Supplementary Information for

Genome-wide CRISPR screen for Zika virus resistance in human neural cells

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Supplementary Materials and Methods

Human PSC cultures

Human iPSC line iPS-wt5 was previously described (1) and cultured on mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) in human iPSC medium, containing DMEM/F12 (Thermo), 20% knockout serum replacement (Thermo), 1% non-essential amino acids (Thermo), 1mM Glutamax (Thermo), 0.1mM b-mercaptoethanol (Sigma) and 12ng/ml bFGF (Thermo). Human ESC line WIBR3 was previously described (2) and cultured on mitomycin C-inactivated MEFs in human ESC medium containing DMEM/F12 (Thermo), 15% fetal bovine serum (Hyclone), 5% knockout serum replacement (Thermo), 1% non-essential amino acids (Thermo), 1mM glutamine (Thermo), 0.1mM b-mercaptoethanol (Sigma) and 4ng/ml bFGF (Thermo). Cultures were passaged manually or with 1mg/ml collagenase type IV (Thermo) every 5-7 days. This and other cell lines were routinely tested for mycoplasma negativity. All experiments involving cells from human subjects were performed in compliance with MIT COUHES protocol 0612002068.

NGD 0.5X

NGD 0.5X was prepared as previously described (3). 500mL of NGD 0.5X consists of a mixture of 475mL of Neurobasal (Thermo), 5mL of Gem21-VitA (0.5X, Gemini Bioproducts), 2.5mL of Neuroplex N2 (0.5X, Gemini Bioproducts), 5mL of 100mM pyruvate, 5mL of Glutamax, 5mL of Pen/strep, 5mL of 5M NaCl, 1g of Albumax I, 3.5ug of Biotin, 85mg of Lactic Acid and 2.5mg of Ascorbic Acid.

NP culture

Differentiation of human PSCs to NPs in 2D adherent culture was performed as previously described (3). Briefly, human PSCs were passaged onto matrigel-coated dishes using PBS without Ca^{2+}/Mg^{2+}, filtered through a 40um cell strainer to remove MEFs, and cultured directly in NGD 0.5X containing dorsomorphin (2.5uM, Stemgent), bFGF (10ng/mL, Thermo) and human insulin (additional 10ng/mL) until super-confluent. bFGF and additional insulin were removed after a week, and NGD 0.5X plus dorsomorphin was replaced every day for 10 days. Cells were subsequently passaged with PBS without Ca^{2+}/Mg^{2+} when rosette lawns were observed throughout the culture. Rho-associated protein kinase (ROCK) inhibitor Y27632 (10mM, Stemgent) was added to the medium during the first 3 passages. After the first passage, NPs were expanded and maintained in NGD 0.5X containing 10ng/ml bFGF and 10ng/mL human insulin. For experiments using iPS-wt5 NPs, two independent NP differentiations were used for the genome-wide screen and focused screen. A third and fourth independent NP

differentiation were used for individual sgRNA experiments, and for compound and interferon treatment experiments, respectively. A fifth independent NP differentiation was used to generate astrocytes. One batch of WIBR3 NPs was used for the focused screen.

Astrocyte culture

NPs were differentiated into astrocytes as previously described (3). Briefly, NPs were plated on matrigel-coated dishes in NGD 0.5X without bFGF and additional insulin. At 4 weeks, PSA-NCAM- and A2B5+ glial progenitors were derived from multipotent NPs, and cultured in NGD 0.5X with the addition of EGF (20ng/mL) and bFGF (20ng/mL) as immature astrocytes. Further maturation was achieved with the withdrawal of bFGF and EGF, and addition of 5% FBS or CNTF (10ng/mL). One batch of iPS-wt5 astrocytes was used for individual sgRNA experiments.

