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(54) **Title**: DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR GENOME EDITING

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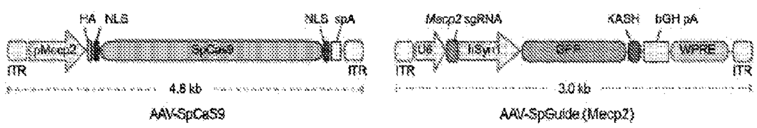

(57) **Abstract**: The invention provides for delivery, engineering and optimization of systems, methods, and compositions for manipulation of sequences and/or activities of target sequences. Provided are delivery systems and tissues or organ which are targeted as sites for delivery. Also provided are vectors and vector systems some of which encode one or more components of a CRISPR complex, as well as methods for the design and use of such vectors. Also provided are methods of directing CRISPR complex formation in eukaryotic cells to ensure enhanced specificity for target recognition and avoidance of toxicity and to edit or modify a target site in a genomic locus of interest to alter or improve the status of a disease or a condition.

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DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR GENOME EDITING

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

[0001] This application claims priority to US provisional patent applications: 61/915,176; 61/915,192; 61/915,215; 61/915,107, 61/915,145; 61/915,148; and 61/915,153 each filed December 12, 2013.

[0002] The foregoing applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, and all documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

FIELD OF THE INVENTION

[0003] The present invention generally relates to the delivery, engineering, optimization and therapeutic applications of systems, methods, and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and components thereof. In particular, the present invention relates to *in vitro*, *ex vivo* and/or *in vivo* systems, methods, and compositions for delivery of the CRISPR-Cas system to achieve therapeutic benefits *via* genome editing in animals, including mammals.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0004] This invention was made with government support under the NIH Pioneer Award (1DP1MH100706) awarded by the National Institutes of Health and grant 1DP1OD009552 also provided by National Insitutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0005] Recent advances in genome sequencing techniques and analysis methods have significantly accelerated the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. Precise genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology, biotechnological, and medical applications. Although genome-editing techniques such as designer zinc fingers (ZFN), transcription activator-like effectors (TALEs), or homing meganucleases are available for producing targeted genome perturbations, there remains a need for new genome engineering technologies that are affordable, easy to set up, scalable, and amenable to targeting multiple positions within the eukaryotic genome.

SUMMARY OF THE INVENTION

[0006] Despite valid therapeutic hypotheses and strong efforts in drug development, there have only been a limited number of successes using small molecules to treat diseases with strong genetic contributions. Thus, there exists a pressing need for alternative and robust systems for therapeutic strategies that are able to modify nucleic acids within disease-affected cells and tissues. Adding the CRISPR-Cas system to the repertoire of therapeutic genome engineering methods significantly simplifies the methodology and accelerates the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases, develop animal models for genetic diseases, and develop safe, effective therapeutic alternatives. To utilize the CRISPR-Cas system effectively for genome editing without deleterious effects, it is critical to understand aspects of engineering, optimization and cell-type/tissue/organ specific delivery of these genome engineering tools, which are aspects of the claimed invention. Aspects of this invention address this need and provide related advantages.

[0007] An exemplary CRISPR complex may comprise a CRISPR enzyme (*e.g.*, Cas9) complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence. Applicants have optimized components of the CRISPR-Cas genome engineering system, including using SaCas9 from *Staphylococcus aureus*. Various delivery means may be employed for delivering components of the CRISPR-Cas sytem to cells, tissues and organs, ex vivo and/or in vivo. Applicants have effectively packaged CRISPR-Cas system components (e.g., comprising SaCas9) into a viral delivery vector, e.g., AAV, and have demonstrated that it can be used to modify endogenous genome sequence in mammalian cells *in vivo*. A key feature of Applicants' present invention it that it effectively addresses the challenges of low efficiency of *in vivo* delivery (of therapeutic components) and low efficiency of homology directed repair (HDR) and in particular challenges associated with co-delivery are solved by the small Cas9, SaCas9 from *Staphylococcus aureus*, which can be readily packaged into a single Adeno-associated virus (AAV) vector to express both the Cas9 protein and its corresponding sgRNA(s). Further, importantly, Applicants have shown that introduction of small SaCas9, has reduced the number of viral vectors required to perform HDR from 3 vectors to 2 vectors. In aspects of the invention particles may be used for delivery of one or more components of the CRISPR-Cas system. And the number of particles to be contacted with can be one or two. In one aspect, the invention provides methods for using one or more elements of a CRISPR-Cas system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide in a genomic locus, wherin the genomic locus is associated with a mutation, including muations associated with an aberrant protein expression or with a disease condition or state. The CRISPR complex of the invention has a wide variety of utilities including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide within a genomic locus, including within a copding, non-coding or regulatory element of such a target locus.. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene or genome editing, gene therapy, drug discovery, drug screening, disease diagnosis, and prognosis. Aspects of the invention relate to Cas9 enzymes having improved targeting specificity in a CRISPR-Cas9 system having guide RNAs having optimal activity, smaller in length than wild-type Cas9 enzymes and nucleic acid molecules coding therefor, and chimeric Cas9 enzymes, as well as methods of improving the target specificity of a Cas9 enzyme or of designing a CRISPR-Cas9 system comprising designing or preparing guide RNAs having optimal activity and/or selecting or preparing a Cas9 enzyme having a smaller size or length than wild-type Cas9 whereby packaging a nucleic acid coding therefor into a delivery vector is more advanced as there is less coding therefor in the delivery vector than for wild-type Cas9, and/or generating chimeric Cas9 enzymes. Also provided are uses of the present sequences, vectors, enzymes or systems, in medicine. Also provided are uses of the same in gene or genome editing.

[0008] In the invention, the Cas enzyme can be wildtype Cas9 including any naturally-occurring bacterial Cas9. Cas9 orthologs typically share the general organization of 3-4 RuvC domains and a HNH domain. The 5' most RuvC domain cleaves the non-complementary strand, and the HNH domain cleaves the complementary strand. All notations are in reference to the guide sequence. The catalytic residue in the 5' RuvC domain is identified through homology comparison of the Cas9 of interest with other Cas9 orthologs (from S. pyogenes type II CRISPR locus, S. thermophilus CRISPR locus 1, S. thermophilus CRISPR locus 3, and Franciscilla novicida type II CRISPR locus), and the conserved Asp residue (D10) is mutated to alanine to convert Cas9 into a complementary-strand nicking enzyme. Similarly, the conserved His and Asn residues in the HNH domains are mutated to Alanine to convert Cas9 into a non-complementary-strand nicking enzyme. In some embodiments, both sets of mutations may be made, to convert Cas9 into a non-cutting enzyme. Accordingly, the Cas enzyme can be wildtype Cas9 including any naturally-occurring bacterial Cas9. The CRISPR, Cas or Cas9 enzyme can be codon optimized for human cells, including specific types of human cells, or a modified version, including any chimeras, mutants, homologs or orthologs. In an additional aspect of the invention, a Cas9 enzyme may comprise one or more mutations and may be used as a generic DNA binding protein with or without fusion to a functional domain. The mutations may be artificially introduced mutations or gain- or loss-of-function mutations. The mutations may include but are not limited to mutations in one of the catalytic domains (D10 and H840) in the RuvC and HNH catalytic domains, respectively. Further mutations have been characterized. In one aspect of the invention, the transcriptional activation domain may be VP64. In other aspects of the invention, the transcriptional repressor domain may be KRAB or SID4X. Other aspects of the invention relate to the mutated Cas 9 enzyme being fused to domains which include but are not limited to a transcriptional activator, repressor, a recombinase, a transposase, a histone remodeler, a demethylase, a DNA methyltransferase, a cryptochrome, a light inducible/controllable domain or a chemically inducible/controllable domain. The invention can involve sgRNAs or tracrRNAs or guide or chimeric guide sequences that allow for enhancing performance of these RNAs in cells. The CRISPR enzyme can be a type I or III CRISPR enzyme, preferably a type II CRISPR enzyme. This type II CRISPR enzyme may be any Cas enzyme. A preferred Cas enzyme may be identified as Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 or saCas9. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein.

[0009] It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCas9, St1Cas9 and so forth. Further examples are provided herein. The skilled person will be able to determine appropriate corresponding residues in Cas9 enzymes other than SpCas9 by comparison of the relevant amino acid sequences. Thus, where a specific amino acid replacement is referred to using the SpCas9 numbering, then, unless the context makes it apparent this is not intended to refer to other Cas9 enzymes, the disclosure is intended to encompass corresponding modifications in other Cas9 enzymes. An example of a codon optimized sequence, in this instance optimized for humans (i.e. being optimized for expression in humans) is provided herein, see the SaCas9 human codon optimized sequence. Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species is known. The invention comprehends methods wherein the Cas9 is a chimeric Cas9 proteins. These methods may comprise N-terminal fragment(s) of one Cas9 homolog with C-terminal fragment(s) of one or more other or another Cas9 homolog. It will be appreciated that in the present methods, where the organism is an animal, the modification may occur ex vivo or in vitro, for instance in a cell culture and in some instances not in vivo. In other embodiments, it may occur in vivo. The invention comprehends in some embodiments a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme), wherein the target sequence is flanked at its 3' end by a PAM (protospacer adjacent motif) sequence comprising 5'-motif, especially where the Cas9 is (or is derived from) S. pyogenes or S. aureus Cas9. For example, a suitable PAM is 5'-NRG or 5'-NNGRR (where N is any Nucleotide) for SpCas9 or SaCas9 enzymes (or derived enzymes). It will be appreciated that SpCas9 or SaCas9 are those from or derived from S. pyogenes or S. aureus Cas9.

[0010] In one aspect, the invention provides a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest, wherein the genomic locus is associated with a mutation associated with an aberrant protein expression or with a disease condition or state comprising:

delivering a non-naturally occurring or engineered composition comprising:

A) I. a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a polynucleotide sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA; and

the method may optionally include also delivering a HDR template, e.g., via a viral delivery vector or a particle, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and

optionally the method may include isolating or obtaining cells expressing said aberrant protein from the organism or non-human organism, optionally expanding the cell population, performing contacting of the viral vector or particle(s) with said cells to obtain a modified cell population, optionally expanding the population of modified cells, and optionally administering modified cells to the organism or non-human organism.

[0011] In one aspect, the invention provides a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest, wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising: contacting a cell with a viral vector or particle containing, a non-naturally occurring or engineered composition comprising: I. (a) a guide sequence capable of hybridizing to a target sequence in a HSC, and (b) at least one or more tracr mate sequences, II. a CRISPR enzyme optionally having one or more NLSs, and III. a polynucleotide sequence comprising a tracr sequence, wherein the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and

the method may optionally include also delivering a HDR template, e.g., via a viral delivery vector or a particle, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and

optionally the method may include isolating or obtaining cells expressing said aberrant protein from the organism or non-human organism, optionally expanding the cell population, performing contacting of the viral vector or particle(s) with ssaid cells to obtain a modified cell population, optionally expanding the population of modified cells, and optionally administering modified cells to the organism or non-human organism.

[0012] The delivery can be of one or more polynucleotides encoding any one or more or all of the CRISPR-complex, advantageously linked to one or more regulatory elements for in vivo expression, e.g. via particle(s), containing a vector containing the polynucleotide(s) operably linked to the regulatory element(s). Any or all of the polynucleotide sequence encoding a CRISPR enzyme, guide sequence, tracr mate sequence or tracr sequence, may be RNA. It will be appreciated that where reference is made to a polynucleotide, which is RNA and is said to 'comprise' a feature such a tracr mate sequence, the RNA sequence includes the feature. Where the polynucleotide is DNA and is said to comprise a feature such a tracr mate sequence, the DNA sequence is or can be transcribed into the RNA including the feature at issue. Where the feature is a protein, such as the CRISPR enzyme, the DNA or RNA sequence referred to is, or can be, translated (and in the case of DNA transcribed first).

[0013] In certain embodiments the invention provides a method of modifying an organism, e.g., mammal including human or a non-human mammal or organism by manipulation of a target sequence in a genomic locus of interest e.g., wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising delivering, e.g., via contacting of a non-naturally occurring or engineered composition with a cell or cell population , wherein the composition comprises one or more delivery vectors or particles comprising viral, plasmid or nucleic acid molecule vector(s) (e.g. RNA) operably encoding a composition for expression thereof, wherein the composition comprises: (A) I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, (b) a tracr mate sequence, and (c) a tracr sequence, and II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (or optionally at least one or more nuclear localization sequences as some embodiments can involve no NLS), wherein (a), (b) and (c) are arranged in a 5` to 3' orientation, wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, or (B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and (b) at least one or more tracr mate sequences, II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and III. a third regulatory element operably linked to a tracr sequence, wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; the method may optionally include also delivering a HDR template, e.g., via the delivery vectors or particle contacting the cell or cell population or contacting the cell cell or cell population with another delivery vector or particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining cells expressing said aberrant proteins from the organism or non-human organism, optionally expanding said cell population, performing contacting of the delivery vector or particle(s) with said cells expressing said aberrant proteins to obtain a modified cell population, optionally expanding the population of modified cells and optionally administering modified cells to the organism or non-human organism. In some embodiments, components I, II and III are located on the same vector. In other embodiments, components I and II are located on the same vector, while component III is located on another vector. In other embodiments, components I and III are located on the same vector, while component II is located on another vector. In other embodiments, components II and III are located on the same vector, while component I is located on another vector. In other embodiments, each of components I, II and III is located on different vectors. The invention also provides a viral or plasmid vector system as described herein.

[0014] By manipulation of a target sequence, Applicants also mean the epigenetic manipulation of a target sequence. This may be of the chromatin state of a target sequence, such as by modification of the methylation state of the target sequence (i.e. addition or removal of methylation or methylation patterns or CpG islands), histone modification, increasing or reducing accessibility to the target sequence, or by promoting 3D folding. It will be appreciated that where reference is made to a method of modifying an organism or mammal including human or a non-human mammal or organism by manipulation of a target sequence in a genomic locus of interest, this may apply to the organism (or mammal) as a whole or just a single cell or population of cells from that organism (if the organism is multicellular). In the case of humans, for instance, Applicants envisage, *inter alia*, a single cell or a population of cells and these may preferably be modified *ex vivo* and then re-introduced. In this case, a biopsy or other tissue or biological fluid sample may be necessary. Stem cells are also particularly preferred in this regard. But, of course, *in vivo* embodiments are also envisaged. And the invention is especially advantageous as to ocular cells, retinal cells, vascular cells, epithelial cells, endothelial cells, and cochlear cells.

