SUPPLEMENTARY INFORMATION

Regulation of the hepatitis C virus RNA replicase by endogenous lipid peroxidation.

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SUPPLEMENTARY RESULTS

SKI does not alter SM abundance or viral RNA-dependent RNA polymerase (RdRp) activity

We considered that SKI might regulate HCV replication by modulating the abundance of sphingomyelin (SM) since there is evidence that it plays a key role in membrane localization of NS5B[26]. Consistent with this, inhibition of SM hydrolysis by spiroepoxide, a neutral sphingomyelinase (nSMase) inhibitor, enhanced H77S.3/GLuc replication by 1.8–fold while reducing HJ3-5/GLuc replication by 50% (**Supplementary Fig. 1b**). Inhibition of SM synthesis with D609 also inhibited H77S.3/GLuc by 60% without affecting HJ3-5/GLuc replication. Despite this, extensive mass spectrometry analyses revealed no significant changes in the total cellular abundance of SM species after SKI treatment, either in infected or uninfected cells (**Supplementary Fig. 3a**). SKI also had negligible effects on the abundance of other sphingolipids, cholesterol, triglyceride or lipid droplets, all important HCV host factors (**Supplementary Fig. 3b–d**)[21,23,68]. In addition, swapping an SM-binding domain identified previously in the NS5B polymerase[26] between H77S.3/GLuc and JFH1-QL/GLuc or HJ3-5/GLuc did not alter their responses to SKI (**Supplementary Fig. 4a,b**). Finally, SKI did not regulate the RdRp activities of the isolated H77S.3 or JFH1 NS5B proteins in a cell-based assay (**Supplementary Fig. 4c**)[69]. We conclude from these results that SKI does not regulate HCV replication by modulating the abundance or ability of SM to influence the catalytic activity of NS5B.

Genetic determinants of lipid peroxidation sensitivity in H77S.3 vs. JFH1 virus

We sought to identify individual genetic determinants of sensitivity to lipid peroxidation by swapping segments between the H77S.3 and JFH1 or HJ3-5 genomes. SKI and VE stimulation of H77S.3/GLuc replication was reduced but not eliminated by individually replacing either NS5A or NS5B with JFH1 sequence, while JFH1 resistance to lipid peroxidation could be mapped to the NS3–NS5B segment but not individual swaps of NS4AB, NS5A or NS5B (**Supplementary Fig. 8**). The involvement of NS3 could not be determined because exchanges in this region were not viable, but the phenotype thus appears to be dependent upon multiple components of the HCV replicase.

Mutations responsible for peroxidation resistance in TNcc

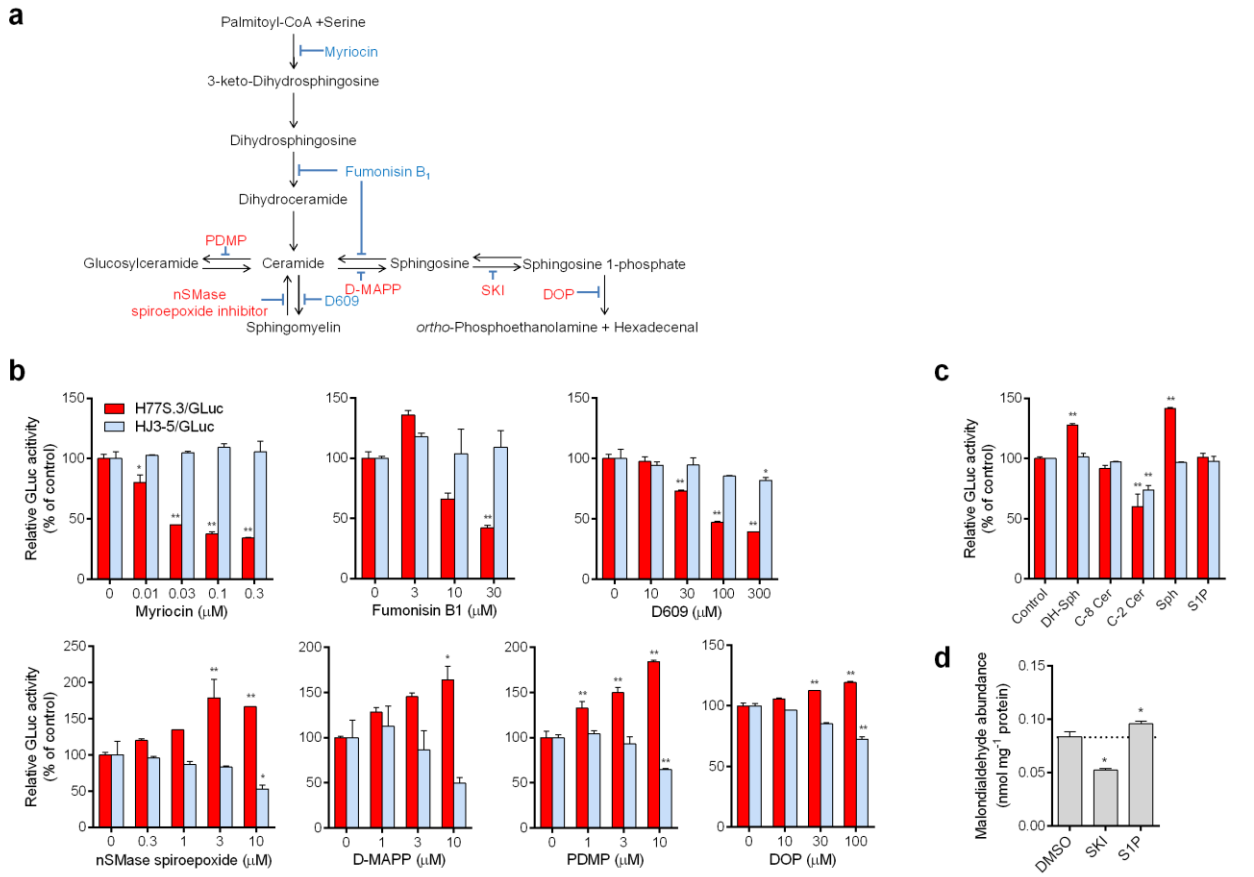
The introduction of individual TNcc mutations, or mutations located within specific nonstructural protein domains, into H77S.3/GLuc$_{IS}$ did not confer resistance, but many of these constructs failed to replicate (**Supplementary Fig. 10c**). To identify mutations that might compensate for the negative effects of the TNcc mutations when placed in the H77S.3$_{IS}$ backbone, we continuously cultured Huh-7.5 cells transfected with H77S.3$_{IS/8mt}$ RNA. Core protein expression increased progressively, and after 32 days a G1909S (NS4B) substitution was identified in 3 of 3 independent cultures (**Supplementary Fig. 11a**). Additional mutations, D2416G (NS5A) and G2963D (NS5B) were present in one culture. Each of these mutations

increased replication fitness when introduced into H77S.3/GLuc$_{IS/8mt}$, with G1909S causing a 70–fold increase, D2416G a 5-fold increase, and G2963D a 17–fold increase in GLuc expression, without altering resistance to lipid peroxidation (**Supplementary Fig. 11b**). Introducing all 3 mutations resulted in an 850–fold increase, exceeding GLuc produced by HJ3-5/GLuc. This RNA, designated H77D (no GLuc insertion), produced yields of infectious virus comparable to HJ3-5 or JFH1-QL that were not increased by VE supplementation (**Fig. 6b**).