Cerebral organoid culture

Cerebral organoids were generated from WIBR3 human ESCs as previously described (4, 5), with modifications. Briefly, human ESCs were dissociated from MEFs using collagenase type IV, and further separated from residual MEFs by gravity separation, before trypsinization to generate single cells. A total of 9000 cells were then plated into each well of a PrimeSurface v-bottom 96-well plate (S-Bio) to form single embryoid bodies (EBs), in medium containing DMEM/F12, 20% KSR, 2mM Glutamax, 1% non-essential amino acids, 50nM b-mercaptoethanol, 4ng/ml bFGF, and 2.5uM dorsomorphin. ROCK inhibitor Y27632 (50uM) was included in the first 24 hours. EBs were maintained in 96-well plates for 6 days, then transferred to ultra-low-attachment 24-well plates (Corning), in neural induction medium containing DMEM/F12, 1X N2 supplement, 1% non-essential amino acids, 2mM Glutamax, 1ug/ml heparin (Stem Cell Technologies), and 2.5uM dorsomorphin. On days 10-12, EBs were embedded in droplets of matrigel, and were allowed to gel at 37C. Embedded EBs were subsequently cultured in neural maturation medium containing 50% DMEM/F12, 50% Neurobasal, 0.5X N2 supplement, 0.5X B27 supplement, 2mM GlutaMAX, 2.5ng/ml human insulin, 0.5% non-essential amino acids, and 25nM b-mercaptoethanol. Droplets were cultured in stationary condition in ultra-low-attachment 6-well plates (Corning) for 4 days, followed by transfer to an orbital shaker (Unimax-1010, Heidolph Brinkmann) rotating continuously at 150rpm. Two independent organoid differentiations were used for immuno-blotting and ZIKV infection.

ZIKV production and infection

ZIKV strain MR766 (Uganda, $ZIKV^{U}$) and PRVABC59 (Puerto Rico, $ZIKV^{PR}$) were obtained from ATCC, and expanded in Vero cells or C6/36 mosquito cells. To establish tittered viral stocks, virus-containing supernatant was harvested and viral titer was determined by infecting Vero cells, followed by flow cytometry analysis of Flavivirus envelope immuno-staining to calculate Vero cell infectious units. MOI used to infect 2D and 3D culture was calculated based on Vero cell infectious units. Virus-containing medium was replaced after 6 or 24 hours with fresh medium. To infect cerebral organoids, the number of cells on the surface was estimated from the diameter of the 3D organoid and was used to calculate the amount of virus applied. Viral inoculum was

washed off and replaced after 24 hours with fresh medium. Organoids were infected by MOI 1 $ZIKV^{PR}$ at day 30 and analyzed 12 days later.

Genome-scale pooled screening

Pooled genome-wide lentiviral sgRNA library (6) (Addgene #1000000100) was prepared as described (7, 8, 9), and 1X10^8 human NPs derived from iPS-wt5 were transduced. Virus containing medium was replaced after 6 hours with fresh NGD 0.5X containing bFGF and insulin. Lentivirus transduction efficiency and MOI was evaluated by selection with puromycin (1ug/ml) on a subset of cells and determined to be between 30% and 50%. 7 days post lentiviral transduction, 125 million NPs were challenged with $ZIKV^{U}$ at MOI 1 for 24 hours. Surviving cells were harvested for genomic DNA extraction 8 days post $ZIKV^{U}$ infection. Genomic DNA was extracted using the Qiagen QIAmp DNA miniprep kit according to manufacturer's instructions, and high-throughput sequencing libraries were prepared as described (7, 8). Mapped reads were given a pseudocount of 1 and normalized per million mapped reads. The log2 fold change in representation of each sgRNA post-$ZIKV^{U}$ versus the initial reference was calculated. The score of the 5th best sgRNA was assigned as the gene score for each gene. The two-sided Kolmogorov-Smirnov test was used to compare the distribution of all sgRNAs for a given gene against the distribution of the entire population of sgRNAs, and the resulting p-values were corrected for multiple comparisons by the Benjamini-Hochberg method. A corrected p-value of < 0.05 was considered significant.

