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Supporting Information

Files in this Data Supplement:

Supporting Figure 5

Fig. 5. Rb is down-regulated posttranslationally in HCV replicon cells. (*A*) Immunoblot of retinoblastoma tumor-suppressor protein (Rb) in extracts of the NNeo/3-5B(RG) subgenomic replicon cell line (lane 5) and its companion NNeo/3-5B(RG)c cured cell line (lane 4). Also shown are immunoblots of lysates of normal Huh7 cells and 2-3 and 2-3c cells. (*B*) Quantitation of replicate immunoblots of Rb in extracts of cycloheximide (CHX)-treated 2-3 and 2-3c cells. Rb abundance was quantified and normalized to actin. Data shown represent mean ± range in two replicate immunoblots. (*C*) Pulse–chase labeling of Rb in 2-3 and 2-3c cells. Cells were pulse-labeled with ^{35}S methionine/cysteine for 30 min, chased with DMEM supplemented with cysteine and methionine, and lysed at the times indicated. Rb was immunoprecipitated from lysates and separated by SDS/PAGE. The ^{35}S label was quantified by PhosphoImager analysis as the percentage recovered from each cell type at 0 h. The calculated half-life of Rb approximated 2 h in the cured 2-3c cells and 1.2 h in the 2-3 replicon cells.

Supporting Figure 6

Fig. 6. The HCV NS5B protein interacts with Rb *in vivo*. Coimmunoprecipitation of endogenous Rb with ectopically expressed NS5B in lysates of normal Huh7 cells transfected with a vector expressing Flag-tagged NS5B (lane 4) or empty vector (lane 3). Lanes 1 and 2 represent 10% of the total protein input in the assays.

Supporting Figure 7

Fig. 7. NS5B-mediated down-regulation of Rb activates an E2F-responsive promoter. (*A*) Mutations within the $LH^{314–318}$ motif and overlapping GDD sequence of NS5B alter regulation of Rb. Shown are immunoblots of Rb, NS5B, and actin in extracts of Huh7 cells transfected with empty vector or vectors expressing the wild-type (wt) NS5B or N316C Con1 NS5B variant, the D318N/D319N, N316A, or the L314A/N316A mutants (see Fig. 3). NS5B proteins were detected with anti-Flag antibody. (*B*) Luciferase promoter activity assays in normal Huh7 cells transfected with p107-luc and cotransfected with vectors expressing wt Rb (pORF9-hRb1) or the R661W mutant, and either NS5B or empty vector. (*C*) Luciferase activity in NNeo/C-5B 2-3 or 2-3c cells transfected with p107-luc or p107mt-luc, a promoter reporter construct with mutations within the two E2F-binding sites. Data in all panels represent means ± SD from at least three independent experiments.

Supporting Figure 8

Fig. 8. Rb inhibits the NS5B RNA polymerase. (*Left*) *In vitro* assays of polymerase activity of purified NS5B in the presence of GST-NS5A (9-fold molar excess), GST-Rb(301–928) (6-fold molar excess), or GST (26-fold molar excess). Polymerase activity was measured by incorporation of BrUMP (A405–A605) into product (see *Materials and Methods* for details). (*Right*) Relative polymerase activities at 14 h in replicate experiments. Data represent means ± SD from three independent experiments. The addition of an ≈6-fold molar excess (relative to NS5B) of GST-Rb(301–928) resulted in a >50% inhibition of polymerase activity relative to that observed in the presence of a 26-fold molar excess of GST. There was no significant suppression of polymerase activity with addition of a 9-fold molar excess of GST-NS5A. These results are consistent with the interaction between Rb and NS5B involving residues within the polymerase active site, with a resultant loss of RNA- dependent RNA polymerase activity.

Supporting Figure 9

Fig. 9. (*A*) Colony-formation assays of U2OS cells transfected with equal amounts of NS5B expression vector or empty vector (see Fig. 4*B*). Shown are mean numbers of colonies ± SD from three independent experiments. Mock, mock transfected. (*B*) Ectopic expression of wt NS5B or the D318N/D319N NS5B mutant does not alter the cell-cycle distribution of Saos-2 cells, which express a functionally inactive Rb mutant. Quantitative analyses of results for two replicate experiments are shown on the right. Compare with the results shown in Fig. 4C.