[0015] The invention in some embodiments comprehends a method of modifying an organism or a non-human organism by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a cell or cell population e.g., wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising delivering, e.g., by contacting the cell or cell population with a delivery vector, e.g., viral vectors or particles comprising a non-naturally occurring or engineered composition comprising :

I. a first CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the first polynucleotide sequence comprises:
   (a) a first guide sequence capable of hybridizing to the first target sequence,
   (b) a first tracr mate sequence, and
   (c) a first tracr sequence,

II. a second CRISPR-Cas system chiRNA polynucleotide sequence, wherein the second polynucleotide sequence comprises:
   (a) a second guide sequence capable of hybridizing to the second target sequence,
   (b) a second tracr mate sequence, and
   (c) a second tracr sequence, and

III. a polynucleotide sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences and comprising one or more mutations, wherein (a), (b) and (c) are arranged in a 5' to 3' orientation; or

IV. expression product(s) of one or more of I. to III., e.g., the the first and the second tracr mate sequence, the CRISPR enzyme;

wherein when transcribed, the first and the second tracr mate sequence hybridize to the first and second tracr sequence respectively and the first and the second guide sequence directs sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively, wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the first tracr mate sequence that is hybridized to the first tracr sequence, wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the second tracr mate sequence that is hybridized to the second tracr sequence, wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of the other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism; and the mthod may optionally include also delivering a HDR template, e.g., via the delivery vector contacting the cell or cell population containing or contacting the cell or cell population with another delivery vector containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining a cell or cell population from the organism or non-human organism, optionally expanding the cell population, performing contacting of the delivery vector or particle(s) with the cell or cell population to obtain a modified cell population, optionally expanding the population of modified cells A method of modeling a disease associated with a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus comprising delivering a non-naturally occurring or engineered composition comprising a viral vector system comprising one or more viral vectors operably encoding a composition for expression thereof, wherein the composition comprises:

(A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the target sequence,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding SaCas9, optionally comprising at least one or more nuclear localization sequences, wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the SaCas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

or

(B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to the target sequence, and

(b) at least one or more tracr mate sequences,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding SaCas9, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the SaCas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence., and optionally administering modified cells to the organism or non-human organism. In some methods of the invention any or all of the polynucleotide sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA. In further embodiments of the invention the polynucleotides encoding the sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA and are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun; but, it is advantageous that the delivery is via a viral vector or a particle. In certain embodiments of the invention, the first and second tracr mate sequence share 100% identity and/or the first and second tracr sequence share 100% identity. In some embodiments, the polynucleotides may be comprised within a vector system comprising one or more vectors. In preferred embodiments of the invention the CRISPR enzyme is a Cas9 enzyme, e.g. SpCas9 or SaCas9. In an aspect of the invention the CRISPR enzyme comprises one or more mutations in a catalytic domain, wherein the one or more mutations, with reference to SpCas9 are selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A, e.g., a D10A mutation. In preferred embodiments, the first CRISPR enzyme has one or more mutations such that the enzyme is a complementary strand nicking enzyme, and the second CRISPR enzyme has one or more mutations such that the enzyme is a non-complementary strand nicking enzyme. Alternatively the first enzyme may be a non-complementary strand nicking enzyme, and the second enzyme may be a complementary strand nicking enzyme. In preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of the other strand near the second target sequence results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0016] With respect to mutations of the CRISPR enzyme, when the enzyme is not SpCas9, mutations may be made at any or all residues corresponding to positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 (which may be ascertained for instance by standard sequence comparison tools). In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. In an aspect the invention provides as to any or each or all embodiments herein-discussed wherein the CRISPR enzyme comprises at least one or more, or at least two or more mutations, wherein the at least one or more mutation or the at least two or more mutations is as to D10, E762, H840, N854, N863, or D986 according to SpCas9 protein, e.g., D10A, E762A, H840A, N854A, N863A and/or D986A as to SpCas9, or N580 according to SaCas9, e.g., N580A as to SaCas9, or any corresponding mutation(s) in a Cas9 of an ortholog to Sp or Sa, or the CRISPR enzyme comprises at least one mutation wherein at least H840 or N863A as to Sp Cas9 or N580A as to Sa Cas9 is mutated; e.g., wherein the CRISPR enzyme comprises H840A, or D10A and H840A, or D10A and N863A, according to SpCas9 protein, or any corresponding mutation(s) in a Cas9 of an ortholog to Sp protein or Sa protein.

[0017] The invention in some embodiments comprehends a method of modifying an organism or a non-human organism by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a cell or cell population e.g., wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, comprising delivering, e.g., by contacting the cells or cell population with a delivery vector or particle(s) comprising a non-naturally occurring or engineered composition comprising :

I. a first regulatory element operably linked to
   (a) a first guide sequence capable of hybridizing to the first target sequence, and
   (b) at least one or more tracr mate sequences,
II. a second regulatory element operably linked to
   (a) a second guide sequence capable of hybridizing to the second target sequence, and
   (b) at least one or more tracr mate sequences,
III. a third regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and
IV. a fourth regulatory element operably linked to a tracr sequence,
V. expression product(s) of one or more of I. to IV., e.g., the the first and the second tracr mate sequence, the CRISPR enzyme;

wherein components I, II, III and IV are located on the same or different vectors of the system, when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the first and the second guide sequence direct sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively, wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of the other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism; and the method may optionally include also delivering a HDR template, e.g., via the delivery vector or particle contacting the cell or cell population containing or contacting the cell or cell population with another particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining a cell or cell population from the organism or non-human organism, optionally expanding the cells, performing contacting of the delivery vector or particle(s) with the cell or cell population to obtain a modified cell population, optionally expanding the population of modified cells, and optionally administering modified HSCs to the organism or non-human organism.

[0018] The invention also provides a vector system as described herein. The system may comprise one, two, three or four different vectors. Components I, II, III and IV may thus be located on one, two, three or four different vectors, and all combinations for possible locations of the components are herein envisaged, for example: components I, II, III and IV can be located on the same vector; components I, II, III and IV can each be located on different vectors; components I, II, II I and IV may be located on a total of two or three different vectors, with all combinations of locations envisaged, etc. In some methods of the invention any or all of the polynucleotide sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA. In further embodiments of the invention the first and second tracr mate sequence share 100% identity and/or the first and second tracr sequence share 100% identity. In preferred embodiments of the invention the CRISPR enzyme is a Cas9 enzyme, e.g. SpCas9. In an aspect of the invention the CRISPR enzyme comprises one or more mutations in a catalytic domain, wherein the one or more mutations with reference to SpCas9 are selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A; e.g., D10A mutation. In preferred embodiments, the first CRISPR enzyme has one or more mutations such that the enzyme is a complementary strand nicking enzyme, and the second CRISPR enzyme has one or more mutations such that the enzyme is a non-complementary strand nicking enzyme. Alternatively the first enzyme may be a non-complementary strand nicking enzyme, and the second enzyme may be a complementary strand nicking enzyme. In a further embodiment of the invention, one or more of the viral vectors are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun; but, viral delivery or particle delivery is advantageous

[0019] In preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of other strand near the second target sequence results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0020] The invention in some embodiments comprehends a method of modifying a genomic locus of interest in cell or cell poulation e.g., wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, by introducing into the cell or cell population, e.g., by contacting the cells or cell population with delivery vectors or particle(s) comprising, a Cas protein having one or more mutations and two guide RNAs that target a first strand and a second strand of the DNA molecule respectively in the cell or cell population, whereby the guide RNAs target the DNA molecule and the Cas protein nicks each of the first strand and the second strand of the DNA molecule, whereby a target in the cell or cell population is altered; and, wherein the Cas protein and the two guide RNAs do not naturally occur together and the mthod may optionally include also delivering a HDR template, e.g., via the delivery vector or particle contacting the cell or cell poulation containing or contacting the cell or population with another delivery vector or particle containing the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining cells from the organism or non-human organism, optionally expanding the cell population, performing contacting of the delivery vector or particle(s) with the cells to obtain a modified cell population, optionally expanding the population of modified cells and optionally administering modified cells to the organism or non-human organism. In preferred methods of the invention the Cas protein nicking each of the first strand and the second strand of the DNA molecule results in a 5' overhang. In embodiments of the invention the 5' overhang is at most

200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs. Embodiments of the invention also comprehend the guide RNAs comprising a guide sequence fused to a tracr mate sequence and a tracr sequence. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9 or SaCas9. In aspects of the invention the Cas protein has one or more mutations in respect of SpCas9 selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A; e.g., a D10A mutation. Aspects of the invention relate to the expression of a gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein.

[0021] The invention in some embodiments comprehends a method of modifying a genomic locus of interest in a cell or cell population e.g., wherein the genomic locus of interest is associated with a mutation associated with an aberrant protein expression or with a disease condition or state, by introducing into the cell or cell population, e.g., by contacting the cell or cell population with a delivery vector or particle(s) comprising,

a) a first regulatory element operably linked to each of two CRISPR-Cas system guide RNAs that target a first strand and a second strand respectively of a double stranded DNA molecule of the cell or cells within the cell population, and

b) a second regulatory element operably linked to a Cas protein, or

c) expression product(s) of a) or b),

wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the DNA molecule of the cells or cells within the cell population and the Cas protein nicks each of the first strand and the second strand of the DNA molecule of the cells or cells within the cell ; and, wherein the Cas protein and the two guide RNAs do not naturally occur together; and the method may optionally include also delivering a HDR template, e.g., via the delivery vector or particle contacting the cell or cell population containing or contacting the cell or cell population with another particle containing, the HDR template wherein the HDR template provides expression of a normal or less aberrant form of the protein; wherein "normal" is as to wild type, and "aberrant" can be a protein expression that gives rise to a condition or disease state; and optionally the method may include isolating or obtaining cells from the organism or non-human organism, optionally expanding said cell population, performing contacting of the delivery vector or particle(s) with the cells to obtain a modified cell population, optionally expanding the population of modified cells, and optionally administering modified cells to the organism or non-human organism. In aspects of the invention the guide RNAs may comprise a guide sequence fused to a tracr mate sequence and a tracr sequence. In an embodiment of the invention the Cas protein is a type II CRISPR-Cas protein. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9 or SaCas9. In aspects of the invention the Cas protein has one or more mutations with reference to SpCas9 selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A; e.g., the D10A mutation. Aspects of the invention relate to the expression of a gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein. In preferred embodiments of the invention the vectors of the system are viral vectors. In a further embodiment, the vectors of the system are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun; and particles are preferred. In one aspect, the invention provides a method of modifying a target polynucleotide in a cell or cell population. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence. In some embodiments, the method further comprises delivering one or more vectors or expression product(s) thereof, e.g., via a delivery vector or particle(s), to said cell or cell population, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence. In some embodiments, said vectors are delivered to a cell or a cell population in a subject. In some embodiments, said modifying takes place in said cell or cell population in a cell culture. In some embodiments, the method further comprises isolating said cell or cell population from a subject prior to said modifying. In some embodiments, the method further comprises returning said cell or cell population and/or cells derived therefrom to said subject.

[0022] In one aspect, the invention provides a method of generating a cell or cell population comprising a mutated disease gene. In some embodiments, a disease gene is any gene associated with an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) introducing one or more vectors or expression product(s) thereof, e.g., via a delivery vector or particle(s), into a cell or cell population, wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; and (b) allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said disease gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, thereby generating a cell or cell population comprising a mutated disease gene. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expression from a gene comprising the target sequence. In some embodiments the modified cell or cell population is administered to an animal to thereby generate an animal model.

[0023] In one aspect, the invention provides for methods of modifying a target polynucleotide in a cell or cell population. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In other embodiments, this invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell that arises from a cell or cell population expressing an aberrant protein. The method comprises increasing or decreasing expression of a target polynucleotide by using a CRISPR complex that binds to the polynucleotide in the cell or cell population; advantageously the CRISPR complex is delivered via a viral delivery vector or particle(s).

[0024] In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a cell or cell population. For example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does.

[0025] In some embodiments, the functional domain is a transcriptional activation domain, preferably VP64.In some embodiments, the functional domain is a transcription repression domain, preferably KRAB. In some embodiments, the transcription repression domain is SID, or concatemers of SID (eg SID4X). In some embodiments, the functional domain is an epigenetic modifying domain, such that an epigenetic modifying enzyme is provided. In some embodiments, the functional domain is an activation domain, which may be the P65 activation domain.

[0026] The invention further comprehends a composition of the invention or a CRISPR complex or enzyme thereof or RNA thereof (including or alternatively mRNA encoding the CRISPR enzyme) for use in medicine or in therapy. In some embodiments the invention comprehends a composition according to the invention or components thereof for use in a method according to the invention. In some embodiments the invention provides for the use of a composition of the invention or a CRISPR complex or enzyme thereof or RNA thereof (including or alternatively mRNA encoding the CRISPR enzyme) in *ex vivo* gene or genome editing, especially in a cell or cell population which optionally may then be introduced into an organism or non-human organism from which the cells or cell population were obtained or another organism or non-human organism of the same species. In certain embodiments the invention comprehends use of a composition of the invention or a CRISPR complex or enzyme thereof or RNA thereof (including or alternatively mRNA encoding the CRISPR enzyme) in the manufacture of a medicament for *ex vivo* gene or genome editing or for use in a method according of the invention. In certain embodiments the invention provides a method of treating or inhibiting a condition caused by a defect in a target sequence in a genomic locus of interest in a subject (e.g., mammal or human) or a non-human subject (e.g., mammal) in need thereof comprising modifying a cell or a cell population of the subject or a non-human subject by manipulation of the target sequence in the cell or cell population and administering the modified cells to the subject or non-human subject, advantageously the modifying of the cells is through contacting the cells with a delivery vector (e.g., viral) or particle containing the CRISPR complex or the components thereof, advantageously in certain embodiments the delivery vector (viral) or particle also provides a HDR template or another particle or a vector provides the HDR template, and wherein the condition is susceptible to treatment or inhibition by manipulation of the target sequence.

[0027] Certain RNA of the CRISPR Cas complex is also known and referred to as sgRNA (single guide RNA). In advantageous embodiments RNA of the CRISPR Cas complex is sgRNA. The CRISPR-Cas9 system has been engineered to target genetic locus or loci in a cell or cell population. Cas9 protein, advantageously codon-optimized for a eukaryotic cell and especially a mammalian cell, e.g., a human cell, (e.g., ocular cell, vascular cell, cochclear cell, etc.) and sgRNA targeting a locus or loci in the cell, e.g., the gene *RHO*, *ATOH1*, *VEGFA* were prepared, and are exemplified herein. These were advantageously delivered via a viral delivery

(AAV). When delivered via particles, the particles are formed by the Cas9 protein and the sgRNA being admixed. The sgRNA and Cas9 protein mixture is admixed with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol, whereby particles containing the sgRNA and Cas9 protein are formed. The invention comprehends so making particles and particles from such a method as well as uses thereof. More generally, particles were formed using an efficient process. First, Cas9 protein and sgRNA targeting a gene or a control gene LacZ are mixed together at a suitable, e.g.,3:1 to 1:3 or 2:1 to 1:2 or 1:1 molar ratio, at a suitable temperature, e.g., 15-30C, e.g., 20-25C, e.g., room temperature, for a suitable time, e.g., 15-45, such as 30 minutes, advantageously in sterile, nuclease free buffer, e.g., 1X PBS. Separately, particle components such as or comprising: a surfactant, e.g., cationic lipid, e.g., 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); phospholipid, e.g., dimyristoylphosphatidylcholine (DMPC); biodegradable polymer, such as an ethylene-glycol polymer or PEG, and a lipoprotein, such as a low-density lipoprotein, e.g., cholesterol were dissolved in an alcohol, advantageously a C_{1-6} alkyl alcohol, such as methanol, ethanol, isopropanol, e.g., 100% ethanol. The two solutions are mixed together to form particles containing the Cas9-sgRNA complexes. In certain embodiments the particle can contain an HDR template. That can be a particle co-administered with sgRNA+Cas9 protein-containing particle, or i.e., in addition to contacting a cell or cell population with an sgRNA+Cas9 protein-containing particle, the cell or cell population is contacted with a particle containing an HDR template; or the HSC is contacted with a particle containing all of the sgRNA, Cas9 and the HDR template. The HDR template can be administered by a separate vector, whereby in a first instance the particle penetrates an HSC cell and the separate vector also penetrates the cell, wherein the HSC genome is modified by the sgRNA+Cas9 and the HDR template is also present, whereby a genomic loci is modified by the HDR; for instance, this may result in correcting a mutation. The particle in the herein discussion is advantageously obtained or obtainable from admixing an sgRNA(s) and Cas9 protein mixture (optionally containing HDR template(s) or such mixture only containing HDR template(s) when separate particles as to template(s) is desired) with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol (wherein one or more sgRNA targets agenetic locus or loci associated with a mutation associated with an aberrant protein xpression or with a disease condition or state).

[0028] In one aspect, the invention provides for methods of modeling a disease associated with a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus comprising delivering a non-naturally occurring or engineered composition comprising :

(A) - I. a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a guide sequence capable of hybridizing to the target sequence,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a polynucleotide sequence encoding Cas9, optionally comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding Cas9 is DNA or RNA,

or

(B) I. polynucleotides comprising:

(a) a guide sequence capable of hybridizing to the target sequence, and

(b) at least one or more tracr mate sequences,

II. a polynucleotide sequence encoding Cas9, and

III. a polynucleotide sequence comprising a tracr sequence,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and the polynucleotide sequence encoding Cas9 is DNA or RNA.

