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Supplemental Information

Dynamic Oscillation of Translation and Stress Granule Formation Mark the Cellular Response to Virus Infection

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture

All cell lines were maintained in Dulbeccos's modified Eagle´s medium (DMEM) supplemented with 2 mM L-glutamine, 1x nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (all from GIBCO, Life Technologies). Huh7 YFP-TIA1 and Huh7 YFP-TIA1 PKR cell lines were derived from the hepatoma cell line Huh7 by retroviral transduction of the corresponding pWPI plasmids. Huh7.5 cells (Blight et al., 2002) were used for production of HCV. Huh7.5[CE1][E2p7NS2] (Steinmann et al., 2008) were additionally supplemented with 5µg/ml blasticidine and used for production of HCV_{TCP} particles used for live-cell imaging. Medium used to culture Huh7.5/RIG-I cells (Binder et al., 2007) was supplemented with 1mg/ml G418 and used for testing of poly(I:C) stability in the ISG56 promoter reporter assay.

Plasmids

The following plasmids were used and are described elsewhere: pFK-J6/C3(Jc1 wt) (Pietschmann et al., 2006) for HCV stock production; pFK JcR-2a (Reiss et al., 2011) for HCV Renilla reporter virus stock production; $pFKI_{389}$Luc-EI/NS3-3′_JFH1_δg encoding a bicistronic subgenomic JFH1 replicon (Luc-NS3-5B) (Steinmann et al., 2008) for characterization of cell line permissiveness.

$pFKI_{389}$neoNS3-3′δg_JFH-1_NS5A-aa2359_mCherry (Schaller et al., 2007) was modified by introduction of a conserved mutation (K1402Q) residing in NS3 (Kaul et al., 2007). This mutation did not affect RNA replication but enhanced infectivity titers of HCV_{TCP} up to 100-fold with peak titers in the range of $1x10^4$ $TCID_{50}$/ml (not shown). The adapted replicon, designated 'mCherry_Neo K1402Q replicon', was used for HCV_{TCP} stock production that were utilized for live-cell imaging. pWPI YFP-TIA1 Neo was generated by AgeI-EcoRI excision of the TIA-1 gene fused N-terminally to YFP, from pEYFP-TIA1 (kindly provoded by Dr. N. Kedersha, Brigham and Women's Hospital Boston) and insertion into the lentiviral transduction vector pWPI carrying a neomycin resistance gene. *EIF2AK2* (PKR) and *PP1R15A* (GADD34) genes were amplified from the ORFeome cDNA clone library (Invitrogen, Life Technologies) and inserted by gateway™ recombination into the gateway compatible vector pWPI Blr and pWPI Puro, respectively.

Retroviral Transduction and Generation of Stable Cell Lines

For production of lentiviral particles $1.2x10^6$ 293T cells were seeded into 6 cm-diameter dishes and transfected using the CalPhos mammalian transfection kit (Becton Dickinson) as recommended by the manufacturer. One hour prior to transfection, medium was replaced. For transfection, 6.4 µg packaging plasmid (pCMVΔ8.91), 6.4 µg transfer vector containing the respective gene of interest and an antibiotic resistance gene (pWPI-based) and 2.1 µg of the VSV envelope glycoprotein expression vector (pMD2.G) were mixed and diluted to a final volume of 438 µl in H_2O. Sixtytwo µl 2 M $CaCl_2$ and 500 µl 2x HBS buffer were added. The mixture was immediately added to the cell culture dish in a drop-wise fashion and the plate was gently swirled to evenly distribute the transfection mixture throughout the plate. After 8h, medium was replaced by 5 ml fresh medium. On the next day, $6.4x10^5$ target cells were seeded into a 10 cm-diameter dish. Twenty-four hours later (48h post transfection), supernatant containing lentiviral particles was harvested and replaced by another 4 ml fresh medium. Supernatant was filtered through a 0.45 µm-pore membrane prior to usage. Transduction of target cells with the lentiviral particles was repeated in three times every 12h to achieve high number of integrates and thus high expression levels. Transduced cell pools were subjected to selection with medium containing the respective antibiotic. Huh7 YFP-TIA1 transduced cells were selected for high expression by using FACS sort.

***In vitro* Transcription**

Ten µg of the respective pFK-based constructs were linearised by restriction with MluI and purified using the Nucleospin Extract II kit (Macherey-Nagel). *In vitro* transcription reaction mixtures contained 80 mM HEPES (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol (DTT), 3.125 mM of each nucleoside triphosphate, 1 U of RNasin (Promega), 0.1 µg plasmid DNA, and 0.6 U of T7 RNA polymerase (Promega) per µl reaction. After incubation for 2h at 37°C, 0.3 U of T7 RNA polymerase per µl reaction mixture was added prior to overnight incubation at 37°C. Transcription was terminated by addition of 1.2 U of RNase-free DNase (Promega) per µg plasmid DNA and 30 min incubation at 37°C. RNA was extracted with acidic phenol and chloroform, precipitated with isopropanol, and dissolved in RNase-free water. RNA integrity was determined by using denaturing agarose gel electrophoresis and concentration was determined by measuring optical density at 260 nm.

Virus Production

Single-cell suspensions of Huh7.5 cells were prepared by trypsinization and washed once with phosphate-buffered saline (PBS). Cells were resuspended at a concentration of 1×10^7 cells per ml in Cytomix (van den Hoff et al., 1995) containing 2 mM ATP and 5 mM glutathione. Ten µg of *in vitro* transcribed RNA was mixed with 400 µl of the cell suspension and transfected by electroporation with a Gene Pulser system (Bio-Rad) in a cuvette with a gap width of 0.4 cm (Bio-Rad) at 975 µF and 270 V. Cells of two transfections were immediately transferred into medium and seeded into a 15 cm-diameter culture dish. Supernatants were collected 24, 48, 72 and 96h post electroporation, filtered through a 0.45 µm-pore membrane and virus particles were concentrated by precipitation with polyethylene glycol (PEG)-8000 in PBS (8% w/v) for 72h at 4°C and 2h centrifugation at 8,000 x g. The pellet was resuspended in medium and stored at -70°C. Infectivity titers of virus stocks were determined by limiting dilution assay using Huh7.5 cells as described elsewhere (Lindenbach et al., 2005).

HCV_{TCP} Production and Purification for Live-Cell Imaging

HCV_{TCP} particles were produced by electroporation of Huh7.5[CE1][E2p7NS2] cells with 10µg mCherry Neo K1402Q replicon RNA generated by *in vitro* transcription as described above. Supernatants were collected 24, 48, 72 and 96h post electroporation, filtered through a 0.45 µm-pore membrane and concentrated by ultrafiltration using a centrifugal filter device (Centricon Plus-70, Millipore) to achieve an around 50-fold volume reduction. Concentrated culture supernatant was loaded on top of a two layer Optiprep cushion (Axis Shield; 6 ml 10% and 3 ml 80%) and centrifuged using a SW28 rotor (Beckman Coulter) at 96,281 x g for 20h at 4°C. Six milliliters, including the 80% cushion, were collected from the bottom of the tube, diluted 1:2 with FCS-free DMEM and concentrated via Amicon Ultra-15 centrifugal filter devices (MWCO 100k, Millipore) at 4,000 x g for 45 min. Concentrated virus suspension was aliquoted and stored at -70°C. Infectivity titers of virus stocks were determined by limiting dilution assay using Huh7.5 cells.

Data Acquisition, Image Analysis, and Statistical Population Analysis

Movies were created from time series maximum intensity Z-projections of 16 to 20 consecutive optical sections spaced by 1.05 to 1.09 µm, acquired for 72h at 1h intervals. A semi-automatic approach was developed for the analysis of single cell and SG dynamics. First, maximum intensity projections along the z-axis were computed for the YFP channel and used for manual annotation of the cell to be quantified. A plugin for the ImageJ software package (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland,

USA; http://rsb.info.nih.gov/ij/) was developed, which provided the possibility to label and track each cell over time with a unique cell ID (corresponding to the mother cell or the sister cells after mother cell division), coordinates of the position of the cell nucleus, infection status (yes/no), and presence of SGs (yes/no). Automatic image analysis was performed by using MATLAB as follows: first, segmentation of the nuclei for all manually annotated cells by using a previously described algorithm (Matula et al., 2009) which was extended to make use of the manually provided cell nuclei positions; second, quantification of the fluorescence intensity of the NS5A-mCherry signal by computing the sum of intensities projected along the z-axis for the mCherry channel; third, definition of a ring around the nucleus of each annotated cell (diameter of 10 pixels) and quantification of the fluorescence intensity within the ring. For each annotated cell, a feature table was created that contained the following statistical information: (i) the total time of presence in the field of view to correct for cells moving out of the field of view or cells that die during the 72h observation period; (ii) the total stress time as determined by detection of SGs; (iii) the number of stress peaks, with a peak being defined as a continuous period of stress preceded or followed by at least one time point without stress; (iv) the SG oscillation frequency, which corresponded to the ratio between the number of stress peaks and the total observation time, multiplied by 24 to obtain the frequency per 24h; (v) the SG oscillation interval or mean of stress peak duration; (vi) the number of generations; (vii) the infection status (yes/no); (viii) "Infection level" corresponding to the sum of the NS5A-mCherry signal per cell in the first time series; and (ix) cell death (yes/no).

