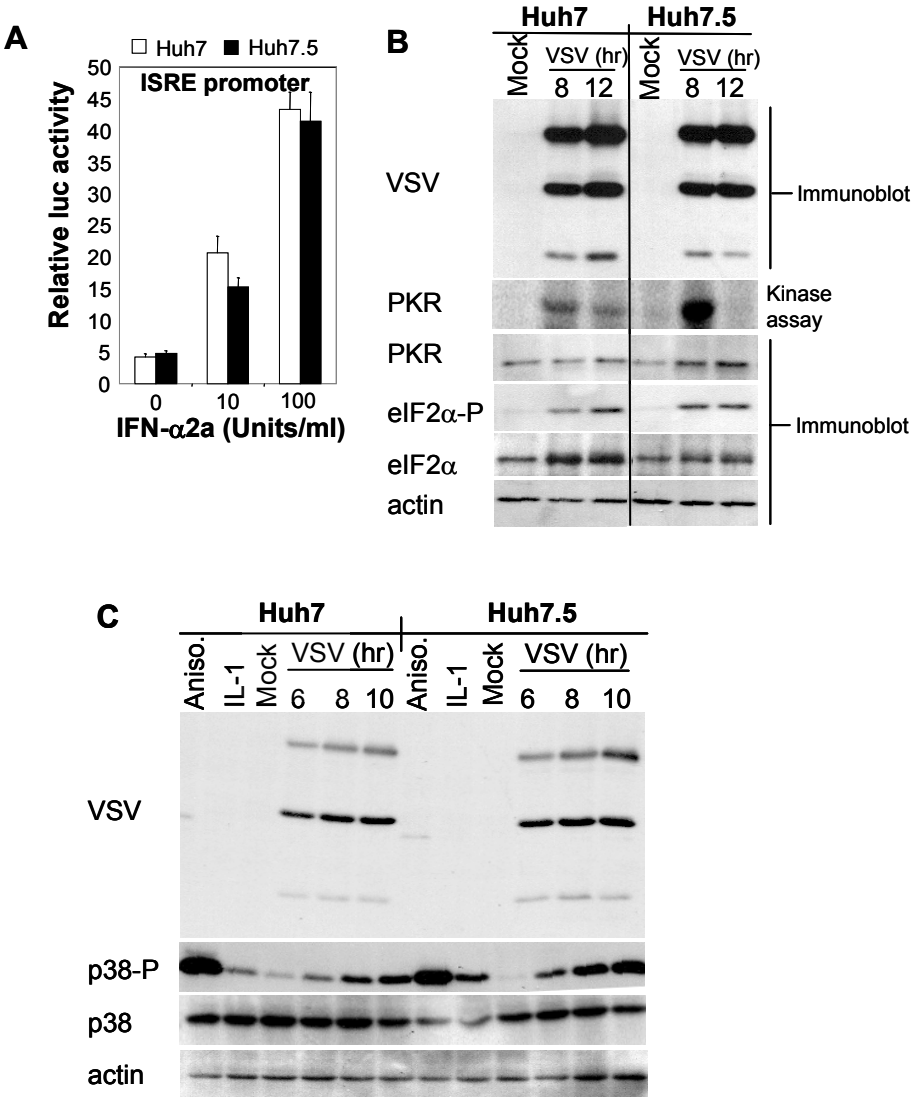
RHEA SUMPTER et al.