[0029] In certain preferred embodiments, the Cas9 is SaCas9.

[0030] In one aspect, the invention provides for methods of modeling a disease associated with a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus comprising delivering a non-naturally occurring or engineered composition comprising a viral vector system comprising one or more viral vectors operably encoding a composition for expression thereof, wherein the composition comprises:

(A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the target sequence,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, (preferably SaCas9) optionally comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

or

(B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to the target sequence, and

(b) at least one or more tracr mate sequences,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence.

[0031] In one aspect the invention provides methods of treating or inhibiting a condition or a disease caused by one or more mutations in a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus in a target sequence in a subject or a non-human subject in need thereof comprising modifying the subject or a non-human subject by manipulation of the target sequence and wherein the condition or disease is susceptible to treatment or inhibition by manipulation of the target sequence comprising providing treatment comprising:

delivering a non-naturally occurring or engineered composition comprising an AAV or lentivirus vector system, comprising one or more AAV or lentivirus vectors operably encoding a composition for expression thereof, wherein the target sequence is manipulated by the composition when expressed, wherein the composition comprises:

(A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, preferably SaCas9, comprising at least one or more nuclear localization sequences,

wherein (A), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,
wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and
wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the arget sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

or

(B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to an target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, preferably SaCas9, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system,
wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and
wherein the CRISPR complex comprises Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence.

[0032] In certain embodiments, the invention provides method of preparing the AAV or lentivirus vector for use in accordance with any of the methods of the invention, comprising transfecting plasmid(s) containing or consisting essentially of nucleic acid molecule(s) coding for the AAV or lentivirus into AAV-infected or lentivirus-infected cells, and supplying AAV AAV or lentivirus rep and/or cap and/or helper nucleic acid molecules obligatory for replication and packaging of the AAV or lentivirus.

[0033] In one aspect, the invention provides a composition for use in any of the methods of invention (e.g., method of modeling a disease associated with a genetic locus in a eukaryotic organism or a non-human organism) comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genetic locus. In certain embodiments, the invention provides for uses of the composition in ex vivo or in vivo gene or genome editing, including therapeutic uses.

[0034] In one aspect, the invention provides a composition for use in the manufacture of a medicament for *in vitro*, *ex vivo or in vivo* gene or genome editing or for use in a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus associated with a disease or in a method of treating or inhibiting a condition or diesease caused by one or more mutations in a genomic locus in a eukaryotic organism or a non-human organism.

[0035] In one aspect, the invention provides a composition comprising:

(A) - I. a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a polynucleotide sequence encoding Cas9, preferably Sa Cas9, optionally comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding Cas9 is DNA or RNA,

or

(B) I. polynucleotides comprising:

(a) a guide sequence capable of hybridizing to an target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a polynucleotide sequence encoding Cas9, preferably SaCas9, and

III. a polynucleotide sequence comprising a tracr sequence, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises SaCas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and the polynucleotide sequence encoding Cas9 is DNA or RNA;

for use in medicine or therapy; or for use in a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus associated with a disease or disorder; or for use in a method of treating or inhibiting a condition caused by one or more mutations in a genetic locus associated with a disease in a eukaryotic organism or a non-human organism.; or for use in *in vitro, ex vivo or in vivo* gene or genome editing.

[0036] In one aspect, the invention provides a therapeutic genome editing method for treating or inhibiting a condition or a disease caused by one or more mutations in a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus in a target sequence in a subject or a non-human subject in need thereof comprising modifying the subject or a non-human subject by manipulation of the target sequence and wherein the condition or disease is susceptible to treatment or inhibition by manipulation of the target sequence comprising providing treatment comprising:

delivering a non-naturally occurring or engineered composition comprising an AAV or lentivirus vector system, comprising one or more AAV or lentivirus vectors operably encoding a composition for expression thereof, wherein the target sequence is manipulated by the composition when expressed, wherein the composition comprises:

(A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, preferably SaCas9, comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the arget sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

or

(B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to an target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding SaCas9, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr.

[0037] In one aspect, the invention provides a method of individualized or personalized treatment of a genetic disease in a subject in need of such treatment comprising:

(a) introducing multiple mutations *ex vivo* in a tissue, organ or a cell line comprising Cas9-expressing eukaryotic cell(s) (preferably Sa Cas9), or *in vivo* in a transgenic non-human mammal having cells that express Cas9, comprising delivering to cell(s) of the tissue, organ, cell or mammal the vector as herein-discussed, wherein the specific mutations or precise sequence substitutions are or have been correlated to the genetic disease;

(b) testing treatment(s) for the genetic disease on the cells to which the vector has been delivered that have the specific mutations or precise sequence substitutions correlated to the genetic disease; and

(c) treating the subject based on results from the testing of treatment(s) of step (b).

[0038] In certain embodiments of any of the aforementioned aspects and embodiments of the invention, the viral vector may be an AAV, e.g., AAV1, AAV2, AAV5, AAV7, AAV8, AAV DJ or any combination thereof.

[0039] In herein discussions concerning the target being associated with a mutation or with a disease condition, such mutation or disease condition can be, for instance a neuronal disease; ocular disease (e.g., retina disease, e.g., retinitis pigmentosa; achromtaopsia; age-related macular degeneration; visual impairment), auditory disease (e.g., cochlear-cell associated disease, hearing impairment, deafness) etc.

[0040] Accordingly, it is an object of the invention to not encompass within the invention any previously known product, process of making the product, or method of using the product such that Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product.

[0041] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as “comprises”, “comprised”, “comprising” and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean “includes”, “included”, “including”, and the like; and that terms such as “consisting essentially of” and “consists essentially of” have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention. It may be advantageous in the practice of the invention to be in compliance with Art. 53(c) EPC and Rule 28(b) and (c) EPC. There are no promises in this document.

[0042] These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0043] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0044] Figure 1A-H shows CRISPR-Cas9 system delivery and targeting of *Mecp2* locus in the mouse brain. (**a**) AAV-SpCas9 and AAV-SpGuide(*Mecp2*) expression vectors. The sgRNA vector contains encoding sequence of the GFP-KASH fusion protein for identification of transduced neurons. (**b**) Expression of HA-Cas9 and GFP-KASH in the dorsal dentate gyrus (DG) of mouse hippocampus. Scale bar, 100 μm. (**c**) Quantification of cells efficiently targeted by the dual-vector Cas9-CRISPR system. (**d**) Graphical representation of the mouse *Mecp2* locus showing Cas9 target location; sgRNA indicated in blue. PAM sequence marked in purple. Representative mutation patterns detected by sequencing of *Mecp2* locus were shown below: green - wild-type sequence; red dashes - deleted bases; red bases: insertion or mutations; red arrowhead indicates CRISPR-Cas9 cutting site. (**e**) SURVEYOR™ assay gel showing modification of the *Mecp2* locus, 2 weeks after AAV delivery in the DG region. (**f**) Western blot analysis of MeCP2 protein expression in the targeted brain region and quantification of MeCP2 protein levels in dorsal DG (*t*-test, $^{**}p<0.001$, n=4 from 3 animals, error bars: s.e.m.). (**g**) Images of the dorsal DG region, 2 weeks after CRISPR-Cas9 targeting of *Mecp2* locus. Scale bar, 150 μm. (**h**) Quantification of MeCP2 positive cells population within all detected cells (DAPI staining) in the targeted brain region in compare to control collateral site (*t*-test, $^{****}p<0.0001$, n=290 and 249 cells from 2 animals, respectively; error bars: s.e.m). (ITR – inverted terminal repeat; HA – hemagglutinin tag; NLS – nuclear localization signal; spA – synthetic polyadenylation signal; U6 – PolIII promoter; sgRNA – single guide RNA; hSyn – human synapsin 1 promoter; GFP- green fluorescent protein; KASH - Klarsicht, ANC1, Syne Homology nuclear transmembrane domain; bGH pA – bovine growth hormone polyadenylatio signal; WPRE – Woodchuck Hepatitis virus posttranscriptional regulatory element).

[0045] Figure 2A-B shows analysis of gene expression in Cas9-mediated MeCP2 knockdown neurons. (**a**) Strategy for cell nuclei purification of CRISPR-Cas9 targeted cells from the mouse brain. (**b**) Hierarchical clustering of differentially expressed genes (*t*-test, $p<0.01$, n=19 populations of sorted nuclei from 8 animals) detected by RNAseq. Relative log2(TPM+1) expression levels of genes are normalized for each row and displayed in red-blue color scale. Each column represents a population of targeted 100 neuronal nuclei FACS sorted from the isolated, dentate gyrus population of cells, either from control or *Mecp2* sgRNA transduced animals, as indicated.

[0046] Figure 3A-E shows cell-autonomous defects in cellular response properties of neurons after CRISPR-mediated MeCP2 knockdown. (a) Cartoon showing *in vivo* experiment configuration from mouse visual cortex and visual stimulation parameter. GFP^+ neuron is shown. Scale bar, 20 μm. (b) Cartoon showing recording configuration in layer 2/3 excitatory neurons that receive both contra- and ipsilateral eye specific input. Genome modified GFP^+ cells are in green whereas unmodified cells are in gray. Normalized spike shape shows regular spiking excitatory neurons. (c,d) Average OSI (c) and evoked FR (d) were measured from GFP^+ cells expressing *Mecp2* and control sgRNA, respectively (*t*-test, *$p<0.05$; numbers in graph indicate numbers of recorded cells; n=2-3 animals; error bars: s.e.m).

[0047] Figure 4A-F shows simultaneous, multiplex gene editing in the mouse brain. (a) Schematic illustration of CRISPR-Cas9 system designed for multiplex genome targeting. (b) Graphical representation of targeted DNMT mouse loci. Guide RNAs are indicated in blue. PAM sequences are marked in purple. (c) SURVEYOR™ assay gel showing modification of DNMTs loci in FACS sorted GFP-KASH positive cells, 4 weeks after AAV delivery in the DG region. (d) Deep sequencing-based analysis of DNMTs loci modification in single cells, showing co-occurrence of modification in multiple loci. (e) Western blot analysis for Dnmt3a and Dnmt1 proteins after in vivo delivery of CRISPR-Cas9 system targeting DNMT family genes (top). Western blot quantification of Dnmt3a and Dnmt1 protein levels in DG after in vivo CRISPR-Cas9 targeting (bottom; t-test, **p<0.001, *p<0.05, Dnmt3a: n=7; Dnmt1: n=5 from 5 animals; error bars: s.e.m). (f) Contextual learning deficits, 8 weeks after targeting of DNMT genes using

SpCas9 in the DG region of hippocampus, tested in training and altered context (t-test, ***p<0.0001, n=18 animals, 2 independent experiments; error bars: s.e.m).

[0048] Figure 5A-F shows cloning and expression of HA-tagged SpCas9 (HA-SpCas9) for AAV packaging. (**a**) Schematic overview of different cloning strategies to minimize SpCas9 expression cassette size using short rat *Map1b* promotor (pMap1b), a truncated version of the mouse *Mecp2* promoter (pMecp2) and a short polyA motif (spA). (**b**) Western blot analysis of primary cortical neuron culture expressing HA-SpCas9 using different SpCas9 expression cassettes. (**c**) *Mecp2* promoter drives HA-SpCas9 (red) expression in neurons (Map1b, NeuN; arrows) but not in astroglia (GFAP, arrowheads). Co-expressioin of HA-SpCas9 with GFP-KASH is shown (bottom). Nuclei were labeled with DAPI (blue). Scale bars, 20 μm. (**d**) Schematic overview of GFP-labeling. Enhanced green fluorescent protein (GFP) fused to the nuclear transmembrane KASH domain and integration of GFP-KASH to the outer nuclear membrane is illustrated. (**e**) Co-infection efficiency calculation, showing populations of cell expressing both HA-SpCas9 and GFP-KASH (n=973 neurons from 3 cultures; error bars: s.e.m). (**f**) Cells were stained with LIFE/DEAD® kit 7 days after virus delivery. Quantification of $DAPI^+$ and dead ($DEAD^+$) cells (control n=518 $DAPI^+$ nuclei; SpCas9/GFP-KASH n=1003 $DAPI^+$ nuclei from 2 cultures; error bars: s.e.m). (ITR – inverted terminal repeat; HA – hemagglutinin tag; NLS – nuclear localization signal; spA – synthetic polyadenylation signal; U6 – PolIII promoter; sgRNA – single guide RNA; hSyn – human synapsin 1 promoter; GFP- green fluorescent protein; KASH - Klarsicht, ANC1, Syne Homology nuclear transmembrane domain; bGH pA – bovine growth hormone polyadenylation signal; WPRE – Woodchuck Hepatitis virus posttranscriptional regulatory element).

[0049] Figure 6A-B shows targeting of *Mecp2* in Neuro-2a cells. (**a**) *Mecp2* targeting sequences and corresponding protospacer adjacent motifs (PAM). (**b**) Evaluation of 6 *Mecp2* sgRNAs co-transfected with SpCas9 into Neuro-2a cells. Locus modification efficiencies were analyzed 48 h after transfection using SURVEYOR™ assay.

[0050] Figure 7A-D shows CRISPR-SpCas9 targeting of *Mecp2* in primary cortical neurons. (**a**) Immunofluorescent staining of MeCP2 (red) in cultured neurons 7 days after AAV-CRISPR transduction (green, GFP-KASH). Nuclei were labeled with DAPI (blue). Scale bar, 20 μm. (**b**) Evaluation of *Mecp2* locus targeting using SpCas9 or dSpCas9, together with *Mecp2* sgRNA or control (targeting bacterial *lacZ* gene) sgRNA, using SURVEYOR™ assay gel. (**c**)

Quantification of MeCP2 positive nuclei in targeted population of neurons (GFP^+). (**d**) Western blot of MeCP2 protein levels after CRISPR-SpCas9 targeting of *Mecp2* locus and quantification of MeCP2 protein levels (*t*-test, $**p<0.001$, n=5 from 3 cultures, error bars: s.e.m).

[0051] Figure 8A-E shows morphological changes in dendritic tree of neurons after SpCas9-mediated MeCP2 knockdown *in vitro*. (a) Reduced complexity of dendritic tree in neurons after CRISPR-SpCas9 targeting of *Mecp2* locus. Scale bar, 20 μm. (b) Changes in dendritic spines morphology in neurons targeted with SpCas9 and *Mecp2* sgRNA. Scale bar, 10 μm. Morphology of cells was visualized with co-transfection with mCherry construct. Cells for morphology analysis were chosen based on the result of Mecp2 staining. (c) Dendritic tree morphology assessed with number of dendritic ends and (d) Sholl analysis (*t*-test, $***p<0.0001$, n=40 from 2 cultures). (e) Spine density quantification (*t*-test, $***p<0.0001$, n=40 from 2 cultures, error bars: s.e.m).

[0052] Figure 9 shows RNAseq of neuronal nuclei from control animals and SpCas9-mediated Mecp2 knockdown. Box plot presenting the number of detected genes across the RNA-seq libraries (19 libraries each of 100 nuclei taken from control sgRNA or *Mecp2* sgRNA transduced nuclei; n=4 animals/group) per quantile of expression level. All genes are divided to 10 quantiles by their mean log2(TPM+1) expression level, then for each quantile the number of genes that are detected (log2(TPM+1)>2) was counted in each sample. The three target sequences shown are SEQ ID NO: ___ , SEQ ID NO: ___ and SEQ ID NO: ___, for Dnmt3a, Dnmt1 and Dnmt3b, respectively.

