



(51) International Patent Classification:

G01N 33/50 (2006.01) A61K 31/00 (2006.01)

C12Q 1/70 (2006.01) A61K 39/00 (2006.01)

A61K 38/00 (2006.01)

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(21) International Application Number:

PCT/US2017/052769

(22) International Filing Date:

21 September 2017 (21.09.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/398,297 22 September 2016 (22.09.2016) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING HEPATITIS E VIRUS

(57) Abstract: The present invention provides a method of inhibiting hepatitis E virus (HEV) release from a cell that is infected with an HEV, and a method of treating a HEV infection in a subject in need thereof. The present invention also provides a method of identifying an agent that inhibits HEV infectivity using a transcomplementation system.



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METHODS AND COMPOSITIONS FOR INHIBITING HEPATITIS E VIRUS

RELATED APPLICATION(S)

This application claims the benefit of U.S. Provisional Application No. 62/398,297, filed September 22, 2016. The entire teachings of the above Application are incorporated herein by reference.

BACKGROUND

[0001] Hepatitis E, transmitted by the hepatitis E virus (HEV), is the leading cause of enterically-transmitted viral hepatitis globally and a major public health threat in developing countries. In the general population, HEV-associated mortality is roughly 1%. However, the risk to pregnant women is significantly higher, reaching as high as 25% amongst women with HEV infections during their third trimester, resulting in 70,000 deaths and 3,000 stillbirths every year. While most HEV infections occur in developing countries, recent epidemiological studies have found a high seroprevalence of anti-HEV antibodies in industrialized countries (Dalton, H. R., *et al. Lancet Infect Dis* 8, 698-709 (2008)), suggesting exposure to the virus from travel to HEV endemic areas or from contact with pigs, a major reservoir of HEV. In a majority of cases, HEV causes an acute infection, but amongst immunocompromised patients – notably organ transplant recipients (Schildgen, O., *et al. N Engl J Med* 358, 2521-2522, author reply 2522 (2008); Kamar, N. *et al. N Engl J Med* 358, 811-817 (2008)) and individuals co-infected with HIV (Dalton, H. R., *et al. N Engl J Med* 361, 1025-1027 (2009)) – HEV can progress to chronicity.

[0002] Pegylated interferon (IFN) and the nucleoside analogue ribavirin (RBV) have been used to treat HEV infection, but the use of these drugs is not recommended in certain patient groups, including pregnant women and organ transplant recipients. Although a vaccine for preventing HEV infection has been developed, it is not available in most countries (Zhang, J. *et al. N Engl J Med* 372, 914-922 (2015)). Moreover, HEV isolates resistant to RBV have been identified (Debing, Y. *et al. Gastroenterology* 147, 1008-1011 e1007; quiz e1015-1006, doi:10.1053/j.gastro.2014.08.040 (2014)).

[0003] Accordingly, there is a significant unmet need for an anti-HEV therapeutic having broad applicability and/or enhanced efficacy.

SUMMARY OF THE INVENTION

[0004] In one aspect, the present invention provides a method of inhibiting hepatitis E virus (HEV) release from a cell that is infected with an HEV, comprising contacting the cell with an agent that inhibits one or more activities of HEV open reading frame 3 (ORF3) protein, wherein the one or more ORF3 activities are selected from, e.g., ion channel activity, multimerization activity, and/or viroporin activity.

[0005] In another aspect, the present invention also provides a method of treating a HEV infection in a subject in need thereof, comprising administering to the subject an effective amount of an agent that inhibits one or more activities of HEV ORF3 protein, wherein the one or more activities are selected from, e.g., ion channel activity, multimerization activity, and/or viroporin activity.

[0006] In other aspects, the present invention provides a method of identifying an agent that inhibits HEV infectivity, wherein the method comprises: a) introducing into a cell culture 1) a nucleic acid that comprises an HEV open reading frame 2 (ORF2) nucleotide sequence, 2) a nucleic acid that comprises an HEV ORF3 nucleotide sequence, and 3) a nucleic acid that i) comprises a reporter gene and an HEV ORF1 nucleotide sequence and ii) lacks HEV ORF2 and ORF3 nucleotide sequences. The method also comprises contacting the cell culture in step a) with an agent to be tested for anti-HEV activity, and further, harvesting the cell culture media from the cell culture that has been contacted with the agent. The harvest media is combined with naïve cells in cell culture. The method further comprises measuring a level of activity of the reporter gene in the naïve cell culture; and comparing the level of reporter activity in the naïve cell culture measured in step e) to a reference activity level, wherein a decrease in measured activity level as compared to the reference activity level indicates that the agent inhibits HEV infectivity.

[0007] The methods described herein facilitate the identification of new HEV therapeutics and provide new therapeutic approaches to the treatment of hepatitis E in HEV-infected patients.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0009] FIGS. 1A-1D demonstrate that ORF3 is a predicted transmembrane protein that associates with ER membranes and can form homo-oligomers. FIG. 1A is a schematic representation of the predicted transmembrane topology (TMHMM Server v. 2.0) of HEV ORF3 protein (Kernow C1/p6). FIG. 1B shows HEV ORF3 protein associates with the ER membrane. HepG2C3A cells lentivirally transduced with HEV ORF3-HA were stained with anti-HA and anti-calnexin antibodies. Nuclei were stained with Hoechst dye. Shown are representative images of at least triplicate experiments. FIG. 1C shows results of a co-immunoprecipitation assay of FLAG-ORF3 and ORF3-HA in HepG2C3A cells. Cells were transduced with FLAG-ORF3 and/or ORF3-HA lentivirus and lysed 72 hours post-transduction. Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody. FIG. 1D is a Western Blot of cell lysates from cells lentivirally transduced to express ORF3-HA or mock transduced, analyzed by blotting with an anti-HA antibody. The monomer and oligomers are indicated with arrowhead and arrows, respectively.

[0010] FIGS. 2A and 2B show that ORF2 and ORF3 are required for releasing viral particles to infect naïve HepG2C3A cells. FIG. 2A shows infection kinetics of transcomplemented HEV in HepG2C3A cells. Cell culture supernatants from naïve HepG2C3A, or HepG2C3A cells transduced with HEV ORF2 or/and ORF3 were collected 5 days post-transfection with rHEV Δ ORF2/3[Gluc] RNA. Naïve HepG2C3A cells were incubated with these supernatants. After 12 hours, cells were washed and Gaussia luciferase (Gluc) activity quantified in the cell culture supernatants at the indicated time points. In FIG. 2B, 5 days following transfection of rHEV Δ ORF2/3[Gluc] RNA into HepG2C3A, or HepG2C3A cells transduced with HEV ORF2 or/and ORF3, lysates were used to infect naïve HepG2C3A cells. Gluc activity was measured in the supernatants 4 days post-infection. Shown are averages and standard deviations of triplicate measurements of three independent experiments.

[0011] FIGS. 3A and 3B indicate that the HEV ORF3 protein has ion channel activity. FIG. 3A shows that IAV M2 can substitute ORF3 function. Cell culture supernatants from naïve HepG2C3A, or HepG2C3A cells transduced with HEV ORF2 and ORF3, M2 or M2(A30P), were collected 5 days post-transfection with rHEV Δ ORF2/3[Gluc] RNA. Naïve HepG2C3A cells were incubated with these supernatants. After 12 hours, cells were washed and Gaussia luciferase activity quantified in the cell culture supernatants 4 days later. Data represent the mean $\pm$ SD (n = 6-9). FIG. 3B shows current-voltage relationship of *X. laevis*

oocytes expressing HEV ORF3, IAV M2, IAV M2(A30P) or control oocytes. During the current recording, the oocytes were bathed in Ringer solution. The standard voltage-clamp protocol consisted of rectangular voltage pulses from -90 to +60 mV in 10-mV increments applied from a holding voltage of -60 mV. Each point represents the steady-state current (average current between 4000-5000 ms) at the corresponding voltage step. Data represent the mean $\pm$ SD (n = 5).

[0012] FIGS. 4A-4C demonstrate that HEV ORF3 ion channel activity is required for release of infectious HEV. FIG. 4A shows the identification via alanine scanning mutagenesis of positions within HEV ORF3 involved in the release of infectious virions. HEV ORF3 mutants generated by changing triplets of amino acids to alanines across the entire ORF3 protein were lentivirally delivered to HepG2C3A cells expressing ORF2. Dually transduced cells were subsequently transfected with rHEV Δ ORF2/3[Gluc] RNA. Five days post-transfection supernatants were collected and used to infect naïve HepG2C3A cells. Gaussia luciferase activity was quantified in the producer cells (bottom panel) and in the cell culture supernatant 4 days post-infection (top panel). The putative transmembrane regions (TM) are underlined. The asterisk marks the serine residue of ORF3, which can be phosphorylated. The two boxes mark the PXXP motifs within ORF3. In FIG. 4B, HepG2C3A cells lentivirally transduced with HA-tagged wild-type or the indicated ORF3 mutant were stained with anti-HA and anti-calnexin antibodies. Nuclei were stained with Hoechst dye. Shown are representative images of triplicate experiments. FIG. 4C shows a current-voltage relationship of *X. laevis* oocytes expressing HEV wild-type or the indicated ORF3 mutants. Experiments were conducted as detailed in FIGS. 3A and 3B. Data represent the mean $\pm$ SD (n = 5).

[0013] FIGS. 5A-5C relate to the transcomplementation system. FIG. 5A is a schematic representation of the wild-type HEV and rHEV Δ ORF2/3[Gluc] genomes and the bicistronic lentiviral constructs for expressing ORF2 and ORF3. FIG. 5B is a schematic representation of the transcomplementation system for packaging HEV virions. FIG. 5C is a representative flowcytometry plot demonstrating efficient ORF2 and ORF3 expression. HepG2C3A cells were transduced with LVX-ORF2-IRES-zsGreen and/or LEX-ORF3-IRES-mCherry or not transduced. Flowcytometric analysis was performed 4 days following transduction to quantify the frequencies of ORF2 and/or ORF3 expressing cells.

[0014] FIGS. 6A-6D illustrate constructs used in ORF3 functional studies and their expression. FIG. 6A is a schematic representation of the bicistronic lentiviral constructs for

expressing HEV ORF2, ORF3, IAV M2 and IAV M2(A30P). FIG. 6B shows representative flowcytometry plots demonstrating efficient ORF2 and ORF3, IAV M2 or M2(A30P) expression. HepG2C3A cells were transduced with LVX-ORF2-IRES-zsGreen and/or LEX-[ORF3 or M2 or M2(A30P)]-IRES-mCherry or not transduced. Flowcytometric analysis was performed 4 days following transduction to quantify the frequencies of ORF2 and/or ORF3 and M2 expressing cells. FIG. 6C is a schematic representation of SP6 driven constructs used for *in vitro* transcription of HEV ORF3, IAV M2 and IAV M2(A30P) mRNAs. FIG. 6D shows that HEV ORF3 expressed in *X. laevis* oocytes localizes to the plasma membrane. Water-injected and HEV ORF3 mRNA-injected oocytes were immunolabeled with polyclonal ORF3 antibody and analyzed by confocal microscopy.

[0015] FIGS. 7A and 7B show expression of ORF3 mutant constructs. In FIG. 7A the cell lysates from HepG2C3A cells lentivirally transduced to express HA-tagged wild-type ORF3, the indicated alanine mutants of ORF3, or mock transduced were analyzed by Western blot with anti-HA or anti-actin antibodies. In FIG. 7B, HepG2C3A cells were lentivirally transduced with HA-tagged wild-type or the indicated ORF3 mutants and stained with anti-HA and anti-calnexin antibodies. Nuclei were stained with Hoechst dye. Shown are representative images of at least triplicate experiments.

[0016] FIGS. 8A-8C illustrate ion channel activity of different ORF3 mutants. FIG. 8A shows that wild-type or alanine mutants of HEV ORF3 expressed in *X. laevis* oocytes localize to the plasma membrane. Water-injected and HEV ORF3 mRNA-injected oocytes were immunolabeled with polyclonal ORF3 antibody and analyzed by confocal microscopy. FIG. 8B shows current-voltage relationship of *X. laevis* oocytes expressing HEV wild-type or the indicated ORF3 mutants. Experiments were conducted as detailed in FIGS. 3A and 3B. Data represent the mean + SD (n = 5). FIG. 8C summarizes the features of triple alanine ORF3 mutants that do not support HEV release.

[0017] FIG. 9 shows the genomic organization of hepatitis E virus.

[0018] FIGS. 10A and 10B depict the development of a cell culture system for producing infectious virions in trans. FIG. 10A is a schematic of transcomplementation platform. FIG. 10B indicates that transfection of rHEV Δ ORF2/3[Gluc] leads to equivalent HEV genome replication in ORF2/3 and non-expressing cells. Supernatants from ORF2/3 and non-expressing HepG2C3A cells that were transfected with rHEV Δ ORF2/3[Gluc] were used to

infect naïve HepG2C3A. Infectious virions were released only in the presence of ORF2/3 in the producer cell.

[0019] FIG. 11 shows that ORF1 and ORF2 from an HEV gt3 strain were transcomplemented with ORF3 derived from HEV gts 1-4. Supernatant was collected and used to infect naïve HepG2C3A cells, producing comparable levels of infection regardless of the ORF3 variant used.

[0020] FIGS. 12A-12C show an experimental overview of testing of known ion channel inhibitors for activity against HEV ORF3. FIG. 12A is a table indicating the seven ion channel inhibitors tested. FIG. 12B shows that HepG2C3a cells transduced with HEV ORF2 and HEV ORF3 were transfected with a recombinant HEV construct, rHEVΔORF2/3[Gluc], expressing HEV ORF1 and a secreted Gaussia luciferase (Gluc) reporter gene. It was hypothesized that treatment with viroporin inhibitors would result in reduced release of infectious virions into supernatant from these “producer” HepG2C3a cells. FIG. 12C describes an experimental design for testing ion channel inhibitors: A single clone-derived cell line highly expressing ORF2 and ORF3 (named HepG2C3a 1F4) was used as a producer cell line to generate infectious virions. The 1F4 cells were pre-treated with each drug in three dilutions for 4-6 hours, and then transfected with rHEVΔORF2/3[Gluc]. The medium from these cells containing progeny virions was collected after 5 days and used to infect naïve HepG2C3a cells. The cells were infected for 12 hours and subsequently washed three times with PBS. On day 5 post-infection, Gluc levels were measured in the supernatant of these cells as a proxy for infection level.

[0021] FIGS. 13A-13C shows the results from viroporin inhibitor testing. Supernatant was collected from producer 1F4 HepG2C3a cells treated with varying dilutions of ion channel inhibitors, and used to infect naïve HepG2C3a cells. The infected cells were washed 12h post infection, and Gluc levels were measured in the supernatants of these cells on day 5 post-infection (shown above). Cells were infected in triplicate in a 24w format. None of the ion channel inhibitors significantly decreased release of infectious particles compared to the positive control (producer cells transfected with p6/Gluc in the absence of drug). Crystal violet staining on drug-treated cells was performed as a rough measure of cytotoxicity.

DETAILED DESCRIPTION OF THE INVENTION

[0022] A description of example embodiments of the invention follows.

[0023] HEV is a quasi-enveloped, positive(+)-sense, single-stranded RNA virus of the *Hepeviridae* family with three open reading frames (ORFs). ORF1 encodes a non-structural polyprotein comprised of a methyltransferase, papain-like cysteine protease, RNA helicase, and RNA-dependent RNA polymerase (Cao and Meng, *Emerging microbes & infections* 1, e17, doi:10.1038/emi.2012.7 (2012)). ORF2 encodes the viral capsid protein and is involved in virion assembly, interaction with the host cell, and immunogenicity. It contains three glycosylation sites necessary for formation of infectious particles (Ahmad, I. *et al.*, *Virus research* 161, 47-58, (2011)). In contrast, ORF3 and its corresponding function(s) have been largely elusive. The smallest ORF of the HEV genome, ORF3 is translated from a subgenomic RNA into a protein of 113-115 amino acids.

[0024] The present invention is based, in part, on the identification of a previously unknown HEV ORF3 function. As described herein, ORF3 forms multimeric complexes associated with intracellular endoplasmic reticulum (ER)-derived membranes via homophilic interactions, which complexes possess ion channel and/or viroporin function. To date, viroporins have been identified in nine different viruses, including six enveloped viruses (HCV, HIV, IAV, rotavirus, Alpha-/Sindbisviruses and Coronaviruses) but only in three non-enveloped viruses (simian virus 40 (SV40), coxsackie B virus (CBV) and polio virus (reviewed in Sze, C. W. & Tan, Y. J. *Viruses* 7, 3261-3284 (2015)). For both enveloped and non-enveloped viruses, viroporin function is frequently linked to virus release, but the underlying mechanism remains incompletely understood.

[0025] As demonstrated herein, the function of HEV ORF3 can be maintained by expressing the well-characterized viroporin influenza A virus (IAV) M2 protein. Further evidence of ORF3's ion channel function has been demonstrated by its ability to increase the flux of cations when expressed in *Xenopus laevis* oocytes. Furthermore, using alanine scanning mutagenesis, amino acid positions in ORF3 that are important for its formation of multimeric complexes, ion channel activity, and release of infectious particles have been identified.

[0026] Methods of Regulating ORF3 Activity

[0027] In one aspect, the present invention provides a method of inhibiting HEV release from a cell that is infected with an HEV, comprising contacting the cell with an agent that inhibits one or more activities of HEV ORF3 protein, wherein the one or more ORF3

activities are selected from ion channel activity, multimerization activity, or viroporin activity, or a combination thereof.

[0028] As used herein, the phrase “inhibiting HEV release” refers to inhibiting HEV release completely or partially from an infected cell.

[0029] As used herein, the phrase “HEV ORF3 protein” refers to a protein encoded by the third open reading frame of a HEV genome. As used herein, “HEV genome” refers to the genome of any one of the various HEV genotypes or quasispecies. As those of skill in the art would appreciate, the methods described herein can be used to inhibit an ORF3 protein of any genotype or quasispecies of HEV. In some embodiments, an ORF3 protein possesses any one or more of ion channel activity, multimerization activity, or viroporin activity. Examples of HEV ORF3 proteins are shown in Table 1 below.

[0030] Table 1. Examples of ORF3 protein sequences

Genotype 3 GenBank: AFD33685.1	MGSPCALGLFCCCSSCFCLCCPRHRPASRLAVVVGGA AAVPA VVSGVTGLILSPSPSIFIQPTSPPI SFHNPGLALGSRPAPLAP LGVTPSPAPPLPPAVDLPQLGLRR (SEQ ID NO: 1)
Genotype 1 ID: P69616	MGSRPCALGLFCCCSSCFCLCCPRHRPVSRLAAVVVGGA AAVP AVVSGVTGLILSPSQSIFIQPTSPPM SPLRPGLDLVFANPPDH SAP LGVTRPSAPPLPHVVDLPQLGPRR (SEQ ID NO: 2)
Genotype 2 ID: Q03499	MGSPPCALGLFCCCSSCFCLCCPRHRPVSRLAAVVVGGA AAVP AVVSGVTGLILSPSQSIFIQPTPLPQTLPLRPGLDLAFANQPGH LAPLGEIRPSAPPLPPVADLPQPGLRR (SEQ ID NO: 3)
Genotype 4 ID: BAG32135.1	MEMPPCALGLFCFCSSCFCLCCPRHRPVSRLAVAAGGA AAVP AVVSGVTGLILSPSPSIFIQPIPSHPTFQPQPGLELALGSQPVHS APLGATNPSAPPLLPVADLPQLGLRR (SEQ ID NO: 4)

[0031] In some embodiment, the HEV ORF3 protein comprises the sequence set forth in SEQ ID NO: 1. However, one of skill in the art would appreciate that the present method can be used to inhibit the activity (e.g., ion channel activity, multimerization activity, or viroporin activity, or a combination thereof) of an HEV ORF3 protein. Thus, in certain embodiments, the HEV ORF3 protein comprises a sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to SEQ ID NO: 1.

[0032] In various embodiments, the HEV protein comprises the sequence set forth in any one of SEQ ID NOs: 2-4. In certain embodiments, the HEV ORF3 protein comprises a

sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to any one of SEQ ID NOs: 2-4.

[0033] Various suitable agents can be used in the present invention. In some embodiments, the agent is a small molecule. Examples of small molecules include organic compounds, organometallic compounds, inorganic compounds, and salts of organic, organometallic and inorganic compounds. Atoms in a small molecule are typically linked together via covalent and/or ionic bonds. The arrangement of atoms in a small organic molecule may represent a chain (*e.g.* a carbon-carbon chain or a carbon-heteroatom chain), or may represent a ring containing carbon atoms, *e.g.* benzene or a polycyclic system, or a combination of carbon and heteroatoms, *i.e.*, heterocycles such as a pyrimidine or quinazoline. Small molecule inhibitors generally have a molecular weight that is less than about 5,000 daltons. For example, such small molecules can be less than about 1000 daltons, less than about 750 daltons or even less than about 500 daltons. Small molecules and other non-peptidic ORF3 inhibitors can be found in nature (*e.g.*, identified, isolated, purified) and/or produced synthetically (*e.g.*, by traditional organic synthesis, bio-mediated synthesis, or a combination thereof). See *e.g.* Ganesan, Drug Discov. Today 7(1): 47-55 (January 2002); Lou, Drug Discov. Today, 6(24): 1288-1294 (December 2001). Examples of naturally occurring small molecules include, but are not limited to, hormones, neurotransmitters, nucleotides, amino acids, sugars, lipids, and their derivatives.

[0034] In some embodiments, the agent can bind to one or more regions of the ORF3 protein. For example, the agent can bind to (or make contact with) the ORF3 protein at any one or more residues selected from the residues at positions 11-13, 29-40, 59-61, 71-73, 80-85, 86-89, or 95-98 of SEQ ID NO: 1.

[0035] As those of skill in the art would appreciate, in certain embodiments, the agent can bind to one or more regions of an ORF3 protein comprising any one of SEQ ID NOs: 2-4, wherein the one or more regions correspond to (*e.g.*, possess equivalent functions to) any one or more residues selected from the residues at positions 11-13, 29-40, 59-61, 71-73, 80-85, 86-89, or 95-98 of SEQ ID NO: 1.

[0036] In certain embodiments, the agent binds to a channel-forming region of an ORF3 protein. In other embodiments, the agent inhibits ORF3 protein ion channel activity, for example, by binding to a region of ORF3 protein comprising, *e.g.*, amino acid positions 11-13 or 59-61, or both, of SEQ ID NO: 1.

[0037] In some embodiments, the agent is contacted with a cell that is infected with HEV to inhibit HEV release from the cell. The present method is suitable for any cell that is infected with an HEV. Examples of such cells include hepatocytes and gastrointestinal cells, as well as cells derived from hepatocytes or gastrointestinal cells.

[0038] In another aspect, the present invention also provides a method of treating a HEV infection in a subject in need thereof. In some embodiments, the method comprises administering to the subject an effective amount of an agent that inhibits one or more activities of a HEV ORF3 protein, wherein the one or more activities are selected from ion channel activity, multimerization activity, or viroporin activity, or a combination thereof.

[0039] As used herein, the terms “treat,” “treating,” or “treatment,” refer to counteracting a medical condition (e.g., a condition related to HEV infection) to the extent that the medical condition is improved according to a clinically-acceptable standard.

[0040] As used herein, “subject” refers to a mammal (e.g., human, non-human primate, cow, sheep, goat, horse, swine, dog, cat, rabbit, guinea pig, rat, mouse). In a particular embodiment, the subject is a human. A “subject in need thereof” refers to a subject (e.g., patient) who has, or is at risk for developing, a disease or condition that can be treated (e.g., improved, ameliorated, prevented) by an agent that inhibits one or more ORF3 protein activities selected from ion channel activity, multimerization activity, or viroporin activity, or a combination thereof. In certain embodiments, the subject is a pregnant female. In other embodiments, the subject is immunocompromised. In some embodiments, the immunocompromised subject is a subject who has undergone an organ transplant or a subject infected with human immunodeficiency virus (HIV), or both. In certain embodiments, the subject is an immunocompromised pregnant female.

