Immunity, Volume *46*

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Supplemental Inventory

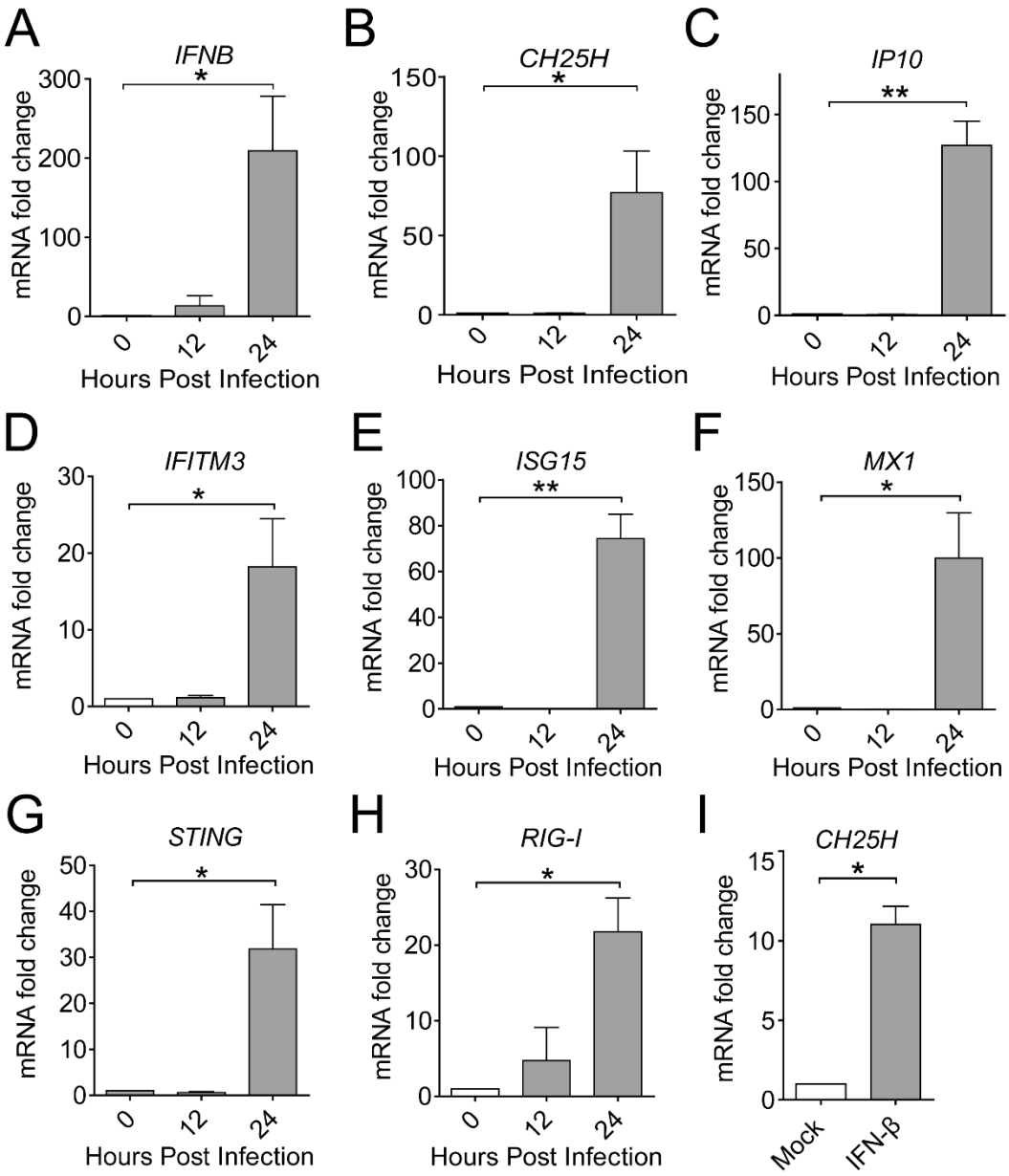

Figure S1. ZIKV infection triggers induction of ISGs in A549 cells, Related to Figure 1

(**A-H**) A549 cells were infected with ZIKV (GZ01/2016 strain, MOI 5) for indicated time, mRNA levels of *IFNB, CH25H, IP10, IFITM3, ISG15, MX1, STING, and RIG-I* in these cells were quantified by RT-qPCR.

(**I**) A549 cells were pretreated with by IFN-β (10ng/ml) for 24 hrs, *CH25H* mRNA expression was quantified by RT-qPCR.

All data are shown as mean ± SEM from three independent experiments, $*p \leq 0.05$, $**p \leq 0.01$, unpaired student *t* test.

