Material and Methods

Cells and Virus

The Huh7 derivative cell line Huh7.5.1 was provided by F. Chisari (Scripps Research Instituite, La Jolla, CA). Cells were maintained at 37C in 5% CO2 in Dulbecco modified Eagle medium containing 10% fetal bovine serum. HSCs were purified from Sprague-Dawley rats (>400 g, Charles River Laboratory, Cambridge, MA, USA) by sequential digestion of the liver with pronase and collagenase, followed by Nycodenz gradient centrifugation as previously described [61]. Cell purity, assessed by examining the autofluorescence properties of the stored retinoids in HSCs, was typically between 90 and 95%. HSCs were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), and 2 mM l-glutamine, and cultured in a 95% air–5% CO2 humidified atmosphere at 37°C. The growth medium was changed every other day. HSCs were activated by culturing on plastic for 7 days. The JFH1 HCV strain was used in tissue culture experiments at an MOI of 0.5 [20].

Reagents

Alpha interferon (IFN-α) was purchased from Fitzgerald and was used to treat cells for 8 hours unless otherwise indicated. (Concord, MA). Poly(IC) (Amersham-Pharmacia) was reconstituted in PBS at 2 mg/mL, denatured at 55C for 30 min, and allowed to anneal to room temperature before use. Huh7.5.1 cells were transfected with 6 μg of poly(IC) in 3.2 μL of Lipofectamine2000 (Invitrogen) for 8 hours unless otherwise indicated. Ribavirin (R9644) , Guanosine (G6264) and Cycloheximide (C4859) were purchased

from Sigma and dissolved in PBS and DMSO, respectively. Loxoribine (tlrl-lox) was purchased from Invivogen (San Diego, Ca) and dissolved in DMSO. MG132 (474790) was purchased from Calbiochem (La Jolla, Ca) and dissolved in DMSO. B18R (14-8185) was purchased from ebioscience ((San Diego, Ca).

RNA Interference

Chemically synthesized 21-nucleotide sense and antisense RNA oligonucleotides were obtained from Dharmacon as On Target Plus SMART pools. Huh7.5.1 cells were plated on twelve-well plates at 90,000 cells per well and transfected with siRNA at a final concentration of 50 ηM per well using Oligofectamine (Invitrogen). Assays were typically performed 72 hours after siRNA treatment, when gene knockdown was found to be maximal.

DNA Microarray analysis

Total RNA was extracted from Huh7.5.1 cells and activated hepatic stellate cells with or without ribavirin treatment (100 μg/mL) for 24 hours with the RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer's instructions. RNA was quantified with a spectrophotometer, and the RNA quality was analyzed with an Agilent (Foster City, CA) bioanalyzer according to the manufacturer's instructions. RNA was then amplified with an Agilent Enzo kit. Amplified complementary RNA was hybridized to an Affymetrix Human 133 Plus 2.0 microarray chip containing 54,675 gene transcripts.

Quantitative PCR

Intracellular and extracellular copy numbers of HCV RNA were determined by real-time quantitative reverse transcription-PCR as described previously [62]. For analysis of endogenous mRNA levels, total RNA was isolated from cells using the RNeasy RNA extraction kit (Qiagen) and cDNA synthesis was performed using 1 μg of total RNA (Roche). Fluorescence real-time PCR analysis was preformed using an ABI 7500 instrument (Applied Biosystems) and TaqMan gene expression assay (Applied Biosystems). Relative amounts of mRNA were normalized to the 18S ribosomal RNA levels in each sample.

Immunohistochemistry

Immunohistochemical staining for IRF7 was performed on 4-μm tissue array sections. Using a kit (Cytomation LSAB plus; DAKO North America, Inc, Carpenteria, California), liver biopsy slides were heated at 60°C for 1 hour and then deparaffinized and dehydrated. After rinsing in automation buffer (DAKO North America, Inc), the slides were subjected to antigen retrieval using 1x citra buffer (6.0 pH; DAKO North America, Inc) at 100 C° (steam) for 30 minutes and were allowed to cool to room temperature. The slides were subjected to a serum-free protein block, a peroxidase block, and an avidin-biotin system block before incubation with IRF7 antibody from Santa Cruz at 1:50 dilution overnight at 4°C in a humidified chamber. A nonspecific negative control consisted of normal colon tissue incubated with a standard negative control antibody (negative control IgG1 antibody; DAKO North America, Inc) at 1:50 dilution. A universal secondary antibody and streptavidin–horseradish peroxidase (LSAB2 kit,