[0041] An agent that inhibits one or more activities of HEV ORF3 protein (and thereby treats a condition related to HEV infection) can be administered to a subject in need thereof by a variety of routes of administration including, for example, oral, dietary, topical, transdermal, or parenteral (e.g., intra-arterial, intravenous, intramuscular, subcutaneous injection, intradermal injection) routes of administration, depending on the agent. Administration can be local or systemic. The chosen mode of administration can vary depending on the particular agent selected. The actual dose of a therapeutic agent and treatment regimen can be determined by a skilled physician, taking into account the nature of the condition being treated, and patient characteristics.

[0042] As defined herein, an “effective amount” refers to an amount of an agent that, when administered to a subject, is sufficient to achieve a desired therapeutic effect (treats a condition related to HEV infection) in the subject under the conditions of administration, such as an amount sufficient to inhibit one or more activities of HEV ORF3 protein (e.g., ion channel activity, multimerization activity, or viroporin activity) in the subject. Various methods of assessing the effectiveness of the agent for treating a condition related to HEV infection are known in the art. For example, the titer can be measured in the sera of a patient according to methods known in the art. Measuring the titer can provide an indication of infectivity. Other methods of assessing infectivity of HEV from patient sera are known in the art.

[0043] An effective amount of the agent to be administered can be determined by a clinician of ordinary skill using the guidance provided herein and other methods known in the art, and is dependent on several factors including, for example, the particular agent chosen, the subject’s age, sensitivity, tolerance to drugs and overall well-being. For example, suitable dosages for a small molecule can be from about 0.001 mg/kg to about 100 mg/kg, from about 0.01 mg/kg to about 100 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.01 mg/kg to about 1 mg/kg body weight per treatment. Determining the dosage for a particular agent, patient and stage of HEV infection is well within the abilities of one of skill in the art.

[0044] An agent that inhibits one or more activities of HEV ORF3 protein (e.g., ion channel activity, multimerization activity, or viroporin activity) can be administered in a single dose or as multiple doses, for example, in an order and on a schedule suitable to achieve a desired therapeutic effect (e.g., treatment of a condition related to HEV infection). Suitable dosages and regimens of administration can be determined by a clinician of ordinary skill.

[0045] In some embodiments, the method comprises administering an effective amount of an agent that inhibits one or more activities of a HEV ORF3 protein (e.g., ion channel activity, multimerization activity, or viroporin activity) in combination with one or more additional therapeutic agents (e.g., additional agents that treat a condition related to HEV infection). Such agents are known in the art and include, for example, interferon (e.g., pegylated interferon) or ribavirin. Thus, in some embodiments, the method further comprises administering an effective amount of interferon or ribavirin, or both, to the subject. As those of skill in the art would appreciate, combination therapies that include agents known to have

a negative side effect to a subset of subjects would typically not be administered to that subset of subjects. For example, a combination therapy that includes interferon or ribavirin, or both, would typically not be administered to pregnant females and/or immunocompromised subjects.

[0046] When administered in a combination therapy, the agent can be administered before, after or concurrently with the other therapy (*e.g.*, administration of interferon or ribavirin, or both). When co-administered simultaneously (*e.g.*, concurrently), the agent and other therapy can be in separate formulations or the same formulation. Alternatively, the agent and other therapy can be administered sequentially, as separate compositions, within an appropriate time frame as determined by a skilled clinician (*e.g.*, a time sufficient to allow an overlap of the pharmaceutical effects of the therapies).

[0047] In some embodiments the agent can be administered to a subject in a composition or formulation comprising the agent and one or more pharmaceutically acceptable carriers or excipients. Suitable pharmaceutical carriers typically will contain inert ingredients that do not interact with the agent or nucleic acid. Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's lactate and the like. Formulations can also include small amounts of substances that enhance the effectiveness of the active ingredient (*e.g.*, emulsifying agents, solubilizing agents, pH buffering agents, wetting agents). Methods of encapsulation compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art. For inhalation, the agent can be solubilized and loaded into a suitable dispenser for administration (*e.g.*, an atomizer or nebulizer or pressurized aerosol dispenser).

[0048] The agent can be administered as a neutral compound or as a salt or ester. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic or tartaric acids, and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. Salts of compounds containing an amine or other basic group can be obtained, for example, by reacting with a suitable organic or inorganic acid, such as hydrogen chloride, hydrogen bromide, acetic acid, perchloric acid and the like. Compounds with a quaternary ammonium group also contain a counteranion such as chloride, bromide, iodide, acetate,

perchlorate and the like. Salts of compounds containing a carboxylic acid or other acidic functional group can be prepared by reacting with a suitable base, for example, a hydroxide base. Salts of acidic functional groups contain a counteranion such as sodium or potassium.

[0049] In some embodiments, pharmaceutical compositions comprising an agent that inhibits one or more activities of a HEV ORF3 protein (e.g., ion channel activity, multimerization activity, or viroporin activity) can also include one or more other therapeutic agents (e.g., interferon or ribavirin, or both) known to treat a condition related to HEV infection.

[0050] Standard pharmaceutical formulation techniques can be employed, such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's lactate and the like. Formulations can also include small amounts of substances that enhance the effectiveness of the active ingredient (e.g., emulsifying, solubilizing, pH buffering, wetting agents). Methods of encapsulation compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art. For inhalation, the agent can be solubilized and loaded into a suitable dispenser for administration (e.g., an atomizer or nebulizer or pressurized aerosol dispenser).

[0051] Methods of Identifying Agents that Inhibit HEV Infectivity

[0052] As described herein, the present invention also relates to a system (e.g., a transcomplementation system) that enables the identification of agents that inhibit various aspects of the HEV lifecycle, and which can lead to the identification of agents that inhibit HEV infectivity.

[0053] Thus, in other aspects, the present invention also provides a method of identifying an agent that inhibits HEV infectivity, also referred to herein as a transcomplementation method. In some embodiments, the method comprises introducing into a cell culture 1) a nucleic acid that comprises an HEV open reading frame 2 (ORF2) nucleotide sequence, 2) a nucleic acid that comprises an HEV ORF3 nucleotide sequence, and 3) a nucleic acid that i) comprises a reporter gene and an HEV ORF1 nucleotide sequence and ii) lacks HEV ORF2 and ORF3 nucleotide sequences. The method further comprises, in a subsequent step, contacting the cell culture with an agent to be tested for anti-HEV activity. In some

embodiments, the method comprises harvesting the cell culture media from the cell culture that has been contacted with the agent, combining the harvested media with a naïve cell culture, and measuring a level of activity of the reporter gene in the naïve cell culture. In various embodiments, the method also comprises comparing the level of reporter activity measured in the naïve cell culture to a reference activity level, wherein a decrease in the measured activity level as compared to the reference activity level indicates that the agent inhibits HEV infectivity.

[0054] As used herein, an agent that “inhibits HEV infectivity” refers to an agent that causes a reduction (completely or partially) in HEV function. A reduction in HEV function can result from a variety of inhibitory effects of an agent in any one or more phases of the HEV lifecycle. Generally, the present invention is suitable for identifying an agent that inhibits, e.g., HEV release from a cell, HEV replication, or HEV assembly, or a combination thereof. In certain embodiments, the agent inhibits ORF1 protein activity, ORF2 protein activity, or ORF3 protein activity, or a combination thereof, thereby inhibiting HEV infectivity.

[0055] The term “nucleic acid” refers to a polymer having multiple nucleotide monomers. A nucleic acid can be single- or double-stranded, and can be DNA (*e.g.*, cDNA or genomic DNA), RNA, or hybrid polymers (*e.g.*, DNA/RNA). Nucleic acids can be chemically or biochemically modified and/or can contain non-natural or derivatized nucleotide bases. Nucleic acids can also include, for example, conformationally restricted nucleic acids (*e.g.*, “locked nucleic acids” or “LNAs,” such as described in Nielsen et al., *J. Biomol. Struct. Dyn.* 17:175-91, 1999), morpholinos, glycol nucleic acids (GNA) and threose nucleic acids (TNA).

[0056] Methods of introducing nucleic acids into a cell are well known in the art, and include, for example, transduction, transfection, transformation, and the like. As those of skill in the art would appreciate, the method selected will depend on the particular context (*e.g.*, the nucleic acid to be introduced and/or the cells to be used for introducing the nucleic acid) to achieve, *e.g.*, high efficiency. In certain embodiments, the different nucleic acid components (*e.g.*, the nucleic acid that comprises an HEV ORF2 nucleotide sequence; the nucleic acid that comprises an HEV ORF3 nucleotide sequence; and the nucleic acid that i) comprises a reporter gene and an HEV ORF1 nucleotide sequence and ii) lacks HEV ORF2 and ORF3 nucleotide sequences) to be introduced into a cell culture in the transcomplementation system can be introduced by different methods. For example, nucleic

acid(s) encoding ORF2 and ORF3 can be transduced into the cell culture using a viral vector (e.g., lentivirus), and the nucleic acid comprising the reporter gene and ORF1 can be transfected (e.g., by other non-viral means). As those of skill in the art would appreciate, a variety of combinations (e.g., transduction, transfection, transformation) can be used to introduce the nucleic acids into cells.

[0057] ORF1 and ORF2 nucleotide sequences within a HEV genome that are involved in ORF1 and ORF2 function are known and/or ascertainable in the art. See, e.g., Cao, D. and Meng, X. J. *Emerging microbes & infections* 1, e17, doi:10.1038/emi.2012.7 (2012); and Ahmad, I., et al., *Virus research* 161(1): 47-58 (2011). Tables 2 and 3 below provide examples of ORF1 and ORF2 nucleotide sequences that can be used in the present invention.

[0058] Table 2. Examples of ORF1 Nucleotide Sequences

Genotype 1	ATGGAGGCCCATCAGTTTATCAAGGCTCCTGGCATCACTAC
ID: AF444002.1	TGCTATTGAGCAGGCTGCTCTAGCAGCGGCCAACTCTGCCC
	TTGCGAATGCTGTGGTAGTTAGGCCTTTTCTCTCTCACCAG
	CAGATTGAGATCCTTATTAACCTAATGCAACCTCGCCAGCT
	TGTTTTCCGCCCCGAGGTTTCTGGAACCATCCCATCCAGC
	GTGTTATCCATAATGAGCTGGAGCTTTACTGTCGCGCCCCGC
	TCCGGCCGCTGCCTTGAAATTGGTGCCCAACCCCGCTCAAT
	AAATGACAATCCTAATGTGGTCCACCGTTGCTTCCTCCGTC
	CTGCCGGGCGTGATGTTTCAGCGTTGGTATACTGCCCCCTACC
	CGCGGGCCGGCTGCTAATTGCCGGCGTTCCGCGCTGCGCGG
	GCTCCCCGCTGCTGACCGCACTTACTGCTTCGACGGGTTTT
	CTGGCTGTAACTTTCCCGCCGAGACGGGCATCGCCCTCTAT
	TCTCTCCATGATATGTCACCATCTGATGTCGCCGAGGCTAT
	GTTCCGCCATGGTATGACGCGGCTTTACGCTGCCCTCCACC
	TCCCGCCTGAGGTCCTGTTGCCCCCTGGCACATAACCGCACC
	GCGTCGTA CTTGCTGATCCATGACGGCAGGCGCGTTGTGGT
	GACGTATGAGGGTGACACTAGTGCTGGTTATAACCACGAT
	GTTTCCAACCTGCGCTCCTGGATTAGAACCACTAAGGTTAC
	CGGAGACCACCCTCTCGTCATCGAGCGGGTTAGGGCCATTG
	GCTGCCACTTTGTCCTTTTACTCACGGCTGCCCCGGAGCCA
	TCACCTATGCCCTATGTCCCTTACCCCGGTCTACCGAGGT
	CTATGTCCGATCGATCTTCGGCCCCGGGTGGTACCCCCCTCCC
	TATTTCCAACCTCATGCTCCACCAAGTCGACCTTCCATGCT
	GTCCCTGCCCATATCTGGGACCGTCTCATGTTGTTTCGGGGC
	CACCCTAGATGACCAAGCCTTTTGCTGCTCCCGCCTAATGA
	CTTACCTCCGCGGCATTAGCTACAAGGTTACTGTGGGCACC
	CTTGTTGCCAATGAAGGCTGGAACGCCTCTGAGGACGCTCT
	TACAGCTGTCATCACTGCCGCCTACCTTACCATCTGCCACC
	AGCGGTACCTCCGCACTCAGGCTATATCTAAGGGGATGCGT
	CGCCTGGAGCGGGAGCATGCTCAGAAGTTTATAACACGCC
	TCTACAGTTGGCTCTTTGAGAAGTCCGGCCGTGATTATATC
	CCCGGCCGTCAGTTGGAGTTCTACGCTCAGTGTAGGCGCTG

GCTCTCGGCCGGCTTTTCATCTTGACCCACGGGTGTTGGTTTT
TGATGAGTCGGCCCCCTGCCACTGTAGGACTGCGATTTCGTA
AGGCGGTCTCAAAGTTTTGCTGCTTTATGAAGTGGCTGGGC
CAGGAGTGCACCTGTTTTCTACAACCTGCAGAAGGCGTCGT
TGGCGACCAGGGCCATGACAACGAGGCCTATGAGGGGTCT
GATGTTGACCCTGCTGAATCCGCTATTAGTGACATATCTGG
GTCCTACGTAGTCCCTGGCACTGCCCTCCAACCGCTTTACC
AAGCCCTTGACCTCCCCGCTGAGATTGTGGCTCGTGCAGGC
CGGCTGACCGCCACAGTAAAGGTCTCCCAGGTGCGACGGGC
GGATCGATTGTGAGACCCTTCTCGGTAATAAAACCTTCCGC
ACGTCGTTTTGTTGACGGGGCGGTTTTAGAGACTAATGGCCC
AGAGCGCCACAATCTCTCTTTTGATGCCAGTCAGAGCACTA
TGGCCGCGGGCCCTTTCAGTCTCACCTATGCCGCCTCTGCT
GCTGGGCTGGAGGTGCGCTATGTCGCCGCGGGGCTTGACC
ACCGGGCGGTTTTTGCCCCCGGGCTTTCACCCCGGTCAGCC
CCTGGCGAGGTACCGCCTTCTGTTCTGCCCTATACAGGTT
TAATCGCGAGGCCAGCGCCTTTCGCTGACCGGTAATTTTT
GGTTCATCCTGAGGGGCTCCTTGGCCCCCTTTGCCCGTTTT
CCCCCGGGCATGTTTGGGAGTCGGCTAATCCATTCTGTGGC
GAGAGCACACTTTACACCCGCACTTGGTCGGAGGTTGATGC
TGTTCTAGTCCAGCCCAGCCCGACTTAGGTTTTACATCTG
AGCCTTCTATACCTAGTAGGGCCGCCACACCTACCCCGGCG
GCCCCCTTACCCCCCCTGCACCGGATCCTTCCCCTACTCTC
TCTGCTCCGGCGCGTGGTGAGCCGGCTCCTGGCGCTACCGC
CGGGGCCCCAGCCATAACCCACCAGACGGCCCCGGCATCGC
CGCCTGCTCTTTACCTACCCGGATGGCTCTAAGGTGTTTCGC
CGGCTCGCTGTTTGAGTCGACATGTACCTGGCTCGTTAACG
CGTCTAATGTTGACCACCGCCCTGGCGGTGGGCTCTGTCAT
GCATTTTACCAGAGGTACCCCGCCTCCTTTGATGCTGCCTC
TTTTGTGATGCGCGACGGCGCGGCCGCTACACATTAACCC
CCCGGCCAATAATTCATGCCGTCGCTCCTGATTATAGGTTG
GAACATAACCCAAAGAGGCTTGAGGCTGCCTACCGGGAGA
CTTGCTCCCGCCTCGGTACCGCTGCATACCACTCCTCGGG
ACCGGCATATAACAGGTGCCGATCGGTCCAGTTTTGACGC
CTGGGAGCGGAATCACCGCCCCGGGGACGAGTTGTACCTT
CCTGAGCTTGCTGCCAGATGGTTCGAGGCCAATAGGCCGA
CCTGCCCAACTCTCACTATAACTGAGGATGTTGCGCGGACA
GCAAATCTGGCTATCGAACTTGACTCAGCCACAGACGTCG
GCCGGGCCTGTGCCGGCTGTCGAGTCACCCCCGGCGTTGTG
CAGTACCAGTTTACCGCAGGTGTGCCTGGATCCGGCAAGTC
CCGCTCTATTACCCAAGCCGACGTGGACGTTGTCTGTTGCC
CGACCCGGGAGTTGCGTAATGCCTGGCGCCGCCGCGGCTTC
GCTGCTTTCACCCCGCACACTGCGGCTAGAGTCACCCAGGG
GCGCCGGGTTGTCAATTGATGAGGCCCCGTCCCTTCCCCCTC
ATTTGCTGCTGCTCCACATGCAGCGGGCCGCCACCGTCCAC
CTTCTTGGCGACCCGAATCAGATCCCAGCCATCGATTTTGA
GCACGCCGGGCTCGTTCCCGCCATCAGGCCCCGATTTGGCCC
CCACCTCCTGGTGGCATGTTACCCATCGCTGCCCTGCGGAT

	GTATGTGAGCTAATCCGCGGCGCATACCCCTATGATTTCAGAC CACTAGTCGGGTCCTCCGGTCGTTGTTCTGGGGTGAGCCCG CCGTTGGGCAGAAAGCTAGTGTTACCCAGGCGGCTAAGGC CGCCAACCCCGGTTTCAGTGACGGTCCATGAGGCACAGGGC GCTACCTACACAGAGACTACCATCATTGCCACGGCAGATG CTCGAGGCCTCATTTCAGTCGTCCCGAGCTCATGCCATTGTT GCCTTGACGCGCCACACTGAGAAGTGCGTCATCATTGACGC ACCAGGCCTGCTTCGCGAGGTGGGCATCTCCGATGCAATCG TTAATAACTTTTTCTTGCTGGTGGCGAAATTGGCCACCAG CGCCCATCTGTTATCCCTCGCGGCAATCCTGACGCCAATGT TGACACCTTGCTGCCTTCCCGCCGTCTTGCCAGATTAGCG CCTTCCATCAGTTGGCTGAGGAGCTTGGCCACAGACCTGCC CCTGTGCGGGCTGTTCTACCGCCCTGCCCTGAGCTTGAACA GGGCCTTCTCTACCTGCCCCAAGAACTCACCACCTGTGATA GTGTCGTAACATTTGAATTAACAGATATTGTGCATTGTCTG ATGGCCGCCCCGAGCCAGCGCAAGGCCGTGCTGTCCACGC TCGTGGGCCGTTATGGCCGCCGCACAAAGCTCTACAATGCC TCCCCTCTGATGTTTCGCGACTCTCTCGCCCGTTTTATCCCG GCCATTGGCCCCGTACAGGTTACAACCTGTGAATTGTACGA GCTAGTGGAGGCCATGGTCGAGAAGGGCCAGGACGGCTCC GCCGTCTTGAGCTCGACCTTTGTAGCCGCGACGTGTCCAG GATCACCTTCTTCCAGAAAGATTGTAATAAATTCACCACGG GGGAGACCATCGCCCATGGTAAAGTGGGCCAGGGCATTTC GGCTTGAGTAAGACCTTCTGTGCCCTTTTCGGCCCCCTGGT TCCGTGCTATTGAGAAGGCTATCCTGGCCCTGCTCCCTCAG GGTGTGTTTTATGGGGATGCCCTTTGATGACACCGTCTTCTC GGCGGCTGTGGCCGCAGCAAAGGCATCCATGGTGTGTTGAG AATGACTTTTCTGAGTTTGATTCCACCCAGAATAATTTTTCC TTGGGCCTAGAGTGTGCTATTATGGAGGAGTGTGGGATGCC GCAGTGGCTCATCCGCTTGTACCACCTTATAAGGTCTGCGT GGATTCTGCAGGCCCCGAAGGAGTCCCTGCGAGGGTTTTG GAAGAAACACTCCGGTGAGCCCGGCACCTTCTGTGGAAT ACTGTCTGGAACATGGCCGTTATCACCCACTGTTATGATTT CCGCGATCTGCAGGTGGCTGCCTTTAAAGGTGATGATTCTGA TAGTGCTTTGCAGTGAGTACCGTCAGAGCCCAGGGGCTGCT GTCCTGATTGCTGGCTGTGGCCTAAAGTTGAAGGTGGATTT CCGTCCGATTGGTCTGTATGCAGGTGTTGTGGTGGCCCCCG GCCTTGGCGCGCTTCCTGATGTCGTGCGCTTCGCCGGTCCG CTTACTGAGAAGAATTGGGGCCCTGGCCCCGAGCGGGCGG AGCAGCTCCGCCTCGCTGTGAGTGATTTTCTCCGCAAGCTC ACGAATGTAGCTCAGATGTGTGTGGATGTTGTCTCTCGTGT TTATGGGGTTTTCCCCTGGGCTCGTTCATAACCTGATTGGCA TGCTACAGGCTGTTGCTGATGGCAAGGCTCATTTCACTGAG TCAGTGAAGCCAGTGCTTGACCTGACAAATTCAATTCTGTG TCGGGTGGAATGA (SEQ ID NO: 5)
Genotype 2	ATGGAGGCCACCAGTTCATTAAGGCTCCTGGCATCACTAC TGCTATTGAGCAAGCAGCTCTAGCAGCGGCCAACTCCGCCC TTGCGAATGCTGTGGTGGTCCGGCCTTTCCTTCCCATCAG

