Pulse-chase imaging reveals continuous *de novo* generation of spatially segregated hepatitis C virus replication organelles

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Supplementary Materials and Methods

Reagents and antibodies

2'-C-methyladenosine (2'CMA) was purchased from Carbosynth (Compton, United Kingdom). AL-9 and OSW-1 were kind gifts of Dr. Raffaele De Francesco (INGM, Milano, Italy) and Dr. Matthew Shair (Harvard University, Cambridge, MA), respectively. SNAP-Cell 505 (S_{505}), SNAP-Cell TMR-star (S_{TMR}), and SNAP-Cell Block (S_{block}) were purchased from New England Biolabs (Ipswich, MA). Cycloheximide, nocodazole, and vinblastine and SB 220025were from Sigma (St. Louis, MO). Antibodies used in this study include: HCV core (mouse monoclonal clone 6G7; Dr. Harry Greenberg, Stanford, CA), HCV NS3 (Virogen, Watertown, MA), HCV NS5A (mouse monoclonal clone 9E10; Dr. Charles Rice, Rockefeller University, New York, NY), β-Actin (mouse monoclonal; Sigma-Aldrich, St. Louis, MO), β-tubulin (Thermo Scientific, Rockford, IL), anti-dsRNA (J2, Scicons, Hungary), tetramethylrhodamine (TMR antibody, Life Technologies, Carlsbad, CA**).** TopFluor Cholesterol was purchased from Avanti Polar Lipids (Alabaster, AL). DAPI, BODIPY 493/503, and Alexa Fluor conjugated secondary antibodies for microscopy experiments, protease and phosphatase Inhibitors and RNase inhibitors were purchased from Life Technologies.

Cell culture

Huh7.5.1 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Huh7.5.1 cells containing a genotype 2a JFH-1 subgenomic replicon encoding SNAP-tagged NS5A were grown with the addition of 400 µg/mL G418 (Life Technologies, Carlsbad, CA).

Viruses and viral replicons

We first subcloned the SNAPf sequence [1] into the unique MluI site in the JFH1/NS5A-MluI construct described in [2] to generate a full length HCV genome and a subgenomic replicon, termed here as pFL-JFH1 (NS5A/SNAP) and pSGR-JFH1 (NS5A/SNAP) respectively. . For the luciferase reporter replicons, a Renilla luciferase (RLuc)-Neo fusion gene was amplified from pORN/C-5B/KE[3] and used to replace the Neo gene in pSGR-JFH1 or pSGR-JFH1(NS5A/SNAP) to generate pSGR-Rluc-JFH1 or pSGR-Rluc-JFH1 (NS5A/SNAP). Further details of plasmid construction are available upon request. *In* vitro transcribed RNA was prepared and transfected into Huh7.5.1 cells as described in [4]. Cell lines stably harboring the subgenomic replicon were isolated by G418 selection.

Immunofluorescence staining

Cells grown on poly-D-lysine coated glass coverslips were rinsed in PBS and fixed in 4% paraformaldehyde in PBS for 15 min at RT followed by quenching in 50 mM NH_4Cl in PBS for 10 min at RT. Cells were then permeabilized with 0.5% Triton-X100 at RT for 15 min before they were blocked with 2% BSA. For dsRNA staining, cells were fixed for 20 min in methanol at -20°C and then blocked in 2% BSA. Blocking was followed by incubation with primary antibody in blocking buffer for 1 hr in RT followed by 4 washes in PBS for 5 min each. Secondary antibody detection was performed with Alexa Fluor 488- or 594-conjugated anti-mouse antibodies. After 4 washes in PBS for 5 minutes each, coverslips were mounted with Prolong Gold with DAPI (Life Technologies). Images were taken with a Nikon A1 laser scanning confocal microscope in sequential scanning mode to limit crosstalk between fluorochromes. Quantitative colocalization was analyzed by NIH ImageJ with the JACoP plugin [5].

Luciferase Assay

For transient replication assays, Huh7.5.1 cells transfected with pSGR-Rluc-JFH1 or pSGR-Rluc-JFH1 (NS5A/SNAP) RNA were lysed and luciferase activity was measured at the indicated time points (Renilla Luciferase Assay System; Promega, Madison, WI) using a Synergy 2 plate reader (BioTek, Winooski, VT). For luciferase activity measurement in stable replicon cell lines, Huh7.5.1 cells stably expressing pSGR-Rluc-JFH1 (NS5A/SNAP) were stained with the indicated SNAP substrates for 15 min and then incubated in fresh medium for the indicated time before they were assayed as described above.

Correlative light-electron microscopy

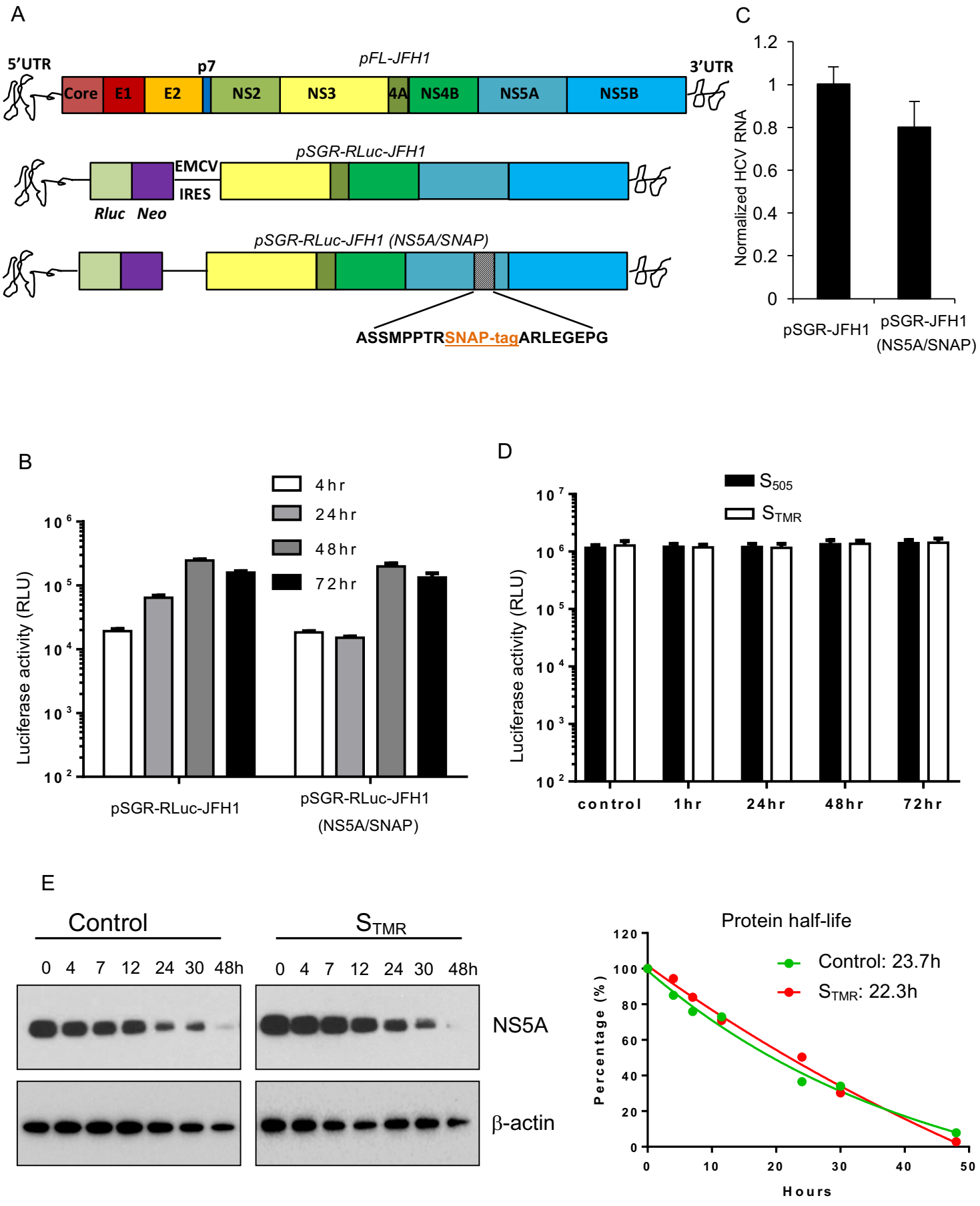
SGR-JFH1(NS5A/SNAP) replicon cells were seeded onto glass bottom dishes with gridded coverslips (MatTek corporation, MA) one day before they were labeled with S_{TMR} for 'old' NS5A. 6 hr later, cells were treated with S_{block} and S_{505} was applied another 16hr later to label 'new' NS5A. Live cells were then evaluated by confocal microscopy to identify cells of interest; their positions were recorded and DIC/confocal fluorescent images were acquired. Cells were immediately fixed with 2.5% glutaraldehyde and embedded with EPON resin. Coverslips were detached from the embedded cell block by sequential immersion of the coverslip into liquid nitrogen followed by water. The cells of interest previously identified by light microscopy were identified on the EPON block; the region of interest was trimmed and processed for sectioning. Ultra-thin sections were collected onto Formvar/carbon-coated copper grids and post stained with uranyl acetate and lead citrate. The sections were viewed on a JEOL JEM-1400 Plus transmission electron microscope at 80 kV. EM images were acquired at varying magnifications for subsequent correlation of fluorescence microscopic images with electron micrographs.