[0053] Figure 10A-B shows multiplex genome targeting of DNMT family members *in vitro*. (**a**) *Dnmt3a*, *Dnmt1* and *Dnmt3b* targeting sequences and corresponding protospacer adjacent motifs (PAM). (**b**) SURVEYOR™ nuclease assay analysis of Neuro-2a cells 48 hours after transfection with SpCas9 and DNMT 3xsgRNA vector targeting *Dnmt3a*, *Dnmt1* and *Dnmt3b* loci. Efficient genome editing of all three targeted genes is shown.

[0054] Figure 11A-C shows next generation sequencing of targeted *Dnmt3a*, *Dnmt1* and *Dnmt3b* loci. Examples of sequencing results of mutated *Dnmt3a* (**a**), *Dnmt1* (**b**) and *Dnmt3b* (**c**) loci after *in vivo* delivery of SpCas9 and DNMT 3xsgRNA into the mouse dentate gyrus. Green: wild-type sequence, red dashes: deleted bases, red bases: insertion or mutations. Red arrowheads indicate CRISPR-SpCas9 cutting site. The full sequences used in this figure are provide as SEQ ID NO: , SEQ ID NO: , and SEQ ID NO: for the Dnmt3a, the Dnmt1 and the Dnmt3b loci, respectively. They are: SEQ ID NO: (Dnmt3a): CCT CCG TGT CAG CGA CCC ATG CCA A, SEQ ID NO: (Dnmt1): CCA GCG TCG AAC AGC TCC AGC CCG and SEQ ID NO: (Dnmt3b) AGA GGG TGC CAG CGG GTA TAT GAG G

[0055] **Figure 12** shows comparison of different programmable nuclease platforms.

[0056] **Figure 13A-C** show types of therapeutic genome modifications. The specific type of genome editing therapy depends on the nature of the mutation causing disease. **a**, In gene disruption, the pathogenic function of a protein is silenced by targeting the locus with NHEJ. Formation of indels on the gene of interest often result in frameshift mutations that create premature stop codons and a non-functional protein product, or non-sense mediated decay of transcripts, suppressing gene function. **b**, HDR gene correction can be used to correct a deleterious mutation. DSB is targeted near the mutation site in the presence of an exogenously provided, corrective HDR template. HDR repair of the break site with the exogenous template corrects the mutation, restoring gene function. **c**, An alternative to gene correction is gene addition. This mode of treatment introduces a therapeutic transgene into a safe-harbor locus in the genome. DSB is targeted to the safe-harbor locus and an HDR template containing homology to the break site, a promoter and a transgene is introduced to the nucleus. HDR repair copies the promoter-transgene cassette into the safe-harbor locus, recovering gene function, albeit without true physiological control over gene expression.

[0057] **Figure 14** shows a schematic representation of *ex vivo* vs. *in vivo* editing therapy. In *ex vivo* editing therapy cells are removed from a patient, edited and then re-engrafted (top panel). For this mode of therapy to be successful, target cells must be capable of survival outside the body and homing back to target tissues post-transplantation. In vivo therapy involves genome editing of cells *in situ* (bottom panels). For *in vivo* systemic therapy, delivery agents that are relatively agnostic to cell identity or state would be used to effect editing in a wide range of tissue types. Although this mode of editing therapy may be possible in the future, no delivery systems currently exist that are efficient enough to make this feasible. *In vivo* targeted therapy, where delivery agents with tropism for specific organ systems are administered to patients are feasible with clinically relevant viral vectors.

[0058] **Figure 15** shows SaCas9 system for ocular gene therapy

[0059] **Figure 16** shows a schematic representation of gene therapy *via* Cas9 Homologous Recombination (HR) vectors.

[0060] **Figure 17** shows an exemplary protocol for ocular gene therapy.

[0061] **Figure 18A-B** show the human *RHO* locus (allele showing P23H mutation). Fig. 7A shows the guide design for RHO locus. Fig. 7B shows the in vitro guide screening results using the SURVEYOR assay.

[0062] **Figure 19** shows RHO HR AAV vector.

[0063] **Figure 20A-B** shows guide selection for CNGA3 and CNGB3. (a) shows human *CNGA3* locus (allele showing two disease mutations) and guide selection. (b) shows human *CNGB3* locus (allele showing disease mutation) and guide selection.

[0064] **Figure 21** shows CNGA3 HR AAV vector.

[0065] **Figure 22** shows CNGB3 HR AAV vector.

[0066] **Figure 23A-B** show guide selection for VEGFA. (a) shows human VEGFA lcous (common region 1); (b) shows human VEGFA lcous (common region 2);

[0067] **Figure 24** shows design of dCas9-based epigenetic modulation system (3 components of the system, dSaCas9, fusion effector, and sgRNA are shown).

[0068] **Figure 25A-C** shows guide selection for ATOH1. (a) shows two higly accessible regions which were selected; (b) shows highly accessible region 1-blue lines indicate guide sequence and magenta lines indicate PAM; (c) shows highly accessible region 2-blue lines indicate guide sequence and magenta lines indicate PAM.

[0069] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[0070] With respect to general information on CRISPR-Cas Systems, components thereof, and delivery of such components, including methods, materials, delivery vehicles, vectors, particles, AAV, and making and using thereof, including as to amounts and formulations, all useful in the practice of the instant invention, reference is made to: US Patents Nos. 8,697,359, 8,771,945, 8,795,965, 8,865,406, 8,871,445, 8,889,356, 8,889,418 and 8,895,308; US Patent Publications US 2014-0310830 (US APP. Ser. No. 14/105,031), US 2014-0287938 A1 (U.S. App. Ser. No. 14/213,991), US 2014-0273234 A1 (U.S. App. Ser. No. 14/293,674), US2014-0273232 A1 (U.S. App. Ser. No. 14/290,575), US 2014-0273231 (U.S. App. Ser. No. 14/259,420), US 2014-0256046 A1 (U.S. App. Ser. No. 14/226,274), US 2014-0248702 A1 (U.S. App. Ser. No. 14/258,458), US 2014-0242700 A1 (U.S. App. Ser. No. 14/222,930), US

2014-0242699 A1 (U.S. App. Ser. No. 14/183,512), US 2014-0242664 A1 (U.S. App. Ser. No. 14/104,990), US 2014-0234972 A1 (U.S. App. Ser. No. 14/183,471), US 2014-0227787 A1 (U.S. App. Ser. No. 14/256,912), US 2014-0189896 A1 (U.S. App. Ser. No. 14/105,035), US 2014-0186958 (U.S. App. Ser. No. 14/105,017), US 2014-0186919 A1 (U.S. App. Ser. No. 14/104,977), US 2014-0186843 A1 (U.S. App. Ser. No. 14/104,900), US 2014-0179770 A1 (U.S. App. Ser. No. 14/104,837) and US 2014-0179006 A1 (U.S. App. Ser. No. 14/183,486), US 2014-0170753 (US App Ser No 14/183,429); European Patent Applications EP 2 771 468 (EP13818570.7), EP 2 764 103 (EP13824232.6), and EP 2 784 162 (EP14170383.5); and PCT Patent Publications WO 2014/093661 (PCT/US2013/074743), WO 2014/093694 (PCT/US2013/074790), WO 2014/093595 (PCT/US2013/074611), WO 2014/093718 (PCT/US2013/074825), WO 2014/093709 (PCT/US2013/074812), WO 2014/093622 (PCT/US2013/074667), WO 2014/093635 (PCT/US2013/074691), WO 2014/093655 (PCT/US2013/074736), WO 2014/093712 (PCT/US2013/074819), WO2014/093701 (PCT/US2013/074800), and WO2014/018423 (PCT/US2013/051418). Reference is also made to US provisional patent applications 61/758,468; 61/802,174; 61/806,375; 61/814,263; 61/819,803 and 61/828,130, filed on January 30, 2013; March 15, 2013; March 28, 2013; April 20, 2013; May 6, 2013 and May 28, 2013 respectively. Reference is also made to US provisional patent application 61/836,123, filed on June 17, 2013. Reference is additionally made to US provisional patent applications 61/835,931, 61/835,936, 61/836,127, 61/836, 101, 61/836,080 and 61/835,973, each filed June 17, 2013. Further reference is made to US provisional patent applications 61/862,468 and 61/862,355 filed on August 5, 2013; 61/871,301 filed on August 28, 2013; 61/960,777 filed on September 25, 2013 and 61/961,980 filed on October 28, 2013. Reference is yet further made to: PCT Patent applications Nos: PCT/US2014/041803, PCT/US2014/041800, PCT/US2014/041809, PCT/US2014/041804 and PCT/US2014/041806, each filed June 10, 2014 6/10/14; PCT/US2014/041808 filed June 11, 2014; and PCT/US2014/62558 filed October 28, 2014, and US Provisional Patent Applications Serial Nos.: 61/915,150, 61/915,301, 61/915,267 and 61/915,260, each filed December 12, 2013; 61/757,972 and 61/768,959, filed on January 29, 2013 and February 25, 2013; 61/835,936, 61/836,127, 61/836,101, 61/836,080, 61/835,973, and 61/835,931, filed June 17, 2013; 62/010,888 and 62/010,879, both filed June 11, 2014; 62/010,329 and 62/010,441, each filed June 10, 2014; 61/939,228 and 61/939,242, each filed February 12, 2014; 61/980,012, filed April 15,2014;

62/038,358, filed August 17, 2014; 62/054,490, 62/055,484, 62/055,460 and 62/055,487, each filed September 25, 2014; and 62/069,243, filed October 27, 2014. Reference is also made to US provisional patent applications Nos. 62/055,484, 62/055,460, and 62/055,487, filed September 25, 2014; US provisional patent application 61/980,012, filed April 15, 2014; and US provisional patent application 61/939,242 filed February 12, 2014. Reference is made to PCT application designating, inter alia, the United States, application No. PCT/US14/41806, filed June 10, 2014. Reference is made to US provisional patent application 61/930,214 filed on January 22, 2014. Reference is made to US provisional patent applications 61/915,251; 61/915,260 and 61/915,267, each filed on December 12, 2013. Reference is made to US provisional patent application USSN 61/980,012 filed April 15, 2014. Reference is made to PCT application designating, inter alia, the United States, application No. PCT/US14/41806, filed June 10, 2014. Reference is made to US provisional patent application 61/930,214 filed on January 22, 2014. Reference is made to US provisional patent applications 61/915,251; 61/915,260 and 61/915,267, each filed on December 12, 2013. Each of these patents, patent publications, and applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, together with any instructions, descriptions, product specifications, and product sheets for any products mentioned therein or in any document therein and incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. All documents (e.g., these patents, patent publications and applications and the appln cited documents) are incorporated herein by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

[0071] Also with respect to general information on CRISPR-Cas Systems, mention is made of the following (also hereby incorporated herein by reference):

- *Multiplex genome engineering using CRISPR/Cas systems*. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., & Zhang, F. *Science* Feb 15;339(6121):819-23 (2013);

- *RNA-guided editing of bacterial genomes using CRISPR-Cas systems*. Jiang W., Bikard D., Cox D., Zhang F, Marraffini LA. Nat Biotechnol Mar;31(3):233-9 (2013);

- *One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering*. Wang H., Yang H., Shivalila CS., Dawlaty MM., Cheng AW., Zhang F., Jaenisch R. Cell May 9;153(4):910-8 (2013);

- *Optical control of mammalian endogenous transcription and epigenetic states*. Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM, Zhang F. Nature. 2013 Aug 22;500(7463):472-6. doi: 10.1038/Nature12466. Epub 2013 Aug 23;

- *Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity*. Ran, FA., Hsu, PD., Lin, CY., Gootenberg, JS., Konermann, S., Trevino, AE., Scott, DA., Inoue, A., Matoba, S., Zhang, Y., & Zhang, F. *Cell* Aug 28. pii: S0092-8674(13)01015-5. (2013*);*

- *DNA targeting specificity of RNA-guided Cas9 nucleases*. Hsu, P., Scott, D., Weinstein, J., Ran, FA., Konermann, S., Agarwala, V., Li, Y., Fine, E., Wu, X., Shalem, O., Cradick, TJ., Marraffini, LA., Bao, G., & Zhang, F. *Nat Biotechnol* doi:10.1038/nbt.2647 (2013);

- *Genome engineering using the CRISPR-Cas9 system*. Ran, FA., Hsu, PD., Wright, J., Agarwala, V., Scott, DA., Zhang, F. Nature Protocols Nov;8(11):2281-308. (2013);

- *Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells*. Shalem, O., Sanjana, NE., Hartenian, E., Shi, X., Scott, DA., Mikkelson, T., Heckl, D., Ebert, BL., Root, DE., Doench, JG., Zhang, F. *Science* Dec 12. (2013). [Epub ahead of print];

- *Crystal structure of cas9 in complex with guide RNA and target DNA*. Nishimasu, H., Ran, FA., Hsu, PD., Konermann, S., Shehata, SI., Dohmae, N., Ishitani, R., Zhang, F., Nureki, O. *Cell* Feb 27. (2014). 156(5):935-49;

- *Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells*. Wu X., Scott DA., Kriz AJ., Chiu AC., Hsu PD., Dadon DB., Cheng AW., Trevino AE., Konermann S., Chen S., Jaenisch R., Zhang F., Sharp PA. *Nat Biotechnol*. (2014) Apr 20. doi: 10.1038/nbt.2889,

- *CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling*, Platt et al., Cell 159(2): 440-455 (2014) DOI: 10.1016/j.cell.2014.09.014,

- *Development and Applications of CRISPR-Cas9 for Genome Engineering*, Hsu et al, Cell 157, 1262-1278 (June 5, 2014) (Hsu 2014),

- *Genetic screens in human cells using the CRISPR/Cas9 system*, Wang et al., Science. 2014 January 3; 343(6166): 80–84. doi:10.1126/science.1246981,

- *Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation*, Doench et al., Nature Biotechnology published online 3 September 2014; doi:10.1038/nbt.3026, and

- *In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9*, Swiech et al, Nature Biotechnology ; published online 19 October 2014; doi:10.1038/nbt.3055.

each of which is incorporated herein by reference, and discussed briefly below:

- Cong *et al.* engineered type II CRISPR/Cas systems for use in eukaryotic cells based on both *Streptococcus thermophilus* Cas9 and also *Streptoccocus pyogenes* Cas9 and demonstrated that Cas9 nucleases can be directed by short RNAs to induce precise cleavage of DNA in human and mouse cells. Their study further showed that Cas9 as converted into a nicking enzyme can be used to facilitate homology-directed repair in eukaryotic cells with minimal mutagenic activity. Additionally, their study demonstrated that multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several at endogenous genomic loci sites within the mammalian genome, demonstrating easy programmability and wide applicability of the RNA-guided nuclease technology. This ability to use RNA to program sequence specific DNA cleavage in cells defined a new class of genome engineering tools. These studies further showed that other CRISPR loci are likely to be transplantable into mammalian cells and can also mediate mammalian genome cleavage. Importantly, it can be envisaged that several aspects of the CRISPR/Cas system can be further improved to increase its efficiency and versatility.
- Jiang *et al.* used the clustered, regularly interspaced, short palindromic repeats (CRISPR)–associated Cas9 endonuclease complexed with dual-RNAs to introduce precise mutations in the genomes of *Streptococcus pneumoniae* and *Escherichia coli.* The approach relied on dual-RNA:Cas9-directed cleavage at the targeted genomic site to kill unmutated cells and circumvents the need for selectable markers or counter-selection systems. The study reported reprogramming dual-RNA:Cas9 specificity by changing the sequence of short CRISPR RNA (crRNA) to make single- and multinucleotide changes carried on editing templates. The study showed that simultaneous use of two crRNAs enabled multiplex mutagenesis. Furthermore, when the approach was used in combination with recombineering, in *S. pneumoniae*, nearly 100% of cells that were recovered using the described approach contained the desired mutation, and in *E. coli,* 65% that were recovered contained the mutation.
- Konermann *et al.* addressed the need in the art for versatile and robust technologies that enable optical and chemical modulation of DNA-binding domains based CRISPR Cas9 enzyme and also Transcriptional Activator Like Effectors

- Cas9 nuclease from the microbial CRISPR-Cas system is targeted to specific genomic loci by a 20 nt guide sequence, which can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. To address this, Ran *et al.* described an approach that combined a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks. Because individual nicks in the genome are repaired with high fidelity, simultaneous nicking *via* appropriately offset guide RNAs is required for double-stranded breaks and extends the number of specifically recognized bases for target cleavage. The authors demonstrated that using paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications that require high specificity.
- Hsu *et al.* characterized SpCas9 targeting specificity in human cells to inform the selection of target sites and avoid off-target effects. The study evaluated >700 guide RNA variants and SpCas9-induced indel mutation levels at >100 predicted genomic off-target loci in 293T and 293FT cells. The authors reported that SpCas9 tolerates mismatches between guide RNA and target DNA at different positions in a sequence-dependent manner, sensitive to the number, position and distribution of mismatches. The authors further showed that SpCas9-mediated cleavage is unaffected by DNA methylation and that the dosage of SpCas9 and sgRNA can be titrated to minimize off-target modification. Additionally, to facilitate mammalian genome engineering applications, the authors reported providing a web-based software tool to guide the selection and validation of target sequences as well as off-target analyses.
- Ran *et al.* described a set of tools for Cas9-mediated genome editing *via* non-homologous end joining (NHEJ) or homology-directed repair (HDR) in mammalian cells, as well as generation of modified cell lines for downstream functional studies. To minimize off-target cleavage, the authors further described a double-nicking strategy using the Cas9 nickase mutant with paired guide RNAs. The protocol provided by the authors experimentally derived guidelines for the selection of target sites, evaluation of cleavage efficiency and analysis of off-target activity. The studies showed that beginning with target design, gene modifications can be achieved within as little as 1–2 weeks, and modified clonal cell lines can be derived within 2–3 weeks.