AAA45730.1	CAGGTTGAGATCCTTATAAATCTCATGCAACCTCGGCAGCT GGTGTTCGTCCTGAGGTTTTTTGGAATCACCCGATTCAAC GTGTTATACATAATGAGCTTGAGCAGTATTGCCGTGCTCGC TCGGGTCGCTGCCTTGAGATTGGAGCCCACCCACGCTCCAT TAATGATAATCCTAATGTCCTCCATCGCTGCTTCTCCACCC CGTCGGCCGGGATGTTGAGCGCTGGTACACAGCCCCGACT AGGGGACCTGCGGCGAACTGTCGCCGCTCGGCACCTTCGTG GTCTGCCACCAGCCGACCGCACTTACTGTTTTGATGGCTTT GCCGGCTGCCGTTTTTGCCGCCGAGACTGGTGTGGCTCTCTA TTCTCTCCATGACTTGCAGCCGGCTGATGTTGCCGAGGCGA TGGCTCGCCACGGCATGACCCGCCCTTTATGCAGCTTTCCAC TTGCCTCCAGAGGTGCTCCTGCCTCCTGGCACCTACCGGAC ATCATCCTACTTGCTGATCCACGATGGTAAGCGCGCGGTTG TCACTTATGAGGGTGACACTAGCGCCGGTTACAATCATGAT GTTGCCACCCTCCGCACATGGATCAGGACAACCTAAGGTTGT GGGTGAACACCCTTTGGTGATCGAGCGGGTGCGGGGTATT GGCTGTCACCTTTGTGTTGTTGATCACTGCGGCCCTGAGCC CTCCCCGATGCCCTACGTTCCCTACCCGCGTTTCGACGGAGG TCTATGTCCGGTCTATCTTTGGGCCCCGGCGGGTCCCCGTCG CTGTTCCCGACCGCTTGTGCTGTCAAGTCCACTTTTCACGCC GTCCCCACGCACATCTGGGACCGTCTCATGCTCTTTGGGGC CACCTTCGACGACCAGGCCTTTTGCTGCTCCAGGCTTATGA CGTACCTTCGTGGCATTAGCTATAAGGTAACCTGTGGGTGCC CTGGTCGCTAATGAAGGCTGGAATGCCACCGAGGATGCGC TCACTGCAGTTATTACGGCGGCTTACCTCACAATATGTCAT CAGCGTTATTTGCGGACCCAGGCGATTTCTAAGGGCATGCG CCGGCTTGAGCTTGAACATGCTCAGAAATTTATTTACAGCC TCTACAGCTGGCTATTTGAGAAGTCAGGTCGTGATTACATC CCAGGCCGCCAGCTGCAGTTCTACGCTCAGTGCCGCCGCTG GTTATCTGCCGGGTTCATCTCGACCCCCGCACCTTAGTTTT TGATGAGTCAGTGCTTGTAGCTGCCGAACCACCATCCGGC GGATCGCTGGAAAATTTTGCTGTTTTATGAAGTGGCTCGGT CAGGAGTGTTCTTGTTTCCTCCAGCCCGCCGAGGGGCTGGC GGGCGACCAAGGTCATGACAATGAGGCCTATGAAGGCTCT GATGTTGATACTGCTGAGCCTGCCACCCTAGACATTACAGG CTCATACATCGTGGATGGTCGGTCTCTGCAAACCTGTCTATC AAGCTCTCGACCTGCCAGCTGACCTGGTAGCTCGCGCAGCC CGACTGTCTGCTACAGTTACTGTTACTGAAACCTCTGGCCG TCTGGATTGCCAAACAATGATCGGCAATAAGACTTTTCTCA CTACCTTTGTTGATGGGGCACGCCCTTGAGGTTAACGGGCCT GAGCAGCTTAACCTCTCTTTTGACAGCCAGCAGTGTAGTAT GGCAGCCGGCCCGTTTTGCCTCACCTATGCTGCCGTAGATG GCGGGCTGGAAGTTCATTTTCCACCGCTGGCCTCGAGAGC CGTGTGTTTTTCCCCCTGGTAATGCCCCGACTGCCCCGCC GAGTGAGGTCACCGCCTTCTGCTCAGCTCTTTATAGGCACA ACCGGCAGAGCCAGCGCCAGTCGGTTATTGGTAGTTTTGTGG CTGCACCCTGAAGGTTTGCTCGGCCTGTTCCCGCCCTTTTCA CCCGGGCATGAGTGGCGGTCTGCTAACCCATTTTGCGGCGA
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	GAGCACGCTCTACACCCGCACTTGGTCCACAATTACAGACA CACCCTTAAGTGTCTGGGCTAATTTCCGGTCATTTGGATGCT GCTCCCCACTCGGGGGGGGCCACCTGCTACTGCCACAGGCC TGCTGTAGGCTCGTCTGACTCTCCAGACCCTGACCCGCTAC CTGATGTTACAGATGGCTCACGCCCTCTGGGGCCCCGTCCG GCTGGCCCCAACCCGAATGGCGTTCCGCAGCGCCGCTTACT ACACACCTACCCTGACGGCGCTAAGATCTATGTCGGCTCCA TTTTCGAGTCTGAGTGCACCTGGCTTGTCAACGCATCTAAC GCCGGCCACCGCCCTGGTGGCGGGCTTTGTCATGCTTTTTT TCAGCGTTACCCTGATTCTGTTGACGCCACCAAGTTTGTGA TGCGTGATGGTCTTGCCGCGTATACCCCTACACCCCGGCCG ATCATTTCATGCGGTGGCCCCGGACTATCGATTGGAACATAA CCCCAAGAGGCTCGAGGCTGCCTACCGCGAGACTTGCGCC CGCCGAGGCACTGCTGCCTATCCACTCTTAGGCGCTGGCAT TTACCAGGTGCCTGTTAGTTTGTAGTTTTGATGCCTGGGAGC GGAACCACCGCCCGTTTGACGAGCTTTACCTAACAGAGCTG GCGGCTCGGTGGTTTGAATCCAACCGCCCCGGTCAGCCAC GTTGAACATAACTGAGGATACCGCCCGTGCGGCCAACCTG GCCCTGGAGCTTGACTCCGGGAGTGAAGTAGGCCGCGCAT GTGCCGGGTGTAAAGTCGAGCCTGGCGTTGTGCGGTATCA GTTTACAGCCGGTGTCCCCGGCTCTGGCAAGTCAAAGTCCG TGCAACAGGCGGATGTGGATGTTGTTGTTGTGCCACTCGC GAGCTTCGGAACGCTTGCGCGCGCCGGGGCTTTGCGGCATT CACTCCGCACACTGCGGCCCGTGCTCACTAGCGGCCGTAGG GTTGTCATTGATGAGGCCCTTCGCTCCCCCACACTTGCT GCTTTTACATATGCAGCGTGCTGCATCTGTGCACCTCCTTG GGGACCCGAATCAGATCCCCGCCATAGATTTTGAGCACAC CGGTCTGATTCCAGCAATACGGCCGGAGTTGGTCCCGACTT CATGGTGGCATGTCACCCACCGTTGCCCTGCAGATGTCTGT GAGTTAGTCCGTGGTGCTTACCCTAAAATCCAGACTACAAG TAAGGTGCTCCGTTCCCTTTTCTGGGGAGAGCCAGCTGTGC GCCAGAAGCTAGTGTTACACAGGCTGCTAAGGCCGCGCA CCCCGGATCTATAACGGTCCATGAGGCCAGGGTGCCACTT TTACCACTACAATAAATTGCAACTGCAGATGCCCGTGGC CTCATAAGTCTCCCGGGCTCACGCTATAGTTGCTCTCAC TAGGCATACTGAAAAATGTGTTATACTTGACTCTCCCGGCC TGTTGCGTGAGGTGGGTATCTCAGATGCCATTGTTAATAAT TTCTTCCTTTCCGGTGGCGAGGTTGGTCACCAGAGACCATC GGTCATTCCGCGAGGCAACCCTGACCGCAATGTTGACGTGC TTGCGGCGTTTCCACCTTCATGCCAAATAAGCGCCTTCCAT CAGCTTGCTGAGGAGCTGGGCCACCGGCCGCGCCGGTGG CGGCTGTGCTACCTCCCTGCCCTGAGCTTGAGCAGGGCCTT CTCTATCTGCCACAGGAGCTAGCCTCCTGTGACAGTGTTGT GACATTTGAGCTAACTGACATTGTGCACTGCCGCATGGCGG CCCCTAGCCAAAGGAAAGCTGTTTTGTCCACGCTGGTAGGC CGGTATGGCAGACGCACAAGGCTTTATGATGCGGGTCACA CCGATGTCCGCGCCTCCCTTGCGCGCTTTATTCCCACTCTCG GGCGGGTTACTGCCACCACCTGTGAACTCTTTGAGCTTGTA
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	GAGGCGATGGTGGAGAAGGGCCAAGACGGTTCAGCCGTCC TCGAGTTGGATTTGTGCAGCCGAGATGTCTCCCGCATAACC TTTTTCCAGAAGGATTGTAACAAGTTCACGACCGGGCAGAC AATTGCGCATGGCAAAGTCGGTCAGGGTATCTTCCGCTGGA GTAAGACGTTTTGTGCCCTGTTTGGCCCCCTGGTTCCGTGCG ATTGAGAAGGCTATTCTATCCCTTTTACCACAAGCTGTGTT CTACGGGGATGCTTATGACGACTCAGTATTCTCTGCTGCCG TGGCTGGCGCCAGCCATGCCATGGTGTGTTGAAAATGATTTT TCTGAGTTTGACTCGACTCAGAATAACTTTTCCCTAGGTCTT GAGTGCGCCATTATGGAAGAGTGTGGTATGCCCCAGTGGC TTGTCAGGTTGTACCATGCCGTCCGGTCGGCGTGGATCCTG CAGGCCCCAAAAGAGTCTTTGAGAGGGTTCTGGAAGAAGC ATTCTGGTGAGCCGGGCAGCTTGCTCTGGAATACGGTGTGG AACATGGCAATCATTGCCCATGCTATGAGTTCGGGGACCT CCAGGTTGCCGCCTTCAAGGGCGACGACTCGGTTCGTCTCT GTAGTGAATACCGCCAGAGCCCAGGCGCCGGTTCGCTTAT AGCAGGCTGTGGTTTGAAGTTGAAGGCTGACTTCCGGCCG ATTGGGCTGTATGCCGGGGTTGTCGTCGCCCCGGGGCTCGG GGCCCTACCCGATGTGCTTCGATTGCGCCGACGGCTTTCGG AGAAGAAGTGGGGGCCTGATCCGGAGCGGGCAGAGCAGCT CCGCCTCGCCGTGCAGGATTTCCCTCCGTAGGTTAACGAATG TGGCCCAGATTTGTGTTGAGGTGGTGTCTAGAGTTTACGGG GTTTCCCCGGGTCTGGTTCATAACCTGATAGGCATGCTCCA GACTATTGGTGATGGTAAGGCGCATTTTACAGAGTCTGTTA AGCCTATACTTGACCTTACACACTCAATTATGCACCGGTCT GAATGA (SEQ ID NO: 6)
Genotype 3 JQ679013.1	ATGGAGGCCACCAGTTCATTAAGGCTCCTGGCATTACTAC TGCCATTGAGCAGGCTGCTCTGGCTGCGGCCAATTCCGCCT TGGCGAATGCTGTGGTGGTTCGGCCGTTTTTATCCCGTCTA CAAAGTGAAGATTCTTATCAATTTGATGCAACCCCGGCAGTT GGTTTTCCGCCCTGAAGTGCTTTGGAATCATCCTATCCAGC GGGTCATACACAATGAAGTAGAACAGTACTGCCGGGGCCCG TGCTGGCCGCTGTTTGGAGGTGCGGAGCCCATCCGAGATCTA TTAATGATAACCCCAACGTCTTGACCCGGTGCTTCCTTAGA CCGGTTGGCAGGGATGTTTACGCGCTGGTACTCTGCCCCAC CCGTGGCCCTGCGGCCAATTGCCGCGCTCCGCGCTGCGTG GCCTTCCCCCGTTGACCGCACCTACTGTTTTGATGGATTCT CCCGCTGTGCTTTTGTGCTGCAGAGACCGGTGTGGCCCTTTAC TCTTTGCATGACCTTTGGCCAGCTGATGTTGCAGAGGCGAT GGCCCGTCATGGGATGACACGGTTGTATGCCGCACTACATC TTCCTCCTGAGGTGCTGCTACCACCCGGCACCTATCACACA ACTTCGTATCTCCTGATTCACGACGGCGATCGCGCCGTTGT AACCTATGAGGGCGATAACAGTGCGGGCTATAACCATGAT GTTTCCATACTTCGTGCGTGGATCCGTACTACTAAAATAGT TGGTGACCACCCGTTGGTTATAGAGCGTGTGCGGGCCATTG GTTGTCATTTTCGTGCTGTTGCTCACCGCGGCCCCCTGAGCCG TCACCTATGCCTTATGTCCCCTACCTCGTTCAACGGAGGT GTATGTTCCGGTCTATATTTGGCCCTGGCGGCTCTCCATCTTT

	GTTTCCGTCAGCCTGCTCTACTAAATCTACCTTCCACGCTGT CCCGGTCCATATCTGGGATCGGCTCATGCTCTTTGGTGCCA CCCTGGATGATCAGGCGTTCTGTTGTTACGACTCATGACT TACCTCCGTGGTATTAGCTACAAGGTCAGTGTGGTGCGCT TGTTGCTAATGAGGGGTGGAACGCCTCTGAAGATGCTCTTA CTGCAGTGATCACTGCGGCTTATCTGACTATCTGCCATCAG CGCTACCTCCGTACCCAGGCGATATCCAAGGGCATGCGCC GGTTGGAGGTTGAGCATGCCCAGAAATTTATCACAAGACT CTACAGTTGGCTATTTGAGAAGTCTGGCCGTGATTACATCC CCGGCCGCCAGCTCCAGTTTTATGCACAGTGCCGACGGTGG CTATCTGCAGGATTCCATCTGGACCCCAGGGTGCTTGT TGATGAATCAGTGCCATGTCGTTGTAGGACGTTTCTGAAGA AAGTCGCGGGTAAATTCTGCTGTTTTATGCGGTGGTTAGGG CAGGAGTGCACCTGCTTCTGGAGCCAGCCGAGGGTTTAGT TGCGGACCATGGCCATGACAATGAGGCTTATGAAGGTTCT GAGGTCGACCAGGCTGAACCTGCCCATCTTGATGTTTCGGG GACTTATGCCGTCCACGGGCACCAGCTTGTAGCCCTCTATA GGGCACTTAATGTCCACATGATATTGCCGCTCGAGCTTCC CGATTAACGGCTACTGTTGAGCTTGTGTCAGGTCCAGACCG CTTGAGTGGCGCACTGTGCTCGGTAATAAGACCTTCCGGA CGACGGTGGTTGATGGCGCCCATCTTGAAGCGAATGGCCC AGAGCAGTATGTCCTGTCATTTGACGCCCTCCTGTCAGTCTA TGGGGGCGGGTCGCACAACCTCACTTATGAGCTCACCCCT GCCGGTTTGCAGGTTAGGATCTCATCTAACGGTCTGGATTG CACTGCTACATTCCCCCCCCGGCGGTGCCCTAGCGCCGCGC CAGGGGAGGTGGCAGCCTTCTGTGCTGCCCTTTACAGATAT AACAGGTTACACCAGCGGCACTCGCTGACCGGTGGACTAT GGTTACACCCTGAGGGATTGCTGGGTATCTTCCCTCCATTC TCCCCTGGGCATATCTGGGAGTCTGCTAACCCCTTTTGCGG GGAGGGGACTTTGTATACCCGGACCTGGTCAACATCTGGCT TTTCTAGTGATTTCTCTCCCCCTGAGGCGGGCCGCCCTGCTT CGGCTGCTGCCCCGGGGCTGCCCCACCCTACCCCGCCTGCT AGTGATATTTGGGCGTTACCACCGCCCTCCGAGGAGTGCTA CACGCGCCTGGGCAACGACTTCCACACGAACAAGCGCGTG TGCGAGGAGATCGCCATTATCCCTAGCAAAAAGCCCCGCA ACAAGATGGCAGGTTATGTCACGCATCTGATGAAGCGAAT TCAGAGAGGCCCAGTAAGAGGTATCTCCATCAAGCTGCAG GAGGAGGCTCAGGTGATGCAGCATCTGTGCCCTTACCCT CGTGCTGCTGGGTGCGCCAGCCCTGTTGTGTACCTTCCC CACCACCACCTCCACCCGTGCGTAAGCCATCAACACCCCCG CCTTCTCGTACCCGTGCGCTCCTCTACACCTACCCCGACGG CGCTAAGGTGTATGCAGGGTCATTGTTTGAATCAGACTGTG ATTGGCTGGTTAACGCCTCAAACCCGGGCCATCGCCCTGGA GGTGGCCTCTGTCACGCCTTTCATCAACGTTTTCCGGAGGC GTTTTATCCGACTGAATTCATTATGCGTGAGGGCCTAGCGG CATACACCCTGACCCCGCGCCCTATCATCCACGCAGTGGCG CCCGACTACAGGGTTGAGCAGAACCCGAAGAGGCTCGAGG CAGCGTACCGGGAAACTTGCTCCCGTCGTGGCACCGCTGCT
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TACCCGCTTTTAGGCTCGGGCATATACCAGGTCCCTGTCAG
CCTCAGTTTTGATGCCTGGGAACGCAATCATCGCCCCGGCG
ATGAGCTTTACTTGACTGAGCCCGCTGCGGCTTGTTTGA
GCTAATAAGCCGGCGCAGCCGGCGCTTACCATAACTGAGG
ATACGGCTCGTACGGCCAGCCTGGCATTAGAGATCGACGC
CGCTACAGAGGTTGGCCGTGCTTGTGCCGGCTGCACTATCA
GTCTGGGATTGTGCACTATCAGTTTACCGCTGGGGTCCCG
GGCTCGGGCAAGTCAAGGTCCATACAACAGGGAGATGTTG
ATGTGGTGGTTGTGCCACCCGGGAGCTCCGTAACAGTTGG
CGCCGCCGGGGTTTCGCGGCTTTCACACCTCACACAGCGGC
CCGTGTTACTAACGGCCGCCGCGTGTGATTGATGAGGCC
CATCTCTCCCGCCACACCTGTTGCTGCTACATATGCAGCGG
GCCTCCTCGGTTACCTACTCGGTGACCCAAATCAGATCCC
TGCTATCGATTTTGAACACGCCGGCCTGGTCCCCGCGATCC
GCCCCGAGCTTGCACCAACGAGCTGGTGGCACGTCACACA
CCGTTGCCCGGCCGATGTGTGCGAACTCATACGCGGGGCCT
ACCCCAAATCCAGACCACGAGCCGTGTGCTACGGTCCCT
GTTTGGGAATGAACCGGCTATCGGCCAGAAGTTGGTTTTTA
CGCAGGCTGCCAAGGCCGCTAACCCTGGTGCATTACGGTT
CACGAAGCTCAGGGTGCCACCTTCACTGAGACCACAGTTAT
AGCCACGGCCGACGCCAGGGGCCTCATTGATCATCCCGG
GCCCATGCTATAGTTGCACTTACCCGCCACACCGAGAAGTG
CGTCATTTTGGATGCTCCCGGCCTGCTGCGTGAAGTCGGTA
TCTCGGATGTGATTGTCAATAATTTTTTCTTGCAGGCGGA
GAGGTCGGCCATCACCGCCCTTCTGTGATACCCCGCGGTAA
CCCCGATCAGAACCTCGGGACTTTACAAGCCTTCCCGCCGT
CCTGCCAGATTAGTGCTTACCACCAGCTGGCTGAGGAATTA
GGCCATCGCCCTGCCCTGTTGCCGCCGCTTGGCCCCCTTG
CCCCGAGCTTGAGCAGGGCCTGCTTTACATGCCACAAGAG
CTTACCGTGTCTGATAGTGTGCTGGTTTTTGAGCTCACGGA
CATAGTCCACTGCCGCATGGCCGCTCCAAGCCAGCGAAAG
GCTGTTCTTTCAACACTTGTGGGGCGGTATGGCCGTAGGAC
GAAGTTATATGAGGCAGCACATTCAGATGTCCGTGAGTCCC
TAGCCAGGTTTATCCCCACTATCGGGCCCGTTCAGGCCACC
ACATGTGAGTTGTATGAGTTGGTTGAGGCCATGGTGGAGA
AGGGTCAGGACGGGTCAGCCGTCTTAGAGCTAGATCTCTG
CAATCGTGATGTCTCGCGCATCACATTTTCCAAAAGGATT
GCAACAAGTTTACAACCTGGTGAGACTATTGCCCATGGCAA
GGTTGGTCAGGGTATATCGGCCTGGAGCAAGACATTCTGC
GCTTTGTTTGGCCCGTGGTTCCGTGCCATTGAGAAAGAAAT
ACTGGCCCTGCTCCCGCCTAATGTCTTTTATGGCGATGCTT
ATGAGGAGTCAGTGCTTGTGCGCGCTGTGTGAGGGGCGGG
GTCATGCATGGTATTTGAAAATGACTTTTCGGAGTTTGATA
GCACCCAGAACAACTTCTCTCTCGGCCTTGAGTGTGTGGTT
ATGGAGGAGTGCGGCATGCCTCAATGGTTAATTAGGTTGTA
TCACCTGGTACGGTCAGCCTGGATTCTGCAGGCGCCAAAG
GAGTCTCTTAAGGGTTTCTGGAAGAAGCATTCTGGTGAGCC
CGGTACCCTTCTTTGGAACACCGTTTGGAAACATGGCAATCA

	TAGCACATTGCTACGAGTTCCGTGACTTTCGTGTTGCTGCC TTTAAGGGTGATGATTCGGTGGTCCTCTGTAGCGACTACCG GCAGAGCCGCAATGCGGCAGCTTTGATTGCTGGCTGTGGG CTTAAATTGAAGGTTGACTATCGCCCCATTGGGCTGTATGC TGGGGTGGTGGTGGCCCCCTGGCTTGGGGACACTGCCTGATG TGGTGCGTTTTGCTGGTGGCTGTCCGAAAAGAATTGGGGC CCCGGCCCGGAACGTGCTGAGCAGCTACGTCTTGCTGTTTG TGATTTCCCTTCGAGGGTTGACGAACGTTGCGCAGGTCTGTG TTGATGTTGTGTCCCGTGTCTATGGAGTTAGCCCCGGGCTG GTACATAACCTTATTGGCATGTTGCAGACCATTGCCGATGG CAAGGCCCACTTTACAGAGACTATTAAACCTGTTCTTGATC TTACAAATTCCATCATACAGCGGGTAGAATGA (SEQ ID NO: 7)
Genotype 4 BAG32133.1	ATGGAGGCCCATCAGTTCATAAAGGCTCCTGGCGTCACTAC TGCTATCGAGCAGGCAGCTCTAGCAGCGGCCAACTCCGCC CTGGCGAATGCTGTGGTGGTTCGGCCTTTCCTTTCCCGGCT ACAGACAGAGATTTTGATAAACCTGATGCAGCCCCGGCAG CTTGTCTTCCGACCTGAGGTTCTGTGGAATCACCCAATCCA GCGCGTAATCCACAACGAGCTTGAGCAGTACTGTCGAGCC CGTGCCGGTCGCTGCCTTGAGGTGGGAGCCCATCCGCGCTC CATTAAATGATAACCCCAATGTTTTGCACCGTTGCTTTTTGA AACCCCGTGGTCGCGACGTTACGCGGTGGTACACCGCCCCC ACCCGCGGCCCTGCAGCGAATTGCCGCCGTTCCGGCTCTTCG TGGACTTCCACCTGTTGACCGGACATACTGTTTTGATGGTT TTTCTGGATGTACGTTTGCTGCTGAGACTGGGGTTGCCCTTT ATTCACTGCACGATCTGTGGCCTGCTGACGTCGCAGAGGCA ATGGCCCCGCCACGGTATGACTCGGCTGTATGCAGCCCTTCA TCTCCCCCGGAGGTGTTACTTCCCTCTGGCACCTATCATA CCACATCATACCTTTTAATTCATGACGGCGATCGTGCTGTG ATTACATATGAGGGTGACTCGAGCGCAGGGTACAATCATG ATGTGTCTATTCTGCGCGCCTGGATCCGCACCACTAAAGTT ACCGGCGACCACCCGTTGGTCATTGAGCGAGTCCGGGGCGG TAGGGTGTCACTTTGTGCTTTTGCTCACAGCCGCGCCTGAA CCATCGCCGATGCCTTATGTCCCATACCCTCGCTCCACTGA GGTCTATGTCCGGTCCATTTTTGGGGCCAGGCGGCTCGCCCT CTCTCTTCCCATCTGCCTGCTCGACTAAGTCTACATTTTCATG CCGTTCCCTGTGCATATCTGGGACAGGCTCATGCTTTTTGGT GCGACCCTTGATGACCAGGCTTTTTGCTGCTCGAGGCTTAT GACGTACCTTCGTGGTATTAGTTATAAGGTTACAGTCGGTG CTCTTGTTGCTAACGAAGGCTGGAACGCCTCCGAGGATGCA CTGACTGCTGTAATTACTGCAGCATATCTCACCATTTGTCA TCAGAGGTACCTCCGCACGCAGGCCATTTGAAAAGGAATG AAGAGGTTGGAGCTTGAACATGCGCAGAAATTTATAACGC GCCTTTATAGTTGGCTGTTTCGAGAAGTCCGGCCGTGATTAC ATCCCCGGCCGTCAGTTGCAGTTTTATGCCCAGTGCCGCCG GTGGTTATCTGCAGGCTTTCATCTTGACCCACGTGTACTTGT TTTTGATGAGGCAGCCCCCTGCCGTTGTCGTAATTTCTTCG GAAAGCCGCCCAAGTTTTGTTGCTTCATGCGGTGGTTAG