Quantitation of NS5A phosphorylation and negative: positive strand RNA ratios

Stable replicon cells were first labeled with S_{block} and S_{TMR} to selectively label 'old' or 'new' NS5A before they were washed once with ice-cold PBS, lysed with 100μM digitonin in PBS containing protease inhibitors, phosphatase inhibitors and RNase inhibitors, and centrifuged for 5 minutes at 12,000 x *g*. TMR-labeled NS5A-SNAP and associated RNA were then isolated by incubating the cell lysate with anti-TMR antibody for 1.5 hr at 4˚C followed with Dynabeads Protein G (Life Technologies) according to the manufacturer's instructions. Immunoisolated material was then subjected to immunoblotting or to strand-specific qRT-PCR as previously described [6].

For strand-specific qRT-PCR, HCV RNA from immunoisolated material was extracted using RNeasy columns (Qiangen) followed by HCV strand-specific quantitative RT-PCR using the primers as described in [6]. *In vitro* transcribed positive-strand and negative-strand HCV RNA were used to generate standard curves for absolute quantification. Results are presented as ratios of positive:negative strand RNA.

Supplementary Figure 1



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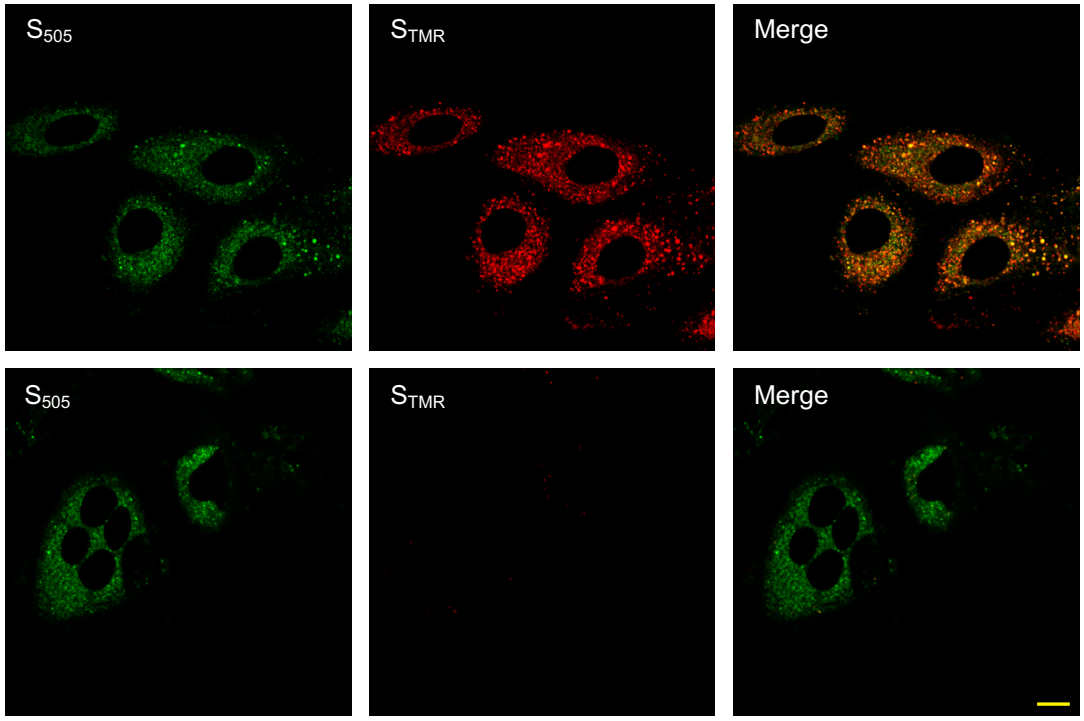
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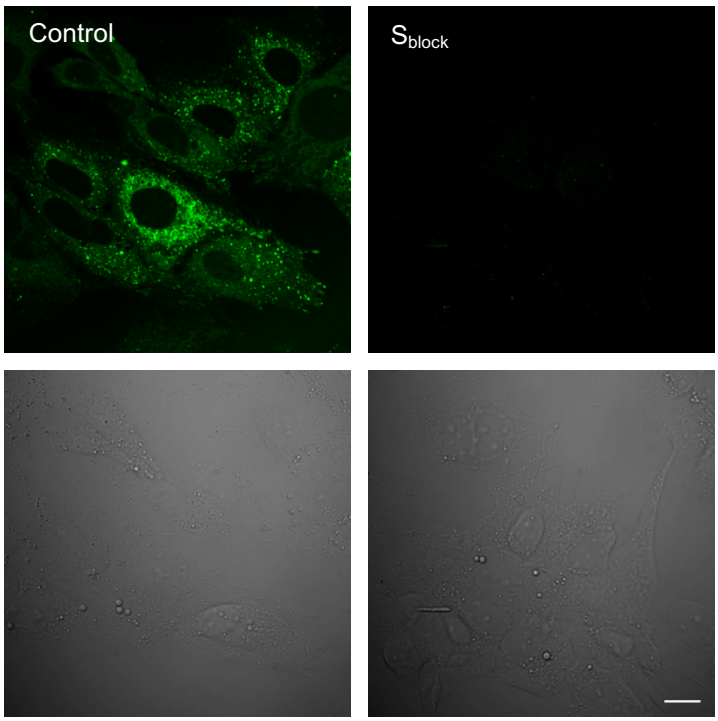
STMR
Merge
Cycloheximide

