

MOLECULAR SYSTEMS AND THERAPIES USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore provisional application no. 10202002623W, filed 20 March 2020, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of molecular biology. In particular, the present invention relates to the gene editing technology for the treatment of diseases.

BACKGROUND OF THE INVENTION

[0003] RNA viruses comprise of a significant group of pathogens that pose a constant health threat and which can lead to global outbreaks and pandemics. An example is seen in the recent SARS-CoV-2 global pandemic which had brought recession in many of the world economies and restricted global human movement to a minimum, hence it is essential that we continue to explore new therapeutics options to treat RNA viruses to avoid the same situation in the future. The recent SARS-CoV-2 pandemic has also shown that conventional therapeutics such as antibodies treatment are ineffective in a global pandemic due to bottlenecks in manufacturing and distribution of such therapeutics and drug therapeutics have shown limited efficacy and usually with undesired side effects.

[0004] Enterovirus 71 (EV71), Coxsackievirus (CAV16 and CAV6) and Parechovirus are RNA viruses that are highly contagious and spread through bodily fluids. EV71 infection is most common in children younger than 5 years of age, with about 50-80% of children tested seropositive for EV71, and has also observed in adults, albeit to a lesser extent. Because of the symptoms associated with the infection, EV71 is considered to be a major contributor to the disease known as hand, foot and mouth disease (HFMD). The infection may occasionally result in severe neurological diseases or death. To date, there is no commercially available vaccine or therapeutics for the prevention or elimination of, for example, EV71 infection. Clinical trials are ongoing only for vaccine modalities and not therapeutic modalities. In recent years, EV71 infections have reached record incidence rates in several countries such as but not limited to Singapore, Malaysia and China, and this highlights a pressing need to develop vaccines and therapeutics against the currently incurable infections.

[0005] Despite many years of research into the potential of using human intravenous immunoglobulin (IV Ig) and monoclonal antibodies (mAb) as therapeutics, progress has been hampered by concerns and risks of, among others, antibody-dependent enhancements (ADE) or antibody-dependent cell-mediated cytotoxicity (ADCC), as well as technical barriers to humanizing monoclonal antibodies and risk of other pathogen spreading through the use of human intravenous immunoglobulin.

[0006] There are currently no commercial vaccines or therapeutics available capable of preventing or treating the diseases disclosed herein. Maintaining good personal hygiene and quarantined of infected individual have been the only measures taken by the public health sector. Thus, there is an unmet need for systems, compositions and methods of detecting and treating the viral diseases disclosed herein.

SUMMARY OF THE INVENTION

[0007] In one aspect, the present disclosure refers to a molecular system comprising (a) an RNA-guided RNA-targeting effector protein, and (b) one or more guide RNA molecule (gRNA); wherein each of the one or more guide RNAs comprise a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to one of the sequences set forth in SEQ ID NO: 1 to 6.

[0008] In another aspect, the present disclosure refers to a molecular system comprising (a) an RNA-guided RNA-targeting effector protein, and (b) a collection of at least 4 different guide RNA molecules (gRNAs); wherein at least 4 of the gRNAs in said collection comprise a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to one of the sequences set forth in SEQ ID NO: 1 to 6.

[0009] In yet another aspect, the present disclosure refers to a method of treating, preventing, suppressing, and/or alleviating a disease associated with or caused by infection, propagation and/or replication of an RNA virus in a subject, comprising administering to a subject in need thereof a molecular system comprising (a) an RNA-guided RNA-targeting effector protein and/or a polynucleic acid encoding said effector protein, and (b) one or more gRNAs and/or one or more polynucleic acids encoding said one or more gRNAs; wherein each of the one or more gRNAs comprises a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95% or 100% identical to one of the sequences set forth in SEQ ID NO: 1-6.

[0010] In a further aspect, the present disclosure refers to a method of treating, preventing, suppressing, and/or alleviating a disease associated with or caused by infection, propagation and/or replication of an RNA virus in a subject, comprising administering to a subject in need

thereof a molecular system comprising (a) an RNA-guided RNA-targeting effector protein and/or a polynucleic acid encoding said effector protein, and (b) a collection of at least 4 different gRNAs and/or one or more polynucleic acids encoding said collection of gRNAs; wherein at least 4 of the gRNAs in said collection comprise a guide sequence that is at least 70%,
5 at least 80%, at least 90%, at least 95% or 100% identical to one of the sequences set forth in SEQ ID NO: 1-6.

[0011] In one aspect, the present disclosure refers to polynucleotides encoding the molecular system as disclosed herein.

[0012] In another aspect, the present disclosure refers to a vector encoding one or more
10 gRNAs, wherein the one or more gRNAs encoded by said vector comprise a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to one of the sequences set forth in SEQ ID NO: 1-6.

[0013] In yet another aspect, the present disclosure refers to a vector encoding the molecular system as disclosed herein.

15 [0014] In one aspect, the present disclosure refers to a composition comprising the polynucleotide as disclosed herein, and/or the vector as disclosed herein.

[0015] In a further aspect, the present disclosure refers to a method of treating, preventing, suppressing, and/or alleviating a disease associated with or caused by pathogenesis, infection, propagation and/or replication of a virus of the Enterovirus genus in a subject, comprising
20 administering to a subject in need thereof the polynucleotides as disclosed herein, and/or the vector as disclosed herein, and/or the composition as disclosed herein.

[0016] In one aspect, the present disclosure refers to a guide RNA molecule (gRNA), comprising a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to any one of the sequences set forth in SEQ ID NO: 1-6.

25 [0017] In another aspect, the present disclosure refers to a guide RNA molecule comprising a guide sequence as set forth in any one of SEQ NO: 1-6.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] The invention will be better understood with reference to the detailed description
30 when considered in conjunction with the non-limiting examples and the accompanying drawings, in which

[0019] **Fig. 1** shows a schematic showing the design and construction of plasmid for AAV packaging of CRISPR-CasRx. Plasmid of pZac2.1-CMV-CasRx-3xHA-PolyA-U6-gRNA. Between the ITR sequences, there is a CMV promoter which drives the expression of CasRx

sequence and there is a 3xHA tag at the end of the CasRx sequence followed by a rabbit polyadenylation sequence. This is followed by the U6 promoter which drives the transcription of the inserted guide sequences followed by another ITR sequence. Guide sequences can be cloned into this backbone using the BbsI restriction enzyme digestion. Sequence of full plasmid and primers for cloning plasmids are shown in Table 1.

[0020] **Fig. 2** shows the results of validation experiments showing the expression of CasRx in AAV-CasRx transduced human cells. To validate that the packaged viruses are able to transduce mammalian cells and express the CasRx cargo, 1×10^5 human immortalized corneal endothelial cells, B4G12, were transduced and plated in a 48-well plate at MOI 100K. Anti-HA staining is carried out at 1:200 for 2 hours and anti-GFP at 1:1500 for 2 hours. Secondary antibodies staining is carried out at 1:1000 for 2 hours. Images are taken at exposure 15 milliseconds and gain set at 6400 for bright field and the 488 channel, exposure is set at 800us and gain at 6400 for the DAPI channel. The results showed that AAVDJ-eGFP is able to transduce B4G12 cells and specific signal of eGFP is detected compared to no transduction control (Fig. 2, row 1 and 2 respectively). The results also confirmed the expression of the CasRx-3xHA protein in the AAVDJ-CMV-CasRx transduced human B4G12 cells (Fig. 2, third row) and that the signal for both the primary and secondary antibodies are specific as shown by the low background signal in the non-infected control (Fig 2, fourth row).

[0021] **Fig. 3** shows an alignment of the position of the guides designed against the GFP gene sequence. Two RNA guides were designed for target GFP gene sequence closer to the 5' end, with the logic that cleavage at the earlier bases would terminate translation more efficiently since protein translation starts from the 5' end (Fig. 3). GFP knockdown will be performed using these guides to validate the RNA cleavage activity of CasRx. Primers for guides cloning are shown in Table 1. GFP guide1 sequence –gtgaacagctcctcgcccttgc (SEQ ID NO: 7); GFP guide2 sequence – gctgaacttggtgccggtttac (SEQ ID NO: 8).

[0022] **Fig. 4** shows an alignment of the position of the guides designed against the EV71 3D protein gene sequence. Six RNA guides were designed for target EV71 3Dpro gene sequence based on highly conserved regions (>85% similarity) from the alignment of six EV71 strains (H8-1, S41, Sin002209, MZ, NJ2017iso2, Shenzhen001-2006) using Benchling Clustal Omega alignment and Lasergene Seqman Pro 15 (DNASTar). Guides sequence and positions with respect to strain41 3Dpro gene sequence are shown in the figure above. Primers for cloning guides are shown in Table 1. Alignments are shown in Figs. 10 to 12. EV71_3D guide1 sequence - gttggtccattgatgttttagtct (SEQ ID NO: 1); EV71_3D guide2 sequence – ttgaaaaacagggtgttcaaaa (SEQ ID NO: 2); EV71_3D guide3 sequence – tagcacgttcctccatgetcat (SEQ ID NO: 3);

EV71_3D guide4 sequence – tatttatccatgtagaatttcatt (SEQ ID NO: 4); EV71_3D guide5 sequence – atgatgttggtgatcattgaatt (SEQ ID NO: 5); EV71_3D guide6 sequence – tctgcaggagtcattggtcaaacatactc (SEQ ID NO: 6).

[0023] **Fig. 5** shows the results of a biopanning of AAV serotypes to identify AAV that efficiently transduce human rhabdomyosarcoma (RD) cells. The human skeletal muscle was proposed to be a target organ that supports EV71 persistent infection and replication. Using the human rhabdomyosarcoma (RD) cell line (ATCC), an AAV panel biopanning experiment was performed to determine the most suitable AAV serotype(s) that can efficiently transduce human muscle cells to deliver the CRISPR-Cas tools for viral RNA targeting. 10K cells were plated into each well of a 96-well plate and transduced with each serotype of AAV at MOI of 100K and the expression of GFP protein was quantitated at 72 hours post- transduction. Experiments were performed in duplicates. The result showed that AAV1, AAV2 and AAVDJ can transduce human rhabdomyosarcoma (RD) cells more efficiently than other AAV serotypes tested, in the order of increasing efficiency as follow, AAV1, AAVDJ, and AAV2 respectively.