GTCAGGATTGCACCTGTTTCCCTCCAACCTATCGAGGGGACGG
GTTGGCGAGCAGGGTTATGATAATGAAGCATTTGAAGGGT
CGGACGTCGACCCTGCTGAGGAGGCCAACTGTGAGTATCTCT
GGGTCATATATTGTAACCTGGTAGCCAGCTACAGCCTCTATA
TCAGGCGCTTGGTATCCCCTCTGATCTTGCTGCCCGTGCCG
GCCGGCTCACTGCCACTGTTGAAGTTTCTGATGCAAATGGC
CGCCTTACCTGCAAAACCATCATGGGCAATAAAACTTTCTC
AACAGTCTTCACTGATGGCGCCCAGCTGGAGGCCAACGGG
CCGGAGCAGTATGTGCTGTCATTCGACCCGATTAAACAAAC
TATGGCCGCCGGCCCGCATAATCTTAGTTATACCTTGACAT
CTGCAGGCCCTTGAAATACATGTCGTCTCCGCCGGGCTTGAT
TGTAAGGCCGTCTTTCCGTCCGGGGTTGCGACCCCGTCTGC
CCCCGGGGAGGTGTCTGCCTTCTGTTTCAGCATTGTACAGAT
TTAACCGCTGTGTCCAGCGGCACTCCCTCATTGGGGGTGTG
TGGTACCACCCTGAGGGGGCTAGTCGGCCTGTTCCCGCCGTT
TTCCCCCGGCCATAGCTGGGAGTCTGCTAACCCTTTTGCG
GGGAGAGTACCCTTTATACCCGTACCTGGTCCGTATCGGGA
TTTTCCAGCTGTTTTTCCCCACTTGAACCGGGGGCCCCGGA
CCCGCCCCCTCTTGTTGAGACTGACACGCCTACAGTTGTTG
ATACTCTGCCTCCAGTTGTTTCAGTACCCTTGAGCAAATA
GTACTTCCACCAGACTCTGTAGATAAGGCAGCCGGCCCGA
CCGCATCTAGCGCCCCTGTTGTACCGCCAGCACCAGTGCAG
TCTGTAGTTCAACCATCTGGGCCTCGCCGGCGGCTGCTTCA
TACTTATCCTGATGGCTCGAAGGTGTATGCTGGCTCCCTTTT
TGAGTCCGACTGTACTTGGCTGGTTAACGCATCTAACCCCTG
GCCACCGCCCTGGTGGCGGCCTCTGCCATGCGTTTTACCAA
CGGTTCCCAGAGTCATTTGATCCCGCTGAGTTTGTATATGTC
AGATGGGTTTGCAGCCTACACCCTGTCTCCCCGGCCCATTA
TTCATGCTGTTGCTCCTGACTATCGGGTTGAACATAACCCT
AAGAGGCTTGAGGCCGCCTATCGGGAGACGTGCTCTCGTCT
CGGGACTGCAGCTTACCCTTTACTTGGCGCCGGTATATATA
AGGTGCCTGTTGGGCTGAGCTTCGATGCCTGGGAACGTAAC
CACCGGCCCGGGGATGAGCTGTATCTGACTGAGCCAGCCG
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ACCATCACTGAGGATACCGCGCGTACGGCGAACCTGGCAT
TGGAGCTAGACTCCGCCACTGAAGTCGGCCGGGCGTGTGC
TGGCTGCCGTGTAGAGCCCGGTGTGCTCCATTATCAGTTTA
CAGCAGGTGTCCCCGGATCAGGTAAATCCCGGTCAGTCCA
GCAGGGGGAGGTGGATGTGGTAGTGGTACCAACTCGTGAG
CTGCGCAATTCGTGGCGGCGCCGCGGGTTTCGCAGCTTATAC
ACCCACACCGCAGCTCGTGTACCCCGTGGTTCGTAGGGTTG
TTATTGATGAGGCCCCCGTCGCTCCCTCCACACTTGCTTTTGC
TGCATATGCAGCGGGCCTCCTCAGTCCATCTCTTAGGTGAT
CCTAATCAAATCCCTGCCATTGACTTTGAGCACGCCGGCCT
TGTTCCAGCTATTCGACCGGAGTTGGTCCCGACAAAATGGT
GGCACGTTACTCATAGATGCCCAGCCGATGTCTGTGAGTTA
ATTCGTGGTGCGTACCCAAAGATCCAGACTGTGAGCCGCGT
ACTCCGCTCTCTGTTCTGGGGGGAGCCCCCGTGGGTCAGA

	AGCTGGTGTTCACCCAGGCGGCGAAGGCCGCCAACCCTGG TGCGATTACAGTCCACGAGGCCAGGGTGCCACATTCAGT AGACTACAATTATCGCCACGGCGGACGCCCCGTGGGCTGAT TCAGTCCTCCAGGGCCCATGCGATTGTGGCTCTGACCCGCC ACACAGAGAAATGTGTGGTTCGTTGACGCCCCGGGCCTCCTC CGCGAGGTTGGTATCTCTGACGCTATTGTTAATAATTTCTTC CTTTCCGGCGGTCAGGTTGGTCAGCACCGCCCCGTGAGTCAT ACCACGCGGCACACTATTGATTGTAATGTTGATACACTTGATG CATTCGCCGCCCTCCTGTCAGTTTAGTGCCTACCACCAGCTT GCCGAGGAGCTTGGCCATCGACCGGCCCGATTGCTGCTGT CTTACCTCCCTGCCCGGAGCTTGAACAGGGCCTGCTTTATA TGCCTCAGGAGCTCACTACATCGGACAGTGTGCTAACATTT GAGCTTACAGATATAGTGCCTGCGGTATGGCGGCGCCTA GTCAGCGCAAGGCAGTCCTGTGCTGCTCTTGTGCGGTAGGTAT GGCCGCCGTACGAAGCTGTATGAAGCTGCTCATGCAGATG TTCGTGGGTCTCTGAATCATTTTATCCCCGAGCTCCGCCCTA TCAGCGTCACTACCTGCGAGCTCTATGAGCTTGTAGAGGCC ATGGTTGAGAAAGGCCAGGACGGCTCCGCGGTTCTGGAGC TCGACTTGTGCAGCCGTGATGTCTCGCGAATAACATTTTC CAGAAAGACTGCAATAAGTTTACAACCTGGCGAAACAATAG CGCATGGCAAAGTTGGGCAGGGGATATCTGCATGGAGTAA AACCTTTTGCGCCCTGTTTGGCCCCCTGGTTCCGTGCTATTGA GAAAGAGATTCTAGCTGTGCTTGCGCCTAACGTATTTTATG GTGATGCATATGAGGACACAGTTTTTGGCCGCTGCCGTGCGA GGAGCCTCCGGCTGTAAGGTTTTTGAAGAATGATTTTTCAGA GTTTGATAGTACCCAAAATAATTTCTCGCTTGGGCTGGAGT GTATAATTATGGAGGAGTGTGGCATGCCGCAGTGGATGAT TCGCCTCTACCATCTCGTCCGCTCCGCCTGGGTCTCCAGG CCCCGAAGGAGTCCTTGCGGGGTTTTTGAAGAAACACTCT GGTGAACCCGGTACTCTGCTCTGGAACACTGTCTGGAATAT GGCAGTTATAGCCCACTGTTATGAGTTCCGTGACCTAAAAG TTGCGGCGTTTAAGGGGGATGATTCTGTTGTGCTCTGTAGC GACTATCGGCAGAGCCGTGATGCAGCTGCCTTGATTGCGG GCTGTGGGTTGAAGCTTAAGGTGGACTTTAGGCCTATTGGG CTGTATGCTGGTGTGTTGTGTTGGCCCCAGGTTTAGGAACCT ACCTGATGTTGTTAGGTTTGCTGGGCGACTCTCAGAGAAAA ACTGGGGGGCCCGTTTGGAGAGGGCAGAGCAGCTACGGCT GGCTGTTTGTGACTTTCTGCGAAGGTAAACGAATGTGGCTC AGGTTTGTGTGGATGTTGTCTCGCAAGTATATGGTGTAGC CCTGGCTTGGTACATAACCTGATTGGGATGCTCCAGACTAT TGCTGATGGTAAGGCCCATTTTACCGAAACAGTTAAACCTG TCCTTGATTTGACCAACTCCATCATATATCGGGTGGATTGA (SEQ ID NO: 8)
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Table 3. Examples of ORF2 Nucleotide Sequences

Genotype 1	ATGCGCCCTCGGCCTATTTTGGCTGTTGCTCCTCATGTTTCTG CCTATGCTGCCCGCGCCACCGCCCCGGTCAGCCGTCTGGCCG
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AAL50056.1	CCGTCGTGGGCGGGCGCAGCGGCGGTTCCGGCGGGTGGTTTCT GGGGTGACCGGGTTGATTCTCAGCCCTTCGCAATCCCCTAT ATTCATCCAACCAACCCCTTCGCCCCCGATGTCACCGCTGC GGCCGGGGCTGGACCTCGTGTTCCGCCAACCCGCCCGACCA CTCGGCTCCGCTTGGCGTGACCAGGCCAGCGCCCCGCCGC TGCTCACGTCTAGACCTACCACAGCTGGGGCCGCGCCGC TAACCGCGGTCTGCTCCGGGCCATGACACCCCGCCAGTGCCT GATGTTGACTCCCGCGGGCGCCATCCTGCGCCGGCAGTATAA CCTATCAACATCTCCCCTCACCTCTTCCGTGGCCACCGGCA CAAATTTGGTTCTTTACGCCGCTCCTCTTAGCCCGCTTCTAC CCCTCCAGGACGGCACCAATACTCATATAATGGCTACAGA AGCTTCTAATTATGCCCAGTACCGGGTTGCTCGTGCCACAA TTCGCTACCGCCCGCTGGTCCCCAACGCTGTTGGTGGCTAC GCTATCTCCATTTTCGTTCTGGCCACAGACCACCACCACCC GACGTCCGTTGACATGAATTCAATAACCTCGACGGATGTCC GTATTTTAGTCCAGCCCGGCATAGCCTCCGAGCTTGTTATT CCAAGTGAGCGCCTACACTATCGCAACCAAGGTTGGCGCT CTGTTGAGACCTCCGGGGTGGCGGAGGAGGAGGCCACCTC TGGTCTTGTCATGCTCTGCATACATGGCTCACCTGTAAATT CTTATACTAATAACCCCTATACCGGTGCCCTCGGGCTGTTG GACTTTGCCCTCGAACTTGAGTTCCGCAACCTCACCCCCGG TAATACCAATACGCGGGTCTCGCGTTACTCCAGCACTGCCC GTCACCGCCTTCGTGCGGGTGACAGATGGGACTGCCGAGCTC ACCACCACGGCTGCTACTCGCTTCATGAAGGACCTCTATTT TACTAGTACTAATGGTGTTGGTGAGATCGGCCGCGGGATA GCGCTTACCCTGTTTAACCTTGCTGACACCCTGCTTGGCGG TCTACCGACAGAATTGATTTTCGTGCGGCTGGTGGCCAGCTGT TCTACTCTCGCCCCGTCGTCTCAGCCAATGGCGAGCCGACT GTTAAGCTGTATACATCTGTGGAGAATGCTCAGCAGGATA AGGGTATTGCAATCCCGCATGACATCGACCTCGGGGAATC CCGTGTAGTTATTCAGGATTATGACAACCAACATGAGCAG GACCGACCGACACCTTCCCCAGCCCCATCGCGTCCCTTTTC TGTCCTCCGAGCTAATGATGTGCTTTGGCTTTCTCTACCGC TGCCGAGTATGACCAGTCCACTTACGGCTCTTCGACCGGCC CAGTCTATGTCTCTGACTCTGTGACCTTGGTTAATGTTGCG ACCGGCGCGCAGGCCGTTGCCCGGTCACTCGACTGGACCA AGGTCACACTTGATGGTCGCCCCCTTCCACCATCCAGCAG TATTCAAAGACCTTCTTTGTCTGCGGCTCCGCGGTAAGCT CTCCTTTTGGGAGGCAGGAACCTACTAAAGCCGGGTACCCTT ATAATTATAACACCACTGCTAGTGACCAACTGCTCGTTGAG AATGCCGCTGGGCATCGGGTTGCTATTTCCACCTACACTAC TAGCCTGGGTGCTGGCCCCGTCTCTATTTCCGCGGTTGCTG TTTTAGCCCCCCTCTGTGCTAGCATTGCTTGAGGATACC ATGGACTACCCTGCCCGCGCCCATCTTTCGATGACTTCTG CCCGGAGTGCCGCCCCCTTGGCCTCCAGGGCTGTGCTTTTC AGTCTACTGTGCTGAGCTTCAGCGCCTTAAGATGAAGGTG GGTAAAACTCGGGAGTTATAG (SEQ ID NO: 9)
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Genotype 2	ATGCGCCCTAGGCCTCTTTTGCTGTTGTTTCCTCTTGTTTCTG
AAA45732.1	CCTATGTTGCCCCGCGCCACCGACCGGTCAGCCGTCTGGCCG
	CCGTCGTGGGCGGGCGCAGCGGCGGTACCGGCGGTGGTTTC
	TGGGGTGACCGGGTTGATTCTCAGCCCTTCGCAATCCCCTA
	TATTCATCCAACCAACCCCTTTGCCCCAGACGTTGCCGCTG
	CGTCCGGGTCTGGACCTCGCCTTCGCCAACCAGCCCCGGCCA
	CTTGGCTCCACTTGGCGAGATCAGGCCCAGCGCCCCCTCCGC
	TGCCTCCCGTCGCCGACCTGCCACAGCCGGGGGCTGCGGGCGC
	TGACGGCTGTGGCGCCTGCCCATGACACCTCACCCGTCCCG
	GACGTTGATTCTCGCGGTGCAATTCTACGCCGCCAGTATAA
	TTTGTCTACTTCACCCCTGACATCCTCTGTGGCCTCTGGCAC
	TAATTTAGTCCTGTATGCAGCCCCCCTTAATCCGCCTCTGCC
	GCTGCAGGACGGTACTAATACTCACATTATGGCCACAGAG
	GCCTCCAATTATGCACAGTACCGGGTTGCCCGCGCTACTAT
	CCGTTACCGGCCCTTAGTGCCTAATGCAGTTGGAGGCTATG
	CTATATCCATTTCTTTCTGGCCTCAAACAACCACAACCCCT
	ACATCTGTTGACATGAATTCCATTACTTCCACTGATGTCAG
	GATTCTTGTTCAACCTGGCATAGCATCTGAATTGGTCATCC
	CAAGCGAGCGCCTTCACTACCGCAATCAAGGTTGGCGCTC
	GGTTGAGACATCTGGTGTGCTGAGGAGGAAGCCACCTCC
	GGTCTTGTCATGTTATGCATACATGGCTCTCCAGTTAACTC
	CTATACCAATAACCCCTTATACCGGTGCCCTTGCTTACTGG
	ACTTTGCCCTTAGAGCTTGAGTTTCGCAATCTCACCACTGT
	AACACCAATACACGTGTGTCCCGTTACTCCAGCACTGCTCG
	TCACTCCGCCCCGAGGGGGCCGACGGGACTGCGGAGCTGACC
	ACAACCTGCAGCCACCAGGTTTCATGAAAGATCTCCACTTTAC
	CGGCCTTAATGGGGTAGGTGAAGTCGGCCGCGGGATAGCT
	CTAACATTACTTAACCTTGCTGACACGCTCCTCGGCCGGGCT
	CCCGACAGAATTAATTTTCGTCGGCTGGCGGGCAACTGTTTT
	ATTCCCGCCCGGTTGTCTCAGCCAATGGCGAGCCAACCGTG
	AAGCTCTATACATCAGTGGAGAATGCTCAGCAGGATAAGG
	GTGTTGCTATCCCCCACGATATCGATCTTGGTGATTTCGCGT
	GTGGTCATTACAGGATTATGACAACCAGCATGAGCAGGATC
	GGCCACCCCGTCGCCTGCGCCATCTCGGCCTTTTTCTGTTT
	TCCGAGCAAATGATGTACTTTGGCTGTCCCTCACTGCAGCC
	GAGTATGACCAGTCCACTTACGGGTCGTCAACTGGCCCGGT
	TTATATCTCGGACAGCGTGACTTTGGTGAATGTTGCGACTG
	GCGCGCAGGCCGTAGCCCGATCGCTTGACTGGTCCAAAGT
	CACCCTCGACGGGGCGGCCCTCCCGACTGTTGAGCAATATT
	CCAAGACATTCTTTGTGCTCCCCCTTCGTGGCAAGCTCTCCT
	TTTGGGAGGCCGGCACAACAAAAGCAGGTTATCCTTATAA
	TTATAATACTACTGCTAGTGACCAGATTCTGATTGAAAATG
	CTGCCGGCCATCGGGTCGCCATTTCAACCTATACCACCAGG
	CTTGGGGCCGGTCCGGTCGCCATTTCTGCGGGCCGCGGTTTT
	GGCTCCACGCTCCGCCCTGGCTCTGCTGGAGGATACTTTTG
	ATTATCCGGGGCGGGCGCACACATTTGATGACTTCTGCCCT
	GAATGCCGCGCTTTAGGCCTCCAGGGTTGTGCTTTCCAGTC
	AACTGTCGCTGAGCTCCAGCGCCTTAAAGTTAAGGTGGGTA

	AAACTCGGGAGTTGTAG (SEQ ID NO: 10)
Genotype 3	ATGTGCCCTAGGGTTGTTCTGCTGCTGTTCTTCGTGTTTCTG
AFD33684.1	CCTATGCTGCCCGCGCCACCGGCCGGCCAGCCGTCTGGCCG
	TCGTTCGTGGGCGGCGCAGCGGCGGTGCCGGCGGTGGTTC
	GGGGTGACAGGGTTGATTCTCAGCCCTTCGCCCTCCCCTAT
	ATTCATCCAACCAACCCCTTCGCCGCCGATATCGTTTCACA
	ATCCGGGGCTGGAACTCGCCCTCGGCAGCCGCCCGCCCC
	TTGGCTCCGCTTGCGGTGACCAGTCCCAGCGCCCTCCGCT
	GCCCCCGCCGTCGATCTGCCCCAGCTGGGGCTGCGCCGT
	GACTGCTGTATCACCAGCCCTGACACAGCCCTGTACCTG
	ATGTTGATTACGTGGTGCTATTCTGCGTCGGCAGTATAAT
	TTGTCCACGTCCCCGCTCACGTTCATCTGTTGCTTCGGGTACC
	AATTTGGTTCTCTACGCTGCCCCGCTAAATCCCCTCTTGCCC
	CTCCAGGATGGCACCAACACCCATATCATGGCTACTGAGG
	CATCCAACCTATGCTCAGTACCGGGTCGTTTCGAGCTACGATC
	CGCTACCGCCCGCTGGTGCCGAATGCTGTTGGTGGTTATGC
	TATTICTATTTCTTTTGGCCTCAAACCTACAACCTACCCCTAC
	TTCTGTTGATATGAATTCTATTACTTCCACTGATGTTAGGAT
	TTTGGTCCAGCCCGGTATTGCCTCCGAGTTAGTCATCCCTA
	GTGAGCGCCTTCATTATCGCAATCAAGGCTGGCGCTCTGTT
	GAGACCACAGGTGTGGCTGAGGAGGAGGCTACCTCCGGTC
	TGGTAATGCTTTGCATTCATGGCTCTCCTGTAACTCTTATA
	CTAATAACACCTTACACTGGTGCGTTGGGGCTCCTTGATTTT
	GCACTAGAGCTTGAATTCAGGAATTTGACACCCGGGAACA
	CCAACACCCGTGTTTCCCGGTATACCAGCACAGCCCGTCAT
	CGGTTGCGTCGCGGTGCTGATGGGACCGCTGAGCTTACTAC
	CACAGCAGCCACACGATTTATGAAGGATCTGCATTTCACTG
	GCACTAATGGCGTTGGTGAGGTGGGTCGCGGTATCGCCCTG
	ACACTGTTCAATCTTGCTGATACGCTTCTAGGTGGTTTACC
	GACAGAATTGATTTTCGTCGGCTGGGGGTGAGTTGTTCTACT
	CCCGCCCTGTTGTCTCGGCCAATGGCGAGCCGACAGTGAA
	GTTATACACATCTGTGGAGAATGCGCAGCAAGACAAGGGC
	ATTACCATCCCACACGATATAGATTTGGGTGACTCCCGTGT
	GGTTATTCAGGATTATGATAATCAGCACGAGCAAGACCGA
	CCCACGCCGTCACCTGCCCCCTCACGCCCTTTCTCAGTCCTT
	CGCGCTAACGATGTTTTGTGGCTCTCCCTCACTGCCGCTGA
	GTACGATCAGGCTACGTATGGGTCGTCTACCAACCCTATGT
	ATGTCTCTGATACAGTTACCTTTGTCAATGTGGCCACTGGT
	GCTCAGGCTGTTGCCCCGCTCTCTTGATTGGTCTAAAGTTAC
	TTTGGATGGTCGCCCCCTTACTACCATTACAGCAGTATTCTA
	AGACATTTTATGTTCTCCCGCTCCGCGGGAAGCTGTCCTTTT
	GGGAGGCTGGCACAACTAGGGCCGGCTACCCATATAACTA
	TAACACCACTGCTAGTGATCAAATTCTGATTGAGAATGCGG
	CCGGCCATCGTGTCGCTATCTCCACCTACACTACCAGCCTG
	GGTGCCGGCCCTGCCTCGATCTCCGCGGTGGGTGTATTAGC
	CCCACACTCGGCCCTTGCTGTTCTTGAGGACACTGTTGATT
	ACCCTGCTCGTGCTCACACTTTTGATGATTTCTGCCCGGAG
	TGTCGTACCCTAGGTTTGCAGGGTTGTGCATTCCAGTCCAC

	TATTGCTGAGCTTCAGCGCCTTAAACGGAGGTAGGCAAA ACCCGGGAGTCTTAA (SEQ ID NO: 11)
Genotype 4 BAG32134.1	ATGTTCTTTTGCTCTTTGTCATGGAGATGCCGCCATGCGCTCT CGGGCTCTTCTGTTTTTGCTCCTCGTGTTTTTGCCTATGCTG CCCGCGCCACCGGCCGGTCAGTCGTCTGGCCGTCGCCGCGG GCGGCGCAGCGGCGGTACCGGCGGTGGTTTCTGGGGTGAC CGGGTTGATTCTCAGCCCTTCGCCCTCCCCCTATATTCATCCA ACCAATCCCTTCGCATCCGACATTCCAACCGCAACCGGGGC TGGAGCTCGCCCTCGGCAGCCAGCCCGTCCACTCGGCTCCG CTTGCGCGACCAATCCCAGCGCCCCGCGCTCCTGCCCCGT CGCCGACCTGCCCCAGCTGGGGCTTCGCCGCTAACAGCTGT TGCCCCAGCCCCCGACACTGCCCCGGTTCCCGACGTGGACT CCCGTGGTGCTATATTGCGCCGCCAGTACAACCTTGTCCACG TCACCGCTTACGTCCACTATCGCTACTGGCACTAATCTTGT GCTATATGCTGCCCCACTGAGCCCTCTGCTCCCTCTCCAGG ATGGGACTAATACTCATATCATGGCCACTGAGGCCTCTAAC TATGCTCAGTATCGCGTTGTCCGTGCCACTATTCGGTACCG GCCTCTGGTGCCGAATGCGGTTGGCGGGTACGCCATATCCA TCTCCTTTTGCCCTCAGACAACAACCAACCCCGACCTCCGTC GACATGAATTCCATCACCTCTACCGATGTCCGTATCCTCGT TCAGCCTGGTATAGCCTCTGAGCTTGTGATCCCTAGTGAGC GCCTGCATTATCGCAATCAGGGTTGGCGCTCGGTTGAGACT TCTGGTGTTGCGGAGGAGGAGGCTACCTCTGGCCTTGTTAT GCTCTGTATTCATGGATCTCCTGTAAATTCCCTACACTAATA CACCCCTATACTGGTGCTCTCGGCTTGCTCGATTTTGCGCTTG AGCTTGAATTTGTAATTTGACACCTGGCAATACGAATACG CGCGTCTCTCGTTATTCTAGTAGTGCGCGCCACAAGTTACG CCGAGGGCCTGATGGCACTGCCGAGTTGACCACCACTGCT GCTACACGTTTCATGAAAGATCTCCATTTTACCGGGACTAA TGGTGTTGGTGAGGTTGGCCGTGGTATTGCGCTAACTCTGT TTAATCTTGCTGATACGCTTCTCGGCGGGCTCCCGACAGAA TTGATTTTCGTGCGGCCGGAGGCCAATTGTTTTACTCACGCC CGTCGTCTCAGCCAATGGCGAGCCGACAGTGAAACTCTAC ACCTCAGTTGAGAATGCCAGCAGGACAAGGGTATAGCCA TTCCACATGATATTGACCTTGGTGAGTCCCGAGTTGTGATT CAGGATTATGATAATCAACACGAGCAAGACCGCCCCACCC CTTCCCCTGCCCCCTCACGTCTTTCTCAGTTCTTCGTGCTA ATGATGTGCTTTGGCTTTCATTGACGGCTGCTGAATACGAT CAAACACTTATGGTTCTTCCACTAATCCTATGTATGTTTCT GACACTGTGACATTTGTTAATGTAGCGACCGGTGCCAGGG GGTTTCTCGTTCCCTGGACTGGTCTAAAGTCACCCCTCGATG GTCGGCCGCTTACAACAATTCAGCAGTATTCTAAGACCTTC TTTGTCCTACCTCTTCGTGGTAAGCTCTCTTTCTGGGAGGCT GGCACTACTAAAGCTGGCTACCCTTATAATTATAACACTAC TGCCAGTGATCAGATCTTAATTGAAAATGCACCTGGTCACC GAGTCTGTATTTCCACCTATACTACTAATCTTGGTTCCGGCC CTGTCTCTATTICTGCCGTTGGTGTCTCGCACCCCATTTCTG TGCTGGCCGCTTTGGAGGATAACCGTTGATTACCCTGCTCGT

	GCTCATACTTTTCGATGATTTCTGCCCTGAGTGCCGTGCGCT CGGTCTCCAGGGCTGCGCTTTTCAATCGACTGTGCGCTGAGC TGCAGCGTCTTAAAATGAAGGTGGGTAAAACCCGGGAGTA TTGA (SEQ ID NO: 12)
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[0059] In one embodiment, the present invention is suitable for identifying an agent that inhibits any one or more activities of HEV ORF3 protein (e.g., ion channel activity, multimerization activity, or viroporin activity), thereby reducing or preventing HEV egress from an infected cell and inhibiting HEV infectivity. In some embodiments, the agent can bind to one or more regions of the ORF3 protein. For example, the agent can bind to (or make contact with) the ORF3 protein at any one or more residues selected from the residues at positions 11-13, 29-40, 59-61, 71-73, 80-85, 86-89, or 95-98 of SEQ ID NO: 1. In certain embodiments, the agent can bind to one or more regions of an ORF3 protein comprising any one of SEQ ID NOs: 2-4, wherein the one or more regions correspond to (e.g., possess equivalent functions to) any one or more residues selected from the residues at positions 11-13, 29-40, 59-61, 71-73, 80-85, 86-89, or 95-98 of SEQ ID NO: 1.