SUPPLEMENTAL FIGURES

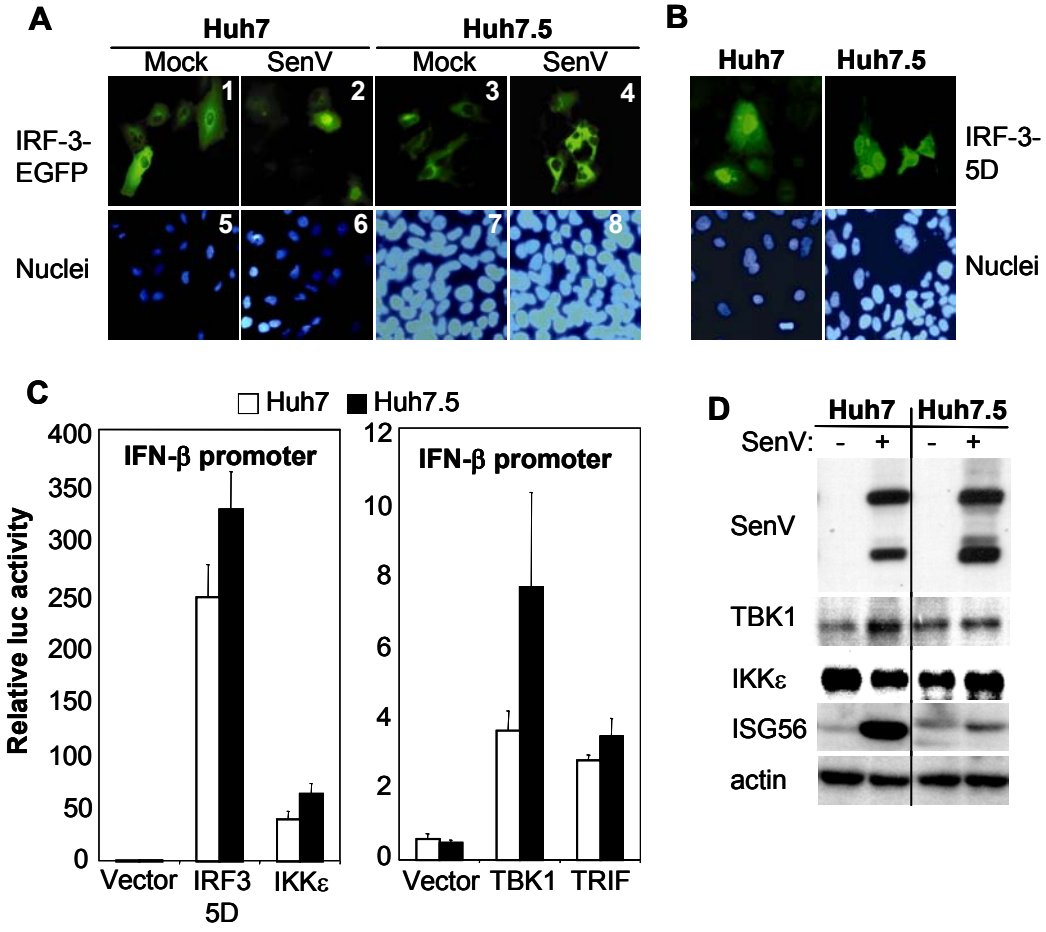
SUPPLEMENTAL FIG. S1



SUPPLEMENTAL FIG. S1. Analysis of IFN and virus-responsive signaling in Huh7 and Huh7.5 cells. (A) Huh7 cells were cotransfected with pISRE-luc and pCMV-*Renilla*

plasmids. 24 hr post transfection, medium was removed and replaced with fresh medium containing 0, 10 or 100 units/ml IFN-α2a. Cells were harvested 8 hr. post-IFN treatment and extracts were subjected to dual luciferase assay. Bars show the average firefly luciferase value and standard deviation relative to the *Renilla* luciferase control. No significant differences in IFN-induced ISRE promoter activation in response to exogenous IFN were observed between Huh7 and Huh7.5 cells. (B) Huh7 or Huh7.5 cells were either mock-infected (mock) for 12 hr or infected with VSV (MOI = 10) for 8 or 12 hr. 20 μg of total cellular protein were subjected to immunoblot analysis for evaluation of VSV, PKR phosphoserine 51 eIF2α (eIF2α-P) and actin abundance. PKR kinase assay was performed in parallel on 200 μg of protein from the corresponding extracts (kinase assay) and the panel shows the presence of active, autophosphorylated PKR. (C) Huh7 and Huh7.5 cells were treated with anisomycin (Ansio; 10 μg/ml for 15 min.), recombinant IL-1 (10 ng/ml for 30 min.) or were mock-infected for 12 hr (mock) or infected with VSV (MOI = 10) for 6, 8 or 10 hr. 20 μg total cellular protein were processed for immunoblot analysis to measure the abundance of VSV proteins, phospho-Thr180/Tyr182-p38 (p38-P), total p38 and β-actin. The data show that Huh7 and Huh7.5 cells have intact virus responsive PKR and p38 Map kinase signaling pathways.

