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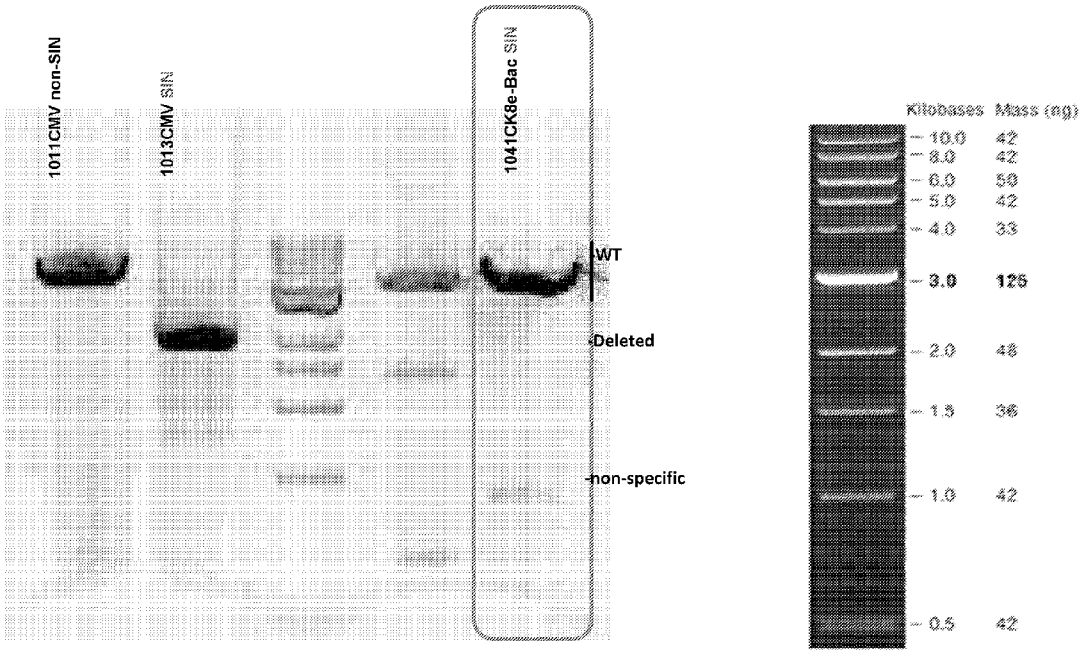
FIG. 1




(57) **Abstract:** Provided herein are self-inactivating (SIN) CRISPR system vectors, methods of preparing said vectors, and methods of using said vectors. The SIN CRISPR system vectors comprise nucleic acid sequence encoding a CRISPR system (*i.e.*, CRISPR nuclease and guide RNA), wherein the sequence encoding the CRISPR nuclease comprises at least one intron comprising a binding site recognized by the CRISPR system.

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# ALL-IN-ONE SELF INACTIVATING CRISPR VECTORS

## FIELD

[0001] The present disclosure relates to self-inactivating vectors encoding a targeting nuclease, methods of preparing said vectors, and methods of using said vectors.

## BACKGROUND

[0002] RNA-guided clustered regularly interspersed short palindromic repeats (CRISPR) systems have emerged as powerful genome modification tools due to their simplicity, target design plasticity, and multiplex targeting capacity. Long term expression of CRISPR nucleases, however, can increase the number of non-specific (off target) cleavage events and/or can elicit immune responses. Thus, it would be beneficial to limit the duration of nuclease expression following delivery.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0003] FIG. **1** presents an image of a gel comprising long range PCR products of various SIN vectors. Lane 1: 1011CMV non-SIN control vector produced using HEK 293T cells showing intact genome; Lane 2:1013CMV SIN vector produced using HEK 293T cells showing rearranged and/or deleted vector genome; Lane 3: Kb ladder; Lane 4 1014Ck8e-AcrA5 - SIN vector produced using HEK293T cell line expressing anti-CRISPR protein AcrIIA5; Lane 5: 1041CK8e-Bac SIN vector produced using baculovirus-Sf9 system showing intact genome.

## SUMMARY

[0004] Among the various aspects of the present disclosure is a nucleic acid comprising a sequence encoding a CRISPR nuclease and a sequence encoding a guide RNA (gRNA), wherein the sequence encoding the CRISPR nuclease comprises at least one intron, and the at least one intron comprises a binding site that is recognized by the CRISPR nuclease and gRNA.

[0005] In some embodiments, the sequence encoding the CRISPR nuclease is codon optimized for expression in eukaryotic cells of interest. In some embodiments, the CRISPR nuclease is linked to at least one nuclear localization signal.

[0006] In other embodiments, the intron in the sequence encoding the CRISPR nuclease is a mammalian intron, an engineered intron, or an artificial intron. In some aspects, the binding site in the intron is about 17 nucleotides to about 23 nucleotides in length and is followed by a protospacer adjacent motif (PAM) sequence.

[0007] In certain embodiments, the sequence encoding the CRISPR nuclease is operably linked to a Pol II promoter, and the sequence encoding a guide RNA is operably linked to a Pol III promoter. In some aspects, the CRISPR nuclease is a Cas9 nuclease or a variant having at least 90% sequence identity to the Cas9 nuclease. In some embodiments, the Cas 9 nuclease is *Staphylococcus aureus* Cas9, *Neisseria meningitidis* Cas9, or *Campylobacter jejuni* Cas9.

[0008] In some embodiments, the sequence encoding the CRISPR nuclease and the sequence encoding the gRNA are flanked by adeno-associated virus (AAV) inverted terminal repeats (ITRs).

[0009] Another aspect of the present disclosure provides a baculovirus expression vector comprising the nucleic acid as described above.

[0010] A further aspect of the present disclosure encompasses a recombinant AAV particle comprising the nucleic acid as described above and at least one capsid protein.

[0011] Still another aspect of the present disclosure provides a method for producing the nucleic acid as described above, wherein the method comprises introducing into a packaging cell a recombinant baculovirus comprising the sequence encoding the CRISPR nuclease, which is interrupted by the intron, and the sequence encoding the guide RNA, which are flanked by the AAV ITRs. In general, upon expression of the recombinant baculovirus encoding the CRISPR nuclease and gRNA, a functional CRISPR nuclease is not produced in the packaging cell. The packaging cell, however, produces AAV particles comprising the nucleic acid described above that is encapsidated by at least one AAV capsid protein.

[0012] In some aspects, the method further comprises expressing AAV replication (Rep) and capsid (Cap) proteins in the packaging cell. For example, a packaging recombinant baculovirus sequence encoding AAV Rep and Cap proteins can be introduced into the packaging cell. Alternatively, the packaging cell comprises sequence encoding AAV Rep and Cap proteins stably integrated into its genome, wherein said sequence encoding AAV Rep and Cap proteins is operably linked to an inducible promoter.

[0013] In further embodiments, the method further comprises introducing into the packaging cell a helper recombinant baculovirus comprising sequence encoding AAV packaging components.

[0014] In some embodiments, the recombinant baculovirus encoding the CRISPR nuclease and gRNA is derived from Autographa californica multiple nucleopolyhedrovirus (AcMNPV). In certain aspects, the packaging cell is a *Spodoptera frugiperda* Sf9 cell.

[0015] A further aspect of the present disclosure encompasses a method for temporally limiting expression of a CRISPR nuclease in eukaryotic cells, wherein the method comprises introducing into the eukaryotic cells the nucleic acid described above or the recombinant AAV particle described above. Upon expression of the nucleic acid, the intron is excised and a CRISPR nuclease is produced, and the CRISPR nuclease complexes with the guide RNA to form a CRISPR system, wherein the CRISPR system cleaves the target genomic locus in the eukaryotic cells leading to an edited genomic locus, and the CRISPR system cleaves the intron in the sequence encoding the CRISPR nuclease in the nucleic acid, thereby inactivating the nucleic acid

[0016] In some embodiments, the nucleic acid or the recombinant AAV particle is inactivated within about 1-3 days after being introduced into the eukaryotic cells, thereby temporally limiting expression of the CRISPR nuclease.

