A Chemically-Disrupted Proximity System for Controlling Dynamic Cellular Processes

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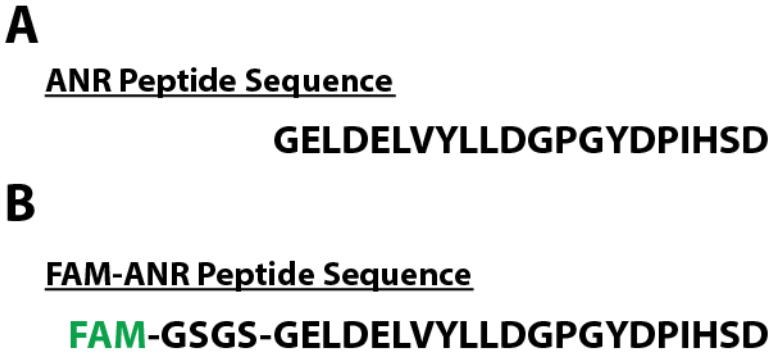


Figure S1. ANR peptide sequence. (A) Amino acid sequence of the ANR portion of the NS3a-based CDP system. ANR is based on the Cp5 peptide scaffold described in Kügler et al. *J. Biol. Chem.* **2012**, *287*, 39224-32. (B) Structure of the ANR probe used in fluorescence polarization assays. The probe contains fluorescein (FAM), connected by a flexible glycine and serine linker, fused to the *N*-terminus of ANR.

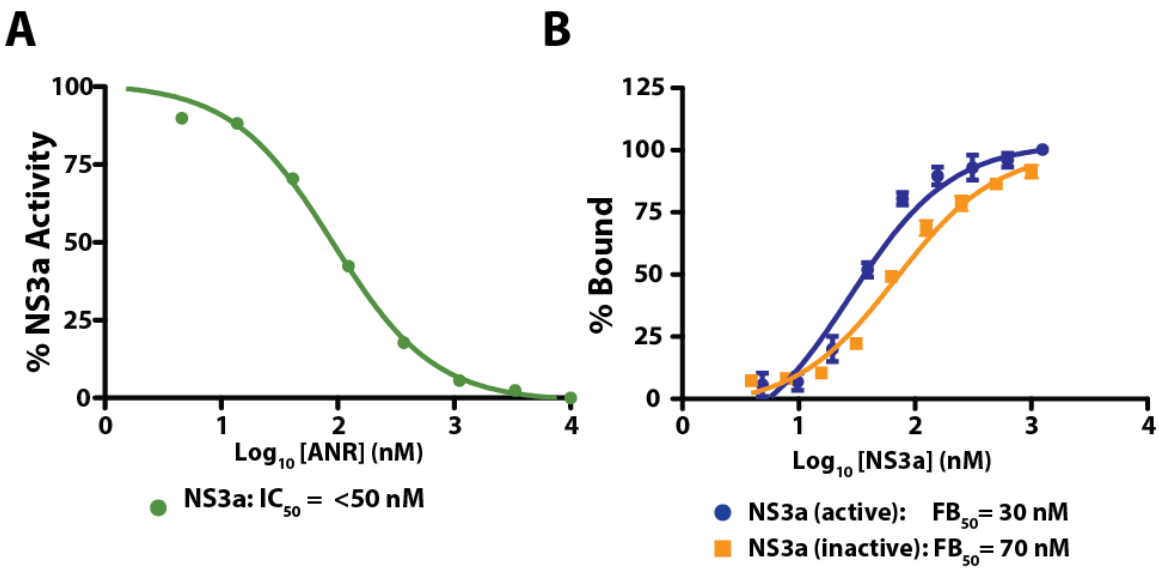
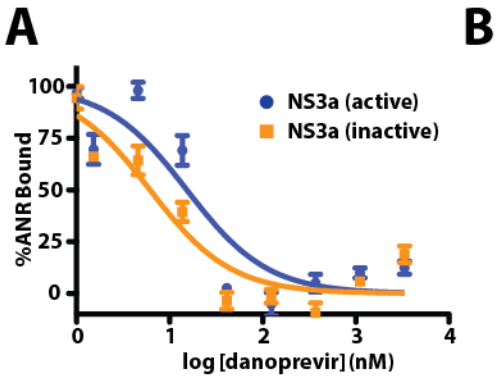


Figure S2. Characterization of ANR's affinity for NS3a. (A) The IC_{50} value of an ANR-GST fusion against NS3a activity in a FRET-based protease assay (Taliani et al *Anal. Biochem.* **1996** *240*, 60-67). The apparent IC_{50} value of ANR is less than the concentration of NS3a protease used in the assay. (B) The 50% fractional binding (FB_{50}) value of FAM-ANR (**Figure S1B**) for catalytically active NS3a (NS3a active) and a catalytically inactive S139A variant (NS3a inactive) determined using a fluorescence polarization binding assay. Values shown are the mean of n=3.



B

Variant	FB_{50} (nM)
NS3a (active)	<75
NS3a (inactive)	<75

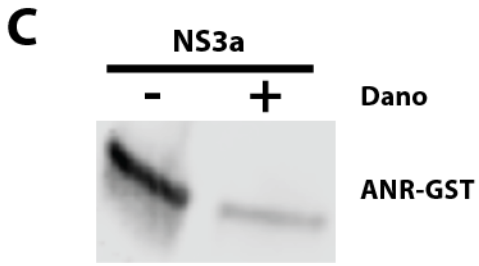


Figure S3. Danoprevir competes with ANR for NS3a binding. (A) Danoprevir titration in a fluorescence polarization competition binding assay with FAM-ANR (n=3). (B) FB_{50} values of danoprevir for active NS3a (NS3a active) and a catalytically inactive S139A variant (NS3a inactive) determined from the titration shown in (A). Danoprevir's apparent IC_{50} is less than the concentration of NS3a active and inactive (75 nM) used in the binding assay. (C) Danoprevir inhibits the ability of immobilized NS3a inactive to pull down ANR-GST. Biotinylated NS3a inactive was immobilized on streptavidin-agarose beads and 5 μM ANR-GST was added with danoprevir (10 μM) or DMSO. Following incubation, beads were washed, and bound ANR-GST was eluted. Eluted samples were subjected to SDS-PAGE and immunoblotting with an anti-GST antibody.

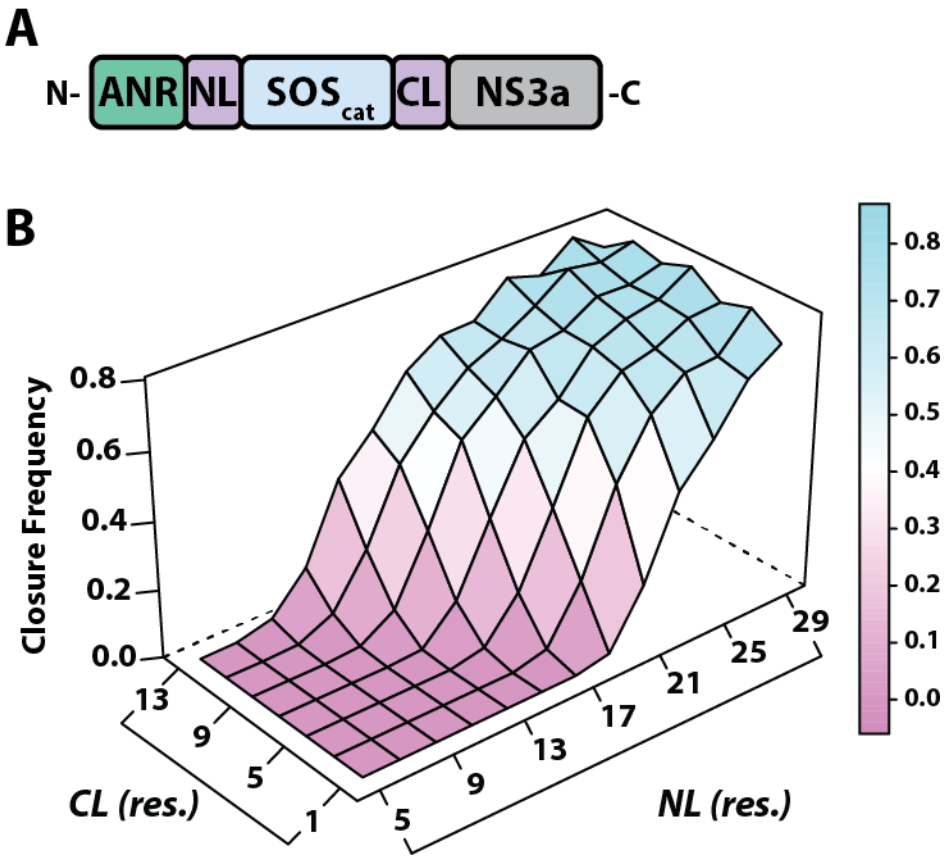


Figure S4. Computational design of NS3a-CIAR. (A) The NS3a-CIAR construct used in computational modeling with RosettaRemodel. The *C*-terminus of ANR is fused to the *N*-terminus of SOScat through a flexible *N*-terminal linker (NL). The *C*-terminus of SOScat is fused to the *N*-terminus of NS3a through a flexible *C*-terminal linker (CL). Combinations of NL and CL lengths ranging from 5-29 residues and 1-13 residues, respectively, were evaluated computationally. (B) RosettaRemodel closure frequency of NS3a-CIAR designs. Closure frequencies of the NS3a-CIAR constructs were determined as a function of NL and CL lengths by RosettaRemodel and plotted as the number of successfully closed trajectories divided by 1000 for each of the linker length pairs. We assigned an arbitrary lower bound on the chain closure frequency at 10%**.** Pairs of linker lengths that give fewer chain closure events would likely not allow intramolecular formation of the NS3a/ANR complex.

Linkers	Mean Center-of-Mass Distance (Å)	SD	Clos.Freq
15-5	17.7502	3.9056	0.020
15-9	18.5625	4.3824	0.265
17-7	18.6433	4.0772	0.309
15-7	18.9188	4.4453	0.106
17-5	19.1694	3.9838	0.140
17-9	19.2955	4.9010	0.490
19-7	19.4044	4.6649	0.500
19-9	19.3501	4.9038	0.576
13-11	19.4370	4.7682	0.201

Figure S5. RosettaRemodel-determined values for the mean center-of-mass distance, standard deviation (SD) of this mean, and closure frequency of exemplary NS3a-CIAR designs. Values obtained from RosettaRemodel (**Figures 2B, 2C**, **S4**) were determined as a function of NL and CL lengths. Linker lengths are represented as NL-CL, with the values shown referring to the number of residues in each linker. We reasoned that the ability of the NS3a/ANR complex to autoinhibit SOScat likely depends on its overlap with the RAS-binding site of SOScat. The mean center-of-mass distance describes the average computed distance between the center-of-mass of SOScat-bound RAS and the NS3a/ANR complex. Designs with the smallest mean center-of-mass distance have the highest relative degree of overlap between the NS3a/ANR complex and SOScat-bound RAS. We used the standard deviation (SD) of this mean to predict the energetic penalty for the NS3a/ANR complex not adopting the average position relative to SOScat. Designs with the smallest SD have the most tightly clustered NS3a/ANR complexes in output PDBs.