G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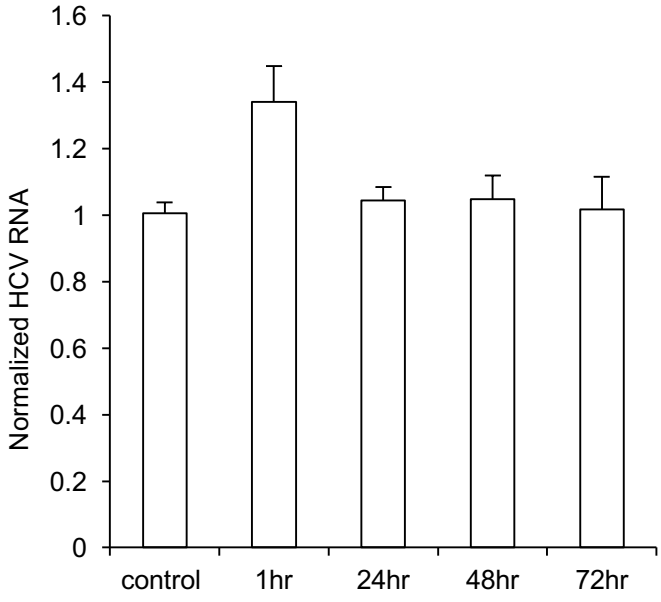
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1.2
1
0.8
0.6
0.4
0.2
0
control
1hr
24hr
48hr
72hr

Supplementary Figure 1, Characterization of SNAP tag staining

(A). Schematic of viral constructs. A SNAP tag was inserted into a known tolerated insertion site within domain III of NS5A in the subgenomic replicon pSGR-RLuc-JFH1 to create pSGR-RLuc-JFH1 (NS5A/SNAP).

(B). Huh7.5.1 cells were transfected with SGR-RLuc-JFH1 or SGR-RLuc-JFH1 (NS5A/SNAP) transcripts before determination of Renilla luciferase activity at the indicated time points. Values (means ±SD) are representatives of 3 independent experiments.

(C). Intracellular HCV RNA levels of Huh7.5.1 cells stably harboring SGR-JFH1 or SGR-JFH1 (NS5A/SNAP) replicons were quantitated by qRT-PCR. Values (means ±SD of 3 independent experiments) are normalized to SGR-JFH1 HCV RNA levels.

(D). Huh7.5.1 cells stably expressing the SGR-RLuc-JFH1 (NS5A/SNAP) replicon were mock-treated or stained with the indicated labeling reagent and then incubated for the indicated times before luciferase activity was measured. Values (means ± SD) are representatives of 2 independent experiments.

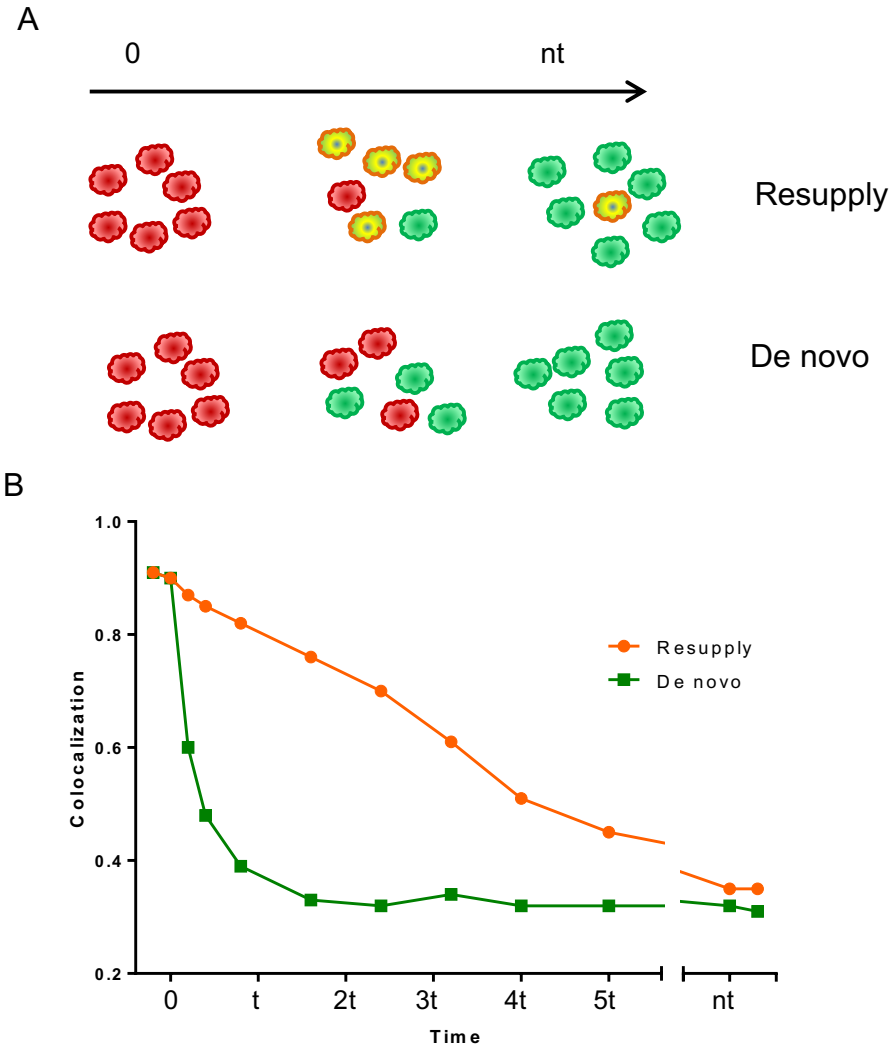
(E). Left panel: SGR-JFH1(NS5A/SNAP) replicon cells were either mock treated or stained with S_{TMR} and then immediately treated with 150 μM cycloheximide (CHX) for the indicated times before they were harvested and NS5A levels analyzed by immunoblotting. Right panel: band densities were quantified with Image J and protein half-lives were calculated using exponential decay curve fitting in GraphPad Prism software.

(F). SGR-JFH1 (NS5A/SNAP) replicon cells were pulse labeled with S_{505} and then immediately treated with S_{block}. Cells were then mock treated or treated with 150 μM CHX for 16 hr to block new NS5A protein synthesis. Newly synthesized NS5A was labeled with S_{TMR}.

(G). SGR-JFH1 (NS5A/SNAP) replicon cells were either mock treated or treated with S_{block} before labeling with S_{505}. Bright field images are included to show the presence of cells. Scale bar, 10μm.

(H). SGR-JFH1 (NS5A/SNAP) replicon cells were either mock treated or stained with S_{block} and then incubated for the indicated time period before HCV RNA was quantitated by qRT-PCR. Values (means ±SEM of 3 independent experiments) are normalized to control cells.