[0024] **Fig. 6** shows results of the validation of knockdown of GFP expression in AAV-CasRx-GFPguides transduced human rhabdomyosarcoma (RD) cells. Human muscle immortalized cell line, RD, were transduced with AAV2-GFP at MOI 10K for expression of GFP. The GFP knockdown efficiency of guide1 only, guide2 only and guide1+guide2 are tested by transduction of the GFP-expressing rhabdomyosarcoma (RD) cells with AAV2-CasRx bearing the guides at MOI 100K (left figure) and MOI 1000K (right figure). The result showed that the knockdown of GFP by individual guide is present but efficiency of knockdown is increased by pooling the two guides. Comparison between two groups was analysed by Student t test (two-tailed) using the software Prism 8. * p<0.05.

[0025] **Fig. 7** shows the results of the inhibition of EV71 replication in AAV-CasRx-EV71_3Dguides transduced EV71-infected human rhabdomyosarcoma (RD) cells. Proof-of-concept experiment for the ability of AAV-CRISPR-CasRx-EV71_3Dguides to inhibit EV71 viral replication. In a 96-well plate, 10×10^3 rhabdomyosarcoma (RD) cells were seeded in each well and transduced with AAVDJ-eGFP with no guides or AAVDJ-CasRx bearing either GFP guide2 or EV71 guide(s) for 72 hours and then subjected to EV71 infection at MOI of 1 for 12h, the supernatant is then harvested for virus plaque assay. The result from the plaque assay counts suggest that individual guide have little inhibitory effect at MOI 100K but pooling the guides (six guides) together can have a strong inhibitory effect on EV71 replication of up to 3 logs of magnitude of virus titre reduction at AAV MOI of 100K. The control using no guides or GFP guide also suggest that the inhibitory effect is specific to the EV71 guides. Comparison between

two groups (CasRx-eGFPg2 and CasRx-EV71_3Dguide(s)) was analysed by Student t test (two-tailed) using the software Prism 8. * $p < 0.05$.

[0026] **Fig. 8** shows the results of inhibition of EV71 replication in EV71-infected human rhabdomyosarcoma (RD) cells transduced with different number of CasRx EV71 3Dpro gene targeted guides. Proof-of-concept experiment for the ability of AAV-CRISPR-CasRx-EV71_3Dguides to inhibit EV71 viral replication. In a 96 well plate, 10×10^3 rhabdomyosarcoma (RD) cells were seeded in each well and transduced with AAV2-eGFP with no guides or AAV2-CasRx bearing either GFP guide2 or EV71 guide(s) for 72 hrs and then subjected to EV71 infection at MOI of 1 for 12h, the supernatant is then harvested for virus plaque assay. The result from the plaque assay counts suggest that all six individual guide have strong inhibitory effect of up to 1 log virus titre reduction at MOI 1000K but pooling the guides gradually together can have an increasing inhibitory effect on EV71 replication of up to 4 logs of magnitude of virus titre reduction at AAV2 MOI of 1000K for the 6 guides pool. The exponential increase in inhibitory effect is stronger in the 4, 5 and 6 guides pool, suggesting either a critical number of guides targets or a synergy between the guides within the pool are important to achieve higher inhibitory efficacy. The control using no guides or GFP guide2 also suggest that the inhibitory effect is specific to the EV71 guides. Comparison between two groups (CasRx-eGFPg2 and CasRx-EV71_3Dguide(s)) was analysed by Student t test (two-tailed) using the software Prism 8. * $p < 0.05$.

[0027] **Fig. 9** shows a schematic of the technology disclosed herein, based on the example of EV71 RNA. In the first step, CRISPR-CasRX components delivered into target cells (in one embodiment, via adeno-associated viruses), resulting in the expression of CasRX and guide RNA(s) in EV71-infected cells. In a next step, the combination of a guide RNA with CasRX results in cleavage of, for example, the EV71 RNA genome to disrupt replication and viral functions, as well as cleavage of, for example, EV71 mRNA (e.g. 3Dpol gene) to disrupt replication and viral functions. It is noted that a multitude of CRISPR-Cas-gRNA complexes can be used within the same cell and/or on the same viral RNA molecule, despite only one exemplary CRISPR complex shown here for clarity.

[0028] **Fig. 10** shows the alignment sequences of strain H8-1 to other Enterovirus strains. The sequences shown in Fig. 10 are present in Table 1.

[0029] **Fig. 11** shows the alignment sequences of LC126150, CAU05876, and an untitled consensus. The sequences shown in Fig. 11 are present in Table 1.

[0030] **Fig. 12** shows the alignment sequences of an Echovirus (Parechovirus) consensus sequence strain to other Echovirus (Parechovirus) sequences. The sequences shown in Fig. 12 are present in Table 1.

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DEFINITIONS

[0031] As used herein, the term “biopanning” refers to the identification or selection of the best-performing AAV serotype(s) amongst a larger library of different AAV serotypes.

[0032] As used herein, the term “RNA-guided RNA-targeting effector protein” refers to a protein which is capable of forming a RNA-targeting complex with a guide RNA or gRNA, said
10 complex is capable of binding with and effecting changes on one or more RNA target molecules. The changes effected on the RNA targets refer to any chemical or physical changes to the components or structure of the RNA molecule, which may include but are not limited to: breaking/cleaving the polynucleotide, substituting one or more nucleotide bases, and inserting or deleting one or more nucleotide bases. In one example, the RNA-guided RNA-targeting effector
15 protein is a Cas protein. In another example, the Cas protein is a Cas nuclease.

[0033] The term “guide RNA” or “gRNA” refers to an RNA molecule comprising a sequence (guide sequence) sufficient complementarity with a target RNA sequence to hybridize with the target RNA sequence and direct sequence-specific binding of an RNA-targeting complex to the target RNA sequence. In some embodiments, the degree of complementarity between a guide
20 sequence (within a guide RNA) and its corresponding target RNA sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 60%, 70%, 80%, 85%, 90%, 95%, 97.5%, 99%, or 100%.

[0034] As used herein, the term “CRISPR-Cas” refers to genome editing technology based on the capability of clustered regularly interspaced palindromic repeats (CRISPR) and, for example,
25 the CRISPR-associated protein nuclease to induce, for example, double-strand (ds) DNA breaks in a specific location that is complementary to the synthetic guide RNA (sgRNA) sequence integrated into the CRISPR-Cas complex/system. This allows the deletion, addition, and/or modification of genes and/or other genomic elements, such as, but not limited to, transcription elements, promoters, promoter enhancers, transcription enhancers, restriction sites, mutations,
30 selection markers, for example antibiotic selection cassettes, and the like. Such nuclease can be isolated from, for example, *Streptococcus pyogenes* (in the case of Cas9).

[0035] The functions of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes are essential in adaptive immunity in select bacteria and archaea, enabling the organisms to respond to and eliminate invading genetic

material. Three types of CRISPR mechanisms have been identified so far, of which type II is the most well studied. Also, other combinations of CRISPR, for example CRISPR-Cpf1 have been developed. Also contemplated herein is the use of CRISPR technologies, wherein the Cas proteins or functionally analogue proteins are not isolated from *S. pyogenes*. Examples of Cas proteins are, but are not limited to, Cas nucleases (for example Cas9 and Cas13 proteins), or proteins with the same functionality, isolated from *S. pyogenes*, *Staphylococcus aureus*, or any representatives of the archaea kingdom. Cas9 proteins can also be substituted with so-called CasX and CasY proteins. In another example, examples of Cpf1 proteins, or proteins with the same functionality are isolated from, but are not limited to, *Acidaminococcus sp.* and *Lachnospiraceae*. In terms of adaptive immunity, the mechanism of CRISPR-Cas-mediated defence is as follows: invading DNA from viruses or plasmids is cut into small fragments and incorporated into a CRISPR locus amidst a series of short repeats (around 20 base pairs). The loci are transcribed, and transcripts are then processed to generate small RNAs (crRNA – CRISPR RNA; also referred to as synthetic guide RNA (sgRNA) in an *in vitro* setting), which are used to guide effector endonucleases that target invading DNA based on sequence complementarity. In terms of gene editing, the CRISPR-Cas system works according to the same principle, with the sgRNA guiding the effector nucleases to the desired sections of the DNA, in which the excision is to be made.

[0036] The term “vector” as used herein refers to a macromolecule or association of macromolecules that comprises or associates with a polynucleotide and which can be used to mediate delivery of the polynucleotide to a cell. Illustrative vectors include, for example, plasmids, viral vectors (virus or the viral genome thereof), liposomes, and other gene delivery vehicles.

[0037] As used herein, the term “AAV” refers to an Adeno-associated virus. The term AAV may be used to refer to the virus itself, or derivatives thereof, for example, but not limited to, the viral capsid, the viral genome, viral particles, viral fragments and combinations thereof. The term “AAV” encompasses all subtypes, both naturally occurring and recombinant forms, and variants thereof except where required otherwise. Naturally occurring forms of AAV refer to any adeno-associated virus or derivative thereof comprising a viral capsid that consists of viral capsid proteins that occur in nature. Non-limiting examples of naturally occurring AAV include AAV type 1 (AAV-1), AAV type 2 (AAV-2), AAV type 3 (AAV-3), AAV type 4 (AAV-4), AAV type 5 (AAV-5), AAV type 6 (AAV-6), AAV type 7 (AAV-7), AAV type 8 (AAV-8), AAV9, AAV10, AAV11, AAV12, AAV13, rh10, avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV. “Primate AAV” refers to AAV that infect

primates, “non-primate AAV” refers to AAV that infect non-primate mammals, “bovine AAV” refers to AAV that infect bovine mammals, etc. “Recombinant AAV”, or “rAAV” includes any AAV that comprises a heterologous polynucleotide sequence in its viral genome. Other examples of AAV serotypes and variants useful for use as vectors include, but are not limited to, AAVDJ, 5 AAV-PHP.S, AAV-PHP.B, AAV-PHP.eB, and Anc80.