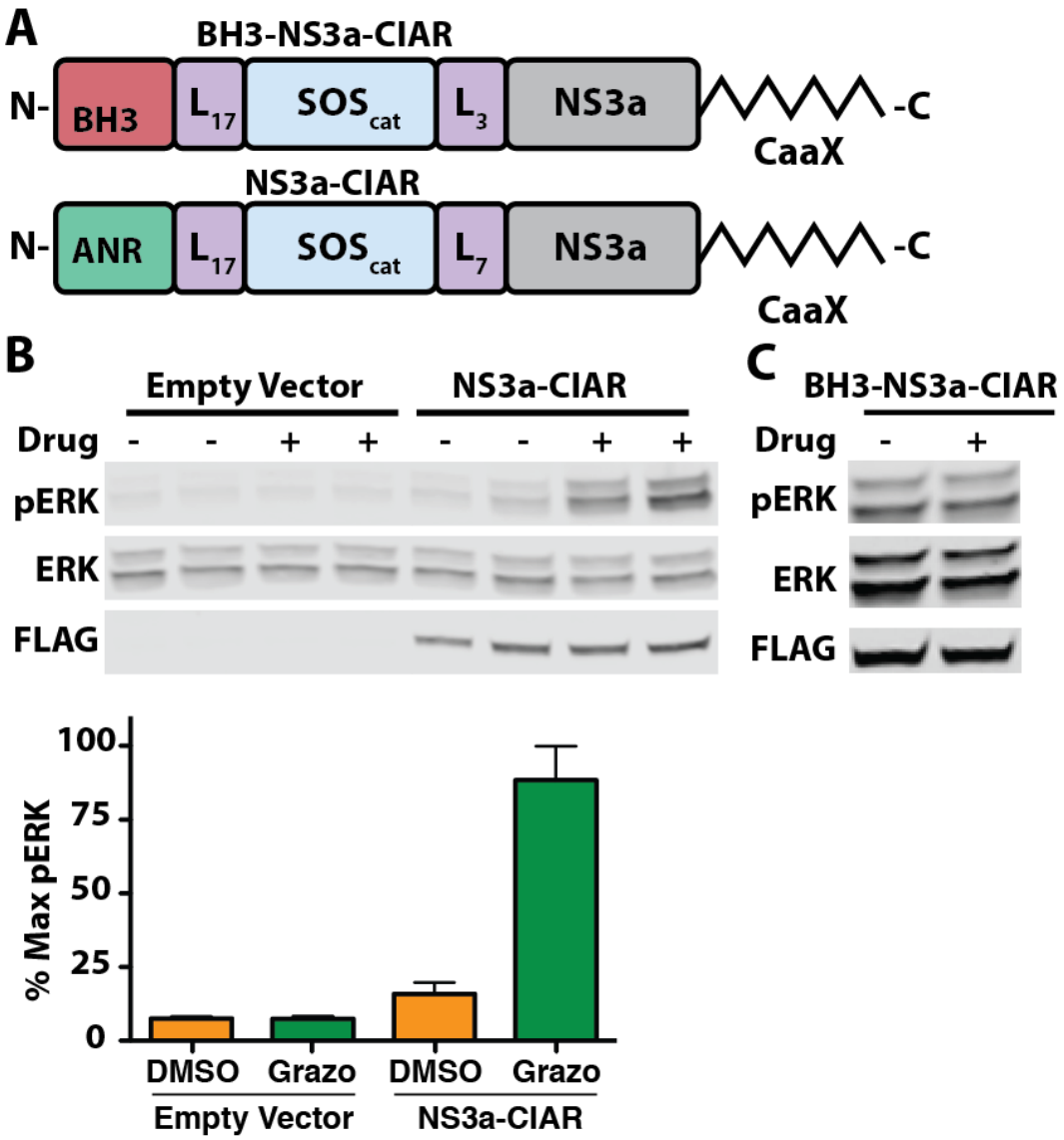


Figure S6. Functional characterization of NS3a-CIAR. (A) Schematic representation of the NS3a-CIAR variants that were tested for RAS/ERK activation in cells. The *top* construct (BH3-NS3a-CIAR) contains a similar architecture as NS3a-CIAR but ANR has been replaced with a peptide (BH3 domain from the protein Bad) that has no detectable affinity for NS3a. The *bottom* construct (NS3a-CIAR) was used in all experiments shown in **Figure 2**. The number of residues in each linker connecting domains are shown as $L_{\#}$. All constructs contain the C-terminal hypervariable region, which includes the CaaX motif, from H-RAS for membrane localization. (B) Phospho-ERK blot (*top*) and quantification (*bottom*) of HEK293 cells transfected with an empty vector or a plasmid containing NS3a-CIAR and treated with DMSO or 1 μM grazoprevir for 60 min. Anti-ERK (middle) and anti-FLAG (bottom) immunoblots are also shown. (C) Phospho-ERK blot of HEK293 cells transfected with a plasmid containing BH3-NS3a-CIAR and treated with DMSO or asunaprevir (10 μM) for 60 min. Anti-ERK (middle) and anti-FLAG (bottom) immunoblots are also shown.

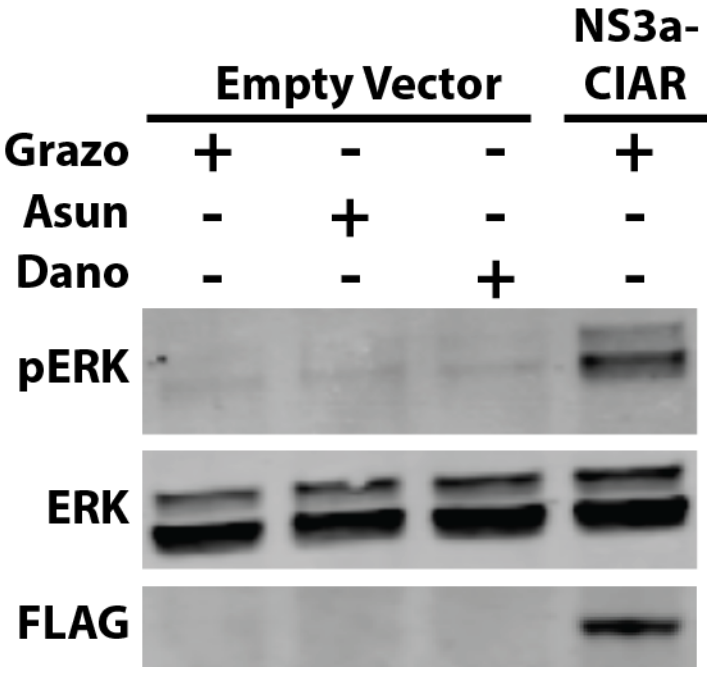


Figure S7. Effects of NS3a inhibitors in cells lacking NS3a-CIAR. Phospho-ERK (*top*), total ERK (*middle*), and FLAG (*bottom*) blots of HEK293 cells transfected with an empty pcDNA5 vector and treated with 1 μM grazoprevir, asunaprevir, or danoprevir or HEK293 cells transfected with the FLAG-tagged NS3a-CIAR construct and treated with 1 μM grazoprevir. Cells were treated with the specified drugs for 60 min.

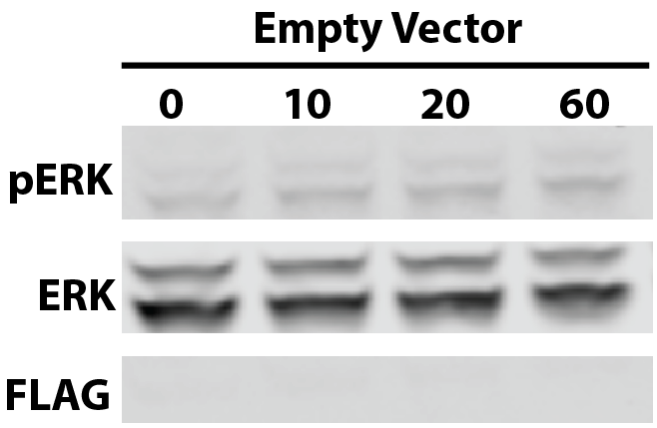
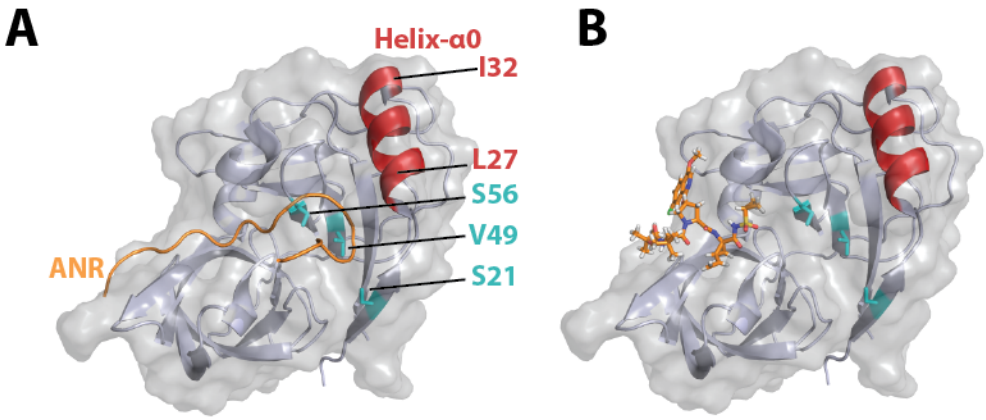


Figure S8. NS3a-CIAR is necessary for temporal activation of the RAS/ERK pathway. Phospho-ERK (*top*), total ERK (*middle*), and FLAG (*bottom*) blots of HEK293 cells transfected with an empty pcDNA5 vector and treated with 10 μM asunaprevir for the time points indicated.