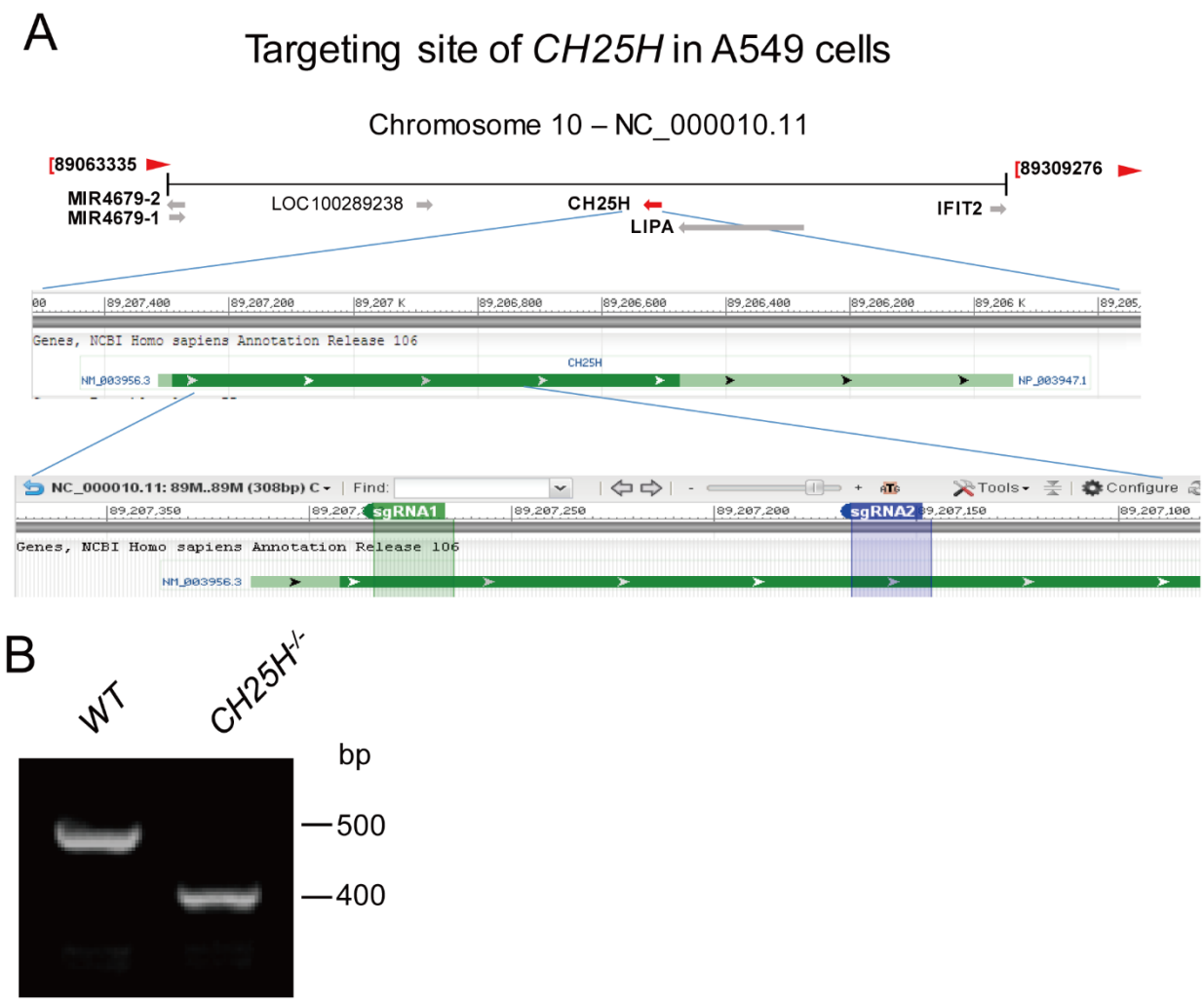

Figure S2. sgRNA targeting sites of *CH25H* and verification of CH25H$^{-/-}$ A549 cells, Related to Figure 1

(**A**) Design of two sgRNAs targeting genome loci of *CH25H* in A549 cells. The two sgRNAs were separated about 100 bp.

(**B**) Deletion of ~100bp genomic DNA in *CH25H*$^{-/-}$ single clone was confirmed by PCR.