[0017] Other aspects and iterations of the present disclosure are described below in more detail.

## DETAILED DESCRIPTION

[0018] The present disclosure provides self-inactivating (SIN) CRISPR system vectors and methods of using the SIN CRISPR system vectors. The SIN CRISPR system vectors comprise nucleic acid sequence encoding a CRISPR nuclease and nucleic acid sequence encoding a guide RNA (gRNA), wherein the nucleic acid sequence encoding the CRISPR nuclease comprises at least one intron. Upon expression of the vector in eukaryotic cells, the intron is spliced out such that a functional CRISPR nuclease is produced, which complexes with the gRNA to form a CRISPR system that cleaves the target locus and also cleaves the intron in the SIN vector, thereby limiting expression of the CRISPR nuclease. In general, the SIN CRISPR system vectors disclosed herein are recombinant AAV vectors.

Moreover, all of the sequences needed for genome editing and self-inactivation are provided within one vector (*i.e.*, all-in-one SIN vector).

[0019] Also provided herein are recombinant baculovirus expression vectors for generating the SIN AAV vectors disclosed herein, wherein the recombinant baculovirus expression vectors comprise nucleotide sequence encoding any of the SIN CRISPR system vectors disclosed herein. Also provided herein are methods for generating the SIN AAV vectors in a baculovirus expression system, wherein the intron in the nucleotide sequence encoding the CRISPR nuclease is not recognized and not excised such that a functional CRISPR nuclease is not generated in the baculovirus expression system.

## (I) Self-Inactivating CRISPR System Vectors

[0020] One aspect of the present disclosure encompasses self-inactivating (SIN) CRISPR system vectors. The SIN CRISPR system vectors provide a CRISPR system for genome editing, and also are able to self-cleave, thereby temporally limiting expression of the CRISPR system in eukaryotic cells.

[0021] A CRISPR system comprises a guide RNA (gRNA) to target the system to a specific DNA sequence and a nuclease that cleaves the targeted DNA sequence. The gRNA drives sequence recognition and specificity of the CRISPR system through Watson-Crick base pairing with a ~20 nucleotide (nt) target sequence in the target locus, wherein the target sequence is adjacent to a specific short DNA motif referred to as a protospacer adjacent motif (PAM). The gRNA forms an RNA-duplex and stem-loop structure that is bound by the CRISPR nuclease to form the catalytically active ribonucleoprotein (RNP) CRISPR system. Once the CRISPR system is bound to DNA at a target sequence, two independent nuclease domains within the CRISPR nuclease cleave opposite strands of the DNA leaving a double-strand break (DSB).

[0022] The SIN CRISPR system vectors disclosed herein comprise a nucleic acid sequence encoding a CRISPR nuclease and a nucleic acid sequence encoding a gRNA, wherein the nucleic acid sequence encoding the CRISPR nuclease comprises at least one intron. The intron in the sequence encoding the CRISPR nuclease comprises a binding site (also called a SIN site) that is recognized and bound by the CRISPR system. Thus, during expression of the vector in eukaryotic cells, the intron is spliced out of the sequence encoding the CRISPR nuclease, thereby producing a functional CRISPR nuclease, which complexes

with the gRNA to form a CRISPR system. The CRISPR system 1) is directed to and cleaves the target genomic locus for genome editing, and 2) is targeted to and cleaves the intron in the vector, thereby inactivating the vector in eukaryotic cells. Moreover, all the sequences needed for genome editing and inactivation of the vector are present within the same vector. As such, the self-inactivating vector disclosed herein is an all-in-one SIN vector.

[0023] The SIN CRISPR system vectors disclosed herein are designed to be generated in baculovirus expression systems. During propagation of a SIN CRISPR system vector in a baculovirus expression system, the intron in the sequence encoding the CRISPR nuclease is not recognized and a functional CRISPR nuclease is not generated. Thus, the SIN CRISPR system vector is not cleaved and not inactivated in the baculovirus expression system, but the SIN CRISPR vector system can be propagated in the baculovirus expression system.

**(a) Sequence encoding CRISPR nuclease**

[0024] The SIN vectors disclosed herein comprise a nucleic acid sequence encoding a CRISPR nuclease. As described above, a nuclease is an enzyme that introduces a break in a double stranded nucleic acid sequence. The break can be double stranded or single stranded. In specific embodiments, the CRISPR nuclease introduces a double stranded break by cleaving both strands of the double stranded nucleic acid sequence. In other embodiments, the CRISPR nuclease can be a nickase in which one of the two nuclease domains within the CRISPR protein is inactivated such that the CRISPR nickase cleaves one strand of the double stranded nucleic acid sequence. The CRISPR nuclease can be naturally occurring, a variant thereof, or a modified or engineered version thereof. For example, the CRISPR nuclease can be modified or engineered to have altered activity, specificity, and/or stability.

[0025] In general, the CRISPR nuclease encoded by the vectors disclosed herein is a Cas9 nuclease or a variant/version thereof having at least 85%, at least 90%, at least 92%, at least 94%, at least 96%, at least 98%, or at least 99.9% sequence identity with a Cas9 nuclease. Furthermore, the Cas9 nuclease encoded by said vectors generally is a small Cas9 protein, e.g., the Cas9 nuclease contains less than about 1200 amino acids (aa). In specific embodiments, the Cas9 nuclease can be *Staphylococcus aureus* Cas9 (SauCas9; 1053 aa), *Neisseria meningitidis* Cas9 (NmeCas9; 1082 aa), or *Campylobacter jejuni* Cas9 (CjeCas9;

984 aa). In other embodiments, the Cas9 nuclease can be *Azospirillum* B510 Cas9 (1168 aa), *Campylobacter lari* CF89-12 Cas9 (1103 aa), *Corynebacter diphtheriae* Cas9 (1084 aa), *Eubacterium ventriosum* Cas9 (1107 aa), *Gluconacetobacter diazotrophicus* Cas9 (1150 aa), *Lactobacillus farciminis* Cas9 (1126 aa), *Neisseria cinerea* Cas9 (1082 aa), *Nitratitractor salsuginis* DSM 16511 Cas9 (1132 aa), *Parvibaculum lavamentivorans* Cas9 (1037 aa), *Roseburia intestinalis* Cas9 (1128 aa), *Sphaerochaeta globus* Cas9 (1179 aa), *Streptococcus pasteurianus* Cas9 (1130 aa), *Streptococcus thermophilus* CRISPR1 (1121 aa), or *Streptococcus thermophilus* LMD-9 Cas9 (1132 aa). In specific embodiments, the Cas9 coded by the SIN vector can be SauCas9 or variant thereof.

[0026] The CRISPR nuclease can be engineered by one or more amino acid substitutions, deletions, and/or insertions to have improved targeting specificity, improved fidelity, altered PAM specificity, decreased off-target effects, and/or increased stability. Non-limiting examples of one or more mutations that improve targeting specificity, improve fidelity, and/or decrease off-target effects include N497A, R661A, Q695A, K810A, K848A, K855A, Q926A, K1003A, R1060A, and/or D1135E (with reference to the numbering system of SpyCas9).

[0027] The CRISPR nuclease generally is linked to at least one nuclear localization signal (NLS) at the or within about 50 amino acids of N-terminal end, at or within about 50 amino acids of the C-terminal end, or both. NLSs are well known in the art. For example, the NLS can be the SV40 Large T-antigen NLS, nucleoplasmin NLS, c-Myc NLS, or derivatives thereof. The linkage between the CRISPR nuclease and the NLS can be a direct or it can be indirect via an intervening linker sequence. Suitable linker sequences are well known in the art.

