Ribonucleotide reductase M2 promotes RNA replication of hepatitis C virus by protecting NS5B protein from hPLIC1-dependent proteasomal degradation

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Running title: RRM2 regulates HCV replication by stabilizing NS5B

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ABSTRACT

Hepatitis C virus (HCV) establishes a chronic infection that can lead to cirrhosis and hepatocellular carcinoma. The HCV life cycle is closely associated with host factors that promote or restrict viral replication, the characterization of which could help to identify potential therapeutic targets. To this end, here we performed a genome-wide microarray analysis and identified ribonucleotide reductase M2 (RRM2) as a cellular factor essential for HCV replication. We found that RRM2 is up-regulated in response to HCV infection in quiescent hepatocytes from humanized chimeric mouse livers. To elucidate the molecular basis of RRM2 expression in HCV-infected cells, we used HCV-infected hepatocytes from chimeric mice and hepatoma cells infected with the HCV strain JFH1. Both models exhibited increased RRM2 mRNA and protein expression levels. Moreover, siRNA-mediated silencing of RRM2 suppressed HCV replication and infection. Of note, RRM2 and RNA polymerase nonstructural protein 5B (NS5B) partially co-localized in cells and co-immunoprecipitated, suggesting that they might interact. RRM2 knockdown reduced NS5B expression, which was dependent on the protein degradation pathway, as NS5B RNA levels did not decrease and NS5B protein stability correlated with RRM2 protein levels. We also found that RRM2 silencing decreased levels of hPLIC1 (human homolog 1 of protein linking integrin-associated protein and cytoskeleton), a ubiquitin-like protein that interacts with NS5B and promotes its degradation. This finding suggests that there is a dynamic interplay between RRM2 and the NS5B–hPLIC1 complex that has an important function in HCV replication. Together, these results identify a role of host RRM2 in viral RNA replication.

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus belonging to the Flaviviridae family (1). HCV infects and replicates within quiescent hepatocytes, establishing a chronic infection that can lead to cirrhosis and hepatocellular carcinoma (HCC). Although
effective anti-HCV drugs have been developed, their low barrier to viral resistance and inability to prevent HCC development represent major clinical challenges (2).

The HCV life cycle proceeds exclusively in the cytoplasm of host cells. Upon decapsidation, the viral genome is released into the cytoplasm, where it is translated into a large polyprotein that is processed by cellular and viral proteases to yield mature structural (core, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (3). Following translation, these proteins become associated with a membranous web derived from the endoplasmic reticulum (ER), in which viral genome replication takes place. NS3 through NS5B constitute the replication complex, and the positive-strand RNA genome serves as a template for the RNA-dependent RNA polymerase NS5B to produce the negative-sense replicative intermediate necessary for the generation of new positive-sense RNA genomes. These are in turn used as templates for further RNA replication, translated to produce new viral proteins, or packaged into virions (3). The HCV life cycle is closely associated with host factors that promote or restrict viral replication; characterization of these factors may therefore help to identify potential therapeutic targets.

To this end, we performed a genome-wide microarray analysis and identified ribonucleotide reductase M2 (RRM2) as a novel cellular factor related to HCV infection. RRM2 catalyzes the conversion of ribonucleotides to deoxyribonucleotides (dNTPs), which are necessary for DNA replication and repair (4). Interestingly, given its role in de novo dNTP biosynthesis, RRM2 has recently been identified as an important cellular factor supporting the viral DNA synthesis of highly pathogenic viruses, including hepatitis B virus (5), human papillomavirus (6), and Kaposi sarcoma-associated herpesvirus (7). However, the relationship between RRM2 expression and viral RNA synthesis is unknown. Our findings provide insight into a novel cellular pathway controlling HCV replication.

Results

Identification of new cellular factors related to HCV infection

To identify cellular factors involved in HCV infection, we performed a genome-wide microarray analysis in humanized chimeric quiescent mouse hepatocytes (8) infected with HCV (Fig. 1A). In the sera of these mice, the HCV RNA load was $2.9 \times 10^6$ copies/ml, whereas the human albumin concentration was $1.1 \times 10^7$ ng/ml (i.e., > 90% of hepatocytes were replaced). In contrast, uninfected control mice showed no viral load and had a human albumin concentration of $1.1 \times 10^7$ ng/ml. Cy3- and Cy5-labeled probes were used to identify genes upregulated in HCV-infected hepatocytes (Fig. S1 and Fig. S2). Of these, the top 5 most upregulated genes are shown in Table 1.

First, Cy3-labeled probes revealed that RRM2 was upregulated 14.9-fold and was the second most upregulated gene. Next, in a reversed experiment, Cy5-labeled probes similarly identified RRM2 to be upregulated 10-fold as the second most upregulated gene. Although RRM2 overexpression is reportedly linked to various types of cancer (9-11), its involvement in HCV infection or hepatitis C pathogenesis has not been previously described.

To clarify the mechanism by which RRM2 contributes to HCV infection, we compared the expression of RRM2 between HCV-infected and uninfected quiescent human hepatocytes in chimeric mice via western blotting and found that RRM2 expression was induced in HCV-infected cells (chimeric mice #4 and #25) but not in uninfected cells (chimeric mice #16 and #20) (Fig. 1B).

We also measured RRM2 mRNA levels in HuH-7 cells and HCV subgenomic replicon-cured K4 cells (which are both HCV-negative) and in HuH-7 cells persistently infected with the HCV isolate JFH-1 [i.e., JFH/K4 cells, which are HCV-positive (12)] (Fig. 1C). Expression of RRM2 mRNA and its corresponding protein was markedly increased in the presence of HCV, indicating that the HCV genome is required for RRM2 expression. In addition, expression of RRM2 mRNA and its protein was downregulated upon siRNA-mediated silencing of the HCV genome in JFH/K4 cells (Fig. 1D). These results indicate that HCV infection induces RRM2 expression.