Supplementary Figure 2

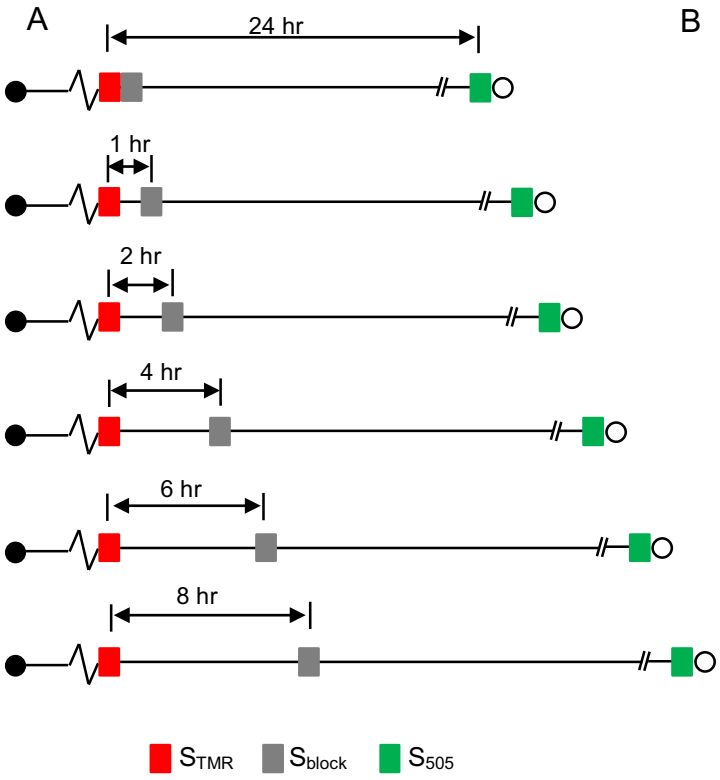


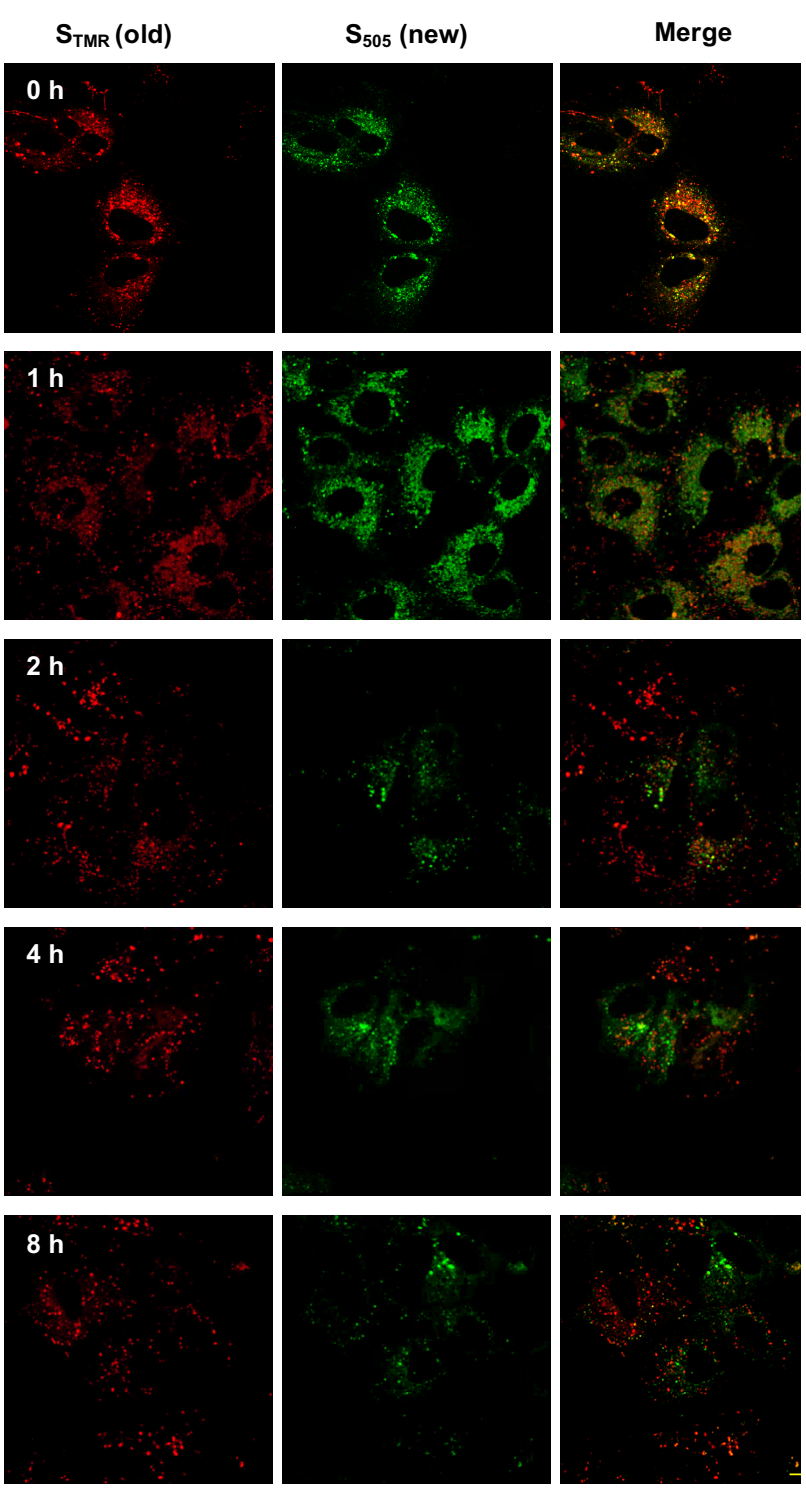
Supplementary Figure 2. Predicted models to distinguish ‘resupply’ vs ‘de novo’ replication organelle formation using quantitative colocalization data over multiple timepoints.

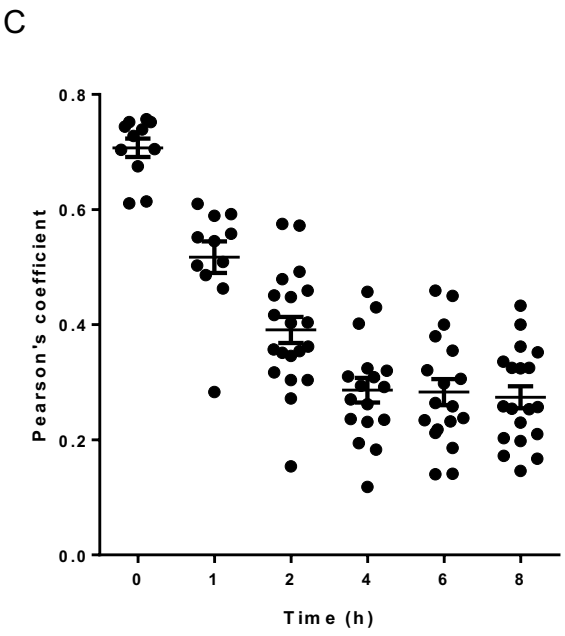
(A). Diagrams of ‘resupply’ and ‘de novo’ formation of replication organelles. In the ‘resupply’ model, newly synthesized NS5A is directed to preformed or ‘old’ replication organelles, while in the ‘de novo’ model, newly synthesized NS5A is appears at sites distinct from ‘old’ replication organelles.

(B). A predicted schematic illustrating expected colocalization data for ‘resupply’ and ‘de novo’ models. A resupply model might be associated with a linear decline in colocalization between “new” and “old” NS5A, while a “de novo” model would be expected to result in a sharp decline.