- Shalem *et al.* described a new way to interrogate gene function on a genome-wide scale. Their studies showed that delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeted 18,080 genes with 64,751 unique guide sequences enabled both negative and positive selection screening in human cells. First, the authors showed use of the GeCKO library to identify genes essential for cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, the authors screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic that inhibits mutant protein kinase BRAF. Their studies showed that the highest-ranking candidates included previously validated genes NF1 and MED12 as well as novel hits NF2, CUL3, TADA2B, and TADA1. The authors observed a high level of consistency between independent guide RNAs targeting the same gene and a high rate of hit confirmation, and thus demonstrated the promise of genome-scale screening with Cas9.
- Nishimasu *et al.* reported the crystal structure of *Streptococcus pyogenes* Cas9 in complex with sgRNA and its target DNA at 2.5 A° resolution. The structure revealed a bilobed architecture composed of target recognition and nuclease lobes, accommodating the sgRNA:DNA heteroduplex in a positively charged groove at their interface. Whereas the recognition lobe is essential for binding sgRNA and DNA, the nuclease lobe contains the HNH and RuvC nuclease domains, which are properly positioned for cleavage of the complementary and non-complementary strands of the target DNA, respectively. The nuclease lobe also contains a carboxyl-terminal domain responsible for the interaction with the protospacer adjacent motif (PAM). This high-resolution structure and accompanying functional analyses have revealed the molecular mechanism of RNA-guided DNA targeting by Cas9, thus paving the way for the rational design of new, versatile genome-editing technologies.
- Wu *et al.* mapped genome-wide binding sites of a catalytically inactive Cas9 (dCas9) from *Streptococcus pyogenes* loaded with single guide RNAs (sgRNAs) in mouse embryonic stem cells (mESCs). The authors showed that each of the four sgRNAs tested targets dCas9 to between tens and thousands of genomic sites, frequently characterized by a 5-nucleotide seed region in the sgRNA and an NGG protospacer adjacent motif (PAM). Chromatin inaccessibility decreases dCas9 binding to other sites with matching seed sequences; thus 70% of off-target sites are associated with genes. The authors

showed that targeted sequencing of 295 dCas9 binding sites in mESCs transfected with catalytically active Cas9 identified only one site mutated above background levels. The authors proposed a two-state model for Cas9 binding and cleavage, in which a seed match triggers binding but extensive pairing with target DNA is required for cleavage.

- Hsu 2014 is a review article that discusses generally CRISPR-Cas9 history from yogurt to genome editing, including genetic screening of cells, that is in the information, data and findings of the applications in the lineage of this specification filed prior to June 5, 2014. The general teachings of Hsu 2014 do not involve the specific models, animals of the instant specification.

[0072] Mention is also made of Tsai et al, "Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing," Nature Biotechnology 32(6): 569-77 (2014) which is not believed to be prior art to the instant invention or application, but which may be considered in the practice of the instant invention.

[0073] In addition, mention is made of concurrently filed PCT application ______________, Attorney Reference 47627.99.2060 and BI-2013/107 entitiled "DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING PARTICLE DELIVERY COMPONENTS (claiming priority from one or more or all of US provisional patent applications: 62/054,490, filed September 24, 2014; 62/010,441, filed June 10, 2014; and 61/915,118, 61/915,215 and 61/915,148, each filed on December 12, 2013) ("the Particle Delivery PCT"), incorporated herein by reference, with respect to a method of preparing an sgRNA-and-Cas9 protein containing particle comprising admixing a mixture comprising an sgRNA and Cas9 protein (and optionally HDR template) with a mixture comprising or consisting essentially of or consisting of surfactant, phospholipid, biodegradable polymer, lipoprotein and alcohol; and particles from such a process. For example, wherein Cas9 protein and sgRNA were mixed together at a suitable, e.g.,3:1 to 1:3 or 2:1 to 1:2 or 1:1 molar ratio, at a suitable temperature, e.g., 15-30C, e.g., 20-25C, e.g., room temperature, for a suitable time, e.g., 15-45, such as 30 minutes, advantageously in sterile, nuclease free buffer, e.g., 1X PBS. Separately, particle components such as or comprising: a surfactant, e.g., cationic lipid, e.g., 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); phospholipid, e.g., dimyristoylphosphatidylcholine (DMPC); biodegradable polymer, such as an ethylene-glycol polymer or PEG, and a lipoprotein, such as a low-density lipoprotein, e.g., cholesterol were dissolved in an alcohol, advantageously a C_{1-6} alkyl alcohol, such as methanol, ethanol, isopropanol, e.g., 100% ethanol. The two solutions were mixed together to form particles containing the Cas9-sgRNA complexes. Accordingly, sgRNA may be pre-complexed with the Cas9 protein, before formulating the entire complex in a particle. Formulations may be made with a different molar ratio of different components known to promote delivery of nucleic acids into cells (e.g. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (DMPC), polyethylene glycol (PEG), and cholesterol) For example DOTAP : DMPC : PEG : Cholesterol Molar Ratios may be DOTAP 100, DMPC 0, PEG 0, Cholesterol 0; or DOTAP 90, DMPC 0, PEG 10, Cholesterol 0; or DOTAP 90, DMPC 0, PEG 5, Cholesterol 5. DOTAP 100, DMPC 0, PEG 0, Cholesterol 0. That application accordingly comprehends admixing sgRNA, Cas9 protein and components that form a particle; as well as particles from such admixing. Aspects of the instant invention can involve particles; for example, particles using a process analogous to that of the Particle Delivery PCT, e.g., by admixing a mixture comprising sgRNA and/or Cas9 as in the instant invention and components that form a particle, e.g., as in the Particle Delivery PCT, to form a particle and particles from such admixing (or, of course, other particles involving sgRNA and/or Cas9 as in the instant invention).

[0074] The invention relates to the engineering and optimization of systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to the CRISPR-Cas system and components thereof. In advantageous embodiments, the Cas enzyme is Cas9, preferably SpCas9 or SaCas9.

[0075] An advantage of the present methods is that the CRISPR system avoids off-target binding and its resulting side effects. This is achieved using systems arranged to have a high degree of sequence specificity for the target DNA.

[0076] Recent advances in the development of genome editing technologies based on programmable nucleases such as zinc finger nucleases, transcription activator like effector nucleases, and CRISPR-Cas9 have significantly improved Applicants' ability to make precise changes in the genomes of eukaryotic cells. Genome editing is already broadening Applicants' ability to elucidate the contribution of genetics to disease by facilitating the creation of more accurate cellular and animal models of pathological processes. A particularly tantalizing application of programmable nucleases is the potential to directly correct genetic mutations in affected tissues and cells to treat genetic diseases that are refractory to traditional therapies. Applicants provide a discussion herein of the current progress towards developing programmable nuclease-based therapies as well as future prospects and challenges.

[0077] Of the approximately 25,000 annotated genes in the human genome, mutations in over 3,000 genes have already been linked to disease phenotypes (www.omim.org/statistics/geneMap), and more disease-relevant genetic variations are being uncovered at a staggeringly rapid pace. Now, due to sharp drops in sequencing cost, the completion of the human genome project, and the exponential growth of genome sequencing data from patients, the role of genetics in human health has become a major area of focus for research, clinical medicine and the development of targeted therapeutics [Lander, E.S. Nature 470, 187-197 (2011)]. These advances in Applicants' understanding of the genetic basis of disease have improved Applicants' understanding of disease mechanisms and pointed toward potential therapeutic strategies. However, despite valid therapeutic hypotheses and strong efforts in drug development, there have only been a limited number of successes using small molecules to treat diseases with strong genetic contributions [Thoene, J.G. Small molecule therapy for genetic disease, (Cambridge University Press, Cambridge, UK ; New York, 2010)]. Thus, alternative approaches are needed. Emerging therapeutic strategies that are able to modify nucleic acids within disease-affected cells and tissues hold enormous potential for treatment. Monogenic, highly penetrant diseases, such as severe-combined immunodeficiency (SCID), haemophilia, and certain enzyme deficiencies have been the focus of such therapies due to their well-defined genetics and often lack of safe, effective therapeutic alternatives.

[0078] Two of the most powerful genetic therapeutic strategies developed thus far are viral gene therapy, which enables complementation of missing gene function via transgene expression, and RNA interference (RNAi), which mediates targeted repression of defective genes by knockdown of the target mRNA (reviewed in Kay, M.A. Nature reviews. Genetics 12, 316-328 (2011) andVaishnaw, A.K., et al. Silence 1, 14 (2010)). Viral gene therapy has been used to successfully treat monogenic recessive disorders affecting the hematopoietic system, such as SCID and Wiskott-Aldrich syndrome, by semi-randomly integrating functional copies of affected genes into the genome of hematopoietic stem/progenitor cells [Gaspar, H.B., et al. Science translational medicine 3, 97ra79 (2011), Howe, S.J., et al. The Journal of clinical investigation 118, 3143-3150 (2008), Aiuti, A., et al. Science 341, 1233151 (2013)]. RNAi has been used to repress the function of genes implicated in cancer, age related macular degeneration and TTR-amyloidosis among others, to generate a therapeutic effect in clinical trials (www.clinicaltrials.gov, trial numbers: NCT00689065, NCT01961921 and NCT00259753. Despite promise and recent success, viral gene therapy and RNAi have limitations that prevent their utility for a large number of diseases. For example, viral gene therapy may cause insertional mutagenesis and dysregulated transgene expression [Howe, S.J., et al. The Journal of clinical investigation 118, 3143-3150 (2008)]. Alternatively, RNAi can only repress the expression of target genes, therefore limiting its use to targets where knockdown is beneficial. Also, RNAi often cannot fully repress gene expression, and is therefore unlikely to provide a benefit for diseases where complete ablation of gene function is necessary for therapy. An exciting alternative that might overcome these limitations would be precise modification of the genomes of target cells, resulting in the removal or correction of deleterious mutations or the insertion of protective mutations. Cartier.

[0079] Watts, "Hematopoietic Stem Cell Expansion and Gene Therapy" Cytotherapy 13(10):1164–1171. doi:10.3109/14653249.2011.620748 (2011), incorporated herein by reference along with the documents it cites, as if set out in full, discusses hematopoietic stem cell (HSC) gene therapy, e.g., virus-mediated hematopoetic stem cell (HSC) gene thereapy, as an highly attractive treatment option for many disorders including hematologic conditions, immunodeficiencies including HIV/AIDS, and other genetic disorders like lysosomal storage diseases, including SCID-X1, ADA-SCID, β-thalassemia, X-linked CGD, Wiskott-Aldrich syndrome, Fanconi anemia, adrenoleukodystrophy (ALD), and metachromatic leukodystrophy (MLD).

[0080] Williams, "Broadening the Indications for Hematopoietic Stem Cell Genetic Therapies," Cell Stem Cell 13:263-264 (2013), incorporated herein by reference along with the documents it cites, as if set out in full, report lentivirus-mediated gene transfer into HSC/P cells from patients with the lysosomal storage disease metachromatic leukodystrophy disease (MLD), a genetic disease caused by deficiency of arylsulfatase A (ARSA), resulting in nerve demyelination; and lentivirus-mediated gene transfer into HSCs of patients with Wiskott-Aldrich syndrome (WAS) (patients with defective WAS protein, an effector of the small GTPase CDC42 that regulates cytoskeletal function in blood cell lineages and thus suffer from immune deficiency with recurrent infections, autoimmune symptoms, and thrombocytopenia with abnormally small and dysfunctional platelets leading to excessive bleeding and an increased risk of leukemia and lymphoma). In contrast to using lentivirus, with the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to MLD (deficiency of arylsulfatase A (ARSA)) using a CRISPR-Cas9 system that targets and corrects the mutation (deficiency of arylsulfatase A (ARSA)) (e.g., with a suitable HDR template that delivers a coding sequence for ARSA). In contrast to using lentivirus, with the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to WAS using a CRISPR-Cas9 system that targets and corrects the mutation (deficiency of WAS protein) (e.g., with a suitable HDR template that delivers a coding sequence for WAS protein); specifically, the sgRNA can target mutation that gives rise to WAS (deficient WAS protein), and the HDR can provide coding for proper expression of WAS protein.

[0081] With the knowledge in the art and the teachings in this disclosure the skilled person can correct HSCs as to immunodeficiency condition such as HIV / AIDS comprising contacting an HSC with a CRISPR-Cas9 system that targets and knocks out CCR5. An sgRNA (and advantageously a dual guide approach, e.g., a pair of different sgRNAs; for instance, sgRNAs targeting of two clinically relevant genes, B2M and CCR5, in primary human CD4+ T cells and CD34+ hematopoietic stem and progenitor cells (HSPCs)) that targets and knocks out CCR5-and-Cas9 protein can be introduced into HSCs. The cells can be administered; and optionally treated / expanded; cf. Cartier. *See also* Kiem, "Hematopoietic stem cell-based gene therapy for HIV disease," Cell Stem Cell. Feb 3, 2012; 10(2): 137–147; incorporated herein by reference along with the documents it cites; Mandal et al, "Efficient Ablation of Genes in Human Hematopoietic Stem and Effector Cells using CRISPR/Cas9," Cell Stem Cell, Volume 15, Issue 5, p643–652, 6 November 2014; incorporated herein by reference along with the documents it cites. Mention is also made of Ebina, "CRISPR/Cas9 system to suppress HIV-1 expression by editing HIV-1 integrated proviral DNA" SCIENTIFIC REPORTS | 3 : 2510 | DOI: 10.1038/srep02510, incorporated herein by reference along with the documents it cites, as another means for combatting HIV/AIDS using a CRISPR-Cas9 system.

[0082] Genome editing technologies based on programmable nucleases such as zinc finger nuclease (reviewed in Urnov, F.D., et al. Nature reviews. Genetics 11, 636-646 (2010)), transcription activator-like effector nucleases (reviewed in Bogdanove, A.J. & Voytas, D.F. Science 333, 1843-1846 (2011)), and clustered regularly interspaced short palindromic repeat

(CRISPR)-associated nuclease Cas9 (reviewed in Hsu, P.D., et al. Cell 157, 1262-1278 (2014)) are opening the possibility of achieving therapeutic genome editing in diseased cells and tissues. Applicants provide a recent review herein.