RRM2 is one of two small regulatory subunits that, along with two large catalytic subunits of RRM1, constitute the ribonucleotide reductase (RR) enzyme complex (4). However, according to the microarray analysis of HCV-infected chimeric mouse hepatocytes, expression of RRM1 was not significantly altered (Fig. S1 and S2).
We also examined RRM1 protein levels in HCV-positive JFH/K4 cells and uninfected HuH-7 cells. As shown in Fig. 1E, levels of RRM1 in JFH/K4 cells were similar to those seen in uninfected cells.

We next sought to determine the effect of HCV infection on RR enzymatic activity. Since cellular dNTP pools are regulated primarily by changes in RR activity (4), we measured dCTP levels as representative of dNTP levels and therefore RR activity in HCV-infected JFH/K4 cells. No significant difference was observed in dCTP levels between HCV-infected JFH/K4 cells and uninfected HuH-7 cells (Fig. 1F).

**RRM2 is required in HCV RNA replication**

To clarify the role of RRM2 in HCV replication, we used the HCV subgenomic replicon cell lines R6FLR-N (13) and FLR3-1 (14) (Fig. 2A), as well as persistently HCV JFH-1-infected JFH/K4 cells (12) (Fig. 2B). R6FLR-N HCV subgenomic replicon cells were treated with RRM2 siRNA (Fig. 3A); 72 h after RRM2 knockdown, we observed that HCV RNA levels were significantly decreased to levels comparable to those in cells treated with HCV genotype 1b-targeting siRNA (HCV R5). In JFH/K4 cells (Fig. 3B), HCV RNA expression was decreased in both cells and culture supernatant following treatment with RRM2 siRNA. A decrease in HCV NS5B protein levels was also observed in R6FLR-N and JFH/K4 cells following RRM2 siRNA treatment (Fig. 3C). These results indicate that RRM2 is involved in HCV replication and persistent infection.

To assess the significance of RRM2 in HCV replication, FLR3-1 HCV replicon cells were also treated with SMARTpool RRM2 siRNAs (consisting of a pool of 4 different RRM2 siRNAs; ON-TARGETplus, Fig. 4A). After 3 days, the half-maximal inhibitory concentration (IC50) value of RRM2 siRNA was 0.15 nM. Therefore, RRM2 silencing inhibits HCV replication with minimal cytotoxicity, suggesting that this approach has therapeutic potential for the treatment of HCV infection.

To confirm the specificity of RRM2 siRNA, we attempted to rescue HCV replication using an RRM2 expression vector (Fig. 4B). In R6FLR-N replicon cells, HCV replication was suppressed by RRM2 knockdown and rescued by RRM2 overexpression without affecting cell viability (Fig. 4C). Thus, RRM2 siRNA specifically suppressed HCV replication.

**RRM2 specifically interacts with HCV RNA polymerase NS5B**

To investigate the mechanisms by which RRM2 modulates HCV replication, we first examined the suppressive effects of RRM2 on HCV proteins. RRM2 siRNA was transfected into HuH-7 cells transfected with an HCV protein-expressing plasmid (pcDNA6-E1, E2, core NS2, NS3, NS4B, NS5A, or NS5B). RRM2 knockdown markedly decreased NS5B protein levels (Fig. 5, bottom right) but had no significant effect on the expression of other proteins (Fig. 5). Therefore, RRM2 expression is specifically relevant to NS5B expression.

We further characterized the interaction between RRM2 and NS5B (Fig. 6). JFH/K4 cells were transfected with the pcDNA6-myc-His-RRM2 expression vector (Fig. 6A–E). Immunoprecipitation with anti-Myc antibodies yielded myc-tagged RRM2 (Fig. 6A) and HCV NS5B (Fig. 6B) co-precipitates. Furthermore, NS5B antibodies yielded co-precipitation of NS5B (Fig. 6C) and RRM2 (Fig. 6D), showing that NS5B interacts with RRM2. To confirm that RRM2 interacts with HCV NS5B, HEK293 cells were transfected with pcDNA6-myc-His-NS5B or empty pcDNA6-myc-His vector and purified by His column. Then, purified NS5B was reacted with RRM2 (Fig. 6A). In addition, JFH/K4 cells were transfected with either pcDNA6-myc-His-RRM2 plasmid or empty pcDNA6-myc-His vector, and purified RRM2 was reacted with NS5B (Fig. 6B). RRM2 and NS5B proteins were co-purified, suggesting a direct interaction between these two proteins.

We further verified the localization of RRM2 and NS5B in JFH/K4 cells using an immunofluorescence assay. Co-labeling of JFH/K4 cells with antibodies against RRM2 and NS5B showed a high degree of co-localization of the two proteins, especially in the perinuclear region (Fig. 6F, G). In addition, G0/G1 phase-arrested HCV-infected cells expressed higher amounts of RRM2 than HCV-uninfected cells (Fig. 6H).