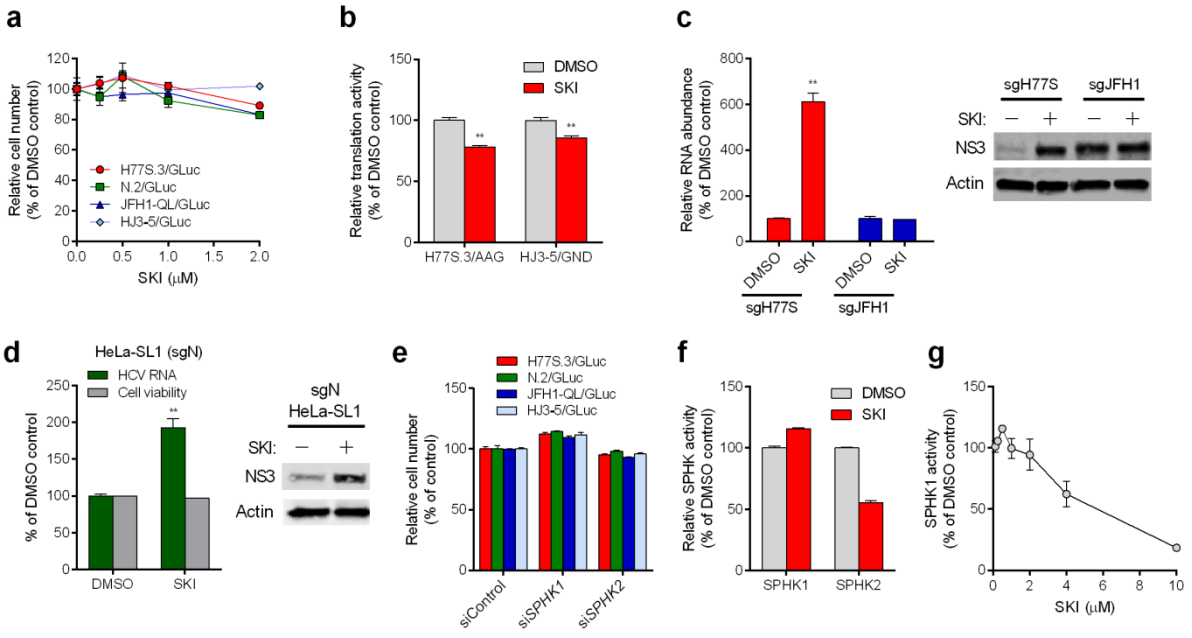
SUPPLEMENTARY REFERENCES

68. Herker, E., et al. Efficient hepatitis C virus particle formation requires diacylglycerol acyltransferase-1. *Nat. Med.* **16**, 1295-1298 (2010).
69. Ranjith-Kumar, C.T., Wen, Y., Baxter, N., Bhardwaj, K. & Cheng Kao, C. A cell-based assay for RNA synthesis by the HCV polymerase reveals new insights on mechanism of polymerase inhibitors and modulation by NS5A. *PLoS One* **6**, e22575 (2011).