Statistical Significance

Statistical analysis was performed by using the GraphPad Prism software (GraphPad). Statistical significance was calculated by performing a two-tailed Student's t test (***, $p < 0.0001$; **, $p < 0.001$; *, $p < 0.01$).

Characterization of PB Docking to SGs and SG Volume

Confocal Z-stacks of YFP-TIA1 (514 nm excitation) and Hedls (561 nm excitation) signals were acquired using a 63x Plan-Apochromat N.A. 1.4 oil immersion objective (Leica) for magnification with a 4x zooming factor, a digital image resolution of 1024 x 1024 and a scanning speed frequency of 100 Hz. Z-stacks of 35 to 45 consecutive optical sections spaced by 0.13µm were acquired. A semi-automatic approach was developed for the analysis of SG volume and PB docking. Image analysis was performed as follows: first, YFP-TIA1 channel

was first filtered using 3D isotropic Gaussian filter (sigma = 0.15 microns), and the background subtracted slice by slice using the 'rolling ball' algorithm implemented in ImageJ's 'Subtract Background' command. Second, Hedls channel was preprocessed using a 3D Difference-of-Gaussians filter (sigma = 0.15 and 0.24 microns) to help separate the peaks of individual PB from surrounding regions. Both channels were then thresholded, where the thresholds were chosen manually in an effort to isolate neighbouring structures while preserving accurate volumes. The thresholded images were cleaned using 3D morphological opening. Objects in each image were finally detected and analyzed using a '3D Objects Counter' originally developed by Bolte and Cordelières (Bolte and Cordelieres, 2006). The centroid of each detected PB was located, and considered to be docking if falling within a SG detected in the corresponding channel. Apart from thresholding, all other steps were automated in Fiji (http://fiji.sc/wiki/index.php/Fiji) using a Beanshell script.

For 3D images, deconvolution was calculated by using the Huygens Suite software (Scientific Volume Imaging) applying 40 iteration cycles and assuming a signal-to-noise ratio between 10 and 15, and finally visualized using the surface renderer.

Immunofluorescence Microscopy and Antibodies

For immunostaining cells were fixed for 15 min with 4% (w/v) paraformaldehyde in PBS, permeabilized by 5 min-treatment with PBS containing 0.5% Triton X-100 and incubated in blocking buffer (5% goat serum, 5% sucrose in PBS) for 30 min. Cells were sequentially incubated with primary and secondary antibodies diluted in blocking buffer. For SG-specific stainings, cells were further permeabilized with 100% ice-cold methanol for 10 min at -20°C prior to treatment as described above. Cells were stained with goat polyclonal anti-eIF3B (Santa Cruz; 1:500), rabbit polyclonal anti-eIF4G (Santa Cruz; 1:500), mouse monoclonal anti-HuR (Santa Cruz; 1:500), mouse monoclonal anti-PCBP2 (Abnova; 1:500), goat polyclonal anti-TIA-1 (Santa Cruz, 1:500), mouse monoclonal anti-G3BP-1 (Santa Cruz; 1:1,000), anti-ADAR-1 (k88#2). Mouse monoclonal anti-S6kinase/p70 (Santa Cruz; 1:1,000) cross-reacting with Hedls was used for detection of PBs. Coverslips were mounted by using Prolong Gold Antifade Reagent (Molecular Probes, Life Technologies).

HCV proteins were detected by using the following antisera: rabbit polyclonal anti-core (C830 (Koch and Bartenschlager, 1999); 1:200), rabbit polyclonal anti-E2 (J6E2 (Owsianka et al., 2001); 1:200), rabbit polyclonal anti-NS3 (N4H3 (Bartenschlager et al., 1994); 1:400), rabbit polyclonal anti-NS4B (#86 (Paul et al., 2011); 1:400), mouse monoclonal anti-NS5A

(9E10 (Lindenbach et al., 2005); 1:2,000, kindly provided by Dr. C.M. Rice), sheep polyclonal anti-NS5A (1:400, kindly provided by Dr. M. Harris).

Images were taken with an Olympus IX70 microscope or a Nikon Ti Eclipse microscope and fluorescence signals were quantified by using the analySIS 3.1 or the NIS-Element AR software package, respectively. Confocal pictures were acquired with a Leica TCS SP5 microscope, analyzed with the LAS AF program and processed by using the ImageJ software package, respectively.

In situ Hybridization and Detection of polyA mRNAs

For *in situ* hybridization cells were fixed for 15 min with 4% (w/v) paraformaldehyde in PBS, permeabilized with 100% ice-cold methanol for 10 min at -20°C prior to treatment. All following steps were performed at room temperature. Cells were washed three times with 2x SSC buffer for 10 min and hybridized for 1h with 0.02 nM Alexa555-oligo-dT50 (MWG Biotech) in hybridization buffer pH 7.0 (0.1mg/ml yeast RNA, 0.4x SSC, 20% Formamide, 0.2%BSA, 10% dextrane sulfate). Cells were washed three times for 10 min with 2x SSC and coverslips were mounted by using Prolong Gold Antifade Reagent (Molecular Probes, Life Technologies). When combined with immunofluorescence staining, *in situ* hybridization was perfomed following the secondary antibody incubation step.

Cell Line Characterization and Luciferase Assay

Quantification of luciferase reporter activity was used to determine transient HCV RNA replication as described previously (Steinmann et al., 2008). In brief, $4x10^6$ Huh7 YFP-TIA1 cells were electroporated with 5µg *in vitro* transcript of the subgenomic JFH1 replicon (Luc-NS3-5B) and resuspended in 20 ml medium. Two milliliters of the cell suspension were seeded into each well of a 6-well plate in duplicate, and lysed 4, 24, 48, 72 and 96h after electroporation by addition of lysis buffer (0.1% Triton X-100, 25 mM glycyl-glycine, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT, pH 7.8). For detection of Firefly luciferase activity, 100 µl lysate was mixed with 360 µl assay buffer (25 mM glycyl-glycine, 15 mM $MgSO_4$, 4 mM EGTA, and 15 mM potassium phosphate pH 7.8) containing 1 mM DTT, 2 mM ATP and 70 µM D-luciferin (P.J.K). For detection of Renilla luciferase activity, 20 µl lysate was mixed with 100 µl assay buffer containing 1.4 µM coelenterazine (P.J.K). All measurements were done in duplicate by using a tube luminometer (Berthold). Replication efficiency was determined by normalization to the 4h values reflecting transfection efficiency.

Infectivity Assays

To test the permissiveness of different cell lines a HCV full-length reporter virus harboring a Renilla luciferase gene was used (Reiss et al., 2011). Into each well of a 24-well plate, $5x10^4$ Huh7 cells were seeded and 24h later infected for 4h at an MOI of 0.5 $TCID_{50}$ per cell. Twenty four, 48, 72 and 96h post infection, cells were harvested and Renilla luciferase activities were determined as described above.

RNAi-Based Replication Rescue Screen

Gene silencing in Huh7 cells was achieved by two consecutive transfections. For each siRNA, $3x10^5$ cells were seeded into each well of a 6 cm-diameter dish and allowed to attach for 1h at 37°C. siRNAs (10nM) were transfected by using the Hiperfect transfection reagent (Qiagen) according to the instructions of the manufacturer. Seventy-two hours post transfection, cells were trypsinized, and $6x10^4$ cells were seeded into a total of 6 wells of a 24-well plate. Cells were allowed to attach for one hour at 37°C and transfected with siRNA in the same way. Eight hours post silencing, cells were infected with the HCV Renilla reporter virus at an MOI of 0.5 $TCID_{50}$ per cell for 24h and subsequently 3 wells were left untreated and 3 wells were treated with 25 IU/ml IFN-α for 60h. Luciferase activities were measured in the plate by adding 400 µl Renilla luciferase assay buffer to each well prior to measurement in a Berthold plate luminometer. Five independent repetitions of the RNAi-based screen were performed. Raw data were subjected to statistical analysis.

