Supporting Information

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SI Text

Sequences of the shRNA. The sequence of shRNA-49 against Beclin-1 (accession number: MN_003766) insert in the vector is CCGGCCCGTGGAATGGAATGAGATTCTCGAGAATCTCATTCCATTCCACGGGTTTTTG, shRNA-50 against Beclin-1 is: CCGGCCGACTTGTTCCTTACGGAAACTCGAGTTTCCGTAAGGAACAAGTCGGTTTTTG, shRNA-92 against Atg12 (accession number: MN_004707) is: CCGGCCAAGGACTCATTGACTTCATCTCGAGATGAAGTCAATGAGTCCTTGGTTTTT, shRNA-800 against Atg4B (accession number: MN_013325) is: CCGGGAAAGATTCTTCGACTCAGAACTCGAGTTCTGAGTCGAAGAATCTTTCTTTTTG, shRNA-801 against Atg4B is: CCGGCGGTTTGCTGAGTTTGAAGATCTC GAGATCTTCAAACTCAGCAAACCGTTTTTG.

In Vitro Transciption of HCV RNA and Introduction by Electroporation. The in vitro transcription of RNA was accomplished using the T7 MEGAscript kit (Ambion). In vitro transcribed RNAs were introduced into Huh-7 cells by electroporation as previously described (1), and the level of delivered HCV RNA were assessed by RT-qPCR 5 h post-electroporation.

Immunofluorescence Analysis. LC3-GFP transfected Huh-7 cells were plated on chambered coverglass (Lab-tek, Thermo Fisher Scientific). Infection were performed at a MOI of 10 for 24 h, and after washing twice with PBS (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) and the cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, the cells were incubated for 30 min with blocking solution (10% FBS and 3% BSA in PBS) and permeabilized with 0.3% Triton X-100. Then the cells were incubated with the primary antibodies. The antibodies used were specific for Core (Affinity Bioreagents); E2 (C1); NS5A (MS5); and NS4A/NS4B (PR12). The fluorescent secondary antibodies were Alexa-555-conjugated anti-human, anti-mouse or anti-rabbit IgG (Molecular Probes). Nuclei were labeled with Hoechst dye (Molecular Probes). Analyses were performed on a Leica SP2 confocal microscope (Leica).

1. Zhong J, et al. (2005) Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 102:9294–9299.

2. Lavillette D, et al. (2005) Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. *Hepatology* 41:265–274.

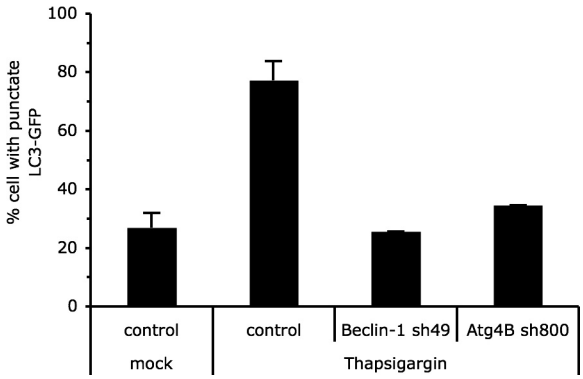

Fig. S1. Reduced Beclin-1 and Atg4B expression compromises autophagic vesicle formation. Results display the quantification of autophagic vesicle formation in Beclin-1 or Atg4B deficient or control Huh-7 cells, as measured by the frequency of cells containing punctate LC3-GFP. After LC3-GFP transfection, Huh-7 cells were grown in normal medium (mock), or were treated with 500 nM of Thapsigargin for 16 h to induce autophagy.