Focused library pooled screening

Oligonucleotide pools containing ~30 sgRNAs per candidate gene (Supplemental Table 3) were synthesized by CustomArray and cloned as described (8), except that pLentiCRISPRv2 was used as the vector. Pooled lentivirus was prepared as for the genome-wide library, and included a spike-in of a library containing 499 intergenic- (WIBR3 NPs) or 5191 intergenic- and protein coding-targeted sgRNAs (iPS-wt5 NPs) that were not predicted to modulate flavivirus infection as a control (Supplemental Table 3). We observed robust survival in the WIBR3 NPs focused screen, so we included additional controls in the iPS-wt5 NPs and astrocytes focused screen to allow us to better distinguish the relative strength of validated candidates. Human NPs derived from WIBR3, iPS-wt5, and astrocytes derived from iPS-wt5 were transduced at an MOI of 0.3 with the focused lentiviral sgRNA library. Virus containing medium was replaced after 6 hours with fresh NGD 0.5X, followed by puromycin (1ug/ml) selection for 5-7 days. 2 million NPs were challenged with $ZIKV^{U}$ at MOI 2. Surviving cells were harvested for genomic DNA extraction 8-9 days post ZIKV infection. Genomic DNA was extracted using a Qiagen QIAamp DNA blood miniprep kit according to manufacturer's instructions. Sequencing libraries and data analysis were performed as for the genome-wide screen, except that the following forward primer was used during PCR amplification in order to amplify from pLentiCRISPRv2: AATGATACGGCGACCACCGAGATCTACACCCCACTGACGGGCACCGGA.

Generation of sgRNA modified cell lines

Individual sgRNAs (Supplemental Table 4) were cloned into the BsmBI site of pLentiCRISPRv2 (Addgene #52961) according to the depositor's protocol. Lentivirus

was generated as described (9). Human NPs and astrocytes derived from iPS-wt5 were transduced with individual sgRNA lentivirus. Virus containing medium was replaced after 6 hours with fresh NGD 0.5X followed by puromycin (1ug/ml) selection for 5-7 days. To transduce WIBR3 human ESCs with ISG15 sgRNA and Cas9 containing lentivirus, human ESCs cultured on MEFs were first dissociated from MEFs using collagenase type IV, and further dissociated using Trypsin/EDTA to generate single cells. Human ESCs were plated onto matrigel-coated dishes and fed mTeSR medium containing 10uM ROCK inhibitor Y27632 and lentivirus particles. Virus-containing medium was replaced after 24hrs followed by puromycin (1ug/ml) selection for 7 days on matrigel-coated dishes in mTeSR, and subsequently on DR4 MEFs for 7days at clonal density. Individual human ESC clones were picked and expanded. PCR on genomic DNA and Sanger sequencing were performed to identify homozygous mutant clones.

Histology and imaging

Cells and tissues were fixed with 4% (w/v) paraformaldehyde in PBS, or in 1:1 acetone / methanol fixative. Paraffin sections were prepared from organoids. Following membrane permeabilization with PBS containing 0.3% triton, cells and organoid sections were blocked with 3% normal donkey serum. Primary antibodies were against Cleaved-caspase 3 (Cell Signaling), Flavivirus (Santa Cruz), GFAP (Abcam), Sox2 (R&D Systems), and visualized by secondary antibodies conjugated with Alexa 488, 568, 594, 647 (Thermo), followed by counter-staining with DAPI (Thermo). Phase contrast images were captured on a wide-field Nikon Ti2000 mounted with a SPOT RT monochrome camera. Fluorescent images of immuno-staining were captured on a Zeiss LSM-700 confocal microscope.

Statistics

All data values were presented as mean +/- SEM. Student's t tests were applied to data with two groups. ANOVA analyses were used for comparisons of data with greater than two groups. Post hoc group comparisons were performed with Bonferroni test. A value of $p<0.05$ was considered significant. Statistics used for CRISPR screens are described in the CRISPR screening methods.