[0060] In some embodiments, the agent is a small molecule, as described herein.

[0061] As described herein, in some embodiments, the method comprises introducing into a cell culture 1) a nucleic acid that comprises an HEV open reading frame 2 (ORF2) nucleotide sequence, 2) a nucleic acid that comprises an HEV ORF3 nucleotide sequence, and 3) a nucleic acid that i) comprises a reporter gene and an HEV ORF1 nucleotide sequence and ii) lacks HEV ORF2 and ORF3 nucleotide sequences. As described herein, various methods for introducing (e.g., transfecting, transducing, transforming) nucleic acids into cells of a cell culture are known in the art and can be used in the methods disclosed herein.

[0062] In some embodiments, the cell culture comprises, or is derived from, a hepatocyte. In certain embodiments, the cell culture comprises, or is derived from, a gastrointestinal cell. However, other suitable types of cells can also be used in the present transcomplementation system, as determined by those of skill in the art.

[0063] In some embodiments, the nucleic acids encoding ORF2 and ORF3 are introduced into the cells in the cell culture on a single vector. However, as will be apparent to those of skill in the art, ORF2 and ORF3 can be introduced into the cells in the cell culture on separate

vectors. In various embodiments, the nucleic acid components introduced into the cells are introduced as an RNA molecule (e.g., in vitro-transcribed HEV RNA).

[0064] In certain embodiments, the nucleic acid that i) comprises ORF1 and a reporter gene and ii) lacks ORF2 and ORF3 is introduced as an RNA molecule (e.g., in vitro-transcribed HEV RNA). The nucleic acid can lack either all or a portion of the ORF2 and ORF3 sequences, provided no functional ORF2 and/or ORF3 proteins are expressed. In a particular embodiment, the nucleic acid lacks ORF2 and ORF3 nucleotide sequences entirely.

[0065] Any suitable reporter gene known in the art can be used in the present invention. In various embodiments, the reporter gene encodes, e.g., a fluorescent reporter, a bioluminescence reporter, a chemiluminescence reporter, or a selectable marker. An example of a bioluminescence reporter includes, e.g., a luciferase enzyme. Examples of selectable markers include, e.g., puromycin, blasticidin, HSK-thymidine kinase, and the like.

[0066] In some embodiments, the method further comprises contacting the cell culture (i.e., the cells introduced with 1) a nucleic acid that comprises an HEV ORF2 nucleotide sequence, 2) a nucleic acid that comprises an HEV ORF3 nucleotide sequence, and 3) a nucleic acid that i) comprises a reporter gene and an HEV ORF1 nucleotide sequence and ii) lacks HEV ORF2 and ORF3 nucleotide sequences) with a candidate agent to be tested for anti-HEV activity (e.g., a reduction, completely or partially, in HEV function), as described herein. Generally, the candidate agent is contacted with the cell culture into which nucleic acid has been introduced by adding the candidate agent into the medium in which the cells are cultured. Methods of determining a suitable concentration range to be tested will depend on the agent to be tested, according to systematic methods typically used in the art.

[0067] In some embodiments, the method also comprises harvesting the cell culture media from the cell culture that has been contacted with the agent to collect the HEV released by the cells (e.g., transfected or transduced cells) into the cell culture media. In certain embodiments, it may be desirable to lyse the cells (e.g., transfected or transduced cells) to determine the proportion of HEV that did not properly release into the cell culture media.

[0068] In some embodiments, the method further comprises combining the harvested media (collected from the cell culture of the cells that have been, e.g., transduced or transfected) with a naïve cell culture to infect the naïve cell culture with the HEV released from the cells (e.g., transduced or transfected). The harvested media can be processed prior to combining (infecting) with the naïve cells to, e.g., filter or concentrate the collected HEV.

[0069] As used herein, a “naïve” cell culture refers to a cell culture comprised of cells that are not infected with HEV and/or have not been introduced with full-length or subgenomic variants (e.g., fragments) of the HEV genome. In certain embodiments, the naïve cells are of the same cell-type as the cells (e.g., transduced or transfected cells) from which the HEV has been obtained.

[0070] In some embodiments, the method further comprises measuring a level of activity of the reporter gene in the naïve cell culture. Methods of measuring the level of activity of reporter genes such as a fluorescent reporter, a bioluminescence reporter, a chemiluminescence reporter are readily available and well-known in the art. For example, as described herein, a luciferase assay can be performed to measure the level of activity of a luciferase gene, according to standard manufacturer’s protocol.

[0071] In various embodiments, the method also comprises comparing the level of reporter activity measured in the naïve cell culture, also referred to herein as “the measured activity level,” to a reference activity level, wherein a decrease in measured activity level as compared to the reference activity level indicates that the agent inhibits HEV infectivity. In some embodiments, the reference activity level is obtained from a naïve cell culture that has been infected with HEV particles harvested from cells that were not contacted with the agent to be tested for anti-HEV activity.

[0072] Alternatively, or in addition to measuring and comparing the level of reporter activity in the naïve cell culture to a reference level, the method comprises comparing the level of released HEV or component thereof (e.g., capsid protein) measured in the naïve cell culture to a reference level of released HEV or component thereof, wherein a decrease in measured HEV level as compared to the reference level indicates that the agent inhibits HEV infectivity. In some embodiments, the level of a released HEV can be measured by ELISA (e.g., by detecting the capsid protein with a capture antibody). In some embodiments, the reference level of released HEV is obtained from a naïve cell culture that has been infected with HEV particles harvested from cells that were not contacted with the agent to be tested for anti-HEV activity.

[0073] In certain embodiments, a decrease in measured activity level of at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at

least 95, or 100% as compared to the reference level indicates that the agent inhibits HEV infectivity.

[0074] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, *Short Protocols in Molecular Biology* (1999) 4th Ed, John Wiley & Sons, Inc. which are incorporated herein by reference) and chemical methods.

EXEMPLIFICATION

[0075] *Materials and methods*

[0076] Cell line and animals

[0077] HEK293T cells (ATCC, Manassas, VA, USA) and HepG2C3A cells (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 IU/ml Penicillin and Streptomycin, in a humidified 5% CO₂ incubator at 37°C. All experiments involving oocytes derived from *Xenopus laevis* were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee at Rutgers University.

[0078] Plasmids construction

[0079] To construct lentiviral construct that encoding Kernow C1/p6 ORF2 or Kernow C1/p6 ORF3, the Kernow C1/p6 ORF2 or Kernow C1/p6 ORF3 cDNA was amplified by PCR from HEV Kernow C1/p6 construct (a kind gift from Dr. Suzanne Emerson, NIH) and then cloned into pLVX-IRES-zsGreen1 or pLEX-IRES-mCherry vectors using In-Fusion® HD Cloning Kit (Clontech, Mountain View, CA, USA). To construct the pLEX-IAV M2-IRES-mCherry vector, cDNA encoding Influenza A virus M2 (A/Puerto Rico/8/34/Mount Sinai/Wi(H1N1)) was synthesized by IDT with gBlock, and then cloned into pLEX-IRES-mCherry vectors using In-Fusion® HD Cloning Kit (Clontech). IAV M2 mutants were generated by Quikchange (Stratagene) site-directed mutagenesis. All the constructs were verified by DNA sequencing analysis.

[0080] *Generation of ORF3 alanine mutants.* Kernow C1/p6 ORF3 alanine triplet mutants were synthesized by GenScript (Piscataway, NJ) or by PCR amplification with primers containing the desired changes. Primary PCR products containing the engineered mutations were assembled and cloned into the pLEX-IRES-mCherry vector using In-Fusion® HD Cloning Kit (Clontech) in according to the instruction.

[0081] *In vitro transcription (IVT) and viral RNA transfection*

[0082] HEV Kernow-C1 p6/Gluc plasmid (genotype 3 subgenomic replicon expressing Gaussia luciferase; a gift from Dr. Suzanne Emerson, NIH) was linearized by MluI and the viral capped RNAs were transcribed *in vitro* from linearized plasmid using mMACHINE mMACHINE® T7 Ultra Kit (Ambion, Austin TX, USA). The IVT reaction mixture of 20 µl was assembled by adding DNA template (1 µg), T7 Reaction Buffer, T7 NTP/ARCA, GTP and T7 Enzyme Mix. The IVT reaction mixture was incubated at 37°C for 3hr. To remove the template DNA, 1µl TURBO DNase (from MEGAscript® T7 Kit) was added to the IVT reaction mixture and incubated for 15 min at 37°C. Then, the viral RNA was purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). Viral RNA was transfected into HepG2C3A cells using TransIT®-mRNA Transfection reagent (Mirus Bio LLC, Madison, WI, USA) in according to the instruction.

[0083] *Gaussia Luciferase assay*

[0084] *Gaussia luciferase activity was measured with the Renilla Luciferase Assay System* (Promega, Madison, WI, USA). Ten microlitres of harvested cell culture medium was added per well of a 96-well black, flat-bottom microplate (Corning, NY, USA), followed by the addition of Renilla luciferase assay substrate and the detection of luminescence using a Berthold luminometer.

[0085] *Lentiviral particles production and infection*

[0086] VSV-G pseudotyped lentiviruses were produced by transient co-transfection of the 3rd generation packaging plasmids pMD2G, psPAX2 and transfer vector with XtremeGENE™ HP DNA Transfection Reagent (Sigma-Aldrich, St. Louis, MO, USA) into HEK293T cells. The media was changed 6 h post-transfection. Supernatants were collected at 48 and 72 hr after transfection, pooled, passed through a 0.45µm filter and frozen at -80°C. For lentiviral transduction, 1×10^5 cells/well were seeded in 6 well tissue culture plates and infected the following day with lentiviruses. Cells were trypsinized and processed for FACS analysis after 3 days of infection to determine the transduction efficiency.

[0087] Co- Immunoprecipitation and Western blot assay

[0088] Cell lysates were prepared from 5×10^6 cells in a 10 mM Tris buffer (pH, 7.5) containing 0.1% NP-40 and 1mM EDTA, along with a cocktail of protease and phosphatase inhibitors. Lysates were then incubated with Flag (M2, sigma) antibody (1 μ g, at 4 °C for 6hr), after which the immune-complexes were precipitated with protein A-sepharose. These immunoprecipitates were resolved on a 12% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and then analyzed by Western blot using anti-Flag (M2, Sigma) or anti-HA(Clone HA-7, Sigma) antibodies. Membranes were then washed three times with TBS-T for 15 min total. Membranes were incubated with goat anti-mouse DyLight800-conjugated antibody (Thermo Fisher Scientific) diluted 1:5000 for 30 min and washed with TBS-T three times for 5 min each. Membranes were visualized using the Odyssey® CLx Imaging System and images were processed using Image Studio Lite Ver5.0.

[0089] Cell culture derived HEV generation and infection

[0090] HEV Kernow C1/p6 viral RNA was generated with in vitro transcription kit and transfected into HepG2C3A cells as describe before. Medium was removed from transfected cells after 7 days post transfection and cells were trypsinized and centrifuged at 800 rpm for 5 min. PBS (1ml) suspended the cell pellet and the sample was subjected to three rounds of freezing and thaw. Debris was removed by centrifugation at 13,200 rpm for 5 min and the supernatant was stocked at -80°C. As for the infection assay, the HepG2C3A cells were seeded into a 6-well plate, and cells were infected by HEV in the following day, medium was changed at 12hs after the infection. After 6 days, cells were fixed and stained for immunofluorescence microscopy.

[0091] Confocal microscopy

[0092] HepG2C3A cells were washed with PBS 3 days after lentiviral transduction with HA-tagged wild type or mutant ORF3 constructs, fixed with 4% paraformaldehyde (PFA) and then permeabilized with 0.3% Triton X-100. The cells were blocked with 2% bovine serum albumin (BSA) and immunolabeled with mouse anti-HA (Clone HA-7, Sigma) and rabbit anti-calnexin(Abcam, Cambridge, MA, USA) antibodies for 1h at room temperature. Cells were washed with phosphate-buffered saline (PBS) and incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody (Thermo Fisher Scientific) or Alexa Fluor 647-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific) for 1h. Nuclei were stained with Hoechst dye. To observe the localization of ORF3 in the *Xenopus laevis* oocytes, the

oocytes were collected at 48 hrs after ORF3 mRNA injection for immunostaining assay as described above, rabbit anti-ORF3 polyclonal antibody (a kind gift from Dr. Suzanne Emerson, NIH) and Alexa Fluor 555-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific) were used. Images were taken using a Nikon A1 Spectral Confocal Microscope. ImageJ analysis was done using ImageJ software (National Institutes of Health).

[0093] Voltage clamp experiments

[0094] The ORF3 or M2 cDNA was cloned into pSP64-polyA vector with restriction sites for HindIII (5' end) and BamHI (3' end) enzymes. The plasmid was linearized by EcoRI digestion and transcribed *in vitro* to synthesized mRNA using the mMESSAGE mMACHINE high-yield capped RNA transcription SP6 kit (Ambion, USA). Healthy *Xenopus laevis* oocytes in stage V to VI were injected with 20 ng of mRNA per oocyte and incubated at 16°C in an ND-96 solution. Two-electrode voltage clamp (OC-725C, Warner Instrument Corp.) was used to record the currents at 48 hr post of injection. The oocytes were first bathed in standard Ringer solution (115mM NaCl, 2mM KCl, 1.8mM CaCl₂ and 5mM HEPS, pH7.4) at room temperature and impaled with microelectrodes filled with 3 M KCl. Currents were generated by applying the rectangular voltage protocol from -90 to +60 mV in 10 mV increments with a holding voltage of -60 mV. Current recording and analysis were performed by pClamp 10.3 software package (Axon Instruments).

[0095] Flow cytometric analysis

[0096] Expression of lentivirally-delivered transgenes was analyzed by flow cytometry. Kernow C1/p6 ORF2 or ORF3 (IAV M2 or its mutant) were transduced into target cells by bicistronic lentiviruses expressing zsGreen or mCherry. After 3 days transduction, cells were fixed in PFA 4%/PBS for 15min, then washed with PBS. The efficiencies of transduction of Kernow C1/p6 ORF2 and ORF3 (IAV M2 or its mutant) were determined by simultaneous expression zsGreen and mCherry. All samples were analyzed on a BD LSRII flow cytometer using FlowJo Software (FlowJo, LLC, Ashland, OR, USA).

[0097] Quantification of intracellular virus infectivity

[0098] The Kernow C1/p6-ΔORF2/3[Gluc] RNA transfected HepG2C3A cells were cultured in the 12-well cell culture plate and cells were trypsinized and washed with PBS, and then lysed by adding 1ml H₂O per well and put on the ice for 20 min, vortexing intermittently every 5 min. The samples were centrifuged at 13,000 × g for 10 min to remove

cellular debris, and 0.9 ml of the supernatant was collected and 0.1ml of 10× concentrated PBS was added to infect cells.

[0099] Statistical Analysis

[00100] Student's t test was used to test for statistical significance of the differences between the different group parameters. *p* values of less than 0.05 were considered statistically significant.

[00101] Results

[00102] Characterization of ORF3 as a pore-forming protein

[00103] Bioinformatic analysis indicated that HEV ORF3 contained a putative transmembrane domain (FIG. 1A). This prediction was corroborated following expression of HEV ORF3 in HepG2C3A cells, a commonly used human hepatoma cell line permissive to HEV infection. In these cells, colocalization of ORF3 with the ER-associated protein calnexin was observed (FIG. 1B), suggesting ORF3 associates with intracellular membranes likely derived from the ER. Based on the punctate pattern in which ORF3 was expressed, it was determined whether the protein forms larger complexes. FLAG-tagged ORF3 immunoprecipitated with an anti-HA antibody in lysates derived from HepG2C3A cells co-expressing FLAG- and HA-tagged ORF3 (FIG. 1C). Additionally, larger protein complexes were observed in Western blots of HepG2C3A lysates even under denaturing conditions, suggesting multimerization (FIG. 1D). Collectively, these data confirm that HEV ORF3 is a transmembrane protein localized at ER membranes (Tyagi, S., et al. *J Biol Chem* 279, 29308-29319 (2004)) and forms multimeric complexes, likely through homophilic interactions.

[00104] The present study indicated that ORF3 shares some similarities to known viroporins, (virally-encoded protein complexes that serve as functional ion channels). Like other viroporins, ORF3 is a small hydrophobic protein that tends to oligomerize in ER-derived membranes. Similar to class IA viroporins such as IAV M2 (Pinto, L. H., et al. *Cell* 69, 517-528 (1992)), HIV-1 Vpu (Cordes, F. S., et al. *Biochemistry* 41, 7359-7365 (2002)), or the coronavirus E protein (Wilson, L., et al. *Virology* 353, 294-306, (2006)), ORF3 has a short tail at the N-terminus that resides in the ER lumen and a long cytosolic tail at the C-terminus that is prone to phosphorylation at a serine in position 70 (Zafrullah, M., et al. *J Virol* 71, 9045-9053 (1997); Emerson, S. U., et al. *J Virol* 80, 10457-10464 (2006)). To enable direct analysis of ORF3's function, ORF2 and/or ORF3 were expressed lentivirally in HepG2C3A cells (FIG. 5), into which was subsequently transfected *in vitro* transcribed RNA

from a recombinant HEV genome derived from the KernowC1/p6 genome (Shukla, P. *et al. Proc Natl Acad Sci U S A* 108, 2438-2443 (2011)) in which ORF2 and 3 were replaced by a secreted version of Gaussia luciferase (Gluc), termed rHEV Δ ORF2/3[Gluc] (see also FIG. 5A). Supernatants collected from these cultures at day 5 post-transfection were then used to infect naïve HepG2C3A cells. The recombinant HEV subgenome replicated equally efficiently in all cells, irrespective of ORF2/3 expression (data not shown). In cells expressing ORF2 only, infectious virions assembled but were retained intracellularly (FIG. 2B). Only supernatants collected from HepG2C3A cells expressing HEV ORF2 and 3 together and not separately led to robust re-infection, as indicated by an approximately 35-45 fold increase in luciferase activity over background (FIG. 2A). These data are consistent with previous reports showing that ORF2 is essential for packaging and ORF3 for release of infectious particles (Yamada, K. *et al. J Gen Virol* 90, 1880-1891 (2009); Emerson, S. U., *et al. J Virol* 80, 10457-10464 (2006)).

[00105] Next, it was determined whether ORF3's essential function in HEV release could be replaced by IAV M2, a well-characterized class IA viroporin (Pinto, L. H., *et al. Cell* 69, 517-528 (1992)). Notably, infection of HepG2C3A cells with supernatants from HepG2C3A cells in which transfection of rHEV Δ ORF2/3[Gluc] was complemented *in trans* with HEV ORF2 and IAV M2 resulted in a 5-8 fold increase in Gluc activity. This signal was approximately two fold lower than the 8-15 fold increase in Gluc activity resulting from infection with particles packaged in cells expressing HEV ORF2 and 3 (FIG. 3A). Furthermore, IAV M2-mediated HEV particle release was dependent on M2's ion channel activity as the IAV M2(A30P) mutant, which abolishes its ion channel activity (Holsinger, L. J., *et al. J Virol* 68, 1551-1563 (1994)), did not support HEV particle egress. Taken together, these data suggest that HEV ORF3 functions as an ion channel.

[00106] To directly test the ability of ORF3 to facilitate the flux of ions across membranes, a two-electrode voltage-clamp procedure was used in *Xenopus (X.) laevis* oocytes injected with wild-type ORF3, wild-type M2, or mutant M2(A30P) mRNAs. Cells were cultured for 2 days, and total membrane currents were measured. Immunofluorescence imaging confirmed expression of ORF3 (FIG. 6D) and the M2 proteins (data not shown) on the outer oocyte membrane. The currents of oocytes expressing the wild-type ORF3, M2, or M2 mutant proteins were studied by holding the membrane voltage of the oocytes at -60 mV and then changing the membrane voltage (between -90 and +60 mV) with a voltage-clamp pulse. This

hyperpolarization induced an inward current with minimal time dependence that increased to a steady value immediately after the hyperpolarization pulse was applied. This current was significantly larger than the endogenous current evoked by identical changes of membrane voltage in control oocytes injected with M2 mutant (A30P) mRNA or mock injected but was very similar to that observed in oocytes injected with wild-type M2 mRNA (FIG. 3B).

Together, these data demonstrate that HEV ORF3 serves as a functional ion channel.

[00107] Regions of ORF3 important for ion channel and viroporin function

[00108] Regions within a HEV ORF3 protein important for release of infectious HEV particles and ion channel function were systematically identified. Alanine scanning mutagenesis was performed to change triplets of amino acids to alanine across the entire ORF3 protein. Lentiviral delivery of individual ORF3 mutants, along with unmodified ORF2, led to high expression of the proteins in the majority of HepG2C3A cells (data not shown). Dually transduced cells were subsequently transfected with rHEV Δ ORF2/3[Gluc] RNA and supernatants collected 5 days thereafter. To assess whether any of the ORF3 mutants affected release of HEV, supernatants were used to infect naïve HepG2C3A cells. Several positions led to $\geq 90\%$ reduction in particle release as compared to HEV released from cells expressing wild-type ORF2 and ORF3 (FIG. 4A, top panel, FIG. 8C). In particular, the present study confirmed that ORF3 residues 86-89 and 95-98, each containing a PXXP motif previously shown to be necessary for HEV release (Kenney, S. P. *et al. J Virol* 86, 5637-5646 (2012); Kenney, S. P., et al. *Virology* 486, 198-208 (2015); Emerson, S. U. *et al. J Virol* 84, 9059-9069 (2010); Nagashima, S. *et al. J Gen Virol* 92, 269-278 (2011)), serve essential functions. In addition, mutations in positions 11-13, 29-40, 59-61, 71-73 and 80-85 reduced virion release by 80-95%. Gluc levels in the transfected cells were equivalent across all experimental conditions, demonstrating that the observed differences cannot be simply attributed to differences in RNA transfection and/or HEV replication efficiency in the different producer cells (FIG. 4A, bottom panel).