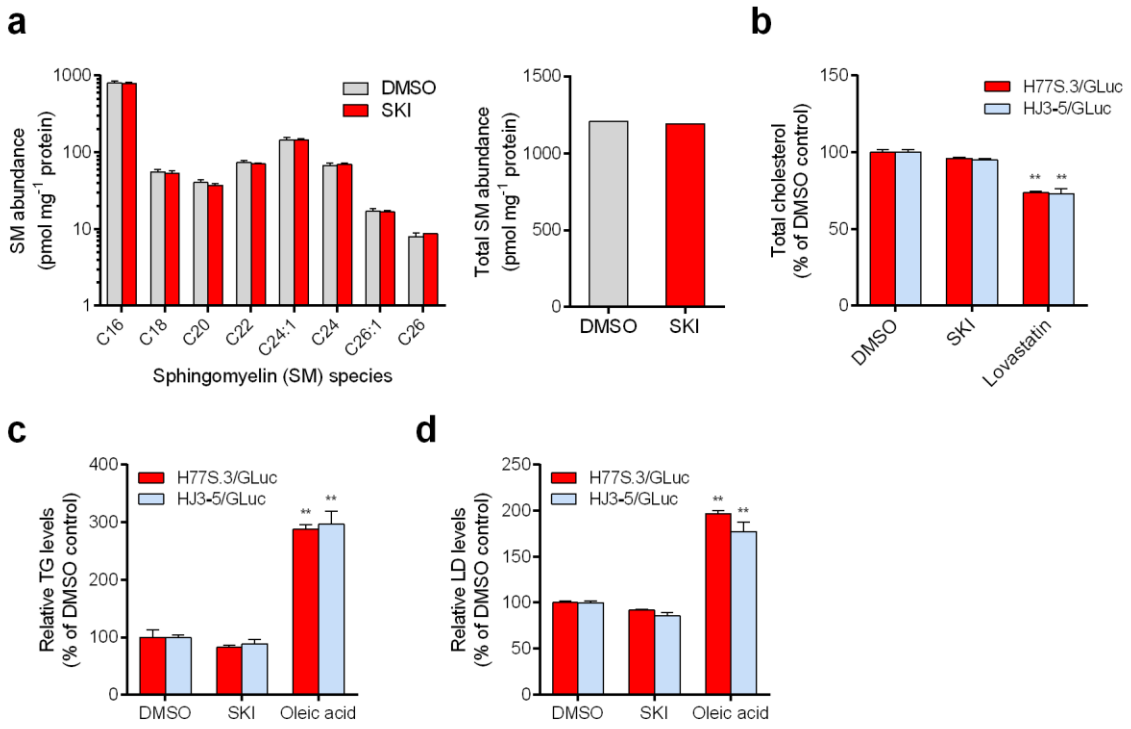
SUPPLEMENTARY FIGURES



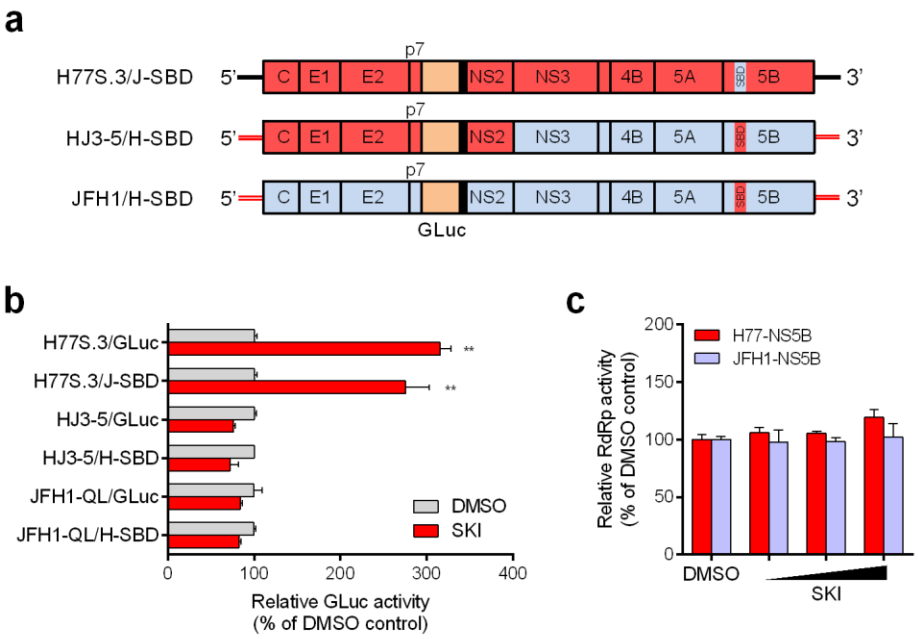
Supplementary Figure 1. Differential sensitivity of HCV strains to inhibitors targeting sphingolipid metabolism. (**a**) Scheme of sphingolipid metabolic pathways and inhibitors targeting the converting enzymes. Compounds shown in light blue and red indicate inhibitors and enhancers, respectively, of genotype 1a H77S.3 replication. (**b**) Effects of sphingolipid converting enzyme inhibitors on GLuc expression from Huh-7.5 cells transfected with either H77S.3/GLuc or HJ3-5/GLuc RNAs. GLuc secreted between 48–72 h after transfection is shown for D609 (SM synthase inhibitor), N-[2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl]-decanamide (PDMP, glucosylceramide synthase inhibitor), and 4-deoxypyridoxine (DOP, sphingosine 1-phosphate lyase inhibitor), and between 72–96 h for myriocin (serine palmitoyltransferase inhibitor), fumonisin B1 (ceramide synthase inhibitor), spiroepoxide (neutral SMase inhibitor), and D-erythro-2-tetradecanoylamino-1-phenyl-1-propanol (D-MAPP, ceramidase inhibitor). PDMP, D-MAPP, spiroepoxide, SKI (sphingosine kinase inhibitor), and DOP enhanced H77S.3/GLuc but suppressed HJ3-5/GLuc replication in a dose-dependent fashion, whereas myriocin and D609 inhibited H77S.3/GLuc without significantly affecting HJ3-5/GLuc replication (see also **Fig. 1b**). (**c**) Effects of sphingolipid supplementation on GLuc expression. Cells were transfected as in panel **b**, and culture media were collected and refed daily with fresh media containing 5 μM dihydrosphingosine (DH-Sph), C-2 or C-8 ceramides (Cer), sphingosine (Sph), or sphingosine 1-phosphate (S1P). Enrichment with sphingosine or dihydrosphingosine, but not S1P, significantly enhanced H77S.3 replication (~1.4–fold). In panels **b** and **c**, GLuc activities are shown as % relative to control, represent the mean ± s.e.m. from two replicate cultures, and are representative of two independent experiments. (**d**) Huh-7.5 cells were grown in the presence of DMSO, 1 μM SKI, or 5 μM S1P, and analyzed for intracellular malondialdehyde abundance at 48 h. Data shown represent the mean ± s.e.m. from three replicate cultures. (*P < 0.05, **P < 0.01 by two-way ANOVA).



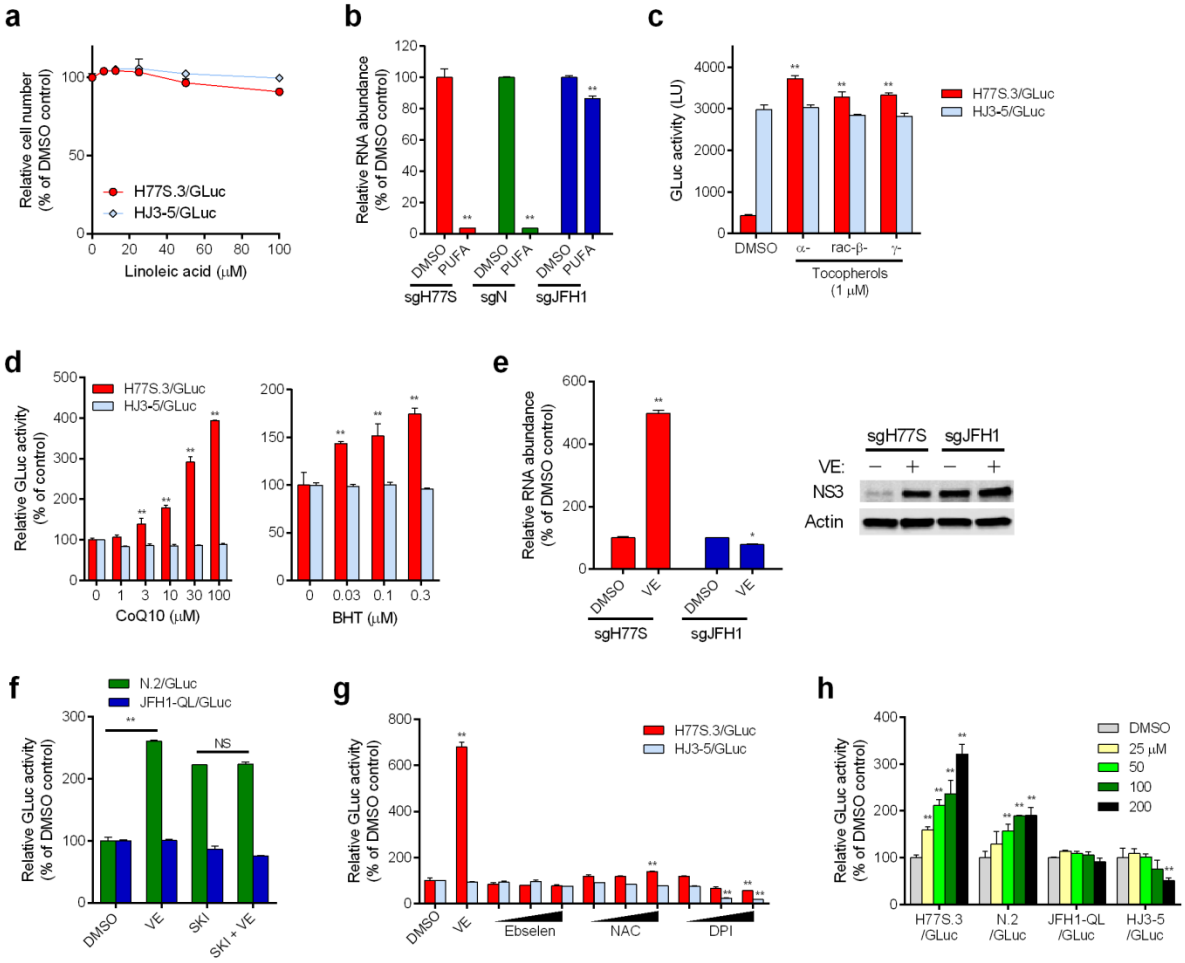
Supplementary Figure 2. SKI acts on the genotype 1 HCV replicase independently of the structural proteins without altering the efficiency of viral IRES-mediated translation and cell proliferation. (**a**) Relative cell numbers of Huh-7.5 cells transfected with mutated HCV/GLuc RNAs as indicated and treated with various concentrations of SKI for 72 h with daily replacement of media containing SKI. Results represent the mean ± s.e.m. from two replicate cultures. (**b**) Effect of SKI on IRES-mediated translation of H77S.3/GLuc and HJ3-5/GLuc RNAs containing lethal mutations in the NS5B RNA polymerase active site (GDD to AAG and GND, respectively). Huh-7.5 cells electroporated with 10 μg RNA were distributed into 6-well plates and incubated in media containing DMSO or 1 μM SKI and media containing GLuc harvested at 6 h after electroporation. Results are means ± s.e.m. from four replicate experiments (***P* < 0.01). (**c**) Effect of SKI on subgenomic H77S (sgH77S) and JFH1 (sgJFH1) replicons. Huh-7.5 cells harboring sgH77S or sgJFH1 were treated with DMSO (vehicle control) or 1 μM SKI for 48 h with daily replacement of media containing SKI. Viral RNA or protein abundance was determined by qRT-PCR (left panel) and immunoblotting with anti-NS3 antibody (right panel). Results represent the mean ± s.e.m. from three replicate cultures (***P* < 0.01). (**d**) Effect of SKI on subgenomic N replicon in non-hepatic (HeLa) cells. The cells were treated and analyzed as in **c** (***P* < 0.01). (**e**) Effect of *SPHK* knockdown on cell proliferation. Huh-7.5 cells transfected with HCV/GLuc RNA were split at 6 h after transfection, retransfected with siRNA at 24 h and grown for additional 72 h with daily replacement of the media. Relative cell numbers were presented as % relative to the cells transfected with nontargeting siRNA control (siControl). Results represent the mean ± s.e.m. from two replicate cultures, and are representative of two independent experiments. (**f**) SKI does not suppress SPHK1 activity at concentrations that enhance genotype 1 HCV replication. Recombinant SPHK1 and SPHK2 proteins (10 nM final) were incubated with DMSO or 1 μM SKI and the activity analyzed in the presence of 100 nM sphingosine as a substrate. (**g**) Huh-7.5 cells were treated with increasing doses (0.125, 0.25, 0.5, 1, 4, 10 μM) of SKI for 24 h, harvested into SPHK assay buffer, and analyzed for SPHK1 activity. Data are presented as % relative to DMSO control. Results represent the mean ± s.e.m. from two replicate experiments (***P* < 0.01).