[0038] As used herein, the term “Enterovirus 71” (EV71), also known as Enterovirus A71 (EV-A71), refers to a virus of the genus Enterovirus in the *Picornaviridae* family, notable for its role in causing epidemics of severe neurological disease and hand, foot, and mouth disease (HFMD) in children. Enterovirus 71 has also been reported to infrequently cause polio-like 10 syndrome permanent paralysis.

[0039] As used herein, the term “Coxsackievirus” refers to a small number of related enteroviruses that belong to the *Picornaviridae* family of non-enveloped, linear, positive-sense single-stranded RNA viruses, as well as its genus Enterovirus, which also includes poliovirus and echovirus. Enteroviruses are among the most common human pathogens. Ordinarily, its 15 members are transmitted by the faecal-oral route. Coxsackieviruses share many characteristics with poliovirus. Coxsackieviruses are also known to be among the leading causes of aseptic meningitis, along with echovirus and mumps virus.

[0040] As used herein, the term “Parechovirus”, also referenced as “Echovirus”, refers to a polyphyletic group of viruses associated with enteric disease in humans. The name is derived 20 from “enteric cytopathic human orphan virus”. These viruses were originally not associated with disease but many have since been identified as disease-causing agents. The term “echovirus” was used in the scientific names of numerous species. Thus, the term “Echovirus” has been replaced with the term “Parechovirus” to denote viruses belonging to this sub-species of *Picornaviridae* family, while other viruses previously considered to be Echoviruses have been reassigned to 25 other species, also within the *Picornaviridae* family. Nevertheless, all echoviruses are now recognized as strains of various species, most, if not all, of which are in the *Picornaviridae* family. It is noted that the terms “Echovirus” and “Parechovirus” have been used interchangeably herein. Examples, of a Parechovirus are, but are not limited to, Parechovirus A, Parechovirus B, Parechovirus C, Parechovirus D, Parechovirus E, and Parechovirus F.

30 [0041] As used herein, the term “hand, foot, and mouth disease” (HFMD) refers to a febrile disorder usually caused by Coxsackievirus A16, Enterovirus 71, or other enteroviruses. Infection causes a vesicular eruption on the hands, feet, and oral mucosa. Atypical HFMD due to Coxsackievirus A6 often causes high fever with papulovesicular lesions progressing to vesicobullous lesions and bullae that are widely distributed on the body. Viruses causing HFMD

are of the *Picornaviridae* family, with Coxsackievirus A16 is the most common cause of HFMD. Enterovirus 71 (EV-71) is the second-most common cause. Many other strains of Coxsackievirus and enterovirus can also be responsible.

[0042] As used herein, the term “transduction or “transduced” refers to a vector-mediated gene transfer procedure, which is usually used to describe both bacteria and/or virus-mediated methods.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0043] Enterovirus 71, Coxsackie CAV16 and CAV6, and Parechoviruses are viruses belonging to the *Picornaviridae* family, which by definition consists of non-enveloped, positive, single-stranded RNA viruses. These viruses are most well-known for their manifestation in young children as hand, foot and mouth disease (HFMD), presenting with blisters or lesions observable in the oral cavity, and with lesions or blisters occasionally occurring on the palms of the hand and the soles of the feet. In more severe cases, hand, foot and mouth disease also presents with more severe symptoms, such as but not limited to, paralysis, aseptic meningitis and encephalitis or even death.

[0044] There are currently no commercial vaccines or therapeutics available which are capable of preventing or treating the diseases disclosed herein. Maintaining good personal hygiene and quarantined of infected individual have been the only measures taken by the public health sector.

[0045] Thus, disclosed herein is a method of treating, preventing, suppressing, and/or alleviating a disease associated with or caused by pathogenesis, infection, propagation and/or replication of a virus of the Enterovirus genus in a subject. In another example, the method disclosed herein comprises administration to a subject in need thereof the polynucleotides of as disclosed herein, and/or the vector as disclosed herein, and/or the composition as disclosed herein.

[0046] CRISPR-Cas presents itself as an alternative in antiviral therapeutics development. CRISPR stands for clustered regularly interspaced short palindromic repeats, and was first discovered in the bacterial immune system. However, it was not until 2012 that the CRISPR-Cas system was found to have the capability of precisely editing DNA in eukaryotes. Before the emergence of CRISPR, research efforts had been focused on mainly the development of humanized monoclonal antibodies targeting neutralizing epitopes as antivirals since the success story of Herceptin, an anti-HER2 humanized monoclonal antibody used for the treatment of breast cancer. Traditional drug screenings to short-list for compounds or small molecules are

also popular approaches for therapeutics development; however, these screening methods often have an inhibitory effect on viral replication. Despite drug screening and monoclonal antibodies studies, there are currently no prospective ongoing clinical trials nor any commercialized therapeutics for HFMD treatment.

5 [0047] The use of CRISPR-Cas has been limited by an efficient delivery system *in vivo*, and current FDA approved drugs were based on adeno-associated virus (AAV) delivery, which is considered safe for human applications as it does not elicit an acute inflammatory immune response. However, the maximum cargo size of adeno-associated virus is roughly 4.7 kilo base pairs (kbp), which is insufficient for the delivery of most known Cas enzymes, as these usually
10 exceed the stated limit, especially after cloning in the mammalian promoter sequence.

[0048] Thus, the molecular systems disclosed herein can be comprised or encoded by a polynucleic acid encoding said effector protein and/or the one or more polynucleic acids encoding said gRNAs are comprised in one or more vectors. As used herein, the term “vector” refers to any molecule (for example, but not limited to, nucleic acid, plasmid, or virus) used to
15 transfer coding information into a host cell.

[0049] As used in the art, viral vectors are tools used to deliver genetic material into cells. This process can be performed inside a living organism (*in vivo*) or in cell culture (*in vitro*). Specifically, viruses have evolved specialized molecular mechanisms to efficiently transport their genomes inside the cells they infect. Delivery of genes or other genetic materia by a vector
20 is termed transduction and the infected cells are described as transduced. In addition to their use in molecular biology research, viral vectors are used for gene therapy and the development of vaccines.

[0050] Thus, in one example, the vector is a viral vector. In another example, the viral vector is, but is not limited to, an adenoviral vector, an Adeno-associated virus (AAV) vector, a
25 lentiviral vector, or a retroviral vector.

[0051] By way of an example of a vector used in the presently disclosed application, the adeno-associated virus (AAV) is a small virus that infects humans and some other primate species. The adeno-associated virus is not currently known to cause disease, and upon infection, has been shown to cause only a mild immune response. The adeno-associated virus is capable of
30 infecting both dividing and non-dividing cells and may incorporate its genome into that of the host cell. Moreover, the adeno-associated virus mostly stays as episomal (that is to say, it can replicate within a host without incorporation of its payload into the host chromosome); performing long and stable expression. These features make the adeno-associated virus a suitable candidate for creating viral vectors for gene therapy. Thus in one example, the viral vector is an

adeno-associated virus (AAV) vector. In another example, the adeno-associated virus (AAV) vector is but is not limited to, AAV type 1 (AAV-1), AAV type 2 (AAV-2), AAV type 3 (AAV-3), AAV type 4 (AAV-4), AAV type 5 (AAV-5), AAV type 6 (AAV-6), AAV type 7 (AAV-7), AAV type 8 (AAV-8), AAV9, AAV10, AAV11, AAV12, AAV13, rh10, AAVDJ, AAV-PHP.S, AAV-PHP.B, AAV-PHP.eB, and Anc80. In one example, the adeno-associated virus (AAV) vector is an AAV2, or AAVDJ, or AAV1 vector.

[0052] In one example, there is disclosed a vector encoding one or more gRNAs as disclosed herein. In such an example, the one or more gRNAs encoded by said vector comprise a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to one of the sequences set forth in SEQ ID NO: 1-6. In another example, the vector encodes at least 4 different gRNAs, wherein each of the gRNAs comprises a guide sequence that is set forth in SEQ ID NO: 1-6. In a further example, the vector encodes at least 6 different gRNAs, wherein each of the gRNAs comprises a guide sequence that is set forth in SEQ ID NO: 1-6.

[0053] Further disclosed herein are polynucleotides encoding the molecular system as disclosed herein, as well as polynucleotides encoding components required by the molecular system or composition disclosed herein.

[0054] Using bioinformatics to mine for Cas enzymes, it was previously found the CasRx, the smallest type VI CRISPR-Cas enzyme, has high RNA-guided RNA-targeting activity. In one example, the CRISPR-Cas modality used herein belongs to the Type VI-D Cas family which is a RNA-guided RNA-targeting Cas modality. This enables delivery of the CRISPR-Cas system into mammalian cells *in vivo*, via an adeno-associated virus (AAV) delivery system, which in turn allows for RNA targeting of the (foreign) viral RNA genome for cleavage.

[0055] Thus, in one example, the RNA-guided RNA-targeting effector protein is an RNA-guided RNA-targeting Cas protein, or a modified variant thereof. In another example, the method or molecular system disclosed herein comprises a RNA-guided RNA-targeting effector protein, wherein the RNA-guided RNA-targeting effector protein is an RNA-guided RNA-targeting Cas protein, or a modified variant thereof.

[0056] With regard to the safety profile of CRISPR-Cas technologies, these technologies have been proven to be safe for use in, for example, gene therapies and CRISPR-Cas therapeutics. This is also the result of higher specificity of CRISPR-Cas technologies compared to other gene editing methods known in the art. It is of note that any RNA editing resulting from type IV CRISPR-Cas used in the present disclosure is not permanent and will terminate once the exogenous DNA has degraded, or after the cell divides. The highly specific activity of the CRISPR-Cas therapeutics will also avoid common issues of “side-effects” from drug compound

or small molecules usage or antibody-dependent enhancement (ADE) or antibody-dependent cellular cytotoxicity (ADCC) issues as seen in antibody therapies.