DAKO North America, Inc) was applied to each slide after several washes with buffer. The IRF7 antibody was visualized using 3,3-diaminobenzidine at room temperature for 5 minutes. The slides were counterstained with hematoxylin, dehydrated, cleared with xylene, and coverslipped using a mounting medium (Permount; Fisher Science, San Francisco, California).

Luciferase Assays

NF-κB (#219077) and ISRE (#219092) luciferase constructs were obtained from Stratagene and luciferase assays were performed according to manufacturers instructions (PathDetect cis-Reporting System).

Liver Biopsies

Liver biopsies analyzed in this studied were obtained from an IRB approved protocol at UNC Chapel Hill and have already been described [1].

Cell Death

Internucleosomal DNA fragmentation was quantitatively assayed by antibody-mediated capture and detection of cytoplasmic mononucleosome- and oligonucleosome-associated histone-DNA complexes (Cell Death Detection ELISA plus kit; Roche Molecular Biochemicals, Mannheim, Germany) that accumulated in dying Huh 7.5.1 cells with intact cell membranes. Briefly, Huh 7.5.1 cells (1×10^4 cells in 200 µL supplemented DMEM medium) 24 hours after treatment were washed, resuspended in 200 µL of the lysis buffer supplied by the manufacturer, and incubated for 30 minutes at room

temperature. After pelleting (200g, 10 minutes), 20 µL of the supernatant (cytoplasmic fraction) was used in the enzyme-linked immunosorbent assay (ELISA) following the manufacturer's standard protocol. Finally, absorbance at 405 nm and 490 nm (reference wavelength), upon incubating with a peroxidase substrate for 5 minutes, was determined with a microplate reader (Bio-Tec Instruments, Winooski, VT). Signals in the wells containing the substrate only were subtracted as background. IRF7 staining was quantitated using the ImageJ analysis software (http://rsbweb.nih.gov/ij/).

Statistical Analysis

Data from repeated experiments were averaged and are expressed as means ± the standard deviations. Statistical analysis was performed using Mann-Whitney U test. For microarray experiments, the signal intensity from gene transcripts was compared between treated and untreated samples. Affymetrix CEL files were imported into Partek Genomics Suite™ 6.3 (Partek Inc., St. Louis, MO) using the default Partek normalization parameters. Probe-level data were pre-processed, including background correction, normalization, and summarization, using robust multi-array average (RMA) analysis. RMA adjusts for background noise on each array using only the PM probe intensities; and subsequently normalizes data across all arrays using quantile normalization and followed by median polish summarization to generate a single measure of expression. These expression measures were then log-transformed, base 2. ANOVA analysis was performed for comparisons using Partek Pro software (Partek Inc.). Before the samples were compared, the signal-to-noise ratio was evaluated by a source of variance analysis. Only genes for which a signal was detected in at least 50% of the samples were included.

Expression differences of at least 1.5-fold with $P < 0.01$ were considered significant.

Technical Assistance

We wish to thank Richard A. Rippe for technical assistance in hepatic stellate cell isolation and Weiping Chen for assistance with the microarray analysis.

Author contribution (see explanation of footnotes below);