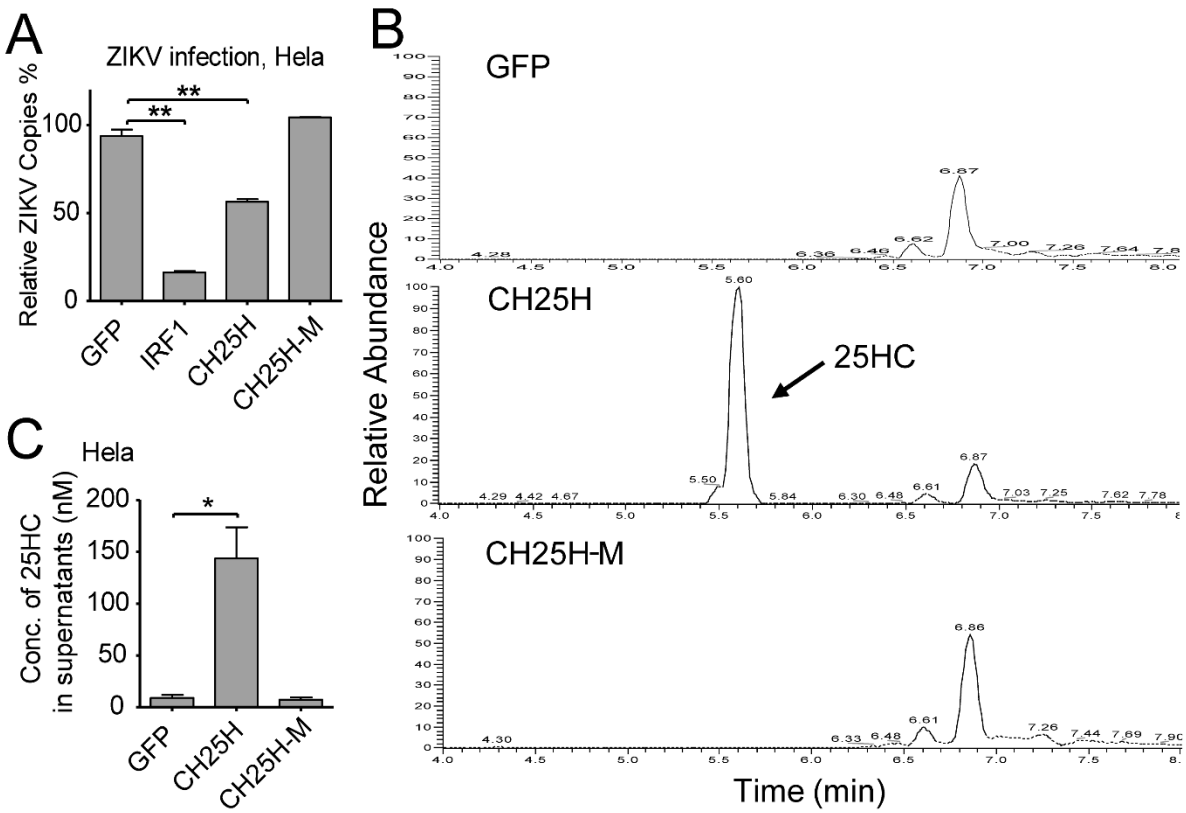

Figure S3. CH25H inhibits ZIKV infection by production of 25HC in Hela cells, Related to Figure 1

(A) Hela cells were pretreated with conditional medium from the indicated construct-transfected Hela cells for 12 hrs, and then the pretreated cells were infected with ZIKV (GZ01/2016 strain, MOI 0.1) for another 48 hrs. ZIKV genomic RNA in the cell lysates were quantified by RT-qPCR.

(**B**-**C**) Hela cells were transfected with GFP, wild-type CH25H, and CH25H-M for 24 hrs, 25HC production was detected by MS (**B**), and 25HC concentration was quantified (**C**). Data of (**A**) and (**C**) are shown as mean ± SEM from three independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, unpaired student *t* test.