Statistical Analysis of RNAi-Based Screen

In case of IFN-α treatment two datasets, absolute values and ratio, were considered for statistical evaluation. They were generated as follows: (i) the median of 9 values obtained by measurement of empty wells (reflecting the background of the assay) was subtracted from all other values measured in the screen. (ii) Values of IFN-α treated cells were divided by the values obtained with untreated cells in order to calculate HCV replication ratio. (iii) All datasets were log-transformed. (iv) Values were normalized to the negative control (non targeting siRNA) by subtracting the median value of the negative control from each measurement and dividing by the median absolute deviation of the negative control. Replicates were summarized by using the mean Z-score per siRNA. (v) Hit candidate selection was performed with a one-sample Wilcoxon rank-sum test with the null hypothesis "mean of distribution of siRNA is smaller or equal to zero". A value was considered as significant in case of a p-value <0.05 and a Z-score >2 for each dataset. In case of IFN-α

treatment, a perturbation was defined as a hit when fulfilling the defined criteria for both absolute values and the corresponding ratio.

siRNA Cytotoxicity Measurement

siRNA transfected cells were seeded into 96-well plates and 96h later cells were incubated with 10 µl per well of the cell proliferation reagent WST1 as recommended by the manufacturer (Roche Applied Science). After 4h incubation, fluorescence was measured at 440 nm with a reference wavelength >600 nm using a Tecan microplate reader.

Quantification of Gene Silencing and HCV RNA by qRT-PCR

Total RNA was extracted by using NucleoSpin RNA II (Macherey-Nagel) according to the manufacturer's instructions. mRNA levels were determined by using the 2x green DYE Master Mix (P.J.K). GAPDH mRNA was used for normalization of input RNA. RT-PCR data were analyzed by using the $\Delta\Delta C_T$ method described previously (Livak and Schmittgen, 2001). The following primers were used: STAT2 forward primer (for) 5′-GCCTGGACTTAGAGCCACTG-3′, reverse primer (rev) 5′-AGATCACAGGGCAAATCTGG-3′; ADAR1-for 5′-GTCAACTGGTGTCTGGCTGA-3′, -rev 5′-TAGTCACGGGCAGCTTTCTT-3′; G3BP1-for 5′-ACCTGCCTCATGAAGTGGAC -3′, -rev 5′-ACATTCAGACGGACCTCACC-3′; HuR-for 5′-CCGAATTTGTGAACCTGCTT-3′, -rev 5′-AGGATATTCACGCAGGATGG-3′; TIA-1-for 5′-CATGGAACCAGCAAGGATTT-3′, -rev 5′-CACTCCCTGTAGCCTCAAGC-3′; TIAR-for 5′-TATACGGGCAACCATGGAAT-3′, -rev 5′-TTAGAGTCCCGGCTCACTGT-3′; Caf1a/b-for 5′-GGTCATTTGTATGGCCTTGG-3′, -rev 5′-GTTCGAGGGATTCAACCAGA-3′; CNOT1-for 5′-CGAGCCAAGTGCTATCACAA-3′, -rev 5′-TTCGATGGTAGGGAAGTTGC-3′; GW182-for 5′-CCAGAATTTCGTCCTGGTG-3′, -rev 5′-AGGATGAGGATGACCCACTG-3′; Lsm1-for 5′-AACAAAGGGTGGAACAGCAG-3′, -rev 5′-GTGATCAAATGCGTGAGGTG-3′; Lsm4-for 5′-ACGGATCTGCTCAGAAAGGA-3′, -rev 5′-TAGGAATCCAGCCAGCTTTG-3′; Pat1a-for 5′-TTCTGAGGAGGAGCTGGTGT-3′, -rev 5′-TGGACAGCACCAAGTACAGC-3′; Pat1b-for 5′-GATGATCGGGACCTTTCTGA-3′, -rev 5′-CAGGATAACGAGGTGGCATT-3′; Upf1-for 5′-AGGTACCGACAGTCCCTGTG-3′, -rev 5′-CAATGACACCACACCCTCAG-3′; Xrn1-for 5′-CGAGGCACCATCATAGGAAT-3′, -rev 5′-AGAGCGACTCCCATGAGAAA-3′; CPEB1-for 5′-CCAGAAGAACCGAGATTCCA-3′, -rev 5′-TCAGCAAGTGCAAAGGTGAC-3′; Rck1-for 5′-ACAGAAAGCTGCTTGCCATT-3′, -rev 5′-CTCACTCCTTTTGCCTGGAG-3′; Ago2-for 5′-ATGGACATCCCCAAAATTGA-3′, -rev 5′-AGGGGCATGGCTGTGTATAG-3′; PCBP2-for 5′-AGGCAGGTTACCATCACTGG-3′, -rev 5′-CTGGAATCGCTGACTGTTCA-3′; GADD34-for 5′-

CAGAAACCCCTACTCATGATCC-3′, -rev 5′-AAATGGACAGTGACCTTCTCG-3′; PKR-for 5′-GCCGCTAAACTTGCATATCTTCA-3′, -rev 5′-TCACACGTAGTAGCAAAAGAACC-3′; ISG56-for 5′-GAAGCAGGCAATCACAGAAA-3′, -rev 5′-TGAAACCGACCATAGTGGAA-3′; IFN-α-for 5′-CGCCGCATTGACCATCTA-3′, -rev 5′-GACATTAGCCAGGAGGTTCTC-3′; GAPDH-for 5′-GAAGGTGAAGGTCGGAGTC-3′, -rev 5′-GAAGATGGTGATGGGATTTC-3′.

Viral RNA was quantified by qRT-PCR using the One-step RT-PCR kit (Qiagen) as described elsewhere (Kaul et al., 2007). In brief, 15 µl of reaction mixture contained 0.6 µl enzyme mixture, 1.5 mM of $MgCl_2$, 1.3 µM of each HCV-specific primer (for 5′-TCTGCGGAACCGGTGAGTA-3′; rev 5′-GGGCATAGAGTGGGTTTATCCA-3′), 0.67 mM of each dNTP, 0.27 µM of HCV-specific probe, 5 µl of template RNA and RNase-free water. To determine absolute RNA amounts, a serial dilution of an RNA standard (10^2 to 10^7 HCV RNA copies per reaction) was processed in parallel. Reactions were performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems) using the following program: 50°C for 30 min, 95°C for 15 min and 40 cycles as follows: 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s.

Metabolic Labeling of Proteins and Quantification of Fluorescence Intensities

De novo synthesized proteins were quantified by using the Click-iT Metabolic Labeling Reagent for Protein Kit (Molecular Probes, Life Technologies) and a modified protocol adapted from Dieterich and colleagues (Dieterich et al., 2010). In brief, $1x10^5$ Huh7 cells were infected with HCV at an MOI of 5 $TCID_{50}$ per cell for 24h and subsequently treated with 100 IU/ml IFN-α to induce SGs. Cells were washed and incubated 1h at 37°C in methione/cysteine-free DMEM (Gibco, Life Technologies) in presence or absence of 200 µg/ml cycloheximide (CHX, Sigma-Aldrich) to deplete methionine pools. Click-iT metabolic labeling reagent L-azidohomoalanine (AHA) was diluted into methione/cysteine-free DMEM to a final concentration of 500 µM in presence or absence of 200 µg/ml CHX and added to the cells for 2h at 37°C. Cells were washed twice with ice-cold PBS, fixed with 4% paraformaldehyde containing 4% sucrose in PBS for 15 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 5 min. For the detection reaction 2 µM of the corresponding alkyne-tagged Alexa Fluor 594 molecule was added to the cells that were treated with the Click-iT Cell Reaction Buffer Kit (Molecular Probes, Life Technologies). The reaction was performed overnight at room temperature. Cell were further stained for eIF3B (Alexa Fluor 488) and coverslips were mounted as described above.

Fluorescence intensities were quantified by using the ImageJ software package. In brief, a region of interest (ROI) as determined by the eIF3B (green)-specific signal was fixed. Intensity of the AHA-594 (red)-specific signal was measured for each ROI.

Western Blot Analysis

For western blot analysis cells seeded into 6-well plates were washed once with PBS and lysed with 100 µl ice cold NP40 lysis buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 15 mM $MgCl_2$, 1% NP40) supplemented with protease inhibitor cocktail ('Complete Mini EDTA free', Roche) and phosphatase inhibitors (0.5 mM NaV, 20 mM NaF, 15 mM 4-NPP). Samples were boiled in 4x Laemmli sample buffer, separated by SDS-PAGE and transferred to a PVDF membrane. It was blocked by overnight incubation at 4°C in Tris-buffered saline containing 0.01% Tween 20 (Sigma) and 5% skim milk or 5% BSA. Immunostaining was performed in the same buffer using appropriate first and secondary antibodies. Proteis were detected by using the ECL Plus Western Blotting Detection System (Pierce, GE Healthcare) according to the instructions of the manufacturer. The following primary antibodies and corresponding blocking buffers were used: rabbit polyclonal anti-phospho-eIF2α (Cell signaling; BSA; 1:500), mouse monoclonal anti-eIF2α (Abcam; BSA; 1:500), rabbit polyclonal anti-PKR (Santa Cruz; milk; 1:500), rabbit polyclonal anti-phospho-PKR (Epitomics; BSA; 1:500), mouse monoclonal anti-NS5A (9E10; milk; 1:2,000), mouse monoclonal anti-GAPDH (Santa Cruz; milk; 1:5,000).