RNA extraction, reverse transcription and quantitative PCR

Cells were homogenized and total RNA extracted using the RNeasy kit (Qiagen) following manufacturer's instructions. Total RNA concentrations were measured using NanoDrop ND-1000 spectrophotometer. RNA extraction from supernatant was performed using QIAamp Viral RNA mini kit (Qiagen). RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen) with random hexamer primers according to manufacturer's instructions. Transcript representation was determined by quantitative PCR using SYBR Green PCR mix (Applied Biosystems), with primer pairs against ZIKV and GAPDH. Cellular viral RNA raw Ct values were normalized to GAPDH. Supernatant viral RNA raw Ct values were normalized to a standard curve of known viral RNA quantity. For the purpose of quantitative analysis, transcripts not detected were assigned a Ct value of 40.

Protein purification and immuno-blotting

Total protein was extracted from cells and tissues using RIPA lysis buffer (Millipore) containing 50mM Tris-HCL pH 7.4, 150mM NaCL, 0.25% deoxycholic acid 1% NP-40, 1mM EDTA, protease inhibitor cocktail (Roche), phosphotase inhibitor cocktail 2 and 3 (Sigma). Total protein from the supernatant was measured using BCA protein assay (Pierce). Primary antibody against ISG15 (Santa Cruz) was visualized with HRP-conjugated secondary antibodies, followed by LumiGlo Chemiluminescent Kit (KPL) in accordance with the manufacturer's instructions. Membranes were stripped and re-probed with antibody against Actin (Sigma).

Compound and interferon treatment of NPs

NPs were treated with 0, 1nM, 10nM and 100nM of Bafilomycin A1 (Enzo), concomitantly with $ZIKV^{U}$ infection. Samples were collected 24 and 48 hours later. For sodium chlorate experiment, NPs were treated with 0, 5, 10, 25, 50 and 100mM sodium chlorate (Sigma), concomitantly with $ZIKV^{U}$ or $ZIKV^{PR}$ infection. Samples were collected after a 48 hours treatment following $ZIKV^{U}$ infection or after 72 hours following $ZIKV^{PR}$ infection. For interferon gamma experiment, NPs were pre-treatment for 2 hours with 20ng/mL interferon gamma, followed by $ZIKV^{U}$ or $ZIKV^{PR}$ infection, and collected after 48 hours or 72 hours.

RNA Sequencing

Transcriptomic analysis was performed using the strand-specific RNA sequencing protocol described previously (10). Briefly, total RNA was extracted using the RNeasy Mini Kit (Qiagen), 24 hours after infection with MOI1 $ZIKV^{U}$. 5ug of polyA-selected RNA was fragmented and dephosphorylated, followed by ligation of an ssRNA adapter. Reverse transcription was performed using a primer complementary to the RNA adapter, then a DNA adapter was ligated onto the 3' end of the cDNA product. The library was PCR amplified, cleaned, quantified using a TapeStation (Agilent) and sequenced on a HiSeq 2500 (Illumina). All primer sequences for this protocol can be found in (10). Reads were mapped to the reference human genome version hg19 using TopHat. Gene expression comparison graph represents Log2(FPKM+1).