**RRM2 stabilizes the NS5B protein**

We next characterized the role of RRM2 in HCV NS5B expression (Fig. 7). NS5B mRNA
levels were measured in lenti-NS5B-expressing HuH-7 cells 72 h after RRM2 siRNA treatment; no significant effects on NS5B mRNA expression were observed after RRM2 silencing in these cells (Fig. 7A). Therefore, we next examined the effects of suppressing RRM2 on NS5B protein stability (Fig. 7B). To examine whether NS5B degradation is enhanced by the silencing of RRM2, we treated lenti-NS5B-expressing HuH-7 cells with cycloheximide (CHX) and puromycin 48 h after RRM2 siRNA treatment. As shown in Fig. 7B, the decrease in the NS5B protein level was significantly higher in the absence of RRM2. In contrast, NS5B levels did not decrease after treatment with CHX in cells overexpressing RRM2 (Fig. 7C). These results indicate that the presence of RRM2 is important for NS5B protein stability.

It was previously reported that NS5B protein levels are regulated via ubiquitination and proteasomal degradation (15). Therefore, RRM2 may enhance NS5B protein levels by inhibiting these processes. Furthermore, NS5B has been shown to bind to hPLIC1 (human homolog 1 of protein linking integrin-associated protein and cytoskeleton), which is thought to regulate HCV replication (15) and physically associates with E3 ubiquitin protein ligases as well as the proteasome (16). Therefore, we examined whether RRM2 is involved in the NS5B-hPLIC1 interaction. We carried out an immunoprecipitation experiment in which HuH-7 cells were transfected with myc-HispcDNA6-NS5B vector, either alone or with Flag-tagged hPLIC1, followed by immunoprecipitation of hPLIC1 from cell lysates using an anti-Flag antibody (Fig. 8A). Consistent with the previous report (10), NS5B interacted with hPLIC1. Levels of hPLIC1 were decreased in the absence of RRM2, and this decrease occurred in an NS5B-dependent manner, as RRM2 knockdown did not affect hPLIC1 protein levels in cells not-transfected with the NS5B expression plasmid (Fig. 8A, lane 3 vs lane 4). This suggests that the negative regulation of hPLIC1 is likely related to NS5B degradation rather than RRM2 silencing (Fig. 8B).

We further examined NS5B ubiquitination in the presence or absence of RRM2 using an immunoprecipitation assay (Fig. 8C). In this experiment, HuH-7 cells were transfected with the myc-NS5B expression plasmid with or without RRM2 or control siRNA and treatment with or without the proteasome inhibitor MG132. NS5B was immunoprecipitated with anti-Myc antibodies, and ubiquitinated NS5B was detected using anti-ubiquitin linkage-specific K48 antibodies. In MG132-treated cells, the level of NS5B ubiquitination was higher after RRM2 knockdown than after mock or control siRNA treatment (Fig. 8C).

Discussion

In this study, we report for the first time that RRM2 modulates HCV RNA replication \textit{in vitro} and \textit{in vivo}. RRM2 expression was upregulated in quiescent human hepatocytes from chimeric mice infected with HCV. The upregulation of RRM2, a ribonucleotide modification enzyme, in HCV infection is unexpected. In addition, RRM2 silencing suppressed HCV RNA replication in hepatoma cells. Although protein structural information is lacking, we provided interesting evidence in the form of protein–protein interaction experiments. Surprisingly, one of the multiple functions of RRM2 was found to protect the HCV NS5B polymerase from degradation via hPLIC1. The involvement of RRM2 in dNTP synthesis may be irrelevant given that HCV is an RNA virus; however, the role of RRM2 in promoting cell proliferation is significant in terms of HCV replication, as it was previously observed that HCV RNA synthesis was reduced in cells with low rates of proliferation and enhanced in cells during the S phase (17). Regulation of RRM2 expression is cell cycle-dependent and peaks during the S phase (18). Therefore, the upregulation of RRM2 via HCV may serve as a link between the cell cycle and regulation of HCV RNA replication. Although RRM2 is expressed during HCV infection, inhibiting its expression resulted in degradation of the HCV RNA polymerase NS5B. In addition, an interaction between RRM2 and NS5B was confirmed via immunoprecipitation analysis and confocal microscopy. In HCV-infected cells, the NS5B protein level may be partially regulated via a ubiquitin-dependent mechanism. A previous study showed that hPLIC1 physically interacts with the NS5B protein, targeting it for proteasomal degradation (10). However, the precise mechanism of hPLIC1-mediated NS5B degradation remains unclear. Our results showed that a reduction in NS5B protein levels induced by RRM2 silencing is accompanied by a decrease in the level of hPLIC1. This result suggests that the entire NS5B-hPLIC1...
RRM2 regulates HCV replication by stabilizing NS5B complex is subjected to degradation in the absence of RRM2. It is highly likely that other cellular or viral factors are involved in this process given the large amount of evidence showing that hPLIC1 functions as an adaptor protein linking the ubiquitination pathway to the proteasome to affect protein degradation (16). In fact, the yeast homolog of hPLIC1, Dsk2p, may deliver polyubiquitinated proteins to the proteasome (19). Further study is required to elucidate the detailed mechanisms that govern NS5B stability in the absence of RRM2.

Numerous factors associated with NS5B activity have been identified. NS5B exhibits RNA polymerase activity and interacts with other HCV viral proteins to promote viral replication; it also interacts with other host factors, including human vesicle-associated membrane protein-associated protein (hVAP)-33 (20), human eukaryotic initiation factor (heIF) 4AII (21), and hPLIC1 (15). The hVAP-33 protein is involved in vesicle trafficking via the endocytotic, exocytotic, ER-Golgi, and intra-Golgi transport pathways. The heIF4AII protein, which has ATPase/helicase activity, interacts with the C-terminal 495–537 amino acid region of NS5B and facilitates the synthesis of RNA by unwinding its secondary structure, while hPLIC1 is associated with the regulation of NS5B stability. Among these factors, hPLIC1 is the most likely factor regulating NS5B stability via RRM2.