[00109] ORF3 mutants that simply affected protein stability were eliminated from the analysis. Western blots of lysates from HepG2C3A cells expressing HA-tagged ORF3 showed that all mutants except ORF3[RLA29-31AAA] and ORF3[VVV32-34AAA] could readily be detected with an anti-HA antibody (FIG. 7A) and those that did express well, with the exception of ORF3[CCC11-13AAA], still formed higher molecular weight complexes indicative of multimerized ORF3. All stable mutants were then subjected to voltage-clamp

experiments. Notably, ORF3[CCC11-13AAA] and ORF3[IFI59-61AAA] exhibited a significant decrease in ion flux across the membrane as compared to oocytes expressing wild-type ORF3 (FIG. 4C). ORF3[PLA86-88AAA] and ORF3[PSA95-97AAA], which had alanine triplets disrupting the PXXP motifs critical for interactions with components of the ESCRT machinery and thereby interfere with the release of infectious particles through the vacuolar protein secretion pathway (Emerson, S. U. *et al. J Virol* 84, 9059-9069 (2010); Nagashima, S. *et al. J Gen Virol* 92, 269-278 (2011); Yamada, K. *et al. J Gen Virol* 90, 1880-1891 (2009); Kenney, S. P., et al. *Virology* 486, 198-208 (2015); Nagashima, S. *et al. J Gen Virol* 92, 2838-2848 (2011); Surjit, M., et al. *J Biol Chem* 281, 8135-8142 (2006)), did not diminish ion channel activity. Of note, none of these mutants, including ORF3[CCC11-13AAA] and ORF3[IFI59-61AAA], displayed vastly different subcellular localization as compared to wild-type ORF3 (FIG. 4B and FIG. 7B). Thus, residues 11-13 and 59-61 likely reside within regions important for ORF3's ion channel function.

[00110] Altogether, the data shown herein demonstrate that ORF3's ion channel activity is important for particle release, which is an additional, distinct function from the previously described, essential interactions of HEV ORF3 with components of the ESCRT pathway.

[00111] Testing known ion channel inhibitors for activity against HEV ORF3

[00112] Using the transcomplementation platform described herein, it was demonstrated that HEV ORF3 exhibits ion channel activity (see hereinabove and Ding *et al*, PNAS 114(5), 1147-1152 (2017), the contents of which are incorporated herein by reference in their entirety. It was shown that ORF3's viroporin function can be substituted in trans with other class Ia viroporins, specifically the influenza A virus (IAV) M2 protein. Using the transcomplementation platform, ORF3 from different HEV genotypes (gts) known to infect humans (gts 1, 2, 3, or 4) was delivered in trans with ORF1 and ORF2 from gt 3. Interestingly, HEV particle release is equivalent when the genotype of ORF3 is mismatched with ORF1/2 (FIG. 11). These data collectively show that ORF3 operates in a viral genotype-independent fashion.

[00113] Known ion channel inhibitors were tested for anti-viral activity against HEV using the transcomplementation platform (FIGS. 12A-12C). Of the known inhibitors tested, none significantly inhibited viral particle release, suggesting that HEV ORF3-specific targets are likely needed to interfere efficiently with viral egress (FIGS. 13A-13C).

[00114] The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

[00115] While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method of inhibiting hepatitis E virus (HEV) release from a cell that is infected with an HEV, comprising contacting the cell with an agent that inhibits one or more activities of HEV open reading frame 3 (ORF3) protein, wherein the one or more ORF3 activities are selected from ion channel activity, multimerization activity, or viroporin activity.
2. The method of claim 1, wherein the agent is a small molecule.
3. The method of claim 1 or 2, wherein the agent binds to HEV ORF3 protein.
4. The method of any one of the preceding claims, wherein the HEV ORF3 protein comprises the sequence set forth in SEQ ID NO: 1.
5. The method of claim 4, wherein the agent binds to any one or more regions of ORF3 protein comprising SEQ ID NO: 1 at amino acid positions 11-13, 29-40, 59-61, 71-73, 80-85, 86-89, or 95-98 of SEQ ID NO: 1.
6. The method of claims 1-3, wherein the HEV ORF3 protein comprises the sequence set forth in any one of SEQ ID NOs: 2-4.
7. The method of any one of the preceding claims, wherein the agent binds to a channel-forming region of ORF3 protein.
8. The method of any one of the preceding claims, wherein the agent inhibits ORF3 protein ion channel activity.
9. The method of claim 8, wherein the agent binds to a region of ORF3 protein comprising SEQ ID NO: 1 at amino acid positions 11-13 or 59-61 of SEQ ID NO: 1, or both.
10. The method of any one of the preceding claims, wherein the cell is a hepatocyte.
11. The method of any one of the preceding claims, wherein the cell is a gastrointestinal cell.

12. A method of treating a hepatitis E virus (HEV) infection in a subject in need thereof, comprising administering to the subject an effective amount of an agent that inhibits one or more activities of HEV open reading frame 3 (ORF3) protein, wherein the one or more activities are selected from ion channel activity, multimerization activity, or viroporin activity.
13. The method claim 12, wherein the agent is a small molecule.
14. The method of claim 12 or 13, wherein the agent binds to HEV ORF3 protein.
15. The method of any one of claims 12-14, wherein the HEV ORF3 protein comprises the sequence set forth in SEQ ID NO: 1.
16. The method of claim 15, wherein the agent binds to any one or more regions of ORF3 protein comprising amino acid positions 11-13, 29-40, 59-61, 71-73, 80-85, 86-89, or 95-98 of SEQ ID NO: 1.
17. The method of any one of claims 12-14, wherein the HEV ORF3 protein comprises the sequence set forth in any one of SEQ ID NOs: 2-4.
18. The method of any one of claims 12-17, wherein the agent binds to a channel-forming region of ORF3 protein.
19. The method of any one of claims 12-18, wherein the agent inhibits ORF3 protein ion channel activity.
20. The method of claim 19, wherein the agent binds to a region of ORF3 protein comprising SEQ ID NO: 1 at amino acid positions 11-13 or 59-61, or both.
21. The method of any one of claims 12-20, wherein the subject is a pregnant female.
22. The method of any one of claims 12-21, wherein the subject is immunocompromised.
23. The method of claim 22, wherein the immunocompromised subject is a subject who has undergone an organ transplant or a subject infected with human immunodeficiency virus (HIV), or both.

24. The method of any one of claims 12-23, further comprising administering an effective amount of interferon or ribavirin, or both, to the subject.

25. A method of identifying an agent that inhibits hepatitis E virus (HEV) infectivity, comprising:

a) introducing into a cell culture 1) a nucleic acid that comprises an HEV open reading frame 2 (ORF2) nucleotide sequence, 2) a nucleic acid that comprises an HEV ORF3 nucleotide sequence, and 3) a nucleic acid that i) comprises a reporter gene and an HEV ORF1 nucleotide sequence and ii) lacks HEV ORF2 and ORF3 nucleotide sequences;

b) contacting the cell culture in step a) with an agent to be tested for anti-HEV activity;

c) harvesting the cell culture media from the cell culture that has been contacted with the agent;

d) combining the harvested media with a naïve cell culture;

e) measuring a level of activity of the reporter gene in the naïve cell culture; and

f) comparing the level of reporter activity in the naïve cell culture measured in step e) to a reference activity level,

wherein a decrease in measured activity level as compared to the reference activity level indicates that the agent inhibits HEV infectivity.

26. The method of claim 25, wherein the agent is a small molecule.

27. The method of claim 25 or 26, wherein ORF2 and ORF3 are introduced into the cells in the cell culture on a single vector.

28. The method of claims 25 or 26, wherein ORF2 and ORF3 are introduced into the cells in the cell culture on separate vectors.

29. The method of any one of claims 25-28, wherein the nucleic acid that i) comprises ORF1 and a reporter gene and ii) lacks ORF2 and ORF3 is introduced as an RNA molecule.

30. The method of any one of claims 25-29, wherein the reporter encodes a luciferase enzyme.

31. The method of any one of claims 25-30, wherein the cell culture introduced with nucleic acid comprises or is derived from a hepatocyte.
32. The method of any one of claims 25-31, wherein the naïve cell culture comprises or is derived from a hepatocyte.
33. The method of any one of claims 25-32, wherein the reference activity level is obtained from a naïve cell culture that has been infected with HEV particles harvested from cells that were not contacted with the agent to be tested for anti-HEV activity.
34. The method of any one of claims 25-33, wherein the agent inhibits HEV release from a cell, HEV replication, or HEV assembly, or a combination thereof.
35. The method of any one of claims 25-34, wherein the agent inhibits ORF1 protein activity, ORF2 protein activity, or ORF3 protein activity, or a combination thereof.
36. The method of any one of claims 25-35, wherein the agent inhibits ORF3 protein activity.
37. The method of any one of claims 25-36, wherein the agent binds to any one or more regions of ORF3 protein comprising SEQ ID NO: 1 at amino acid positions 11-13, 29-40, 59-61, 71-73, 80-85, 86-89, or 95-98 of SEQ ID NO: 1.

FIG. 1A

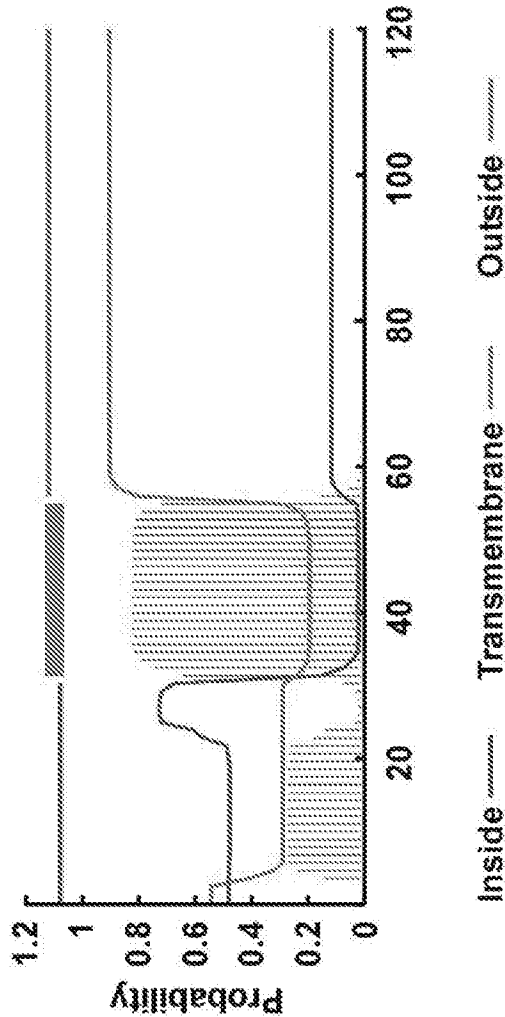


FIG. 1B

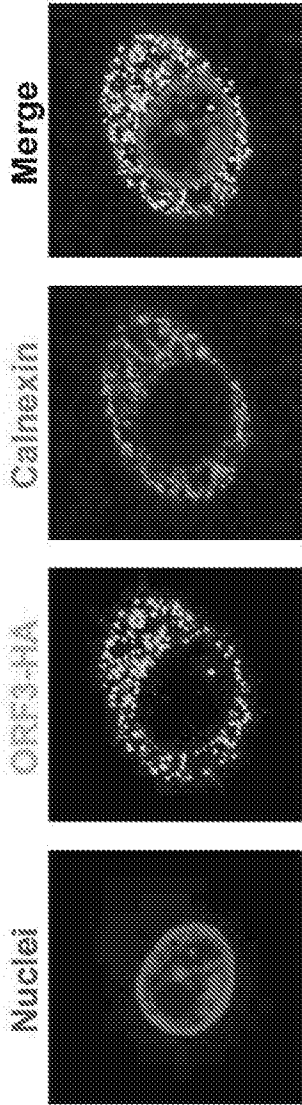


FIG. 1C

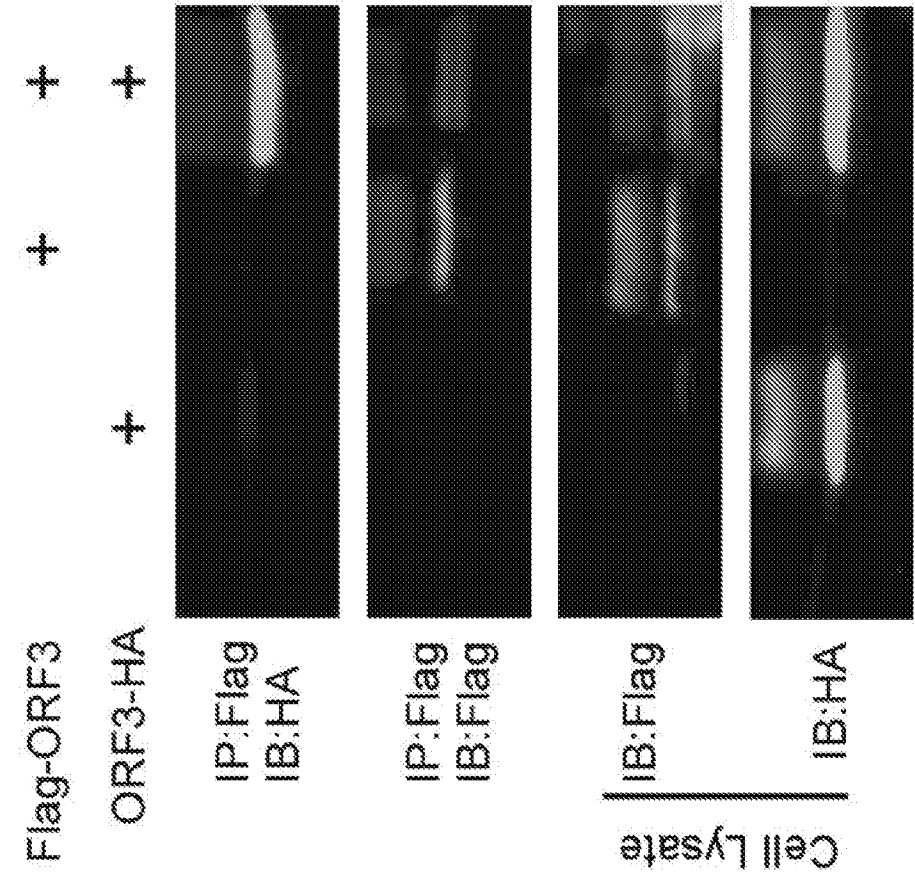
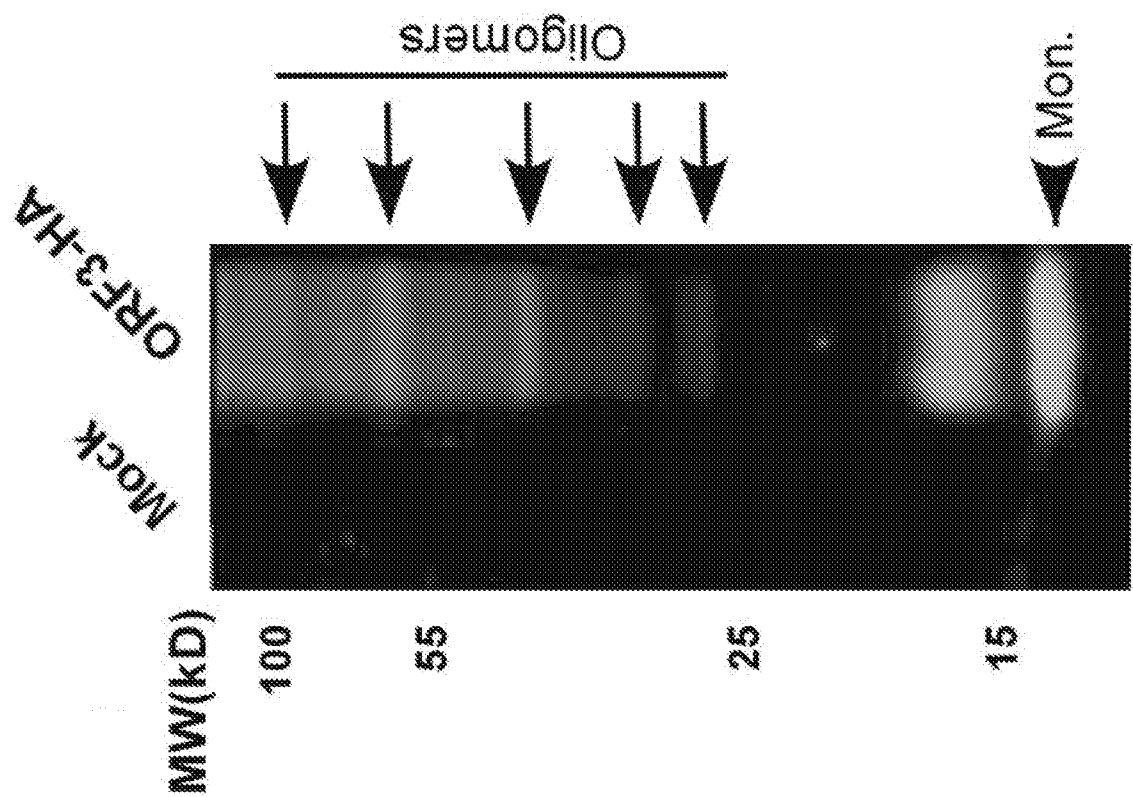