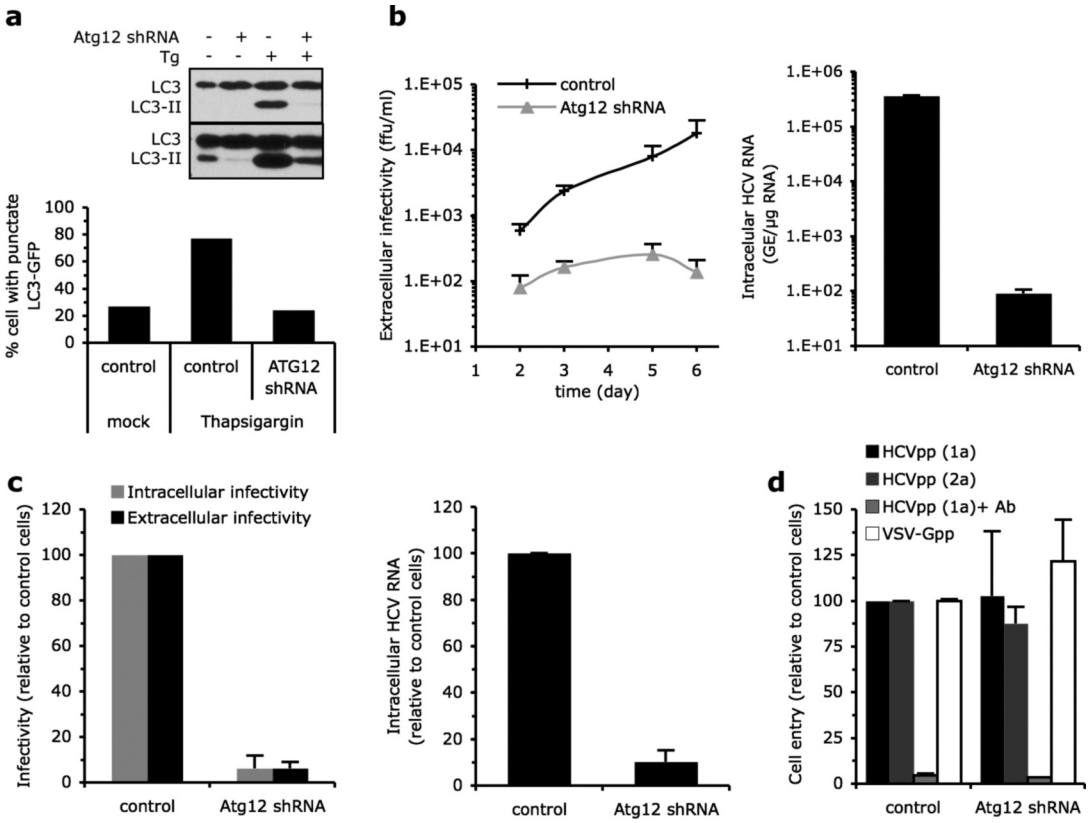

Fig. S2. ShRNA against Atg12 reduces autophagic vesicle content and inhibits HCV infection. Huh-7 cells were transduced with lentiviral vectors expressing shRNA against Atg12. The selected shRNA sequence and MOI used to transduce Huh-7 cells did not compromise cell viability. At 8 days posttransduction, transduced and control cells were harvested and seeded in equal numbers. Twenty and 72 h later MTT was added and cellular proliferation was monitored. The MTT results for the Atg12 transduced cells were 87.27 ± 22.84% of the nontransduced cells, set to 100. (*A*) Analysis of LC3 protein modification and its cellular localization in Atg12-shRNA transduced versus control cells. (*Upper*) Immunoblotting analysis using antibody against LC3 of Atg12-shRNA transduced and control Huh-7 cells treated or not with thapsigargin (Tg) for 16 h. Two different time exposures are presented. (*Lower*) Quantification of autophagic vesicles in ATG12-shRNA transduced or control Huh-7 cells, as measured by the frequency of cells containing punctate LC3-GFP. LC3-GFP expressing cells were treated or not with Tg. (*B*) Atg12-shRNA transduced and control Huh-7 were infected with HCV at an MOI of 0.01. (*Left*) The infectivity of their virus-containing cell supernatants was determined at different times postinoculation, as indicated. Results display average infectious titers, expressed as focus-forming units (ffu)/mL (mean ± SD; $n = 2$). (*Right*) The intracellular HCV RNA levels were determined by RT-qPCR 6 days postinoculation (MOI of 0.01) (mean ± SD; $n = 2$). GE, genome equivalent. (*C*) Extracellular and intracellular infectious virus and intracellular RNA production during 24 h in a single step infection of Atg12-shRNA transduced and control Huh-7 cells at MOI of 10 (mean ± SD; $n = 2$). (*Left*) Accumulation of extracellular and intracellular HCV infectious particles was determined 24 h after inoculation, and expressed as a percentage of that in control cells. (*Right*) Intracellular HCV RNA content was determined at 24 h post inoculation and expressed as a percentage of that in control cells. (*D*) Cell entry of HCVpp harboring E1E2 glycoproteins derived from HCV strains H77 (1a) or JHF-1 (2a) and control particles harboring the VSV-G glycoprotein (VSV-Gpp) expressed as a percentage of that in control cells (mean ± SD; $n = 4$). To assess the specificity of E1E2 glycoprotein-mediated cell entry, anti-E2 antibody (Ab) was added at 20 μg/mL.