Supplementary Figure 3







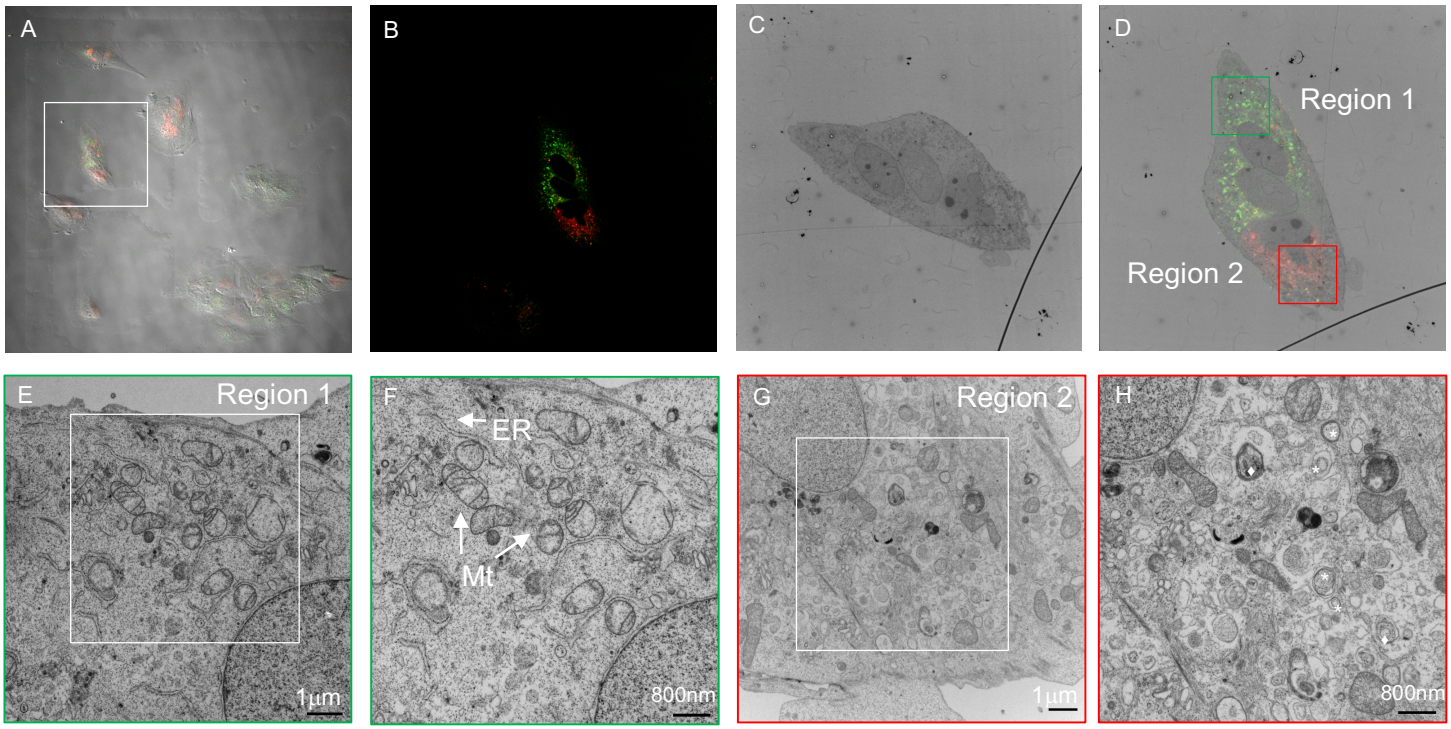
Supplementary Figure 3, Pulse-chase imaging of SNAP-tagged NS5A with a fixed chase time

(A). Schematic of experimental design. Parallel cultures of NS5A-SNAP subgenomic replicon cells were pulse labeled with S_{TMR} before they were treated with S_{block} at the indicated times. Cells were then incubated for another 24 hr before newly synthesized NS5A was labeled with S_{505}.

(B). Representative images of live cells labeled as described above. Scale bar, 10μm.

(C). Quantitation of colocalization between S_{TMR} labeled NS5A and S_{505} labeled NS5A. Each point denotes the Pearson's coefficient calculated from a single cell with summary mean±SD values indicated for each timepoint.

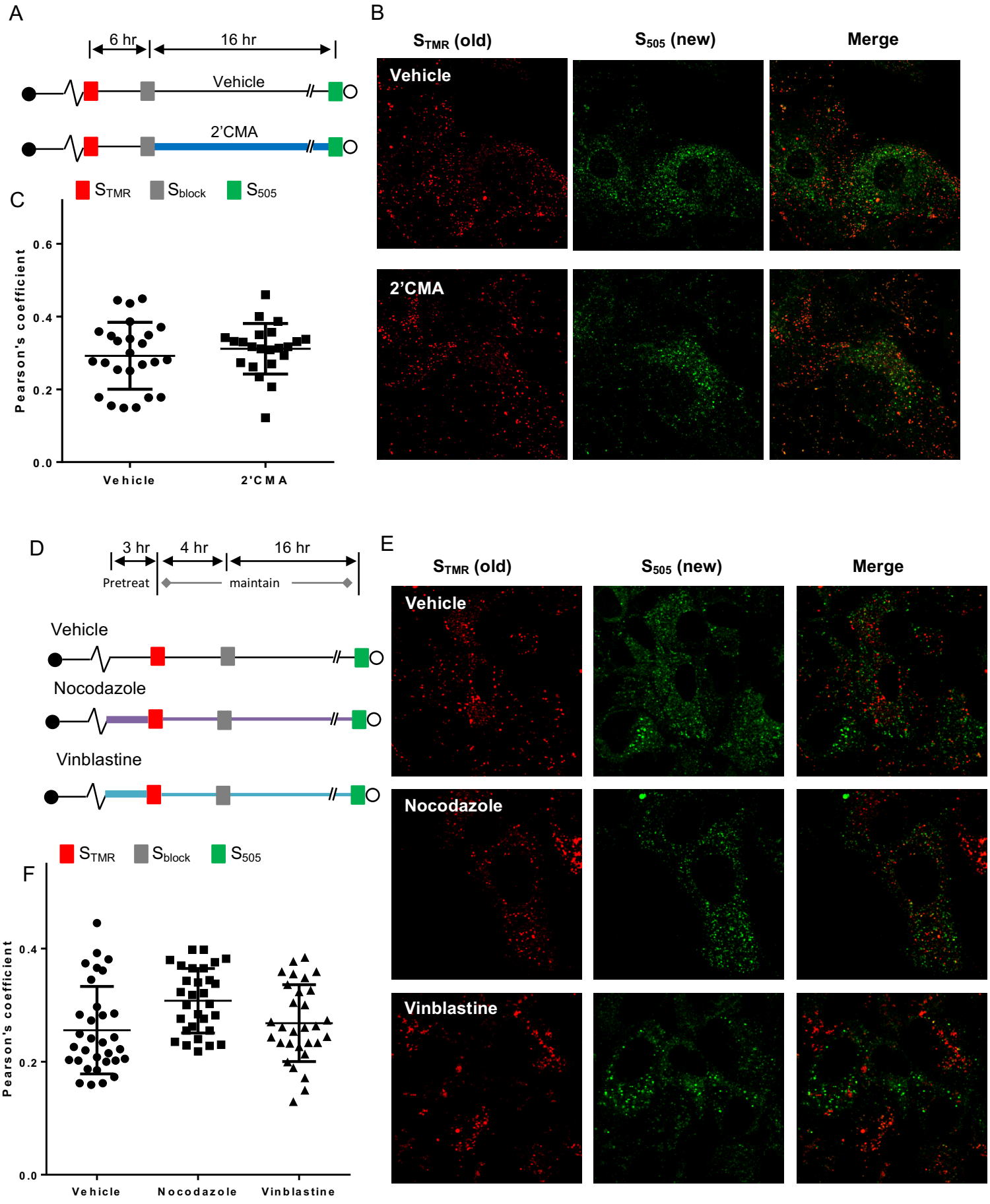
Supplementary Figure 4



Supplementary Figure 4. Correlative light-electron microscopy of cells containing SGR-JFH1(NS5A-SNAP) subgenomic replicon.

Live cells containing the subgenomic replicon grown on gridded coverslips were stained with S_{TMR} (for old foci) and S_{505} (for new foci) before fluorescent images were taken. Cells were then immediately processed for EM. (A) DIC image of live cells on gridded coverslips. (B) Fluorescent image of cells of interest. (C) EM image of cells of interest. (D) EM image overlapped with fluorescent image. (E-F) higher magnification images of region 1 in panel D. (G-H) higher magnification images of region 2 in panel D. ER, endoplasmic reticulum; Mt, mitochondria; DMVs, double membrane vesicles. Asterisks indicate DMVs; diamonds indicate multi-membrane vesicles (MMVs).