Genome Editing Technologies

[0083] Programmable nucleases enable precise genome editing by introducing targeted DNA double strand breaks (DSBs) at specific genomic loci. DSBs subsequently signal DNA damage and recruit endogenous repair machinery for either non-homologous end-joining (NHEJ) or homology directed repair (HDR) to the DSB site to mediate genome editing.

[0084] To date, three major classes of nucleases, zinc finger nucleases (ZFNs, Figure 12, left panel) [Kim, Y.G., et al. Proceedings of the National Academy of Sciences of the United States of America 93, 1156-1160 (1996); Wolfe, S.A., et al. Annual review of biophysics and biomolecular structure 29, 183-212 (2000); Bibikova, M., et al. Science 300, 764 (2003); Bibikova, M., et al. Genetics 161, 1169-1175 (2002); Miller, J., et al. The EMBO journal 4, 1609-1614 (1985); Miller, J.C., et al. Nature biotechnology 25, 778-785 (2007)], transcription activator like effector nucleases (TALENs, Figure 1 middle panel) [Boch, J., et al. Science 326, 1509-1512 (2009); Moscou, M.J. & Bogdanove, A.J. Science 326, 1501 (2009); Christian, M., et al. Genetics 186, 757-761 (2010); Miller, J.C., et al. Nature biotechnology 29, 143-148 (2011)], and the CRISPR-associated nuclease Cas9 (Figure 1, right panel) [Bolotin, A., et al. Microbiology 151, 2551-2561 (2005); Barrangou, R., et al. Science 315, 1709-1712 (2007); Garneau, J.E., et al. Nature 468, 67-71 (2010); Deltcheva, E., et al. Nature 471, 602-607 (2011); Sapranauskas, R., et al. Nucleic acids research 39, 9275-9282 (2011); Jinek, M., et al. Science 337, 816-821 (2012); Gasiunas, G., et al. Proceedings of the National Academy of Sciences of the United States of America 109, E2579-2586 (2012); Cong, L., et al. Science 339, 819-823 (2013); Mali, P., et al. Science 339, 823-826 (2013)], have been developed to enable site-specific genome editing. These three types of nuclease systems can be broadly classified into two categories based on their mode of DNA recognition - whereas ZFN and TALEN achieve specific DNA binding via protein-DNA interactions, Cas9 is targeted to specific DNA sequences via a short RNA guide molecule that base-pairs directly with the target DNA (Fig. 13). ZFNs and TALENs are chimeric enzymes consisting of a DNA binding domain fused to the sequence agnostic nuclease domain, *Fok*I [Kim, Y.G., et al. Proceedings of the National Academy of Sciences of the United States of America 93, 1156-1160 (1996); Christian, M., et al. Genetics

186, 757-761 (2010)]. Re-targeting ZFNs and TALENs require protein engineering of the DNA binding domain, which is particularly challenging for ZFNs and still difficult for TALENs [Isalan, M. Nature methods 9, 32-34 (2012); Sun, N. & Zhao, H. Biotechnology and bioengineering 110, 1811-1821 (2013)]. In contrast, the Cas9 protein is invariant and can be easily retargeted to new genomic loci by changing a small portion of the sequence of an accompanying RNA guide. All three nucleases have been demonstrated to achieve efficient genome editing in a wide range of model organisms and mammalian cells and efforts are now underway in both industry and academia to develop these tools as therapeutics [Tebas, P., et al. The New England journal of medicine 370, 901-910 (2014); Genovese, P., et al. Nature 510, 235-240 (2014); Li, H., et al. Nature 475, 217-221 (2011); Yin, H., et al. Nature biotechnology 32, 551-553 (2014)].

[0085] Once the DSB has been made, the lesion may be repaired by either NHEJ or HDR depending on the cell state and the presence of a repair template. NHEJ may repair the lesion by directly rejoining the two DSB ends in a process that does not require a repair template. Although NHEJ-mediated DSB repair can be accurate, repeated repair of the same DSB by NHEJ machinery due to nuclease activity eventually results in the formation of small insertion or deletion mutations bridging the break site [Bibikova, M., et al. Genetics 161, 1169-1175 (2002)]. Such insertions or deletions (indels) that are introduced into the coding sequence of a gene can cause a frameshift mutations that lead to mRNA degradation via nonsense-mediated decay to deplete the functional gene, or results in the production of nonfunctional truncated proteins [Hentze, M.W. & Kulozik, A.E. Cell 96, 307-310 (1999)]. Thus, NHEJ may be used to suppress gene function similar to RNAi, however, it leads to continued suppression of gene expression in targeted cells by introducing permanent covalent modifications to the genome.

[0086] In comparison, HDR allows researchers to use an exogenous DNA template to specify the outcome of the DSB repair [Bibikova, M., et al. Science 300, 764 (2003); Choulika, A., et al. Molecular and cellular biology 15, 1968-1973 (1995); Bibikova, M., et al. Molecular and cellular biology 21, 289-297 (2001); Krejci, L., et al. Nucleic acids research 40, 5795-5818 (2012); Plessis, A., et al. Genetics 130, 451-460 (1992); Rouet, P., et al. Molecular and cellular biology 14, 8096-8106 (1994); Rudin, N., et al. Genetics 122, 519-534 (1989)]. Upon introduction of a targeted DSB, HDR machinery may use exogenously provided single or double stranded DNA templates with sequence homology to the break site to synthesize DNA that is used to repair the lesion, in the process incorporating any changes encoded in the template DNA. For example, HDR may be used along with an appropriately designed repair template to correct a deleterious mutation directly, thereby restoring gene function while preserving physiological regulation of gene expression.

Considerations for Therapeutic Applications

[0087] The first consideration in genome editing therapy is the choice of sequence-specific nuclease. Each nuclease platform possesses its own unique set of strengths and weaknesses, many of which must be balanced in the context of treatment to maximize therapeutic benefit (Figure 12).

[0088] Thus far, two therapeutic editing approaches with nucleases have shown significant promise: gene disruption and gene correction. Gene disruption involves stimulation of NHEJ to create targeted indels in genetic elements, often resulting in loss of function mutations that are beneficial to patients (Figure13A). In contrast, gene correction uses HDR to directly reverse a disease causing mutation, restoring function while preserving physiological regulation of the corrected element (Figure 13B). HDR may also be used to insert a therapeutic transgene into a defined 'safe harbor' locus in the genome to recover missing gene function (Figure 13C).

[0089] For a specific editing therapy to be efficacious, a sufficiently high level of modification must be achieved in target cell populations to reverse disease symptoms. This therapeutic modification 'threshold' is determined by the fitness of edited cells following treatment and the amount of gene product necessary to reverse symptoms.

Cell Fitness and Outcome

[0090] With regard to fitness, editing creates three potential outcomes for treated cells relative to their unedited counterparts: increased, neutral, or decreased fitness. In the case of increased fitness, for example in the treatment of SCID-X1, modified hematopoietic progenitor cells selectively expand relative to their unedited counterparts. SCID-X1 is a disease caused by mutations in the IL2RG gene, the function of which is required for proper development of the hematopoietic lymphocyte lineage [Leonard, W.J., et al. Immunological reviews 138, 61-86 (1994); Kaushansky, K. & Williams, W.J. Williams hematology, (McGraw-Hill Medical, New York, 2010)]. In clinical trials with patients who received viral gene therapy for SCID-X1, and a rare example of a spontaneous correction of SCID-X1 mutation, corrected hematopoietic progenitor cells were able to overcome this developmental block and expand relative to their diseased counterparts to mediate therapy [Bousso, P., et al. Proceedings of the National Academy of Sciences of the United States of America 97, 274-278 (2000); Hacein-Bey-Abina, S., et al. The New England journal of medicine 346, 1185-1193 (2002); Gaspar, H.B., et al. Lancet 364, 2181-2187 (2004)]. In this case, where edited cells possess a selective advantage, even low numbers of edited cells can be amplified through expansion, providing a therapeutic benefit to the patient. In contrast, editing for other hematopoietic diseases, like chronic granulomatous disorder (CGD), would induce no change in fitness for edited hematopoietic progenitor cells, increasing the therapeutic modification threshold. CGD is caused by mutations in genes encoding phagocytic oxidase proteins, which are normally used by neutrophils to generate reactive oxygen species that kill pathogens [Mukherjee, S. & Thrasher, A.J. Gene 525, 174-181 (2013)]. As dysfunction of these genes does not influence hematopoietic progenitor cell fitness or development, but only the ability of a mature hematopoietic cell type to fight infections, there would be likely no preferential expansion of edited cells in this disease. Indeed, no selective advantage for gene corrected cells in CGD has been observed in gene therapy trials, leading to difficulties with long-term cell engraftment [Malech, H.L., et al. Proceedings of the National Academy of Sciences of the United States of America 94, 12133-12138 (1997); Kang, H.J., et al. Molecular therapy: the journal of the American Society of Gene Therapy 19, 2092-2101 (2011)]. As such, significantly higher levels of editing would be required to treat diseases like CGD, where editing creates a neutral fitness advantage, relative to diseases where editing creates increased fitness for target cells. If editing imposes a fitness disadvantage, as would be the case for restoring function to a tumor suppressor gene in cancer cells, modified cells would be outcompeted by their diseased counterparts, causing the benefit of treatment to be low relative to editing rates. This latter class of diseases would be particularly difficult to treat with genome editing therapy. X-linked Chronic granulomatous disease (CGD) is a hereditary disorder of host defense due to absent or decreased activity of phagocyte NADPH oxidase. From this disclosure and knowledge in the art, the skilled person is enabled to use a CRISPR-Cas9 system that targets and corrects the mutation (absent or decreased activity of phagocyte NADPH oxidase) (e.g., with a suitable HDR template that delivers a coding sequence for phagocyte NADPH oxidase); specifically, the sgRNA can target mutation that gives rise to CGD (deficient phagocyte NADPH oxidase), and the HDR can provide coding for proper expression of phagocyte NADPH oxidase.

[0091] In addition to cell fitness, the amount of gene product necessary to treat disease also influences the minimal level of therapeutic genome editing that must be achieved to reverse symptoms. Haemophilia B is one disease where a small change in gene product levels can result in significant changes in clinical outcomes. This disease is caused by mutations in the gene encoding factor IX, a protein normally secreted by the liver into the blood, where it functions as a component of the clotting cascade. Clinical severity of haemophilia B is related to the amount of factor IX activity. Whereas severe disease is associated with less than 1% of normal activity, milder forms of the diseases are associated with greater than 1% of factor IX activity [Kaushansky, K. & Williams, W.J. Williams hematology, (McGraw-Hill Medical, New York, 2010); Lofqvist, T., et al. Journal of internal medicine 241, 395-400 (1997)]. This suggests that editing therapies that can restore factor IX expression to even a small percentage of liver cells could have a large impact on clinical outcomes. A study using ZFNs to correct a mouse model of haemophilia B shortly after birth demonstrated that 3-7% correction was sufficient to reverse disease symptoms, providing preclinical evidence for this hypothesis [Li, H., et al. Nature 475, 217-221 (2011)].

[0092] Disorders where a small change in gene product levels can influence clinical outcomes and diseases where there is a fitness advantage for edited cells, are ideal targets for genome editing therapy, as the therapeutic modification threshold is low enough to permit a high chance of success given the current technology.

[0093] Targeting these diseases has now resulted in successes with editing therapy at the preclinical level and a phase I clinical trial (see Table below). Improvements in DSB repair pathway manipulation and nuclease delivery are needed to extend these promising results to diseases with a neutral fitness advantage for edited cells, or where larger amounts of gene product are needed for treatment. The Table below shows examples of applications of genome editing to therapeutic models.

Disease Type	Nuclease Platform Employed	Therapeutic Strategy	References
Hemophilia B	ZFN	HDR-mediated insertion of correct gene sequence	Li, H., et al. Nature 475, 217-221 (2011)
HIV	ZFN and CRISPR	NHEJ-mediated inactivation of CCR5	Tebas, P., et al. The New England journal of medicine 370, 901-910 (2014). Holt, N., et al. Nature biotechnology 28, 839-

Disease Type	Nuclease Platform Employed	Therapeutic Strategy	References
			847 (2010), Perez, E.E., et al. Nature biotechnology 26, 808-816 (2008), Ye, L., et al. Proceedings of the National Academy of Sciences of the United States of America 111, 9591-9596 (2014)
DMD	CRISPR and TALEN	NHEJ-mediated removal of stop codon, and HDR-mediated gene correction	Ousterout, D.G., et al. Molecular therapy : the journal of the American Society of Gene Therapy 21, 1718-1726 (2013), Long, C., et al. Science 345, 1184-1188 (2014)
HBV	TALEN and CRISPR	NHEJ-mediated depletion of viral DNA	Bloom, K., et al. Molecular therapy : the journal of the American Society of Gene Therapy 21, 1889-1897 (2013), Lin, S.R., et al. Nucleic acids 3, e186 (2014)
SCID	ZFN	HDR-mediated insertion of correct gene sequence	Genovese, P., et al. Nature 510, 235-240 (2014)
Cataract	CRISPR	HDR-mediated correction of mutation in mouse zygote	Wu, Y., et al. Cell stem cell 13, 659-662 (2013)
Cystic fibrosis	CRISPR	HDR-mediated correction of CFTR in intestinal stem cell organoid	Schwank, G., et al. Cell stem cell 13, 653-658 (2013)
Hereditary tyrosinemia	CRISPR	HDR-mediated correction of mutation in liver	Yin, H., et al. Nature biotechnology 32, 551-553 (2014)

[0094] An embodiment comprehends contacting a Hemophilia B, SCID (e.g., SCID-X1, ADA-SCID) or Hereditary tyrosinemia mutation-carrying hematopoetic stem cell with an sgRNA and Cas9 protein targeting a genomic locus of interest as to Hemophilia B, SCID (e.g., SCID-X1, ADA-SCID) or Hereditary tyrosinemia (e.g., as in Li, Genovese or Yin); advantageously with a suitable HDR template to correct the mutation.

Efficiency of DSB Repair Pathways

[0095] The activity of NHEJ and HDR DSB repair varies significantly by cell type and cell state. NHEJ is not highly regulated by the cell cycle and is efficient across cell types, allowing for high levels of gene disruption in accessible target cell populations. In contrast, HDR acts primarily during S/G2 phase, and is therefore restricted to cells that are actively dividing, limiting treatments that require precise genome modifications to mitotic cells [Ciccia, A. & Elledge, S.J. Molecular cell 40, 179-204 (2010); Chapman, J.R., et al. Molecular cell 47, 497-510 (2012)].

[0096] The efficiency of correction via HDR may be controlled by the epigenetic state or sequence of the targeted locus, or the specific repair template configuration (single vs. double stranded, long vs. short homology arms) used [Hacein-Bey-Abina, S., et al. The New England journal of medicine 346, 1185-1193 (2002); Gaspar, H.B., et al. Lancet 364, 2181-2187 (2004); Beumer, K.J., et al. G3 (2013)]. The relative activity of NHEJ and HDR machineries in target cells may also affect gene correction efficiency, as these pathways may compete to resolve DSBs [Beumer, K.J., et al. Proceedings of the National Academy of Sciences of the United States of America 105, 19821-19826 (2008)]. HDR also imposes a delivery challenge not seen with NHEJ strategies, as it requires the concurrent delivery of nucleases and repair templates. In practice, these constraints have so far led to low levels of HDR in therapeutically relevant cell types. Clinical translation has therefore largely focused on NHEJ strategies to treat disease, although proof-of-concept preclinical HDR treatments have now been described for mouse models of haemophilia B and hereditary tyrosinemia [Li, H., et al. Nature 475, 217-221 (2011); Yin, H., et al. Nature biotechnology 32, 551-553 (2014)].