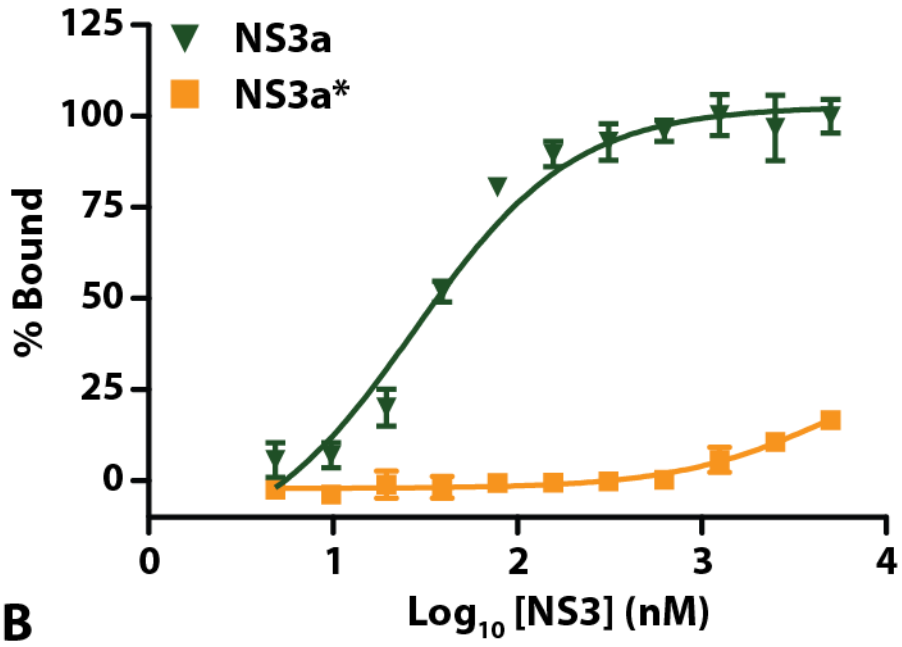


C

		Tyr-Finger Pocket Residues		
Name	Helix-α0 Sequence (L27-I32)	S21	V49	S56
NS3a	LLGIII	Ser	Val	Ser
NS3a*	EEGCQE	Ala	Ile	Thr
H7	EEGCQE	Ser	Val	Ser
H6	LEGCIE	Ser	Val	Ser
H5	LLGCII	Ser	Val	Ser
H4	LLGCIE	Ser	Val	Ser
H3	LLGCQE	Ser	Val	Ser
H2	ELGCQE	Ser	Val	Ser
H1	LEGCQE	Ser	Val	Ser

Figure S9. NS3a/NS3a* chimeras. (A) Crystal structure of ANR bound to NS3a (PDB: 4A1X). Previous work (Brass, V.; Berke, J. M.; Montserret, R.; Blum, H. E.; Penin, F.; Moradpour, D. *Proc. Natl. Acad. Sci. U.S.A.* **2008,** *105*, 14545-50) has demonstrated that NS3a interacts with membranes through an amphipathic helix (helix-α0) and that this helix is partially responsible for the insolubility of recombinant NS3a. A variant of NS3a optimized for solubility (NS3a*) has been previously reported (Wittekind, M. et al. US Patent 6333186. **2004**). However, NS3a* fails to bind ANR effectively (**Figure S10**). Regions of NS3a that appear to make critical contacts with ANR and that differ between NS3a and NS3a* are shown in red [helix-α0 (residues 27-32)] and cyan [Tyr-finger pocket (residues 21, 49, and 56)]. (B) Crystal structure of NS3a bound to Asunaprevir (PDB: 4WF8). (C) Table depicting all NS3a/NS3a* chimeras that were generated and tested in the mitochondrial colocalization assay (**Figure 2A**). The sequences of two regions [helix-α0 (residues 27-32) and the Tyr-finger pocket (residues 21, 49, and 56)] that differ between NS3a and NS3a* are shown in the first two rows. Chimeras were generated by introducing the sequences shown for both regions into NS3a*. All sequences for NS3a, NS3a*, and NS3a*/NS3a chimeras are provided in the methods section. Chimera's are henceforth referred to as NS3a(H#).

A

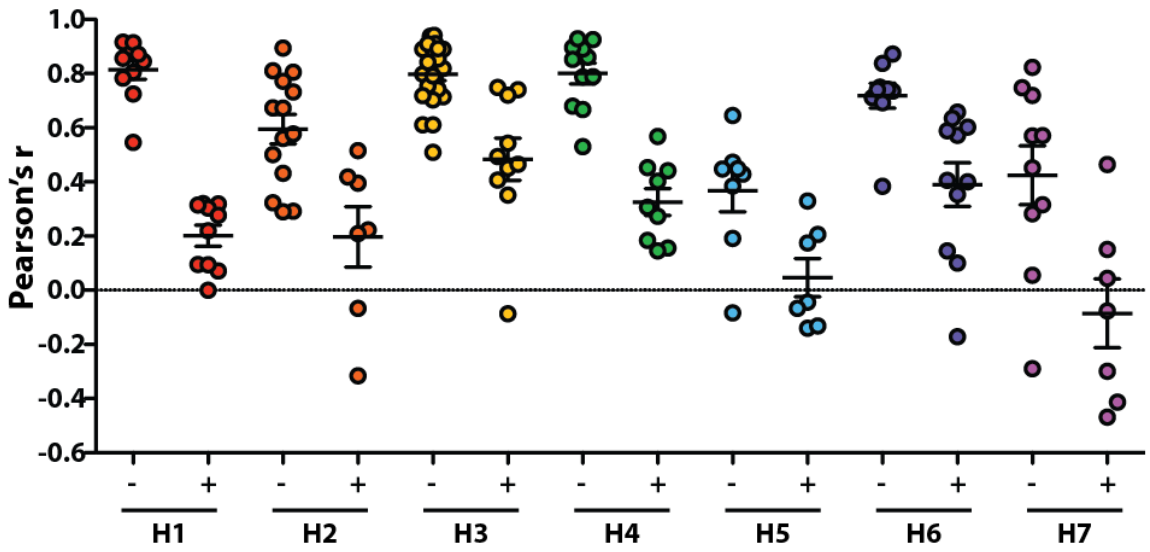


B

Variant	FB_{50} (nM)
NS3a	30
NS3a*	>5000

Figure S10. *In vitro* characterization of the solubility optimized NS3a variant NS3a*. (**A**) 50% fractional binding (FB_{50}) curves of NS3a and NS3a* for FAM-ANR determined using a fluorescence polarization assay (NS3a titration data from **Figure S3b** is re-plotted for reference). Values shown for each concentration of NS3a and NS3a* are the mean +/- sem of n=3. (B) FB_{50} values of NS3a and NS3a* for FAM-ANR.

A



B

	Number of Cells						
	H1	H2	H3	H4	H5	H6	H7
DMSO	10	14	23	11	8	9	10
Asunaprevir (10μM)	10	7	10	9	7	11	7
p-Value	<0.0001	0.0017	<0.0001	<0.0001	0.01	0.0037	0.0082

Figure S11. Screening of NS3a chimeras in a mitochondrial colocalization assay.
(A) Pearson's r-correlation coefficients of mCherry and GFP fluorescence determined by confocal fluorescence microscopy in NIH-3T3 cells. Cells were co-transfected with EGFP-ANR$_2$ and a mitochondrially localized mCherry-NS3a-chimera (Tom20-mCherry-NS3a(H#), sequences shown in **Figure S9C**) and treated with 10 μM asunaprevir or DMSO for 30 minutes followed immediately by fixation and analysis by confocal fluorescence microscopy. Pearson's r-correlation coefficients were determined using ImageJ and unpaired two-sided student's t-tests were calculated using Graphpad Prism. (B) Cell counts and statistics for both drug and DMSO treated cells for each NS3a-chimera. Only cells expressing both mCherry and EGFP were imaged and analyzed.

A Mitochondria

Drug	Number of Cells	p Value
DMSO	19	<0.0001
Asun	19	

B Plasma Membrane

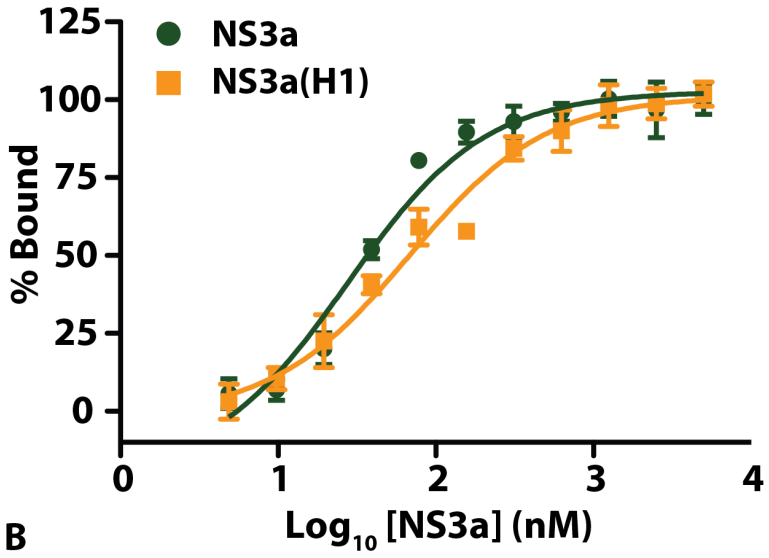
Drug	Number of Cells	p Value
DMSO	32	<0.0001
Asun	32	

C Nucleus

Time	Number of Cells	p Value
0	18	0.001
5	13	
15	9	<0.0001
60	6	<0.0001

Figure S12. Cell numbers and statistics for the colocalization experiments quantified in **Figure 3**. Cells expressing EGFP and mCherry were imaged and analyzed. Pearson's r-correlation coefficients were determined in ImageJ and unpaired two-sided student's t-tests were calculated using Graphpad Prism. (A) Number of cells analyzed per condition and statistics for mitochondrial colocalization (data shown in **Figure 3C**). (B) Number of cells analyzed per condition and statistics for plasma membrane colocalization (data shown in **Figure 3E**). (C) Number of cells analyzed per time point and statistics for nuclear colocalization (data shown in **Figure 3G**)

A



B

Variant	FB_{50} (nM)
NS3a	30
NS3a(H1)	70

Figure S13. *In vitro* characterization of the NS3a(H1) chimera. (A) 50% fractional binding (FB_{50}) curves of NS3a and NS3a(H1) for FAM-ANR determined using a fluorescence polarization assay. Values shown for each concentration of NS3a and NS3a* are the mean +/- sem of n=3. (B) Mean FB_{50} values of NS3a and NS3a(H1) for FAM-ANR (NS3a titration data from **Figure S3b** is re-plotted for reference).