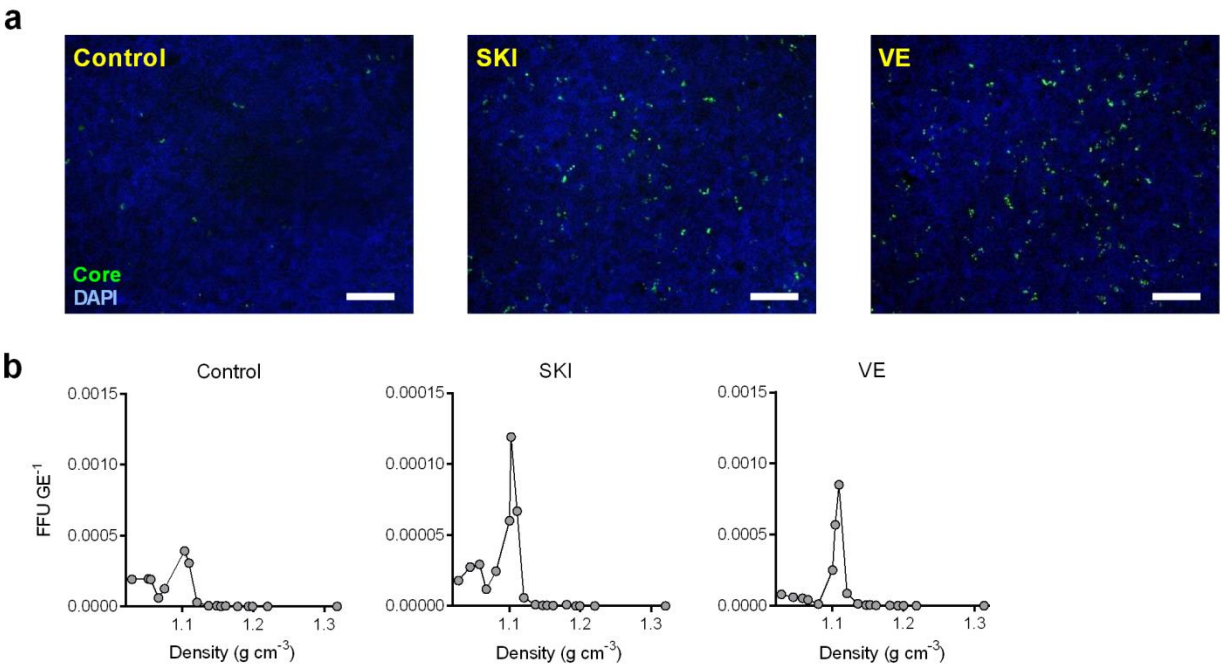
Supplementary Figure 3. Cellular sphingomyelin (SM), cholesterol, triglyceride (TG), and lipid droplet (LD) abundance is not affected by SKI. (**a**) Huh-7.5 cells transfected with H77S.3/GLuc RNA were treated with DMSO (vehicle control) or 1 μM SKI for 72 h with daily replacement of media containing SKI. Lipids were then extracted and sphingomyelin (SM) species determined by LC-MS/MS. Numbers indicate chain length followed by the number of double bonds in the fatty acid. Total SM abundance is shown on the right. Results represent the mean ± s.e.m. from three replicate cultures. (**b**) Huh-7.5 cells transfected with either H77S.3/GLuc or HJ3-5/GLuc RNA were treated with DMSO or 1 μM SKI for 72 h and total cholesterol levels determined by Amplex Red Cholesterol Assay Kit. Lovastatin (50 μM) was used as a positive control that reduces cholesterol abundance. The data are expressed as % relative to control. Results represent the mean ± s.e.m. from two replicate experiments (***P* < 0.01). (**c**) Cellular triglyceride (TG) abundance in Huh-7.5 cells treated as in **a** is presented as % relative to control. (**d**) Lipid droplets (LD) in Huh-7.5 cells treated as in **a** were stained with a lipophilic fluorescence dye, BODIPY 493/503, and analyzed by flow cytometry. Mean fluorescence intensities are presented as % relative to control. Results represent the mean ± s.e.m. from three replicate experiments (***P* < 0.01).



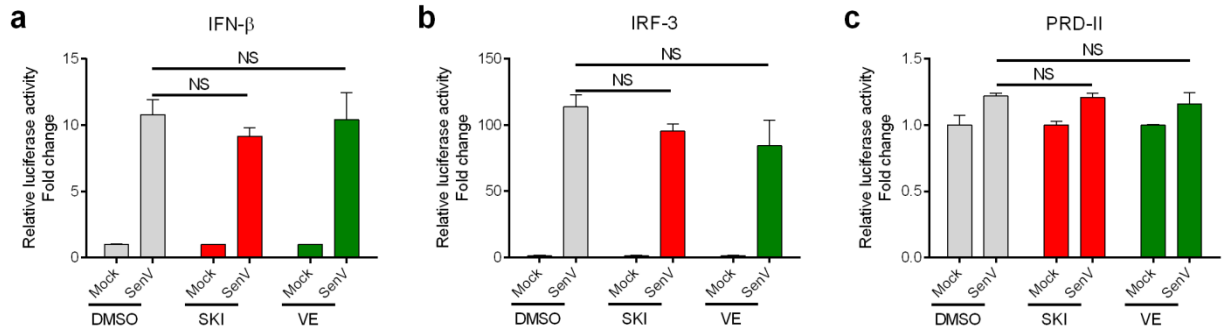
Supplementary Figure 4. The sphingomyelin-binding domain (SBD) in NS5B does not control sensitivity to SKI. (**a**) Schematic representation of SBD mutants. SBD derived from H77S.3 is shown in red (H-SBD) and that from JFH1 in blue (J-SBD). (**b**) Relative GLuc activity secreted between 24–48 h after transfection. Media containing DMSO (vehicle control) or 1 μM SKI were added to cells at 6 h after transfection and replaced at 24 h. GLuc activities are shown as % relative to DMSO control. Results represent the mean ± s.e.m. from two replicate cultures (**$P < 0.01$). (**c**) RNA-dependent RNA polymerase (RdRp) activity determined as described previously[69]. Increasing concentrations of SKI (0.1, 0.3 and 1 μM) were added to Huh-7.5 cells transfected with plasmids encoding H77c and JFH1 NS5B for a total of 48 h with replacement of media containing the same concentrations of SKI at 24 h. Results represent the mean ± s.e.m. from three replicate experiments.