RRM2 is involved in the replication of HCV, as well as that of other viruses. The human papillomavirus E7 protein induces RRM2 overexpression, which enhances hypoxia-inducible factor-1α and vascular endothelial growth factor expression via activation of extracellular signal-regulated kinase 1/2 signaling through the production of reactive oxygen species in cervical cancer cells (22). In this mechanism, the E7 protein interacts with the retinoblastoma tumor suppressor protein (pRb), leading to the release of E2F from the pRb complex; E2F then binds to the RRM2 promoter to activate RRM2 gene transcription (22). Similarly, RRM2 expression was found to be induced by hepatitis B virus (HBV) as a result of E2F1 accumulation (23), while an RRM2 inhibitor suppressed HBV replication (5). We previously showed that expression of the full HCV genome activates cyclin-dependent kinase (CDK)-Rb-E2F signaling in hepatocytes and increases their tumorigenicity during passaging (24). Also, NS5B contributes to suppress Rb and activates E2F (25).

It is plausible that HCV upregulates RRM2 expression via these pathways in hepatocytes.

Taken together, we propose a model for HCV RNA replication involving RRM2, NS5B, and hPLIC1, shown in Fig. 9. In this model, (i) expression of RRM2 is induced by HCV infection in quiescent hepatocytes; (ii) an RNA replication scaffold is formed by RRM2, NS5B, and hPLIC1 (15), and a stable HCV replication system is established; (iii) when the expression of RRM2 is suppressed, the NS5B and hPLIC1 proteins are degraded by hPLIC1-dependent proteasomal degradation (19). Thus, RRM2 is essential for HCV RNA replication via protection of the NS5B protein from hPLIC1-dependent proteasomal degradation.

### Experimental Procedures

#### Ethics statement

Chimeric mice were purchased from PhoenixBio Co. and maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Protocols for animal experiments were pre-approved by the local ethics committees of PhoenixBio Co. (approval no. H06-323) and Kagoshima University (approval no. 24002). Chimeric mice were infected with plasma isolated before 2003 from an HCV-positive patient in accordance with the Declaration of Helsinki.

#### Animals and HCV infection

Chimeric mice were established by transplanting human hepatocytes into severe combined immunodeficiency (SCID) mice carrying a urokinase plasminogen activator (uPA) transgene controlled by an albumin promoter (8). Chimeric mice were infected with plasma isolated before 2003 from an HCV-positive patient (sample code name R6; genotype 1b AY46460) (26). uPA-Tg/SCID mice were transplanted with human hepatocytes and infected with HCV R6 (10^6 copies/mouse) (n = 3) after 55 days or left uninfected as a negative control (n = 3) (Fig. 1A). RNA levels reached 2.9 × 10^6 copies/ml in mouse sera after 84 days (data not shown), at which point the mice were sacrificed via bleeding under anesthesia for analysis. Negative control mice were similarly sacrificed. HCV RNA and human albumin levels were determined at the time of sacrifice. Human serum albumin levels in the blood of chimeric mice were measured using an Alb-II kit.
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HCV RNA levels in sera and JFH/K4 cells were measured by real-time PCR as previously described (27). HCV RNA or total RNA from human hepatocytes in chimeric mouse livers and sera were isolated from flash-frozen liver tissue or from sera by means of a guanidine thiocyanate (GTC) (Sigma-Aldrich)–phenol/chloroform extraction method.

**Microarray analysis**

Chimeric mice with humanized livers (8) were infected with HCV R6 and then sacrificed as described above (Fig. 1A). Uninfected control mice showed no viral load and a human albumin concentration of $1.1 \times 10^7$ ng/ml. Total liver RNA was extracted with GTC and an RNaseasy kit (Qiagen), and RNA integrity was assessed with a bioanalyzer (Agilent Technologies). cRNA targets from HCV-infected and uninfected humanized mouse livers were conjugated with Cy3 or Cy5 and hybridized to the Human Genome CGH Microarray 44K (G4410B; Agilent Technologies) according to the manufacturer's instructions.

**Cells and reagents**

Two HCV subgenomic replicon cell lines were used in this study: FLR3-1 and R6FLR-N, containing the HCV genotype 1b replicon in HuH-7 cells (13,14) (Fig. 2). In addition, JFH/K4 cells (12), which are persistently infected with the HCV JFH-1 strain (28), were used. HuH-7 and cured K4 (29,30), both HCV-negative cell lines, served as controls. HuH-7 cells were obtained from the Japanese Collection of Research Bioreresources Cell Bank (JCRB Cell Bank, Osaka, Japan). All cells were determined to be free of mycoplasma infection (data not shown). HCV replicon cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with GlutaMAX-I (Life Technologies) containing 10% fetal calf serum (FCS) (Sigma-Aldrich). JFH/K4 cells were transfected with a lentiviral vector expressing NS5B (31) or pcDNA6 plasmids carrying the coding sequence (HCV strain R6 (24)) of the core, E1, E2, NS2, NS3/4A, NS4B, NS5A, or NS5B proteins were maintained in DMEM supplemented with 10% FCS.