Supporting Materials and Methods

Cells. Human hepatoma Huh7 cells and human osteosarcoma U2OS and SAOS-2 cells were grown in DMEM (Cellgro) supplemented with 10% (vol/vol) heat-inactivated FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% (vol/vol) CO_2. Huh7 cell lines containing autonomously replicating, genome-length or subgenomic, dicistronic, selectable hepatitis C virus (HCV) RNAs derived from the genotype 1b HCV-N strain (1) were cultured with 500 μg/ml G418 (Cellgro). HCV RNA-free cured cell lines were prepared and maintained as described in ref. 2, except C-5B/2-3 PostSCH6, which was treated with SCH6, an inhibitor of the HCV NS3/4A serine protease that was kindly provided by Stephan Bogen and Bruce Malcolm of the Schering–Plough Research Institute, Kenilworth, NJ (3).

Drug Treatment of Cell Cultures. For *in vivo* protein-stability assays, cycloheximide (CHX, Calbiochem), prepared as a solution in DMSO, was added to the medium at a final concentration of 100 μg/ml. NNeo/C-5B 2-3 and 2-3c-cured cells were seeded into six-well plates and grown to 50% confluence. CHX or DMSO as a control was added to the culture media, and the cells were incubated for up to 3 h, followed by preparation of cell extracts for quantitative immunoblots. To eliminate replicating HCV from 2-3 cells with a small-molecule inhibitor of the virus protease, the cells were cultured in the presence of 1 μM SCH6 and in the absence of G418 for 2 weeks. The cells were tested for Rb abundance 2 weeks after removal of the drug from the culture media.

Plasmids. Full-length cDNA encoding amino acids 1–928 of human Rb, cloned into the pORF9 mammalian expression vector designated pORF9-hRB1, was purchased from InvivoGen (San Diego). pORF9-hRB1 was digested with EcoRI and AvrII to remove the 1.9-kb fragment encoding amino acids 301–928 of Rb and religated to generate pORF9-hRB1(1–301). The 1.9-kb fragment was subcloned into the pBSIIKS(+) (Stratagene) vector to generate pTM-008, which was digested with EcoRI and NotI to obtain cDNA encoding amino acids 301–928 of Rb that was subcloned into the pGEX-4T-3 (Amersham Pharmacia Biosciences) bacterial expression vector to generate pGEX-hRB1(301–928). A vector expressing the R661W mutant of

human RB1 was constructed by PCR mutagenesis with the primers 5'-tgg cta aat aca ctt tgt gaa cgc ctt c-3' and 5'-gag ata ggc tag ccg ata cac ttt ttt a-3' to generate pORF9-hRB1(R661W).

cDNAs encoding the NS3/4A, NS4B, NS5A, and NS5B proteins of HCV-N were subcloned into the pCMV-tag4 mammalian expression vector (Stratagene) in-frame with a C-terminal Flag tag to generate pCMV-tag4-NS3/4A, pCMV-tag4-NS4B, pCMV-tag4-NS5A, and pCMV-tag4-NS5B, respectively. NS5B mutants (see Fig. 3*A*) were constructed by using pCMV-tag4-NS5B as a template by PCR mutagenesis using the primers 5'-ggg aac aac ctt gtc gtt atc tgc-3' and 5'-gtt cac gag cat cgt gca gtc c-3' for D318N/D319N; 5'-ggg gac gac ctt gtc gtt atc tgc-3' and 5'-gca cac gag cat cgt gca gtc ctg g-3' for N316C; 5'-ggg gac gac ctt gtc gtt atc tgc-3' and 5'-ggc cac gag cat cgt gca gtc ctg g-3' for N316A; and, 5'-ggg gac gac ctt gtc gtt atc tgc-3' and 5'-ggc cac ggc cat cgt gca gtc ctg gag-3' for L314A/N316A.

Genomic DNA was isolated from Huh7 cells by using a GenElute mammalian genomic DNA miniprep kit (Sigma), and the human p107 promoter region (4) was cloned into pGEMT Easy (Promega) after its amplification by PCR using the primers 5'-gga aga tct ccg cgg gct tca tct ccc-3' and 5'-ccc aag ctt ttc agg ccc cgc ggg ctg-3' to generate pTM-032 and sequenced on both strands. pTM-032 was digested with BglII and HindIII, and the 0.4-kb fragment containing the p107 promoter was subcloned into pGL3(R2.1)-Basic (Promega) to generate the p107-luc reporter plasmid. Site-directed mutagenesis of the two tandem E2F-binding sites in the p107 promoter was carried out as described in ref. 4 to generate the p107mt-luc reporter plasmid.