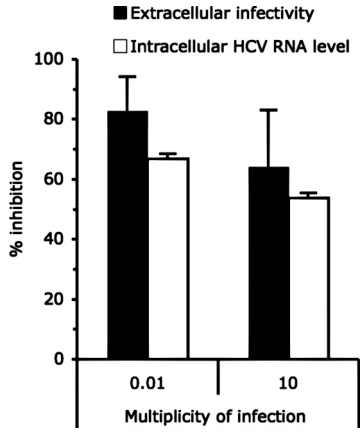

Fig. S3. Inhibition of HCV infection by a dominant-negative ATG5 mutant. Huh-7 cells transfected with a dominant negative mCherry-ATG5 mutant (K130R) and untransfected Huh-7 cells were infected with HCV at an MOI of 0.01 and 10. Huh-7 cells transfected with mCherry-ATG5 served as a transfection control. Results display the average inhibition of extracellular infectivity and intracellular HCV RNA levels in the dominant negative mCherry-ATG5 mutant expressing cells relative to mCherry-ATG5 expressing cells (mean ± SD; $n = 2$). Similar results were obtained when the dominant-negative mutant was compared with the untransfected Huh-7 cells.

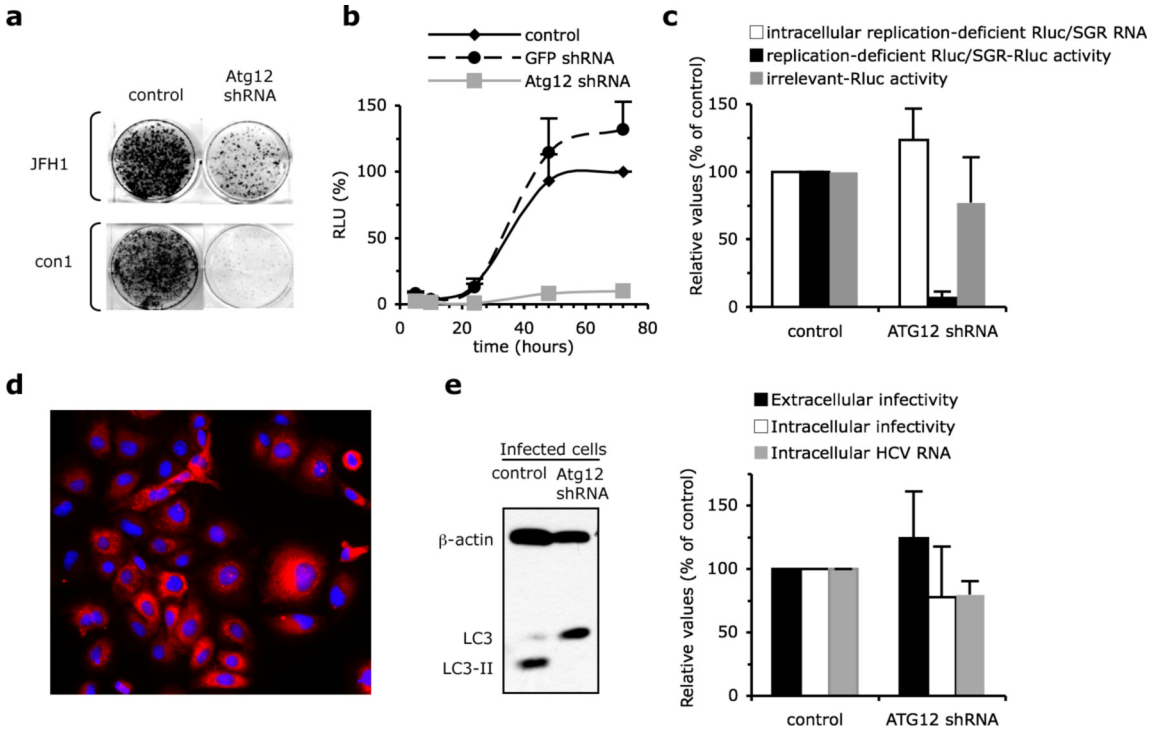

Fig. S4. ShRNA against Atg12 inhibits initial HCV RNA translation. Huh-7 cells were transduced with lentiviral vectors expressing shRNA against Atg12 (*A*) Analysis of JFH1 and Con1 neo/SGR replication in Atg12-shRNA transduced and control Huh-7 cells. Neomycin selected cells were fixed and stained with Crystal Violet. Results are representative of two independent experiments. (*B*) Analysis of Rluc/SGR translation/replication by monitoring Rluc activity in Atg12- and GFP-shRNA transduced and control Huh-7 cells at different times posttransfection. For each independent experiment, Rluc activity was normalized to cell density and expressed as a percentage of that determined in control cells at 72 h posttransfection (mean ± SD; $n = 3$). (*C*) Translation of replication-deficient Rluc/SGR