The **RRM2 siRNA sequence** was 5’-UGGAGCGAUUUGAGCAAGAAGUUCU-3’ (stealth siRNA; Life Technologies). Other siRNAs included ON-TARGETplus siRNAs targeting **RRM2** (a mix of four siRNAs), a non-targeting pool as a negative control (GE Healthcare), HCV R5-targeted siRNA (5’-GUCUCGUAGACCGUGAUCAU-3’), and HCV R7-targeted siRNA (5’-GUCUCGUAGACCGUCACCAUU-3’) (underlined nucleotides represent overhangs). The HCV R5-targeted siRNA was effective against HCV R6 genotype 1b, whereas HCV R7-targeted siRNA was effective against HCV strain JFH-1 (12,13). The **RRM1 siRNA sequence** was 5’-CCCAGUUACUGAAUAGCAGAUCUU-3’. Non-target siRNA #3 (Thermo Fisher Scientific) was used as a negative control. SiRNA transfection was carried out via reverse transfection using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer’s protocol.

**Antibodies and inhibitors**

The following antibodies were used in this study: goat (sc10846) or mouse (sc37693) anti-RRM2 (Santa Cruz Biotechnology), rabbit anti-RRM1 (Abcam), rabbit anti-DDDDK targeting Flag tag (MBL International), rabbit anti-Myc (MBL International or Sigma-Aldrich), goat anti-hPLIC1 (H-20, Santa Cruz Biotechnology), and rabbit anti-ubiquitin linkage-specific K48 (Abcam). Monoclonal anti-actin antibodies (Sigma-Aldrich) were used for normalization. HCV proteins were detected using the originally established anti-core (515), anti-E1 (384), anti-E2 (544), anti-NS4B (4B52), anti-NS5A (5A32), and anti-NS5B (5B14) monoclonal antibodies or rabbit anti-NS2, anti-NS3 (R212), and anti-NS5B (266-A, Virogen Co.) polyclonal antibodies as previously described (24). Appropriate horseradish peroxidase-conjugated secondary antibodies (Dako) were used, and immunoreactivity was detected via enhanced chemiluminescence (GE Healthcare) and a Fusion solo system charge-coupled device (CCD) imager (Vilber-Loumat Co.). MG132, a proteasome inhibitor (Calbiochem) was used.

**Measurement of dCTP levels**

JFH/K4 and HuH-7 cells ($1 \times 10^6$) were seeded in 100-mm dishes and grown for 48 h. dNTP extraction was carried out as previously described (32). Briefly, the cells were collected and resuspended in ice-cold 60% methanol, vortexed
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vigorously, incubated at 95°C for 3 min, and sonicated for 30 s. The extracts were centrifuged to remove cell debris and precipitate protein and DNA. The resultant supernatants were transferred to Amicon Ultra 0.5-ml centrifugal filters (Millipore) to remove macromolecules larger than 3 kDa and were then dried under a centrifugal vacuum at 70°C; the pellet was resuspended in 25 µl nuclease-free water and stored at –80°C. Samples were processed for dCTP quantification using a sensitive fluorescence-based method as previously described (32).

**HCV replicon assay**

The HCV subgenomic replicon cell lines R6FLR-N (genotype 1b, AY045702) (13) and FLR3-1 (genotype 1b, CAB46677.1) (14), as well as HCV JFH-1 persistently infected JFH/K4 cells were treated with RRM2, HCV R5, HCV R7, or control siRNA. Opti-MEM (Life Technologies), containing optimal concentrations of siRNA and Lipofectamine RNAiMAX, was added to the wells of a 96-well tissue culture plate. After 15 min, HCV replicon cells were seeded at a density of 5 × 10³ cells/well. Luciferase activity was measured using the Bright-Glo luciferase assay kit (Promega), and replicon cell viability was evaluated using the water-soluble tetrazolium (WST)-8 cell counting kit (Dojindo) 72 h after transfection. Results are reported as a luminescence value or were calculated as average percentages relative to untreated cells (designated as 100%). RRM2 and GAPDH mRNA levels were measured using the TaqMan Gene Expression assay (Thermo Fisher Scientific). Rescue experiments were performed as previously described (12). The open reading frame of RRM2 was subcloned into the pcDNA6-myc-His vector (Thermo Fisher Scientific) and designated as pcDNA6-myc-His-RRM2. R6FLR-N cells were transfected with RRM2 siRNA (0.1 nM), followed 48 h later by the pcDNA6-myc-His-RRM2 expression vector or pcDNA6-myc-His (0.1 and 0.3 µg) using Lipofectamine LTX (Life Technologies). After 24 h, viral replication was assessed by measuring luciferase activity, and cell viability was determined with the WST-8 assay.

**Immunoprecipitation assays**

JFH/K4 cells were transfected with pcDNA6-RRM2 or empty pcDNA6 vector (5 µg) using Lipofectamine LTX (Life Technologies). At 48 h post-transfection, cell lysates were precipitated with anti-Myc (Sigma-Aldrich) or anti-NS5B antibodies; the expression of RRM2 and viral proteins in precipitates was characterized by western blotting with goat anti-RRM2 (Santa Cruz Biotechnology) or mouse monoclonal anti-NS5B (5B14) (24) primary antibodies, respectively. Monoclonal anti-actin antibodies (Sigma-Aldrich) were used for normalization. Appropriate horseradish peroxidase-conjugated secondary antibodies (Dako) were used, and immunoreactivity was detected via enhanced chemiluminescence (GE Healthcare) and the Fusion solo system CCD imager (Vilber-Loumat Co.).

**Immunofluorescence analysis**

JFH/K4 cells were seeded at 10⁵ cells/well in 24-well culture plates containing glass slides and maintained at 37 °C in a 5% CO₂ incubator. After 24 h, the cells were fixed with 1% paraformaldehyde in phosphate-buffered saline (Ca²⁺/Mg²⁺-free) and reacted with anti-RRM2 and anti-NS5B antibodies (Virogen Co.). Anti-RRM2 and anti-NS5B antibodies were detected with AlexaFluor568-conjugated anti-goat or mouse and AlexaFluor488-conjugated anti-rabbit secondary antibodies, respectively (both from Thermo Fisher Scientific). Labeled cells were observed under a confocal-based high-resolution stimulated emission depletion microscope (Leica) at 592 or 660 nm. Nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI; Thermo Fisher Scientific).