Culture of Primary Human Hepatocytes (PHHs)

PHHs were purchased from Biopredic (Rennes, France). Cells derived from three different donors were seeded into 12-well plates at a density of $7.6x10^5$ per well and cultured in Williams E medium containing Glutamax-I, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml bovine insulin and 50 µM hydrocortisone hemisuccinate. Cells were infected or not with HCV at a MOI of 5 $TCID_{50}$ per cell for 6 h prior to medium removal and extensive washes. Cells were harvested for RNA extraction at time points specified in the main text.

Guanabenz Cytotoxicity Assay

For each time point of the 10h-time course, Huh7 YFP-TIA1 cells ($8x10^3$) were seeded in triplicate wells into 96-well plates. Five, 10, 25, 50, 75 and 100 µM Guanabenz was added to the cells for a 0, 2, 6 or 10h-incubation at 37°C. The CytoTox-ONE Assay was used to determine viability of treated cells by measuring release of total lactate dehydrogenase as

recommended by the manufacturer (Promega). Fluorescence was measured at 560 nm using a Tecan microplate reader.

Poly(I:C) Transfection

Huh7 cells ($3x10^5$) were transfected with 4 μg of the dsRNA analogue poly(I:C) (Sigma-Aldrich) by using the Lipofectamine 2000 transfection reagent (Invitrogen, Life Technologies) according to the manufacturer´s instructions. Three hours post transfection, cells were washed and fresh DMEM or microscopy medium was added. For dsRNA stability assay, total RNA of poly(I:C)-transfected cells was extracted 0, 12, 24, 48 and 72h post transfection by using the TRIzol reagent (Invitrogen, Life Technologies) according to the instructions of the manufacturer.

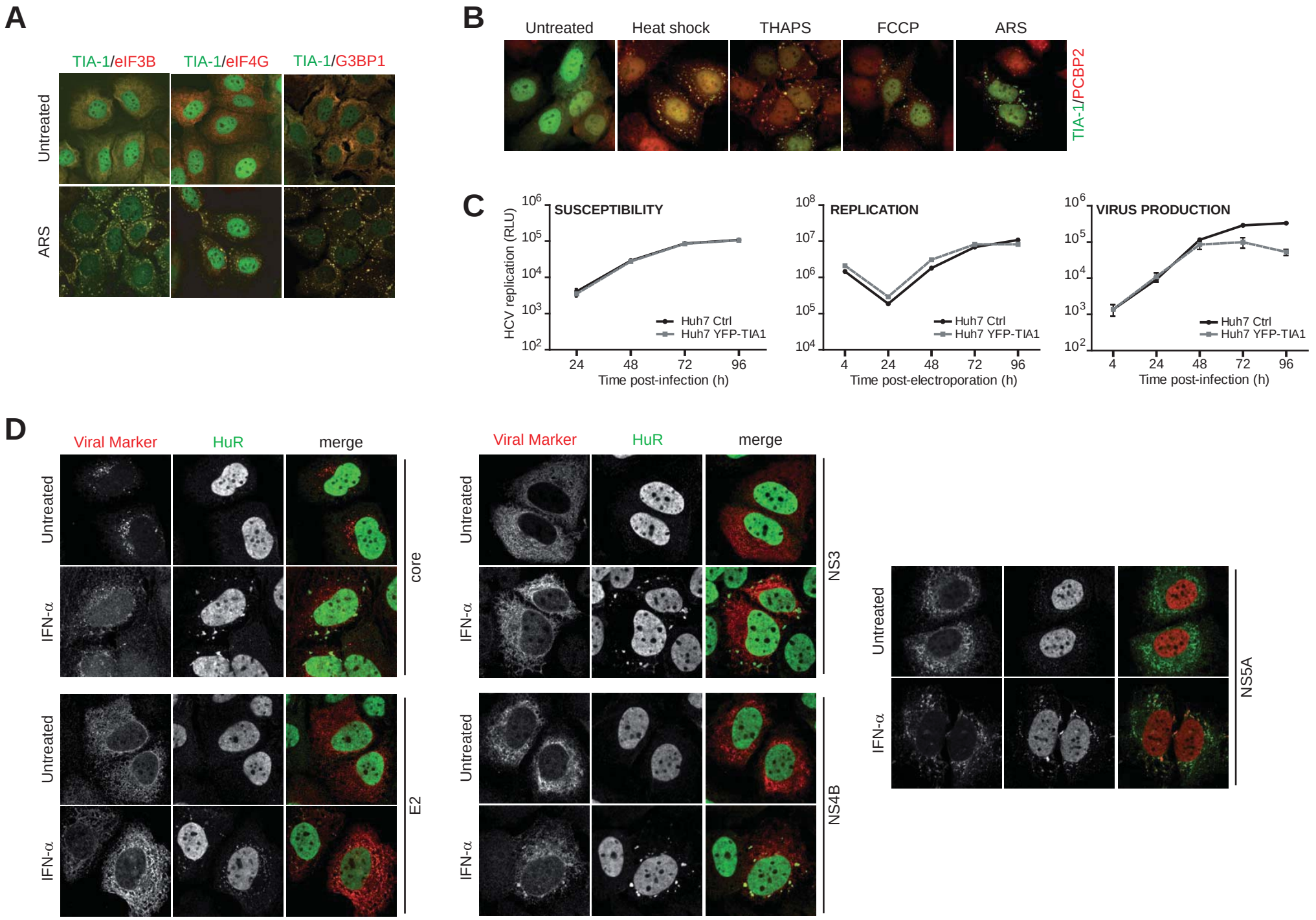
dsRNA Stability and ISG56 Promoter Reporter Assay

The ISG56 promoter reporter assay has been reported previously (Binder et al., 2011). In brief, $2x10^5$ Huh7.5/RIG-I cells were co-transfected with the ISG56 promoter-Firefly luciferase construct pGL3B/561 (kindly provided by Dr. G. Sen, Lerner Research Institute Cleaveland) and the Renilla luciferase plasmid pRL-SV40 (Promega) at a ratio of 3:1 by using the Effectene transfection reagent (Qiagen) according to the manufacturer´s instructions. Ten hours later cells were stimulated by transfection of 1μg TRIzol-purified total RNA extracted from poly(I:C)-transfected Huh7 cells (see above) by using the Lipofectamine 2000 transfection reagent (Invitrogen, Life Technologies). All experiments were performed in triplicate wells. Twelve hours post stimulation, cells were lysed and Firefly and Renilla luciferase activities were measured as described above. Values were corrected for transfection efficiency by normalizing Firefly to Renilla luciferase values. All values were normalized to time point T=0 to calculate ISG56 promoter induction.

Infection with RNA Viruses

Sendai virus (SeV) and NewCastle Disease virus (New Jersey, NDV) were a kind gift of Dr. R. Zawatzki (DKFZ, Heidelberg) and had a HA titer of 1:3200 and 1:1200, respectively. Huh7 or Huh7 YFP-TIA1 cells (3×10^5) were infected with SeV or NDV both at a dilution of 1:25 in PBS containing 0.3% BSA on a tumbler at room temperature. One hour post infection, cells were washed and fresh DMEM or microscopy medium was added. For SG characterization, cells were fixed 18 h after infection and stained for SG markers.

Encephalomyocarditis virus (EMCV) and Semliki Forest virus (SFV) were a kind gift of Dr. G. Kochs (University of Freiburg). DENV New Guinea C strain was grown on BHK-21 and purified on a 20% sucrose cushion. Huh7 or Huh7 YFP-TIA1 cells ($3x10^5$) were infected with an MOI of 5 and 10 $TCID_{50}$ for GADD34 induction experiments and live imaging, respectively.