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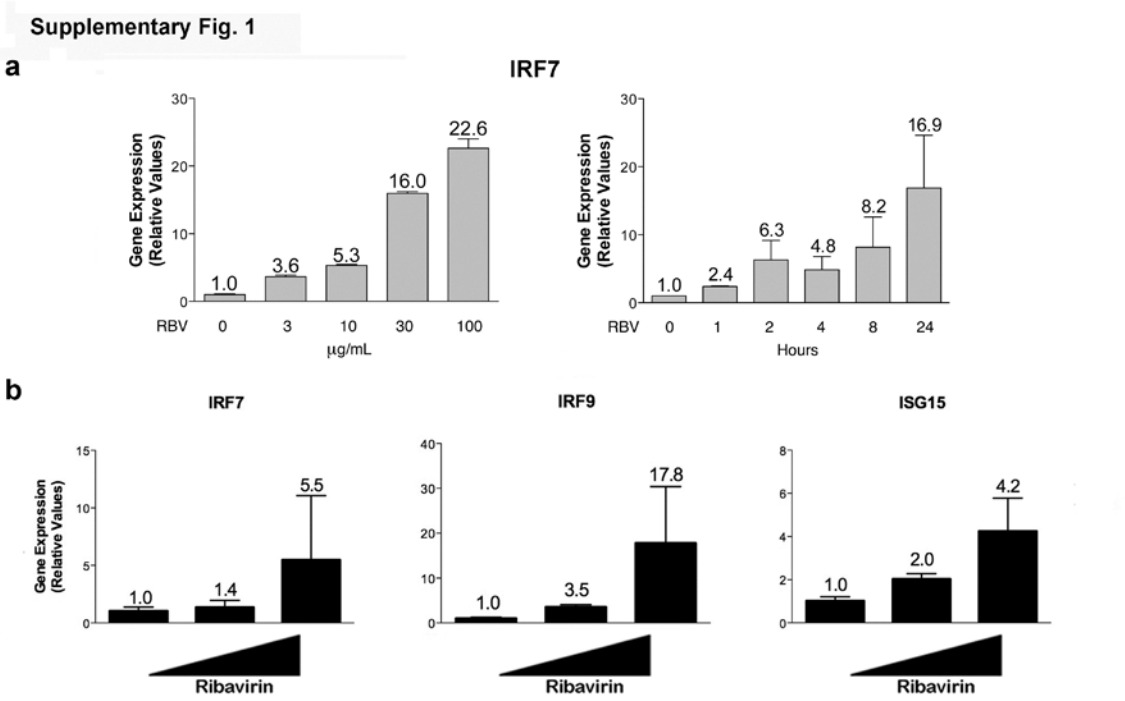
[A] Designed research