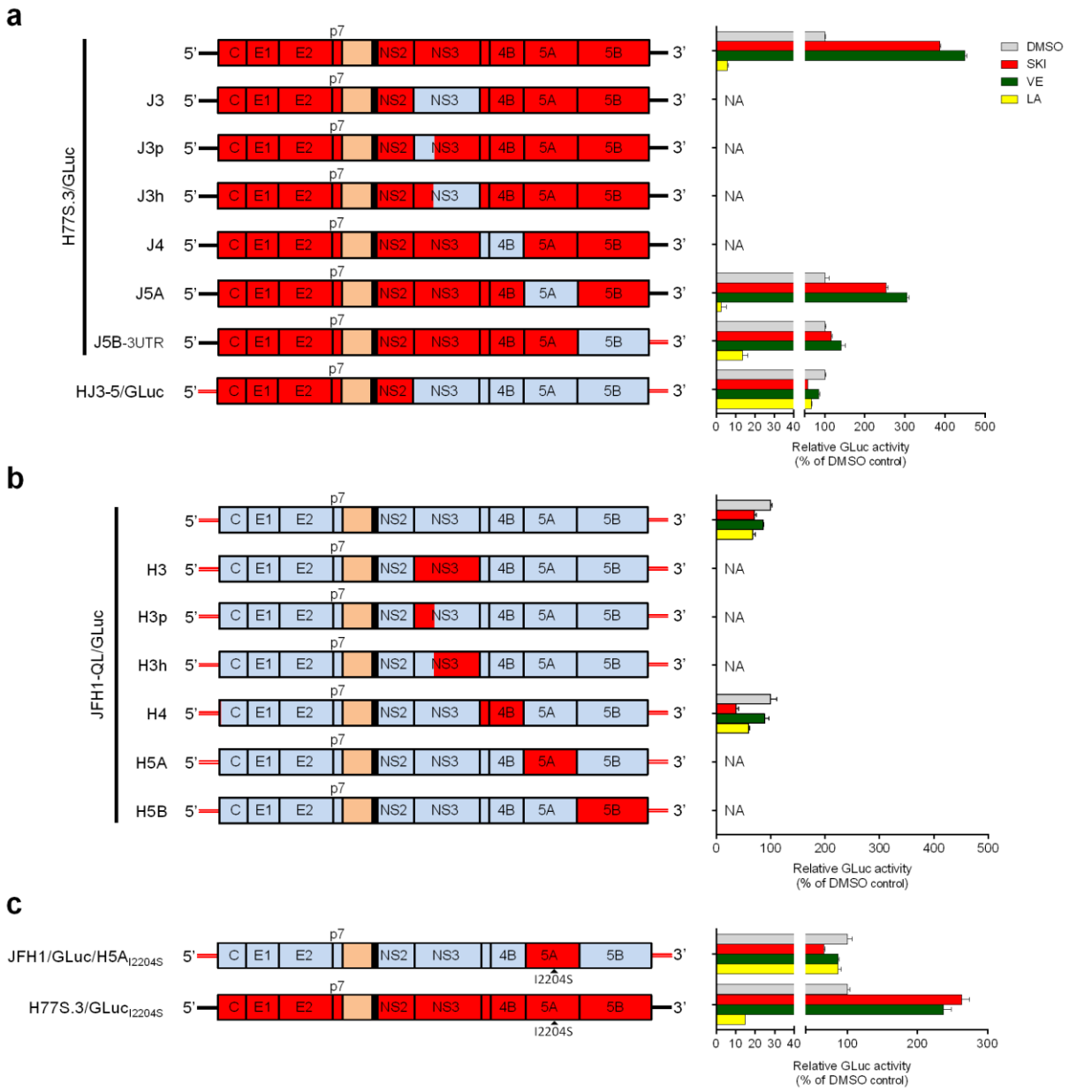
Supplementary Figure 5. Effects of lipid peroxidation on subgenomic HCV replicons and cell viability. (**a**) Effect of polyunsaturated fatty acid (PUFA) on cell proliferation. Huh-7.5 cells transfected with H77S.3/GLuc or HJ3-5/GLuc RNA were enumerated after 72 h growth in media supplemented with linoleic acid with daily replacement of media containing the PUFA. Data are presented as % cells relative to the control. (**b**) Huh-7.5 cells harboring subgenomic H77S (sgH77S), N (sgN), or JFH1 (sgJFH1) replicons were treated with 50 μM linoleic acid (PUFA) for 72 h with daily replacement of media containing PUFA. HCV RNA present in total cellular RNA was quantified by qRT-PCR. Data are expressed as percent RNA abundance relative to control. (**c,d**) Effects of various lipophilic antioxidants on H77S.3/GLuc and HJ3-5/GLuc replication. (**c**) α-, rac-β-, or γ-tocopherols, (**d**) coenzyme Q10 (CoQ10), or butylated hydroxytoluene (BHT) were added to Huh-7.5 cells transfected with the indicated reporter virus RNA with daily replacement of media containing each compound. Results shown represent the percent GLuc secreted between 48–72 h after transfection relative to control. (**e**) Effect of VE on subgenomic replicons. Huh-7.5 cells harboring sgH77S or sgJFH1 replicons were treated with 1 μM VE as in **b**. Relative HCV RNA abundance was determined by qRT-PCR (mean ± s.e.m. from 3 replicate cultures, left panel) and NS3 protein expression determined by immunoblotting (right panel). (**f**) Huh-7.5 cells transfected with N.2/GLuc or JFH1-QL/GLuc RNAs were treated with a combination of SKI and VE and analyzed as in **Fig. 2g** (right panel) in the main manuscript. (**g,h**) The effect of increasing doses of (**g**) water-soluble antioxidants, ebselen (1, 3, and 10 μM) and N-acetyl cysteine (NAC) (0.3, 1, and 3 mM), and the NADPH oxidase inhibitor diphenyleneiodonium (DPI) (0.3, 1, and 3 μM), and 1 μM VE for comparison, and (**h**) a monounsaturated fatty acid (oleic acid, 25–200 μM) on replication of different HCVs. Huh-7.5 cells were transfected with viral RNAs and treated with compounds in lieu of PUFA as in **a**. Results represent the mean ± s.e.m. from two (**a**, **c**–**h**) or three (**b**) replicate experiments.