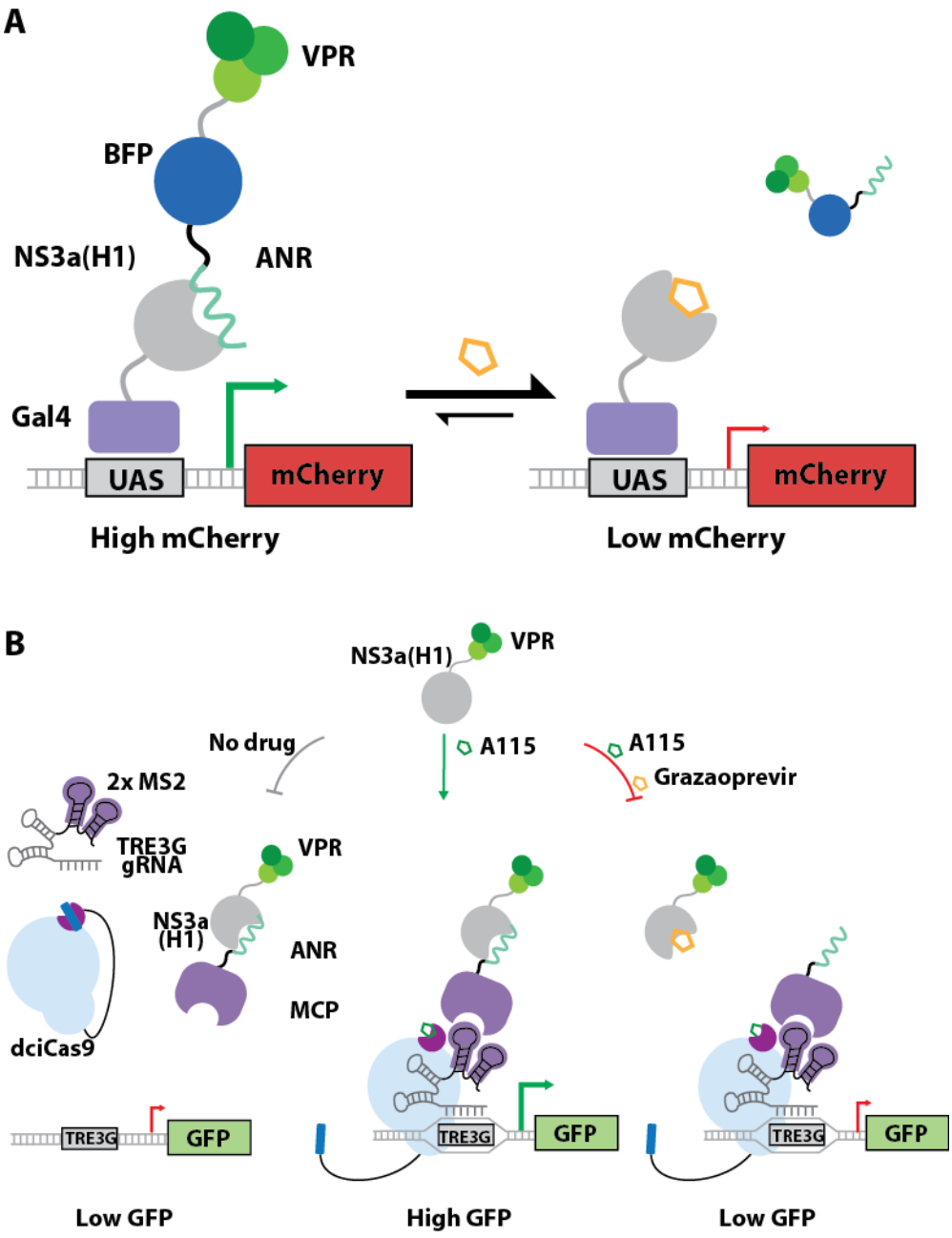


Figure S14. Expanded schematics representations from Figure 4A, C.

Methodss

1. Computational design of NS3a-CIAR

The NS3a-CIAR construct was modeled after a previously developed BCL-xL/BH3 autoinhibited SOScat fusion design wherein a BH3 peptide was fused to the *N*-terminus (residue 574) of SOScat and BCL-xL was fused to the *C*-terminus (residue 1020). Due to similarities in the topology between the BCL-xL/BH3 complex and the NS3a/ANR complex, we limited our computational modeling to a construct composed of SOScat (574-1029) containing ANR fused to the *N*-terminus and NS3a fused to the *C*-terminus. ANR and NS3a were fused to SOScat through flexible linkers.

The NS3a/ANR complex (PDB 4A1X) was modeled using the RosettaRemodel conformational sampling protocol described previously (Rose, J. C. et al. *Nat. Chem. Biol*. **2017**, *13*, 119-126.). Briefly, the NS3a/ANR autoinhibitory complex was treated as a single rigid-body between the *N*- and *C*- termini of SOScat (PDB 1XD2). To allow this setup, the SOScat structure was circularly permuted, with a chain break introduced arbitrarily, away from the termini. This scheme allows for treatment of the NS3a/ANR complex across the termini as a loop closure problem, wherein a break is randomly introduced into one of the linkers to be reconnected via both random fragment moves and chain-closure algorithms guided by the Rosetta energy function; trajectories that properly reconnected the chain were considered successful.

Linkers were assigned the identity of repeating glycine-serine/threonine residues. We tested *N*-terminal linkers between 1 and 13 residues in length at 2 residue increments, and *C*-terminal linkers between 5 and 29 residues in length at 2 residue increments, giving 91 different linker length combinations.

The definition for running lengths 5 and 5 on either side of the insertion is given below; only the residues with assignments in the blueprint file are given; in the actual blueprint, all the entries listed in the brackets were present.

Residue entries 1–36:

37 E .

38 P L PIKAA P

0 x L PIKAA G

0 x L PIKAA T

0 x L PIKAA G
0 x L PIKAA T
0 x L PIKAA G
0 x I NATRO *(209 lines corresponding to NS3a/ANR complex)*
0 x L PIKAA G
0 x L PIKAA S
0 x L PIKAA G
0 x L PIKAA T
0 x L PIKAA G
49 D L PIKAA D
50 V .
(residue entries from 51 and on).

The flags to run the calculations are as follows:

-database (Rosetta database location)
-s [circularly permuted template PDB file]
-remodel:blueprint (blueprint files)
-insert_segment_from_pdb (PDB formatted ANR/NS3a complex)
-num_trajectory 10
-save_top 10
-remodel:quick_and_dirty
-use_clusters false
-vall debug1000vall
-overwrite
-max_linear_chainbreak 0.2

1,000 independent trajectories were sampled in 100 parallel runs that used the flags above. The lowest energy model from each successful trajectory was saved as a PDB file.

2. Plasmid construction

Bacterial expression constructs

Non-biotinylated NS3a variants and ANR-GST fusions were obtained as double stranded DNA G-Blocks (IDT) containing Gibson Assembly overhangs designed in NEBuilder (NEB). ANR was designed with an *N*-terminal hexahistidine tag and a *C*-terminal Glutathione S-Transferase domain. NS3a protease genes were sub-cloned into the pMCSG7 vector backbone by PCR linearization of the vector, then Gibson assembly of the vector with the gene insert (NEB, product number E2611L). All NS3a constructs contained an N-terminal hexahistidine tag. This NS3a fusion was used for all *in vitro* experiments with NS3a except for the protease assay shown in **Figure S2A** and the pulldown experiments shown in **Figure S3C**.

NS3a for biotinylation was cloned into the pDW363 vector. NS3a was *N*-terminally fused to AviTag biotin acceptor peptide followed by a hexahistidine tag. The pDW363 vector contains a bi-cistronic BirA biotin ligase. Avi-tagged NS3a was cloned into pDW363 via PCR-linearization of the vector, followed by Gibson assembly with the gene insert, obtained as double stranded DNA G-Blocks containing Gibson Assembly overhangs designed in NEBuilder.

Mammalian expression constructs

All constructs for NS3a-CIAR and sub-cellular colocalization microscopy experiments were obtained as codon-optimized, double-stranded DNA G-Blocks (Integrated DNA Technologies) containing Gibson Assembly overhangs designed in NEBuilder (NEB). NS3a-CIAR constructs contain the C-terminal hyper variable region (HVR), which includes the CaaX motif, from HRAS. All genes were sub-cloned into pcDNA5/FRT/TO vector (Thermo Fisher Scientific) by PCR linearization of the vector, then Gibson Assembly of the vector with the gene insert. ANR and NS3a sequence variants were obtained via Quikchange mutagenesis.

Plasmids containing single-guide RNAs (TRE3G) were generated by cloning into gRNA Cloning Vector (gifts from George Church (Addgene plasmid #41824)). DNA corresponding to the guide target was ordered as a single stranded oligonucleotide containing Gibson assembly overhangs complementary to the vector and assembled with AflII-digested gRNA vector. A scaffold RNA (scRNA) targeting TRE3G containing two MS2 hairpins was cloned into dual insert vectors derived from pSico, expressing the scaffold RNA under a U6 promoter and the protein inserts under a CMV promoter:

pJZC34 (MS2/MCP) (gift from Jesse Zalatan). All MS2 fusions were expressed as P2A-BFP fusions instead of the IRES-mCherry fusions in the original vectors.

The parental pLenti Gal4 reporter plasmid 'G143' (UAS-mCherry/CMV-Gal4-ERT2-VP16-P2A-Puro) was a gift from Doug Fowler. The ERT2-VP16 and Puromycin resistance cassette was exchanged for NS3a(H1)-P2A-ANR-BFP-NLS-VPR. Fragments were obtained from the previously mentioned pcDNA5/FRT/TO expression systems by PCR and restriction digesting G143 with BamHI and SexAI. Fragments and digested vector were assembled using Gibson Assembly.

All PCR reactions (vector linearizations, Gibson assembly insert preparation, and Quikchanges) were performed with Q5 polymerase (New England Biolabs). All Gibson assembly reactions were performed with NEBuilder HiFi Assembly Master Mix (New England Biolabs). Oligonucleotides and Gene Blocks used for cloning were synthesized by Integrated DNA Technologies. Correct insertion of the genes and vector preparations were verified by whole gene sequencing (Genewiz). Protein sequences for all constructs used are provided in **Supplemental Table 1**.

3. Protein expression and purification

SNAPtag-NS3a

The SNAPtag-NS3a-His_6 plasmid was transformed into BL21(DE3) *E. coli* cells. One colony was used to inoculate 5 mL of LB broth with ampicillin (100 μg/mL). 18 hours post inoculation, the entirety of the 5 mL culture was used to inoculate 500 mL of LB both with ampicillin (100 μg/mL). Cultures were grown at 37 °C to on OD_{600} of 0.8, cooled to 18 °C and induced with 0.25 mM IPTG. Protein was expressed at 18 °C overnight. Cells were harvested by centrifugation and pellets stored at -80 °C. For SNAPtag-NS3a purification, the pellets were thawed on ice and re-suspended in 10 mL of LS-His_6 Lysis Buffer (50 mM HEPES pH 7.8, 100 mM NaCl, 20% (w/v) glycerol, 20 mM imidazole, 5 mM DTT). The re-suspended cell pellet was lysed via sonication and the lysate was cleared by centrifugation. The cleared lysate was purified using Ni-NTA agarose (Qiagen) by rotating at 4 °C for 1 hour. The resin was subsequently washed with 10 mL of LS-Lysis Buffer and the protein was eluted in 3 mL of LS-Elution Buffer (50 mM HEPES pH 7.8, 100 mM NaCl, 20% (w/v) glycerol, 200 mM Imidazole, 5 mM DTT). Purified protein was dialyzed twice into 1000 mL LS-Storage Buffer (50 mM

HEPES pH 7.8, 100 mM NaCl, 20% (w/v) glycerol, 5 mM DTT, 0.6 mM lauryldimethylamine-N-oxide). Protein was stored by snap-freezing aliquots and storing at -80 °C.