[0057] It is of note that, in the art, a therapeutic drug that can precisely target for the pathogen genomic material for destruction has yet to be found. The application of the CRISPR-Cas in antiviral therapeutics shows its effect by eliminating viral replication by directly breaking down the viruses itself. Due to the direct-targeting mechanism, these therapeutics do not rely on the immune system to assist in eliminating the viruses, and can therefore be used with equal potency in patients that are immune-compromised or in young and aged patients with weaker immunity.

[0058] The guide RNAs (gRNAs) as disclosed herein are based on a highly conserved region with a similarity of at least 85% sequence identity between six Enterovirus 71 (EV71) strains (Fig. 4). The guide RNAs disclosed herein are capable of targeting multiple strains. This conservation of sequences further implies that these sequences are of functional importance to the viruses. Without being bound by theory, it is thought that less conserved sequences imply the ability of viruses strains to mutate at those (less conserved sites), thereby escaping CRISPR targeting. Thus, conserved regions of the circulating pathogenic strains are targeted by the (molecular) systems, compositions and uses of the compositions disclosed herein. Specifically, in one example, the target chose was the functionally important polymerase 3D gene.

[0059] Thus, disclosed herein is a molecular system based on the CRISPR-Cas system that is capable of targeting RNA. Such a molecular system comprises, for example, (a) an RNA-guided RNA-targeting effector protein, and (b) one or more guide RNA molecule (gRNA). The person skilled in the art would appreciate that the guide RNAs can be customised and tailored to the RNA target that the molecular system is to affect.

[0060] In one example, the molecular system disclosed herein is for use in treating a disease associated with or caused by the infection, propagation and/or replication of an RNA virus.

[0061] Also disclosed herein are specific guide RNAs (gRNAs) with defined sequences which have been empirically validated to have antiviral activity. In one example, the guide strains are as disclosed herein.

[0062] The term “sequence identity” means that two polynucleotide or amino acid sequences are identical (i.e., on a nucleotide-by-nucleotide or residue-by-residue basis) over the comparison window. The term “percentage of sequence identity” is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) or residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window (i.e., the window size), and multiplying the result

by 100 to yield the percentage of sequence identity. The terms “substantial identity” as used herein denotes a characteristic of a polynucleotide or amino acid sequence, wherein the polynucleotide or amino acid comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 18 nucleotide (6 amino acid) positions, frequently over a window of at least 24-48 nucleotide (8-16 amino acid) positions, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the comparison window. The reference sequence may be a subset of a larger sequence.

[0063] Thus, also disclosed herein is a guide RNA molecule (gRNA), comprising a guide sequence that is at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any one of the sequences set forth herein. In one example, the sequence identity of the guide RNA molecules is in comparison to the SEQ ID Nos 1-6 as disclosed herein. Also disclosed herein is a guide RNA molecule comprising a guide sequence as set forth in any one of SEQ NO: 1-6.

[0064] In another example, the one or more guide RNAs comprise a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to one of the sequences set forth in SEQ ID Nos 1 to 6. In another example, the guide RNAs are as set forth in SEQ ID Nos 1 to 6.

[0065] It is further disclosed in the scope of the present disclosure that multiple RNAs can be targeted by using a combination or collection of multiple guide RNA molecules, wherein the number of guide RNA molecules is the same as the number of RNA targets that the user wishes to target.

[0066] In one example, the molecular system disclosed herein comprises a collection of at least 4 different guide RNA molecules (gRNAs). This collection of guide RNAs are capable of targeting the same or different targets on the viral RNA, whereby the target is defined by the sequence of the guide RNA. In one example, at least 4 of the gRNAs in said collection comprise a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to one of the sequences set forth in SEQ ID NO: 1 to 6. In another example, the collection comprises at least 4 of the gRNAs disclosed herein, wherein the gRNAs comprise a guide

sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to one of the sequences set forth in SEQ ID NO: 1 to 4. In another example, the collection disclosed herein comprises at least 6 different gRNAs, wherein 6 of the gRNAs in said collection comprise a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95%, or 100% identical to one of the sequences set forth in SEQ ID NO: 1 to 6.

[0067] This disclosure describes a technology and strategy for targeting and eliminating foreign RNA, for example RNA viruses, using type VI CRISPR-Cas systems programmed to directly cleave the foreign RNA (for example, RNA viral genomes).

[0068] Also disclosed herein are (molecular) systems, compositions and uses of the compositions described herein in neutralising a virus infection. Furthermore, the (molecular) systems, compositions and uses of the compositions described herein and also be used to reduce viral replication and thus reduce viral titre in a subject.

[0069] It is understood that a subject being infected with a virus may not necessarily display symptoms of the disease that the virus is known to cause. Such subjects are known as viral carriers, and are usually asymptomatic. In such subject, the (molecular) systems, compositions and uses of the compositions described herein are used to reduce viral titres or suppress viral replication, thereby preventing manifestation of disease symptoms in the subject.

[0070] Also disclosed herein are (molecular) systems, compositions and uses of the compositions described herein in for the treatment and/or prevention of hand, foot, and mouth disease (HFMD). Also disclosed herein is the use of the disclosed composition and molecular systems in the reduction of the amount of viral RNA in the subject. This viral RNA can be, but is not limited to, viruses that cause hand, foot, and mouth disease (HFMD). In one example, the viruses causing hand, foot, and mouth disease (HFMD) is, but are not limited to, Enterovirus 71 (for example, EV71), Coxsackievirus (for example, CAV16 and CAV6), Parechovirus (formerly referred to as Echovirus, including, but not limited to Parechovirus A, Parechovirus B, Parechovirus C, Parechovirus D, Parechovirus E, and Parechovirus F), and combinations thereof.

[0071] The viral genome is deactivated by cleavage of the viral RNA genome using the CRISPR-Cas system as disclosed herein. . In another example, the Cas protein or nuclease is Cas13 or Cas13d. In one example, the Cas13d nuclease is isolated from *Ruminococcus flavefaciens* (CasRX). In another example, Cas13d is the Cas13d orthologue of *Ruminococcus flavefaciens*. The type VI CRISPR-Cas systems target only the RNA viral genomes without risk of undesired activity against the host DNA genome.

[0072] Thus, based on the disclosure herein, in one example, the wherein the RNA-guided RNA-targeting effector protein is an RNA-guided RNA-targeting Cas protein, or a modified

variant thereof. In another example, the Cas protein is but is not limited to, a Cas13a, a Cas13b, a Cas13c, and a Cas13d protein.

[0073] It is noted that in the context of the present disclosed, the claimed system would not function as intended with Cas9 as a nuclease, as Cas9 is a DNA-targeting modality. The application disclosed herein requires a RNA-targeting nuclease for direct targeting and cleavage of the viral genome. Conversely, DNA viruses (such as, for example, herpesviruses, poxviruses) can be targeted by Cas9, but not by Cas13. Thus, in one example, the Cas nuclease is one which targets RNA.

[0074] Also disclosed herein is a set of guide RNAs (gRNAs) against Enterovirus 71 (EV71), Cocksackievirus and Parechovirus (Echovirus) viral genomes. Use of these guide RNAs in the claimed system has been shown to eliminate the target viruses. This has been shown by the more than 1000x reduction of viral titres in infected cells, when compared to individual gRNAs that do not show antiviral activity when used individually. Exemplary data for the reduction of viral titres in infected cells is shown in Fig. 7, showing data based on the example of Enterovirus 71 (EV71).

[0075] In one example, the molecular system as disclosed herein comprises a Cas protein, wherein the Cas protein is a CasRx; and a collection comprises 6 different guide RNA molecules (gRNAs), wherein each of the gRNAs comprised in the collection is a guide sequence that is set forth in SEQ ID NO: 1 to 6.

[0076] The disclosure also describes compositions that function both as a prophylactic, that is to say, as compound that prevents primary infection, and as a therapeutic (meaning that the composition eliminates or reduces an existing infection).

[0077] In one example, there is disclosed a molecular system as disclosed herein for use in therapy. In another example, there is disclosed a method of treating, preventing, suppressing, and/or alleviating a disease associated with or caused by infection, propagation and/or replication of an RNA virus in a subject. Further disclosed herein is a composition as disclosed herein for use in therapy.

[0078] As disclosed herein, the methods of treatments disclosed herein can comprise administering to a subject in need thereof a molecular system comprising (a) an RNA-guided RNA-targeting effector protein and/or a polynucleic acid encoding said effector protein, and (b) one or more gRNAs and/or one or more polynucleic acids encoding said one or more gRNAs; wherein each of the one or more gRNAs comprises a guide sequence that is at least 70%, at least 80%, at least 90%, at least 95% or 100% identical to one of the sequences set forth in SEQ ID NO: 1-6. In another example, the methods of treatment disclosed herein can comprise

administering to a subject in need thereof a molecular system comprising (a) an RNA-guided RNA-targeting effector protein and/or a polynucleic acid encoding said effector protein, and (b) a collection of at least 4 different gRNAs and/or one or more polynucleic acids encoding said collection of gRNAs; wherein at least 4 of the gRNAs in said collection comprise a guide
5 sequence that is at least 70%, at least 80%, at least 90%, at least 95% or 100% identical to one of the sequences set forth in SEQ ID NO: 1-6.

[0079] The present disclosure also includes use of any one of the following in the manufacture of a medicament for treatment of a disease disclosed herein, the following being the molecular system, the vectors, the compositions, or the guide RNA molecules as disclose herein,
10 or combinations thereof.

[0080] In the context of this specification, the term “treatment”, refers to any and all uses which remedy a disease state or symptoms, prevent the establishment of disease, or otherwise prevent, hinder, retard, or reverse the progression of disease or other undesirable symptoms in any way whatsoever.