RNAs in Atg12-shRNA transduced and control Huh-7 cells. Intracellular Rluc/SGR RNA levels and replication-deficient SGR Rluc activity were determined at 6 h posttransfection. In parallel transfections, Rluc activity expressed from pRL-TK plasmid (irrelevant-Rluc) was determined. For each independent experiment, Rluc activity was normalized to cell density and expressed as a percentage of that determined in control cells (mean ± SD; $n = 3$). (*D*) Immunostaining of E2 (red channel) in Huh-7 cells infected by HCV, before transduction with lentiviral vectors expressing shRNAs against autophagy proteins for the experiments shown in *E* and Fig. 5*B*. The nuclei were stained by Hoechst solution (blue). (*E*) Huh-7 cells that were virtually all infected by HCV were transduced with a lentiviral vector expressing shRNA against Atg12. (*Left*) LC3 lipidation detected by immonublotting analysis. β-actin expression was examined as a protein loading control. (*Right*) Extracellular and intracellular infectivity and intracellular HCV RNA levels were determined 8 days posttransduction and are expressed as a percentage of those in control cells (mean ± SD; $n = 2$).

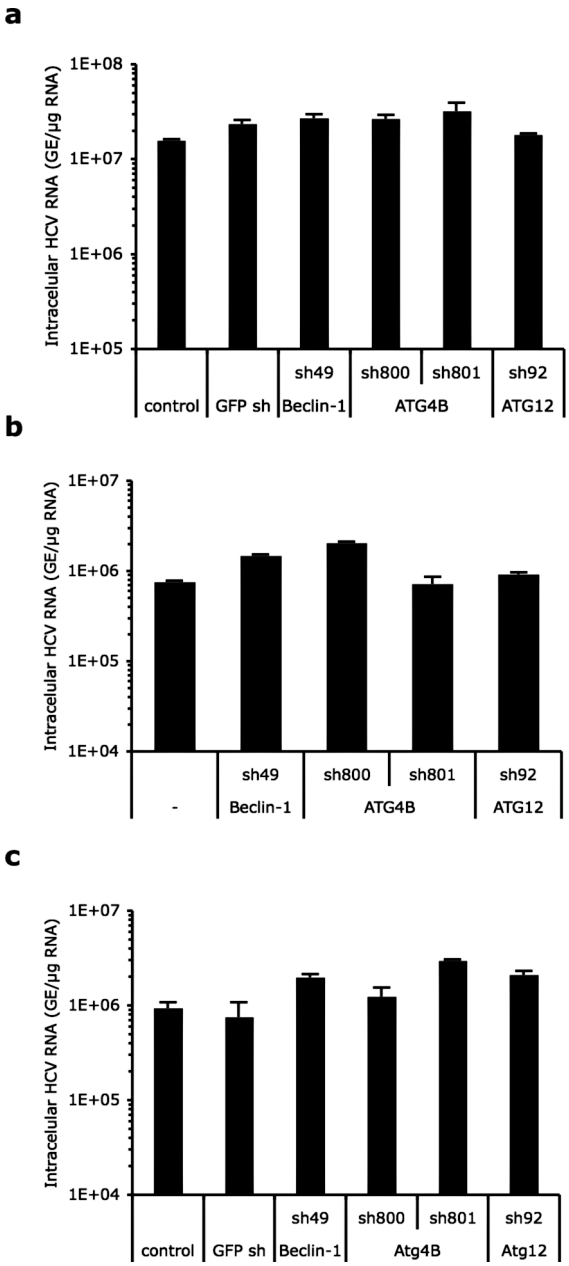

Fig. S5. Efficiency of RNA transfection in autophagy-protein deficient and control Huh-7 cells. Intracellular SGR RNA levels were determined at 6 h posttransfection by RT-qPCR. GE, genome equivalent. (*A*) JFH1 neo/SGR RNA transfection. (*B*) con1 neo/SGR RNA transfection. (*C*) Rluc/SGR RNA transfection.