Cell and Tissue Targeting

[0097] Any given genome editing application may comprise combinations of proteins, small RNA molecules, and/or repair templates, making delivery of these multiple parts substantially more challenging than small molecule therapeutics. Two main strategies for delivery of genome editing tools have been developed: *ex vivo* and *in vivo*. In *ex vivo* treatments, diseased cells are removed from the body, edited and then transplanted back into the patient (Figure 14, top panel). *Ex vivo* editing has the advantage of allowing the target cell population to be well defined and the specific dosage of therapeutic molecules delivered to cells to be specified. The latter consideration may be particularly important when off-target modifications are a concern, as titrating the amount of nuclease may decrease such mutations (Hsu et al., 2013). Another advantage of *ex vivo* approaches is the typically high editing rates that can be achieved, due to the development of efficient delivery systems for proteins and nucleic acids into cells in culture for research and gene therapy applications.

[0098] However, there are two large drawbacks with *ex vivo* approaches that limit their application to a small number of diseases. First, target cells must be capable of surviving manipulation outside the body. For many tissues, like the brain, culturing cells outside the body is a major challenge, because cells either fail to survive, or lose properties necessary for their function *in vivo*. Thus, *ex vivo* therapy is largely limited to tissues with adult stem cell populations amenable to *ex vivo* culture and manipulation, such as the hematopoietic system. Second, cultured cells often engraft poorly upon re-introduction into a patient, decreasing the effectiveness of treatment. However, engraftment may be enhanced by ablative conditioning regimens that deplete host cells prior to transplantation, which are clinically feasible but introduce significant risks to patients [Bunn, H.F. & Aster, J. Pathophysiology of blood disorders, (McGraw-Hill, New York, 2011)]

[0099] *In vivo* genome editing involves direct delivery of editing systems to cell types in their native tissues (Figure 14, bottom panels). *In vivo* editing allows diseases in which the affected cell population is not amenable to *ex vivo* manipulation to be treated. Furthermore, delivering nucleases to cells *in situ* allows for the treatment of multiple tissue and cell types. These properties probably allow *in vivo* treatment to be applied to a wider range of diseases than *ex vivo* therapies.

[00100] To date, *in vivo* editing has largely been achieved through the use of viral vectors with defined, tissue-specific tropism. Such vectors are currently limited in terms of cargo carrying capacity and tropism, restricting this mode of therapy to organ systems where transduction with clinically useful vectors is efficient, such as the liver, muscle and eye [Kotterman, M.A. & Schaffer, D.V. Nature reviews. Genetics 15, 445-451 (2014); Nguyen, T.H. & Ferry, N. Gene therapy 11 Suppl 1, S76-84 (2004); Boye, S.E., et al. Molecular therapy: the journal of the American Society of Gene Therapy 21, 509-519 (2013)].

[00101] A major potential barrier for *in vivo* delivery is the immune response that may be created in response to the large amounts of virus necessary for treatment, but this phenomenon is not unique to genome editing and is observed with other virus based gene therapies [Bessis, N., et al. Gene therapy 11 Suppl 1, S10-17 (2004)]. It is also possible that peptides from editing nucleases themselves are presented on MHC Class I molecules to stimulate an immune response, although there is little evidence to support this happening at the preclinical level. Another major difficulty with this mode of therapy is controlling the distribution and consequently the dosage of genome editing nucleases *in vivo*, leading to off-target mutation profiles that may be difficult to predict.

Examples of Successful Genome Editing Therapeutic Strategies

Ex Vivo Editing Therapy

[00102] The long standing clinical expertise with the purification, culture and transplantation of hematopoietic cells has made diseases affecting the blood system such as SCID, Fanconi anemia, Wiskott-Aldrich syndrome and sickle cell anemia the focus of *ex vivo* editing therapy. Another reason to focus on hematopoietic cells is that, thanks to previous efforts to design gene therapy for blood disorders, delivery systems of relatively high efficiency already exist. Despite these advantages, the often low efficiency of cell engraftment upon transplantation necessitates that this mode of therapy be applied to diseases where edited cells possess a fitness advantage, so that a small number of engrafted, edited cells can expand and treat disease.

[00103] Fanconi anemia: Mutations in at least 15 genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1/BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ/BACH1/BRIP1*, *FANCL/PHF9/POG*, *FANCM*, *FANCN/PALB2*, *FANCO/Rad51C*, and *FANCP/SLX4/BTBD12*) can cause Fanconi anemia. Proteins produced from these genes are involved in a cell process known as the FA pathway. The FA pathway is turned on (activated) when the process of making new copies of DNA, called DNA replication, is blocked due to DNA damage. The FA pathway sends certain proteins to the area of damage, which trigger DNA repair so DNA replication can continue. The FA pathway is particularly responsive to a certain type of DNA damage known as interstrand cross-links (ICLs). ICLs occur when two DNA building blocks (nucleotides) on opposite strands of DNA are abnormally attached or linked together, which stops the process of DNA replication. ICLs can be caused by a buildup of toxic substances produced in the body or by treatment with certain cancer therapy drugs. Eight proteins associated with Fanconi anemia group together to form a complex known as the FA core complex. The FA core complex activates two proteins, called FANCD2 and FANCI. The activation of these two proteins brings DNA repair proteins to the area of the ICL so the cross-link can be removed and DNA replication can continue. the FA core complex. More in particular, the FA core complex is a nuclear multiprotein complex consisting of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM, functions as an E3 ubiquitin ligase and mediates the activation of the ID complex, which is a heterodimer composed of FANCD2 and FANCI. Once monoubiquitinated, it interacts with classical tumor suppressors downstream of the FA pathway including FANCD1/BRCA2, FANCN/PALB2, FANCJ/BRIP1, and FANCO/Rad51C and thereby contributes to DNA repair via homologous recombination (HR). Eighty to 90 percent of FA cases are due to mutations in one of three genes, *FANCA*, *FANCC*, and *FANCG*. These genes provide instructions for producing components of the FA core complex. Mutations in such genes associated with the FA core complex will cause the complex to be nonfunctional and disrupt the entire FA pathway. As a result, DNA damage is not repaired efficiently and ICLs build up over time. Geiselhart, "Review Article, Disrupted Signaling through the Fanconi Anemia Pathway Leads to Dysfunctional Hematopoietic Stem Cell Biology: Underlying Mechanisms and Potential Therapeutic Strategies," Anemia Volume 2012 (2012), Article ID 265790, http://dx.doi.org/10.1155/2012/265790 discussed FA and an animal experiment involving intrafemoral injection of a lentivirus encoding the FANCC gene resulting in correction of HSCs in vivo. From this disclosure and the knowledge in the art, one can use a CRISPR-Cas9 system that targets and one or more of the mutations associated with FA, for instance a CRISPR-Cas9 system having sgRNA(s) and HDR template(s) that respectively targets one or more of the mutations of *FANCA*, *FANCC*, or *FANCG* that give rise to FA and provide corrective expression of one or more of FANCA, FANCC or FANCG.

[00104] One such disease is HIV, where infection results in a fitness disadvantage to CD4+ T cells.

[00105] The rationale for genome editing for HIV treatment originates from the observation that individuals homozygous for loss of function mutations in CCR5, a cellular co-receptor for the virus, are highly resistant to infection and otherwise healthy, suggesting that mimicking this mutation with genome editing could be a safe and effective therapeutic strategy [Liu, R., et al. Cell 86, 367-377 (1996)]. This idea was clinically validated when an HIV infected patient was given an allogeneic bone marrow transplant from a donor homozygous for a loss of function CCR5 mutation, resulting in undetectable levels of HIV and restoration of normal CD4 T-cell counts [Hutter, G., et al. The New England journal of medicine 360, 692-698 (2009)]. Although bone marrow transplantation is not a realistic treatment strategy for most HIV patients, due to cost and potential graft vs. host disease, HIV therapies that convert a patient's own T-cells are realistic.

[00106] Early studies using ZFNs and NHEJ to knockout CCR5 in humanized mouse models of HIV showed that transplantation of CCR5 edited CD4 T cells improved viral load and CD4 T-cell counts [Perez, E.E., et al. Nature biotechnology 26, 808-816 (2008)]. Importantly, these models also showed that HIV infection resulted in selection for CCR5 null cells, suggesting that editing confers a fitness advantage and potentially allowing a small number of edited cells to create a therapeutic effect.

[00107] As a result of this and other promising preclinical studies, genome editing therapy that knocks out CCR5 in patient T cells has now been tested in humans [Holt, N., et al. Nature biotechnology 28, 839-847 (2010); Li, L., et al. Molecular therapy : the journal of the American Society of Gene Therapy 21, 1259-1269 (2013)]. In a recent phase I clinical trial, CD4+ T cells from patients with HIV were removed, edited with ZFNs designed to knockout the CCR5 gene, and autologously transplanted back into patients [Tebas, P., et al. The New England journal of medicine 370, 901-910 (2014)]. Early results from this trial suggest that genome editing through ZFNs of the CCR5 locus is safe, although the follow up time is too short to fully understand the risks and efficacy of treatment.

[00108] *Ex vivo* editing therapy has been recently extended to include gene correction strategies. The barriers to HDR *ex vivo* were overcome in a recent paper from Genovese and colleagues, who achieved gene correction of a mutated IL2RG gene in hematopoietic stem cells (HSCs) obtained from a patient suffering from SCID-X1 [Genovese, P., et al. Nature 510, 235-240 (2014)]. Genovese *et. al.* accomplished gene correction in HSCs using a multimodal strategy. First, HSCs were transduced using integration-deficient lentivirus containing an HDR template encoding a therapeutic cDNA for IL2RG. Following transduction, cells were electroporated with mRNA encoding ZFNs targeting a mutational hotspot in IL2RG to stimulate HDR based gene correction. To increase HDR rates, culture conditions were optimized with small molecules to encourage HSC division. With optimized culture conditions, nucleases and HDR templates, gene corrected HSCs from the SCID-X1 patient were obtained in culture at therapeutically relevant rates. HSCs from unaffected individuals that underwent the same gene correction procedure could sustain long-term hematopoiesis in mice, the gold standard for HSC function. HSCs are capable of giving rise to all hematopoietic cell types and can be autologously transplanted, making them an extremely valuable cell population for all hematopoietic genetic disorders [Weissman, I.L. & Shizuru, J.A. Blood 112, 3543-3553 (2008)]. Gene corrected HSCs could, in principle, be used to treat a wide range of genetic blood disorders making this study an exciting breakthrough for therapeutic genome editing.

In Vivo Editing Therapy

[00109] *In vivo* editing therapy faces similar challenges to *ex vivo* strategies and is also limited by the small number of efficient delivery systems. Inefficient modification of target loci are compounded by any inefficiencies in delivery, making tissues lacking robust delivery platforms particularly difficult to treat with this mode of therapy. For organ systems where delivery is efficient, however, there have already been a number of exciting preclinical therapeutic successes.

[00110] The first example of successful *in vivo* editing therapy was demonstrated in a mouse model of haemophilia B [Li, H., et al. Nature 475, 217-221 (2011)]. As noted earlier, Haemophilia B is an X-linked recessive disorder caused by loss-of-function mutations in the gene encoding Factor IX, a crucial component of the clotting cascade. Recovering Factor IX activity to above 1% of its levels in severely affected individuals can transform the disease into a significantly milder form, as infusion of recombinant Factor IX into such patients prophylactically from a young age to achieve such levels largely ameliorates clinical complications [Lofqvist, T., et al. Journal of internal medicine 241, 395-400 (1997)]. Thus, only low levels of HDR gene correction would be necessary to change clinical outcomes for patients. In addition, Factor IX is synthesized and secreted by the liver, an organ that can be transduced efficiently by viral vectors encoding editing systems. With the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to Haemophilia B using a CRISPR-Cas9 system that targets and corrects the mutation (X-linked recessive disorder caused by loss-of-function mutations in the gene encoding Factor IX) (e.g., with a suitable HDR template that delivers a coding sequence for Factor IX); specifically, the sgRNA can target mutation that give rise to Haemophilia B, and the HDR can provide coding for proper expression of Factor IX.

[00111] Using hepatotropic adeno-associated viral (AAV) serotypes encoding ZFNs and a corrective HDR template, up to 7% gene correction of a mutated, humanized Factor IX gene in the murine liver was achieved [Li, H., et al. Nature 475, 217-221 (2011)]. This resulted in improvement of clot formation kinetics, a measure of the function of the clotting cascade, demonstrating for the first time that *in vivo* editing therapy is not only feasible, but also efficacious.

[00112] Building on this study, other groups have recently used *in vivo* genome editing of the liver with CRISPR-Cas9 to successfully treat a mouse model of hereditary tyrosinemia and to create mutations that provide protection against cardiovascular disease. These two distinct applications demonstrate the versatility of this approach for disorders that involve hepatic dysfunction [Yin, H., et al. Nature biotechnology 32, 551-553 (2014); Ding, Q., et al. Circulation research 115, 488-492 (2014)]. Application of *in vivo* editing to other organ systems are necessary to prove that this strategy is widely applicable. Currently, efforts to optimize both viral and non-viral vectors are underway to expand the range of disorders that can be treated with this mode of therapy [Kotterman, M.A. & Schaffer, D.V. Nature reviews. Genetics 15, 445-451 (2014); Yin, H., et al. Nature reviews. Genetics 15, 541-555 (2014)].

Specificity of Editing Nucleases

[00113] The specificity of genome editing tools is one of the main safety concerns for clinical application. Genetic modifications are permanent, and deleterious off-target mutations have the potential to create cells with oncogenic potential and other undesirable side effects. Furthermore, oncogenic mutations resulting from off-target editing may lead to expansion of the edited the cells, thus, even low levels of off-target mutagenesis may have devastating consequences.

[00114] Two issues remain outstanding: evaluating and reducing off-target effects. A number of studies have attempted to evaluate the targeting specificity of ZFN, TALEN, and Cas9 nucleases. The limited number of studies characterizing ZFN [Pattanayak, V., et al. Nature methods 8, 765-770 (2011); Gabriel, R., et al. Nature biotechnology 29, 816-823 (2011)] and TALEN [Guilinger, J.P., et al. Nature methods 11, 429-435 (2014)] specificity have only highlighted the challenges of detecting ZFN and TALEN off-target activity. Of note, the two independent studies attempting to characterize the off-target profile of the same pair of CCR5-targeting ZFNs have returned distinct and non-overlapping off-target sites, which highlights the challenges associated with analysis of nuclease specificity.

[00115] Many studies have attempted to evaluate the specificity of Cas9, partly owing to the simplicity of the RNA-guided DNA targeting mechanism of Cas9, which makes it significantly easier to establish hypotheses regarding possible off-targeting mechanisms based on Watson-Crick base pairing rules. While initial bacterial [Sapranauskas, R., et al. Nucleic acids research 39, 9275-9282 (2011)], biochemical [Jinek, M., et al. Science 337, 816-821 (2012); Gasiunas, G., et al. Proceedings of the National Academy of Sciences of the United States of America 109, E2579-2586 (2012)], and mammalian [Cong, L., et al. Science 339, 819-823 (2013)] experiments have suggested that the 3` 8-12bp seed region of the guide sequence can be sensitive to single base mismatches, further work have shown that this rule-of-thumb is not necessarily accurate, especially in situations where there is high concentration of Cas9 and guide RNA [Fu, Y., et al. Nature biotechnology 31, 822-826 (2013); Cho, S.W., et al. Genome research 24, 132-141 (2014); Hsu, P.D., et al. Nature biotechnology 31, 827-832 (2013); Mali, P., et al. Nature biotechnology 31, 833-838 (2013); Pattanayak, V., et al. Nature biotechnology 31, 839-843 (2013)]. Many of these studies were carried out in cell lines and examined Cas9-mediated mutagenesis at genomic sites bearing high levels of homology to the on-target sequence and found that, unsurprisingly, subsets of highly homologous off-target sites were significantly mutated by the nuclease. However, the scope of possible off-target sites evaluated by these studies was limited to computationally predicted sites. More recently, whole-genome sequencing of Cas9-edited cell lines revealed low incidence of off-target mutation, which suggests that Cas9-mediated genome editing can be specific [Veres, A., et al. Cell stem cell 15, 27-30 (2014)]. Despite these studies, unbiased assessment of genome wide off-targeting using more advanced methods like direct capture of DSBs [Crosetto, N., et al. Nature methods 10, 361-365 (2013)] and techniques that can detect larger structural variations (i.e. translocations) potentially imposed by nuclease treatment remains an urgent need and should be undertaken to understand the true risk of mutagenesis imposed by programmable nucleases. It is worth noting that off-target effects may be cell-type specific; for example off-target effects in transformed cell lines with dysergulated DSB repair pathways may overestimate off-target effects that would be observed in primary healthy cells.