[0028] Typically, the sequence encoding the CRISPR nuclease is codon optimized for expression in eukaryotic cells of interest. For example, the sequence can be codon optimized for expression in human cells. Codon optimization programs are widely available.

[0029] The sequence encoding the CRISPR nuclease in the vectors disclosed herein is operably linked to a promoter sequence for expression in the cells of interest. In general, the sequence encoding the CRISPR nuclease is operably linked to a Pol II promoter. In some embodiments, the Pol II promoter can be a constitutively active promoter, for example, a cytomegalovirus (CMV) promoter, CMV immediate early promoter (CMVIE), a

CAG promoter (a hybrid comprising the CMV enhancer fused to the chicken beta-actin promoter), actin promoters, elongation factor (EF)-1 alpha promoter, SV40 early promoter, mouse mammary tumor virus long terminal repeat promoter, adenovirus major late promoter (Ad MLP), herpes simplex virus (HSV) promoter, Rous sarcoma virus (RSV) promoter, synthetic promoters, hybrid promoters, and the like. In other embodiments, the promoter can be a tissue-specific promoter that is active selectively or preferentially in particular cell populations over other cell populations. Non-limiting examples of suitable tissue-specific promoter include muscle-specific promoters such as muscle creatine kinase (MCK), CK8e, or C5-12 promoters, CNS-specific promoters such as Synapsin 1, CaMKII alpha, GAD67, GAD65, or VGAT promoters, liver-specific promoter such as albumin promoter, lung-specific promoter such as SP-B, endothelial cell-specific promoter such as ICAM, hematopoietic cell-specific promoter such as IFN beta or CD45, and osteoblast-specific promoter such as OG-2. In still other embodiments, the promoter can be an inducible promoter that responds to the presence of a specific compound, such as tetracycline.

[0030] In some embodiments, the nucleic acid sequence encoding the CRISPR nuclease can also be operably linked to polyadenylation signal (*e.g.*, SV40 late polyadenylation signal, rabbit globin polyadenylation signal), enhancer sequences, and/or transcriptional termination signal (*e.g.*, woodchuck hepatitis virus post-transcriptional regulatory element).

**(b) Intron**

[0031] The sequence encoding the CRISPR nuclease is interrupted by at least one intron. For example, the sequence encoding the CRISPR nuclease can comprise one intron and two exons. In other embodiments, the sequence encoding the CRISPR nuclease can be interrupted by two or more introns.

[0032] The intron can be a mammalian intron (*e.g.*, present in a mammalian protein-coding gene), an engineered (*e.g.*, chimeric or hybrid) intron, or an artificial intron, provided the intron comprises the elements required for splicing. Essential splicing elements include a donor site (5' end of the intron), a branch site (near the 3' end of the intron), and an acceptor site (3' end of the intron). Non-limiting examples of suitable introns include 1) a chimeric intron comprising 5′-donor site from the first intron of the human β-globin gene and the branch and 3′-acceptor site from the intron of an immunoglobulin gene heavy chain

variable region (*e.g.*, pCI-Neo vector); 2) a hybrid intron comprising an adenovirus splice donor and an immunoglobulin G splice acceptor (Choi et al., Mol Cell Biol, 1991, 11(6):3070-3074); 3) a chimeric intron comprising the first intron of the human beta globin gene and an artificial sequence (*e.g.*, pSF-CMV-Intron1 expression plasmid); 4) human β-globin intron 1; 5) mouse immunoglobulin heavy chain precursor V intron; and 6) rabbit β-globin intron 2. In specific embodiments, the intron can be 1) listed above.

[0033] The length of the intron can and will vary. In general, the intron can range in length from about 50 bp to about 250 bp. In certain embodiments, the length of the intron can range from about 50 bp to about 100 bp, from about 100 bp to about 150 bp, from about 150 bp to about 200 bp, or from about 200 bp to about 250 bp.

[0034] The intron can be inserted anywhere in the sequence encoding the CRISPR nuclease. In one embodiment, the intron can be introduced at the codon coding for Asn 580 of a Cas9 nuclease. Means for combining heterologous sequences are well known in the art.

[0035] Additionally, the intron is engineered to contain a binding site that can be recognized by the CRISPR nuclease and gRNA encoded by the vector. The binding site in the intron comprises a target sequence that is followed by a PAM sequence specific for the CRISPR nuclease, wherein the gRNA of the CRISPR system can hybridize to the complement of the target sequence in the intron. The target sequence in the intron can range in length from about 17 nucleotides (nt) to about 23 nt, from about 18 nt to about 22 nt, or from about 19 nt to about 21 nt. In specific embodiments, the target sequence can be about 20 nt. The length of the PAM sequence can range from about 2 nt to about 10 nt. For example, SauCas9 recognizes a 5 nt PAM (NGRRT), NmeCas9 recognizes a 8 nt PAM (NNNNGATT), and CjeCa9 recognized a 8 nt PAM (NNNNRYAC), wherein N is A, C, G, or T; R is A or G; and Y is C or T. As mentioned above, persons skilled in the art are familiar with means for combining sequences of interest.

**(c) gRNA**

[0036] The SIN vectors disclosed herein also comprise a nucleic acid sequence encoding a gRNA. A gRNA comprises a CRISPR repeat sequence (crRNA) comprising a spacer or guide sequence that hybridizes the complement of a target sequence, and a trans-activating crRNA (tracrRNA) sequence. A portion of the crRNA sequence base pairs with a

portion of the tracrRNA sequence to form a duplex, and the rest of the tracrRNA sequence can form secondary structure, e.g., at least one stem-loop structure(s), that mediates binding of gRNA to the CRISPR nuclease. The crRNA can comprise an optional spacer extension sequence at the 5' end, and the tracrRNA can comprise an optional tracrRNA extension sequence at the 3' end. A gRNA can be a single molecule (i.e., single molecule gRNA or sgRNA) or can comprise two separate molecules (*e.g.*, crRNA and tracrRNA). In general, the gRNAs encoded by the vectors disclosed herein are single molecule gRNAs.

[0037] The spacer sequence at the 5' end of the gRNA is a sequence that defines the target sequence of the target nucleic acid (e.g., a target genomic locus or the target intron sequence). As mentioned above, the target sequence is followed by a PAM sequence and the target sequence is cleaved by the CRISPR system. The "target nucleic acid" is a double-stranded molecule; one strand comprises the target sequence and is referred to as the "PAM strand," and the other complementary strand is referred to as the "non-PAM strand." One of skill in the art recognizes that the gRNA spacer sequence hybridizes to the complement of the target sequence, which is located in the non-PAM strand of the target nucleic acid. The spacer sequence of a gRNA interacts with a target nucleic acid in a sequence-specific manner via hybridization (i.e., base pairing). The nucleotide sequence of the spacer thus varies depending on the target sequence of the target locus.

[0038] In some embodiments, the spacer sequence of the gRNA can range in length from about 15 nt to about 25 nt. In various embodiments, the spacer sequence can range in length from about 16 nt to about 24 nt, from about 17 nt to about 23 nt, from about 18 nt to about 22 nt, from about 19 nt to about 21 nt. In some embodiments, the spacer sequence is 20 nt long. In general, the spacer sequence has at least about 90%, at least about 95%, or at least about 99% sequence identity to the target sequence in the target nucleic acid. In certain embodiments, the spacer sequence has 100% sequence identity to the target sequence.