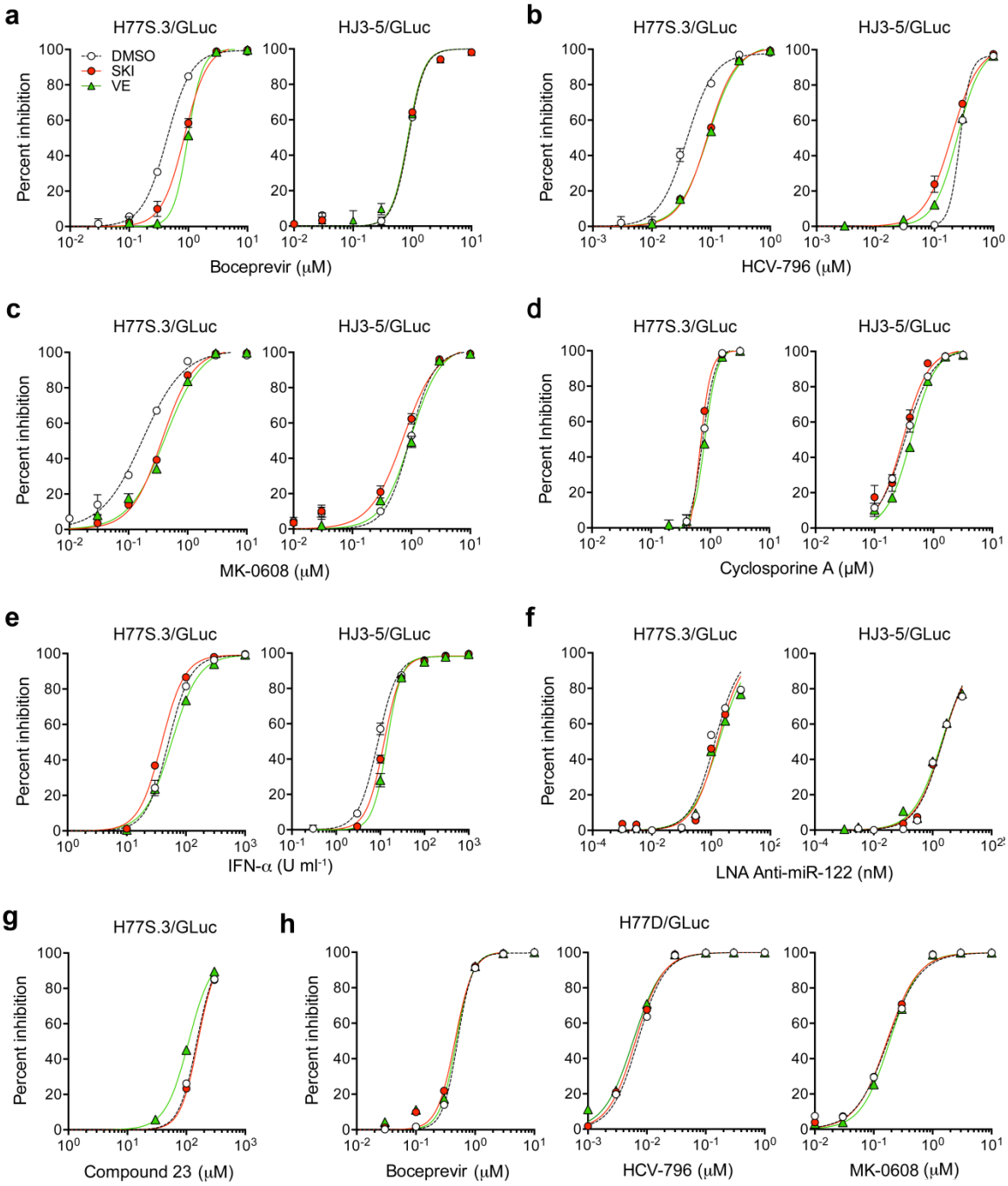
Supplementary Figure 6. SKI and VE increase yields of infectious virus particles from H77S.3 RNA-transfected cells and enhance specific infectivity of H77S.3. (**a**) Infectivity of infectious H77S.3 virus particles produced from H77S.3 RNA-transfected Huh-7.5 cells. Infectious H77S.3 particles produced between 72–96 h after transfection were inoculated on naïve Huh-7.5 cells and stained for core protein expression (green) at 72 h after infection. Nuclei were counterstained with DAPI (blue). Scale bar, 400 μm. 'Control' = DMSO. (**b**) SKI and VE treatment enhance specific infectivity of H77S.3. Data are expressed as the virus titer (FFU count) divided by RNA copy number (GE = genome equivalent) in each fraction of an isopycnic iodixanol gradient, plotted according to density. FFU and RT-PCR results on each fraction are means from triplicate assays. Results shown are representative of two independent experiments.



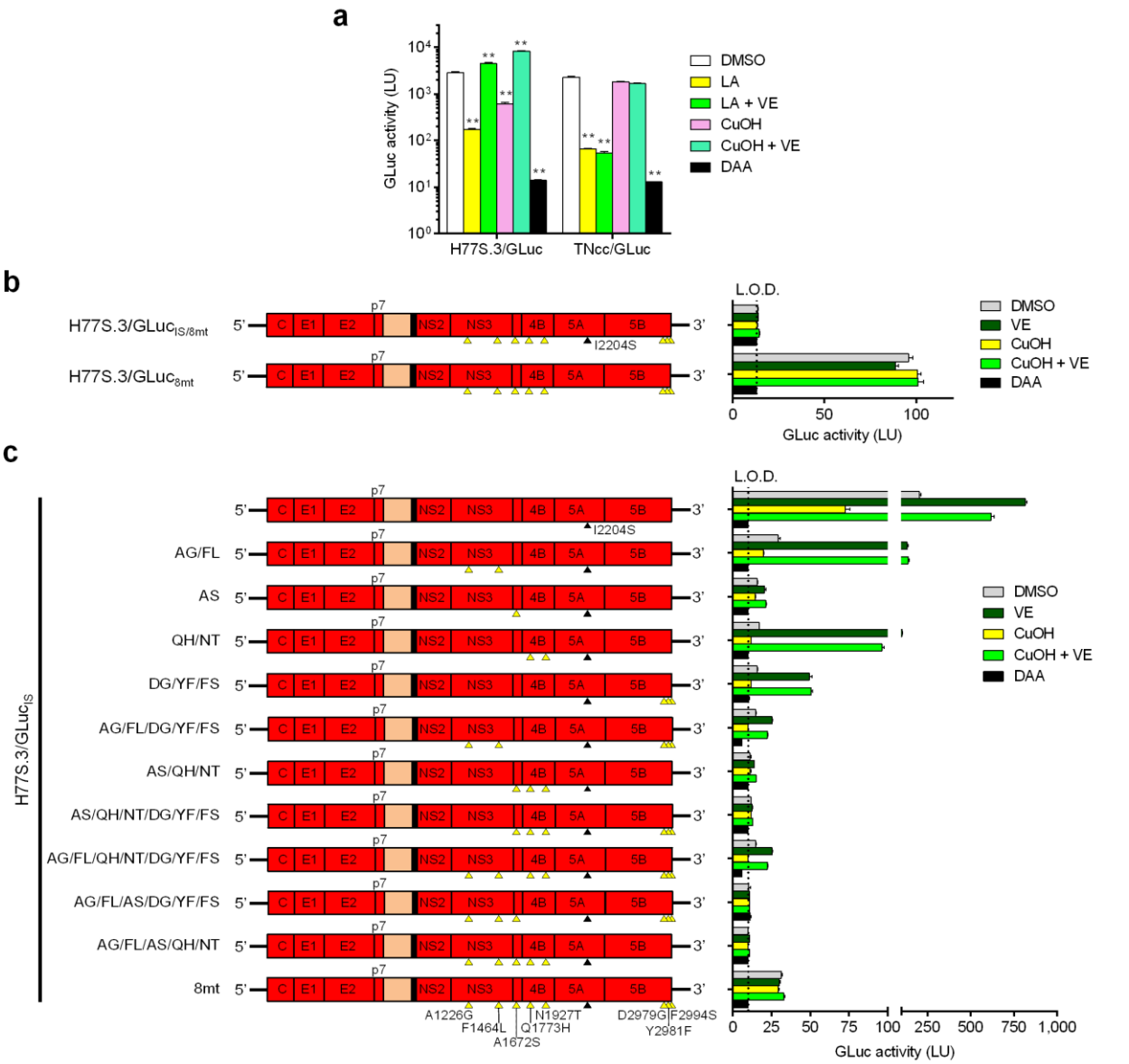
Supplementary Figure 7. SKI and VE supplementation have no significant effect on retinoic acid-inducible gene I (RIG-I)-mediated antiviral signaling pathways. FT3-7 cells, a clonal derivative of Huh-7 cells, grown in the presence of DMSO (vehicle control, open bars), 1 μM SKI (red bars) or 1 μM VE (green bars) in 24-well plates were co-transfected with 200 ng of interferon-β promoter reporter plasmids: (**a**) pIFN-β-Luc, (**b**) p4xIRF-3-Luc, or (**c**) pPRD-II-Luc, together with 0.4 ng pRL-Luc (transfection control), then challenged 24 h later by infection with Sendai virus (SenV, 100 HAU ml^{-1}). Cells were harvested 16 h later for dual luciferase assay. Data are expressed as fold-change in normalized luciferase activity relative to mock-infected samples, and represent the mean ± s.e.m. from three replicate experiments.