**NS5B stability assays**

HuH-7 lenti-NS5B cells were transfected with RRM2 or control siRNA for 48 h and then treated with CHX (100 µg/ml, Wako) and puromycin (50 µg/ml, InvivoGen) for 6 h before harvesting. During the time course assay, HuH-7 lenti-NS5B cells were spread and 1 day later transfected with pcDNA6-myc-His RRM2 or empty pcDNA6 vector; after 3 days, cells were treated with CHX (100 µg/ml) for the indicated amount of time. Cell lysates were analyzed by western blotting using anti-NS5B, anti-RRM2, and anti-actin antibodies.

The open reading frame of NS5B was subcloned into the pcDNA6-myc-His vector and designated as pcDNA6-myc-His-NS5B. The pCS2-Flag-hPLIC1 expression vector was purchased...
from Addgene. HuH-7 cells were transfected with pcDNA6-myc-His-NS5B (2 µg) with or without Flag-hPLIC1 (2 µg) using Lipofectamine LTX for 48 h. Reverse transfection of siRNAs was performed 24 h prior to plasmid DNA transfection. Six hours before harvest, cells were treated with the MG132 proteasome inhibitor (5 µM). Cells were lysed with lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM pyrophosphate, 1 mM β-glycerophosphate, 1 mM ortho-vanadium, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] and precipitated with anti-Flag antibodies (MBL International). Precipitates were characterized by western blotting using specific antibodies.

**NS5B ubiquitination assay**

HuH-7 cells were transfected with the pcDNA6-myc-His-NS5B expression plasmid using Lipofectamine LTX for 48 h. Reverse transfection with control or RRM2 siRNA was performed 24 h prior to NS5B plasmid transfection. MG132 (10 µM) was added to cells 6 h prior to harvest. Cells were lysed with lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM pyrophosphate, 1 mM β-glycerophosphate, 1 mM ortho-vanadium, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride], and NS5B protein was immunoprecipitated with anti-Myc antibodies. Ubiquitinated NS5B protein was detected using rabbit anti-ubiquitin linkage-specific K48 antibodies. Cell lysates were analyzed via western blotting for NS5B, RRM2, and β-actin.

**Statistical analysis**

Experiments were performed in triplicate, and differences between groups were evaluated with Student’s *t*-test. *P* < 0.05 was considered statistically significant.
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The abbreviations used are: RRM2, ribonucleotide reductase subunit M2; HCV, hepatitis C virus; siRNA, short interfering RNA, NS5B, RNA polymerase nonstructural protein 5B, hPLIC1, human homolog 1 of protein linking integrin-associated protein and cytoskeleton; HCC, hepatocellular carcinoma; ER, endoplasmic reticulum; dNTP, deoxyribonucleotide; CHX, cycloheximide; Rb, retinoblastoma tumor suppressor protein; CDK, cyclin-dependent kinase; SCID, severe combined immunodeficiency; uPA, urokinase plasminogen activator; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum
**Table 1**

Analysis of upregulated genes in HCV-infected hepatocytes from humanized chimeric mouse.

| Gene   | Systematic Name | Description                                                                 | Fold Change |
|--------|-----------------|-----------------------------------------------------------------------------|-------------|
| EDN2   | NM_001956       | Homo sapiens endothelin 2 (EDN2)                                            | 19.5        |
| CXCL9  | NM_002416       | Homo sapiens chemokine (C-X-C motif) ligand 9 (CXCL9)                        | 10.7        |
| RRM2   | NM_001034       | Homo sapiens ribonucleotide reductase M2 polypeptide (RRM2)                 | 14.9        |
| RRM2   | NM_001034       | Homo sapiens ribonucleotide reductase M2 polypeptide (RRM2)                 | 10.0        |
| AF23191# | NM_001956     | Homo sapiens medium-chain 2-hydroxy acid oxidase (HAOX2) (HAOX2) mRNA       | 14.0        |
| H19    | NR_002196       | Homo sapiens H19, imprinted maternally expressed untranslated mRNA (H19) on chromosome 11 | 9.8         |
| ID4    | NM_001546       | Homo sapiens inhibitor of DNA binding 4, dominant negative helix-loop-helix protein (ID4) | 10.8        |
| ARMCX2 | NM_014782      | Homo sapiens armadillo repeat containing, X-linked 2 (ARMCK2)              | 8.6         |
| HLA-DQB1 | NM_002127     | Homo sapiens major histocompatibility complex, class II, DP beta 3 (HLA-DQB1) | 8.7         |

Microarray analysis shown in EXPERIMENTAL PROCEDURES.

Left column: cRNA targets from HCV-infected and uninfected humanized mouse livers were conjugated with Cyanine 3 (Cy3) and Cyanine 5 (Cy5), respectively.

Right column: Contrary to the left column, Cy5 and Cy3 conjugation were used for cRNA targets from HCV-infected and uninfected humanized livers, respectively.

