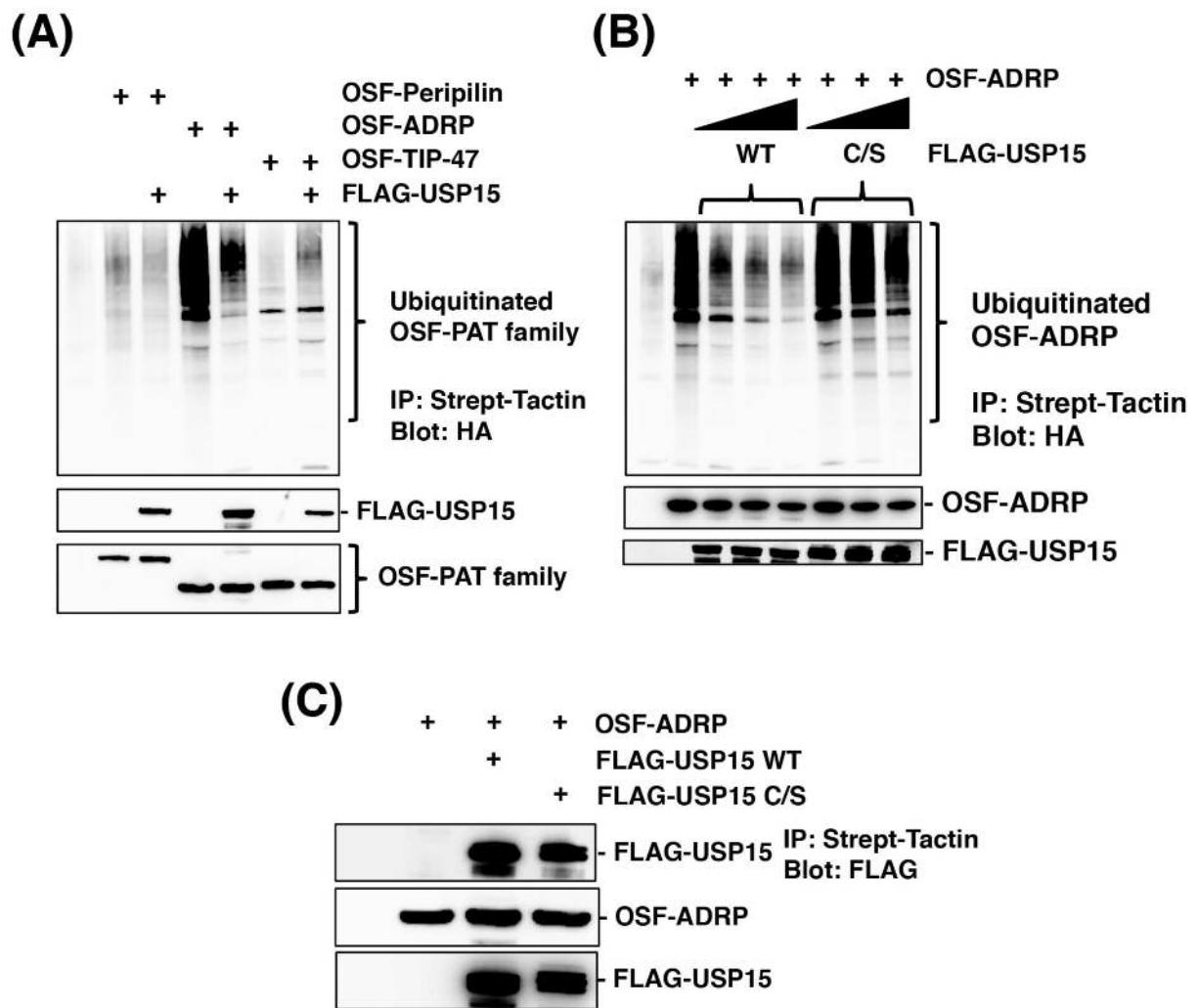


Figure 21

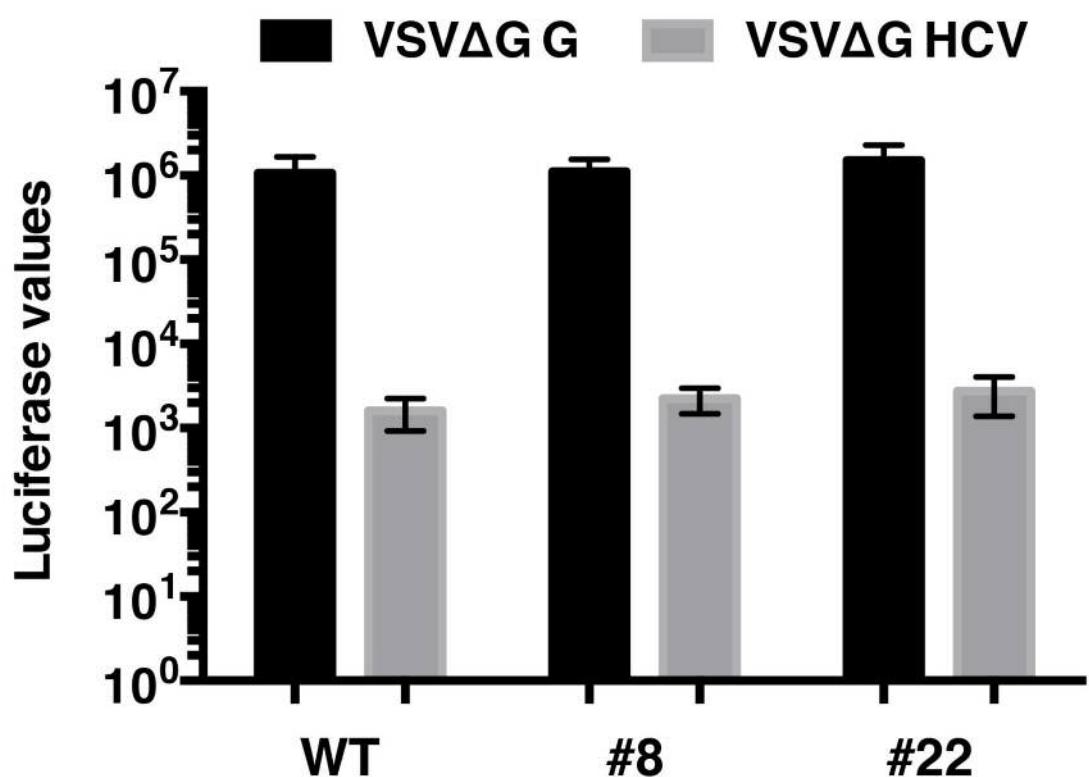
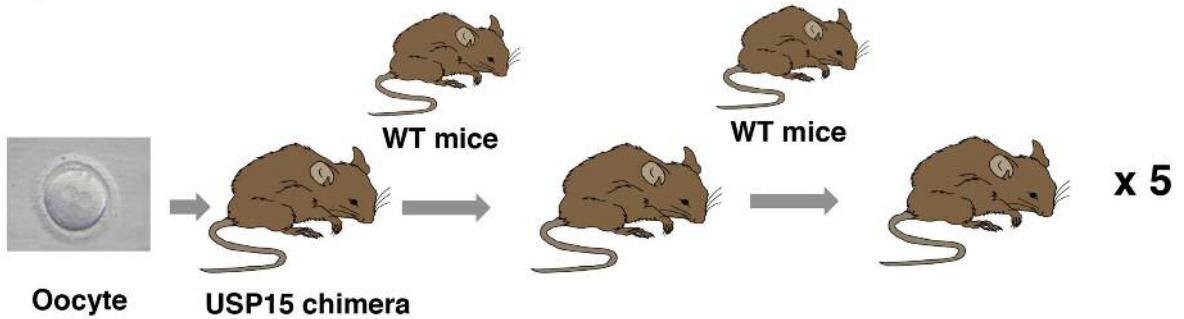
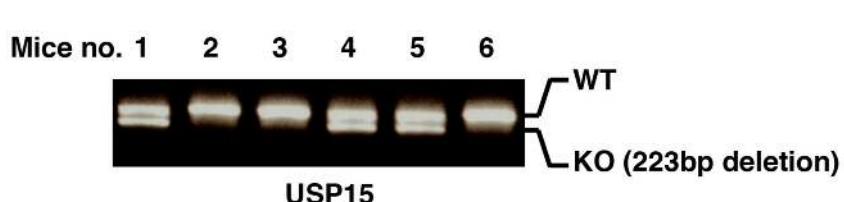


Figure 22

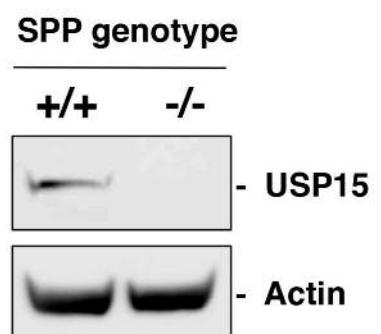
(A)



(B)



(C)



(D)