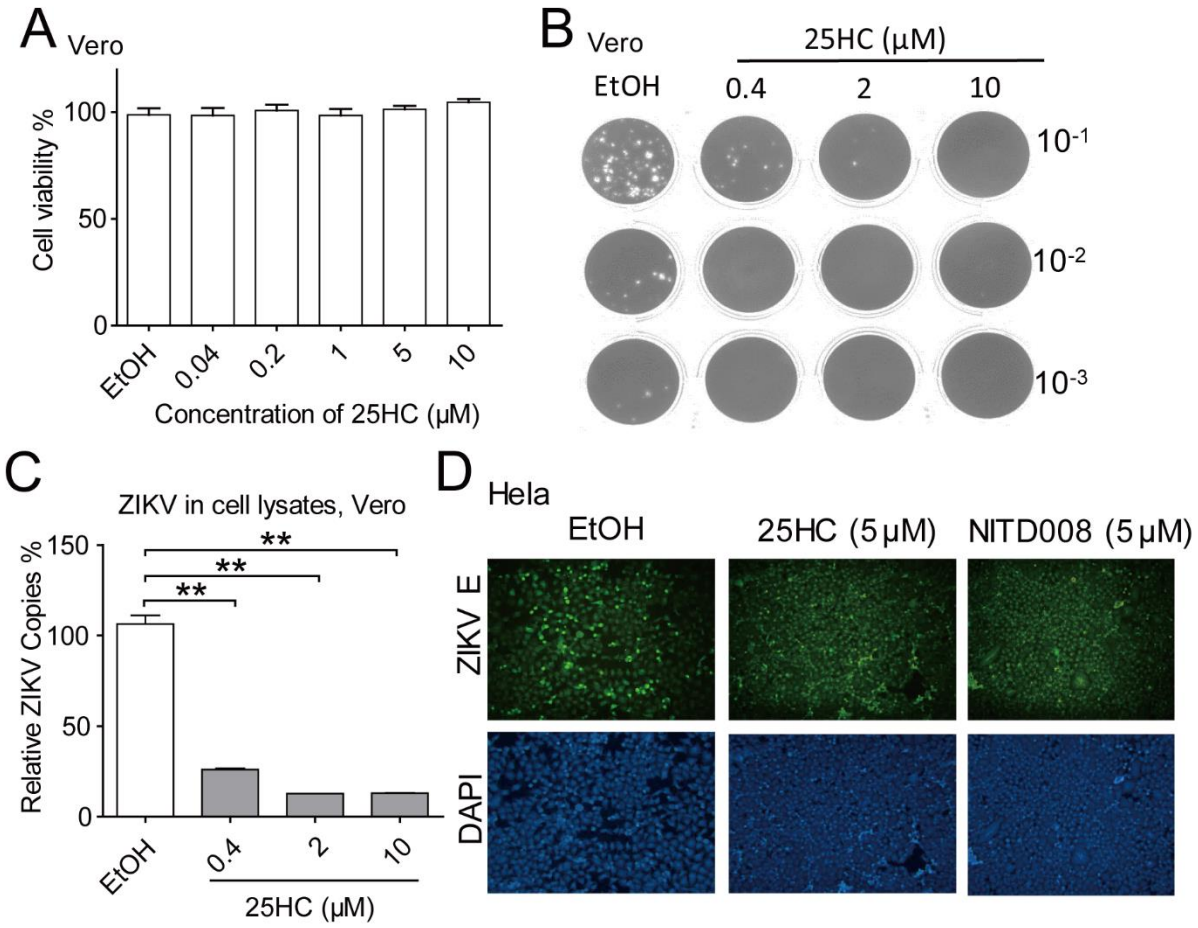

Figure S4. 25HC protects host cells against ZIKV infection *in vitro*, Related to Figure 2

(**A**) Vero cells were pretreated with indicated concentration of 25HC for 36 hrs, then MTT assay was performed to determine the cell toxicity of 25HC. Cell viability in samples with EtOH was set to 100%.

(**B-C**) Vero cells were pretreated with indicated concentration of 25HC or EtOH for 8 hrs, and then these cells were infected with ZIKV (GZ01/2016 strain) at MOI 0.1 (**B**) or MOI 1 (**C**). 48 hpi, Supernatant ZIKV were quantified by plaque assay (**B**). ZIKV genomic RNA copies in cell lysates were quantified by RT-qPCR (**C**).

(**D**) Hela cells were pretreated with EtOH, 25HC, and NITD008 for 8 hrs, and then these cells were infected with ZIKV (GZ01/2016 strain, MOI 0.1) for 48 hrs. ZIKV in the drug treated and ZIKV-infected was qualified by immunofluorescence assay with 4G2 antibody targeting ZIKV E protein

Data of (**B**) and (**D**) are one representative of three independent experiments, data of (**A**) and (**C**) are shown as mean ± SEM from three independent experiments, $^{**}p \leq 0.01$, unpaired student *t* test.