Supplementary Figure 5



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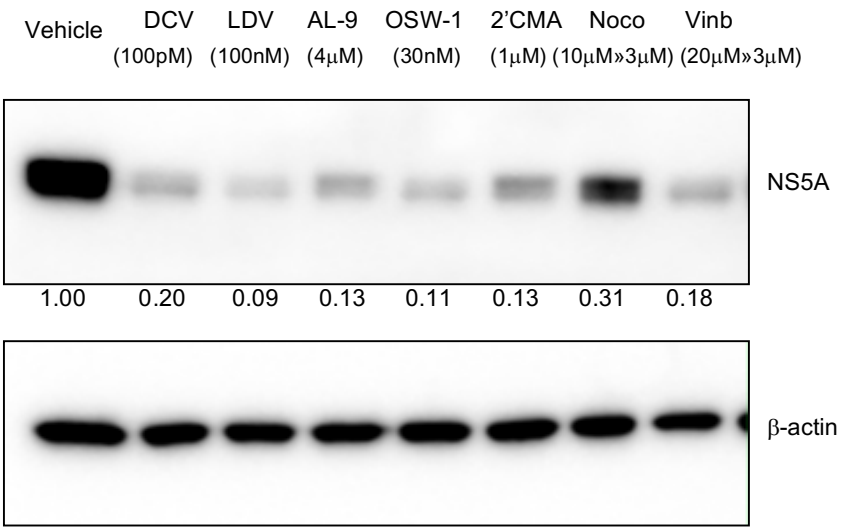
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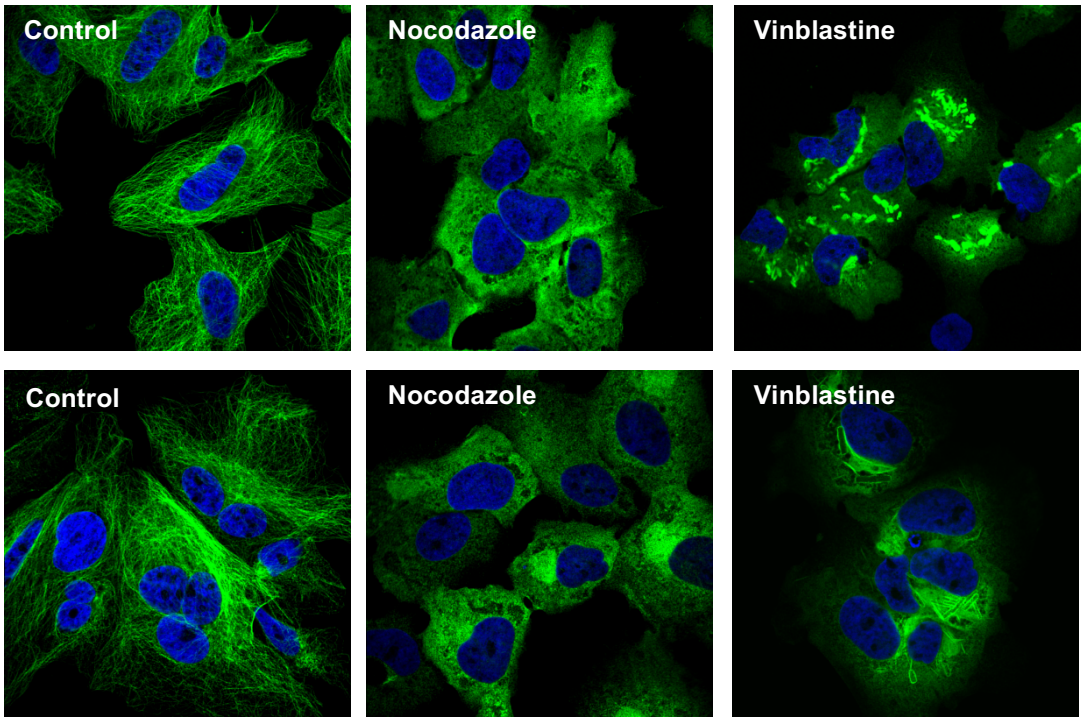
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Nocodazole
Vinblastine
Control
Nocodazole
Vinblastine

Supplementary Figure 5, Formation of new NS5A foci does not depend on NS5B or microtubules.

(A). Schematic of experimental design. SGR-JFH1 (NS5A/SNAP) subgenomic replicon cells were pulse labeled with S_{TMR} and treated with S_{block} 6 hr later. Cells were then incubated with vehicle or 1 μM 2'CMA for another 16 hr before newly synthesized NS5A was labeled with S_{505}.

(B). Representative live-cell images of cells labeled as described above. Scale bar, 10 μm.

(C). Quantitation of colocalization between S_{TMR}-labeled and S_{505}-labeled NS5A. Each point denotes the Pearson's coefficient calculated from a single cell. Summary mean±SD values representative of 3 independent experiments are indicated.

(D). Schematic of experimental design. pSGR-JFH1 (NS5A/SNAP) subgenomic replicon cells were either mock treated or pretreated with 10 μM nocodazole or 20 μM vinblastine for 3 hr before labeling with S_{TMR}. Cells were then maintained in 3 μM nocodazole, or 3 μM vinblastine for the next 20 hrs. "New" NS5A was visualized by sequential S_{block} and S_{505} labeling at the indicated time points.

(E). Representative live-cell images of cells labeled as described above. Scale bar, 10 μm.