[B] Performed research

[C] Contributed new reagents or analytic tools

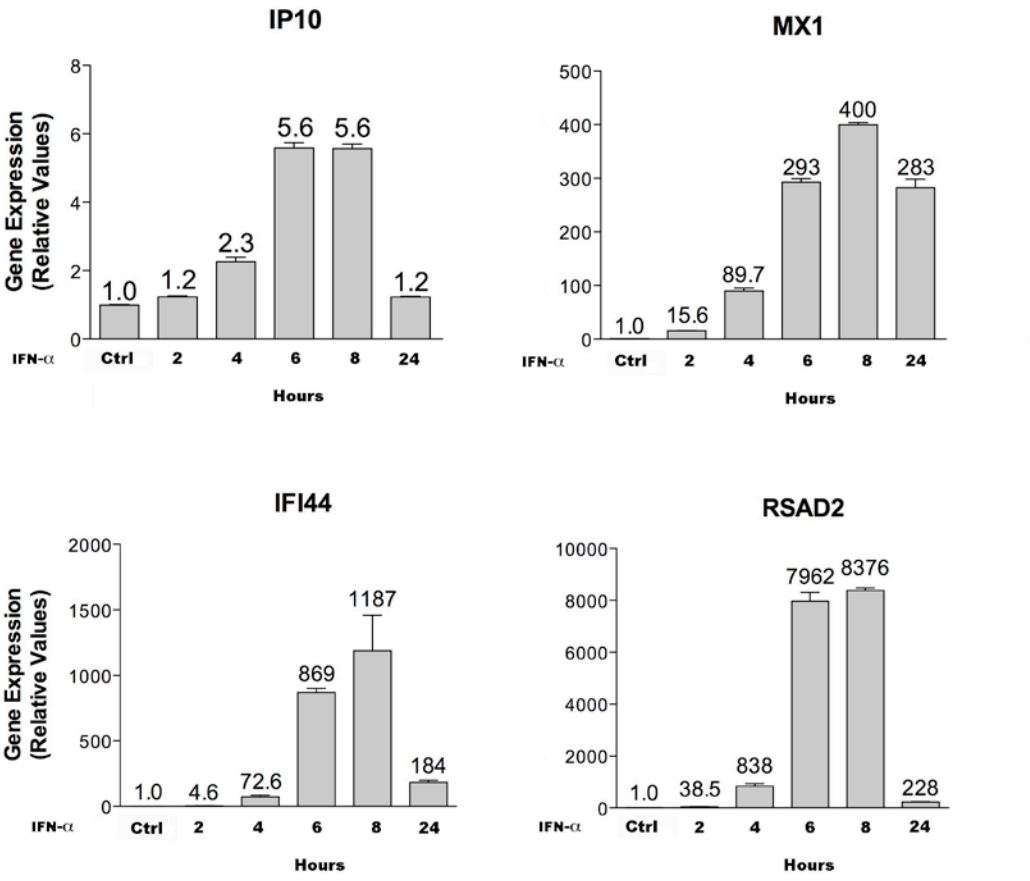
[D] Analyzed data

[E] Wrote the paper

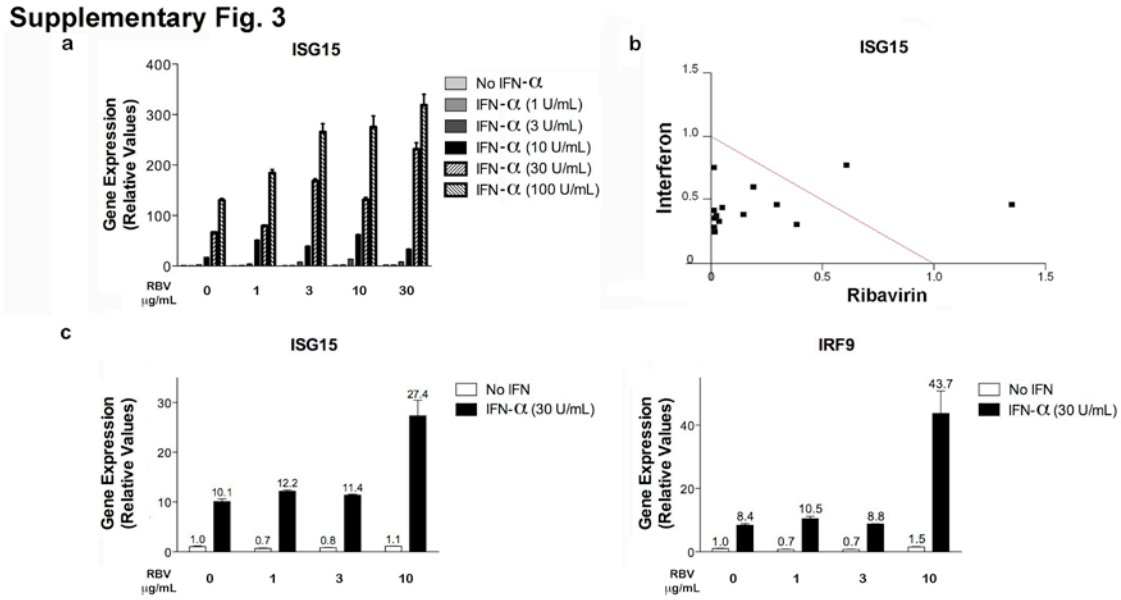


Supplementary Figure 1: Upregulation of IRF7 mRNA levels with increasing doses and duration of treatment with ribavirin. (a) Huh7.5.1 cells were treated with ribavirin for 24 hours with increasing doses or with 100 µg/mL for increasing durations and harvested for qPCR determination of IRF7 levels. (b) qPCR was performed on the HepG2 cell line 24 hours after treatment with ribavirin at doses of 0, 10, and 100 µg/mL for IRF7, IRF9 and ISG15. Data is obtained from experiments performed in triplicate.