The human MAD2 promoter region (5) was isolated from Huh7 genomic DNA by PCR using the primers 5'-gga aga tct acg aat tcc caa agc atc tag cgg aag-3' and 5'-cca agc ttg gcc agg gac aca aac aaa agc acg-3', cloned into pGEM-T (Promega) to generate pTM-087, and sequenced on both strands. pTM-087 was digested with BglII and HindIII, and the 0.5-kb fragment containing the MAD2 promoter was subcloned into pGL3(R2.1)-Basic (Promega) to generate the MAD2-luc reporter plasmid.

Transfections. Cells were seeded into six-well plates or 10-cm dishes 24 h before transfection and grown to 50% confluence. Before transfection, the culture medium was replaced with fresh medium without antibiotics. For determination of the activity of HCV nonstructural proteins to negatively regulate Rb, Huh7 cells in six-well plates were transiently transfected with 1 μg of pCMV-tag4 (empty vector), pCMV-tag4-NS3/4A, pCMV-tag4-NS4B, pCMV-tag4-NS5A, pCMV-tag4-NS5B wt, or individual NS5B mutant expression vectors, as indicated, by using FuGENE 6 reagents (Roche Diagnostics). Forty-eight h posttransfection, protein extracts were prepared for immunoblots.

Northern Blot Analysis. Total RNA was extracted from Huh7 cells or their derivatives by using a GenElute mammalian total RNA miniprep kit (Sigma), and RNA concentration was determined by spectrometry at 260 nm. Equal amounts of RNA (10 μg) were separated by 1% denaturing agarose-formaldehyde gel electrophoresis and transferred to a positively charged nylon membrane with a NorthernMax kit (Ambion) according to the manufacturer's instructions. Transferred RNAs were immobilized by UV cross-linking (Stratagene) and stained with ethidium bromide to locate 28S rRNA on the membrane. HCV RNA and *RB* mRNA were hybridized to biotinylated DNA probes specific for nucleotides 8213–8821 of HCV-N genomic RNA and nucleotides 195–714 of human RB1 protein coding sequence, respectively. Bound probes were detected by streptavidin-alkaline phosphatase, followed by reaction with CDP-Star (Ambion) and exposure to x-ray film. Where indicated, RNA bands were scanned and quantified by using nih image software.

Immunoblot Analysis. Cells were washed three times with chilled PBS and incubated in chilled lysis buffer (20 mM Tris•HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA-2Na, 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 2 mM DTT, 1 mM PMSF, and 2 μg/ml aprotinin) for 30 min at 4°C. Cell debris was pelleted by centrifugation at 13,000 × *g* for 30 min at 4°C, and supernatants were used as soluble fractions. Protein concentrations were determined by the modified Bradford assay with BSA as a standard (Bio-Rad). Equal amounts of proteins were separated on SDS-9% or 4–20% gradient polyacrylamide gels and electrophoretically transferred to PVDF membranes, followed by blocking with 5% nonfat dry milk in TBS-T. Membranes were incubated with mouse monoclonal antibodies against β-actin (AC-15, Sigma), Flag (M2, Sigma), GFP (JL-8, BD Biosciences), GST (B-14, Santa Cruz Biotechnology), and Rb (G3-245, BD Biosciences; 4H1, Cell Signaling Technology, Beverly, MA), rabbit polyclonal antibodies against p107 (sc-318, Santa Cruz Biotechnology), p130 (sc-317, Santa Cruz Biotechnology), and NS5B (a generous gift of Craig E. Cameron, Pennsylvania State University, University Park, PA) or goat polyclonal antibodies against HCV NS5A/B (B65980G, BioDesign, Kennebunk, ME). After washing with TBS-T, membranes were probed with anti-mouse, rabbit, or goat Ig secondary antibodies conjugated with horseradish peroxidase (HRP), and bound antibodies were visualized by ECL Plus reagents (Amersham Pharmacia Biosciences) and exposed to x-ray films. Where indicated, immunoreactive bands were scanned and quantified by using nih image software.

For quantitative immunoblots of Rb and actin, anti-mouse Ig secondary antibodies conjugated with DyLight 647 (Pierce) were used, and the images were captured and quantified on a Storm 860 PhosphoImager (Molecular Dynamics).