[0039] In some embodiments, a single molecule gRNA can comprise, in the 5' to 3' direction, an optional spacer extension sequence, a spacer sequence, a minimum crRNA sequence, a single-molecule guide linker, a minimum tracrRNA sequence, a 3' tracrRNA sequence and an optional tracrRNA extension sequence. The optional tracrRNA extension may comprise elements that contribute additional functionality (*e.g.*, stability) to the guide RNA. The guide linker links the minimum CRISPR repeat and the minimum tracrRNA

sequence to form a hairpin structure. The optional tracrRNA extension may comprise one or more hairpins. The overall length of a single molecule gRNA can range from about 80 nt to about 250 nt.

[0040] The gRNA, in some embodiments, can comprise one or more uracil residues at the 3' end of the gRNA sequence. For example, the gRNA may comprise one (U), two (UU), three (UUU), four (UUUU) or more uracils at the 3' end of the gRNA sequence. In some embodiments the gRNA comprises 5, 6, 7, or 8 uracils at the 3' end of the gRNA sequence. In some embodiments the gRNA comprises 1 to 8, 2 to 8, 3 to 8, or 4 to 8 uracils at the 3' end of the gRNA sequence.

[0041] The nucleic acid sequence encoding the gRNA in the vectors disclosed herein is operably linked to a promoter sequence for expression in the cells of interest. In general, the sequence encoding the gRNA is operably linked to a Pol III promoter. Non-limiting examples of suitable Pol III promoters include mammalian U6, U3, H1, and 7SK promoters.

[0042] In some embodiments, the nucleic acid sequence encoding the gRNA can be operably linked to a Pol III transcription termination sequence (*e.g.*, a short run of T residues).

[0043] The sequence encoding the gRNA (and any operably linked sequence) can be in the same orientation or in the opposite orientation as the sequence encoding the CRISPR nuclease (and any operably linked sequence) in the SIN vectors disclosed herein.

**(d) Additional sequence elements**

[0044] Typically, the nucleic acid sequences encoding the CRISPR nuclease and the gRNA are flanked by adeno-associated virus (AAV) inverted terminal repeats (ITRs). Thus, the SIN vectors disclosed herein are linear vectors comprising 5' and 3' ITRs. Stated another way, the SIN vectors disclosed herein are recombinant AAV (rAAV) vectors.

[0045] The 5' and 3' ITRs flanking the CRISPR system coding sequence can be derived from any natural or recombinant AAV serotype. The 5' and 3' ITRs can be derived from the same or different AAV serotypes. Non-limiting examples of suitable serotypes include AAV1, AAV10, AAV106.1/hu.37, AAV11, AAV114.3/hu.40, AAV 12, AAV127.2/hu.41, AAV127.5/hu.42, AAV128.1/hu.43, AAV128.3/hu.44, AAV130.4/hu.48, AAV145.1/hu.53, AAV145.5/hu.54, AAV145.6/hu.55, AAV16.12/hu.11, AAV16.3,

AAV16.8/hu.10, AAV161.10/hu.60, AAV161.6/hu.61, AAVl-7/rh.48, AAVl-8/rh.49, AAV2, AAV2.5T, AAV2-15/rh.62, AAV223.1, AAV223.2, AAV223.4, AAV223.5, AAV223.6, AAV223.7, AAV2-3/rh.61, AAV24.1, AAV2-4/rh.50, AAV2-5/rh.51, AAV27.3, AAV29.3/bb.1, AAV29.5/bb.2, AAV2G9, AAV-2-pre-miRNA-101, AAV3, AAV3.1/hu.6, AAV3.1/hu.9, AAV3-11/rh.53, AAV3-3, AAV33.12/hu.17, AAV33.4/hu.15, AAV33.8/hu.16, AAV3-9/rh.52, AAV3a, AAV3b, AAV4, AAV4-19/rh.55, AAV42.12, AAV42-10, AAV42-11, AAV42-12, AAV42-13, AAV42-15, AAV42-1b, AAV42-2, AAV42-3a, AAV42-3b, AAV42-4, AAV42-5a, AAV42-5b, AAV42-6b, AAV42-8, AAV42-aa, AAV43-1, AAV43-12, AAV43-20, AAV43-21, AAV43-23, AAV43-25, AAV43-5, AAV4-4, AAV44.1, AAV44.2, AAV44.5, AAV46.2/hu.28, AAV46.6/hu.29, AAV4-8/r11.64, AAV4-8/rh.64, AAV4-9/rh.54, AAV5, AAV52.1/hu.20, AAV52/hu.19, AAV5-22/rh.58, AAV5-3/rh.57, AAV54.1/hu.21, AAV54.2/hu.22, AAV54.4R/hu.27, AAV54.5/hu.23, AAV54.7/hu.24, AAV58.2/hu.25, AAV6, AAV6.1, AAV6.1.2, AAV6.2, AAV7, AAV7.2, AAV7.3/hu.7, AAV8, AAV-8b, AAV-8h, AAV9, AAV9.11, AAV9.13, AAV9.16, AAV9.24, AAV9.45, AAV9.47, AAV9.61, AAV9.68, AAV9.84, AAV9.9, AAV A3.3, AAV A3.4, AAVA3.5, AAV A3.7, AAV-b, AAVC1, AAVC2, AAVC5, AAVCh.5, AAVCh.5R1, AAVcy.2, AAVcy.3, AAVcy.4, AAVcy.5, AAVCy.5R1, AAVCy.5R2, AAVCy.5R3, AAVCy.5R4, AAVcy.6, AAV-DJ, AAV-DJ8, AAVF3, AAVF5, AAV-h, AAVH-1/hu.1, AAVH2, AAVH-5/hu.3, AAVH6, AAVhE1.1, AAVhER1.14, AAVhEr1.16, AAVhEr1.18, AAVhER1.23, AAVhEr1.35, AAVhEr1.36, AAVhEr1.5, AAVhEr1.7, AAVhEr1.8, AAVhEr2.16, AAVhEr2.29, AAVhEr2.30, AAVhEr2.31, AAVhEr2.36, AAVhEr2.4, AAVhEr3.1, AAVhu.1, AAVhu.10, AAVhu.11, AAVhu.12, AAVhu.13, AAVhu.14/9, AAVhu.15, AAVhu.16, AAVhu.17, AAVhu.18, AAVhu.19, AAVhu.2, AAVhu.20, AAVhu.21, AAVhu.22, AAVhu.23.2, AAVhu.24, AAVhu.25, AAVhu.27, AAVhu.28, AAVhu.29, AAVhu.29R, AAVhu.3, AAVhu.31, AAVhu.32, AAVhu.34, AAVhu.35, AAVhu.37, AAVhu.39, AAVhu.4, AAVhu.40, AAVhu.41, AAVhu.42, AAVhu.43, AAVhu.44, AAVhu.44R1, AAVhu.44R2, AAVhu.44R3, AAVhu.45, AAVhu.46, AAVhu.47, AAVhu.48, AAVhu.48R1, AAVhu.48R2, AAVhu.48R3, AAVhu.49, AAVhu.5, AAVhu.51, AAVhu.52, AAVhu.53, AAVhu.54, AAVhu.55, AAVhu.56, AAVhu.57, AAVhu.58, AAVhu.6, AAVhu.60, AAVhu.61, AAVhu.63, AAVhu.64, AAVhu.66, AAVhu.67, AAVhu.7, AAVhu.8, AAVhu.9, AAVhu.t19, AAVLG-10/rh.40, AAVLG-4/rh.38, AAVLG-9/hu.39, AAVLG-9/hu.39, AAV-LK01, AAV-LK02,