Supplementary Fig. 2

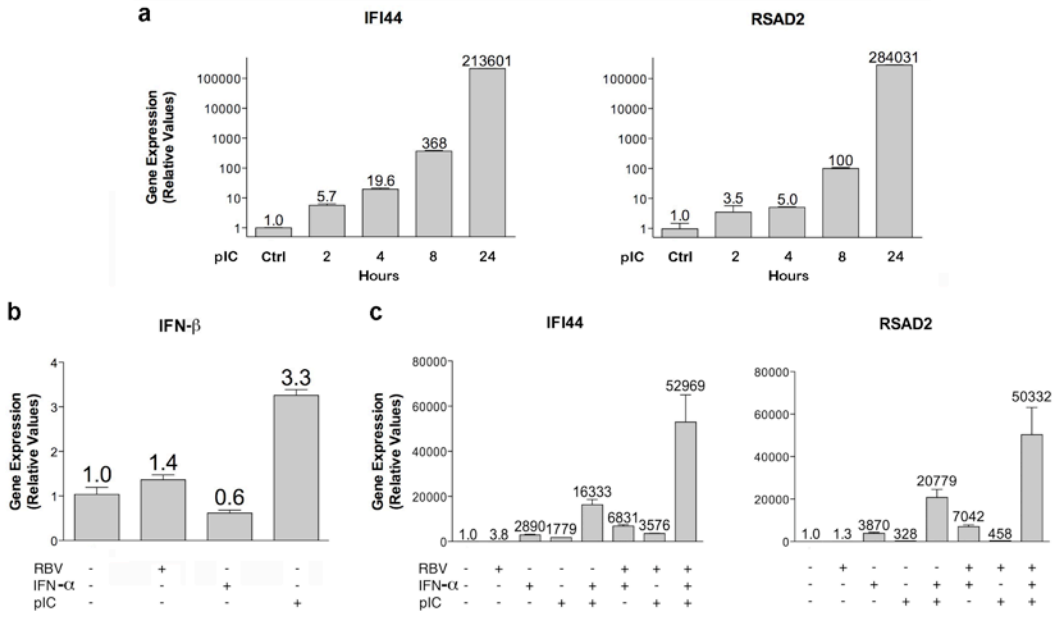


Supplementary Figure 2: Induction of ISGs by interferon-α is greatest at 8 h. Huh7.5.1 cells were treated with 100 U/mL interferon-α and harvested at different time points for qPCR analysis of IP10, MX1, IFI44 and RSAD2 expression levels. Data is obtained from experiments performed in triplicate.