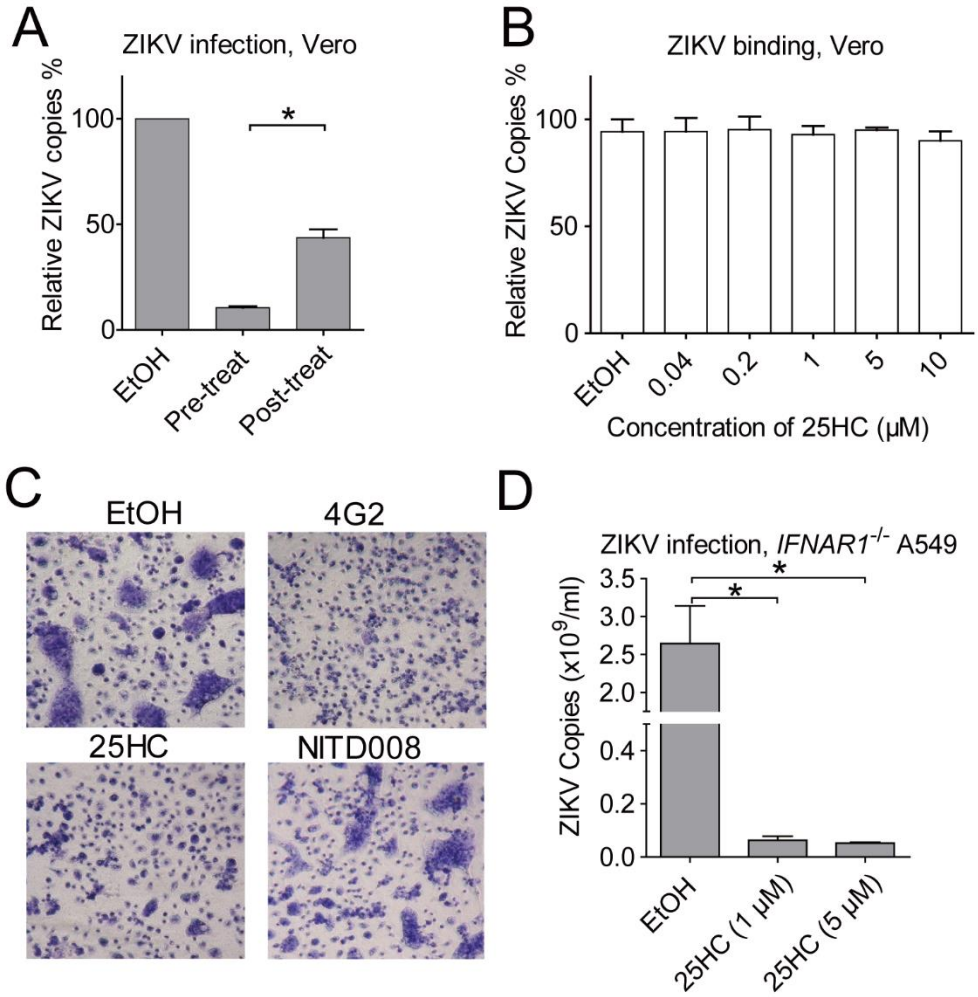

Figure S5. 25HC can not inhibit ZIKV replicon activity, Related to Figure 3

(**A**) Vero cells were treated with 25HC 8 hrs before (pre-treat) or 1 hrs after (post-treat) ZIKV infection (GZ01/2016 strain, MOI 0.1). Relative ZIKV genomic RNA copies in cell lysates were quantified by RT-qPCR.

(**B**) Vero cells were pretreated with indicated concentration of 25HC and then infected with ZIKV (GZ01/2016 strain, MOI 1) in 4°C for 1 hr. Then relative ZIKV genomic RNA copies in the cell lysates were quantified by RT-qPCR.

(**C**) The inhibitory function of 25HC to DENV mediated cell-cell fusion. Figure legends were as same as that in Fig. 3B. Data was one representative of three independent experiments.

(**D**) *IFNAR1*$^{-/-}$ A549 cells were pretreated with 25HC for 12hrs and subsequently infected with ZIKV (MOI 0.1) for 1 hr. The viral RNA in supernatants was quantified by RT-qPCR at 48 hpi. Data of (**A, B, D**) are shown as mean ± SEM from three independent experiments, *$p \leq 0.05$, unpaired student *t* test.