The top 5 most induced genes are shown.
Figure 1. mRNA expression in quiescent human hepatocytes of chimeric mice with or without HCV infection and the effects of HCV on RRM2 expression. (A) Timeline of HCV infection in chimeric mice with humanized livers. Human hepatocytes were transplanted into three uPA-Tg/SCID mice, which were then infected with HCV R6 (genotype 1b) after > 90% of mouse hepatocytes had been replaced, as determined by serum human albumin levels and histochemical analysis (see Methods).
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for details). (B) RRM2 protein expression in quiescent human hepatocytes from uninfected and HCV-infected chimeric mice. Average expression levels in four chimeric mice (#16 and #20 were HCV-negative, #4 and #25 were HCV-positive) were normalized to that of actin. HCV core protein was also detected. (C) Quantification of RRM2 mRNA levels relative to that of GAPDH in HuH-7, cured K4, and JFH/K4 cells. RRM2 protein expression was verified via western blotting using anti-RRM2 antibodies (lower panel). The densitometric RRM2/actin ratios are shown below the blots. (D) Quantification of RRM2 mRNA as in (C) in JFH/K4 cells treated with RRM2 siRNA, HCV R7 siRNA, or control (Cont.) siRNA. Values in untreated cells (Non) are shown for comparison. Expression of RRM2 and β-actin proteins was verified via western blotting (lower), as described in (C). Results in (C) and (D) are an average of triplicate samples, and vertical bars indicate SD from three independent experiments (n=3). *P < 0.05. (E) HCV-infected JFH/K4 cells and uninfected HuH-7 cells were analyzed via western blotting for RRM1, HCV core, and β-actin. (F) dCTP pool size was measured in JFH/K4 and HuH-7 cells using a fluorescence-based assay. Results shown as fold change in dCTP levels relative to that in uninfected HuH-7 cells. Values represent the average fold changes ± SD (vertical bars) from three independent experiments (n=3).
Figure 2. Genomic structure of HCV replicons used in this study. (A) R6FLR-N: human hepatoma cell line HuH-7 harboring a subgenomic HCV replicon derived from HCV strain R6 [genotype 1b (24)]. FLR3-1: HuH-7 cells harboring a subgenomic HCV replicon derived from HCV strain Con-1 [genotype 1b (33)]. (B) HCV JFH-1 [genotype 2a (28)] genome structure. UTR, untranslated region; C, truncated HCV core region; Luc, firefly luciferase gene; 2A, 2A genes of foot-and-mouth disease virus; Neo, neomycin resistance gene; EMCV, encephalomyocarditis virus; IRES, internal ribosomal entry site; NS, HCV nonstructural protein.
Figure 3. Effect of RRM2 on HCV replication. HCV RNA copy numbers in (A) R6FLR-N HCV replicon cells and (B) cells and supernatant of JFH/K4 cells persistently infected with HCV and treated with RRM2 siRNA. Results are presented as means ± SD (vertical bars) of three independent experiments (n=3). *P < 0.05. (C) Immunoblot analyses of NS5B and RRM2 expression in R6FLR-N HCV replicons and JFH/K4 cells using specific antibodies against RRM2, NS5B, and β-actin. Cells were transfected with control (cont.) or RRM2 siRNA (5, 10 nM). The densitometric NS5B/actin ratios are shown below the blots. Blots shown are representative of four independent experiments (n=4).
Figure 4. HCV utilizes RRM2 during replication. (A) Efficacy of RRM2 siRNA compared to that of HCV R5 siRNA and control siRNA. FLR3-1 replicon cells were incubated with the indicated constructs for 3 days, and replication was measured as luciferase activity (ratio of replication inhibition). Cell viability was determined with the water-soluble tetrazolium (WST) assay. (B) R6FLR-N cells pretreated with RRM2 siRNA (0.1 nM) and transfected with mock (Non) or pcDNA6-myc-His-RRM2 vectors (0.3 µg each), analyzed via western blotting with anti-Myc or anti-RRM2 antibodies. (C) R6FLR-N replicon cells transfected with RRM2 siRNA (0.1 nM) followed by pcDNA6-myc-His-RRM2 expression vector or empty pcDNA6-myc-His-vector at 0.1 or 0.3 µg. Luminescence is shown as relative luminescence units (RLUs). Cell viability was measured by WST assay (OD450). Results are presented as means ± SD (vertical bars) of three independent experiments (n=3). *P < 0.05.
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Figure 5. Effect of RRM2 silencing on HCV protein expression. RRM2 or control siRNA was transfected into HuH-7 cells with pcDNA6 plasmids carrying the coding sequence of core, E1, E2, NS2, NS3, NS4B, NS5A, or NS5B. At 72 h post-transfection, cells were harvested and subjected to western blotting analysis using the originally established anti-core (515), anti-E1 (384), anti-E2 (544), anti-NS2, anti-NS4B (4B52), anti-NS5A (5A32), and anti-NS5B (5B14) monoclonal antibodies or rabbit anti-NS3 (R212) polyclonal antibodies (18). Protein levels were measured via an imager; the protein to actin ratio was calculated, and its ratio to mock-treated (non) cells is indicated. Blots shown are representative of three independent experiments (n=3).
Figure 6. Interaction of RRM2 with HCV RNA polymerase NS5B. (A–D) HCV-JFH-1-infected HuH-7 cells were transfected with pcDNA6-myc-His-RRM2 (RRM2) or pcDNA6-myc-His (vector) or mock-transfected (Non) and precipitated with anti-Myc or anti-NS5B antibodies.
Proteins were analyzed via western blotting with anti-RRM2 (A, D) or anti-NS5B (B, C) antibodies. Asterisk indicates the IgG light chain. (E) Inputs of each cell lysate are shown. Blots shown are representative of three independent experiments (n=3). (F) JFH/K4 cells were labeled with anti-RRM2 and -NS5B antibodies, which were detected with AlexaFluor568- and AlexaFluor488-conjugated secondary antibodies, respectively. Labeled cells were observed via stimulated emission depletion (STED) microscopy, and co-localization (merged area) is indicated by the yellow signal (2400×, bar = 0.5 µm). Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). (G) An enlarged view of the merged image is shown (indicated by the white square in panel F). (H) JFH/K4 cells and HuH-7 cells were treated with 0.1% FCS DMEM for 24 h and stained with anti-NS5B and anti-RRM2 antibodies, which were detected using Alexa568- and Alexa488-conjugated secondary antibodies, respectively. NS indicates the reaction with normal rabbit sera and secondary antibodies conjugated with Alexa488 or Alexa568. Labeled cells were observed using a BZ-X700 (Keyence Co., Japan). Nuclei were stained with DAPI. Bars indicate 50 µm. Yellow arrowheads indicate the merged image of NS5B and upregulated RRM2. Upon overexpression of RRM2, RRM2 expression correlated with NS5B expression in merged image. All confocal images are representative of three independent experiments (n=3).
Figure 7. RRM2 regulates RNA polymerase NS5B protein stability. (A) Quantification of NS5B mRNA levels relative to those of GAPDH in HuH-7 lenti-NS5B cells treated with RRM2 or control siRNA or mock-treated (Non) cells for 72 h. Data are shown as an average value of three independent experiments. Vertical bars indicate SD (n=3). (B) HuH-7 lenti-NS5B cells were transfected with RRM2 or control siRNA for 48 h and then treated with cycloheximide (CHX, 100 µg/ml) and puromycin (Puro, 50 µg/ml) for 6 h. Cells were harvested and analyzed via western blotting using anti-NS5B, anti-RRM2, and anti-actin antibodies. (C) Lenti-NS5B-expressing HuH-7 cells were transfected with the pcDNA6-myc-His-RRM2 vector. An empty vector pcDNA6-myc-His was used as a negative control. At 48 h post-transfection, cells were treated with CHX (100 µg/ml) for the indicated times. The cells were harvested and analyzed via western blotting for NS5B, RRM2, and β-actin as reported in (B). Blots shown are representative of three independent experiments (n=3).
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### Figure A