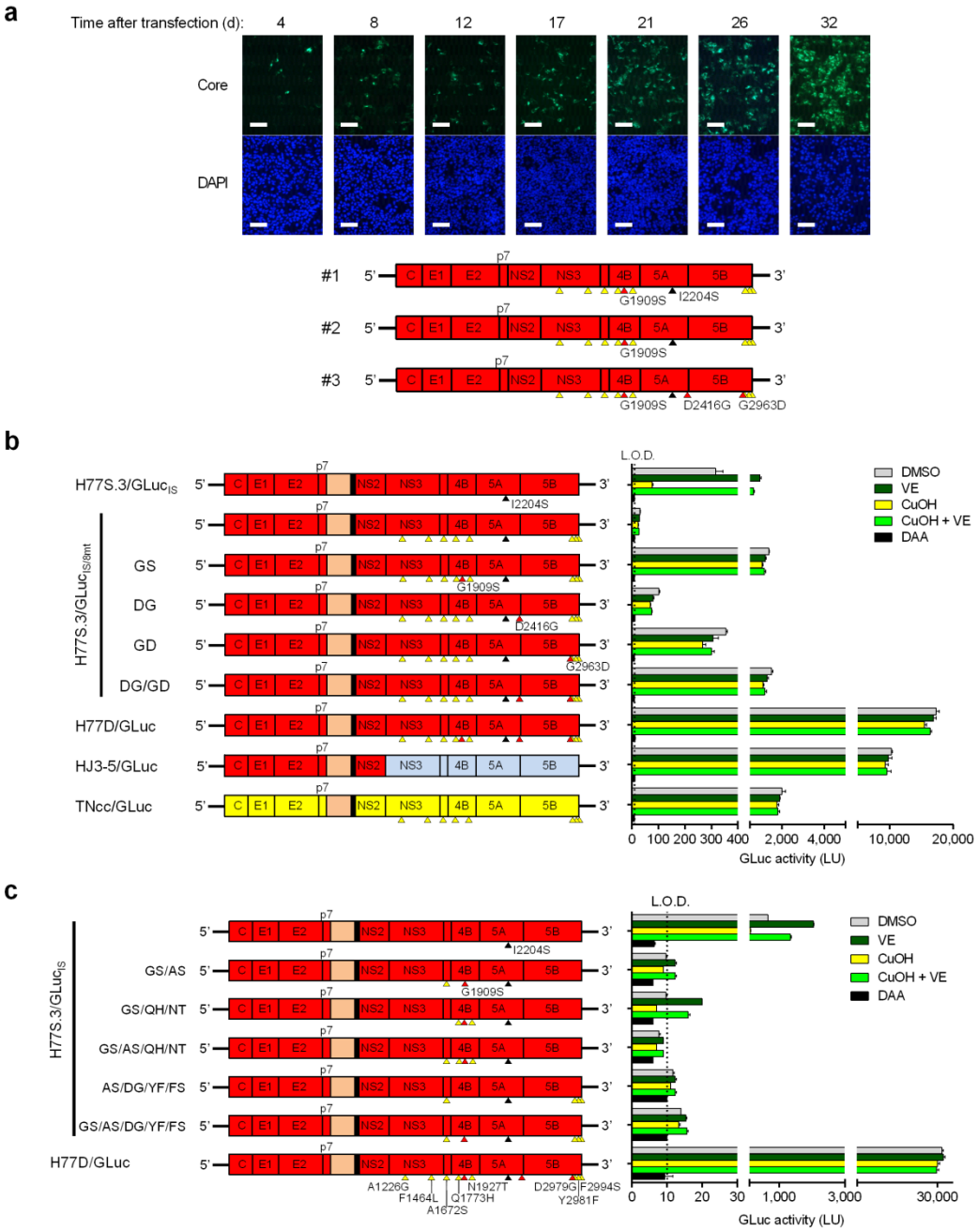
Supplementary Figure 8. Impact of swapping nonstructural protein coding regions between H77S.3/GLuc and JFH1-QL/GLuc on viral sensitivity to lipid peroxidation. All mutants were constructed in the background of GLuc-expressing reporter genomes. (**a**) H77S.3/GLuc mutants that express individual nonstructural proteins derived from JFH1 were constructed as shown on the left. Media containing DMSO (vehicle control), 1 μM SKI, 1 μM VE, or 50 μM linoleic acid (LA) were added to cells 6 h after RNA transfection and replaced at 24 h. GLuc activity secreted between 24–48 h after the addition of compounds is shown as the percent relative to the DMSO control. NA, not available because the chimeric genome is not viable due to sequence incompatibilities. (**b**) JFH1-QL/GLuc mutants expressing nonstructural proteins derived from H77S.3. The experiments were carried out as in **a**. Only JFH1/H4 was capable of replication. It remains non-responsive to SKI and VE and resistant to LA. (**c**) A JFH1/GLuc chimera with substitution of NS5A with sequence from H77S.3 became replication competent with a point mutation, I2204S (H77 polyprotein coordinates) reversing a cell culture-adaptive mutation in H77S.3. NS5A$_{I2204S}$ mutants were constructed in both the H77S.3/GLuc and JFH1-QL/GLuc/H5A genetic backgrounds and assessed for sensitivity to lipid peroxidation as in **a**. Results represent the mean ± s.e.m. from two replicate cultures.