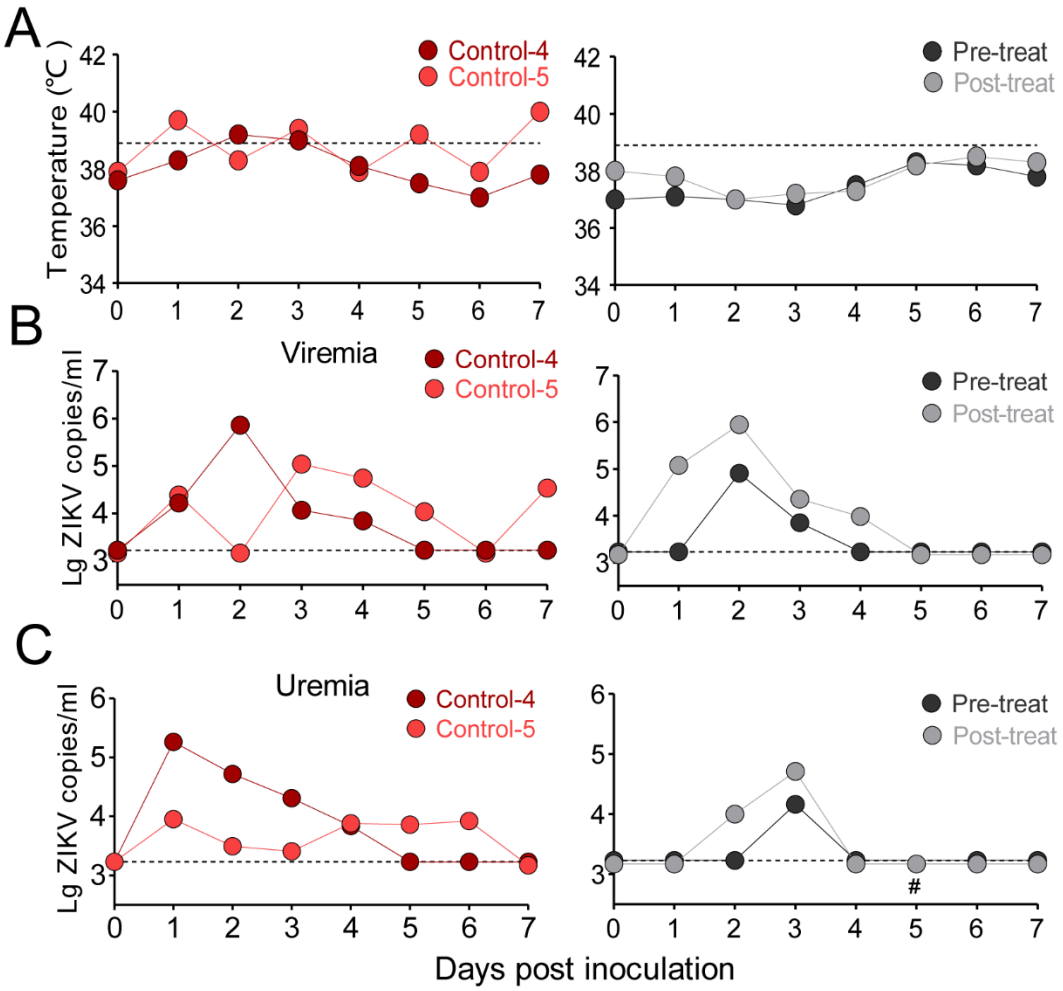

Figure S6. 25HC attenuated ZIKV titers in urine of rhesus monkeys, Related to Figure 5

(**A-C**) Rhesus monkeys were infected with 1x105 PFU of ZIKV (GZ01/2016 strain) intramuscularly as shown in Fig. 5, of which two animals received 25HC, one prior to- (pre-treat) and one post-ZIKV challenge (post-treat), the other two monkeys served as control.

(**A**) Underarm temperature of these EtOH/25HC-pretreated and 25HC-treated and ZIKV-infected ($1x10^5$ PFU) monkeys was measured by thermometers.

Virus titers in serum (**B**) and urine (**C**) of monkeys challenged by $1x10^5$ PFU ZIKV were determined by RT-qPCR. #: we failed to collect the urine sample from the 25HC-pretreated monkey on day 5 post infection.