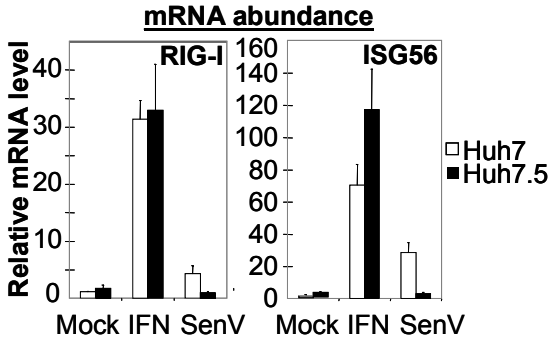
SUPPLEMENTAL FIG. S2



SUPPLEMENTAL FIG. S2. Localization of the IRF-3 signaling defect in Huh7.5 cells. (A) Huh7 and Huh7.5 cells were transfected with plasmids encoding either a C-terminal EGFP-tagged IRF-3 (IRF-3-EGFP) and allowed to recover for 24 hr. IRF-3-EGFP transfected cells were then mock-infected (mock) or infected with SenV for 24 hr, fixed, permeablized and nuclei were stained with DAPI. The subcellular localization of IRF-3-EGFP (upper panel set) and nuclei (lower panel set) were then assessed by direct

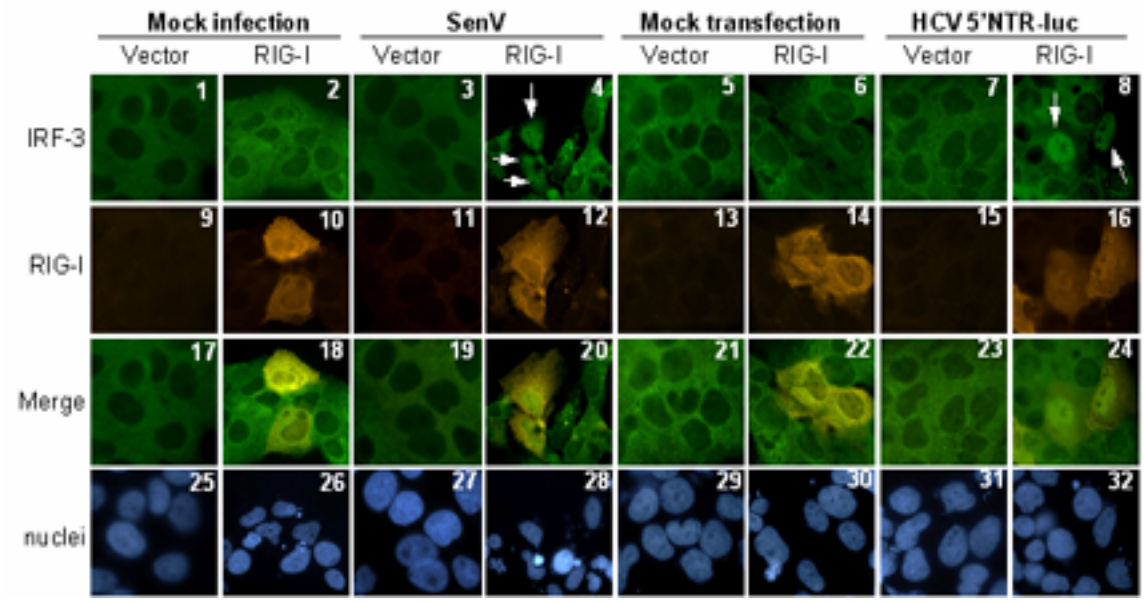
fluorescent microscopy using 40X magnification. Nuclear translocation of IRF-3-EGFP in response to SenV infection was observed in Huh7 but not in Huh7.5 cells. (B) Huh7 and Huh7.5 cells were transfected with a constitutively active phosphomimetic IRF-3 mutant (IRF-3 5D) and allowed to recover for 24 hr. Cells were processed for immunostaining and DAPI staining. IRF-3 5D (upper panels) was visualized with a rabbit polyclonal anti-IRF-3 serum followed a FITC-conjugated donkey anti-rabbit secondary antibody. Nuclei are shown in the lower panels. Magnification was 40X. IRF3 5D constitutively localized to the nucleus in both Huh7 and Huh7.5 cells. (C) Huh7 and Huh7.5 cells were cotransfected with pIFN-β luc and pCMV-*Renilla* plasmids and plasmids directing the expression of IRF-3 5D, IKKε, TBK1 or TRIF. Cells were harvested 24-hr later and extracts were subjected to dual luciferase assay. Bars show the average firefly luciferase value and standard deviation relative to the *Renilla* luciferase control. Activation of the IFN-β promoter in response to each stimulus was not significantly different between Huh7 and Huh7.5 cells. (D) Huh7 and Huh7.5 cells were mock-infected (-) or infected with SenV for 24 hr. 20 μg total cellular proteins were processed for immunoblot analysis using antisera specific for SenV proteins, TBK1, IKKε, ISG56 or β-actin. The levels of endogenous TBK1 and IKKε were similar in Huh7 and Huh7.5 cells but only Huh7.5 cells exhibited a defect in virus-induced ISG56 expression.