NS3a variants

NS3a variant expressions were performed in BL21 (DE3) *E. coli* by growing cells at 37 °C to an O.D.600 of 0.5-1.0, then moved to 18 °C. Immediately following transfer to 18 °C, protein expression was induced with 0.5 mM IPTG overnight. For biotinylated constructs, 12.5 mg of D(+)-biotin/L was added simultaneously during inoculation with the overnight culture. Following 16-20 hours overnight growth, cultures were subsequently harvested, and cell pellets frozen at -80 °C. Cell pellets were then re-suspended in 20 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole, 1 mM DTT, 0.1% Tween-20. All buffers for NS3a variant purifications included 10% v/v glycerol. Cells were lysed by sonication, and the supernatant was incubated with Ni-NTA resin (Qiagen) for a minimum of 1 hour at 4 °C. Ni-NTA resin was then washed with three volumes of "NS3a-Wash Buffer" (20 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, 10% glycerol), and proteins were eluted with "NS3a Elution Bufer" (20 mM Tris pH 8.0, 500 mM NaCl, 300 mM imidazole, 10% glycerol). Purified protein was dialyzed twice (3.5 kDa mwco Slide-A-Lyzer dialysis cassettes, Thermo Scientific) into 1000 mL NS3a-Storage Buffer (50 mM HEPES pH 7.8, 100 mM NaCl, 10% (w/v) glycerol, 5 mM DTT, 0.6 mM lauryldimethylamine-N-oxide). Protein was stored by snap-freezing aliquots in liquid nitrogen and storing at -80 °C. Biotinylated constructs were then further purified by size exclusion chromatography on a Superdex-75 10/300 GL column (GE Healthcare) in a buffer of in 20 mM Tris pH 8.0, 300 mM NaCl, 1 mM DTT, 10% glycerol.

ANR-GST

His_6-ANR-GST plasmid was expressed in BL21(DE3) *E. coli* cells. 18 hours post inoculation, the entirety of the 5 mL culture was used to inoculate 250 mL of LB both with ampicillin (100 µg/mL). Cultures were grown at 37 °C to on OD_{600} of 0.8, cooled to 18 °C and induced with 0.5 mM IPTG. Protein was expressed at 18 °C overnight. Cells

were harvested by centrifugation and pellets stored at -80 °C. For ANR-GST purification, the pellet was thawed on ice and re-suspended in 10 mL of His_6 Lysis Buffer (50 mM HEPES pH 7.8, 100 mM NaCl, 20 mM imidazole, 5 mM DTT) supplemented with PMSF (1 mM). The re-suspended cell pellet was lysed via sonication and the lysate was cleared by centrifugation. The cleared lysate was purified using Ni-NTA agarose (Qiagen) by rotating at 4 °C for 1 hour. The resin was subsequently washed with 10 mL of Lysis Buffer and the protein was eluted in 3 mL of Elution Buffer (50 mM HEPES pH 7.8, 100 mM NaCl, 200 mM Imidazole, 5 mM DTT). Purified protein was dialyzed twice into 1000 mL Storage Buffer (50 mM HEPES pH 7.8, 100 mM NaCl, 5 mM DTT). Protein was stored by snap-freezing aliquots and storing at -80 °C.

Inhibitor sources

Grazoprevir was purchased from MedChem Express (MK-5172, product #: HY-15298). Asunaprevir (BMS-650032, product #: A3195) and Danoprevir (RG7227, product #: A4024) were both purchased from ApexBio. A-115463 was purchased from ChemieTek (Product #: CT-A115).

4. Fluorescence polarization assays

A. Determination of FB_{50}s

The affinities of the NS3a variants for ANR were determined using a fluorescence polarization assay. Fluorescently labeled ANR (FAM-ANR, **Figure S1B**) was obtained as a crude mixture from GenScript and purified by HPLC. Titrations of recombinant NS3a variants (3-fold serial dilutions, starting at 5 μM) were diluted in FP-Buffer (50 mM HEPES, pH 7.8, 100 mM NaCl, 5 mM DTT, 1% Glycerol, 0.01% Tween, 5% v/v DMSO). These dilutions were added to a wells containing FAM-ANR (final concentration = 10 nM). FAM-ANR/NS3a solutions were incubated at room temperature in the dark for 1 hour. Fluorescence polarization was measured on a Perkin Elmer EnVision fluorometer (excitation, 495 nm; emission 520 nm). All measurements were carried out in black 96-well plates (Corning, product #: 3720) and run in triplicate. Anisotropy values were obtained and a nonlinear regression model was used to determine binding constants in GraphPad Prism.

B. Fluorescence polarization competition assay

Fluorescence polarization competition assays were used to determine the ability of danoprevir to displace ANR. A 75 nM solution of NS3a in FP-Buffer was incubated with 50 nM FAM-ANR in a black 96-well plate for 1 hour in the dark. 3-fold serial dilutions of danoprevir were prepared in FP-Buffer such that, when added to the NS3a/FAM-ANR solution, the highest concentration of danoprevir tested was 10 μM. Plates were incubated for 1 hour in the dark. Fluorescence polarization was measured at 22 °C on a Perkin Elmer EnVision fluorometer (excitation, 495 nm; emission 520 nm). Each measurement was carried out in triplicate. Anisotropy values were obtained and a nonlinear regression model was used to fit curves with GraphPad Prism.

5. NS3a protease inhibition assay

The potency of ANR against NS3a protease was determined via a FRET assay. Titrations of ANR-GST (3-fold serial dilutions starting at 10 μM) were added to a black 96-well plate (Corning, product number 3720) containing 50 nM SNAPtag-NS3a. Reactions were incubated with NS3a-SNAPtag at room temperature for 1 hour. To each well was simultaneously added substrate M-2235 (Bachem) to a final concentration of 5 μM and reactions were monitored by measuring the fluorescence intensity every minute for 30 minutes at 22 °C on a Perkin Elmer EnVision fluorometer (excitation, 360 nm; emission 460 nm). Each measurement was carried out in triplicate. Slopes of the fluorescence increase were compared to a no-protease control. A nonlinear regression model was used to fit curves using GraphPad Prism.

6. ANR-GST pulldown

Pierce high-capacity streptavidin beads (Thermo-Fisher #PI20359) were prepared by washing three times with Buffer PDA (TBS + 0.05% tween + 0.5 mg/mL BSA). For each condition and each replicate, beads were washed and incubated separately. The wash was performed by adding 200 μL Buffer PDA to 30 μL of a 50/50 bead slurry, inverting to mix, and spinning down (2500 x g for 2 min). The supernatant was removed

by pipetting, and the wash was repeated two more times to end with a 50/50 slurry of beads in wash buffer.

Purified biotinylated NS3a was prepared at a 50x final concentration and 10 µL were added to a 490 µL 50/50 slurry of streptavidin beads and Buffer PD for final NS3a concentration of 125 nM. Beads were incubated and rotated at 4 °C. After one hour, beads were harvested and washed three times as described previously, ending in a 50/50 bead/buffer slurry. ANR was added to all samples at a final concentration of 5 µM. For the danoprevir treated samples, danoprevir was added to a final concentration of 10 µM. Buffer PD was added to a final volume of 500 µL, and the beads were incubated and rotated at 4 °C. After 1 hour, beads were pelleted and washed three times in Buffer FDB (TBS buffer + 0.05% Tween) with 5 minute incubations between washes on a rotator at 4 °C. To obtain final bound protein, beads were pelleted and supernatant was aspirated, resulting in a final volume of beads of 20 µL. 10 µL 3x SDS loading dye was added directly to beads and boiled at 90 °C for 10 min. Bead mixture was pelleted and supernatants were loaded directly onto a polyacrylamide gel for Western Blot analysis (Mini-PROTEAN TGX Any kD, Bio-Rad #456-9036).

7. Mammalian cell culture

A. NIH-3T3 cell culture and transient transfection conditions

NIH-3T3 cells were maintained in DMEM (Gibco, product number 11065092) supplemented with 10% FBS (Gibco, product number A3160602). All transient transfections were done using LipoFectamine3000 (ThermoFisher, product number L3000015) at a ratio of 3:2:1 LipoFectamine3000:p3000Reagent:DNA (µg) prepared in OptiMem (Gibco, product number 11058021) 16-20 hours after plating of cells. Transfections were allowed to proceed for 24 hours before experiments were performed. Cells were tested and found free of mycoplasma monthly.

B. Confocal microscopy of protein colocalization

24 hours prior to transfection, $3x10^4$ 3T3 cells were plated onto 18 mm glass cover slips (Fisher, product number 12-546) in a standard 12-well plate. After co-transfection with the appropriate NS3a/ANR pairs (Tom20-mCherry-NS3a(H#)/EGFP-ANR_2, Myr-mCherry-ANR_2/EGFP-NS3a(H1), or NLS_3-BFP-ANR_2/EGFP-NS3a(H1)),

cells were allowed to recover for 24 hours before treatment with 10 μM asunaprevir or DMSO (0.5% DMSO final concentration). Cells were incubated with drug for the stated time points before media was aspirated, then washed once with chilled PBS, and immediately fixed in 4% paraformaldehyde (Electron Microscopy Services, product number 15710). Paraformaldehyde solution was prepared in 1x PBS and cells were allowed to fix for 15 minutes. Paraformaldehyde was removed and cells were washed twice with chilled PBS. Slides were mounted onto glass cover slips using Fluoromount G (Southern Biotechnology, product number 0100-01) and sealed. Images were generated using a Leica SP8X Confocal Microscope. UV lasers at 405 nm was used for BFP. White lasers (488 nm and 587 nm) were used for EGFP and mCherry, respectively. BFP fluorescence emissions were recorded using a PMT detector. EGFP and mCherry fluorescence emissions were recorded by separate HyD detectors. Images were acquired using a 63x oil objective at 512x512 resolution. Only images of cells exhibiting both mCherry and EGFP (or both BFP and EGFP for nuclear colocalization) were collected. The degree of colocalization was measured as Pearson's r-correlation coefficients. Pearson's r coefficients were determined using ImageJ.