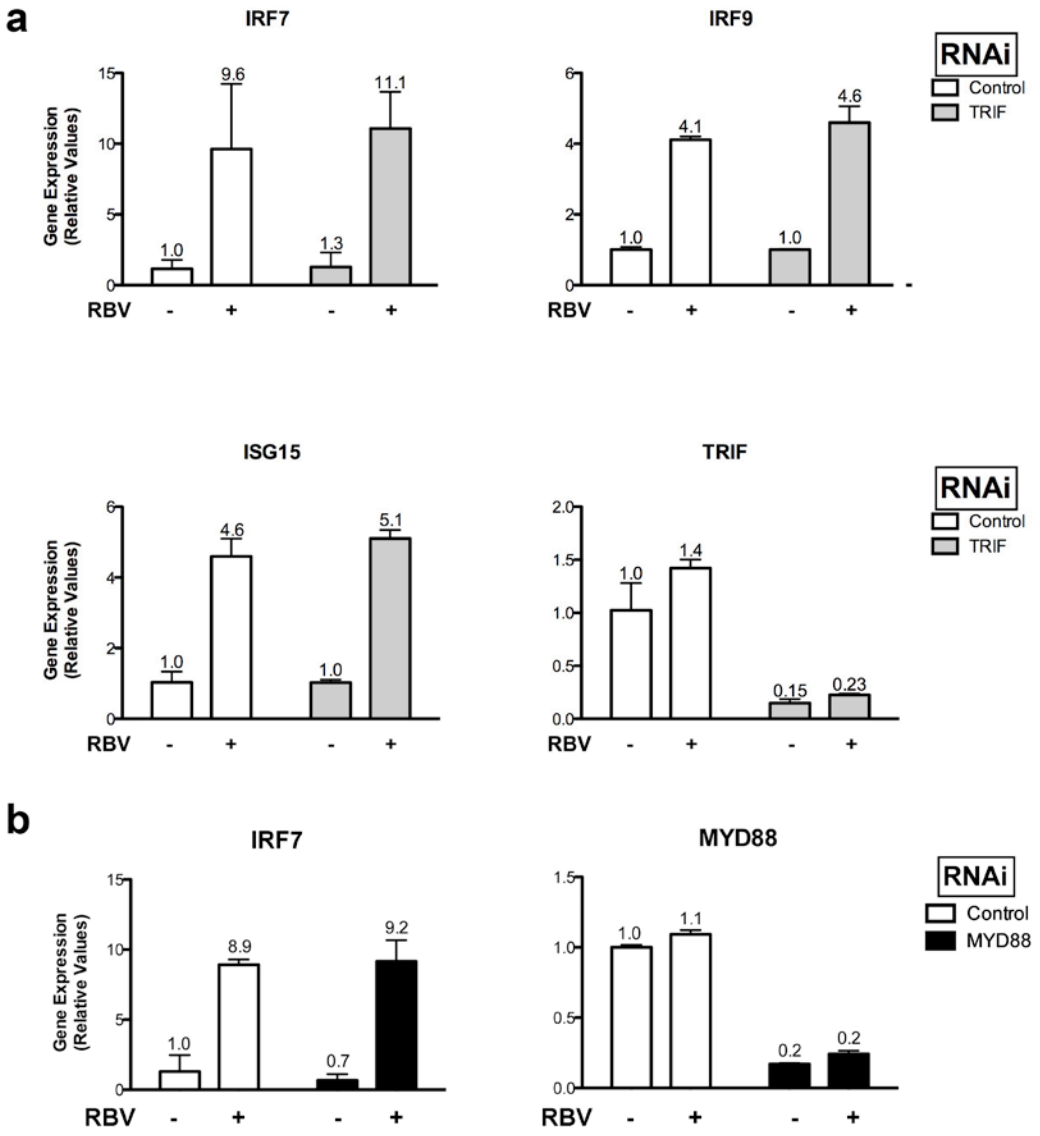
Supplementary Figure 3: Synergistic induction of ISG15 by ribavirin and interferon-α. (a) qPCR analysis of ISG15 induction after treatment with ribavirin and interferon at various concentrations in Huh 7.5.1 cells. (b) Normalized isobologram demonstrating synergy between ribavirin (1, 3, 10, 30 μg/mL for 24 h) and interferon-α (1, 3, 10, 30, 100 U/mL for 8 h) on ISG15 mRNA levels for 14 combinations of the two drugs generated with the Calcusyn analysis software. (c) qPCR analysis of ISG15 and IRF9 induction after treatment with ribavirin (1, 3, 10 μg/mL for 24 h) and interferon-α (30 U/mL for 8 h) in Huh 7 cells. Data is obtained from experiments performed in triplicate.

Supplementary Fig. 4



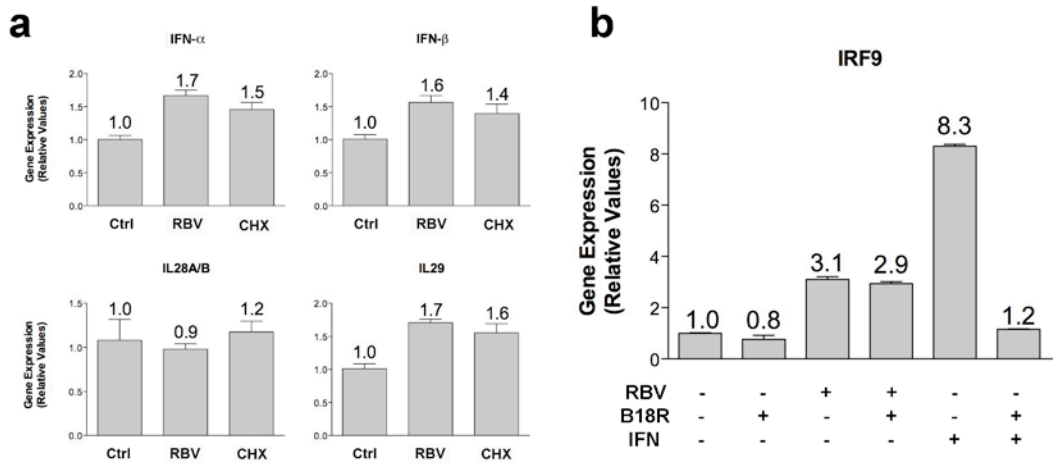
Supplementary Figure 4: Gene induction by ribavirin, interferon-α and poly(IC). qPCR analysis of (a) IFI44 and RSAD2 expression levels in Huh7.5.1 cells transfected with poly(IC) (6 μg/mL) at various time points. (b) Interferon-β (c) IFI44 and RSAD2 mRNA levels in Huh7.5.1 cells after treatment with ribavirin (100 μg/mL for 24 hours), interferon-α (100 U/mL for 8 hours) and poly(IC) (6 μg/mL for 8 hours). Data is obtained from experiments performed in triplicate.