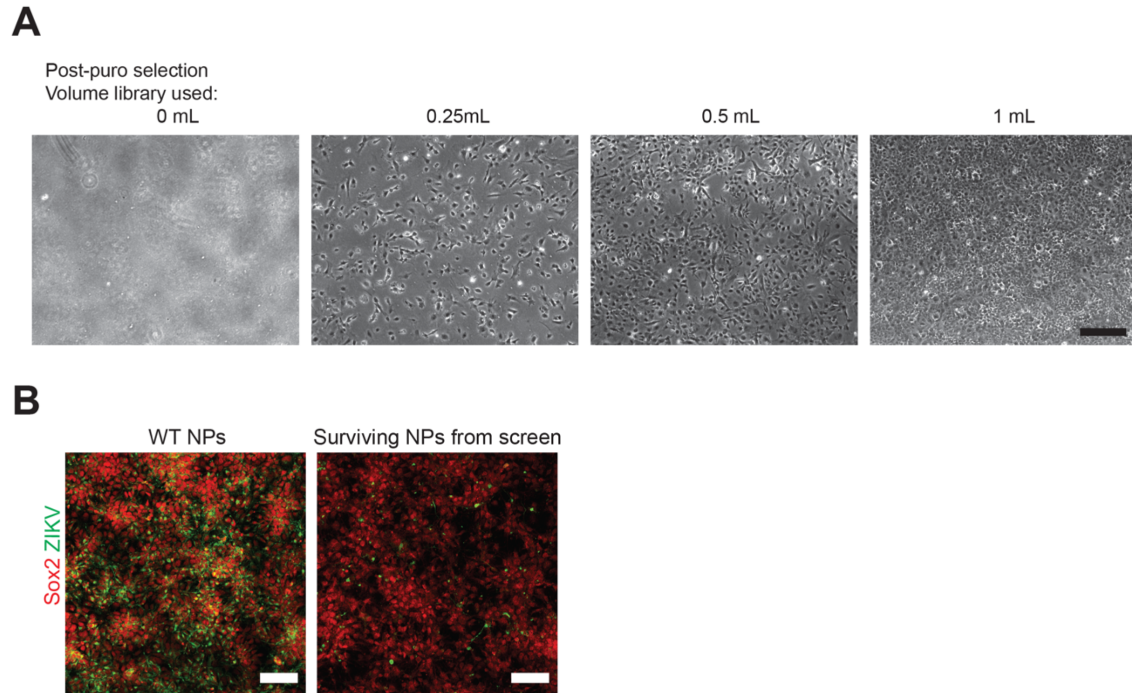

Fig. S1. Human NPs used for genome-wide CRISPR screen.
(A) Transduction of iPS-wt5 NPs with the genome-wide CRISPR knockout lentiviral library, measured by resistance to puromycin treatment. Scale bars: 100 μm.
(B) Surviving NPs displayed minimal level of ZIKV protein (green), and continue to be positive for canonical marker Sox2 (red). Control NPs were infected with MOI 1 $ZIKV^{U}$ and collected at 48hrs post infection. Scale bars: 100 μm.