	USP15 ^{+/+}	USP15 ^{+/-}	USP15 ^{-/-}
Postnatal (~2wk)	7	14	4

Figure 23

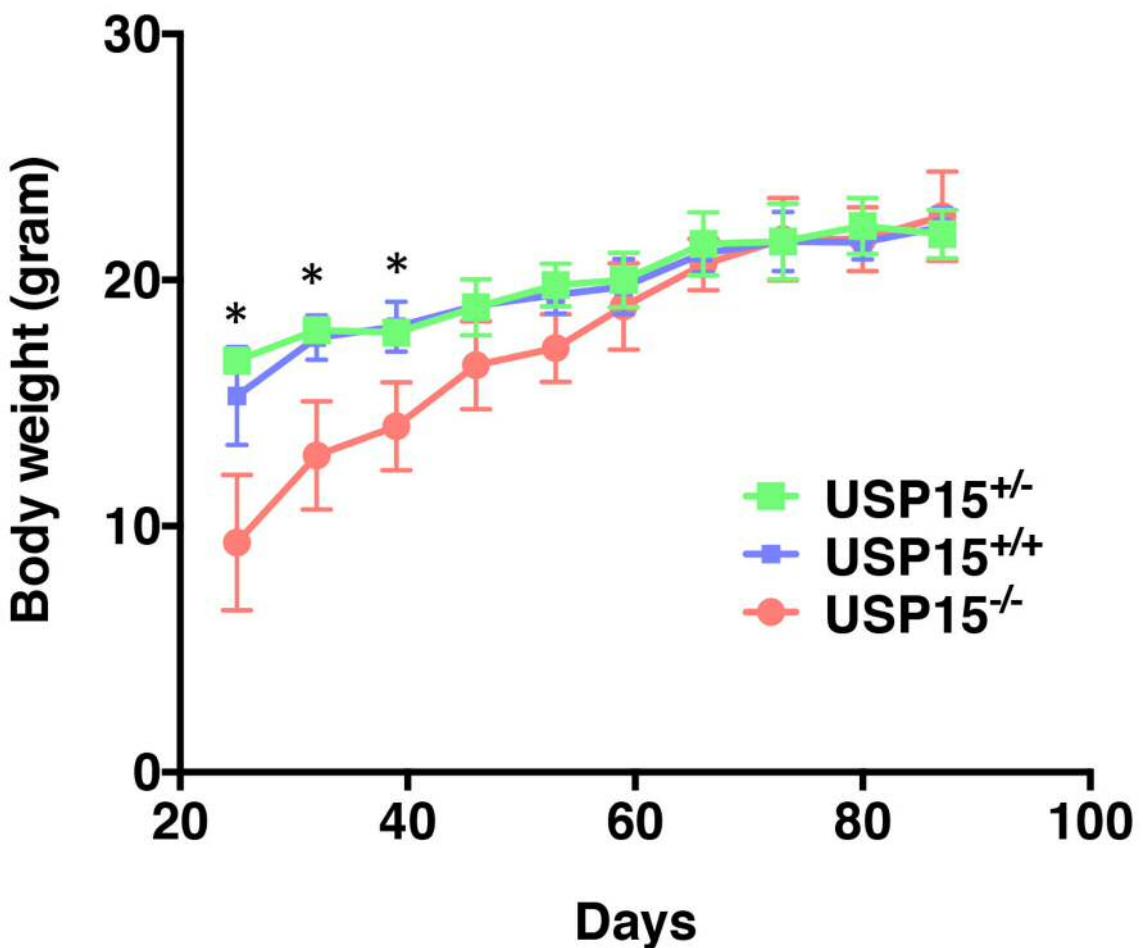


Figure 24

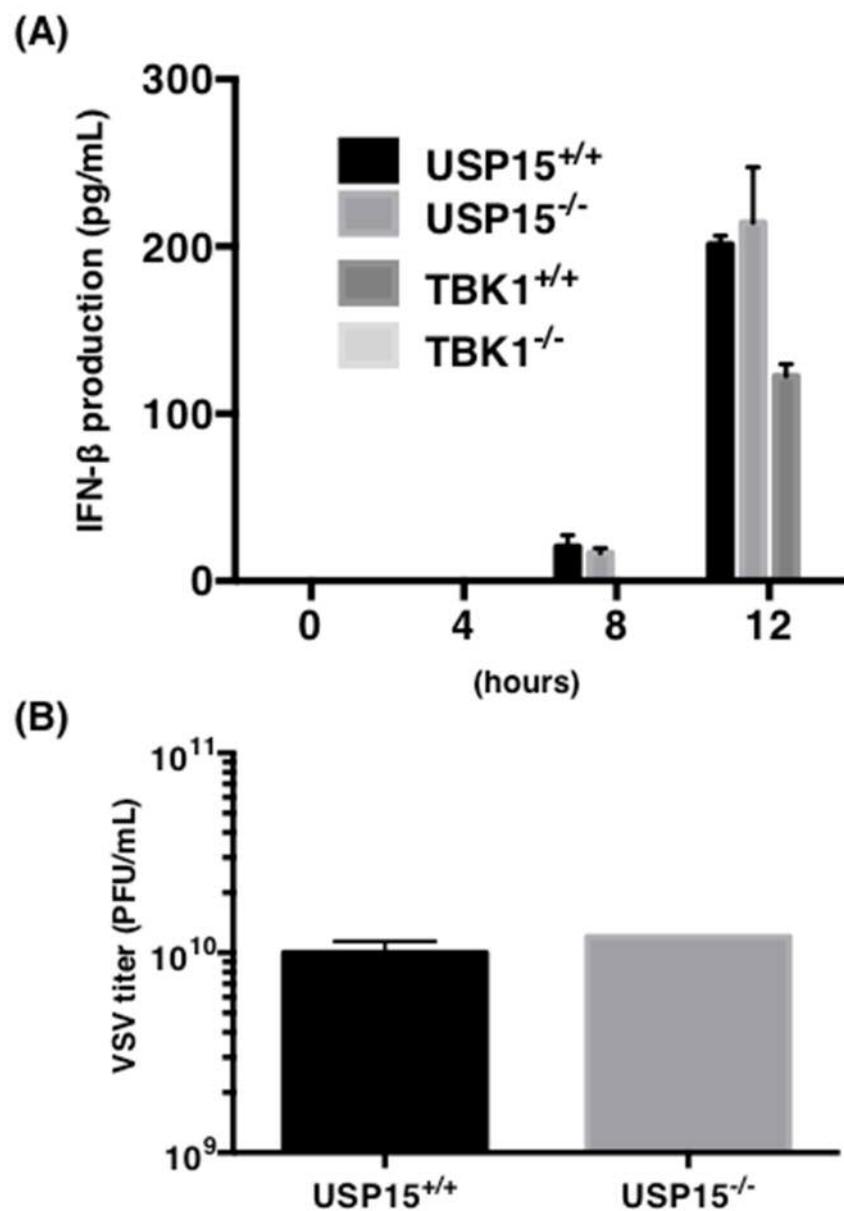


Figure 25

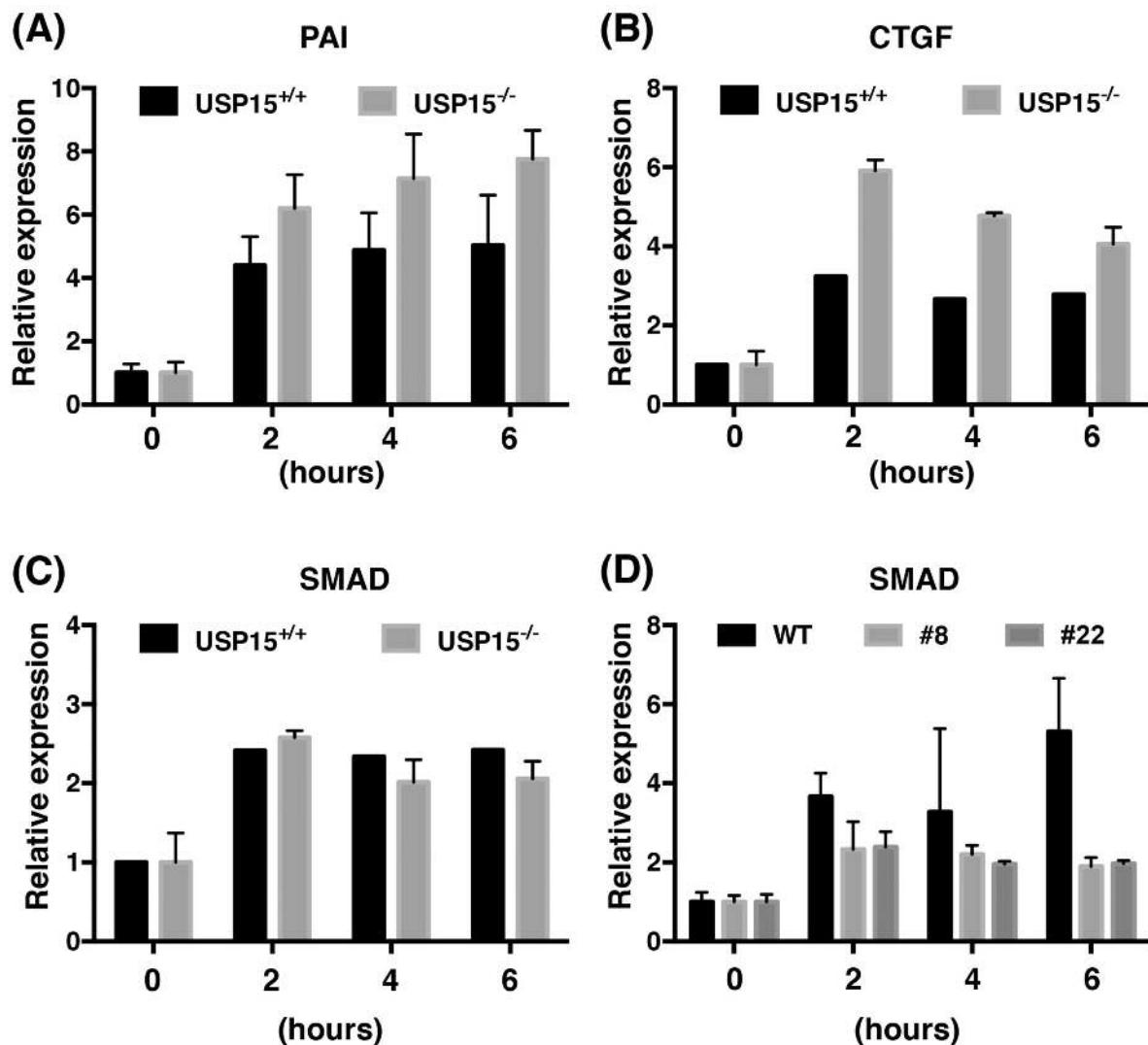


Figure 26

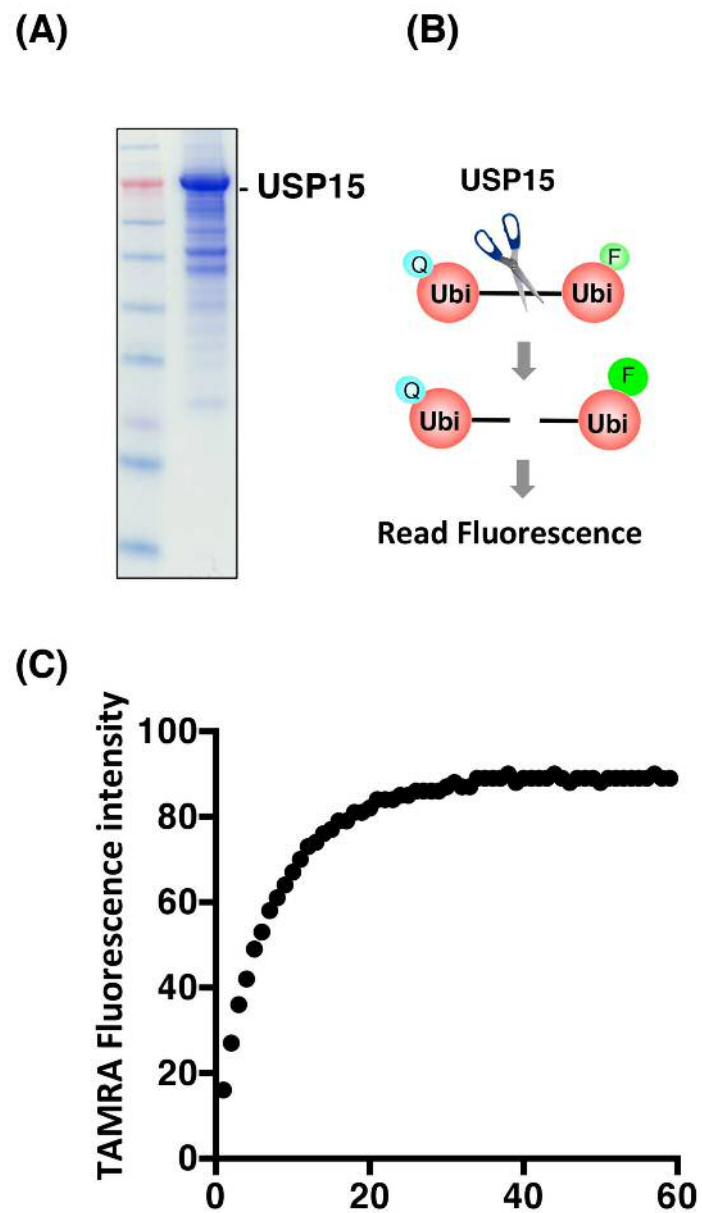


Figure 27

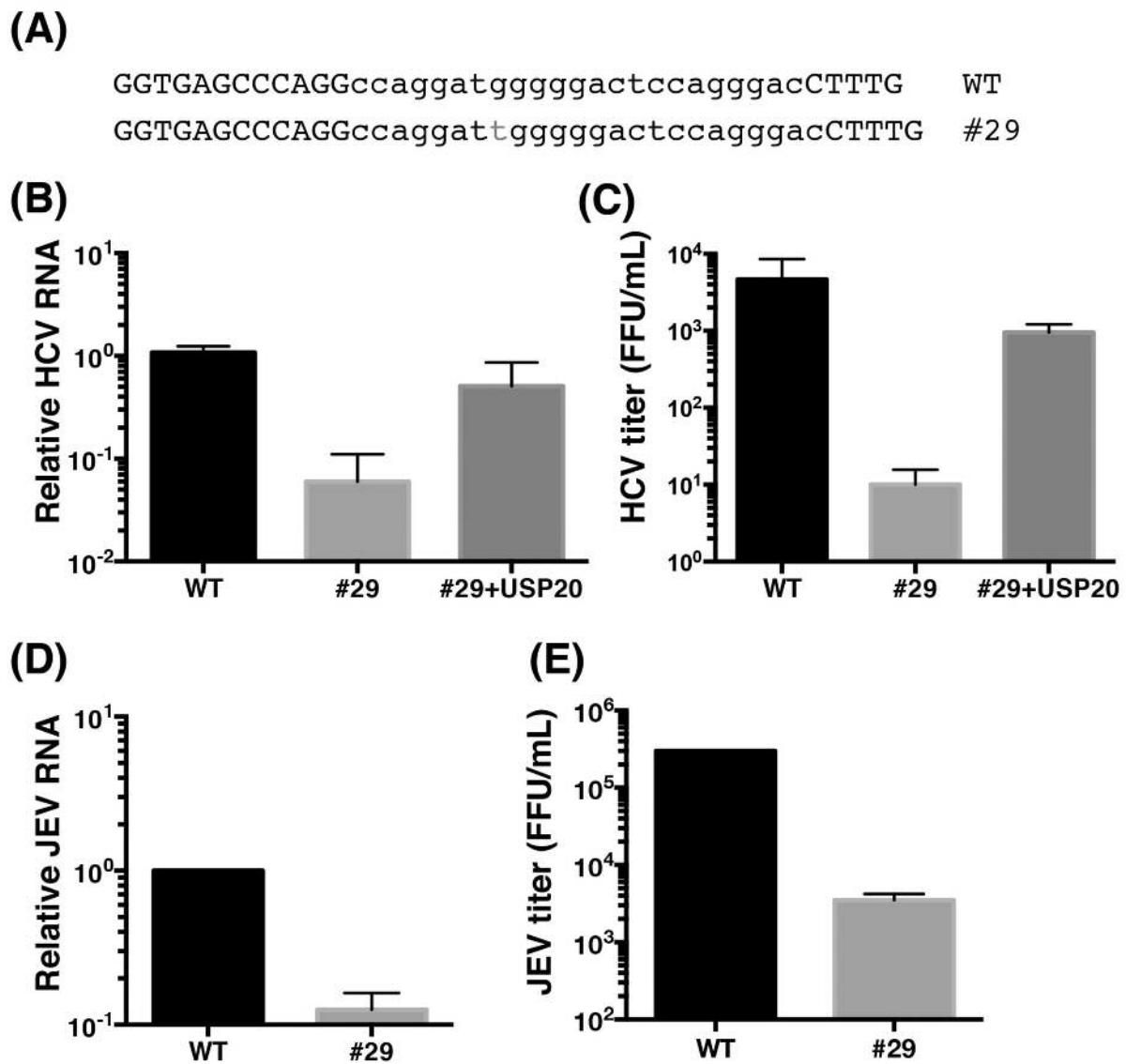


Figure legends

Fig. 1. Schematic of hepatitis viruses.

Fig. 2. Classification of hepatitis viruses.