15 [0081] Thus, also disclosed herein is a composition comprising the polynucleotide as disclosed herein and/or the vector as disclosed herein. Such a composition as disclosed herein includes within its scope a pharmaceutically acceptable composition. The pharmaceutical composition is therefore suitable for use in the methods of treatment disclosed herein, as well as being suitable for use in the manufacture of a medicament as disclosed herein.

20 [0082] In one example, such a composition as disclosed herein further comprises an RNA-guided RNA-targeting effector protein. Such a guide effector protein can be chosen from the RNA-targeting effector proteins disclosed herein, such as for example, but not limited to Cas13a, Cas13b, Cas13c, and Cas13d.

[0083] In the context of this specification the terms “therapeutically effective amount” and
25 “diagnostically effective amount”, include within their meaning a sufficient but non-toxic amount of a compound or composition of the invention to provide the desired therapeutic or diagnostic effect. The exact amount required will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being treated, the particular agent being administered, the mode of
30 administration, and so forth. Thus, it is not possible to specify an exact “effective amount”. However, for any given case, an appropriate “effective amount” may be determined by one of ordinary skill in the art using only routine experimentation.

[0084] It is shown in the data disclosed herein that viral replication (of, for example, the viruses disclosed herein) is inhibited via direct targeting of the viral RNA genome for cleavage.

[0085] Disclosed herein is a DNA construct that comprising: a promoter that drives transgene expression in mammalian cells, as would be understood by a person skilled in the art; a transgene encoding an RNA-guided RNA-targeting protein present downstream of the promoter, such that the transgene is expressed into its encoded transcripts and proteins within a mammalian cell; a polyA sequence downstream of said transgene that terminates transcription, as would be understood by a person having ordinary skill in the art. In one example, the RNA-guided RNA-targeting protein is a Type VI CRISPR-Cas. In another example, the Cas nuclease is a Cas13. In yet another example, the Cas nuclease is Cas13d from *Ruminococcus flavefaciens* (CasRX).

[0086] Also disclosed herein is a second promoter that drives the gRNA expression in a mammalian system (such as, but not limited to, the U6 or H1 promoters). Further disclosed herein is a sequence downstream of the second promoter, encoding the gRNA that complexes with the expressed CRISPR-Cas protein and guides it to a defined RNA sequence in the RNA viral genome. In one example, the one or more of guide RNA (gRNA)-encoding sequences, result in the expression of one or more guide RNAs (gRNAs) within the same cell. This allows for the targeting of multiple, unrelated sites within the viral genome in one expression construct. In another example, the guide RNAs (gRNAs) are as disclosed in Table 1.

[0087] In one example, the vector comprises two AAV2 inverted terminal repeats (ITRs) that allow for the expressed DNA construct to be packaged into adeno-associated virus (AAV) particles. When transduced into mammalian cells, the packaged AAV viruses allow for the expression of the Cas proteins and gRNAs.

[0088] Also disclosed herein is a method of determining gRNA efficiency, as well as a method of selecting gRNAs against viral RNA. In one example, the viral RNA is RNA from, but not limited to, the EV71, Coxsackievirus and Parechovirus (Echovirus) genomes. In another example, the methods disclosed herein are suitable for antiviral applications, such as, but not limited to, treatment of and prophylaxis against hand, foot, and mouth disease (HFMD). This is done by targeting the viral genome of, for example, but not limited to, Enterovirus 71. In one example, targets within the EV71 genome include, but is not limited to, the non-coding sequences that include the 5'UTRs and 3'UTRs, and the coding sequences of 2Apro, 2BC, 2B, 2C, 3AB, 3A, 3B (VPg), 3CDpro, 3Cpro, 3Dpol, VP1, VP2, VP3 and VP4. The Genbank Accession number for the referenced gene sequence EV-A71 is AF316321.2.

[0089] This disclosure describes technology and compositions for targeting and eliminating RNA viruses. The system disclosed herein utilises type VI CRISPR-Cas systems programmed to directly cleave the RNA viral genomes. Other types of CRISPR-Cas systems can be used in a similar manner. The composition and use here provide for the treatment and/or prevention of

hand, foot, and mouth disease (HFMD); and/or reduction of Enterovirus 71 (EV71), Coxsackievirus (CAV16 and CAV6) and Parechovirus (Echovirus; Parechovirus A, Parechovirus B, Parechovirus C, Parechovirus D, Parechovirus E, and Parechovirus F), the viruses that cause hand, foot, and mouth disease (HFMD), in a subject, by cleavage of the RNA genome with the system disclosed herein. In one example, the system comprises CRISPR-Cas13. In another example, the Cas13d from *Ruminococcus flavefaciens* (CasRX). The type VI CRISPR-Cas systems target only the RNA viral genomes without risk of undesired activity against the host DNA genome.

[0090] The technology disclosed herein can be applied to any antiviral therapeutic application for RNA viruses by changing the guide sequences.

[0091] Thus, in one example, the target virus is an RNA virus. In one example, the RNA virus is a virus of the Picornaviridae family, which is a family of viruses comprising related non-enveloped RNA viruses which infect vertebrates, including mammals and birds. They are viruses that represent a large family of small, positive-sense, single-stranded RNA viruses with a 30-nm icosahedral capsid. The viruses of this family can cause a range of diseases including, but not limited to, the common cold, poliomyelitis, meningitis, hepatitis, and paralysis. Thus, in one example, the disease to be treated is, but is not limited to, polio; mild respiratory illness (the common cold); hand, foot, and mouth disease (HFMD); acute hemorrhagic conjunctivitis; aseptic meningitis; myocarditis; severe neonatal sepsis-like disease; acute flaccid paralysis; acute flaccid myelitis; Bornholm disease; epidemic pleurodynia; Herpangina; and chronic fatigue syndrome. In one example, the disease to be treated is hand, foot, and mouth disease (HFMD).

[0092] In another example, the RNA virus is a virus of the Enterovirus genus, which is a genus of positive-sense single-stranded RNA viruses associated with several human and mammalian diseases. Enteroviruses are named by their transmission-route through the intestine (enteric meaning intestinal). In another example, the molecular systems, methods of treatments, vectors, guide RNA molecules and CRISPR-Cas systems disclosed herein target at least one virus. The virus can be, but is not limited to Enterovirus, Coxsackie virus and Parechovirus. In another example, the virus can be, but is not limited to, Enterovirus 71, Coxsackie virus CAV16, Coxsackie virus CAV6, Parechovirus A, Parechovirus B, Parechovirus C, Parechovirus D, Parechovirus E, Parechovirus F, and combinations thereof.

[0093] Also contemplated in the present disclosure is the use of the described technology as a screening tool for comparative study of Cas enzyme proteins against RNA virus sequences.

[0094] The CRISPR-Cas therapeutics offer a new class of anti-viral therapeutics that can be easily manufactured on a large scale basis for distribution. The results shown herein show that by

targeting, for example, EV71 nucleic acid using crRNA, the RNA-targeting RNase CasRX would be able to significantly inhibit the replication of the viruses. This is a demonstration of AAV-packaged anti-viral CRISPR-Cas tools that are shown to work effectively against RNA viruses, based on the example of EV71, as shown in the data presented herewith. This approach
5 allows delivery of therapeutics into an *in vivo* system, such as a mammalian or human body, for targeting the virus replication in the virus-infected cells throughout the body. In the selection of the guide molecules disclosed herein, the crRNA guides have been selected to avoid any possible off-target excisions to the human genome.

[0095] The data presented herewith also shows that this antiviral approach is dependent on
10 the MOI of AAV-therapeutics that were used for the transduction, as well as the dynamics of the pooled crRNA guides. This, a pool of six crRNA guides has been identified as a cocktail for the effective inhibition of the EV71 virus replication.

[0096] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for
15 example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the
20 invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0097] As used in this application, the singular form "a," "an," and "the" include plural
25 references unless the context clearly dictates otherwise. For example, the term "a genetic marker" includes a plurality of genetic markers, including mixtures and combinations thereof.

[0098] As used herein, the term "about", in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated
30 value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

[0099] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed

ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[00100] Certain embodiments may also be described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the embodiments with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00101] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00102] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

[00103] **Design and synthesis of CasRX construct.** The CasRX sequence was obtained from Addgene (pXR001: EF1a-CasRX-2A-EGFP, plasmid no. 109049). The CasRX was cloned with a HA tag and a rabbit polyA tail (CasRX-HA-polyA) driven under a mammalian CMV promoter. Immediately downstream of the expression cassette, the gRNA backbone driven by human U6 promoter was cloned in and the sequence was obtained from Addgene (pXR003: CasRX gRNA cloning backbone, plasmid no. 109053).