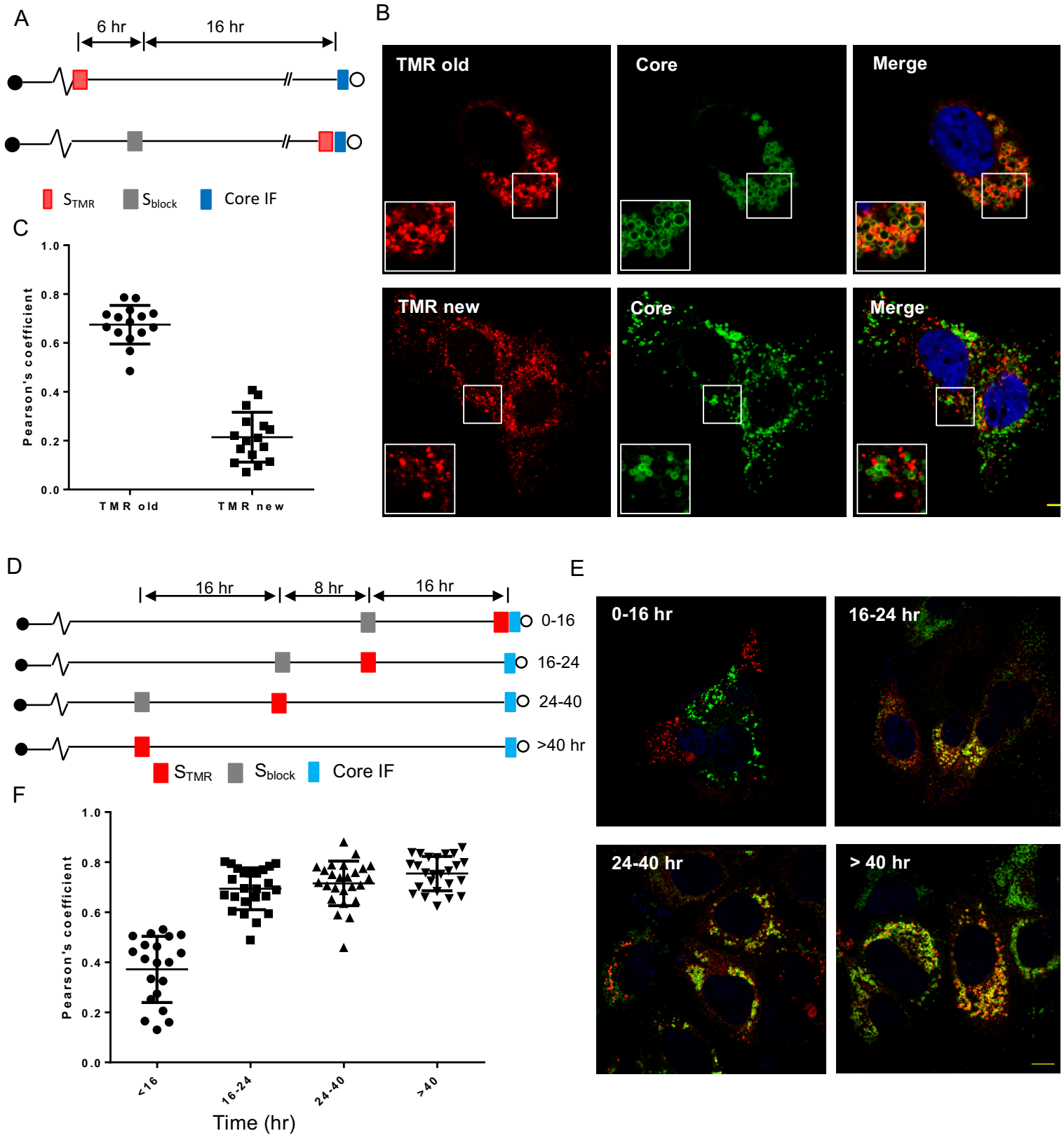
(F). Quantitation of colocalization between S_{TMR} labeled NS5A and S_{505} labeled NS5A. Each point denotes the Pearson's coefficient calculated from a single cell. Summary mean±SD values representative of 3 independent experiments are indicated.

(G). SGR-JFH1 (NS5A/SNAP) subgenomic replicon cells were either mock treated or treated with the indicated inhibitors before they were lysed and subjected to immunoblotting for NS5A and β-actin. Chemiluminescence was quantitated with a Li-Cor

Odyssey imaging system and the relative amounts of NS5A in this representative immunoblot are shown below. DCV, daclatasvir; LDV, ledipasvir; noco, nocodazole; vinb, vinblastine.

(H). Upper panels: Preformed microtubules are disrupted by pharmacologic inhibitors. SGR-JFH1 (NS5A/SNAP) subgenomic replicon cells were either mock treated or treated with 10 μM nocodazole, or 20 μM vinblastine for 3 hr. Cells were then fixed and immunostained with β-tubulin. Lower panels: Maintenance of microtubule disruption by pharmacologic inhibitors. pSGR-JFH1 (NS5A/SNAP) subgenomic replicon cells were either mock treated or treated with 10 μM nocodazole or 20 μM vinblastine for 3 hr before they were maintained in media containing 3 μM nocodazole or 3 μM vinblastine for another 20 hr. Cells were then fixed and immunostained for β-tubulin. Scale bar, 10 μm.

Supplementary Figure 6



Supplementary Figure 6, Progressively increasing association of NS5A over time with core protein

(A). Schematic of experimental design to visualize colocalization of 'new' and 'old' NS5A foci with HCV core protein. Huh-7.5.1 cells infected with full-length JFH1(NS5A/SNAP) were either pulse labeled with S_{TMR} (for old foci) or treated first with S_{block} and 16 hr later with S_{TMR} (for new foci). Cells were then fixed and immunostained for HCV core protein.

(B). Representative images of cells labeled as described. Scale bar, 10 μm.

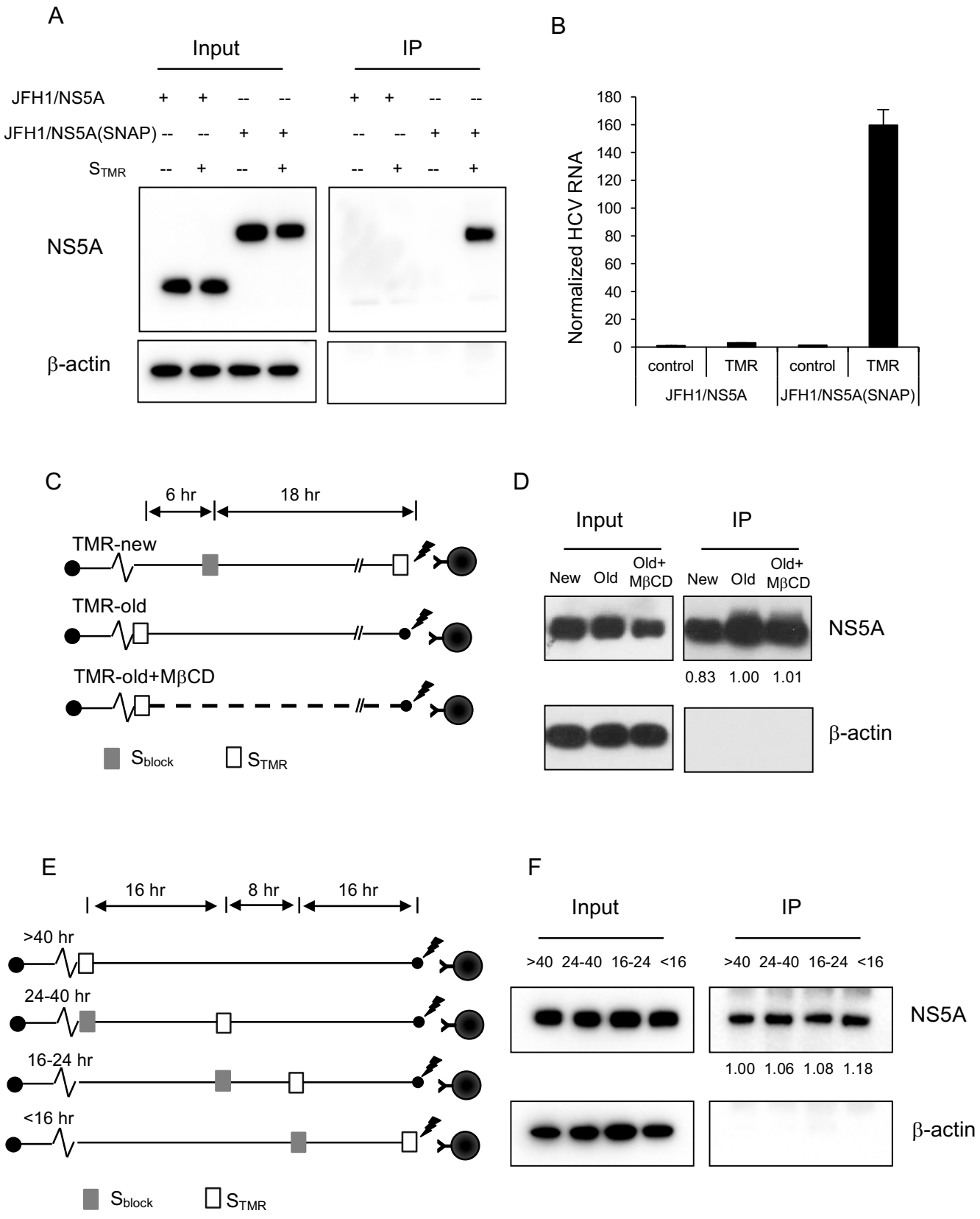
(C). Quantitation of colocalization between S_{TMR} labeled NS5A and core protein. Each point denotes the Pearson's coefficient calculated from a single cell. Summary mean±SD values representative of 3 independent experiments are indicated.