Fig. 3. Current situation of liver cancer and HCV infection. (A) Rank of cancer incidence in Japan according to sex or total (2012). (B) About 70% patients of liver cancer were derived from HCV infection. (C) About 70% of HCV infected patients has genotype 1b typed HCV. (D) Current therapy such as PEG-IFN/RVB can achieve 50% SVR. However, the other 50% of patients cannot be effective to PEG-IFN/RVB therapy.

Fig. 4. HCV genome and viral proteins. HCV encodes 10 different viral proteins in the genome.

Fig. 5. HCV life cycles.

Fig. 6. HCV core protein plays a major role in HCV induced liver diseases. Liver-specific core transgenic mice (CoreTG) develop steatosis and hepatocellular carcinoma. On the other hands, CoreTG show larger size of pancreatic islets showing as insulin resistance.

Fig. 7. Molecular mechanisms of core induced liver diseases. Core protein upregulates SREBP-1c to enhance fatty acids synthesis. PA28 γ mediated proteasomal degradation of core is required for core-induced diseases in transgenic mice.

Fig. 8. Maturation of core protein by signal peptide peptidase (SPP). Core proteins are processed by SPP. Transmembrane domain and N-terminus hydrophobic region are necessary for recognition of SPP.

Fig. 9. HCV receptor candidates and species-specificity for HCV infection. (A) Reported HCV receptor candidates. (B) Human CD81 and human OCLD are essential for successful HCV infection in mice.