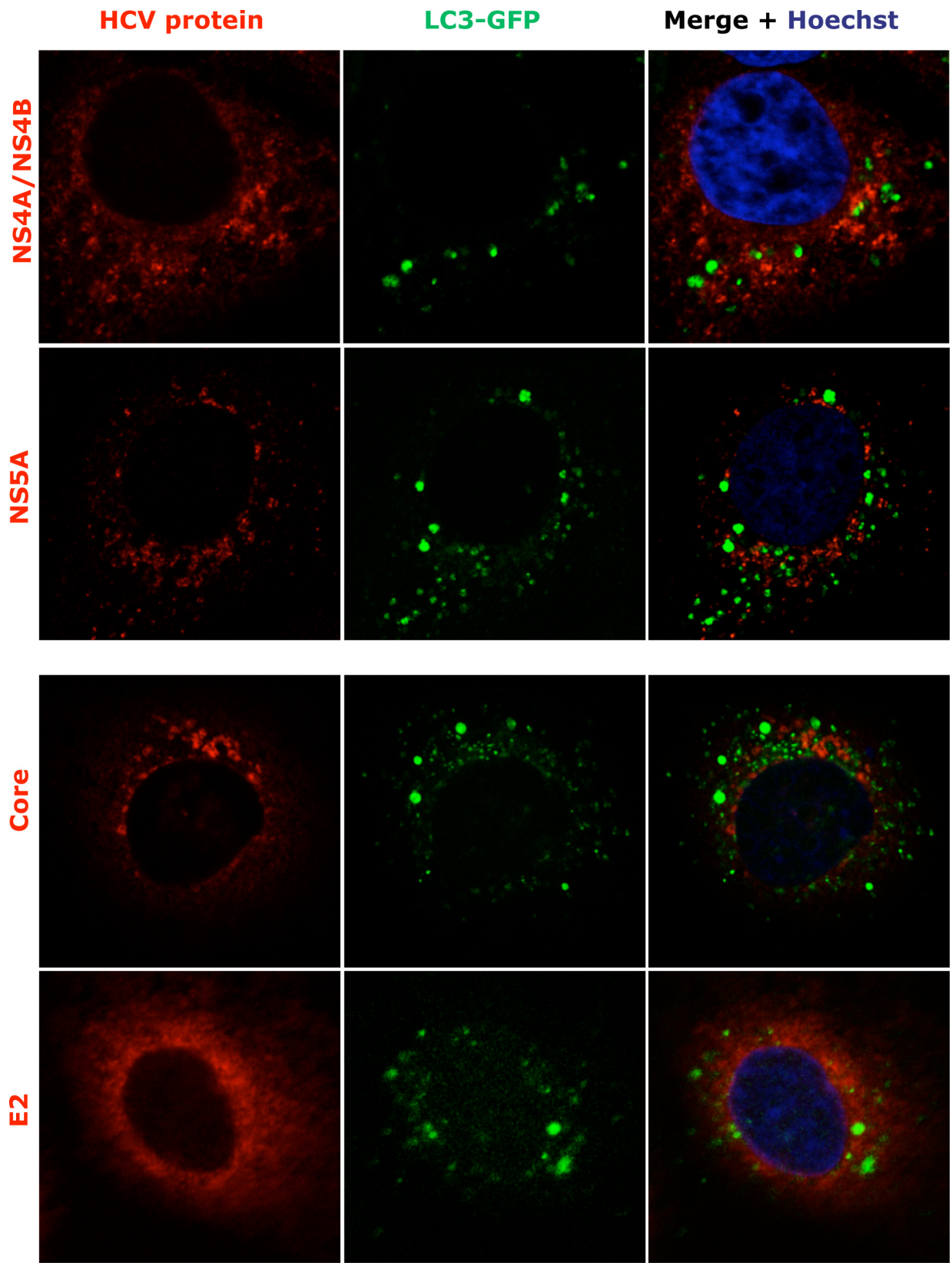

Fig. S6. HCV structural and nonstructural proteins do not co-localize with the autophagosome. Forty-eight hours after transfection with a plasmid encoding LC3-GFP, Huh-7 cells were infected with HCV, and 24 h later, they were stained with antibodies against E2, core, NS4A/NS4B and NS5A protein, as indicated (red channel). The nuclei were stained by Hoechst solution (blue).