AAVLK03, AAV-LK03, AAV-LK04, AAV-LK05, AAV-LK06, AAV-LK07, AAV-LK08, AAV-LK09, AAV-LK10, AAV-LK11, AAV-LK12, AAV-LK13, AAV-LK14, AAV-LK15, AAV-LK17, AAV-LK18, AAV-LK19, AAVN721-8/rh.43, AAV-PAEC, AAV-PAEC11, AAV-PAEC12, AAV-PAEC2, AAV-PAEC4, AAV-PAEC6, AAV-PAEC7, AAV-PAEC 8, AAVpi.1, AAVpi.2, AAVpi.3, AAVrh.10, AAVrh.12, AAVrh.13, AAVrh.13R, AAVrh.14, AAVrh.17, AAVrh.18, AAVrh.19, AAVrh.2, AAVrh.20, AAVrh.21, AAVrh.22, AAVrh.23, AAVrh.24, AAVrh.25, AAVrh.2R, AAVrh.31, AAVrh.32, AAVrh.33, AAVrh.34, AAVrh.35, AAVrh.36, AAVrh.37, AAVrh.37R2, AAVrh.38, AAVrh.39, AAVrh.40, AAVrh.43, AAVrh.44, AAVrh.45, AAVrh.46, AAVrh.47, AAVrh.48, AAVrh.48, AAVrh.48.1, AAVrh.48.1.2, AAVrh.48.2, AAVrh.49, AAVrh.50, AAVrh.51, AAVrh.52, AAVrh.53, AAVrh.54, AAVrh.55, AAVrh.56, AAVrh.57, AAVrh.58, AAVrh.59, AAVrh.60, AAVrh.61, AAVrh.62, AAVrh.64, AAVrh.64R1, AAVrh.64R2, AAVrh.65, AAVrh.67, AAVrh.68, AAVrh.69, AAVrh.70, AAVrh.72, AAVrh.73, AAVrh.74, AAVrh.8, AAVrh.8R, AAVrh8R, AAVrh8R A586R mutant, AAVrh8R R533A mutant, BAAV, BNP61 AAV, BNP62 AAV, BNP63 AAV, bovine AAV, caprine AAV, Japanese AAV 10, true type AAV (ttAAV), UPENN AAV 10, AAV-LK16, AAAV, AAV Shuffle 100-1, AAV Shuffle 100-2, AAV Shuffle 100-3, AAV Shuffle 100-7, AAV Shuffle 10-2, AAV Shuffle 10-6, AAV Shuffle 10-8, AAV SM 100-10, AAV SM 100-3, AAV SM 10-1, AAV SM 10-2, and/or AAV SM 10-8. In specific embodiments, the ITRs can be derived from AAV-8 or variant thereof.

**(II) Recombinant AAV Particles**

[0046] Another aspect of the present disclosure encompasses recombinant AAV (rAAV) particles (also called virions) comprising any one of the SIN CRISPR system nucleic acid vectors described above in section (I) that is encapsidated by at least one AAV capsid (Cap) protein. Typically, all the Cap proteins of an AAV are present in the particle. The Cap proteins can be wildtype AAV Cap proteins or can be variant AAV Cap proteins that may have altered and/or enhanced tropism towards one or more cell types. The rAAV particles can be generated as described below in section (IV).

**(III) Baculovirus Expression Vectors**

[0047] A further aspect of the present disclosure comprises recombinant baculovirus expression vectors comprising sequence encoding any one of the SIN CRISPR system nucleic acid vectors described above in section (I). The recombinant baculovirus expression vector comprises the nucleic acid sequence encoding the CRISPR nuclease, which is interrupted by the intron, and the nucleic acid sequence encoding the guide RNA, wherein the CRISPR system coding sequences are flanked by AAV ITRs. In general, the baculovirus expression vectors disclosed herein are derived from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). Means for producing baculovirus expression vectors are well known in the art (*see, e.g.*, Chambers et al., Current Protocols in Protein Science, 2018, 91, 5.4.1-5.4.6. doi: 10.1002/cpps.47).

**(IV) Methods for Producing SIN CRISPR System Vectors or rAAV Particles**

[0048] Still another aspect of the present disclosure encompasses methods for producing the SIN CRISPR system vectors described above in section (I). The methods comprise introducing into packaging cells one of the recombinant baculovirus vectors described above in section (III).

[0049] The method further comprises expressing AAV replication (Rep) and capsid (Cap) proteins in the packaging cell. In one embodiment, a packaging recombinant baculovirus sequence encoding AAV Rep and Cap proteins is introduced into the packaging cell, such that the packaging cells expresses said AAV Rep and Cap proteins. In another embodiment, the packaging cell comprises sequence encoding AAV Rep and Cap proteins stably integrated into its genome. The integrated sequence encoding said AAV Rep and Cap proteins is operably linked to an inducible promoter, such that upon induction of the promoter, the packaging cell expresses said AAV Rep and Cap proteins.

[0050] The method further comprises introducing into the packaging cell a helper recombinant baculovirus comprising sequence encoding AAV packaging components. For example, the helper recombinant baculovirus can encode E2A, E4, and VA genes.

[0051] The various recombinant baculoviruses can be introduced into the packaging cells by any suitable means, e.g., via transfection, electroporation, or the like. The method further comprises culturing the packaging cells under suitable conditions such that the packaging cells produce rAAV particles comprising a SIN AAV nucleic acid vector

encapsidated by Cap proteins. The rAAV particles can be harvested and purified using conventional means (e.g., chromatography).

[0052] In general, the packaging cell cells are insect cells. Non-limiting examples of suitable insect cells include those derived from *Spodoptera frugiperda*, e.g., Sf9, Sf-21 cells, or derived from *Trichoplusia ni*, e.g., Tn-368, BTI-TN-5B1-4. In specific embodiments, the packaging cells can be Sf9 cells.

## (V) Methods for Temporally Limiting Expression of CRISPR Nuclease

[0053] Yet another aspect of the present disclosure encompasses methods for temporally limiting expression of a CRISPR nuclease in eukaryotic cells, wherein the methods comprise introducing into eukaryotic cells any of the SIN CRISPR system nucleic acids described above in section (I) or the rAAV particles comprising the SIN CRISPR system nucleic acids described above in section (II).

[0054] Upon transcription of a SIN CRISPR system nucleic acid vector in the eukaryotic cells, the intron in the RNA encoding the CRISPR nuclease is spliced out such that a functional CRISPR nuclease is produced. The CRISPR nuclease complexes with the guide RNA to form a CRISPR system, wherein the CRISPR system cleaves the target genomic locus in the eukaryotic cell leading to an edited genomic locus, and the CRISPR system cleaves the intron in the nucleic acid sequence encoding the CRISPR nuclease in the SIN CRISPR system vector leading to degradation and inactivation of the SIN CRISPR system vector. In general, expression of the SIN CRISPR system vector is limited to the first several days after being introduced in to the eukaryotic cells.

[0055] The SIN CRISPR system nucleic acids or rAAV particles comprising the SIN CRISPR system nucleic acids can be introduced into the eukaryotic cells by a variety of methods. Non-limiting examples of suitable methods include transfection, nucleofection, electroporation, lipofection, sonoporation, and the like.

[0056] Cleavage of the target genomic locus can lead to insertion, deletion, and or substitution of at least nucleotide during cell mediated repair of the double stranded break (*e.g.*, non-homologous end joining, NHEJ, or microhomology-mediated end joining, MMEJ), such that small deletions and insertions can occur at the cleavage site. Such insertions and/or deletions (also called indels) can inactivate the target genomic locus such that functional gene product is not produced, thereby creating a "knockout." In other embodiments, the resultant

indels can reduce expression or lead to expression of a modified product from the targeted genomic locus, leading to a "knockdown."

**(a) Optional donor template**

[0057] In some embodiments, the method can further comprise introducing into the eukaryotic cells a donor template, wherein the donor template comprises a donor sequence that is flanked by sequence homologous to the target locus. During homology-directed repair (HDR) of double stranded break, the donor sequence can be integrated into or exchange with sequence at the target genomic locus, such that exogenous or modified sequences can be integrated into the genome.