Supplementary Fig. 5

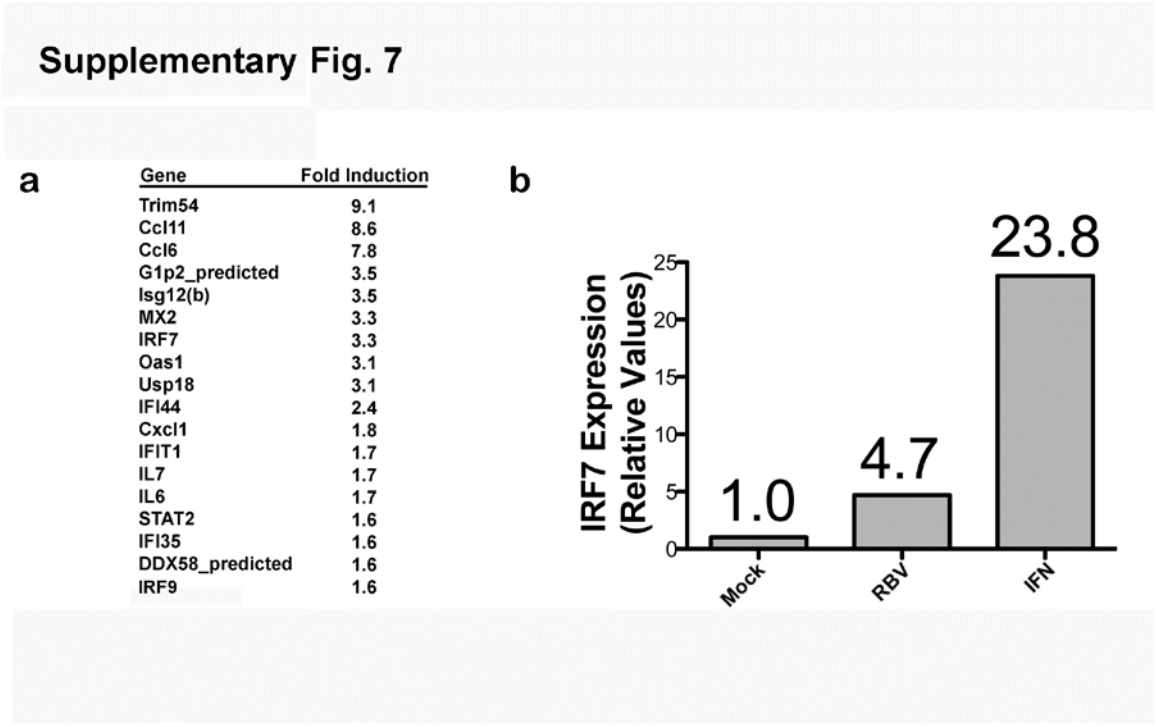


Supplementary Figure 5: Gene induction by ribavirin does not involve the TLR signaling pathway. qPCR analysis of (a) IRF7, IRF9, and ISG15 mRNA levels in Huh7.5.1 cells first treated with siRNAs for three days targeting TRIF (with quantification of siRNA mediated gene suppression) and (b) qPCR analysis of IRF7 in MYD88 siRNA treated cells (with quantification of siRNA mediated gene suppression) and then treated with or without ribavirin (100 mg/mL for 24 h). Data is obtained from experiments performed in triplicate.