Statistics

All P-values are from unpaired, two-sided *t*-tests, computed using Graphpad Prism 5.

C. HEK293 and HEK293T cell culture and transient transfection conditions

HEK293 and HEK293T cells were maintained in DMEM (Gibco, #11065092) supplemented with 10% FBS (Gibco, product number A3160602). Transient transfections for all experiments were carried out using TurboFectin8.0 (Origene) at a ratio of 3:1 TurboFectin:DNA (μg) prepared in OptiMem (Gibco, #11058021) 16-20 hours after plating of cells. Transfections were allowed to proceed for 18-24 hours before experiments were performed or media was exchanged. Cells were tested and found free of mycoplasma monthly.

Activation of NS3a-CIAR

18-24 hours prior to transfection, $3.0x10^5$ HEK293 cells were plated onto poly-D-lysine 12 well plates. Immediately prior to transfection, media was aspirated and cells were washed with 1 mL of pre-warmed (37 °C) PBS, then serum starved with FBS-free DMEM. Following serum starvation, cells were transfected with 1 µg of FLAG-tagged NS3a-CIAR, BH3-NS3a-CIAR, or an empty pCDNA5 vector. Transfected cells were allowed to serum stave for 18-20 hours prior to drug treatment. For drug treatment, serum-free media was prepared with DMSO, 10 µM asuneprevir (**Figure 2F**, **S6**, **S8**), or 1 µM danoprevir, grazoprevir, or asunaprevir (**Figure 2D,E**, **S7**). Media was aspirated, washed once with pre-warmed DPBS, then treated with drug/DMSO media for the requisite amount of time. Media was subsequently aspirated and the cells were washed twice with 1 mL chilled PBS, then lysed with 75 µL Mod. RIPA buffer (50 mM Tris, pH 7.8, 1% IGEPAL CA-630, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 30 mM NaF, Pierce Protease Inhibitor Tablet). Cleared lysates were subjected to SDS-PAGE and transferred to nitrocellulose. Blocking and antibody incubations were done in TBS with 0.1% Tween-20 (v/v) and blocking buffer (Odyssey). Primary antibodies were all purchased from Cell Signaling Technologies and were diluted as follows: Total ERK (1:2500, #9107), phosphorylated ERK (1:2500, #4370), FLAG (1:2,500, #D6W5B). Blots were washed three times in TBS with 0.1% Tween-20. Antibody binding was detected by using near-infrared-dye-conjugated secondary antibodies and visualized on the LI-COR Odyssey scanner. Blots were quantified via densitometry with Image Studio (LI-COR).

Chemically-disruptable Gal4(DBD)-NS3a(H1)/ANR-VPR transcriptional regulation

18-24 hours prior to transfection, HEK293T cells were plated in a 12-well plate at a density of $1.25x10^5$ cells/mL. Cells were subsequently transfected with 1 µg of the Gal4 reporter plasmid (UAS-mCherry/CMV-Gal4-NS3a(H1)-P2A-ANR-Myc-BFP-VPR-NLS) in OptiMem. For the negative control experiment, 500 ng of a plasmid where ANR was replaced with the non-NS3a binding protein DNCR2 (UAS-mCherry/CMV-Gal4-NS3a(H1)-P2A-DNCR2-Myc-VPR-NLS was co-transfected with 500 ng of a BFP expressing reporter plasmid in OptiMem. 16 hours post transfection, cells were washed with 1 mL DPBS. Complete media containing 1 µM danoprevir, 1 µM grazoprevir, or DMSO was subsequently added to each well. 24 hours after drug treatment, media was

removed and cells were washed with 1 mL DPBS, then detached with 200 µL Versene (Sigma-Aldrich, 15-040-066). Cells were then re-suspended with 500 µL DPBS, and pelleted at 2500 rpm for 3 min at room temperature. Supernatant was subsequently removed and the cells were re-suspended in 400 µL DPBS and analyzed on a FACS LSRII (BD Biosciences).

For Gal4/NS3a-CDP mediated transcriptional activation FACS experiments, 10,000 single cell events were collected for each of the samples run. Of these 10,00 single cell events, the median mCherry fluorescence signal is reported only for cells exhibiting BFP signal greater than that of non-transfected cells. The gathered FACS data were analyzed using FlowJo (v.10.1). See **Supplementary Methods Figure 1** for mammalian cell gating strategies.

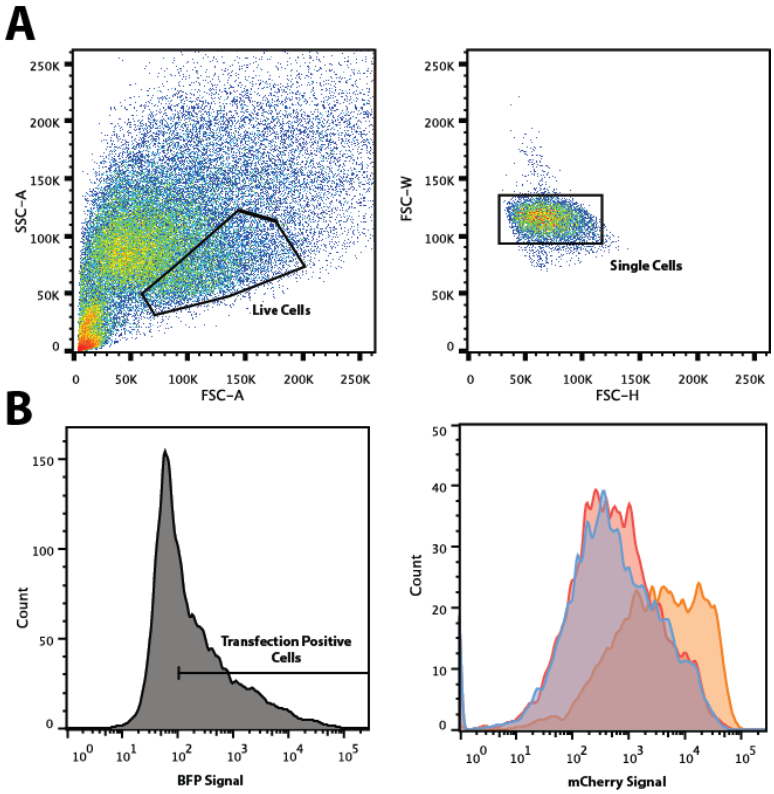
dciCas9-mediated transcription

GFP expression experiments were performed in a HEK293T cell line with GFP stably integrated downstream of a tetracycline-inducible landing pad (7x-TRE3G operator) created in a similar manner as a previously reported Tet-Bxb1-BFP HEK293T cell line (Matreyek et al. Nucleic Acids Res. **2017**, *45*, e102.). For the dciCas9-mediated transcriptional activation experiment, $6x10^4$ cells/well were plated in 12-well plates on day 1 and transfected with 1 µg total DNA on day 2 (0.3 µg dciCas9 vector, 0.3 µg NS3a(H1)-VPR vector, and 0.4 µg NLS-MCP-ANR_2/TRE3G scaffold RNA vector). 18 hours after transfection, media was replaced with complete DMEM containing DMSO, 10 µM A115, or 10 µM A115 and 10 µM grazoprevir. 48 hours post drug treatment, media was aspirated and cells were washed with 1 mL pre-warmed DPBS, then detached and analyzed as described in the chemically-disruptable Gal4(DBD)-NS3a(H1)/VPR-ANR/transcriptional regulation experiment.

For FACS analysis, 10,000 single cell events were collected for each of the samples run. Of these 10,00 single cell events, the median GFP fluorescence signal is reported only for cells exhibiting BFP signal greater than that of non-transfected cells. The gathered FACS data were analyzed using FlowJo (v.10.1). See **Supplementary Methods Figure 1** for mammalian cell gating strategies.

Statistics

All P-values are from unpaired, two-sided *t*-tests, computed using Graphpad Prism 5.



Supplementary Methods Figure 1. FACS Gating Strategies. (A) HEK293T gating from all events from live cells (left) then single cell populations (right). (B) From the single cell populations in (A), a third gate for BFP positive cells (left) was set then mCherry or EGFP signal was measured (mCherry example shown on right).

Supplementary Table 1. Sequences of proteins and guide RNAs

Sequence ID	**Description**	**Sequence (NS3a sequences are shown in bold. ANR sequences are shown in italics. Regions of note are underlined or highlighted).**
ANR-GST	His_6-*ANR*- GST (*E. coli*)	MHHHHHHGSGTGS*GELGRLVYLLDGPGYDPIHSD*GTGSSPILGYWKIKGLVQPTRLLL EYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKH NMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRL CHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKS SKYIAWPLQGWQATFGGGDHPPKSDLVPR
$SNAP_{tag}$-**NS3a**	$SNAP_{tag}$-**NS3a**-His_6 (*E. coli*)	MDKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPAAVLGGPEP LMQATAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQ LAALAGNPAATAAVKTALSGNPVPILIPCHRVVSSSGAVGGYEGGLAVKEWLLAHEGH RLGKPGLGGTGT**AKGSVVIVGRINLSGDTAYSQQTRGLLGIIITSATGRDKNQVDGEV QVLSTATQSFLATCVNGVCWTVYHGAGSKTLAGPKGPITQMYTNVDQDLVGWPAPPGA RSMTPCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPVSYLKGSSGGPLLCPSGH VVGIFRAAVCTRGVAKAVDFIPVESMETTMR**GSHHHHHH
NS3a (inactive)	Avi-His_6-**NS3a** solubility optimized S139A (*E. coli*)	MAGGLNDIFEAQKIEWHEDTGGSSHHHHHHGSGSGS**MAKGSVVIVGRINLSGDTAYSQ QTRGLLGCIITSATGRDKNQVDGEVQVLSTATQSFLATCVNGVCWTVYHGAGSKTLAG PKGPITQMYTNVDQDLVGWPAPPGARSMTPCTCGSSDLYLVTRHADVIPVRRRGDSRG SLLSPRPVSYLKGSAGGPLLCPSGHVVGIFRAAVCTRGVAKAVDFIPVESMETTMR**
NS3a (active)	Avi-His_6-**NS3a** solubility optimized catalytically active (*E. coli*)	MAGGLNDIFEAQKIEWHEDTGGSSHHHHHHGSGSGS**MAKGSVVIVGRINLSGDTAYSQ QTRGLLGCIITSATGRDKNQVDGEVQVLSTATQSFLATCVNGVCWTVYHGAGSKTLAG PKGPITQMYTNVDQDLVGWPAPPGARSMTPCTCGSSDLYLVTRHADVIPVRRRGDSRG SLLSPRPVSYLKGSSGGPLLCPSGHVVGIFRAAVCTRGVAKAVDFIPVESMETTMR**
NS3a* (inactive)	His_6-**NS3a**	MAGGSSHHHHHHGSGSGS**MKKKGSVVIVGRINLSGDTAYAQQTRGEEGCQETSQTGRD**