Fig. 10. MicroRNA is involved in HCV replication. (A) Schematic diagram of microRNA production. MicroRNA plays a role in regulation of mRNA by suppression of translation or cleavage of RNA. (B) HCV genome contains 2 miR-122 binding sites in IRES region. (C) Non-hepatic cells introduced miR-122 can achieve HCV replication.

Fig. 11. HCV replication alters morphology of ER membrane to prevent host antiviral responses. (A) HCV replicating cells show accumulation of small membrane cavity derived from ER membrane called membranous web. (B) One membrane cavity is formed by double membrane structure. DMV: double membrane vesicle (C) HCV infection induced phosphorylation of phosphatidylinositol through PI4KIII α or PI4KIII β . Phosphatidylinositol-4-phosphate (PI4P) is essential for HCV replication. Lipid transfer proteins such as CERT and OSBP play a role in transfer PI4P to ER membrane.

Fig. 12. Host factors involved in HCV replication.

Fig. 13. IL28B SNPs can predict outcome of PEG-IFN/RVB therapy.

Fig. 14. HCV infection suppresses ubiquitination of cellular proteins. (A) Huh7.5.1 cells were infected with HCV at moi of 1 and harvested at indicated time points. Ubiquitinated proteins were purified by TUBE agarose beads and subjected to immunoblot analysis by using anti-ubiquitin antibody. (B) Huh7.5.1 cells infected with HCV were treated with PR-619 or DMSO at 24h

post-infection and intracellular viral RNA and cell viability at 24h post-treatment were determined by qPCR and PI exclusion, respectively. Data represent means \pm SD from 2 independent experiments.

Fig. 15. RNAi screening to identify DUBs participate in HCV replication. (A) DNA microarray analysis of the expression of DUBs in Huh7.5.1 cells. (B) Sixty five DUB-knockdown Huh7.5.1 cell lines were infected with HCV at moi of 1 and intracellular HCV RNA were determined by qPCR at 2 days post-infection. Data were presented as relative value compared to Huh7.5.1 cells expressing shRNA against LacZ. (C) Huh7.5.1 cells infected with HCV at moi of 1 were harvested at indicated time points and expression of USP15 mRNA (left) and HCV RNA (right) was determined by qPCR. Data represent means \pm SD from 2 independent experiments.

Fig. 16. Establishment of USP15 knockout Huh7 cells by CRISPR/Cas9 system. Schematic representation of strategy to generate USP15 knockout Huh7 cells. Target sequence for USP15 sgRNA was inserted into EGFP coding sequence and designated as pCAG EGxxFP hsUSP15. In cells co-transfected with pX330 hsUSP15 and pCAG EGxxFP hsUSP15, sgRNA binds to the target sequences in both genomic USP15 and pCAG EGxxFP hsUSP15, and then Cas9 cleaves both target sequences. Cells in which USP15 target sequence were efficiently cleaved, EGFP gene was rescued by the homologous recombination and express EGFP. In EGFP strong positive cell population sorted by FACS, high level of USP15 knockdown cells were concentrated. Huh7 cells transfected with pCAG EGxxFP hsUSP15 together with pX330 hsUSP15 or pX330 empty were collected by using FACS at 7 days post-transfection and seeded on culture dishes to form single

colonies. Cellular DNAs were isolated from each cell clones and mutation was confirmed by sequencing. USP15 knockout Huh7 cell lines #8 has one nucleotide (T) insertion into sgRNA targeting genome. #22 has 11 nucleotides (gctccggaaa) deletion in one chromosome and one nucleotide (T) insertion in the other chromosome.

Fig. 17. Characterization of USP15 knockout Huh7 cells. (A) Expression of USP15 in parental, USP15 knockout clone #8 and #22 Huh7 cells was determined by specific antibody. (B) Cell growth curves of parental, USP15 knockout clone #8 and #22 Huh7 cells were determined by MTT assay. (C) Expression of miR-122 in parental, USP15 knockout clone #8 and #22 Huh7 cells was determined by qPCR.

Fig. 18. USP15 specifically participates in the propagation of HCV. (A) *In vitro* transcribed HCV subgenomic replicon RNA was electroporated into parental, USP15 knockout clone #8, and #22 Huh7 cells, incubated for 3 weeks in the presence of 1mg/ml of G418. Colonies were visualized by staining with Giemsa (Merck) at 3 weeks post-electroporation. Parental, USP15 knockout clone #8, and #22 Huh7 cells were infected with HCV at moi of 5 and intracellular viral RNA (B) and infectious titers in the culture supernatants (C) were determined by qPCR and plaque assay at 4 days post-infection. Parental, USP15 knockout clone #8, and #22 Huh7 cells were infected with JEV at moi of 3 and intracellular viral RNA (D) and infectious titers in the culture supernatants (E) were determined by qPCR and plaque assay at 2 days post-infection. Parental, USP15 knockout clone #8, and #22 Huh7 cells were transfected with a plasmid encoding HBV and intracellular viral DNA was determined by qPCR at 3 days post-transfection.