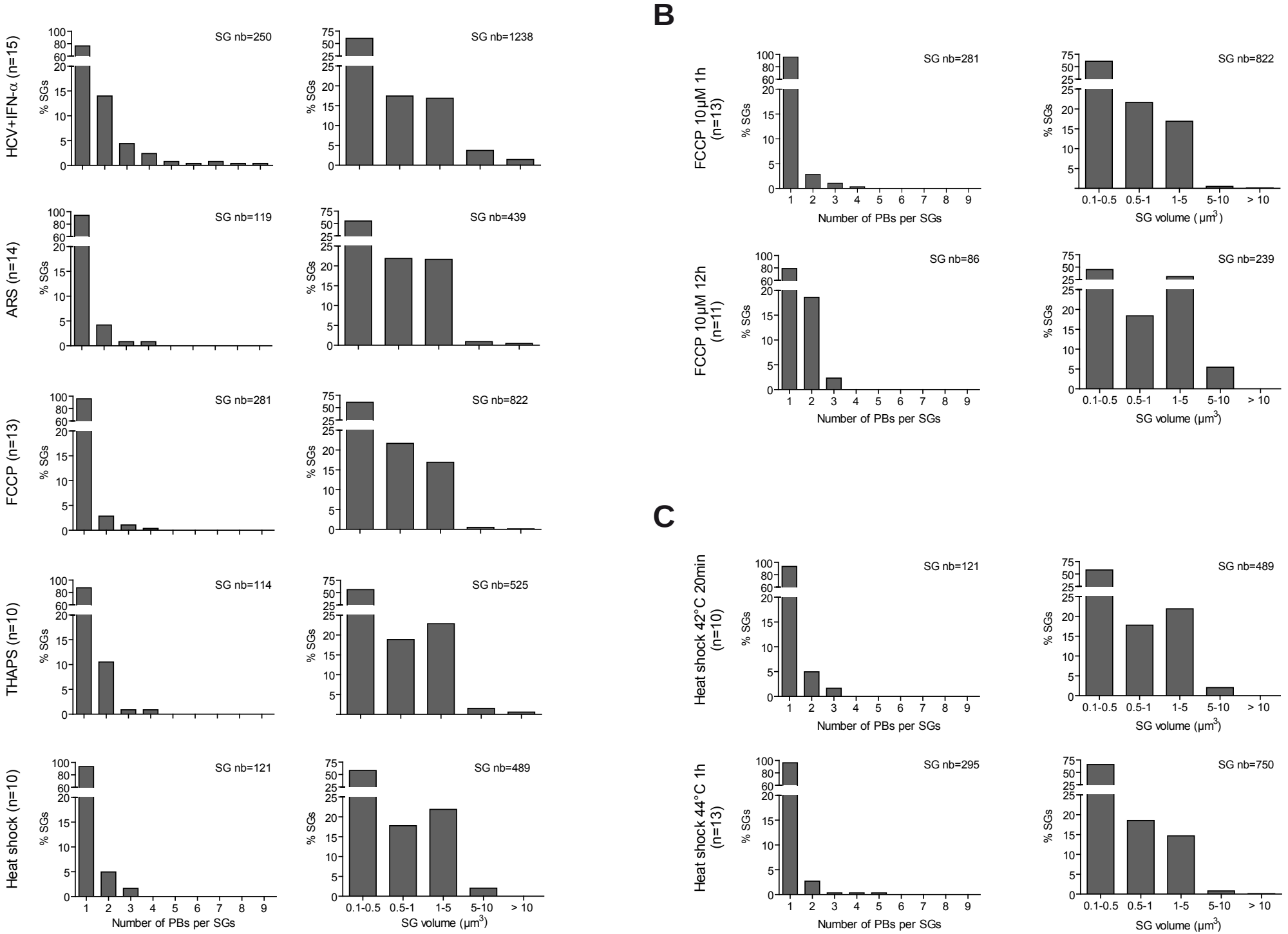
Supplemental Figure S1

Figure S1, Related to Figure 1. Characterization of the Huh7 YFP-TIA1 Cell Line

(A) Cells were subjected to oxidative stress by 1h treatment with 0.5μM Na-arsenite (ARS), and stained for SG markers eIF3B, eIF4G and G3BP1 (red). Shown are merges of representative fluorescence micrographs.

(B) Induction of SGs by various stresses. Cells were subjected to different stress conditions by using the following treatments: 1h with 0.5 μM Na-arsenite (ARS, oxidative stress); 1h in glucose-free DMEM containing 10 μM FCCP (FCCP; metabolic stress); 1h with 10 μM thapsigargin (THAPS; ER stress); 20 min at 42°C (Heat shock). Cells were fixed and stained for the SG marker PCBP2 (red). Shown are merges of representative fluorescence micrographs. (C) Ectopic expression of YFP-TIA1 does not affect permissiveness for HCV. Susceptibility of Huh7 YFP-TIA1 cells for HCV as compared to the parental Huh7 cells (Ctrl) was determined by infecting cells with the HCV Renilla reporter virus at a MOI of 0.5 $TCID_{50}$ per cell. Cells were lysed at time points specified in the bottom and Renilla luciferase activities were determined (Relative Light Units, RLU). To assess RNA replication cells were transfected with *in vitro* transcripts of the same HCV Renilla reporter virus, lysed 4, 24, 48, 72 and 96h later and luciferase activities were determined. To assess efficiency of virus production in Huh7 YFP-TIA1 cells they were transfected in the same way, supernatants were collected 4, 24, 48, 72 and 96h after transfection and used to infect naive Huh7.5 cells for 72h. Cells were lysed 4, 24, 48, 72 and 96h post infection and Renilla luciferase activity was determined (Relative Light Units, RLU).

(D) HCV proteins do not localize to SGs to a detectable extent. Huh7 cells stably expressing YFP-TIA1 (green) were infected with HCV (5 $TCID_{50}$ per cell) for 24h and subsequently treated or not with 100 IU/ml IFN-α. Cells were fixed 18h post-IFN-α addition and stained for HCV core protein, the E2 envelope protein, non-structural proteins NS3, NS4B and NS5A (red) and for the SG marker HuR (green). Shown are representative confocal micrographs.

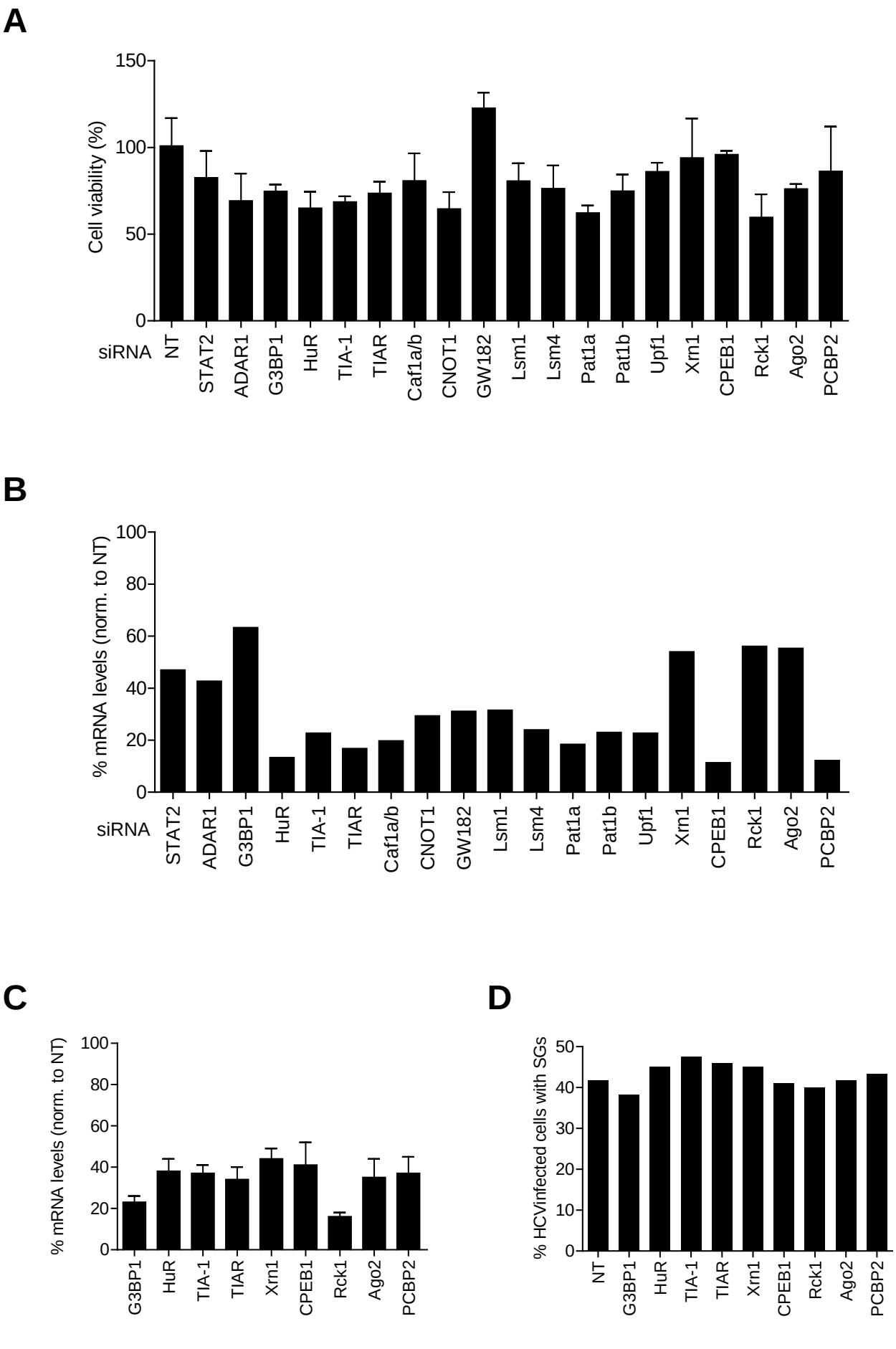


Supplemental Figure S2

Figure S2, Related to Figure 1. Characterization of SG size and PB Association to SGs