(D).Schematic of time-course experimental design. Huh-7.5.1 cells infected with full-length JFH1(NS5A/SNAP) were treated with S_{block} or S_{TMR} at the indicated time points. Cells were then fixed and immunostained with core antibody before cell imaging.

(E). Representative images of cells labeled as described. Scale bar, 10 μm.

(F). Quantitation of colocalization between S_{TMR} labeled NS5A and core protein. Each point denotes the Pearson's coefficient calculated from a single cell. Mean and errors (SD) are indicated.

Supplementary Figure 7



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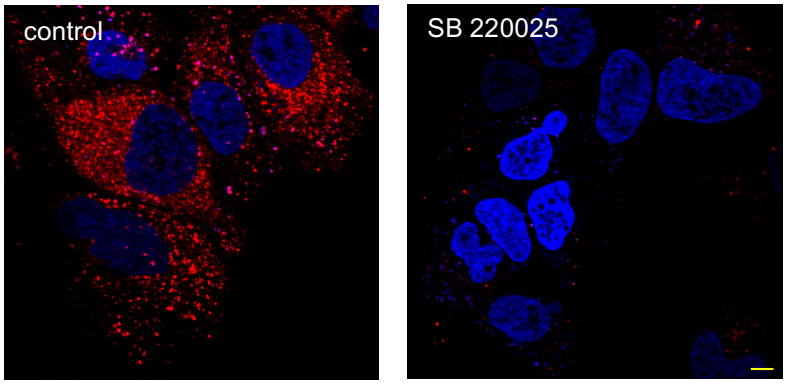

H

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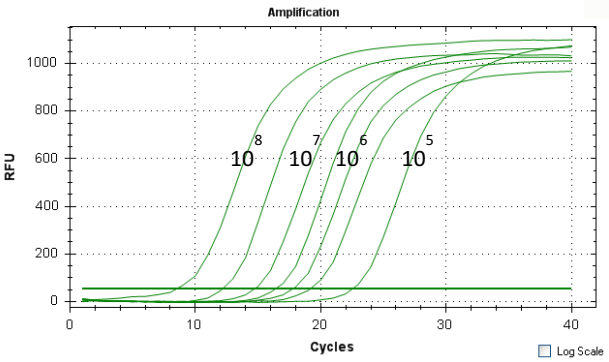
+ strand quantitation



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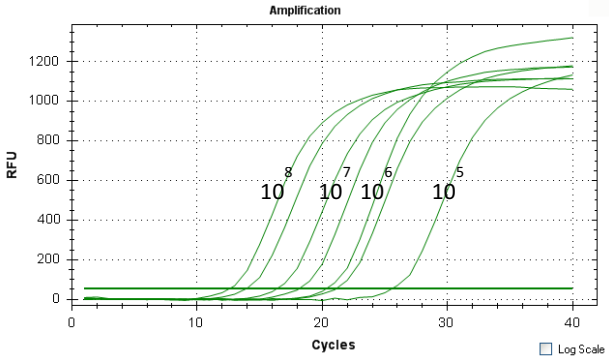

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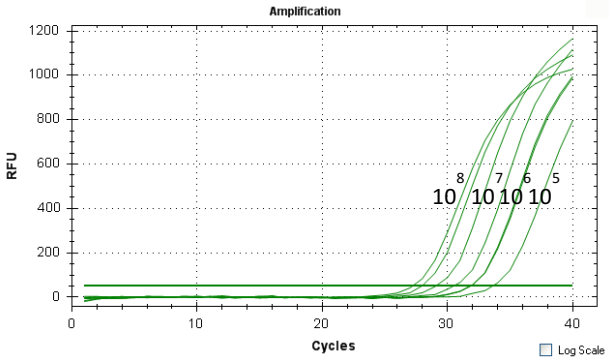

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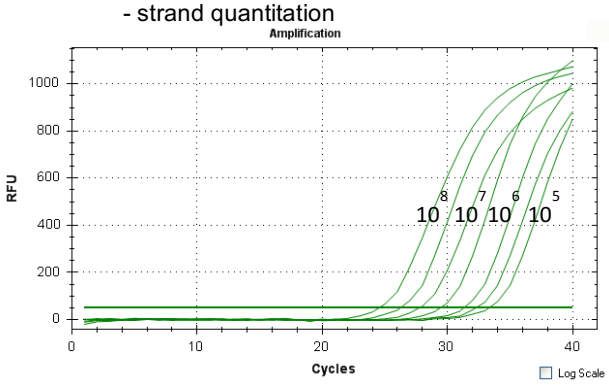

Supplementary Figure 7. 'Old' replication organelles contain more hyperphosphorylated NS5A and a higher ratio of positive:negative strand genomes.

(A). Huh 7.5.1 cells containing SGR-JFH1 or SGR-JFH1(NS5A/SNAP) replicons were either mock treated or treated with S_{TMR} before they were lysed and immunoprecipitated with anti-TMR antibody. The whole cell lysates and immunoprecipitates were subjected to immunoblotting for NS5A and β-actin.

(B). Huh 7.5.1 cells containing SGR-JFH1 or SGR-JFH1(NS5A/SNAP) replicons were treated and immunoprecipitated as described above and the HCV RNA associated with the anti-TMR immunoprecipitates was quantitated by qRT-PCR. Values (means ±SD) are representatives of 2 independent experiments and normalized to control SGR-JFH1 HCV RNA levels.

(C). Schematic of experimental design. SGR-JFH1(NS5A/SNAP) subgenomic replicon cells were either treated first with S_{block} and 18 hr later with TMR (for 'new' NS5A), pulse labeled with S_{TMR} (for 'old' NS5A) or pulse labeled with S_{TMR} and then treated with 1 mM MβCD (for 'old+MβCD') before lysis and immunoprecipitation of TMR-labeled NS5A with anti-TMR antibody.

(D). Cell lysates (left panel) and immunoprecipitated TMR-NS5A (right panel) obtained as described above were subjected to immunoblotting for NS5A and β-actin. Band densities were quantified with Image J and the relative amounts of NS5A in this representative immunoblot are shown below.

(E). Schematic of experimental design. pSGR-JFH1(NS5A/SNAP) subgenomic replicon cells were treated with S_{block} or S_{TMR} at the indicated time points before lysis and immunoprecipitation of TMR-labeled NS5A with anti-TMR antibody.

(F). Cell lysates (right panel) and immunoprecipitated TMR-NS5A (left panel) obtained as described above were subjected to immunoblotting for NS5A and β-actin. Chemiluminescence was quantitated with Odyssey Imaging system and the relative amounts of NS5A in this representative immunoblot are shown below.

(G). Huh 7.5.1 cells containg SGR-JFH1(NS5A/SNAP) were either mock treated or treated with 5 μM SB 220025 for 24 hr before they were labeled with S_{TMR}. Nuclei were counterstained with DAPI. Scale bar, 10 μm.

(H). Specificity of strand-specific qRT-PCR. The left-sided plots show the results of the positive-strand quantitation assay using the indicated masses of positive-strand (upper left) and negative-strand (lower left) synthetic RNA generated by *in vitro* transcription. The right-sided plots show the results of the negative-strand quantitation assay using the indicated masses of negative-strand (upper right) and positive-strand (lower right).

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