FIG 1D



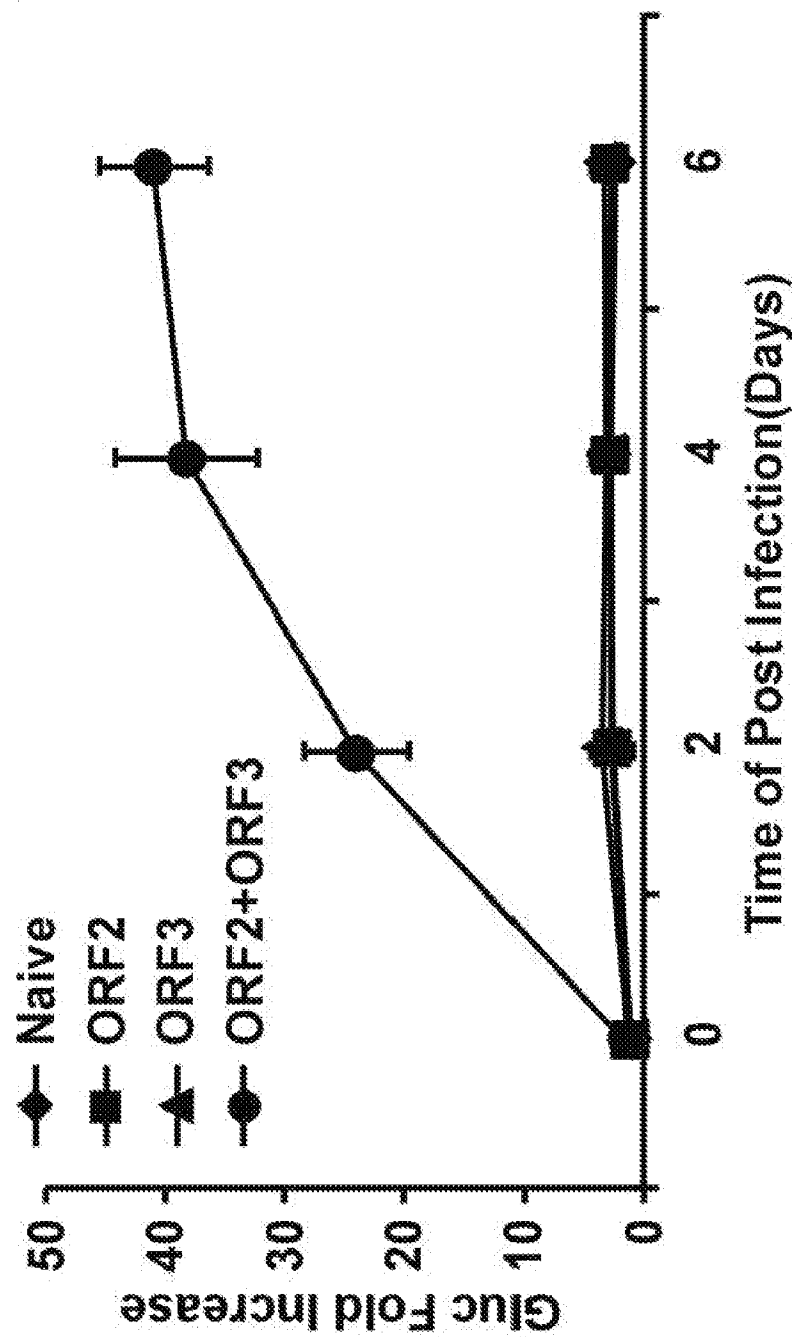


FIG. 2A

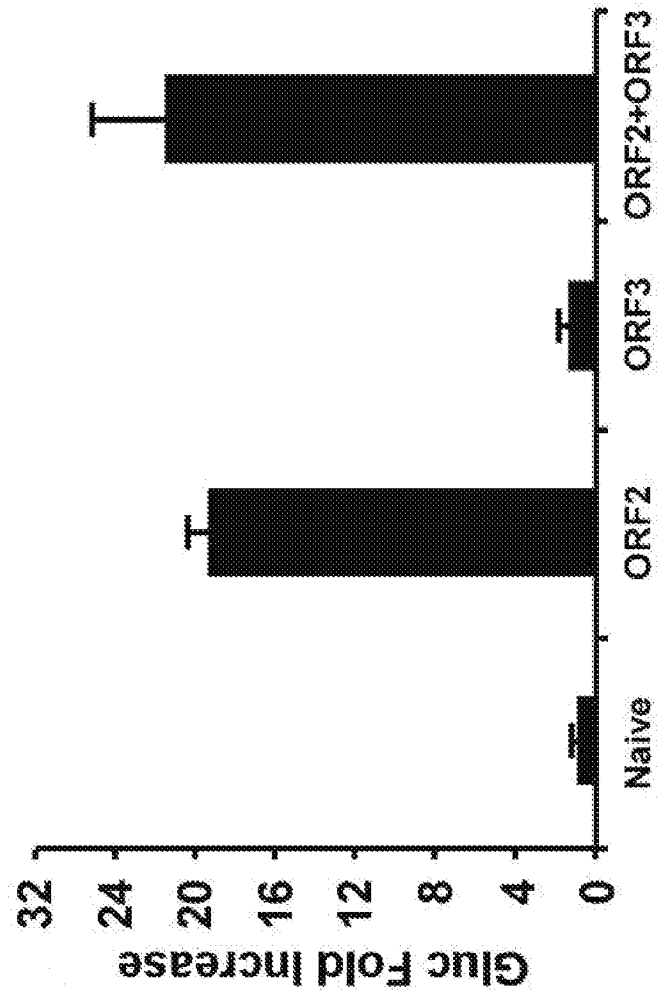


FIG. 2B

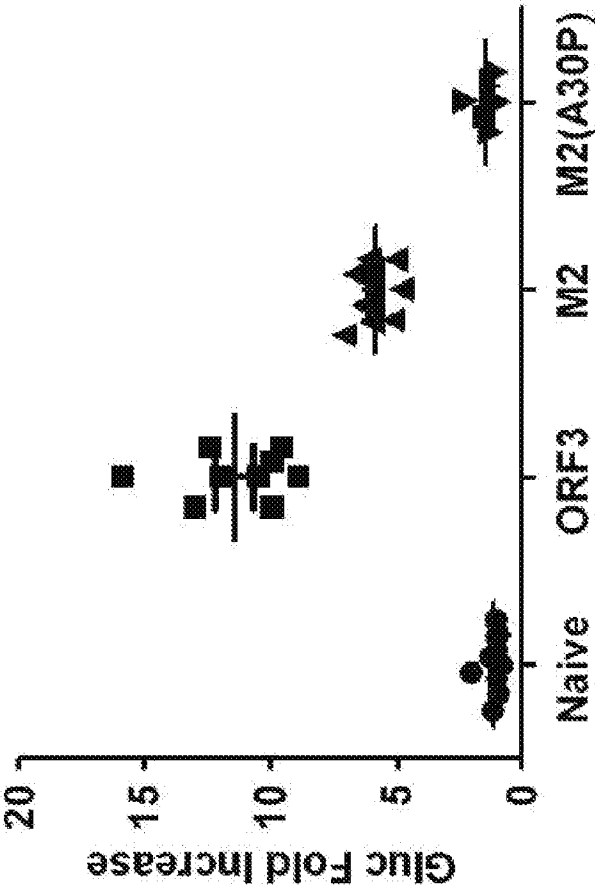


FIG. 3A

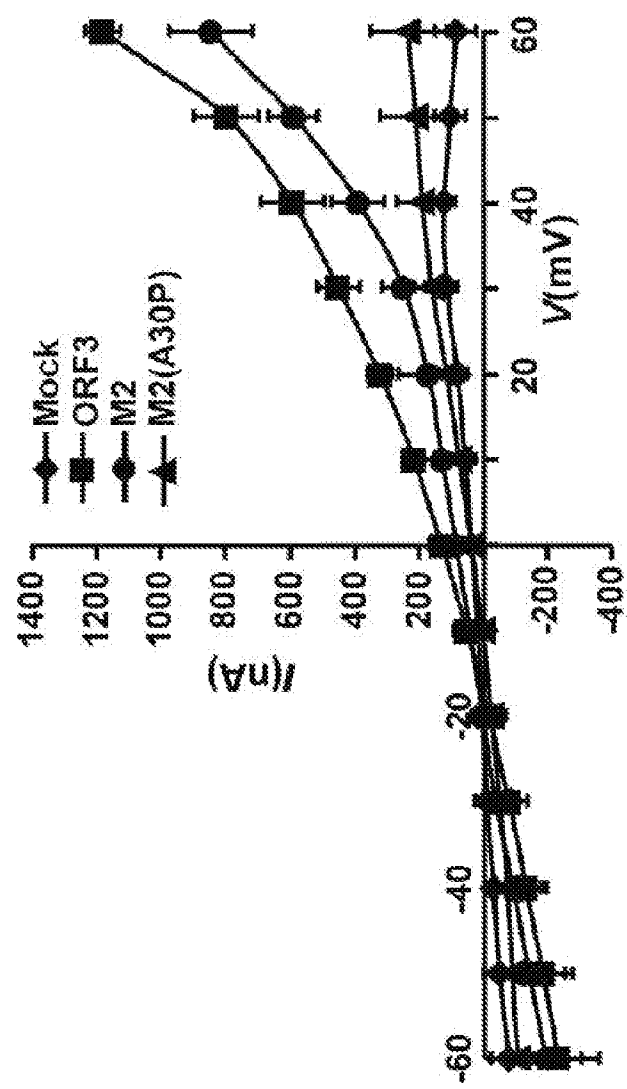


FIG. 3B

FIG. 4A

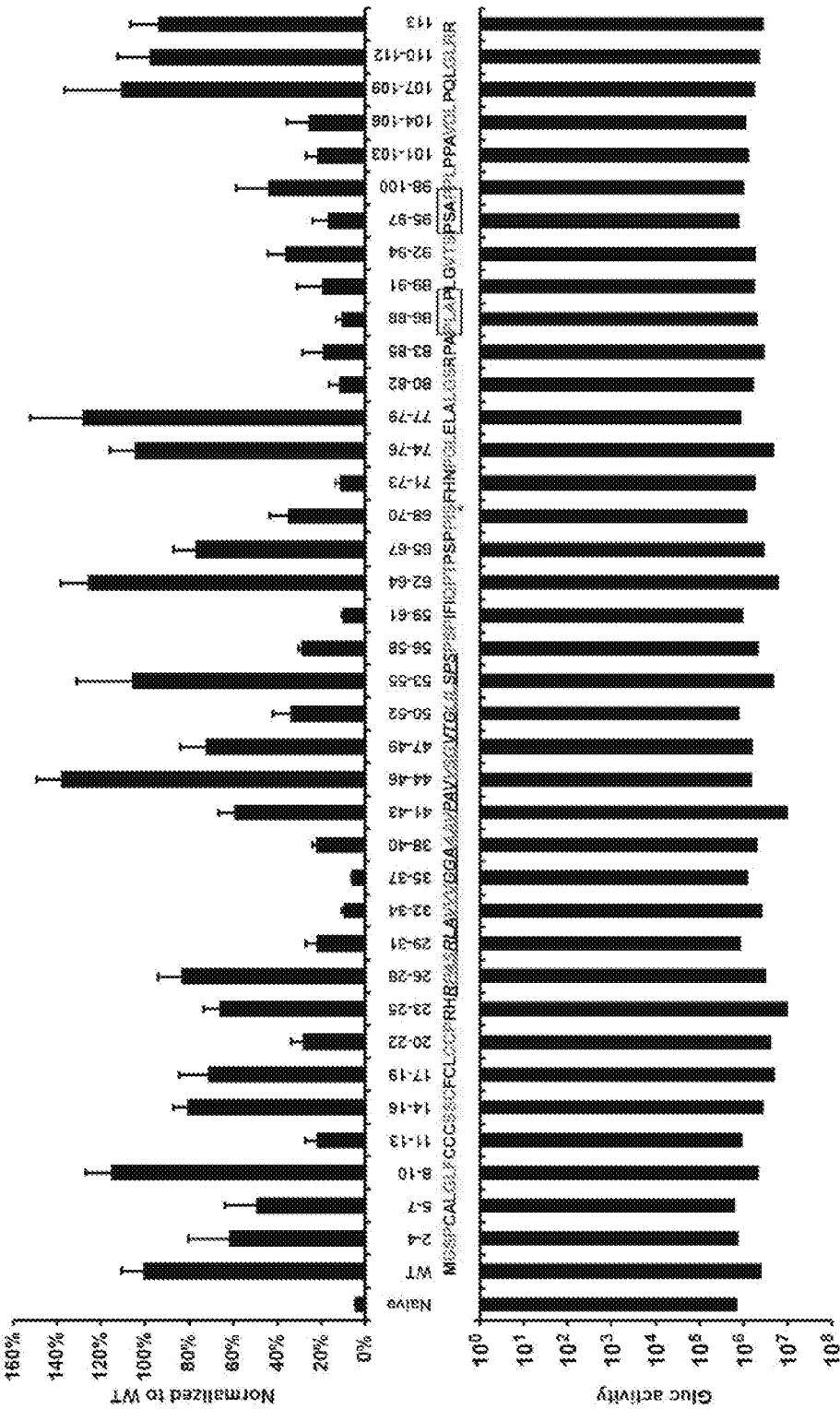


FIG 4C

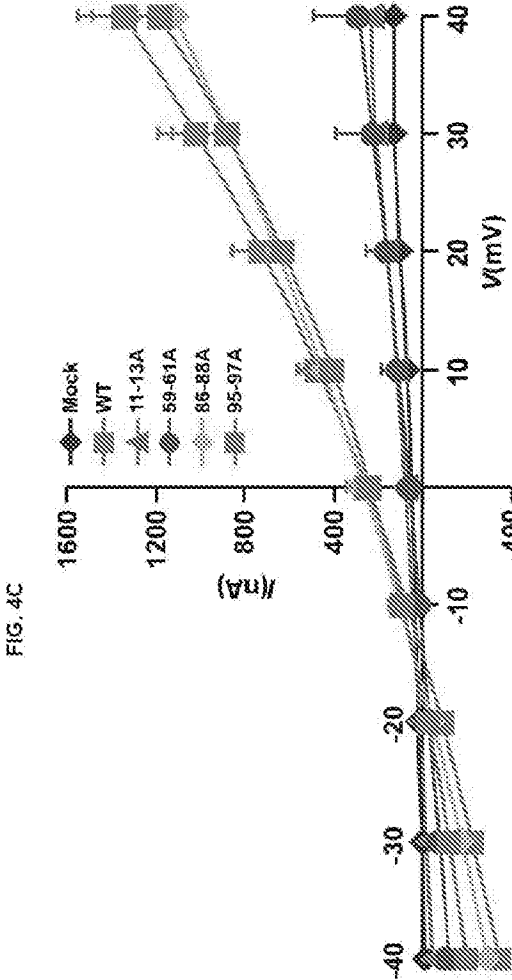


FIG 4B

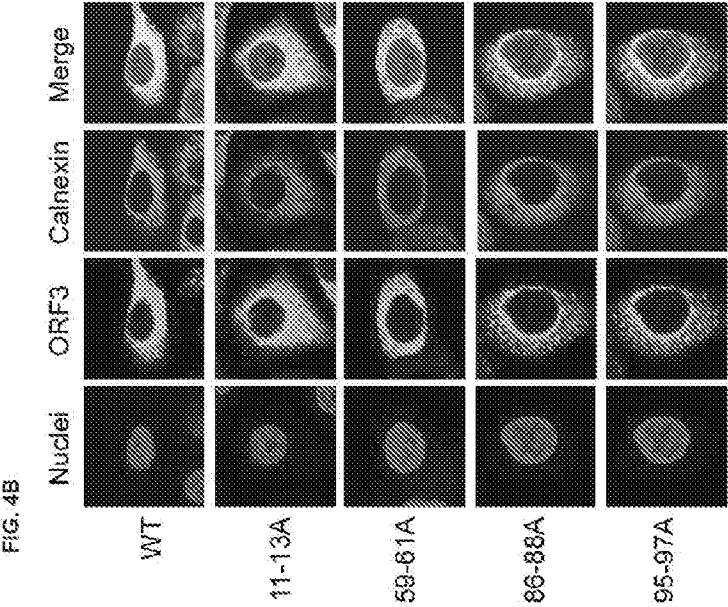


FIG.5A

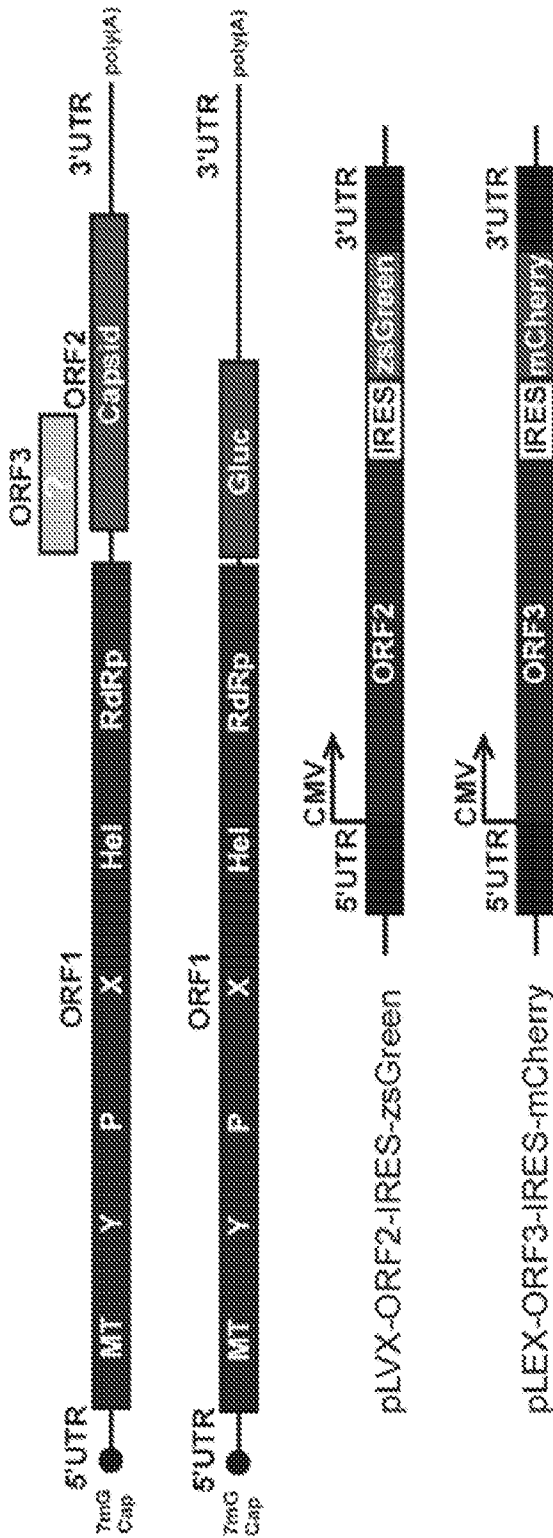


FIG. 5B

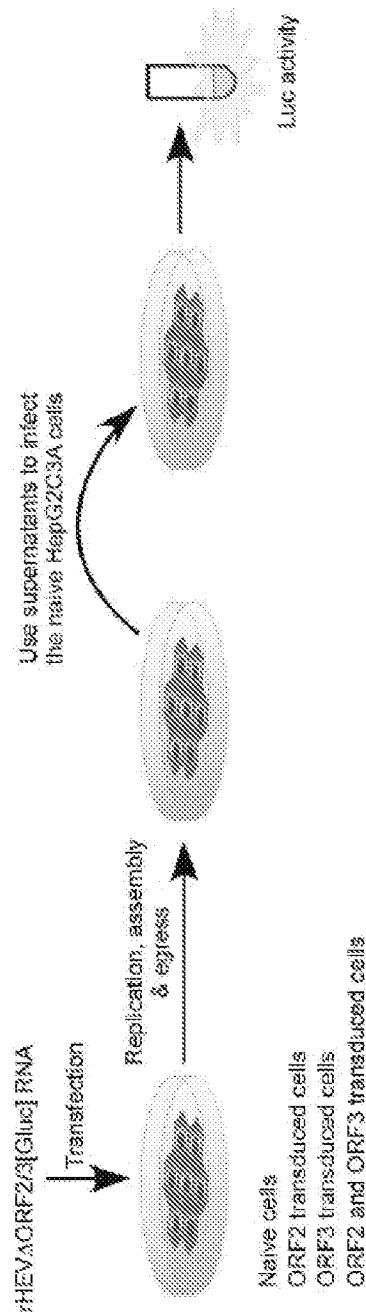


FIG. 5C

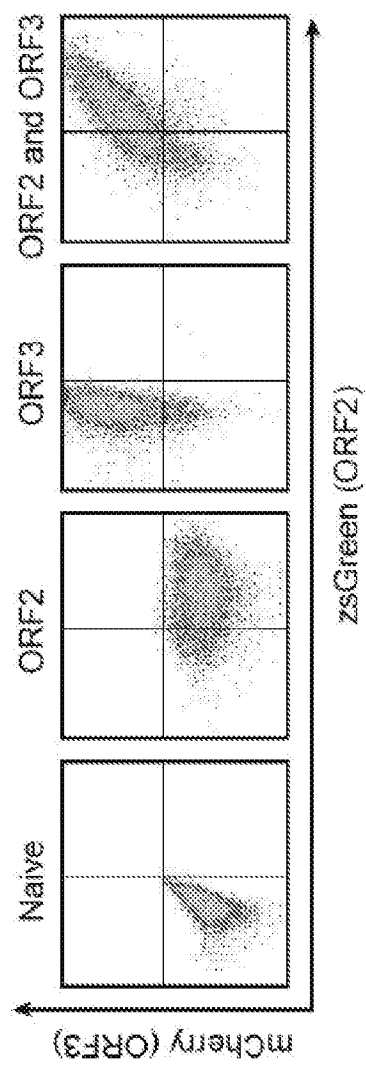


FIG.6A

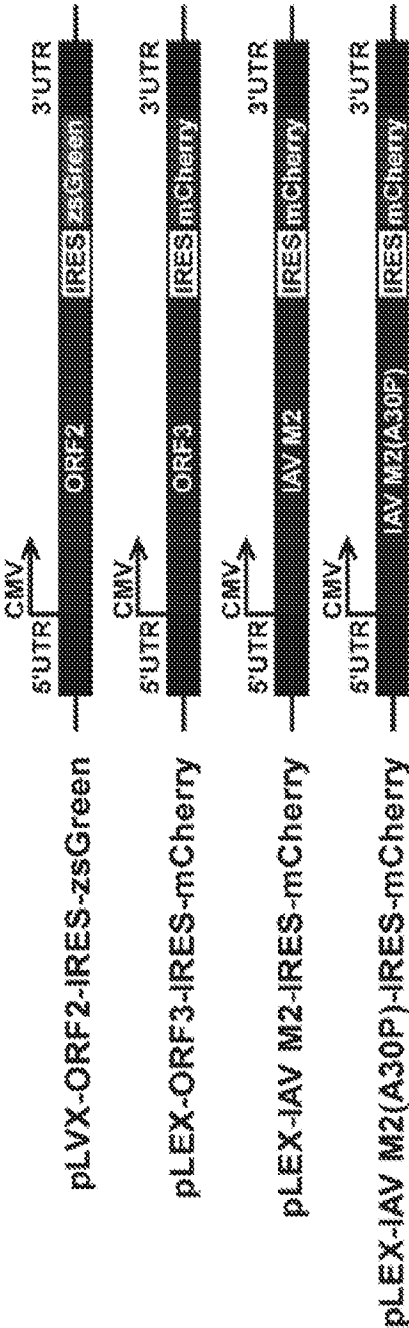


FIG.6B

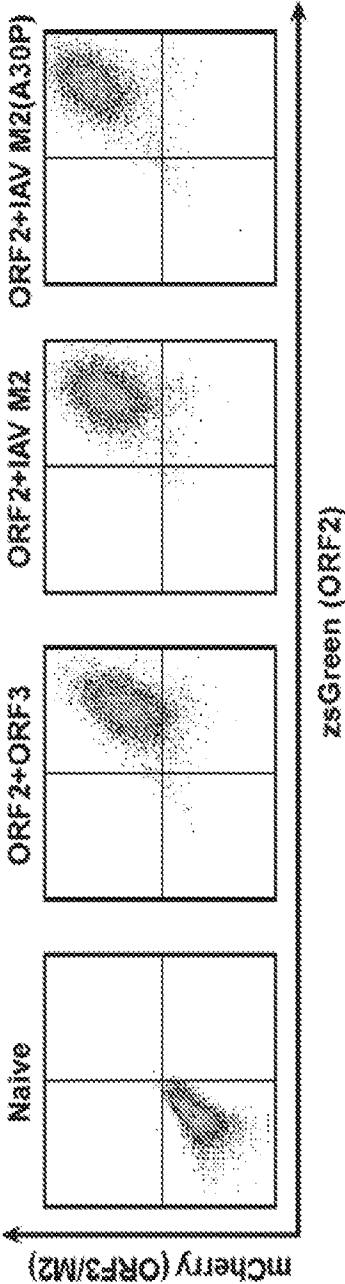


FIG. 6C

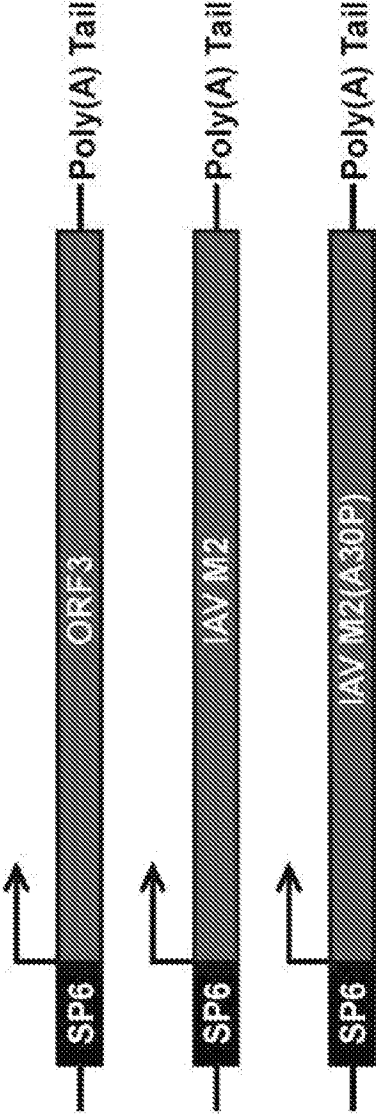


FIG. 6D

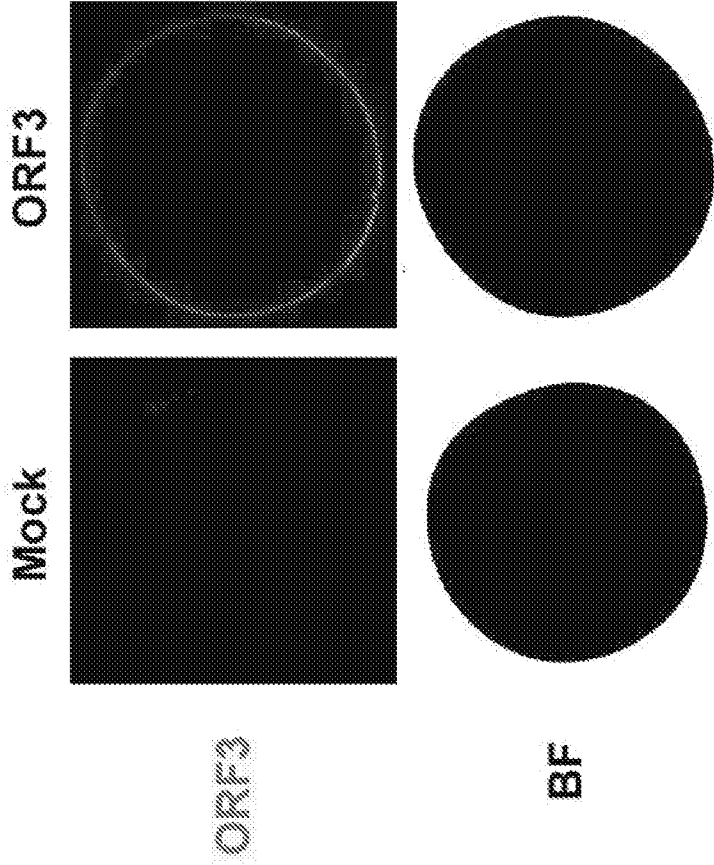
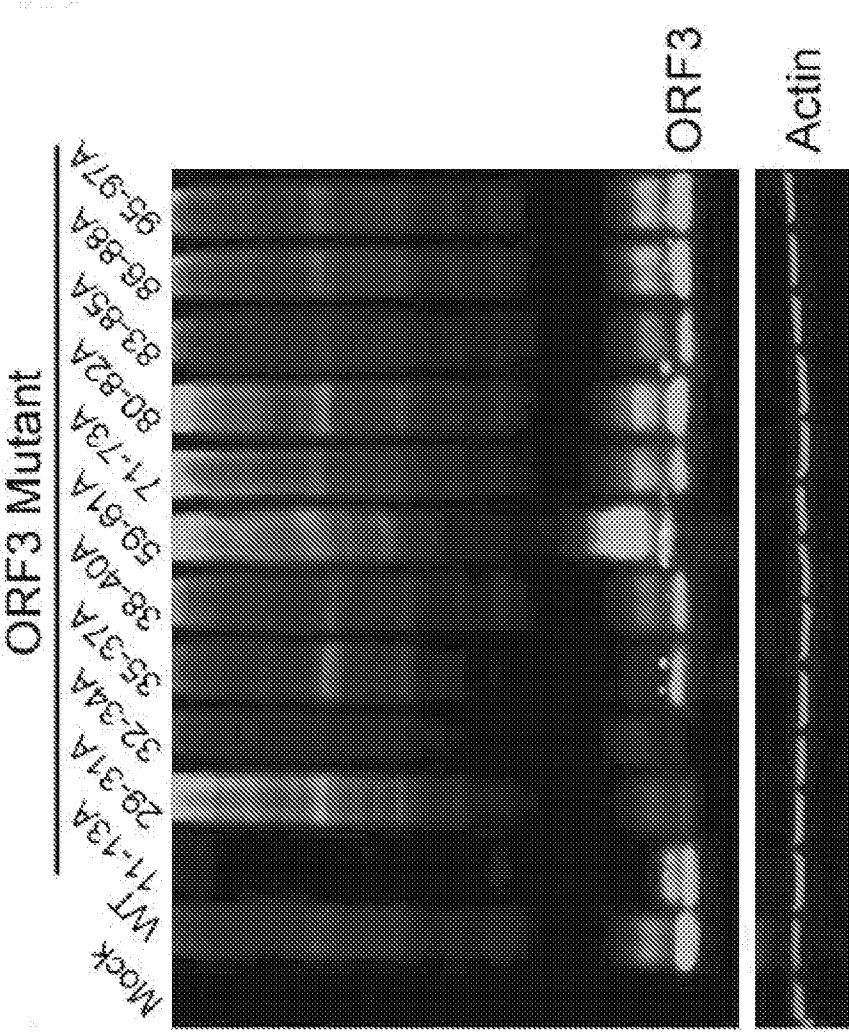