(A) Quantification of PB association to HCV-induced SGs in cells treated with 100 IU/ml IFN-α for 18h and comparison to SGs induced by the following stresses: 1h treatment with 0.5µM Na-arsenite (ARS; oxidative stress); 1h treatment in glucose-free DMEM containing 10µM FCCP (metabolic stress); 1h treatment with 10µM thapsigargin (THAPS; ER stress) and 20 min treatment at 42°C (Heat shock). Huh7 cells stably expressing YFP-TIA1 were fixed and immunostained with the PB marker Hedls. Z-stack sections were acquired with a confocal microscope and analyzed using a semi-automated PB docking detection tool (see material and method section). n is the number of cells analyzed for each condition. A quantification of PBs docking per SGs is shown in the histograms of the left panel (SGs free of PBs docking were excluded from the analysis). Histograms representing SG volume are shown in the middle panel. SGs (with or without PB docking) were grouped by size into 5 categories: 0.1 to 0.5 μm^3, 0.5 to 1 μm^3, 1 to 5 μm^3, 5 to 10 μm^3 and >10 μm^3 (SGs with a volume <0.1 μm^3 were excluded).

(B and C) Modification of stress conditions and effect on SG volume and PB docking. (B) Treatment with 10µM FCCP in glucose-free DMEM for 1h (upper panel) or 12h (lower panel). (C) Heat shock for 20 min at 42°C (upper panel) or 1h at 44°C (lower panel).

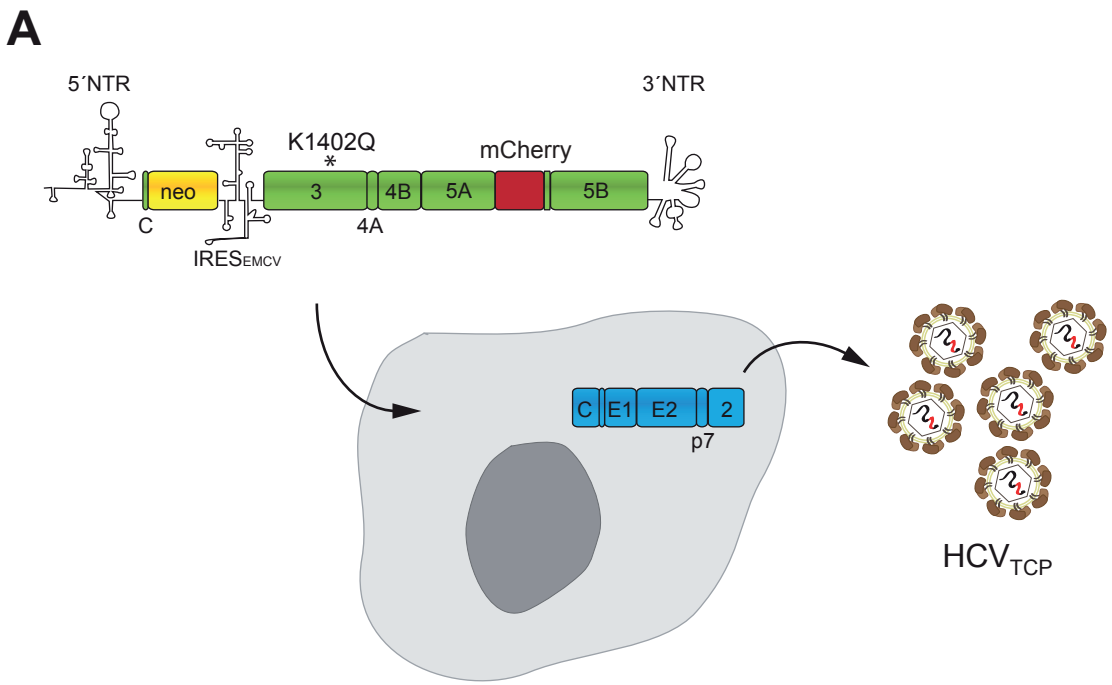


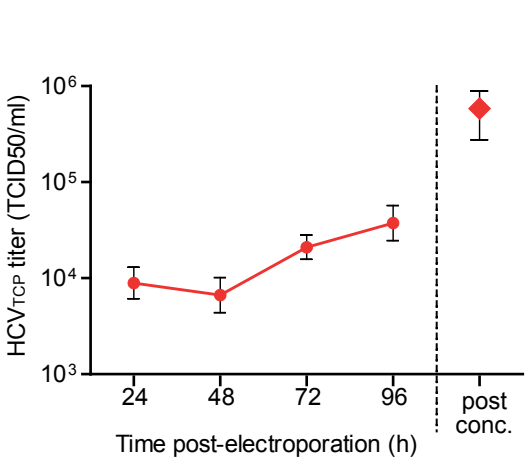
Supplemental Figure S3

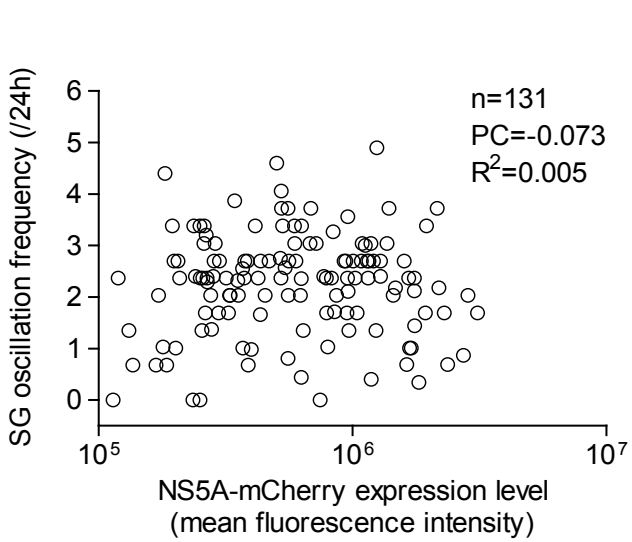
Figure S3, Related to Figure 2. RNAi-Based Screen

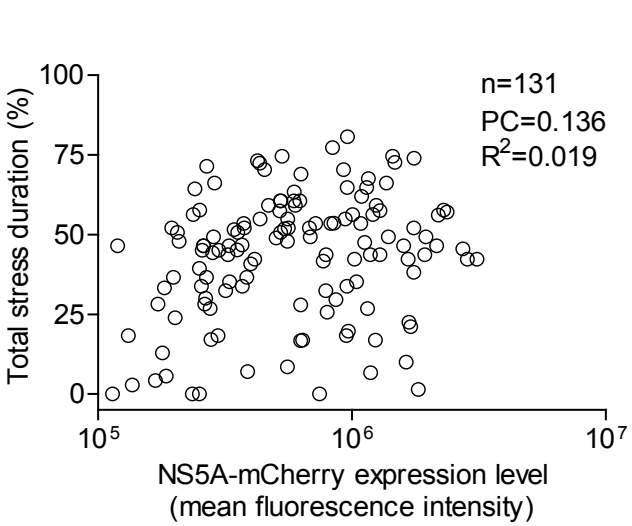
(A and B) The antiviral activity of genes specified in the bottom of each panel was determined by using a RNAi-based screen monitoring the rescue of HCV replication in cells transfected with a given siRNA prior to treatment with IFN-α (cf. Figure 2 in the main text). Target genes included those involved in the formation of SGs (ADAR1, G3BP1, HuR, TIA-1, and TIAR), described as components of PBs (Caf1a/b, CNOT1, GW182, Lsm1, Lsm4, Pat1a, Pat1b, and Upf1) or residing in PBs and SGs (Xrn1, CPEB1, Rck1, Ago2, and PCBP2). A siRNA directed against STAT2 (green box), a central component of the IFN-α-signaling, and a non-targeting siRNA (NT, red box) were included as positive and negative control, respectively. (A) Quantification of viability of cells after transfection of siRNAs specified in the bottom. Cell proliferation was measured by using the WST1 assay. All values were normalized to non-targeting siRNA (NT) values and are given as percentage of cell viability. (B) Determination of the knock-down efficiency. mRNA amounts of siRNA target genes specified in the bottom were quantified by using qRT-PCR. All values were normalized to GAPDH mRNA levels and results are presented as fold induction relative to NT siRNA-transfected cells.