SUPPLEMENTAL FIG. S3



SUPPLEMENTAL FIG. S3. RIG-I and ISG56 mRNA abundance was quantified by real-time PCR of cDNA derived from cells that were mock-treated, treated with IFN-α2a (10 units/ml) for 20 hr or infected with SenV for 20 hr. Bars show average and SD RNA level relative to the GAPDH control from triplicate experiments. These results show that RIG-I is an ISG and is expressed at a low, basal level in human hepatocytes.

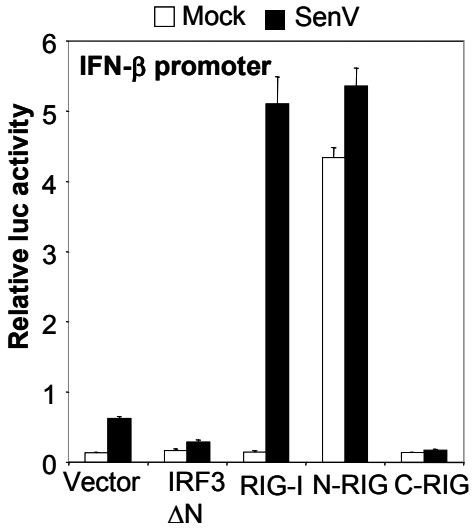
SUPPLEMENTAL FIG. S4.



SUPPLEMENTAL FIG. S4. RIG-I expression restores virus and HCV RNA-induced IRF-3 nuclear translocation in Huh7.5 cells. Huh7.5 cells, cultured on a glass slide, were transfected with vector control or a plasmid encoding full-length RIG-I. 24 hr post transfection the cells were mock-infected or infected with SenV for 24 hr, mock-transfected or transfected with in vitro transcribed HCV 5'NTR-luc RNA for 8 hr as indicated. Cells were then processed for dual immunofluorescence microscopy using rabbit polyclonal anti-IRF-3 serum and monoclonal anti-FLAG M2 antibody followed by FITC-conjugated donkey anti-rabbit and Rhodamine-conjugated donkey anti-mouse secondary antibodies to visualize IRF-3 and ectopic RIG-I. Panels 1-8 show IRF-3 staining. Panels 9-16 show staining of ectopic RIG-I. Panels 17-24 show a composite merged image of IRF-3 and ectopic RIG-I staining. Panels 25-32 show DAPI-stained

nuclei. White arrows point to the cells in which IRF-3 accumulated in the nucleus. Magnification was 40X. For Huh7.5 cells, nuclear accumulation of IRF-3 was restricted to only those cells that expressed ectopic RIG-I.

SUPPLEMENTAL FIG. S5



SUPPLEMENTAL FIG. S5. Regulation of the IFN-β promoter by distinct domains of RIG-I. Cultures of Huh7 cells were cotransfected with pIFN-β luc and pCMV-*Renilla* along with plasmids directing the expression of IRF-3 ΔN (dominant negative IRF3; a kind gift from Dr. J. Hiscott), RIG-I full length, N-RIG or C-RIG. 24 hr post transfection the cells were either mock infected or infected with SenV for 20 hr. Cells were harvested 24 hr later and subjected to the dual luciferase assay. Bars show the average firefly luciferase value and SD relative to the *Renilla* control for three experiments. Ectopic expression of full-length RIG-I results in increased IFN-β promoter activity upon virus infection. Expression of N-RIG (encoding the amino-terminal CARD homology domain) or C-RIG (encoding the helicase domain) directs the constitutive activation or dominant negative repression of the IFN-β promoter, respectively.