[0058] In certain aspects, the donor sequence of the donor template can be a modified version of the target locus. For example, the donor sequence can be essentially identical to sequence at the target locus, but which comprises at least one nucleotide change. Thus, upon integration or exchange of the donor sequence into the target locus, the sequence at the target locus comprises at least one nucleotide change. For example, the nucleotide change can be an insertion of one or more nucleotides, a deletion of one or more nucleotides, a substitution of one or more nucleotides (e.g., modify a SNP), or combinations thereof. As a consequence of the "gene correction" integration of the donor sequence, the cell can produce a modified gene product from the targeted chromosomal sequence (i.e., target locus). In other embodiments, the nucleotide change can be a deletion of one or more nucleotides, such that the reading frame is interrupted and the cell no longer produces the gene product, thereby creating a "knockout" or "knockdown".

[0059] In other aspects, the donor sequence of the donor polynucleotide can be an exogenous sequence. As used herein, an "exogenous" sequence refers to a sequence that is not native to the cell, or a sequence whose native location is in a different location in the genome of the cell. For example, the exogenous sequence can comprise protein coding sequence, which can be operably linked to an exogenous promoter control sequence such that, upon integration into the genome, the cell is able to express the protein coded by the integrated sequence. Alternatively, the exogenous sequence can be integrated in-frame into the genome such that expression of the exogenous sequence is regulated by an endogenous promoter control sequence. In such cases, integration of an exogenous sequence into a chromosomal sequence is termed a "knock in." In other iterations, the exogenous sequence

can be a transcriptional control sequence, another expression control sequence, an RNA coding sequence, and so forth.

[0060] As can be appreciated by those skilled in the art, the length of the donor sequence can and will vary. For example, the donor sequence can vary in length from several nucleotides to hundreds of nucleotides to hundreds of thousands of nucleotides.

[0061] The donor template can be single stranded or double stranded, linear or circular. The donor template can be RNA or DNA. In some embodiments, the donor template can be a single stranded DNA oligonucleotide. In other embodiments, the donor template can be double stranded DNA provided to the cell as part of a vector.

[0062] In embodiments in which the donor template is part of a vector, suitable vectors include plasmid vectors, DNA minicircles, adenovirus vectors, adeno-associated virus vectors, alphavirus vectors, baculovirus vectors, enterovirus vectors, Epstein Barr virus vectors, herpesvirus vectors, lentiviral vectors, papovavirus vectors, pestivirus vectors, poxvirus vectors; retroviral vectors, vaccinia virus, and combinations thereof. In some embodiments, the vector can be an adeno-associated virus (AAV) vector or a recombinant AAV vector, examples of which are detailed above in section (I)(d).

**(b) Cells**

[0063] A variety of eukaryotic cells can be used in the methods disclosed herein. In some embodiments, the cells are mammalian cells. In specific embodiments, the cells are human cells.

[0064] In some embodiments, the cells can be *in vitro* (*e.g.*, cell line cells, cultured cells, primary cells). In other embodiments, the cells can be *ex vivo* cells isolated from an organism. In still other embodiments, the cells can be *in vivo* cells within an organism.

[0065] In some embodiments, the cells may be stem cells (*e.g.*, embryonic stem cells, fetal stem cells, amniotic stem cells, or umbilical cord stem cells). In certain embodiments, the stem cells may be adult stem cells isolated from bone marrow, adipose tissue, or blood. In still other embodiments, the cells may be induced pluripotent stem cells (*e.g.*, human iPSCs).

[0066] In particular embodiments, the cells may be hematopoietic stem and progenitor cells (HSPCs) or hematopoietic stem cells (HSCs). HSPCs give rise to all blood

cell types, including erythroid (erythrocytes or red blood cells (RBCs)), myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, megakaryocytes / platelets, and dendritic cells), and lymphoid (T-cells, B-cells, NK-cells). Blood cells are produced by the proliferation and differentiation of a very small population of pluripotent HSCs that also have the ability to replenish themselves by self-renewal. During differentiation, the progeny of HSCs progress through various intermediate maturational stages, generating multi-potential and lineage-committed progenitor cells prior to reaching maturity. Bone marrow (BM) is the major site of hematopoiesis in humans and, under normal conditions, only small numbers of HSPCs can be found in the peripheral blood (PB). Treatment with cytokines (in particular granulocyte colony-stimulating factor; G-CSF), some myelosuppressive drugs used in cancer treatment, and compounds that disrupt the interaction between hematopoietic and BM stromal cells can rapidly mobilize large numbers of stem and progenitors into the circulation. The cell surface glycoprotein CD34 is routinely used to identify and isolate HSPCs.

[0067] In other embodiments, the cells may be mesenchymal stem cells (*e.g.*, multipotent stromal cells that can differentiate into a variety of cell types). Mesenchymal stem cells (MSCs) are adult stem cells found in the bone marrow, or isolated from other tissues such as cord blood, peripheral blood, fallopian tube, and fetal liver and lung. As multipotent stem cells, MSCs differentiate into multiple cell types including adipocytes, chondrocytes, osteocytes, and cardiomyocytes. Mesenchymal stem cells are a distinct entity to the mesenchyme, embryonic connective tissue, which is derived from the mesoderm and differentiates to form hematopoietic stem cells (HPCs).

[0068] In still other embodiments, the cells may be immune cells such as T cells, B cells, natural killer (NK) cells, NKT cells, mast cells, eosinophils, basophils, macrophages, neutrophils, or dendritic cells.

[0069] In further embodiments, the cells may be primary cells isolated directly from human or animal tissue. Non-limiting examples of suitable primary cells include adipocytes, astrocytes, blood cells (e.g., erythroid, lymphoid), chondrocytes, endothelial cells, epithelial cells, fibroblasts, hair cells, hepatocytes, keratinocytes, melanocyte, myocytes, neurons, osteoblasts, skeletal muscle cells, smooth muscle cells, stem cells, or synoviocytes.

[0070] In additional embodiments, the cells can be (immortalized) mammalian cell line cells. Non-limiting examples of suitable mammalian cell lines include human embryonic kidney cells (HEK293, HEK293T); human cervical carcinoma cells (HELA);

human lung cells (W138); human liver cells (Hep G2); human U2-OS osteosarcoma cells, human A549 cells, human A-431 cells, and human K562 cells; Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells; mouse myeloma NS0 cells, mouse embryonic fibroblast 3T3 cells (NIH3T3), mouse B lymphoma A20 cells; mouse melanoma B16 cells; mouse myoblast C2C12 cells; mouse myeloma SP2/0 cells; mouse embryonic mesenchymal C3H-10T1/2 cells; mouse carcinoma CT26 cells, mouse prostate DuCuP cells; mouse breast EMT6 cells; mouse hepatoma Hepa1c1c7 cells; mouse myeloma J5582 cells; mouse epithelial MTD-1A cells; mouse myocardial MyEnd cells; mouse renal RenCa cells; mouse pancreatic RIN-5F cells; mouse melanoma X64 cells; mouse lymphoma YAC-1 cells; rat glioblastoma 9L cells; rat B lymphoma RBL cells; rat neuroblastoma B35 cells; rat hepatoma cells (HTC); buffalo rat liver BRL 3A cells; canine kidney cells (MDCK); canine mammary (CMT) cells; rat osteosarcoma D17 cells; rat monocyte/macrophage DH82 cells; monkey kidney SV-40 transformed fibroblast (COS7) cells; monkey kidney CVI-76 cells; African green monkey kidney (VERO-76) cells. An extensive list of mammalian cell lines may be found in the American Type Culture Collection catalog (ATCC, Manassas, VA).

**DEFINITIONS**

[0071] All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

[0072] The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

[0073] It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

[0074] In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases “consisting of” and “consisting

essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

[0075] The terms "about" and "substantially" preceding a numerical value mean ±10% of the recited numerical value.