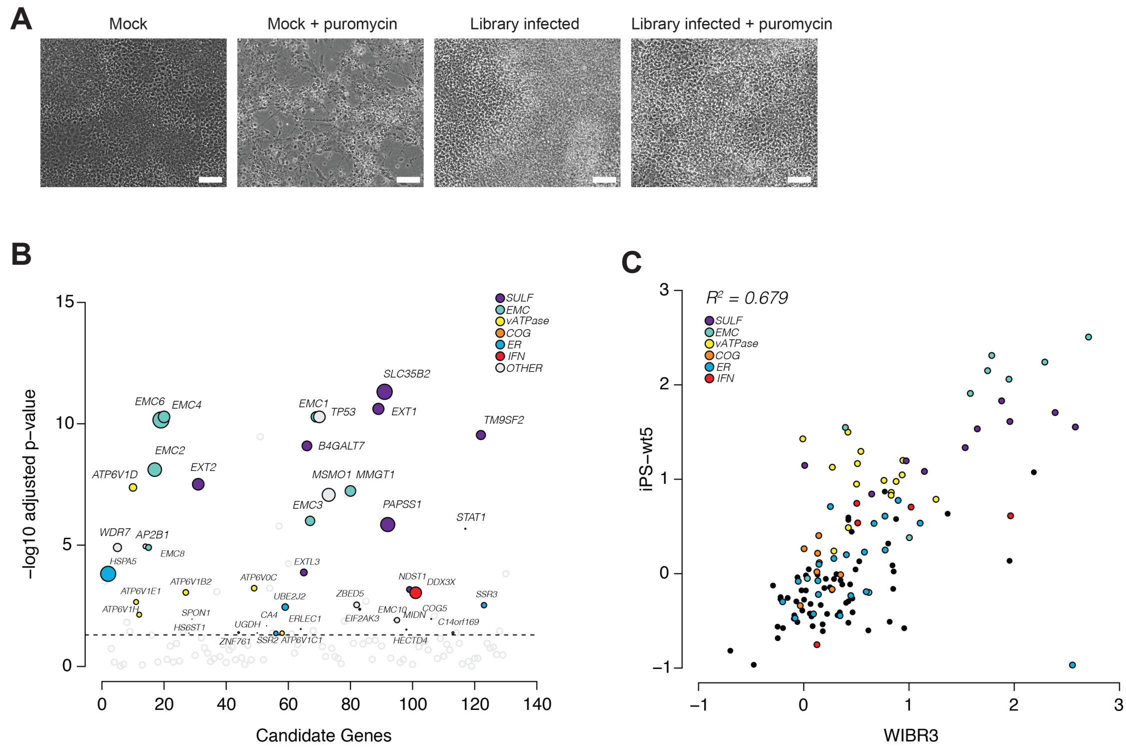

Fig. S2. CRISPR screen using a focused library.
(A) Human NPs transduced with the focused lentiviral library showed resistance to puromycin treatment. Scale bars: 100 μm.
(B) Focused screen in WIBR3 NPs identified most of the hits previously identified in the genome-wide screen. The size of the circles represents the magnitude of the increase in sgRNA representation after $ZIKV^U$ exposure.
(C) Correlation of log2 fold change of 5th best sgRNA for candidate genes in the two focused screens, with Pearson R2 value indicated. Donor backgrounds are indicated on the axes.