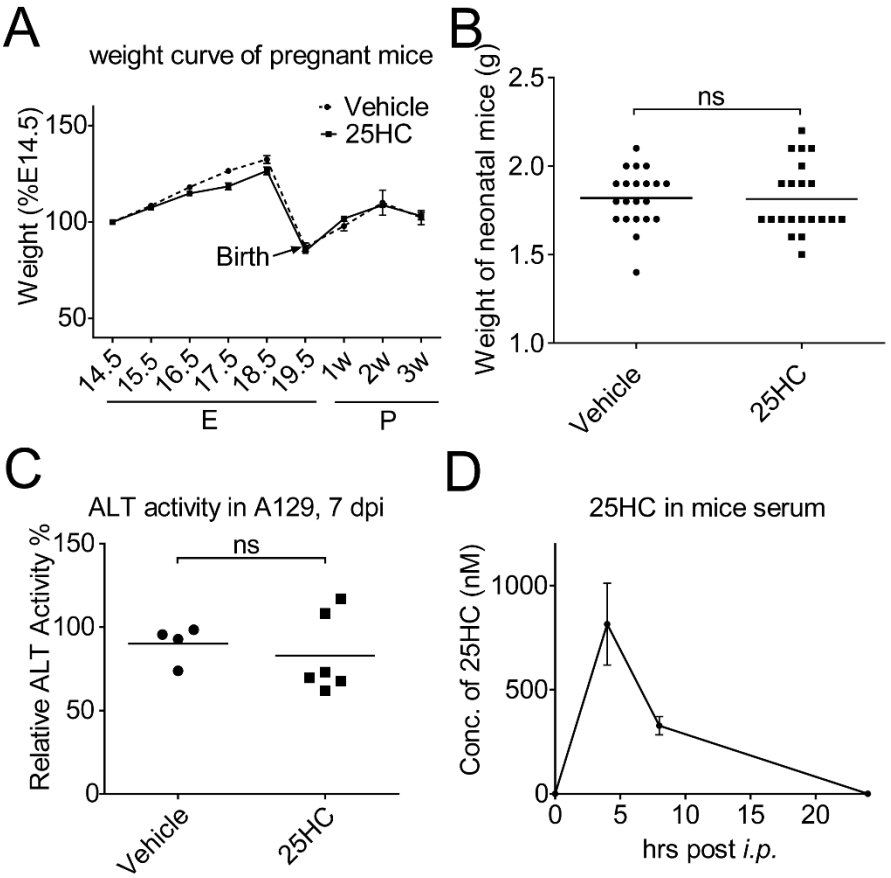

Figure S7. The safety and toxicity tests of 25HC in BALB/c mice, Related to Figure 7

(**A-B**) Vehicle or 25HC (50mg/kg) was injected into the peritoneal cavity of pregnant mice once daily for 5 days from 14.5 day of embryonic. E, embryonic; P, postnatal. **(A)** The weights of pregnant mice before and after birth of neonatal mice. (**B**) The weights of neonatal mice at P1. Vehicle/25HC: n=21. (**C**) The Alanine aminotransferase (ALT) activity in serum of mice as shown in Figure 4D. Vehicle: n=4; 25HC: n=6. (**D**) 25HC concentration in serum of 4 weeks old BALB/c mice, which were injected with 25HC (50mg/kg) before the MS analysis. *i.p.* =intraperitoneal.

Supplemental Experimental Procedures

RNA isolation, reverse transcription and real time quantitative PCR

Total RNA from cells, tissues or viruses was extracted with the Purelink RNA Extraction kit (Thermo Fisher). Viral RNA copies were determined by quantitative reverse transcription PCR (RT-qPCR) (Johnson et al., 2005) with the OneStep PrimeScript RT-PCR Kit (Takara, Beijing). ZIKV primers and TaqMan probes were described previously (Deng et al., 2016). Primers used to amplify ISGs were obtained from Primer Bank (https://pga.mgh.harvard.edu/primerbank/) and the primer sequences are available upon request. SYRB Green qPCR mix (TransGen Biotech, Beijing) was used to analyze the mRNA level of the ISGs.

RNA sequencing

Total RNA was extracted from ZIKV (GZ01 strain, MOI 5)-infected A549 cells at 24 hrs post-infection (hpi). RNA concentration was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher. The quality of extracted RNA was evaluated using an Agilent Technology 2100 Bioanalyzer. RNA libraries were constructed using a TruSeq Stranded mRNA Sample Prep Kit (RS-122-2001, Illumina). The quantity and quality of the libraries were also assessed by Qubit and Agilent 2100 Bioanalyzer, respectively and their molar concentration was validated by qPCR for library pooling. Sequencing was performed on the Illumina NextSeq 500 platform using PE150 chemistry (Illumina) in the sequencing core facility of Suzhou Institute of Systems Medicine.

Cell Transfection

Hela cells were transfected with 700 ng of plasmids expressing the genes of interest in a 24-well plate with Lipofectamine 2000 (Thermo Fisher), according to the manufacturer's instructions. At 24 hrs post-transfection, cells were infected with ZIKV at an MOI of 0.1.

Gene knockout by CRISPR/CAS9

To generate a *CH25H* knockout A549 cell line, two sgRNAs (with 117-bp gap sequence) targeting the CH25H gene were designed and cloned into sgRNA-expressing vectors under control of the U6 promoter. They were co-transfected into A549 cells with CAS9-expressing

plasmids, followed by puromycin selection as described previously (Cong et al., 2013; Mali et al., 2013). The *CH25H*$^{-/-}$ single clone was isolated by serial dilution and confirmed by sequencing and PCR genotyping. The target sequences of the two sgRNAs were: 5'-CCACAACTGCTCCGACCCCC-3' and 5'-TTCTCCATCACCACATACGT-3'. *IFNAR1*$^{-/-}$ A549 cells were obtained with the same method and the target sequences of the two sgRNAs were: 5'-GAAACACTTCTTCATGGTATG-3' and 5'-GATAATTGGATAAAATTGTC-3'. The *IFNAR1*$^{-/-}$ single clone was confirmed by sequencing and PCR genotyping.