[0076] As used herein, the terms "complementary" or "complementarity" refer to the association of double-stranded nucleic acids by base pairing through specific hydrogen bonds. The base paring may be standard Watson-Crick base pairing (e.g., 5'-A G T C-3' pairs with the complementary sequence 3'-T C A G-5'). The base pairing also may be Hoogsteen or reversed Hoogsteen hydrogen bonding. Complementarity is typically measured with respect to a duplex region and thus, excludes overhangs, for example. Complementarity between two strands of the duplex region may be partial and expressed as a percentage (e.g., 70%), if only some (e.g., 70%) of the bases are complementary. The bases that are not complementary are "mismatched." Complementarity may also be complete (i.e., 100%), if all the bases in the duplex region are complementary.

[0077] A "gene," as used herein, refers to a DNA region (including exons and introns) encoding a gene product, as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites, and locus control regions.

[0078] The terms "nuclease" and "endonuclease" are used interchangeably herein, and refer to an enzyme that cleaves both strands of a double-stranded nucleic acid sequence.

[0079] The terms "nucleic acid" and "polynucleotide" refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogs of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (e.g., phosphorothioate backbones). In general, an analog of a particular nucleotide has the same base-pairing specificity; i.e., an analog of A will base-pair with T.

[0080] The term “nucleotide” refers to deoxyribonucleotides or ribonucleotides. The nucleotides may be standard nucleotides (i.e., adenosine, guanosine, cytidine, thymidine, and uridine), nucleotide isomers, or nucleotide analogs. A nucleotide analog refers to a nucleotide having a modified purine or pyrimidine base or a modified ribose moiety. A nucleotide analog may be a naturally occurring nucleotide (e.g., inosine, pseudo uridine, etc.) or a non-naturally occurring nucleotide. Non-limiting examples of modifications on the sugar or base moieties of a nucleotide include the addition (or removal) of acetyl groups, amino groups, carboxyl groups, carboxymethyl groups, hydroxyl groups, methyl groups, phosphoryl groups, and thiol groups, as well as the substitution of the carbon and nitrogen atoms of the bases with other atoms (e.g., 7-deaza purines). Nucleotide analogs also include dideoxy nucleotides, 2’-O-methyl nucleotides, locked nucleic acids (LNA), peptide nucleic acids (PNA), and morpholinos.

[0081] The term "sequence identity" as used herein, indicates a quantitative measure of the degree of identity between two sequences of substantially equal length. The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequence and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, Wis.) in the “BestFit” utility application. Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+GenBank CDS translations+Swiss protein+Spupdate+PIR. Details of these programs can be found on the GenBank website.

[0082] The term "targeted deletion" refers to a deletion of at least one nucleotide base pair at a specific site in a target nucleic acid by a gene editing system that is engineered to target the specific site.

[0083] The terms "target sequence," "target site," and "site" are used interchangeably to refer to the specific sequence in a target nucleic acid to which the gene editing system is targeted.

[0084] The terms "treating" or "treatment," as used herein, refer to alleviating, ameliorating, or inhibiting the symptoms of a disease or disorder; reversing, inhibiting, or slowing the progression of a disease or disorder; and/or preventing or delaying the onset of a disease or disorder.

[0085] Where a range of values is provided, each value between the upper and lower ends of the range are specifically contemplated and described herein.

## EXAMPLES

[0086] The following examples illustrate various non-limiting embodiments of the present disclosure.

### Example 1. All-in-One SIN AAV8 Vector

[0087] A chimeric intron from pCI-Neo vector (Promega) was inserted at amino acid Asn 580 (AA^C) of 580) of the nucleotide sequence encoding SauCas9. The chimeric intron was modified to contain a gRNA binding site (e.g., 21 nt followed by PAM specific for Cas9). The Cas9 coding sequence was codon optimized and linked one NLS at each end. A recombinant AAV vector was constructed by inserting the Cas9 coding sequence operably linked to a muscle-specific promoter (*e.g.*, CK8e) and gRNA expression sequence operably linked to a Pol III promoter between the ITRs of AAV8 vector.

### Example 2. Baculoviral-Sf9 System for Generation of All-in-One SIN AAV8

[0088] All-in-one vector sequences were first cloned into pFastBac and transferred into bacmids in Bac-to-Bac DH10Bac cells. Similarly, bacmids containing the capsid and helper proteins were generated using Bac-to-Bac method. Baculoviruses were generated in insect Sf9 cells. AAV vector were generated by coinfection of Sf9 cells with the resulting baculoviruses. The infected cells were harvested at 72 hours post-infection and

lysed using a standard chemical method. The lysates were clarified by centrifugation and concentrated by tangential flow filtration. The vectors were further purified using affinity chromatography and buffer exchange was performed by ultrafiltration using a hollow fiber module.

[0089] Long-range PCR analysis confirmed that the genome remained intact (*e.g.*, no rearrangements and/or insertions/deletions in the vector genome) (see FIG. **1**, lane 5, labeled 1041CK8e-Bac SIN). In contrast, a rearaanged/deleted vector genome was observed when 1013CMV-SIN vector was produced using HEK 293T cells (FIG. **1**, lane 2). Nucleotide sequence verification of long-range PCR product confirmed intactness of genome of the all-in-one SIN AAV vector produced using the baculovirus-Sf9 method.

[0090] Quantitative PCR revealed that the yield of the all-in-one SIN Cas9/gRNA vector was about 1E13 vg/mL.

## CLAIMS

**What is claimed is:**

1. A nucleic acid comprising a sequence encoding a CRISPR nuclease and a sequence encoding a guide RNA (gRNA), wherein the sequence encoding the CRISPR nuclease comprises at least one intron, and the at least one intron comprises a binding site that is recognized by the CRISPR nuclease and gRNA.

2. The nucleic acid of claim 1, wherein the sequence encoding the CRISPR nuclease is codon optimized for expression in an eukaryotic cell of interest.

3. The nucleic acid of claim 1 or 2, wherein the CRISPR nuclease is linked to at least one nuclear localization signal.

4. The nucleic acid of any one of claims 1 to 3, wherein the intron is a mammalian intron, an engineered intron, or an artificial intron.

5. The nucleic acid of any one of claims 1 to 4, wherein the binding site in the intron is about 17 nucleotides to about 23 nucleotides in length and is followed by a protospacer adjacent motif (PAM) sequence.

6. The nucleic acid of any one of claims 1 to 5, wherein the sequence encoding the CRISPR nuclease is operably linked to a Pol II promoter, and the sequence encoding a guide RNA is operably linked to a Pol III promoter.

7. The nucleic acid of any one of claims 1 to 6, wherein the CRISPR nuclease is a Cas9 nuclease or a variant having at least 90% sequence identity to the Cas9 nuclease.

8. The nucleic acid of claim 7, wherein the Cas 9 is *Staphylococcus aureus* Cas9, *Neisseria meningitidis* Cas9, *Campylobacter jejuni* Cas9, *Azospirillum* B510 Cas9, *Campylobacter lari* CF89-12 Cas9, *Corynebacter diphtheriae* Cas9, *Eubacterium ventriosum* Cas9, *Gluconacetobacter diazotrophicus* Cas9, *Lactobacillus farciminis* Cas9, *Neisseria cinerea* Cas9, *Nitratitractor salsuginis* DSM 16511 Cas9, *Parvibaculum lavamentivorans* Cas9, *Roseburia intestinalis* Cas9, *Sphaerochaeta*

*globus* Cas9, *Streptococcus pasteurianus* Cas9, *Streptococcus thermophilus* CRISPR1, or *Streptococcus thermophilus* LMD-9 Cas9.