[00104] **Cell lines and viruses.** Human rhabdomyosarcoma (RD) cells were purchased from the American Type Culture Collection (ATCC). Human B4G12 cells were purchased from Creative Bioarray. The cells were grown in media recommended by the ATCC and Creative Bioarray respectively. AAVs were generated in-house. Briefly, AAV were packaged via a triple transfection of 293AAV cell line (Cell Biolabs AAV-100) that were plated in a HYPERFlask 'M' (Corning) in growth media consisting of DMEM+glutaMax+pyruvate+10%FBS (Thermo

Fisher), supplemented with 1x MEM non-essential amino acids (Gibco). Confluency at transfection was between 70 to 90%. Media was replaced with fresh pre-warmed growth media before transfection. For each HYPERFlask 'M', 200 µg of pHHelper (Cell Biolabs), 100 µg of pRepCap [encoding capsid proteins for serotype DJ or 2], and 100 µg of pZac-CASI-GFP or pZac-CMV-CasRX were mixed in 5 ml of DMEM, and 2 mg of PEI "MAX" (Polysciences) (40 kDa, 1 mg/ml in H₂O, pH 7.1) added for PEI: DNA mass ratio of 5:1. The mixture was incubated for 15 minutes, and transferred drop-wise to the cell media. The day after transfection, media was changed to DMEM+glutamax+pyruvate+2%FBS. Cells were harvested 48 to 72 hours after transfection by scrapping or dissociation with 1xPBS (pH7.2) + 5 mM EDTA, and pelleted at 1500 g for 12 minutes. Cell pellets were re-suspended in 1–5 ml of lysis buffer (Tris HCl pH 7.5, 2 mM MgCl, 150 mM NaCl), and freeze-thawed 3 times between dry-ice-ethanol bath and 37°C water bath. Cell debris was clarified via 4000 g for 5 minutes, and the supernatant collected. The collected supernatant was treated with 50 U/ml of Benzonase (Sigma-Aldrich) and 1 U/ml of RNase cocktail (Invitrogen) for 30 min at 37°C to remove unpackaged nucleic acids. After incubation, the lysate was loaded on top of a discontinuous density gradient consisting of 6 ml each of 15%, 25%, 40%, 60% Optiprep (Sigma-Aldrich) in an 29.9 ml Optiseal polypropylene tube (Beckman-Coulter). The tubes were ultra-centrifuged at 54000 rpm, at 18°C, for 1.5 hours, on a Type 70 Ti rotor. The 40% fraction was extracted, and dialyzed with 1xPBS (pH 7.2) supplemented with 35 mM NaCl, using Amicon Ultra-15 (100 kDa MWCO) (Millipore). The titer of the purified AAV vector stocks were determined using real-time qPCR with ITR-sequence-specific primers and probe, referenced against the ATCC reference standard material 8 (ATCC). The Enterovirus strain used is EV-A71 strain 5865/sin/000009.

[00105] **Immunofluorescence assay.** 10,000 human immortalized corneal endothelial cells, B4G12, were plated on glass slides in a 48-well plate and transduced with AAVDJ-CasRX or AAVDJ-GFP at MOI 100K. At 3 days post-transduction, cells were fixed and permeabilized using methanol for 10mins and blocked using 1X PBS with 5% BSA for 1 hour. This is followed by primary antibody anti-HA (abcam) incubation at 1:200 dilution for 2 hours at room temperature or anti-GFP (abcam) at 1:1500 for 2 hours at room temperature. Secondary antibodies staining is carried out at 1:1000 (Thermo Fisher) for 2 hours at room temperature. Slides were then washed 3 times with 1X PBS and mounted on to the slide using ProLong mounting medium (Thermo). Images are taken using an Olympus microscope, exposure set at 15 milliseconds and gain set at 6400 for bright field and the 488 channel, exposure is set at 800us and gain set at 6400 for the DAPI channel.

[00106] **Biopanning assay for selection of AAV serotype.** Immortalized human rhabdomyosarcoma (RD) cells were seeded in a 48-well plate at a density of 10,000 cells per well in 200 μ l DMEM containing 10% FBS. The cells were cultured overnight at 37°C and allowed to adhere to the well. A panel of AAV (1, 2, 6, 7, 8, 9, rh10, DJ and Anc80) were used to transduce the cells at MOI of 100K, in triplicates. At 72 hours post-transduction, the cells were harvested and the total GFP protein is quantitated using a GFP quantification kit (Biovision) on a multi-well plate reader (Tecan).

[00107] **RNA gene interference activity in AAVDJ-CasRX transduced cells.** Immortalized human rhabdomyosarcoma (RD) cells were seeded in a 48-well plate at a density of 10,000 cells per well in 200 μ l DMEM containing 10% FBS. The cells were transduced with AAV2-GFP at MOI of 10K for expression of GFP. The GFP knockdown efficiency of guide1 only, guide2 only and guide1+guide2 are tested by transduction of the GFP-expressing rhabdomyosarcoma (RD) cells with AAV2-CasRX bearing the respective guides at of MOI 100K and MOI 1000K. At 72 hours post-transduction, the cells were harvested and the total GFP protein is quantitated using a GFP quantification kit (Biovision) on a multi-well plate reader (Tecan).

[00108] ***In vitro* antiviral plaque assay with EV71.** For screening of anti-EV71 activity, rhabdomyosarcoma (RD) cells were seeded in 96-well plates at a density of 10^4 cells per well and incubated overnight in an incubator. Perform dilution of AAV2-CasRX bearing the different guides for transduction individually or in pooled format at MOI 1K, 10K, 100K and 1000K in a 100ul volume. At 72 hours post-transduction, the cells were infected with EV-A71 virus at MOI of 1. The plate was washed twice with 1X PBS and incubated with DMEM with 2% FBS for 12h. The supernatant from each well were harvested at 12 hours post-infection and used for subsequent virus plaque assay. For virus plaque assay, rhabdomyosarcoma (RD) cells were seeded in 24-well plates and incubated with 10 to 10^6 fold serially diluted supernatant samples in a 100 ul volume. Plates were washed twice with PBS and overlay media were added to each well and incubated with 15mins rocking interval for an hour before leaving it in the incubator for 4 days. After 4 days of incubation, the overlay media was removed and crystal violet stain was added to visualize the plaques for counting.

TABLE 1: TABLE OF SEQUENCES

All sequences are provided in 5'-3', unless otherwise denoted.

SEQ ID NO:	Comments/Annotations	Sequence
1	EV71_3D guide1 sequence	gttgggccattgatgttagtct
2	EV71_3D guide2 sequence	ttggaaaacaggcctgttcaaa
3	EV71_3D guide3 sequence	tagcacgcttctccatgctcat
4	EV71_3D guide4 sequence	tattatccatgtagaattcat
5	EV71_3D guide5 sequence	atgatgttgatgacattgaatt
6	EV71_3D guide6 sequence	tctgcaggagtcattggtcaaacatactc
7	GFP guide1 sequence	gtgaacagctcctcgcccttgct
8	GFP guide2 sequence	gctgaactgtggccgtttac
9	GG001; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCCATCTGCTTATATAGACCTCCC ACCGT
10	GG002; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCAAGATGGGCGTGAAGTCCACA CT
11	GG003; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCCTATCCGGATCCGGAATTGCCG
12	GG004; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCGGATACCCATACGATGTTCCAG ATTACGCT
13	GG005; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	TAGGTCTCACATTAGGCATAGTCGGGGAC ATCATATG
14	GG006; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCCAATGGCTAATAAAGGAAATT TATTTTCATTGCAATAGTGTG
15	GG007; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCACTCGATCTCCATAAGAGAAG AGGGACAGCT
16	GG008; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCCCGAGGGCCTATTTCCCATGAT TCCTTC
17	GG009; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCAATTCACGACACCTGAAATGG AAGAAA
18	GG0010; Primer for cloning pZac-CMV-CasRx-polyA-U6-gRNA	ATGGTCTCGGAATGGGCGTGAAGTCCACA CT
19	EK19001; Primer for cloning EV71_3D_guide1 into pZac backbone	CCTACCAACTGGTCGGGGTTTGGTTGGTCC ATTGATGTTTAGTCT

20	EK19002; Primer for cloning EV71_3D_guide1 into pZac backbone	GGAGAAAATACCGCATCAGAATTCAAAAG ACTAAACATCAATGGACCAAC
21	EK19003; Primer for cloning EV71_3D_guide2 into pZac backbone	CCTACCAACTGGTCGGGGTTTGTGTTGAAA ACAGGGCTTGTTCAAA
22	EK19004; Primer for cloning EV71_3D_guide2 into pZac backbone	GGAGAAAATACCGCATCAGAATTCAAATT TGAACAAGCCCTGTTTCCAA
23	EK19005; Primer for cloning EV71_3D_guide3 into pZac backbone	CCTACCAACTGGTCGGGGTTTGTAGCACG CTTCCTCCATGCTCAT
24	EK19006; Primer for cloning EV71_3D_guide3 into pZac backbone	GGAGAAAATACCGCATCAGAATTCAAAAT GAGCATGGAGGAAGCGTGCTA
25	EK19007; Primer for cloning EV71_3D_guide4 into pZac backbone	CCTACCAACTGGTCGGGGTTTGTATTTATC CATGTAGAATTTTCAT
26	EK19008; Primer for cloning EV71_3D_guide4 into pZac backbone	GGAGAAAATACCGCATCAGAATTCAAAAT GAAATTCTACATGGATAAATA
27	EK19009; Primer for cloning EV71_3D_guide5 into pZac backbone	CCTACCAACTGGTCGGGGTTTGTATGATGTT GTTGATCATTGAATT
28	EK19010; Primer for cloning EV71_3D_guide5 into pZac backbone	GGAGAAAATACCGCATCAGAATTCAAAAA TTCAATGATCAACAACATCAT
29	EK19011; Primer for cloning EV71_3D_guide6 into pZac backbone	CCTACCAACTGGTCGGGGTTTGTCTGCAGG AGTCATGGTCAAACCATACTC
30	EK19012; Primer for cloning EV71_3D_guide6 into pZac backbone	GGAGAAAATACCGCATCAGAATTCAAAGA GTATGGTTTGACCATGACTCCTGCAGA
31	SV038; primer for cloning GFP_guide1 into pZac backbone	AACTGGTCGGGGTTTGGTGAACAGCTCCT CGCC
32	SV039; primer for cloning GFP_guide1 into pZac backbone	GAAAATACCGCATCAGAATTCAAAAAAAA GCAAGGGCGAGGAGC
33	SV040; primer for cloning GFP_guide2 into pZac backbone	AACTGGTCGGGGTTTGGCTGAACTTGTGG CCGTT
34	SV041; primer for cloning GFP_guide2 into pZac backbone	CCGCATCAGAATTCAAAAAAAGTAAACGG CCACAAGTTCAG