| anti-Flag IP | Myc-NS5B | Flag-hPLIC1 |
|-------------|----------|-------------|
| cont.       | RRM2     | RRM2        |
| non         | siRNA    | siRNA       |
|             |          |             |

- **myc-NS5B**
  - lane 1: 70kDa
  - lane 2: 55kDa

- **Flag-hPLIC1**
  - lane 1: 70kDa
  - lane 5: 40kDa

### Figure B

#### Control siRNA
- NS5B
- hPLIC1
- Nucleus
- Merge

#### RRM2 siRNA
- NS5B
- hPLIC1
- Nucleus
- Merge

### Figure C

| siRNA | non | cont. | RRM2 |
|-------|-----|-------|------|
|       |     |       |      |
|       |     |       |      |
|       |     |       |      |

- **MG132**
  - 70kDa
  - 55kDa

- **myc-NS5B**
  - 70kDa
  - 55kDa

- **RRM2**
  - 70kDa
  - 55kDa

- **Actin**
  - 40kDa
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Figure 8. RRM2 may regulate RNA polymerase NS5B protein stability through the ubiquitin/proteasome-dependent protein degradation system. (A) HuH-7 cells were transfected with pcDNA6-myc-His-NS5B either alone or with Flag-hPLIC1 in the presence or absence of RRM2 siRNA. Six hours before harvest, cells were treated with the proteasome inhibitor MG132 (5 µM). Cell lysates were immunoprecipitated with anti-Flag antibody and probed with anti-NS5B (upper left) or -Flag antibodies (lower right). Each cell lysate was immunoblotted with anti-NS5B (arrow), -Flag, -RRM2, and -actin antibodies (right). (B) Levels of NS5B and hPLIC1 following RRM2 knockdown. JFH/K4 cells were stained with anti-NS5B and anti-hPLIC1 antibodies, which were detected using Alexa488- and Alexa568-conjugated secondary antibodies, respectively. Labeled cells were observed using a BZ-X700 (Keyence Co., Japan). Nuclei were stained with DAPI. Bars indicate 50 µm. Representative confocal images from three independent experiments are shown (n=3). (C) Ubiquitination assay and western blotting of lysates from HuH-7 cells co-transfected with pcDNA6-myc-His-NS5B and non, control or RRM2 siRNA for 48 h. MG132 (10 µM) was added to cells for 6 h prior to harvesting. NS5B was immunoprecipitated with anti-Myc antibodies. Ubiquitinated NS5B was detected using rabbit anti-ubiquitin linkage-specific K48 antibodies. Cell lysates were subjected to western blotting analysis with anti-NS5B, anti-RRM2, and anti-actin antibodies. *asterisks indicate non-specific reaction. Blots shown are representative of three independent experiments (n=3).
Figure 9. Schematic illustration of the proposed role of RRM2 in HCV replication. (i) RRM2 is induced by HCV infection in quiescent hepatocytes. (ii) RRM2 is essential for NS5B stability. hPLIC1 promotes the ubiquitin/proteasome-mediated degradation of NS5B, which is blocked by interaction with RRM2. (iii) Silencing of RRM2 activates ubiquitination and decreases the stability of NS5B and its interaction with hPLIC1. On the other hand, when NS5B is not present, degradation of hPLIC1 was suppressed even without RRM2. This may be due to the fact that no scaffold for the ubiquitination machinery is formed.
Ribonucleotide reductase M2 promotes RNA replication of hepatitis C virus by protecting NS5B protein from hPLIC1-dependent proteasomal degradation
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