9. The nucleic acid of claim 7, wherein the Cas 9 nuclease is *Staphylococcus aureus* Cas9, *Neisseria meningitidis* Cas9, or *Campylobacter jejuni* Cas9.

10. The nucleic acid of any one of claims 1 to 9, wherein the sequence encoding the CRISPR nuclease and the sequence encoding the gRNA are flanked by adeno-associated virus (AAV) inverted terminal repeats (ITRs).

11. The nucleic acid of claim 10, wherein the AAV ITRs are derived from AAV-8.

12. The nucleic acid of claim 11, wherein the sequence encoding the CRISPR nuclease is operably linked to a muscle specific promoter.

13. The nucleic acid of any one of claims 1 to 12, wherein the nucleic acid self-inactivates within about one to three days after being introduced into an eukaryotic cell.

14. A baculovirus expression vector comprising the nucleic acid of any one of claims 1 to 13.

15. A recombinant AAV particle comprising the nucleic acid of any one of claims 1 to 13 and at least one capsid protein.

16. A method for producing the nucleic acid of any one of claims 1 to 13, the method comprising introducing into a packaging cell a recombinant baculovirus comprising the sequence encoding the CRISPR nuclease, which is interrupted by the intron, and the sequence encoding the guide RNA, which are flanked by the AAV ITRs.

17. The method of claim 16, further comprising expressing AAV replication (Rep) and capsid (Cap) proteins in the packaging cell.

18. The method of claim 17, wherein a packaging recombinant baculovirus sequence encoding AAV Rep and Cap proteins is introduced into the packaging cell.

19. The method of claim 17, wherein the packaging cell comprises sequence encoding AAV Rep and Cap proteins stably integrated into its genome, wherein said sequence encoding AAV Rep and Cap proteins is operably linked to an inducible promoter.

20. The method of any one of claims 16 to 19, further comprising introducing into the packaging cell a helper recombinant baculovirus comprising sequence encoding AAV packaging components.

21. The method of any one of claims 16 to 20, wherein, upon expression of the recombinant baculovirus encoding the CRISPR nuclease and gRNA, a functional CRISPR nuclease is not produced.

22. The method of any one of claims 16 to 21, wherein the recombinant baculovirus encoding the CRISPR nuclease and gRNA is derived from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV).

23. The method of any one of claims 16 to 22, wherein the packaging cell is a *Spodoptera frugiperda* Sf9 cell.

24. The method of any one of claims 16 to 23, wherein the packaging cell produces AAV particles comprising the nucleic acid of any one of claims 1 to 13 that is encapsidated by at least one AAV Cap protein.

25. A method for temporally limiting expression of a CRISPR nuclease in an eukaryotic cell, the method comprising introducing into the eukaryotic cell the nucleic acid of any one of claims 1 to 13 or the recombinant AAV particle of claim 15.

26. The method of claim 25 wherein, upon expression of the nucleic acid, the intron is excised and a CRISPR nuclease is produced, and the CRISPR nuclease complexes with the guide RNA to form a CRISPR system, wherein the CRISPR system cleaves the target genomic locus in the eukaryotic cell leading to an edited genomic locus, and the CRISPR system cleaves the intron in the sequence encoding the CRISPR nuclease in the nucleic acid, thereby inactivating the nucleic acid.

27. The method of claims 25 or 26, wherein the nucleic acid or the recombinant AAV particle is inactivated within about 1-3 days after being introduced into the eukaryotic cell, thereby temporally limiting expression of the CRISPR nuclease.

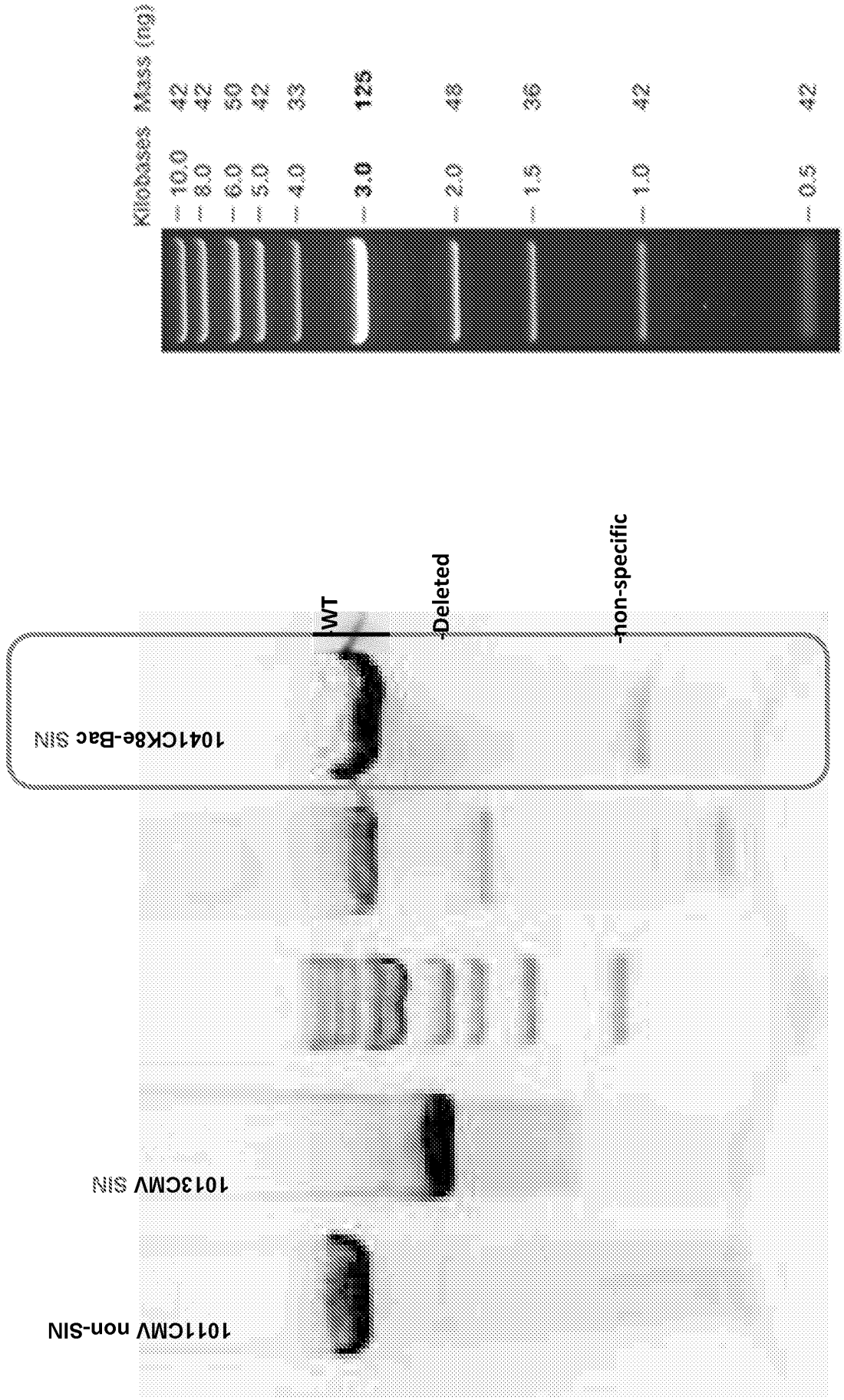
Kilobases
Mass (ng)
10.0
8.0
6.0
5.0
4.0
3.0
2.0
1.5
1.0
0.5
42
42
60
42
33
125
48
36
42
42
WT
Deleted
non-specific
1041CK8e-Bac SIN
1013CMV SIN
1011CMV non-SIN

FIG. 1

# INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2020/058683

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/22 C12N15/11
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)
C12N

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, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 16 December 2020 | 18/01/2021 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Sprinks, Matthew |

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