35	3D_protein_guides_desig 1395 bp ds-DNA FEATURES: Location/Qualifiers CDS: 1..1392 mat_peptide: 7..1392 /label="Geneious name: 3D peptide" misc_feature: 46..68 /label="Guide1" misc_feature: 166..188 /label="Guide2" misc_feature: 289..311 /label="Guide3" misc_feature: 427..449 /label="Guide4" misc_feature: 898..920 /label="Guide5" misc_feature: 1054..1082 /label="Guide6"	atgcaaggtgagatccaatgggtgaagcctaacaaggaaactggca gactaaacatcaatggaccaactcgcactaagttggagcctagtgtatt tcatgatgtgttgaaggcaacaaggaccagcagtttaacaagtaa agaccctagattggaggtcgacttgaacaagccctgtttccaagtat gtgggcaatgtttacacgagcccgatgaatatgtgactcaagctgcc ctccactatgcgaatcaactaaacaattggacataaactagcaag atgagcatggagggaagcgtgctatggcactgaaaacctggaagcaa tagacctctgcactagtctgggtatccatacagtgccttggtatcaa gaaaagagacattctcagcccataaccagggatgtgtctaatgatga aattctacatggataaacggactagatctgccatacttacctatgtg aaggatgaacttagatctctggataaaatcaagaaaggaaatcacg cctgatagaggccagcagcttgaatgactctgtctacctcagaatgac tttgggcacctttacgaggtgtttcatgctaacctgggtactgtgactg gctcagcagtaggttgaaccagacgtgtttggagtaaactaccga ttctgctgcctgggtcactctttgccttggactactcaggatatgatgta gtctcagcccggtatggttcagggtcttagaagttgtgttacgggaga ttgggtattcagaggaggccgtgtccctaataagaaggaaatcaaccac accacatgtgtaccggaataaaacatactgtgtacttgggtggatg ccctcaggggtgctctggtacttccattctcaattcaatgatcaacaacat catcattagaaccttttgatcaaaaccttaagggaatagacctggat gagttgaacatggtggcctatggggacgatgtgctggccagttacctt tttctattgattgcctgaattggctaagactggcaagagtatgtgttg accatgactcctgcagacaaatcacctgtttcaatgaagtaacatgg gagaatgctaccttctgaagagagggttcttggcagaccaccaattt ccattcttaattcacctacgatgcccatgagagagatccatgagtcca ttcgatggactaaggacgcgcgtaacaccaaggatcacgtgcgctcc ctgtgtctattggcatggcacaatggaaggatgaatatgaaaagtgtg gagtgaatttagatcagttccagttggaaaagcgttggccattcctaac tttgagaatctgagaagaaattggctcgaattgttttaa
36	GFP_guides_design 746 bp ds-DNA circular FEATURES: Location/Qualifiers CDS: 1..746/label="eGFP" CDS: 1..746/label="Translation 1-746" misc_feature: 7..29 /label="Knockdown_Guide1" misc_feature: 67..87 /label="Knockdown_Guide2"	Atggtgagcaagggcgaggagctgttcaccggggtggtgccatc ctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgt ccggcgagggcgagggcgatgccactacggcaagctgacctga agttcatctgcaccaccggcaagctgcccgtgccctggcccaccctc gtgaccaccctgacctacggcgtgcagtgtctcagccgctacccega ccacatgaagcagcacgacttctcaagtccgcatgcccgaaggct acgtccaggagcgcaccatcttctcaaggacgacggcaactacaag accgcgcggaggtgaagttcgaggcgacacctggtgaaccgc atcgagctgaagggcatcgacttcaaggaggacggcaacatcctgg ggcacaagctggagtacaactacaacagccacaacgtctatatcatg gccgacaagcagaagaacggcatcaagggtgaacttcaagatccgcc acaacatcgaggacggcagcgtgcagctcgccgaccactaccagc agaacacccccatcggcgacggccccgtgctgctgcccgacaacc actacctgagcaccagtcgcccctgagcaaaagaccccaacgagaa gcgcgatcacatggtcctgctggagttcgtgaccgccggggatca ctctggcatggacgagctgtacaagtaaATCACGACgacga gctgtacaagtaa

37	See end of table	See end of table
38	primer complement(121..144) /label="2225" of SEQ ID NO: 37	GGGAGAGGCGGTTTGCGTATTGGG
39	primer 534..557 /label="EK18011" of SEQ ID NO: 37	gccatgctctaggaagatcgggac
40	primer 534..593 /label="EK18001" of SEQ ID NO: 37	gccatgctctaggaagatcgggacattgattattgactagtattataatag taatcaatta
41	primer 622..645 /label="1075" of SEQ ID NO: 37	CGGAGTTCCGCGTTACATAACTTAC
42	primer 722..741/label="1324" of SEQ ID NO: 37	ACGCCAATAGGGACTTTCCA
43	primer 811..830/label="2394" of SEQ ID NO: 37	AAGTACGCCCCCTATTGACG
44	primer complement(1052..1077) /label="EK18015" of SEQ ID NO: 37	gagttgttacgacattttggaaagtc
45	primer complement(1111..1134) /label="GG001" of SEQ ID NO: 37	ATGGTCTCCatctgcttatatagacctcccaccgt
46	primer complement(1111..1134) /label="SV036" of SEQ ID NO: 37	tgtggacttcacgcccatggtggcctgcttatatagacctcccaccgt
47	primer 4018..4044/label="GG004" of SEQ ID NO: 37	ATGGTCTCGgataccatacagatgtccagattacgct
48	primer complement(4076..4100)/label= "GG005" of SEQ ID NO: 37	TAGGTCTCAcattaggcatagtcggggacatcatg
49	primer 4102..4140 /label="GG006" of SEQ ID NO: 37	ATGGTCTCCAatggctaataaaggaaatttatttcattgcaata gtgtg
50	primer complement(4462..4488) /label="GG007" of SEQ ID NO: 37	ATGGTCTCActcgatctcataagagaagaggacagct
51	primer 4489..4510 /label="U6_fwd_seqchk" of SEQ ID NO: 37	gagggcctatttcccatgattc
52	primer 4489..4514 /label="GG008" of SEQ ID NO: 37	ATGGTCTCCgagggcctatttcccatgattccttc
53	Primer complement(4490..4511) /label="13 EF1a_R (out)" of SEQ ID NO: 37	ggaatcatgggaaataggccct
54	primer complement(4713..4738) /label="736" of SEQ ID NO: 37	CGGTGTTTCGTCCTTTCCACAAGATA

55	primer 4743..4759 /label="EK_GS_F1" of SEQ ID NO: 37	cctaccaactggtcggg
56	primer 4743..4764 /label="EK18100" of SEQ ID NO: 37	cctaccaactggtcggggtttg
57	primer complement(4791..4817) /label="EK18101" of SEQ ID NO: 37	GGAGAAAATACCGCATCAGAATTCAAA
58	primer complement(4796..4817) /label="EK_GS_R1" of SEQ ID NO: 37	ggagaaaataccgcatcagaat
59	primer complement(4843..4895) /label="EK18008" of SEQ ID NO: 37	gctacttatctacgtagccatgcgaattactatgggtgctttgacgtatgc gg
60	primer complement(4866..4895) /label="EK18012" of SEQ ID NO: 37	gctacttatctacgtagccatgcgaattac
61	primer complement(5206..5225) /label="2452" of SEQ ID NO: 37	TTCAGGCTGCGCAACTGTTG
62	primer 5824..5849 /label="1625" of SEQ ID NO: 37	ATGAGTATTCAACATTTCCGTGTTCG
63	primer complement(7226..7246) /label="2372" of SEQ ID NO: 37	GCTCACGCTGTAGGTATCTCA
64	consensus sequence - untitled consensus; and aligned sequences - EV71- SHENZEN001-2006; and EV71-NJ2017iso2 of FIG. 10	agactcaacatcaatggaccaac
65	aligned sequence - Strain H8-1 of FIG. 10	agactcaacatcaatggccaac
66	aligned sequences - HEVA71- Strain41, Enterovirus- sin002209, and nucleic acid sequence of Guide1 of FIG. 10	agactaaacatcaatggaccaac
67	aligned sequence - EV71 MZ of FIG. 10	agactcagcatcaatggaccaac
68	Guide1 protein sequence of FIG. 10	RLNINGPT
69	consensus sequence - untitled consensus of FIG. 10	tttgaacaagccctgttctcaa
70	aligned sequence - Strain H8-1 of FIG. 10	tttgaacaggccctgttctcaa
71	aligned sequence - EV71- SHENZEN001-2006 of FIG. 10	tttgaacaagccctgttctcaa

72	aligned sequences - HEVA71-Strain41, Enterovirus-sin002209, and nucleic acid sequence of Guide2 of FIG. 10	tttgaacaagccctgttttccaa
73	aligned sequences - EV71 MZ, and EV71-NJ2017iso2 of FIG. 10	tttgaacaggccctgttctctaa
74	Guide2 protein sequence of FIG. 10	FEQALFSK
75	consensus sequence - untitled consensus; and aligned sequences -Strain H8-1, EV71-SHENZEN001-2006, EV71-MZ, and EV71-NJ2017iso2 of FIG. 10	atgagcatggaggaggcctgcta
76	aligned sequences - HEVA71-Strain41, Enterovirus-sin002209, and nucleic acid sequence of Guide3 of FIG. 10	atgagcatggaggaagcgtgcta
77	Guide3 protein sequence of FIG. 10	MSMEEACY
78	consensus sequence - untitled consensus; and aligned sequences -Strain H8-1, and EV71-SHENZEN001-2006 of FIG. 10	atgaagttctacatggacaaata
79	aligned sequences - HEVA71-Strain41, Enterovirus-sin002209, and nucleic acid sequence of Guide4 of FIG. 10	atgaaattctacatggataaata
80	aligned sequences - EV71-MZ and EV71-NJ2017iso2 of FIG. 10	atgaagttttacatggacaagta
81	Guide4 protein sequence of FIG. 10	MKFYMDKY
82	consensus sequence - untitled consensus; and aligned sequences - Strain H8-1 and EV71-SHENZEN001-2006 of FIG. 10	aactcaatgatcaacaacattat
83	aligned sequence - HEVA71-Strain41 and nucleic acid sequence of Guide5 of FIG. 10	aattcaatgatcaacaacatcat
84	aligned sequence - EV71-MZ of FIG. 10.	aactcaatgatcaataacattat
85	aligned sequence - Enterovirus-sin002209 of FIG. 10	aattcaatgatcaataacatcat
86	aligned sequence - EV71-NJ2017iso2 of FIG. 10	aattcaatgatcaacaacattat