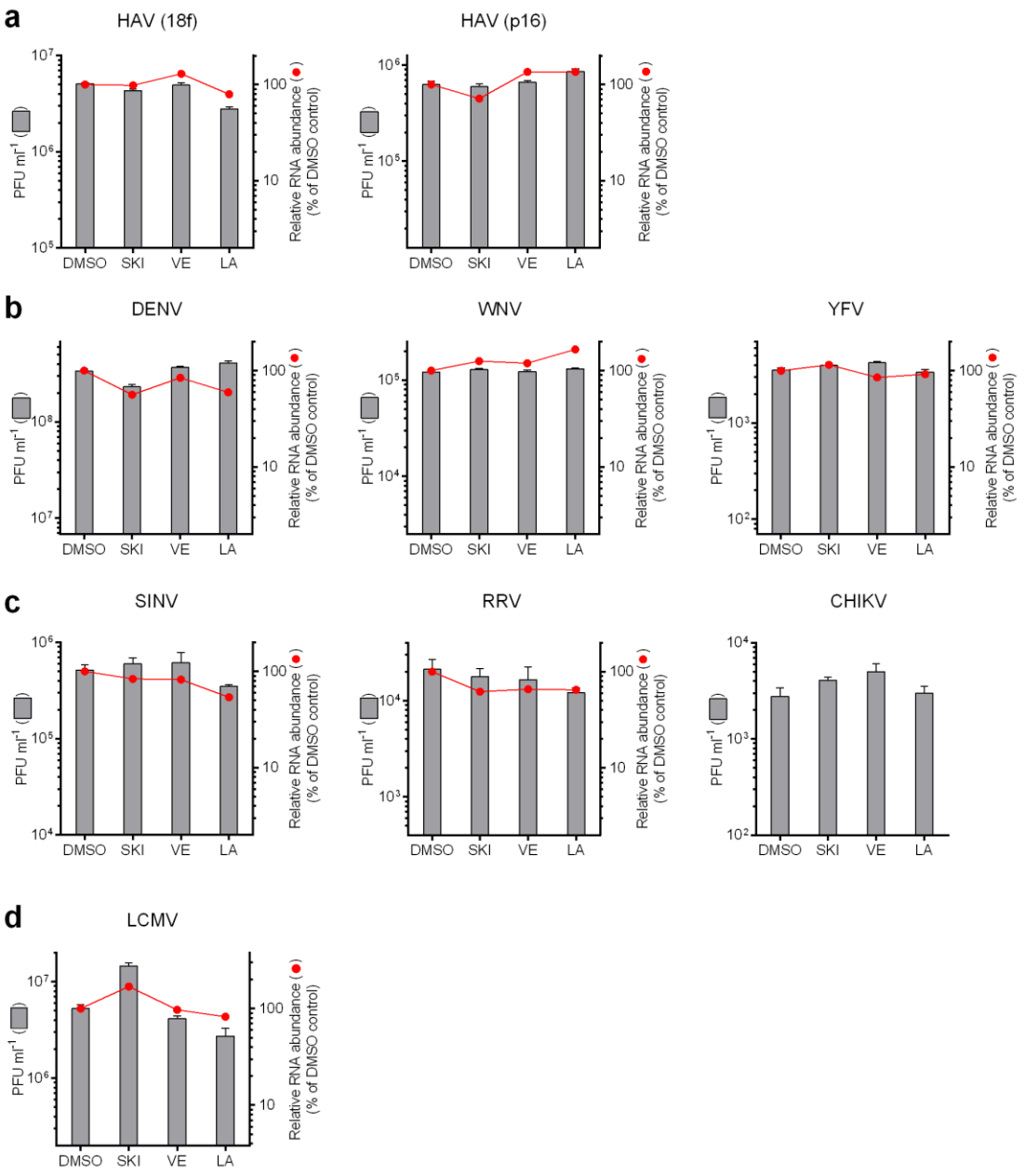
Supplementary Figure 9. (**a**–**g**) Activity of direct-acting and indirect, host-targeting antivirals against H77S.3/GLuc and HJ3-5/GLuc viruses in the presence of SKI or VE (1 μM each) versus DMSO vehicle. (**a**) Boceprevir, a linear ketoamide inhibitor of the NS3/4A protease. (**b**) HCV-796, an allosteric inhibitor of the NS5B RNA-dependent RNA polymerase. (**c**) MK-0608, a nucleoside inhibitor of NS5B. (**d**) Cyclosporine A, an inhibitor of HCV replication that targets the essential host factor, cyclophilins. (**e**) Recombinant human interferon (IFN)-α. (**f**) LNA anti-miR-122, a locked nucleic acid (LNA) antagomir of miR-122. (**g**) Compound 23, a PI4KIIIα inhibitor. (**h**) Inhibition of the lipid peroxidation-resistant H77D/GLuc virus by DAAs: boceprevir, HCV-796, and MK-0608. Compare with the peroxidation-sensitive H77S.3 virus in panels (**a**–**c**). Assays were carried out as described in the legend to **Fig. 5d** in the main manuscript. Nonlinear regression curves were fit according to a four-parameter (variable slope) vs. log concentration inhibitor model. Results represent the mean ± s.e.m. of triplicate cultures.



Supplementary Figure 10. Mapping TNcc-derived mutations that confer resistance to lipid peroxidation. (**a**) PUFA suppresses TNcc replication independently of induction of lipid peroxidation. The TNcc genome was modified to incorporate the GLuc sequence between the p7 and NS2 coding regions and the replication of TNcc/GLuc was compared with H77S.3/GLuc. Huh-7.5 cells were treated with 50 μM linoleic acid (LA), LA plus 1 μM VE, 10 μM CuOH and CuOH plus VE, or a DAA (sofosbuvir, 30 μM). Data shown represent GLuc secreted between 48–72 h. Because co-treatment with VE did not reverse the inhibitory effect of LA, indicating that LA suppresses replication of this virus independently of lipid peroxidation, CuOH was used in subsequent experiments to assess sensitivity to lipid peroxidation. (**b**) Removal of the cell culture-adaptive S2204I mutation (I2204S substitution) is required for compatibility with TNcc-derived mutations in H77S.3. Cells transfected with H77S.3 mutant RNAs were treated with VE, CuOH, CuOH plus VE, or DAA. H77S.3 RNA with 8 TNcc-derived 8 mutations (yellow arrowheads) was not viable without the I2204S substitution. Data shown represent GLuc activity secreted into the cell culture media between 48–72 h. L.O.D. = limit of detection. (**c**) Cells transfected with H77S$_{IS}$ mutants with individual TNcc-derived mutations or the different combinations of them were transfected and treated as in **b**. Results shown represent GLuc activity secreted between 48–72 h and are the mean ± s.e.m. values from triplicate cultures and are representative of two (**a**) or three (**b,c**) independent experiments. L.O.D. = limit of detection.



Supplementary Figure 11. Selection of compensatory mutations that enable efficient replication of H77S.3$_{IS}$ bearing TNcc-derived mutations. (**a**) Adaptation of H77S.3$_{IS/8mut}$ virus to replication in Huh-7.5 cells over a period of 32 d following transfection with the viral RNA. (top) Cells were immunostained for core protein expression (green) at the indicated times. Nuclei were counterstained with DAPI (blue). Scale bar, 200 μm. (bottom) Amino acid substitutions encoded by viral RNAs isolated from 3 independent cultures at d 32. Red arrowheads indicate newly identified compensatory mutations. The I2204S (black arrowhead) substitution and TNcc-derived mutations (yellow arrowheads) are also indicated. (**b,c**) Huh-7.5 cells transfected with H77S.3/GLuc$_{IS}$ RNAs into which compensatory mutations identified in panel **a** were introduced. Cells were treated with 1 μM SKI, 1 μM VE, 10 μM CuOH, CuOH plus VE, 30 μM sofosbuvir (DAA), or DMSO (vehicle control) beginning 6 h after RNA transfection. Data shown represent mean ± s.e.m GLuc secreted between 48–72 h from triplicate cultures and are representative of two independent experiments. L.O.D. = limit of detection.



Supplementary Figure 12. Other RNA viruses are resistant to endogenous and PUFA-induced lipid peroxidation in Huh-7.5 cells. (**a**) Huh-7.5 cells were inoculated with cytopathic (18f, left panel) and noncytopathic (p16, right panel) cell culture-adapted variants of the HM175 strain of hepatitis A virus (HAV) at an m.o.i of 1 for 2 h, followed by addition of media supplemented with DMSO (vehicle control), 1 μM SKI, 1 μM VE, or 50 μM LA with daily replacement of media until harvest after 72 h of infection. Intracellular HAV RNA levels were determined by qRT-PCR and are shown as % relative to DMSO control. Infectious virus titers in supernatant fluids after 72 h of infection were determined by an infectious focus formation assay and presented as focus-forming units (FFU) ml^{-1}. (**b**) Flaviviruses, including dengue virus (DENV, serotype 2 strain 16681), West Nile virus (WNV, NY99 strain), and yellow fever virus (YFV, 17D strain), (**c**) alphaviruses, including Sindbis virus (SINV, AR86 strain), Ross River virus (RRV, T48 strain), and Chikungunya virus (CHIKV, SL15649 strain), and (**d**) lymphocytic choriomeningitis virus (LCMV, Clone 13 strain) do not respond to changes in cellular lipid peroxidation. Huh-7.5 cells were inoculated with viruses at an m.o.i of 1 (DENV and LCMV), 0.5 (WNV), 0.1 (YFV), or 0.01 (alphaviruses), then treated as in **a**. Results represent the mean ± s.e.m. from three replicate cultures.