	solubility optimized S139A (*E. coli*)	**KNQVEGEVQIVSTATQTFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLV GWQAPQGSRSLTPCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSAGG PLLCPAGHAVGIFRAAVSTRGVAKAVDFIPVESLETTMRSP**
NS3a* (active)	His_6-**NS3a** solubility optimized -catalytically active (*E. coli*)	MAGGSSHHHHHHGSGSGS**MKKKGSVVIVGRINLSGDTAYAQQTRGEEGCQETSQTGRD KNQVEGEVQIVSTATQTFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLV GWQAPQGSRSLTPCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGG PLLCPAGHAVGIFRAAVSTRGVAKAVDFIPVESLETTMRSP**
NS3a(H1) (active)	His_6-**NS3a(H1)** Chimera -catalytically active (*E. coli*)	MGGSSHHHHHHGSGSGS**MKKKGSVVIVGRINLSGDTAYSQQTRGLEGCQETSQTGRDK NQVEGEVQVVSTATQSFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVG WQAPQGSRSLTPCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGP LLCPAGHAVGIFRAAVSTRGVAKAVDFIPVESLETTMRSP**
Tom20-mCherry-NS3a(H1)	Mitochondrial-localized **NS3a/NS3a* chimera H1** -catalytically active, pcDNA5/FRT/TO	MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFGSGGSMVSKGEEDNMAIIKEFMRF KVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAY VKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKGSGSTGTSGSGSGTG SGSGTG**MKKKGSVVIVGRINLSGDTAYSQQTRGLEGCQETSQTGRDKNQVEGEVQVVS TATQSFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLT PCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGI FRAAVSTRGVAKAVDFIPVESLETTMRSP**GSGTGSGTSGSTGTGSTGDYKDDDDK
Tom20-mCherry-NS3a(H2)	Mitochondrial-localized **NS3a/NS3a* chimera H2** -catalytically active, pcDNA5/FRT/TO	MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFGSGGSMVSKGEEDNMAIIKEFMRF KVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAY VKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKGSGSTGTSGSGSGTG SGSGTG**MKKKGSVVIVGRINLSGDTAYSQQTRGELGCQETSQTGRDKNQVEGEVQVVS TATQSFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLT PCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGI FRAAVSTRGVAKAVDFIPVESLETTMRSP**GSGTGSGTSGSTGTGSTGDYKDDDDK
Tom20-mCherry-NS3a(H3)	Mitochondrial-localized **NS3a/NS3a* chimera H3** -catalytically active, pcDNA5/FRT/TO	MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFGSGGSMVSKGEEDNMAIIKEFMRF KVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAY VKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKGSGSTGTSGSGSGTG SGSGTG**MKKKGSVVIVGRINLSGDTAYSQQTRGLLGCQETSQTGRDKNQVEGEVQVVS TATQSFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLT PCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGI FRAAVSTRGVAKAVDFIPVESLETTMRSP**GSGTGSGTSGSTGTGSTGDYKDDDDK
Tom20-mCherry-NS3a(H4)	Mitochondrial-localized **NS3a/NS3a* chimera H4** -catalytically active, pcDNA5/FRT/TO	MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFGSGGSMVSKGEEDNMAIIKEFMRF KVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAY VKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKGSGSTGTSGSGSGTG SGSGTG**MKKKGSVVIVGRINLSGDTAYSQQTRGLLGCIETSQTGRDKNQVEGEVQVVS TATQSFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLT PCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGI FRAAVSTRGVAKAVDFIPVESLETTMRSP**GSGTGSGTSGSTGTGSTGDYKDDDDK
Tom20-mCherry-NS3a(H5)	Mitochondrial-localized **NS3a/NS3a* chimera H5** -catalytically active, pcDNA5/FRT/TO	MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFGSGGSMVSKGEEDNMAIIKEFMRF KVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAY VKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKGSGSTGTSGSGSGTG SGSGTG**MKKKGSVVIVGRINLSGDTAYSQQTRGLLGCIITSQTGRDKNQVEGEVQVVS TATQSFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLT PCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGI FRAAVSTRGVAKAVDFIPVESLETTMRSP**GSGTGSGTSGSTGTGSTGDYKDDDDK
Tom20-mCherry-NS3a(H6)	Mitochondrial-localized **NS3a/NS3a* chimera H6** -catalytically active, pcDNA5/FRT/TO	MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFGSGGSMVSKGEEDNMAIIKEFMRF KVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAY VKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKGSGSTGTSGSGSGTG SGSGTG**MKKKGSVVIVGRINLSGDTAYSQQTRGLEGCIETSQTGRDKNQVEGEVQVVS TATQSFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLT PCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGI FRAAVSTRGVAKAVDFIPVESLETTMRSP**GSGTGSGTSGSTGTGSTGDYKDDDDK

Tom20-mCherry-NS3a(H7)	Mitochondrial-localized **NS3a/NS3a* chimera H7** -catalytically active, pcDNA5/FRT/TO	MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFGSGGSMVSKGEEDNMAIIKEFMRF KVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAY VKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFP SDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYKGSGSTGTSGSGSGTG SGSGTG**MKKKGSVVIVGRINLSGDTAYSQQTRGEEGCQETSQTGRDKNQVEGEVQVVS TATQSFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLT PCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGI FRAAVSTRGVAKAVDFIPVESLETTMRSP**GSGTGSGTSGSTGTGSTGDYKDDDDK
EGFP-NS3a(H1)	EGFP-(ANR-binding-restored) **NS3a/NS3a* chimera H1** -catalytically active pcDNA5/FRT/TO	MVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGP LPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDS SLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKD GGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMD ELYKGSGTGDYKDDDDK**KKKGSVVIVGRINLSGDTAYAQQTRGLEGCQETSQTGRDKN QVEGEVQIVSTATQTFLATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGW QAPQGSRSLTPCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSAGGPL LCPAGHAVGIFRAAVSTRGVAKAVDFIPVESLETTMRSP**
EGFP-ANR$_2$	EGFP-*ANR-ANR* pcDNA5/FRT/TO	MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPW PTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFE GDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIED GSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITL GMDELYKSGSGEQKLISEEDLGSGTGSGTGSGTGTTSGTGTGGSTG*GELDELVYLLDG PGYDPIHSD*GSGTGSGTGSGTGTTSGTGTGGSTG*GELDELVYLLDGPGYDPIHSD*
Myr-mCherry-ANR$_2$	Plasma membrane localized Myr-*ANR-ANR*-mCherry pcDNA5/FRT/TO	MGCGCSSHPEDDGTGSGTGSMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEG EGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGF KWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSER MYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNED YTIVEQYERAEGRHSTGGMDELYKGSGSEQKLISEEDLGSGTGSGTGSGTGTTSGTGT GGSTG*GELDELVYLLDGPGYDPIHSD*GSGTGSGTGSGTGTTSGTGTGGSTGGELDELV YLLDGPGYDPIHSD
NLS$_3$-BFP-ANR$_2$	Nuclear localized 3xNLS-BFP-*ANR-ANR* pcDNA5/FRT/TO	MDPKKKRKVDPKKKRKVDPKKKRKVGSGSELIKENMHMKLYMEGTVDNHHFKCTSEGE GKPYEGTQTMRIKVVEGGPLPFAFDILATSFLYGSKTFINHTQGIPDFFKQSFPEGFT WERVTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFTSNGPVMQKKTLGWEAFTETL YPADGGLEGRNDMALKLVGGSHLIANIKTTYRSKKPAKNLKMPGVYYVDYRLERIKEA NNETYVEQHEVAVARYCDLPSKLGHKLNSGSGEQKLISEEDLGSGTGSGTGSGTGTTS GTGTGGSTG*GELDELVYLLDGPGYDPIHSD*GSGTGSGTGSGTGTTSGTGTGGSTG*GEL DELVYLLDGPGYDPIHSD*
NS3a-CIAR	*ANR*-SOScat-**NS3a**-HVR (hypervariability region from HRAS). Non-solubility optimized NS3a, -catalytically active pcDNA5/FRT/TO	M*GELDELVYLLDGPGYDPIHSD*GSGTGSGTGSGTGSGTGDVYRFAEPDSEENIIFEEN MQPKAGIPIIKAGTVIKLIERLTYHMYADPNFVRTFLTTYRSFCKPQELLSLIIERFE IPEPEPTEADRIAIENGDQPLSAELKRFRKEYIQPVQLRVLNVCRHWVEHHFYDFERD AYLLQRMEEFIGTVRGKAMKKWVESITKIIQRKKIARDNGPGHNITFQSSPPTVEWHI SRPGHIETFDLLTLHPIEIARQLTLLESDLYRAVQPSELVGSVWTKEDKEINSPNLLK MIRHTTNLTLWFEKCIVETENLEERVAVVSRIIEILQVFQELNNFNGVLEVVSAMNSS PVYRLDHTFEQIPSRQKKILEEAHELSEDHYKKYLAKLRSINPPCVPFFGIYLTNILK TEEGNPEVLKRHGKELINFSKRRKVAEILGEIQQYQNQPYCLRVESDIKRFFENLNPM GNSMEKEFTDYLFNKSLEIEPGSGTGSG**MAKGSVVIVGRINLSGDTAYSQQTRGLLGI IITSLTGRDKNQVDGEVQVLSTATQSFLATCVNGVCWTVYHGAGSKTLAGPKGPITQM YTNVDQDLVGWPAPPGARSMTPCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPRPV SYLKGSSGGPLLCPSGHVVGIFRAAVCTRGVAKAVDFIPVESMETTMR**GSGTGSGGSG TGDYKDDDDKQHKLRKLNPPDESGPGCMSCKCVLS
BH3-NS3a-CIAR	BH3-SOScat-**NS3a**-HVR. Non-solubility optimized NS3a, -catalytically active (hypervariability region from HRAS) pcDNA5/FRT/TO	M*APPNLWAAQRYGRELRRMADEGEGSFK*GSGTGSGTGSGTGSGTGDVYRFAEPDSEEN IIFEENMQPKAGIPIIKAGTVIKLIERLTYHMYADPNFVRTFLTTYRSFCKPQELLSL IIERFEIPEPEPTEADRIAIENGDQPLSAELKRFRKEYIQPVQLRVLNVCRHWVEHHF YDFERDAYLLQRMEEFIGTVRGKAMKKWVESITKIIQRKKIARDNGPGHNITFQSSPP TVEWHISRPGHIETFDLLTLHPIEIARQLTLLESDLYRAVQPSELVGSVWTKEDKEIN SPNLLKMIRHTTNLTLWFEKCIVETENLEERVAVVSRIIEILQVFQELNNFNGVLEVV SAMNSSPVYRLDHTFEQIPSRQKKILEEAHELSEDHYKKYLAKLRSINPPCVPFFGIY LTNILKTEEGNPEVLKRHGKELINFSKRRKVAEILGEIQQYQNQPYCLRVESDIKRFF ENLNPMGNSMEKEFTDYLFNKSLEIEPGSG**MAKGSVVIVGRINLSGDTAYSQQTRGLL GIIITSLTGRDKNQVDGEVQVLSTATQSFLATCVNGVCWTVYHGAGSKTLAGPKGPIT QMYTNVDQDLVGWPAPPGARSMTPCTCGSSDLYLVTRHADVIPVRRRGDSRGSLLSPR PVSYLKGSSGGPLLCPSGHVVGIFRAAVCTRGVAKAVDFIPVESMETTMR**DYKDDDDK QHKLRKLNPPDESGPGCMSCKCVLS
dciCas9	Catalytically	MDYKDDDDKDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGA

	inactive ciCas9 (D10A, H840A) pcDNA5/FRT/TO	LLFDSGETAEATRLKRTARRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVE EDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRG HFLIEGDLNPDNSSNRELVVDFLSYKLSQKGYSWSQFSDVEENRTEAPEGTESEMETP SAINGNPSWHLADSPAVNGATGHSSSLDAREVIPMAAVKQALREAGDEFELRYRRAFS DLTSQLHITPGTAYQSFEQVVNELFRDGVNWGRIVAFFSFGGALCVESVDKEMQVLVS RIAAWMATYLNDHLEPWIQENGGWDTFVELYGNNGSGTASGTGSGTGSATGSGTVNTE ITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQ EEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQE DFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKG ASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGE QKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKII KDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGW GRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQG DSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQK NSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRL SDYDVDAIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKL ITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKL IREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESE FVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIET NGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARK KDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDF LEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLA SHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKH RDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGL YETRIDLSQLGGD*SRA*DPKKKRKVTGSGTAPPNLWAAQRYGRELRRMADEGEGSFK
NS3a(H1)-VPR	(ANR-binding-restored) **NS3a/NS3a* chimera H1**-VPR -catalytically active, pcDNA5/FRT/TO	**MKKKGSVVIVGRINLSGDTAYSQQTRGLEGCQETSQTGRDKNQVEGEVQVVSTATQSF** **LATSINGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLTPCTCGS** **SDLYLVTRHADVIPVRRRGDSRGSLLSPRPISYLKGSSGGPLLCPAGHAVGIFRAAVS** **TRGVAKAVDFIPVESLETTMRSP**GSGTGSGEQKLISEEDLEFSSAAGTSDALDDFDLD MLGSDALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLGS*PKKKRKV*GSQYLPDT DDRHRIEEKRKRTYETFKSIMKKSPFSGPTDPRPPPRRIAVPSRSSASVPKPAPQPYP FTSSLSTINYDEFPTMVFPSGQISQASALAPAPPQVLPQAPAPAPAPAMVSALAQAPA PVPVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDDEDLGALLGNSTDPAVFTDL ASVDNSEFQQLLNQGIPVAPHTTEPMLMEYPEAITRLVTGAQRPPDPAPAPLGAPGLP NGLLSGDEDFSSIADMDFSALLSQISSGSGSGSRDSREGMFLPKPEAGSAISDVFEGR EVCQPKRIRPFHPPGSPWANRPLPASLAPTPTGPVHEPVGSLTPAPVPQPLDPAPAVT PEASHLLEDPDEETSQAVKALREMADTVIPQKEEAAICGQMDLSHPPPRGHLDELTTT LESMTEDLNLDSPLTPELNEILDTFLNDECLLHAMHISTGLSIFDTSLF
NLS-MCP-ANR$_2$	Nuclear localized MCP-*ANR-ANR*-P2A-BFP Expressed with TRE3G-2xMS2	M*PKKKRKV*GSMASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCS VRQSSAQNRKYTIKVEVPKGAWRSYLNMELTIPIFATNSDCELIVKAMQGLLKDGNPI PSAIAANSGIYGSGGSGTGSGTGSGTGTTSGTGTGGSTG*GELDELVYLLDGPGYDPIH* *SD*GSGTGSGTGSGTGTTSGTGTGGSTG*GELDELVYLLDGPGYDPIHSD*GSGATNFSLL KQAGDVEENPGPSELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQTMRIKVVE GGPLPFAFDILATSFLYGSKTFINHTQGIPDFFKQSFPEGFTWERVTTYEDGGVLTAT QDTSLQDGCLIYNVKIRGVNFTSNGPVMQKKTLGWEAFTETLYPADGGLEGRNDMALK LVGGSHLIANIKTTYRSKKPAKNLKMPGVYYVDYRLERIKEANNETYVEQHEVAVARY CDLPSKLGHKLN
Gal4(DBD)-NS3a(H1)-P2A-ANR-BFP-VPR-NLS	Gal4-(ANR-binding restored) **NS3a/NS3a* chimera H1**-P2A-*ANR*-Myc-BFP-VPR (internal NLS) -catalytically active pcDNA5/FRT/TO	MKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVE SRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLGTPAAASTAGSGG**MAKGSVVIVG** **RINLSGDTAYSQQTRGLEGCQETSQTGRDKNQVEGEVQVVSTATQSFLATSINGVLWT** **VYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLTPCTCGSSDLYLVTRHAD** **VIPVRRRGDSRGSLLSPRPISYLKGSAGGPLLCPAGHAVGIFRAAVSTRGVAKAVDFI** **PVESLETTMRSP**GSGATNFSLLKQAGDVEENPGPGALSGM*GELDELVYLLDGPGYDPI* *HSD*GVLSGSGTGSGTGSGTGTTSGTGTGGSTGEQKLISEEDLGSGSSELIKENMHMKL YMEGTVDNHHFKCTSEGEGKPYEGTQTMRIKVVEGGPLPFAFDILATSFLYGSKTFIN HTQGIPDFFKQSFPEGFTWERVTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFTSN GPVMQKKTLGWEAFTETLYPADGGLEGRNDMALKLVGGSHLIANIKTTYRSKKPAKNL KMPGVYYVDYRLERIKEANNETYVEQHEVAVARYCDLPSKLGHKLNGSGSDALDDFDL DMLGSDALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLGSPKKKRKVGSQYLPD TDDRHRIEEKRKRTYETFKSIMKKSPFSGPTDPRPPPRRIAVPSRSSASVPKPAPQPY PFTSSLSTINYDEFPTMVFPSGQISQASALAPAPPQVLPQAPAPAPAPAMVSALAQAP APVPVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDDEDLGALLGNSTDPAVFTD LASVDNSEFQQLLNQGIPVAPHTTEPMLMEYPEAITRLVTGAQRPPDPAPAPLGAPGL PNGLLSGDEDFSSIADMDFSALLSQISSGSGSGSRDSREGMFLPKPEAGSAISDVFEG REVCQPKRIRPFHPPGSPWANRPLPASLAPTPTGPVHEPVGSLTPAPVPQPLDPAPAV TPEASHLLEDPDEETSQAVKALREMADTVIPQKEEAAICGQMDLSHPPPRGHLDELTT

		TLESMTEDLNLDSPLTPELNEILDTFLNDECLLHAMHISTGLSIFDTSLF
Gal4(DBD)-NS3a(H1)-P2A-DNCR2-VPR-NLS	Gal4-**NS3a***-P2A-*DNCR2*-VPR (internal NLS) -catalytically inactive pcDNA5/FRT/TO	MKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVE SRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLGTPAAASTLEGGGSAGSGG**KKKG** **SVVIVGRINLSGDTAYAQQTRGEEGCQETSQTGRDKNQVEGEVQIVSTATQTFLATSI** **NGVLWTVYHGAGTRTIASPKGPVTQMYTNVDKDLVGWQAPQGSRSLTPCTCGSSDLYL** **VTRHADVIPVRRRGDSRGSLLSPRPISYLKGSAGGPLLCPAGHAVGIFRAAVSTRGVA** **KAVDFIPVESLETTMRSP**GSGATNFSLLKQAGDVEENPGP*MSSDEEEARELIERAKEA* *AERAQEAAERTGDPRVRELARELKRLAQEAAEEVKRDPSSSDVNEALKLIVEAIEAAV* *DALEAAERTGDPEVRELARELVRLAVEAAEEVQRNPSSSDVNEALHSIVYAIEAAIFA* *LEAAERTGDPEVRELARELVRLAVEAAEEVQRNPSSRNVEHALMRIVLAIYLAEENLR* *EAEESGDPEKREKARERVREAVERAEEVQRDPSGWLNHE*QKLISEEDLDALDDFDLDM LGSDALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLGSPKKKRKVGSQYLPDTD DRHRIEEKRKRTYETFKSIMKKSPFSGPTDPRPPPRRIAVPSRSSASVPKPAPQPYPF TSSLSTINYDEFPTMVFPSGQISQASALAPAPPQVLPQAPAPAPAPAMVSALAQAPAP VPVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDDEDLGALLGNSTDPAVFTDLA SVDNSEFQQLLNQGIPVAPHTTEPMLMEYPEAITRLVTGAQRPPDPAPAPLGAPGLPN GLLSGDEDFSSIADMDFSALLSQISSGSGSGSRDSREGMFLPKPEAGSAISDVFEGRE VCQPKRIRPFHPPGSPWANRPLPASLAPTPTGPVHEPVGSLTPAPVPQPLDPAPAVTP EASHLLEDPDEETSQAVKALREMADTVIPQKEEAAICGQMDLSHPPPRGHLDELTTTL ESMTEDLNLDSPLTPELNEILDTFLNDECLLHAMHISTGLSIFDTSLF
BFP	BFP reporter pcDNA5/FRT/TO	MSELIKENMHMKLYMEGTVDNHHFKCTSEGEGKPYEGTQTMRIKVVEGGPLPFAFDIL ATSFLYGSKTFINHTQGIPDFFKQSFPEGFTWERVTTYEDGGVLTATQDTSLQDGCLI YNVKIRGVNFTSNGPVMQKKTLGWEAFTETLYPADGGLEGRNDMALKLVGGSHLIANI KTTYRSKKPAKNLKMPGVYYVDYRLERIKEANNETYVEQHEVAVARYCDLPSKLGHKL N
TRE3G-2xMS2	scRNA, wt+f6 MS2 expressed with NLS-MCP-ANR-ANR-P2A-BFP	**GTACGTTCTCTATCACTGATA**GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTA AATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGGGAGCACA TGAGGATCACCCATGTGCGACTCCCACAGTCACTGGGGAGTCTTCCCTTTTTTTGTTT TTTATGTCT
TRE3G	TRE3G guide in gRNA_Cloning Vector	GTACGTTCTCTATCACTGATA