(C and D) Effect of individual SG component knock-down on SG formation in HCV-infected cells treated with IFN-α. Components residing exclusively in SGs (G3BP1, HuR, TIA-1, and TIAR) or in both SGs and PBs (Xrn1, CPEB1, Rck1, Ago2, and PCBP2) were silenced. (C) Determination of the knock-down efficiency. mRNA amounts of siRNA target genes specified in the bottom were quantified by using qRT-PCR. All values were normalized to GAPDH mRNA levels and results are presented as fold induction relative to NT siRNA-transfected cells. Shown are means of triplicate measurements ± s.d. of a representative experiment. (D) Quantification of HCV-induced SGs in cells silenced with siRNAs specified in the bottom. Cells treated for 18h with 100 IU/ml IFN-α were fixed. HCV infection was detected by NS5A immunostaining and polyA mRNAs in SGs by *in situ* hybridization of Alexa555-oligo-dT50 and anlyzed by fluorescence microscopy.









Supplemental Figure S4

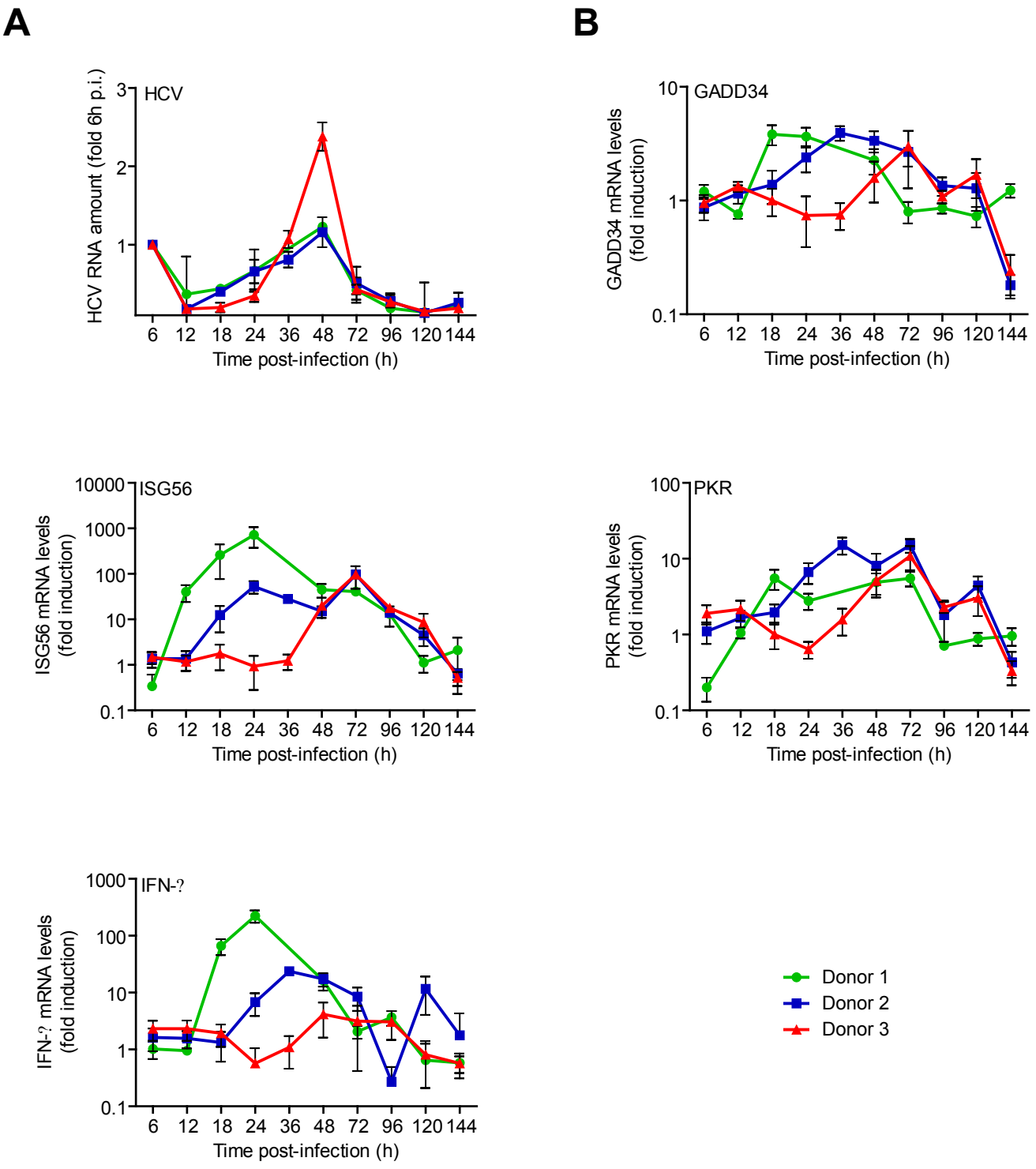
Figure S4, Related to Figure 3. HCV *trans*-Complementation Particle System (HCV_{TCP}) and Lack of Correlation between NS5A-mCherry Expression Level and SG Oscillation Frequency or Stress Interval

(A) Schematic of the HCV_{TCP} system. Huh7.5[CE1][E2p7NS2] cells stably express the structural proteins core (C), envelope glycoproteins E1 and E2 and the assembly factors p7 and NS2 (Steinmann et al., 2008). These cells were transfected with a subgenomic replicon that is composed of the HCV 5' non-translated region (NTR), the selectable marker *neo* that is fused N-terminally to 16 codons of the C gene of HCV, the internal ribosome entry site (IRES) of the encephalomyocarditis virus directing translation of the HCV sequence NS3 to NS5B and the HCV 3'NTR. The position of the mutation in NS3 increasing assembly efficiency (K1402Q) is indicated with a star. Owing to autonomous replication, replicon RNA amplifies and is packaged into virus particles generated from stably expressed HCV proteins. The resulting HCV_{TCP} particles are fully infectious and contain the subgenomic replicon RNA.

(B) Supernatants of Huh7.5[CE1][E2p7NS2] electroporated with mCherry_Neo K1402Q replicon RNA were collected 24, 48, 72 and 96h after transfection and for each time point infectivity titers were determined by limiting dilution assay ($TCID_{50}$/ml). Infectivity titers of concentrated HCV_{TCP} stocks (conc.) were determined in parallel.

(C) Huh7 YFP-TIA1 cells were infected with HCV and treated with 100 IU/ml IFN-α. Scatter plot of correlation between NS5A-mCherry mean fluorescence intensity (reflecting the level of HCV replication) and SG oscillation frequency. n, number of analyzed cells; PC, Pearson´s correlation; R^2, coefficient of determination.

(D) Same as in (C), but showing a scatter plot of correlation between NS5A-mCherry mean fluorescence intensity and total stress time.