Fig. 19. USP15 participates in the lipid droplet formation. (A) Huh7.5.1 infected with HCV at moi of 5 and permeabilized after fixation. Subcellular localization of USP15 and LDs was determined by immunofluorescence after staining with anti-USP15 antibody and HCS LipidTOX Red neutral lipid, respectively. (B) Parental, USP15 knockout clone #8, and #22 Huh7 cells infected HCV were trypsinized, fixed with 4% PFA, permeabilized with 0.3% Saponin in PBS together with HCS LipidTOX Red neutral lipid, and analyzed by FACS. (C) Parental and USP15 knockout Huh7 clone #8 with or without exogenous expression of FLAG-USP15 were permeabilized after fixation and stained with HCS LipidTOX Red neutral lipid.

Fig. 20. ADRP is a specific target for USP15. (A) *In vitro* ubiquitination assay. HA-tagged ubiquitin was expressed in HEK293T together with OSF-tagged PAT family proteins, Perilipin, ADRP or TIP-47 in the presence or absence of FLAG-tagged USP15 and OSF-tagged PAT family proteins were precipitated by Strept-Tactin Beads. Purified proteins were subjected to immunoblot analysis by using anti-HA antibody to assess ubiquitination of the OSF-tagged proteins. (B) HEK293T cells were transfected with expression plasmids encoding HA-tagged ubiquitin, OSF-tagged ADRP and various amounts of FLAG-tagged USP15 of wild type (WT) or C/S mutant (C/S). OSF-tagged ADRP precipitated by Strept-Tactin Beads was assessed by immunoblotting. (C) Interactions between OSF-tagged ADRP and FLAG-tagged USP15 of wild type (WT) or C/S mutant (C/S) was determined by immunoprecipitation analysis. Cell lysates were immunoprecipitated by Strept-Tactin beads and subjected to immunoblotting by using anti-FLAG antibody.

Fig. 21. USP15 does not involve in HCV infection steps. VSV Δ G HCV or VSV Δ G G was infected with Huh7 or USP15 knockout Huh7 cells. After 24 hours, luciferase activity was determined.

Fig. 22. Generation of USP15 knockout mice (A) Schematic diagram of generation of USP15 knockout mice. (B) PCR can confirm USP15 deletion. (C) MEFs were prepared by USP15 $^{+/+}$ or USP15 $^{-/-}$ embryos and confirmed protein expression by western blot. (D) Numbers of offspring derived from USP15 $^{+/+}$ parents.

Fig. 23. Body weight of USP15 $^{-/-}$ female mice. (N=3)

Fig. 24. USP15 does not involve in RIG-I mediated innate immune response. (A) MEFs derived from USP15 $^{+/+}$, USP15 $^{-/-}$, TBK1 $^{+/+}$ or TBK1 $^{-/-}$ embryos were infected with VSV (moi=5). Culture supernatants were quantified IFN- β concentration by ELISA. (B) VSV was infected with USP15 $^{+/+}$ or USP15 $^{-/-}$ MEFs. After 24 hours, viral titers were determined by plaque assay.

Fig. 25. USP15 may involve in TGF- β signaling in cell type specific manner. 1ng/mL of TGF- β was stimulated with USP15 $^{-/-}$, USP15 $^{+/+}$ MEFs, Huh7 and USP15 knockout Huh7 cells. RNAs were extracted and mRNA expression of PAI (A), CTGF (B) and SMAD (C, D) was determined by qPCR.

Fig. 26. Establishment of USP15 DUB activity in vitro. (A) Purified recombinant USP15 (rUSP15) by baculovirus expression system. (B) Schematic diagram of DiUb48 substrate (C) rUSP15 has a DUB activity in vitro. Fluorescent intensity of TAMRA was determined by plate reader.

Fig. 27. USP20 involved in HCV/JEV replication. (A) Generation of USP20 knockout Huh7 cells by CRISPR/Cas9 system. (B, C) Huh7 cells were infected with HCV at moi of 5 and intracellular viral RNA and infectious titers in the culture supernatants were determined by qPCR and plaque assay at 4 days post-infection. (D, E) Parental, USP15 knockout clone #8, and #22 Huh7 cells were infected with JEV at moi of 3 and intracellular viral RNA and infectious titers in the culture supernatants were determined by qPCR and plaque assay at 2 days post-infection.

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Achievements

Meetings

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B型肝炎ウイルスX蛋白質と相互作用する宿主側因子の解析

幸脇貴久, Pham Duc Ngoc, 福原崇介、岡本徹、松浦善治

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