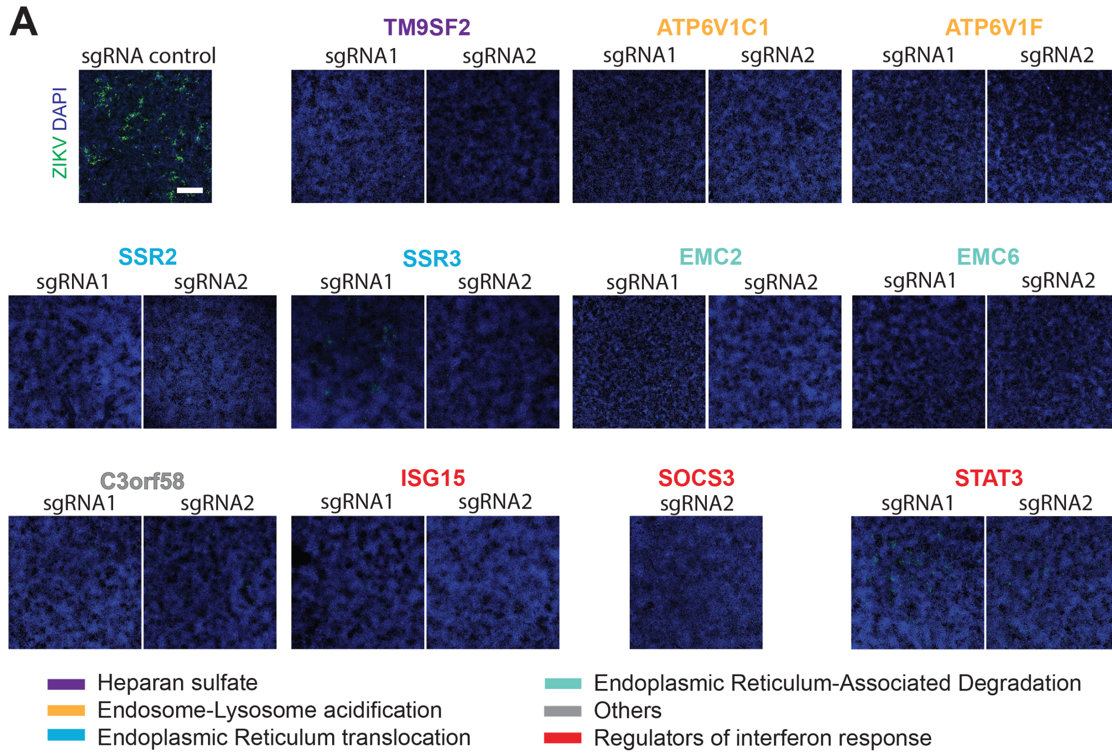

Fig. S3. ZIKV^{U} infection in human NPs targeted with individual sgRNAs.
(A) Immuno-staining for ZIKV envelope (green) and DAPI (blue) showed reduced infection by MOI 0.5 ZIKV^{U} in iPS-wt5 NPs individually targeted for candidate host genes, 24hrs post infection. Scale bars: 1000 μm.

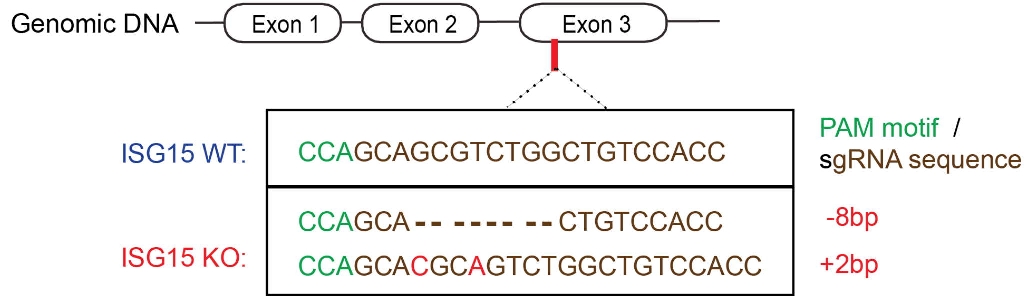

Fig. S4. ISG15 mutant WIBR3 human ESCs.
(A) Diagram of the human ISG15 gene, and generation of ISG15 frameshift mutations using CRISPR/Cas9-mediated gene targeting in WIBR3 human ESCs.