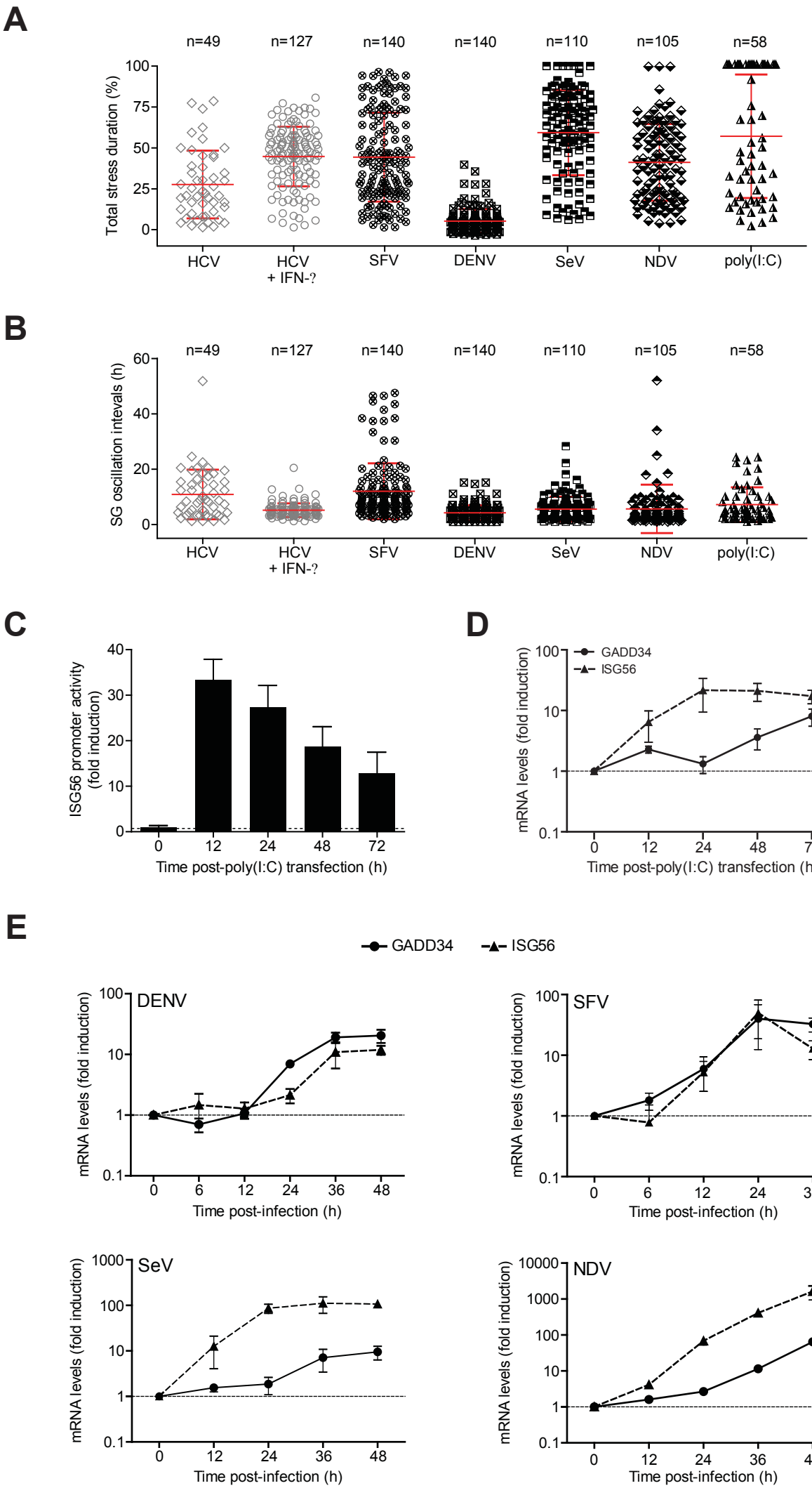
Supplemental Figure S5

Figure S5, Related to Figure 6. HCV Induces GADD34 Expression in Primary Human Hepatocytes (PHHs), an IFN-Competent Cell Model

PHHs of 3 donors were infected or not with HCV (5 $TCID_{50}$ per cell) for 6h prior to medium removal and extensive washes. Cells were harvested for RNA extraction at time points specified on the X-axis. All values were normalized to amounts of GAPDH mRNA present in the same RNA sample. Results are presented as fold induction relative to non-infected cells.

(A) HCV RNA quantifications are given in the upper panel. Lower panels display mRNA quantifications of ISG56 and IFN-α genes, respectively, to demonstrate induction of an IFN response.

(B) GADD34 and PKR mRNA quantification in HCV-infected PHHs.



Supplemental Figure S6

Figure S6, Related to Figure 7. dsRNA Induces Highly Dynamic SGs

(A and B) Scatter plots of total stress duration and SG oscillation intervals. Analyses are based on randomly selected Huh7 YFP-TIA1 cells infected with SFV, DENV SeV and NDV or transfected with 4 µg poly(I:C). Shown in red are mean values ± s.d. The number of cells analyzed (n) is given in the top. Total stress duration and SG oscillation intervals of HCV-infected cells in absence or presence of IFN-α (grey symbols) are shown as reference.

(C) Poly(I:C) stability in transfected cells. Huh7 cells ($3x10^5$) were transfected with 4µg poly(I:C) and collected at different time points post transfection to extract total RNA. One microgram of purified total RNA was transfected into Huh7.5 RIG-I cells (Binder et al., 2007) that were co-transfected with reporter plasmid containing the ISG56 promoter upstream of the Firefly luciferase gene and a Renilla luciferase reporter plasmid that was used to normalize for transfection efficiency. Values were normalized to those obtained after transfection of total RNA from cells that had been harvested immediately after poly(I:C) transfection (T0). Shown are the mean values ± s.d. of 3 independent experiments.

(D) mRNA quantification of GADD34 in Huh7 cells transfected with 4µg poly(I:C). Cells were harvested at time points specified in the bottom and total RNA was extracted. For each time point values were normalized to GAPDH mRNA levels. Induction of ISG56 mRNA expression was included as control for IFN-α response of Huh7 YFP-TIA1 cells. Results are presented as fold induction relative to non-infected cells. Shown are mean values ± s.d. of 3 independent experiments.

(E) Kinetics of induction of ISG56 and GADD34 mRNA levels in Huh7 cells infected with SFV, DENV, SeV and NDV. Cells were collected at different time points after infection to extract total RNA. All values were normalized to GAPDH mRNA levels present in the same RNA sample. Results are presented as fold induction relative to non-infected cells. Shown are mean values ± s.d. of 3 independent experiments.

SUPPLEMENTAL REFERENCES

Bartenschlager,R., Ahlborn-Laake,L., Mous,J., and Jacobsen,H. (1994). Kinetic and structural analyses of hepatitis C virus polyprotein processing. J. Virol. *68*, 5045-5055.

Binder,M., Kochs,G., Bartenschlager,R., and Lohmann,V. (2007). Hepatitis C virus escape from the interferon regulatory factor 3 pathway by a passive and active evasion strategy. Hepatology *46*, 1365-1374.

Blight,K.J., McKeating,J.A., and Rice,C.M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. *76*, 13001-13014.

Bolte,S. and Cordelieres,F.P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. *224*, 213-232.

Dieterich,D.C., Hodas,J.J., Gouzer,G., Shadrin,I.Y., Ngo,J.T., Triller,A., Tirrell,D.A., and Schuman,E.M. (2010). In situ visualization and dynamics of newly synthesized proteins in rat hippocampal neurons. Nat. Neurosci. *13*, 897-905.

Kaul,A., Woerz,I., Meuleman,P., Leroux-Roels,G., and Bartenschlager,R. (2007). Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. J. Virol. *81*, 13168-13179.

Koch,J.O. and Bartenschlager,R. (1999). Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. J. Virol. *73*, 7138-7146.

Lindenbach,B.D., Evans,M.J., Syder,A.J., Wolk,B., Tellinghuisen,T.L., Liu,C.C., Maruyama,T., Hynes,R.O., Burton,D.R., McKeating,J.A., and Rice,C.M. (2005). Complete replication of hepatitis C virus in cell culture. Science *309*, 623-626.

Matula,P., Kumar,A., Worz,I., Erfle,H., Bartenschlager,R., Eils,R., and Rohr,K. (2009). Single-cell-based image analysis of high-throughput cell array screens for quantification of viral infection. Cytometry A *75*, 309-318.

Owsianka,A., Clayton,R.F., Loomis-Price,L.D., McKeating,J.A., and Patel,A.H. (2001). Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. J. Gen. Virol. *82*, 1877-1883.

Paul,D., Romero-Brey,I., Gouttenoire,J., Stoitsova,S., Krijnse-Locker,J., Moradpour,D., and Bartenschlager,R. (2011). NS4B self-interaction through conserved C-terminal elements is required for the establishment of functional hepatitis C virus replication complexes. J. Virol. *85*, 6963-6976.

Reiss,S., Rebhan,I., Backes,P., Romero-Brey,I., Erfle,H., Matula,P., Kaderali,L., Poenisch,M., Blankenburg,H., Hiet,M.S., Longerich,T., Diehl,S., Ramirez,F., Balla,T., Rohr,K., Kaul,A., Buhler,S., Pepperkok,R., Lengauer,T., Albrecht,M., Eils,R., Schirmacher,P., Lohmann,V., and Bartenschlager,R. (2011). Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. Cell Host. Microbe *9*, 32-45.

Schaller,T., Appel,N., Koutsoudakis,G., Kallis,S., Lohmann,V., Pietschmann,T., and Bartenschlager,R. (2007). Analysis of hepatitis C virus superinfection exclusion by using novel fluorochrome gene-tagged viral genomes. J. Virol. *81*, 4591-4603.

Steinmann,E., Brohm,C., Kallis,S., Bartenschlager,R., and Pietschmann,T. (2008). Efficient trans-encapsidation of hepatitis C virus RNAs into infectious virus-like particles. J. Virol. *82*, 7034-7046.

van den Hoff,M.J., Christoffels,V.M., Labruyere,W.T., Moorman,A.F., and Lamers,W.H. (1995). Electrotransfection with "intracellular" buffer. Methods Mol. Biol. *48*, 185-197.