Indirect Immunofluorescence. Huh7 2-3 and 2-3c cells were seeded on eight-well glass chamber slides and grown until 50–60% confluent. After washing twice with PBS, the cells were fixed in methanol–acetone (1:1 [vol/vol]) for 10 min at –20°C, air-dried for 60 min at room temperature, washed twice with PBS, and incubated with blocking buffer (1% BSA in PBS) overnight at 4°C. NS5B was visualized by staining with mouse monoclonal antibody 20A12C7 (a kind gift from Craig Cameron) and Rb protein by staining with mouse monoclonal antibody G3-245. After washing three times with PBS, slides were further incubated with a goat anti-mouse Ig secondary antibody conjugated with either FITC (Rb) or TRITC (NS5B) for 1 h at room temperature. Slides were washed three times with PBS, counterstained with DAPI, mounted in Vectashield mounting medium (Vector

Laboratories), and examined with a Zeiss AxioPlan2 fluorescence microscope.

Luciferase Reporter Assays. Huh7 cells (0.4×10^5 cells per well) were seeded into 24-well plates 24 h before transfection, and, before transfection, the culture medium was exchanged with fresh medium without antibiotics. For coexpression of NS5B and Rb, 300 ng of the p107-luc reporter plasmid DNA was cotransfected with 300 ng of pCMV-tag4 or pCMV-tag4-NS5B and 900 ng of pORF9-hRB1 or pORF9-hRB1(R661W), along with 1 ng of pRL-CMV (Promega) by using FuGENE 6 reagents (Roche Diagnostics). For analysis of NS5B mutants, 300 ng of p107-luc or MAD2-luc reporter was similarly cotransfected with 300 ng of pCMV-tag4, pCMV-tag4-NS5B WT, pCMV-tag4-NS5B D318N/D319N, or pCMV-tag4-NS5B L314A/N316A, along with 1 ng of pRL-CMV. Seventy-two h posttransfection, cells were washed with PBS and incubated in 100 μl of 1× passive lysis buffer (Promega) with gentle rocking for 30 min at room temperature to prepare cell lysates. Lysates were stored at –20°C until use. Dual luciferase assays were carried out with 20 μl of lysate according to the manufacturer's instructions (Promega) by using a TD-20/20 luminometer (Turner, Palo Alto, CA), and firefly luciferase activities were normalized to *Renilla* luciferase activities. For analysis of p107 promoter activity in HCV replicon and cured cells, NNeo/C-5B 2-3 or 2-3c cells in 24-well plates were transiently transfected with 300 ng of p107-luc or p107mt-luc and 1 ng of pRL-CMV. Forty- eight h posttransfection, cell lysates were prepared, and dual luciferase assays were carried out as described above. Comparisons of relative luciferase activities were performed by Student's *t* test by using excel 2003 (Microsoft).

Coimmunoprecipitation Assays. Huh7 cells were grown to 50% confluence in 10-cm dishes and transfected with 5 μg of pCMV-tag4-NS5B or pCMV-tag4. Forty-eight hours posttransfection, cells were lysed in 1 ml of immunoprecipitation (IP) lysis buffer (20 mM Tris•HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA-2Na, 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 1 mM PMSF, and 2 μg/ml aprotinin), and extracts were prepared as described above. Extracts (500 μg) were precleared, mixed with anti-Flag monoclonal antibodies (M2, Sigma) and protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biosciences), and incubated for 3 h at 4°C with gentle rotation. Immune complexes were precipitated and washed three times with IP lysis buffer. Immunoprecipitated proteins were eluted from the resins with 2× SDS sample buffer [100 mM Tris•HCl (pH 6.8), 4 mM EDTA-2Na, 16% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.5 mg/ml bromophenol blue, and 2% (vol/vol) β-mercaptoethanol] and analyzed by immunoblot. For analysis of the interaction in HCV replicon cells, NNeo/C-5B 2-3 and 2-3c-cured cells were lysed in IP lysis buffer, and soluble extracts were prepared. Extracts (500 μg) were mixed with 10 μl of preequilibrated α-Rb agarose (Calbiochem) and incubated overnight at 4°C with gentle rotation. The precipitate was treated with RNase A (final 0.13 mg/ml) before separation by SDS/PAGE and immunoblot analysis.