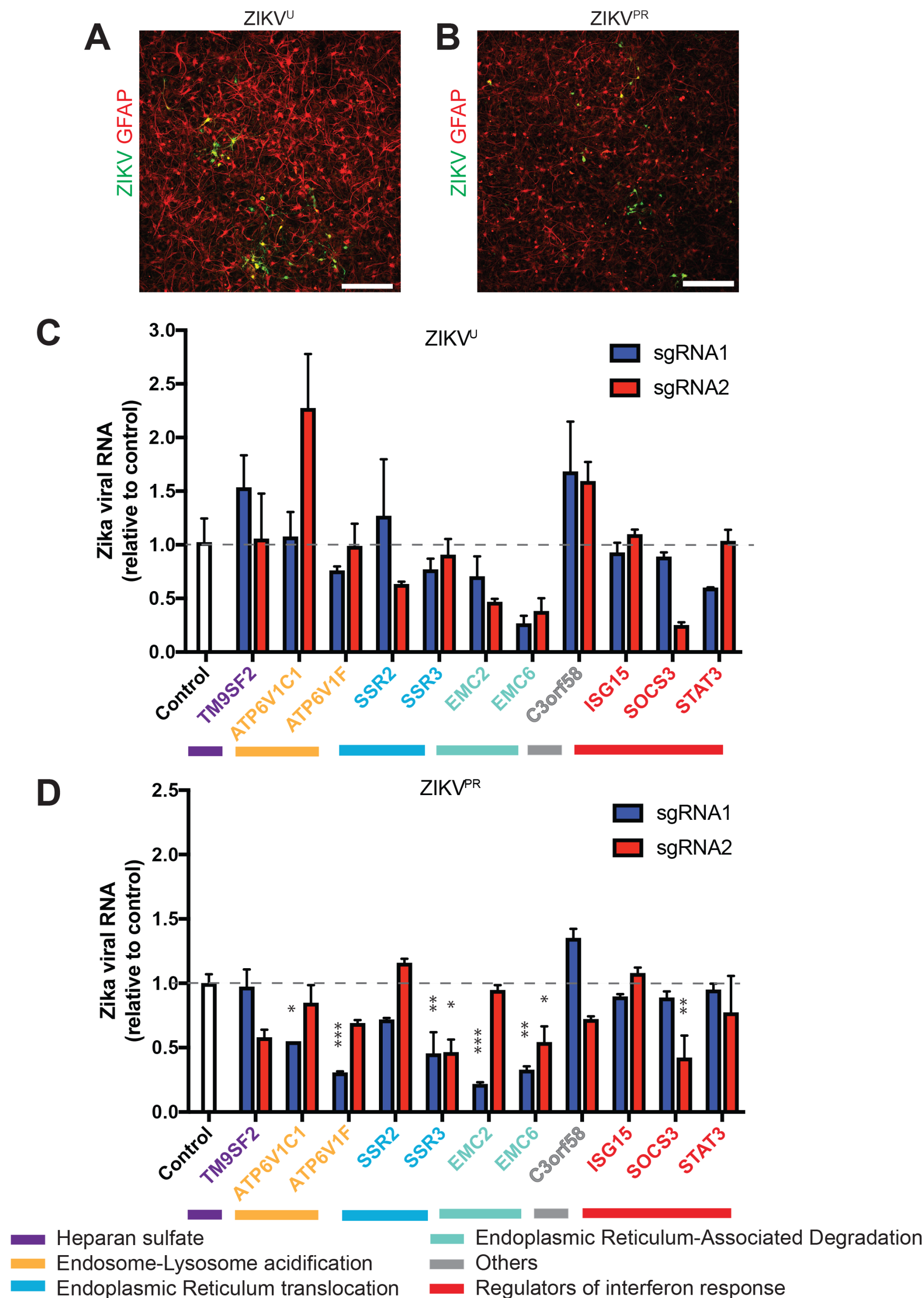

Fig. S5. Human astrocytes used for individual CRISPR gene targeting.
(A-B) Human astrocytes derived from iPS-wt5 expressed astrocytic marker GFAP (red) and were susceptible to $ZIKV^{U}$ and $ZIKV^{PR}$ infection (green). Astrocytes were infected with MOI 0.1 $ZIKV^{U}$ or MOI 0.6 $ZIKV^{PR}$, and collected at 48hrs. Scale bars: 100 μm.
(C-D) Human iPS-wt5 astrocytes individual targeted for candidate ZIKV host genes showed partially reduced infection by $ZIKV^{U}$ (C) and $ZIKV^{PR}$ (D), as measured by viral RNA load. Astrocytes were infected with MOI 1 $ZIKV^{U}$ or $ZIKV^{PR}$, and collected at 72hrs. Experimental sgRNA results that showed significant reduction compared to control sgRNA were noted.
Technical replicate: 2. Results are mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Additional data table S1 (separate file)

CRISPR sgRNA ranking from genome-wide screen.

Additional data table S2 (separate file)

RNA sequencing analysis of mock treated human iPS-wt5 NPs, and NPs infected with $ZIKV^U$ at MOI1, collected at 24 hours post infection.

Additional data table S3 (separate file)

Secondary CRISPR screen with control sgRNAs and sgRNAs against candidate ZIKV host genes. Relevant information includes hits for secondary screen, sgRNA used for screens on iPS-wt5 and WIBR3 NPs, and CRISPR ranking.

Additional data table S4 (separate file)

List of sgRNA used for individual validation.

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