FIG. 7A



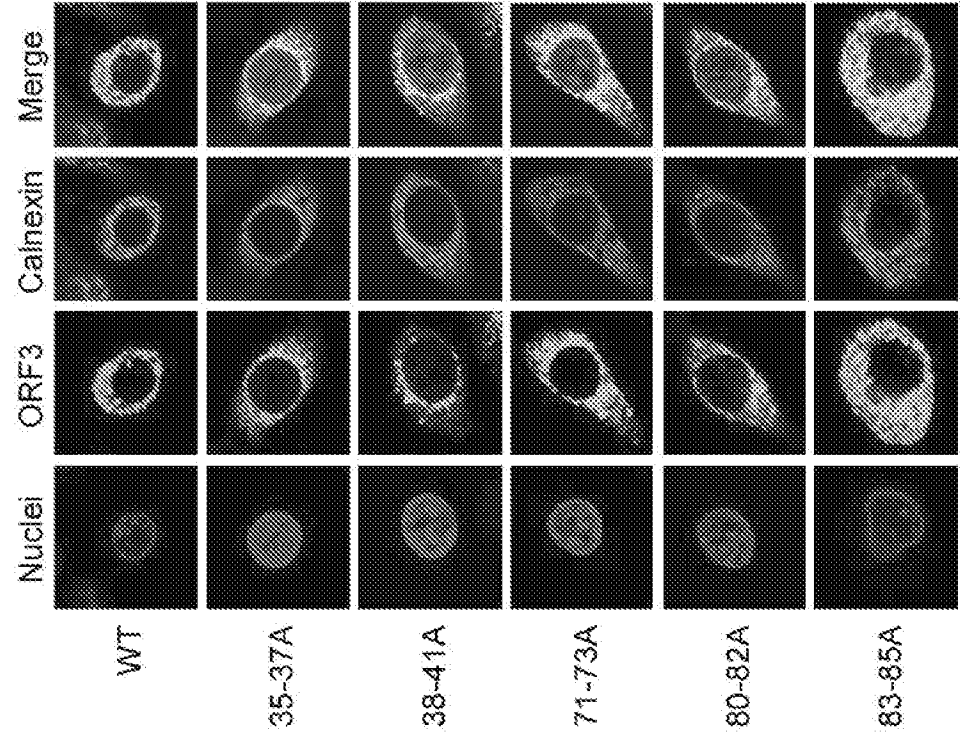


FIG. 7B

FIG. 8A

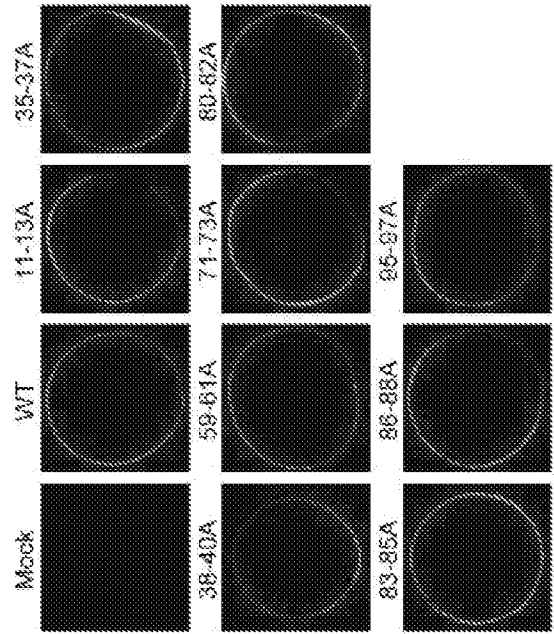


FIG. 8B

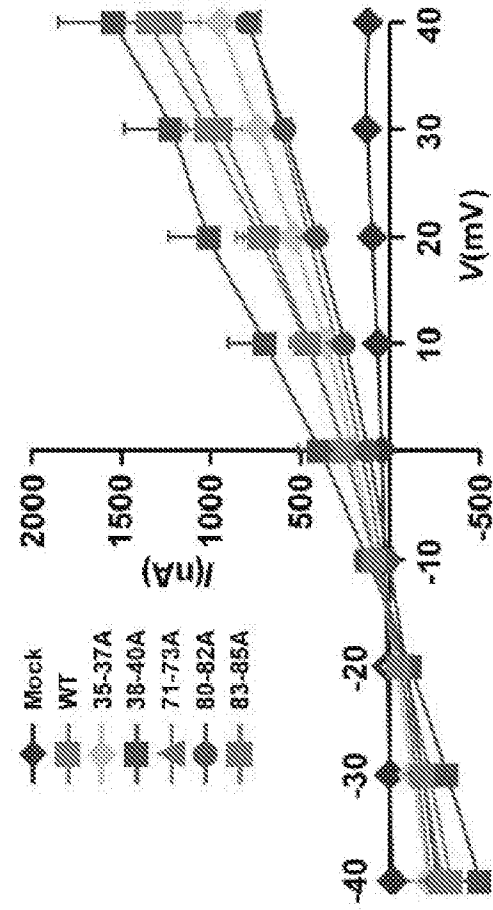


FIG. 8C

Name	HEV egress	Protein Expression	Ion Channel activity
WT	100.0±9.1%	✓	100.0±5.4%
11-13A	20.7±5.3%	✓	10.4±2.0%
29-31A	16.3±7.2%	Not stable	
32-34A	9.3±1.1%	Not stable	
35-37A	5.6±0.5%	✓	70.5±3.8%
38-40A	10.4±3.7%	✓	122.4±10.2%
59-61A	9.6±0.8%	✓	9.4±3.3%
71-73A	10.8±2.8%	✓	55.2±1.0%
80-82A	11.1±5.2%	✓	47.3±7.3%
83-85A	18.7±9.4%	✓	94.0±4.5%
86-88A	10.2±3.0%	✓	90.6±9.2%
95-97A	12.3±0.6%	✓	86.3±4.1%

FIG. 9

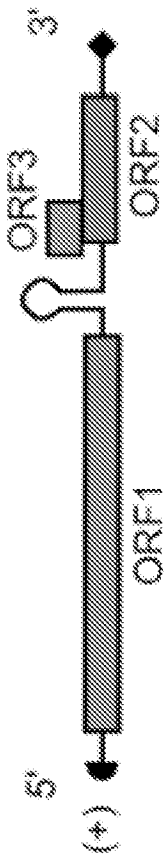


FIG. 10A

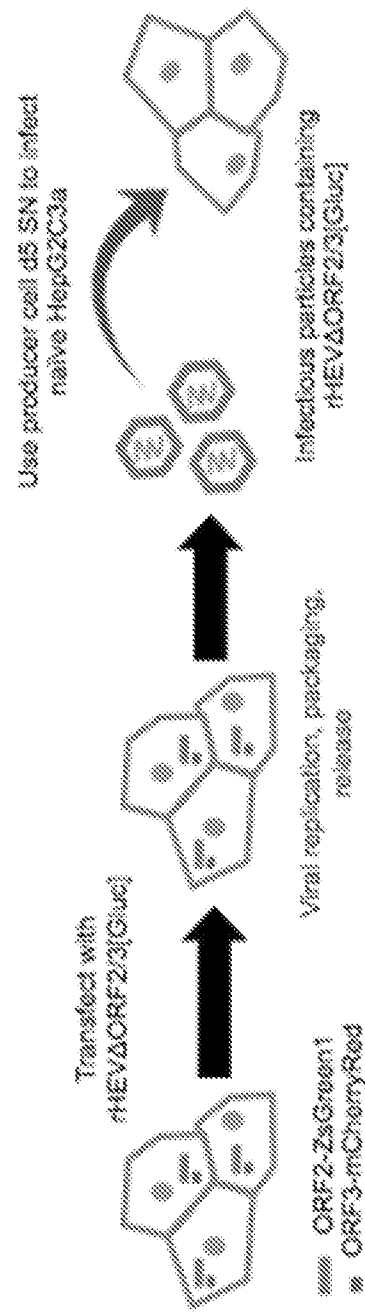


FIG. 10B

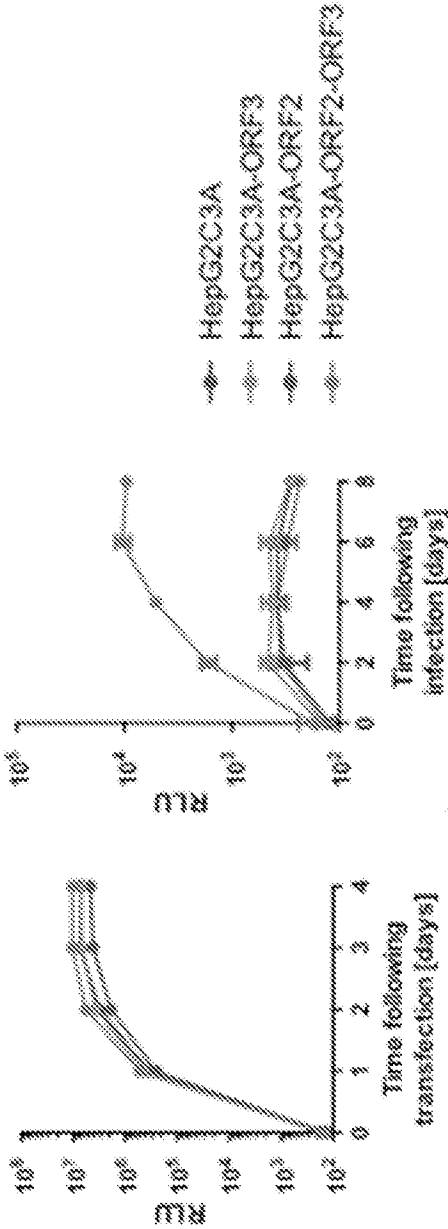


FIG. 11

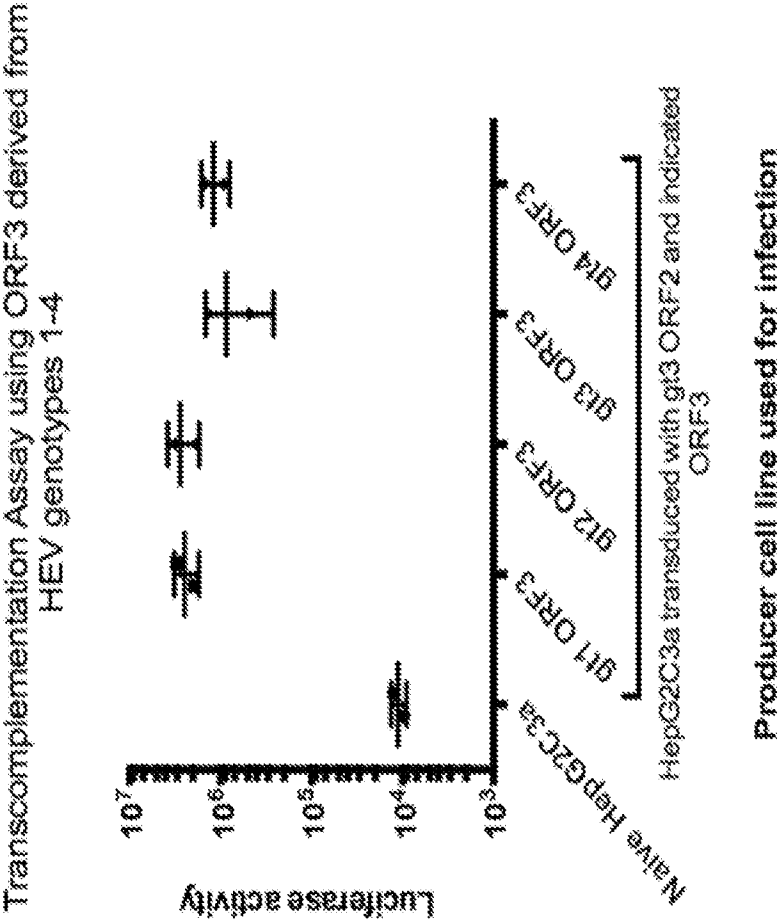


FIG. 12A

compound	chemical	target
amantadine	1-adamantylamine	JAV M2, HCV p7, DENV M
rimantadine	1-[(1-adamantyl)ethanamine]	JAV M2, HCV p7
VN-NDN1	N-nonyl-decynolirmycin	HCV p7
VN-DG1	N-nonyl-decylgalactonolirmycin	HCV p7
HMA	5-(N,N-hexamethylene)amilofide	HCV p7, SARS CoV E, DENV E, HIV Vpu
Emodin	6-methyl-1,3,8-trihydroxyanthraquinone (RS)-2-(3,4-dimethoxyphenyl)-5-[(2-(3,4-dimethoxyphenyl)ethyl)-(methylamino)-2-prop-2-yl]pentanenitrile	SARS CoV 3a
Verapamil	5-pentanenitrile	CSFV p7

FIG. 12B

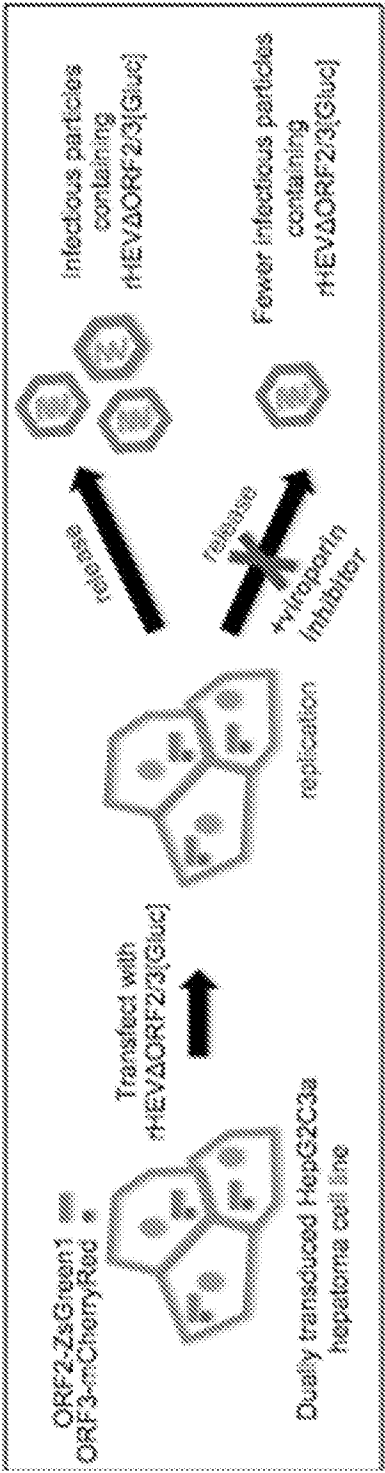


FIG. 12C

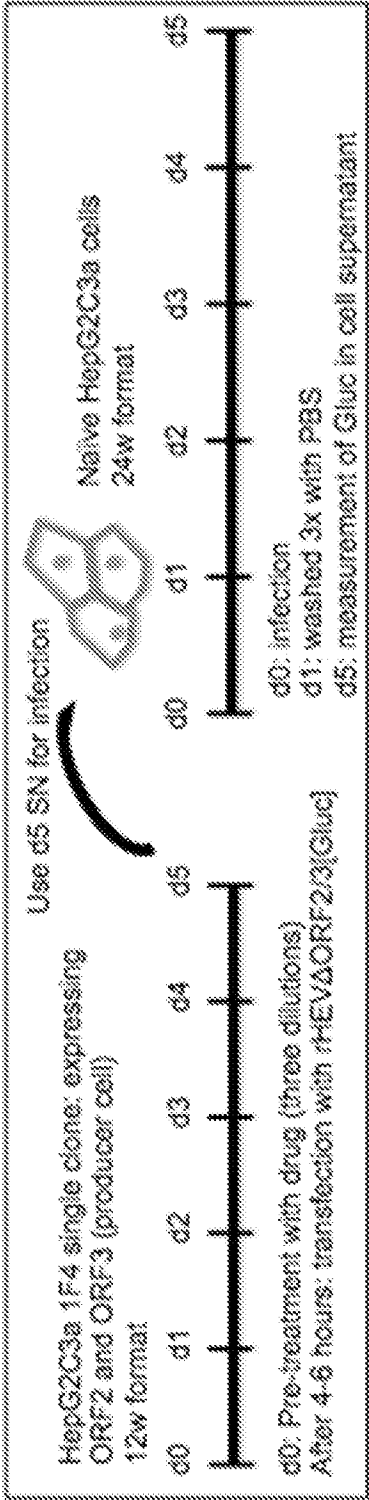


FIG. 13B

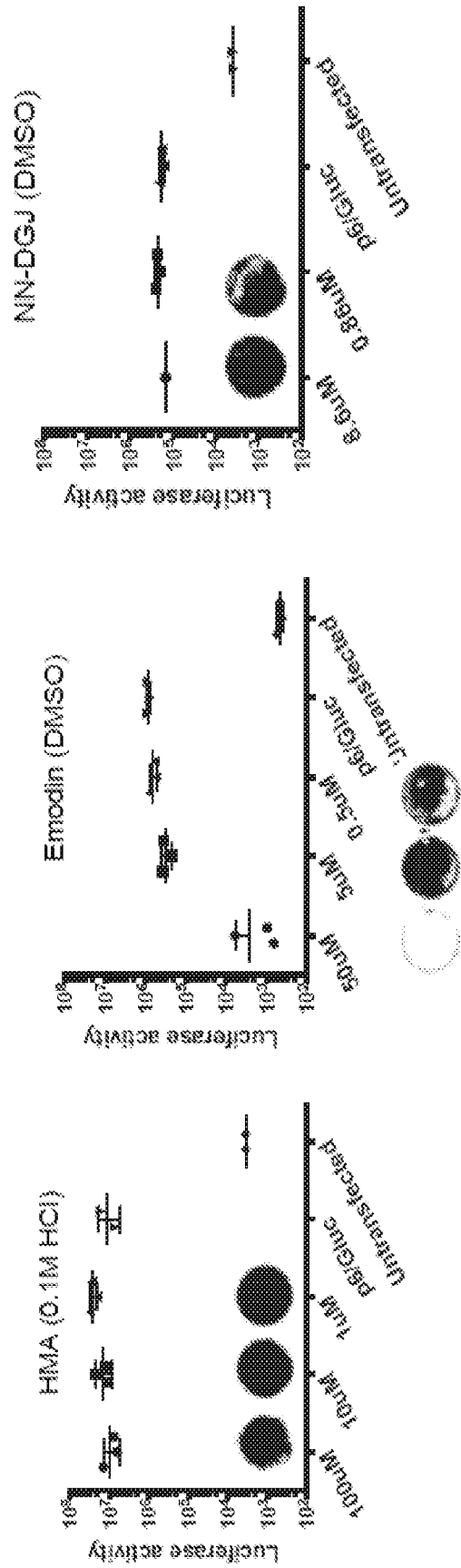
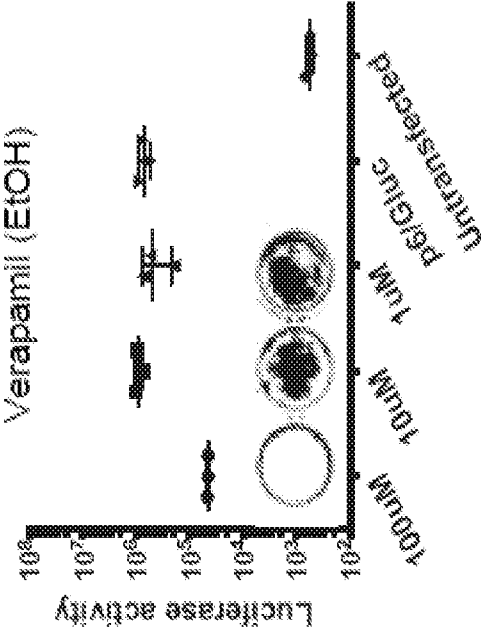


FIG. 13C



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/052769

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50 C12Q1/70 A61K38/00 A61K31/00 A61K39/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	QIANG DING ET AL: "Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles", PROCEEDINGS NATIONAL ACADEMY OF SCIENCES PNAS, vol. 114, no. 5, 17 January 2017 (2017-01-17), pages 1147-1152, XP055433829, US ISSN: 0027-8424, DOI: 10.1073/pnas.1614955114 the whole document ----- -/-	1-37



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 December 2017

Date of mailing of the international search report

02/03/2018

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Fayos, Cécile

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/052769

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCOTT P KENNEY ET AL: "Therapeutic targets for the treatment of hepatitis E virus infection", EXPERT OPINION ON THERAPEUTIC TARGETS, vol. 19, no. 9, 13 June 2015 (2015-06-13), pages 1245-1260, XP055433967, UK ISSN: 1472-8222, DOI: 10.1517/14728222.2015.1056155	1,2,12,13
Y	the whole document	1-37
X	K. YAMADA ET AL: "ORF3 protein of hepatitis E virus is essential for virion release from infected cells", JOURNAL OF GENERAL VIROLOGY, vol. 90, no. 8, 1 April 2009 (2009-04-01), pages 1880-1891, XP055433986, GB ISSN: 0022-1317, DOI: 10.1099/vir.0.010561-0	1,2
Y	the whole document	1-37
X	KENNEY SCOTT P ET AL: "Replacement of the hepatitis E virus ORF3 protein PxxP motif with heterologous late domain motifs affects virus release via interaction with TSG101", VIROLOGY, vol. 486, 2015, pages 198-208, XP029327219, ISSN: 0042-6822, DOI: 10.1016/J.VIROL.2015.09.012	1,2
Y	the whole document	1-37
X	TAO LIU ET AL: "RNA Interference Induces Effective Inhibition of mRNA Accumulation and Protein Expression of SHEV ORF3 Gene In vitro", CURRENT MICROBIOLOGY, SPRINGER-VERLAG, NE, vol. 62, no. 5, 12 January 2011 (2011-01-12), pages 1355-1362, XP019892668, ISSN: 1432-0991, DOI: 10.1007/S00284-010-9863-3	1,2,8,10,11
Y	the whole document	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/052769

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-24, 36, 37(completely); 25-35(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-24, 36, 37(completely); 25-35(partially)

A method of inhibiting hepatitis E virus (HEV) release from a cell that is infected with an HEV or a method of treating a hepatitis E virus (HEV) infection in a subject in need thereof, comprising contacting the cell with/ administering to the subject an agent that inhibits one or more activities of HEV open reading frame 3 (ORF3) protein, wherein the one or more ORF3 activities are selected from ion channel activity, multimerization activity, or viroporin activity and a method of identifying an agent that inhibits ORF3 protein activity and inhibits hepatitis E virus (HEV) infectivity.

2. claims: 25-35(partially)

A method of identifying an agent that inhibits ORF1 protein activity and inhibits hepatitis E virus (HEV) infectivity.

3. claims: 25-35(partially)

A method of identifying an agent that inhibits ORF2 protein activity and inhibits hepatitis E virus (HEV) infectivity.