Pulse–Chase Labeling of Rb. Cells ($0.4–1.0 \times 10^5$), maintained in DMEM with 10% FBS plus antibiotics, were starved for 1 h with methionine/cysteine-free DMEM, incubated with 200 μCi [^{35}S]methionine/cysteine for 30 min, washed three times with PBS, and lysed directly (time = 0 h) or chased in DMEM supplemented with 2 mM methionine and 2 mM cysteine for up to 3 h before lysis. Cell lysates were immunoprecipitated with anti-Rb agarose (Oncogene) overnight at 4°C. After extensive washing, the precipitates were boiled in 20 μl of sample buffer and separated on 10% SDS/PAGE.

^{35}S-Rb recovered in the gel was quantified by PhosphoImager analysis.

***In Vitro* Rb-Binding Assay.** GST and the GST-Rb(301-928) fusion protein were expressed in *Escherichia coli* BL21 (DE3) strains transformed with pGEX-4T-3 and pGEX-hRB1(301-928), respectively, and purified by using a BugBuster GST-Bind purification kit (Novagen). Extracts (40 μg) of Huh7 cells expressing equal amounts of Flag-tagged NS5B wt, D318N/D319N, N316C, N316A, or L314A/N316A proteins were mixed with anti-Flag monoclonal antibodies (M2, Sigma) and protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biosciences), and incubated for 2 h at 4°C with gentle rotation. Immune complexes were precipitated and washed three times with IP lysis buffer to prepare NS5B resins. GST or GST-Rb(301-928) (2.5 μg) was mixed with each NS5B resin and further incubated for 2 h at 4°C with gentle rotation. The resins were washed three times with IP lysis buffer, and bound proteins were eluted with 2× SDS sample buffer and subjected to immunoblot analysis.

Colony-Formation Assay. Colony-formation assays in U2OS osteosarcoma cells (6) (American Type Culture Collection) were carried out with cells seeded into six-well plates, grown to 50% confluence, and transfected with 2 μg of pCMV-tag4 or pCMV-tag4-NS5B WT by using FuGENE 6 reagents (Roche Diagnostics). At 24 h posttransfection, cells were split into 10-cm dishes with selection media containing 200 μg/ml G418. After 3 days, the G418 concentration was increased to 500 μg/ml, with refeeding with fresh media every 3 days. At 3 weeks, cells were fixed in methanol, stained with Coomassie Brilliant blue, and the surviving colonies counted.

FACS Analysis. U2OS or SAOS-2 cells were seeded into 10-cm dishes, grown to 30% confluence, and transfected with 5 μg of pCMV-tag4, pCMV-tag4-NS5B WT, pCMV-tag4-NS5B D318N/D319N, or pCMV-tag4-NS3/4A, along with 1 μg of pEGFP-C1 (Clontech) by using FuGENE 6 reagents (Roche Diagnostics). Two days later, cells were harvested, fixed in 1% paraformaldehyde followed by 75% ethanol. DNA was stained with 100 μg/ml propidium iodide and 50 μg/ml RNase A for 30 min at 37°C in the dark. Samples were filtered through nylon mesh to remove cell aggregates and analyzed by a FACS Canto flow cytometer (BD Biosciences). In each assay, at least 5,000 GFP-positive cells were selected for cell-cycle analysis carried out by using ModFitLT V3.0.

NS5B RNA Polymerase Assays. RNA-dependent RNA polymerase assays were carried out by using a RepliScreen kit (Replizyme, Heslington, York,

U.K.) reagents according to the manufacturer's instructions. Briefly, 0.2 μ g (3.1 pmol) of recombinant NS5B protein with a 21-aa C-terminal deletion was preincubated with 2 μg of GST (80 pmol), GST-NS5A (27 pmol), or GST-Rb(301-928) (21 pmol) at 35°C for 70 min. The polymerase reaction was carried out in 96-well plate with an immobilized poly(rA) template and an oligo-dT_{22} primer and 5-bromouridine-5'-triphosphate (BrUTP) at 35°C for 3 h. The incorporated 5-bromouridine-5'-monophosphate (BrUMP) was detected by using a specific antibody conjugated with alkaline phosphatase, and quantitative colorimetric measurements (A405-A605) of activity were taken at the indicated times by using *p*-nitrophenyl phosphate (pNPP) as a substrate with SpectraMax M2 (Molecular Devices).

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