87	Guide5 protein sequence of FIG. 10	NSMINNII
88	consensus sequence - untitled consensus; and aligned sequences - Strain H8-1 and EV71-SHENZEN001-2006 of FIG. 10	gagtatggtctgacatgactcctgcaga
89	aligned sequence - HEVA71-Strain41, Enterovirus-sin002209, and nucleic acid sequence of Guide6 of FIG. 10	gagtatggtttgacatgactcctgcaga
90	aligned sequence - EV71-MZ of FIG. 10.	gagtatggcttgacatgactcctgctga
91	aligned sequence - EV71-NJ2017iso2 of FIG. 10	gagtatggtctgacatgactcctgctga
92	Guide6 protein sequence of FIG. 10	EYGLTMTPAD
93	consensus sequence - untitled consensus of FIG. 11	ctttgagggcaacaagaaccag
94	aligned sequence - LC126150 of FIG. 11	ctttgagggcaacaaggaaccag
95	aligned sequence - CAU05876; Cox_Guide1 of FIG. 11	ctttgaggggaacaagaaccag
96	consensus sequence - untitled consensus of FIG. 11	ctccaagtatgtaggaacacac
97	aligned sequence - LC126150 of FIG. 11	ctctaagtatgtaggaacacac
98	aligned sequence - CAU05876; Cox_Guide2 of FIG. 11	ctccaagtatgtaggaacacac
99	consensus sequence - untitled consensus of FIG. 11; aligned sequence - CAU05876; Cox_Guide3 of FIG. 11	cactatgcaaatcagttgaagca
100	aligned sequence - LC126150 of FIG. 11	cattatgcaaatcagttgaagca
101	consensus sequence - untitled consensus of FIG. 11	gatgtgagcaagatgaaattcta
102	aligned sequence - LC126150 of FIG. 11	gatgtgagcaagatgaagttcta
103	aligned sequence - CAU05876; Cox_Guide4 of FIG. 11	gatgtgagtaagatgaaattcta
104	consensus sequence - untitled consensus; aligned sequences - LC126150 and CAU05876; Cox_Guide5 of FIG. 11	tgcctcaggctgttcaggaaca
105	consensus sequence - untitled consensus; aligned sequence - CAU05876; Cox_Guide6 of FIG. 11	caactcaatgatcaacaacatca

106	aligned sequence - LC126150 of FIG. 11	caactcaatgattaacaacatca
107	consensus sequence - untitled consensus; aligned sequences - AF465516; Echo_guide1 and X89538_Echo7_3D of FIG. 12	gagacaaattacatcgactacctg
108	aligned sequences - X89554_Echo27_3D and X89548_Echo19_3D of FIG. 12	gagacaaattacattgactacttg
109	aligned sequence - X89541_Echo12_3D of FIG. 12	gagacaaactacatcgactaccta
110	consensus sequence - untitled consensus; aligned sequences - AF465516; Echo_guide2 and X89538_Echo7_3D of FIG. 12	tcccaccacctgtacagagacaa
111	aligned sequence - X89554_Echo27_3D of FIG. 12	tctcaccatctgtacagagataa
112	aligned sequence - X89548_Echo19_3D of FIG. 12	tcccaccacctgtacagagataa
113	aligned sequence - X89541_Echo12_3D of FIG. 12	tctcaccacctgtacagagacaa
114	consensus sequence - untitled consensus; aligned sequences - AF465516; Echo_guide3 and X89538_Echo7_3D of FIG. 12	ttcaggatgattgcatatggtga
115	aligned sequence - X89554_Echo27_3D of FIG. 12	ttcaggatgatagcatatggtga
116	aligned sequence - X89548_Echo19_3D of FIG. 12	tttaggatgatcgcatatggtga
117	aligned sequence - X89541_Echo12_3D of FIG. 12	tttagaatgattgcatatggtga
118	consensus sequence - untitled consensus of FIG. 12	ttgattatgacaccagcagataa
119	aligned sequence - AF465516; Echo_guide4 and X89538_Echo7_3D	ttggttatgacaccagcagataa
120	aligned sequence - X89554_Echo27_3D of FIG. 12	ttgattatgacaccagcagacaa
121	aligned sequence - X89548_Echo19_3D of FIG. 12	ttgatcatgacaccagcagataa
122	aligned sequence - X89541_Echo12_3D of FIG. 12	ctgattatgacaccagcagacaa

SEQ ID NO: 37: pZac2.1-CMV-CasRx-3xHA- 7508 bp ds-DNA circular*Sequence annotation:**Features: Location/Qualifiers*

- 5 primer: complement(121..144) /label="2225" /note="sequence: GGGAGAGGCGGTTTGCCTA
TTGGG" (SEQ ID NO: 38)
- repeat_region: 364..493 /label="ITR"
- 10 primer: 534..557/label="EK18011" /note="sequence: gccatgctctaggaagatcgggac"(SEQ ID NO:
39)
- primer: 534..593/label="EK18001"/note="sequence: gccatgctctaggaagatcgggacattgattattgactagtta
ttaatagtaatacaatta" (SEQ ID NO: 40)
- 15 enhancer: 555..934/label="CMV enhancer"
- CDS: 555..934/label="Translation 555-934"
- 20 primer: 622..645/label="1075" /note="sequence: CGGAGTTCCGCGTTACATAACTTAC"
(SEQ ID NO: 41)
- primer: 722..741/label="1324" /note="sequence: ACGCCAATAGGGACTTTCCA" (SEQ ID
NO: 42)
- 25 primer: 811..830/label="2394" /note="sequence: AAGTACGCCCCCTATTGACG" (SEQ ID
NO: 43)
- promoter: 936..1134/label="CMV promoter"
- 30 primer: complement(1052..1077)/label="EK18015" /note="sequence: gagttgttacgacattttgg
aaagtc" (SEQ ID NO: 44)
- primer: complement(1111..1134) /label="GG001" /note="sequence: ATGGTCTCCatctgcttatatag
acctcccaccgt" (SEQ ID NO: 45)
- 35 primer: complement(1111..1134)/label="SV036" /note="sequence: tgtggacttcacgcccattggtggcctgc
ttatatagacctcccaccgt" (SEQ ID NO: 46)
- 40 misc_feature: 1135..1140 /label="Kozak"
- misc_feature: 1141..4017/label="CasRx"
- CDS: 1141..4017/label="Translation 1141-4017"
- 45 primer: 4018..4044 /label="GG004" /note="sequence: ATGGTCTCGgatacccatcacgatgttcaga
ttacgt" (SEQ ID NO: 47)
- CDS: 4018..4098/label="3xHA"

- primer: complement(4076..4100) /label="GG005" /note="sequence: TAGGTCTCAcattaggcatagt
cggggacatcatatg"(SEQ ID NO: 48)
- 5 primer: 4102..4140 /label="GG006" /note="sequence: ATGGTCTCCAatggctaataaaggaaattttttca
ttgcaatagtgtg" (SEQ ID NO: 49)
- polyA_signal 4102..4488 /label="rabbit b-globin polyA"
- 10 primer: complement(4462..4488) /label="GG007" /note="sequence: ATGGTCTCActcgatctccata
agagaagaggacagct" (SEQ ID NO: 50)
- primer: 4489..4510 /label="U6_fwd_seqchk" /note="sequence: gagggcctatttcccatgattc" (SEQ ID
NO: 51)
- 15 primer: 4489..4514 /label="GG008" /note="sequence: ATGGTCTCCgagggcctatttcccatgatt
ccttc"(SEQ ID NO: 52)
- promoter: 4489..4729/label="U6 promoter"
- 20 primer: complement(4490..4511) /label="13 EF1a_R (out)" /note="sequence: ggaatcatgggaaata
ggccct" (SEQ ID NO: 53)
- primer_bind: 4660..4679 /label="LKO.1 5"
- 25 primer: complement(4713..4738) /label="736" /note="sequence: CGGTGTTTCGTCCTTTCCA
CAAGATA" (SEQ ID NO: 54)
- primer: 4743..4759 /label="EK_GS_F1" /note="sequence: cctaccaactggtcggg"(SEQ ID NO: 55)
- 30 primer: 4743..4764 /label="EK18100" /note="sequence: cctaccaactggtcggggtttg" (SEQ ID NO:
56)
- primer: complement(4791..4817) /label="EK18101" /note="sequence: GGAGAAAATACCGCA
TCAGAATTCAAA" (SEQ ID NO: 57)
- 35 primer: complement(4796..4817) /label="EK_GS_R1" /note="sequence: ggagaaaataccgcatcag
aat" (SEQ ID NO: 58)
- 40 primer: complement(4843..4895) /label="EK18008" /note="sequence: gctacttatctacgtagccatgcg
aattactatggtgctttgacgtatgcgg" (SEQ ID NO: 59)
- primer: complement(4866..4895) /label="EK18012" /note="sequence: gctacttatctacgtagccatg
cgaattac" (SEQ ID NO: 60)
- 45 misc_feature: join(4868..7508,1..554) /label="pZac2.1 BB"
- misc_feature: 4920..5060 /label="ITR"

primer: complement(5206..5225) /label="2452" /note="sequence: TTCAGGCTGCGCAACTGT
TG" (SEQ ID NO: 61)

5 primer: 5824..5849 /label="1625" /note="sequence: ATGAGTATTCAACATTTCCGTGTCGC"
(SEQ ID NO: 62)

primer: complement(7226..7246) /label="2372" /note="sequence: GCTCACGCTGTAGGT
ATCTCA" (SEQ ID NO: 63)

10 *Full sequence:*

tcctcggttatccctgattctgtggataaccgtattaccgctttgagtgagctgataccgctcgccgcagccgaacgaccgagcgagcg
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25 taaaaacggcgctataagatcggaacgcaaatcagccatcctaagggtacgccgtgtgtggtgtaacaacctctgtatacaggaccc
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