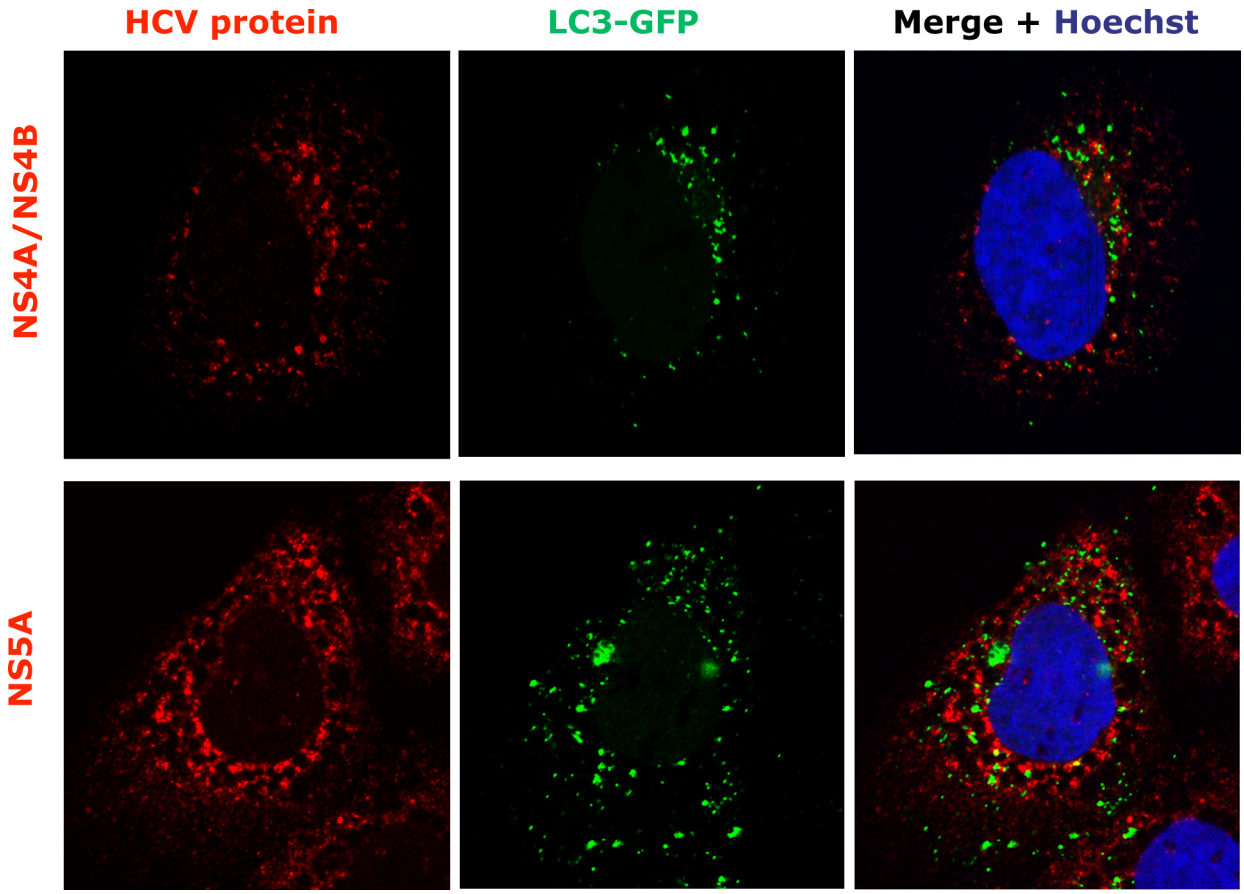

Fig. S7. HCV NS5A and NS4A/NS4B proteins do not co-localize with LC3-GFP in Bafilomycin A1-treated cells. After transfection with a plasmid encoding LC3-GFP, Huh-7 were infected with HCV, and 24 h later they were treated for 6 h with Bafilomycin A1 (200 nM). Cells were stained with antibodies against NS5A and NS4A/NS4B protein, as indicated (red channel). The nuclei were stained by Hoechst solution (blue). The ability of Bafilomycin A1 treatment to block lysosomal acidification was controlled by analyzing cell entry of pseudoparticles harboring the vesicular stomatitis virus (VSV) glycoprotein (VSV-Gpp), which mediates cell entry in a pH-dependent manner (2). As previously reported (2), 200 nM Bafilomycin A1 treatment for 6 h reduced VSV-Gpp cell entry by 97.78% (± 1.9%) compared to untreated cells.