Supplementary Fig. 6

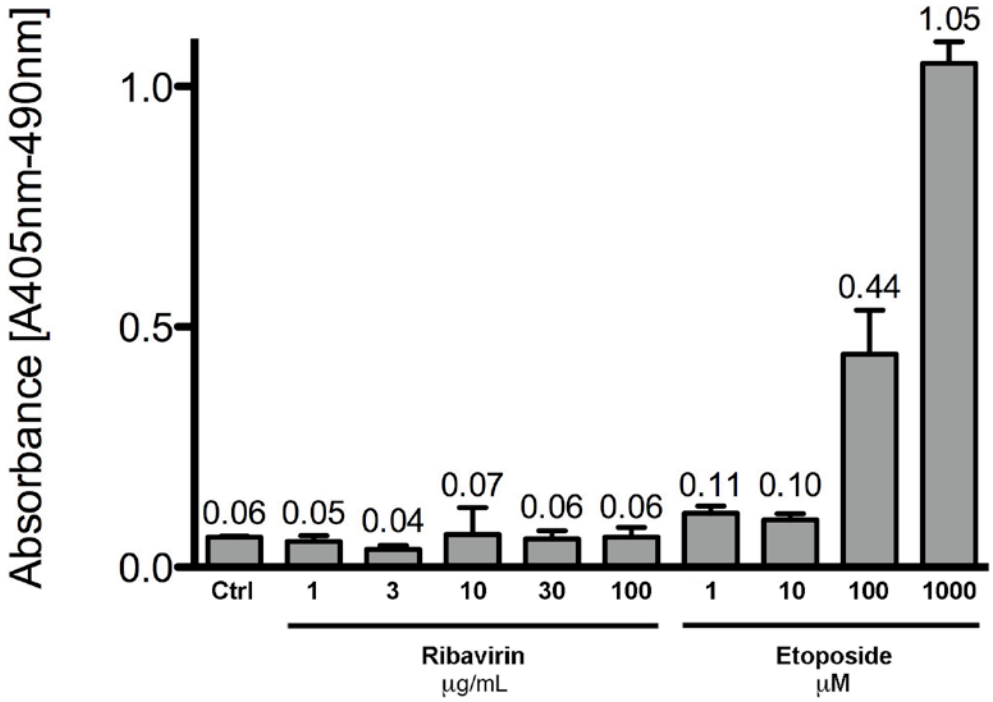


Supplementary Figure 6: Blocking Type I Interferon has no Effect on Gene Induction by Ribavirin. (a) qPCR analysis of IFN-α, IFN-β, IL28A/B, and IL29 following treatment with ribavirin (100 μg/mL for 24 h) and cycloheximide (10 μg/mL for 24h). Data is obtained from experiments performed in triplicate. (b) qPCR analysis of IRF9 mRNA levels after treatment with ribavirin (100 μg/mL for 24 h), B18R (30 ηg/mL for 30h) and IFN-α (100 U/mL for 24 h). Data is obtained from experiments performed in triplicate.



Supplementary Figure 7: Induction of ISGs by ribavirin in hepatic stellate cells. (a) Microarray analysis demonstrating gene induction of several ISGs after treatment of activated hepatic stellate cells with ribavirin for 24 hours at a dose of 100 μg/mL. Primary rat hepatic stellate cells were isolated and activated as described in Material and Methods. **(b)** qPCR analysis of IRF7 mRNA levels from HSCs treated with ribavirin (100 μg/mL) and Interferon-α (100 U/mL) for 24 hours. Microarray analysis was performed on RNA from obtained from the combination of samples from three separate experiments.

Supplementary Fig. 8



Supplementary Figure 8: Treatment with ribavirin does not cause increased cell death. Huh7.5.1 cells were treated with ribavirin and etoposide at the indicated doses for 24 hours, and then subjected to cell toxicity assay as described in Materials and Methods. Data is obtained from experiments performed in triplicate.