25HC extraction and mass spectrometry (MS) analysis

25HC in mice serum was analyzed as described before (Honda et al., 2008). To analysis 25HC in the supernatant from cell cultures, samples was collected and desalted by Waters Colum (Oasis-HLB), re-dissolved in 80% methanol, and analyzed by orbitrap mass spectrometer (Q Exactive, Thermo Fisher). The data were acquired by full MS/ddMS2 scan method in positive and negative ion mode as described before (Tang et al., 2016). The resolutions of 70,000 and 17,500 were selected for full scan and MS/MS, respectively. The ten most intense precursors were isolated for fragmentation with stepped NCE 30+50%. Mass range of m/z 60-900 was used for scanning. The BEH C18 column (Waters) was used for separation at 35°C. Mobile phase A contained 7.5 mM ammonium acetate aqueous buffer at pH 4.2, while mobile phase B contained 5%/95% acetonitrile/methanol. The chromatographic peaks were extracted using Sieve (Thermo Fisher) and metabolites were confirmed using either accurate masses or MS/MS spectrum. 25HC purchased from Sigma (H1015) was used as a standard.

Syncytia formation assay

C6/36 *Aedes albopictus* cells were pretreated with 25HC for 8 hrs and infected with dengue virus at MOI 0.2 for 4 days. The fusion medium (pH 5.8) was used to stimulate virus-mediated cell-to-cell fusion for 2 hrs. The fusion medium was replaced by normal medium for another 24 hrs. Syncytia formation was detected after staining with Giemsa. The number of syncytia was determined as described previously (Liu et al., 2013a).

Liposome-binding assay

EtOH or 5 μM 25HC at the indicated concentrations of recombinant unilamillar liposomes, with

a composition of 7:3 phosphatidylcholine:cholesterol (Encapsula Inc.), were added to A549 cells for 8 hrs. They were subsequently infected with ZIKV (MOI 0.3) for 1 hr, and the supernatants were subjected to plaque assay at 48 hpi.

ZIKV and DENV replicon assay

The DENV replicon was previously described (Liu et al., 2013b), and the ZIKV replicon that carries the Renilla luciferase gene was developed based on ZIKV strain SZ01 as described (Xie et al., 2016).The replicon assay was performed as described (Liu et al., 2013b; Xie et al., 2016) with minor modifications. Briefly, $2x10^5$ BHK-21 cells in a 24-well plate were transfected with 200 ng of the *in vitro* transcribed RNA with Lipofectamine 2000 reagent (Thermo Fisher). 25HC or EtOH were added to the media at 6 hrs post transfection (hpt). The cell lysates were collected at 48 hpt and the renilla luciferase activity was measured by the renilla luciferase assay system (Promega) in a GloMax-96 microplate luminometer. The luciferase activity in samples with EtOH was set to 100%.

Human cortical organoid differentiation and ZIKV infection in organoids

All human embryonic stem cell-based experiments were conducted with prior approval from the UCLA Embryonic Stem Cell Research Oversight (ESCRO) Committee. H9 human embryonic stem cells (Thomson et al., 1998) were obtained from the Stem Cell Core at the UCLA Broad Stem Cell Research Center and tested for mycoplasma negativity and normal chromosome numbers. The H9 cells were confirmed for pluripotency markers (>95% Oct4, Sox2, and Nanog staining), and experiments were performed on cells between passages 40 and 80. The cortical organoid differentiation from hES cells was performed as described (Kadoshima et al., 2013) with minor modifications (Watanabe et al., manuscript in preparation). Zika virus strain PRVABC59 was originally obtained from the Centers for Disease Control and Prevention (CDC) of the United States, and further amplified in C6/36 *Aedes albopictus* cells. Each organoid (~ 80,000 cells/organoid at D20s) was inoculated with 1:2-1:8 diluted viral stock for 2 hrs and then further diluted with a fresh culture media to 1:7. After 24 hrs, the media was replaced with fresh media, and the organoids were analyzed at different time points. 25HC-treatment of organoids was performed with 0.1 to 2.5 μM of 25HC for 15 hrs prior to ZIKV infection. At 2 hpi, 25HC was added to the media again and organoids were subjected to RT-qPCR, plaque assay and immunofluorescence assay at the indicated time points.

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