[00116] In order to reduce the frequency of off-target effects, many groups are rapidly improving the targeting specificity of Cas9. For example, transformation of Cas9 into a single-strand DNA nickase that functions as an obligate heterodimer dramatically reduces off-target indel formation at computationally predicted off target sites [Mali, P., et al. Nature biotechnology 31, 833-838 (2013); Ran, F.A., et al. Cell 154, 1380-1389 (2013)]. Additionally, truncation of the guide RNA as well as RNA-guided FokI nuclease based on fusion between catalytically inactive Cas9 and the FokI nuclease domain are also able to achieve improved levels of targeting specificity [Fu, Y., et al. Nature biotechnology 32, 279-284 (2014); Guilinger, J.P., et al. Nature biotechnology 32, 577-582 (2014); Tsai, S.Q., et al. Nature biotechnology 32, 569-576 (2014)]. These, and future, improved nuclease strategies are considered, whenever possible, for therapeutic applications.

Crispr-Cas systems and compositions for therapeutic applications, e.g., genome editing

[00117] In general, in addition to discussion throughout this document concerning the CRISPR-Cas or CRISPR system, the CRISPR-Cas or CRISPR system is as used in the herein-cited documents, such as WO 2014/093622 (PCT/US2013/074667) and refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), or "RNA(s)" as that term is herein used (e.g., RNA(s) to guide Cas9, e.g. CRISPR RNA and transactivating (tracr) RNA or a single guide RNA (sgRNA) (chimeric RNA)) or other sequences and transcripts from a CRISPR locus. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell. In some embodiments, direct repeats may be identified *in silico* by searching for repetitive motifs that fulfill any or all of the following criteria: 1. found in a 2Kb window of genomic sequence flanking the type II CRISPR locus; 2. span from 20 to 50 bp; and 3. interspaced by 20 to 50 bp. In some embodiments, 2 of these criteria may be used, for instance 1 and 2, 2 and 3, or 1 and 3. In some embodiments, all 3 criteria may be used. In some embodiments it may be preferred in a CRISPR complex that the tracr sequence has one or more hairpins and is 30 or more nucleotides in length, 40 or more nucleotides in length, or 50 or more nucleotides in length; the guide sequence is between 10 to 30 nucleotides in length, the CRISPR/Cas enzyme is a Type II Cas9 enzyme. In embodiments of the invention the terms guide sequence and guide RNA are used interchangeably as in foregoing cited documents such as WO 2014/093622 (PCT/US2013/074667). In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. Preferably the guide sequence is 10 - 30 nucleotides long. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art.A guide sequence may be selected to target any target sequence. In some embodiments, the target sequence is a sequence within a genome of a cell. Exemplary target sequences include those that are unique in the target genome. For example, for the S. pyogenes Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGG where NNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an S. pyogenes Cas9 target site of the form MMMMMMMMMNNNNNNNNNNNXGG where NNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. For the S. thermophilus CRISPR1 Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXXAGAAW where NNNNNNNNNNNNXXAGAAW (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. A unique target sequence in a genome may include an S. thermophilus CRISPR1 Cas9 target site of the form MMMMMMMMMNNNNNNNNNNNXXAGAAW where NNNNNNNNNNNXXAGAAW (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. For the S. pyogenes Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an S. pyogenes Cas9 target site of the form MMMMMMMMMNNNNNNNNNNNXGGXG where NNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. In each of these sequences "M" may be A, G, T, or C, and need not be considered in identifying a sequence as unique. In some embodiments, a guide sequence is selected to reduce the degree secondary structure within the guide sequence. In some embodiments, about or less than about 75%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or fewer of the nucleotides of the guide sequence participate in self-complementary base pairing when optimally folded. Optimal folding may be determined by any suitable polynucleotide folding algorithm. Some programs are based on calculating the minimal Gibbs free energy. An example of one such algorithm is mFold, as described by Zuker and Stiegler (Nucleic Acids Res. 9 (1981), 133-148). Another example folding algorithm is the online webserver RNAfold, developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g.

A.R. Gruber et al., 2008, Cell 106(1): 23-24; and PA Carr and GM Church, 2009, Nature Biotechnology 27(12): 1151-62).

[00118] In general, a tracr mate sequence includes any sequence that has sufficient complementarity with a tracr sequence to promote one or more of: (1) excision of a guide sequence flanked by tracr mate sequences in a cell containing the corresponding tracr sequence; and (2) formation of a CRISPR complex at a target sequence, wherein the CRISPR complex comprises the tracr mate sequence hybridized to the tracr sequence. In general, degree of complementarity is with reference to the optimal alignment of the tracr mate sequence and tracr sequence, along the length of the shorter of the two sequences. Optimal alignment may be determined by any suitable alignment algorithm, and may further account for secondary structures, such as self-complementarity within either the tracr sequence or tracr mate sequence. In some embodiments, the degree of complementarity between the tracr sequence and tracr mate sequence along the length of the shorter of the two when optimally aligned is about or more than about 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97.5%, 99%, or higher. In some embodiments, the tracr sequence is about or more than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or more nucleotides in length. In some embodiments, the tracr sequence and tracr mate sequence are contained within a single transcript, such that hybridization between the two produces a transcript having a secondary structure, such as a hairpin. In an embodiment of the invention, the transcript or transcribed polynucleotide sequence has at least two or more hairpins. In preferred embodiments, the transcript has two, three, four or five hairpins. In a further embodiment of the invention, the transcript has at most five hairpins. In a hairpin structure the portion of the sequence 5' of the final "N" and upstream of the loop corresponds to the tracr mate sequence, and the portion of the sequence 3' of the loop corresponds to the tracr sequence Further non-limiting examples of single polynucleotides comprising a guide sequence, a tracr mate sequence, and a tracr sequence are as follows (listed 5' to 3'), where "N" represents a base of a guide sequence, the first block of lower case letters represent the tracr mate sequence, and the second block of lower case letters represent the tracr sequence, and the final poly-T sequence represents the transcription terminator: (1) NNNNNNNNNNNNNNNNNNNNgtttttgtactctcaagatttaGAAAtaaatcttgcagaagctacaaagataa ggcttcatgccgaaatcaacaccctgtcattttatggcagggtgttttcgttatttaaTTTTTT; (2) NNNNNNNNNNNNNNNNNNNNgtttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccg

aaatcaacaccctgtcattttatggcagggtgttttcgttatttaaTTTTTT; (3)
NNNNNNNNNNNNNNNNNNNNgtttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccg aaatcaacaccctgtcattttatggcagggtgtTTTTTT; (4)
NNNNNNNNNNNNNNNNNNNNgttttagagctaGAAAtagcaagttaaaataaggctagtccgttatcaactt gaaaaagtggcaccgagtcggtgcTTTTTT; (5)
NNNNNNNNNNNNNNNNNNNNgttttagagctaGAAATAGcaagttaaaataaggctagtccgttatcaac ttgaaaaagtgTTTTTTT; and (6)
NNNNNNNNNNNNNNNNNNNNgttttagagctagAAATAGcaagttaaaataaggctagtccgttatcaTT TTTTTT. In some embodiments, sequences (1) to (3) are used in combination with Cas9 from *S. thermophilus* CRISPR1. In some embodiments, sequences (4) to (6) are used in combination with Cas9 from *S. pyogenes*. In some embodiments, the tracr sequence is a separate transcript from a transcript comprising the tracr mate sequence.

[00119] In some embodiments, candidate tracrRNA may be subsequently predicted by sequences that fulfill any or all of the following criteria: 1. sequence homology to direct repeats (motif search in Geneious with up to 18-bp mismatches); 2. presence of a predicted Rho-independent transcriptional terminator in direction of transcription; and 3. stable hairpin secondary structure between tracrRNA and direct repeat. In some embodiments, 2 of these criteria may be used, for instance 1 and 2, 2 and 3, or 1 and 3. In some embodiments, all 3 criteria may be used.

[00120] In some embodiments, chimeric synthetic guide RNAs (sgRNAs) designs may incorporate at least 12 bp of duplex structure between the direct repeat and tracrRNA.

[00121] For minimization of toxicity and off-target effect, it will be important to control the concentration of CRISPR enzyme mRNA and guide RNA delivered. Optimal concentrations of CRISPR enzyme mRNA and guide RNA can be determined by testing different concentrations in a cellular or non-human eukaryote animal model and using deep sequencing the analyze the extent of modification at potential off-target genomic loci. For example, for the guide sequence targeting 5'-GAGTCCGAGCAGAAGAAGAA-3' in the EMX1 gene of the human genome, deep sequencing can be used to assess the level of modification at the following two off-target loci, 1: 5'-GAGTCCTAGCAGGAGAAGAA-3' and 2: 5'-GAGTCTAAGCAGAAGAAGAA-3'. The concentration that gives the highest level of on-target modification while minimizing the level of off-target modification should be chosen for in vivo delivery. Alternatively, to minimize the level of toxicity and off-target effect, CRISPR enzyme nickase mRNA (for example S. pyogenes Cas9 with the D10A mutation) can be delivered with a pair of guide RNAs targeting a site of interest. The two guide RNAs need to be spaced as follows. Guide sequences and strategies to mimize toxicity and off-target effects can be as in WO 2014/093622 (PCT/US2013/074667).

[00122] The CRISPR system is derived advantageously from a type II CRISPR system. In some embodiments, one or more elements of a CRISPR system is derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes*. In preferred embodiments of the invention, the CRISPR system is a type II CRISPR system and the Cas enzyme is Cas9, which catalyzes DNA cleavage. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologues thereof, or modified versions thereof.

[00123] In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, a vector encodes a CRISPR enzyme that is mutated to with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from S. pyogenes converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). Other examples of mutations that render Cas9 a nickase include, without limitation, H840A, N854A, and N863A. As a further example, two or more catalytic domains of Cas9 (RuvC I, RuvC II, and RuvC III or the HNH domain) may be mutated to produce a mutated Cas9 substantially lacking all DNA cleavage activity. In some embodiments, a D10A mutation is combined with one or more of H840A, N854A, or N863A mutations to produce a Cas9 enzyme substantially lacking all DNA cleavage activity. In some embodiments, a CRISPR enzyme is considered to substantially lack all DNA cleavage activity when the DNA cleavage activity of the mutated enzyme is about no more than 25%, 10%, 5%, 1%, 0.1%, 0.01%, or less of the DNA cleavage activity of the non-mutated form of the enzyme; an example can be when the DNA cleavage activity of the mutated form is nil or negligible as compared with the non-mutated form. Where the enzyme is not SpCas9, mutations may be made at any or all residues corresponding to positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 (which may be ascertained for instance by standard sequence comparison tools). In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. The same (or conservative substitutions of these mutations) at corresponding positions in other Cas9s are also preferred. Particularly preferred are D10 and H840 in SpCas9. However, in other Cas9s, residues corresponding to SpCas9 D10 and H840 are also preferred. For instance in Sa Cas9, a mutation at N580, e.g., N580A is advantageous. Orthologs of SpCas9 can be used in the practice of the invention. A Cas enzyme may be identified Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 (*S. pyogenes* Cas9) or saCas9 (*S. aureus* Cas9). StCas9" refers to wild type Cas9 from S. thermophilus, the protein sequence of which is given in the SwissProt database under accession number G3ECR1. Similarly, S pyogenes Cas9 or spCas9 is included in SwissProt under accession number Q99ZW2. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein. It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCa9, St1Cas9 and so forth. Enzymatic action by Cas9 derived from *Streptococcus pyogenes* or any closely related Cas9 generates double stranded breaks at target site sequences which hybridize to 20 nucleotides of the guide sequence and that have a protospacer-adjacent motif (PAM) sequence (examples include NGG/NRG or a PAM that can be determined as described herein) following the 20 nucleotides of the target sequence. CRISPR activity through Cas9 for site-specific DNA recognition and cleavage is defined by the guide sequence, the tracr sequence that hybridizes in part to the guide sequence and the PAM sequence. More aspects of the CRISPR system are described in Karginov and Hannon, The CRISPR system: small RNA-guided defence in bacteria and archaea, Mole Cell 2010, January 15; 37(1): 7. The type II CRISPR locus from *Streptococcus pyogenes* SF370, which contains a cluster of four genes Cas9, Cas1, Cas2, and Csn1, as well as two non-coding RNA elements, tracrRNA and a characteristic array of repetitive sequences (direct repeats) interspaced by short stretches of non-repetitive sequences (spacers, about 30bp each). In this system, targeted DNA double-strand break (DSB) is generated in four sequential steps. First, two non-coding RNAs, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Second, tracrRNA hybridizes to the direct repeats of pre-crRNA, which is then processed into mature crRNAs containing individual spacer sequences. Third, the mature crRNA:tracrRNA complex directs Cas9 to the DNA target consisting of the protospacer and the corresponding PAM via heteroduplex formation between the spacer region of the crRNA and the protospacer DNA. Finally, Cas9 mediates cleavage of target DNA upstream of PAM to create a DSB within the protospacer. A pre-crRNA array consisting of a single spacer flanked by two direct repeats (DRs) is also encompassed by the term "tracr-mate sequences"). In certain embodiments, Cas9 may be constitutively present or inducibly present or conditionally present or administered or delivered. Cas9 optimization may be used to enhance function or to develop new functions, one can generate chimeric Cas9 proteins. And Cas9 may be used as a generic DNA binding protein.

[00124] Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence.

[00125] An example of a codon optimized sequence, is in this instance a sequence optimized for expression in a eukaryote, e.g., humans (i.e. being optimized for expression in humans), or for another eukaryote, animal or mammal as herein discussed; see, e.g., SaCas9 human codon optimized sequence in WO 2014/093622 (PCT/US2013/074667). Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species other than human, or for codon optimization for specific organs is known. In some embodiments, an enzyme coding sequence encoding a CRISPR enzyme is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, or non-human eukaryote or animal or mammal as herein discussed, e.g., mouse, rat, rabbit, dog, livestock, or non-human mammal or primate. In some embodiments, processes for modifying the germ line genetic identity of human beings and/or processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes, may be excluded. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at www.kazusa.orjp/codon/ and these tables can be adapted in a number of ways. See Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" Nucl. Acids Res. 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or

more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a CRISPR enzyme correspond to the most frequently used codon for a particular amino acid.

[00126] In some embodiments, a vector encodes a CRISPR enzyme comprising one or more nuclear localization sequences (NLSs), such as about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs. In some embodiments, the CRISPR enzyme comprises about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the amino-terminus, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the carboxy-terminus, or a combination of these (e.g. zero or at least one or more NLS at the amino-terminus and zero or at one or more NLS at the carboxy terminus). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In a preferred embodiment of the invention, the CRISPR enzyme comprises at most 6 NLSs. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N- or C-terminus. Non-limiting examples of NLSs include an NLS sequence derived from: the NLS of the SV40 virus large T-antigen, having the amino acid sequence PKKKRKV; the NLS from nucleoplasmin (e.g. the nucleoplasmin bipartite NLS with the sequence KRPAATKKAGQAKKKK); the c-myc NLS having the amino acid sequence PAAKRVKLD or RQRRNELKRSP; the hRNPA1 M9 NLS having the sequence NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY; the sequence RMRIZFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV of the IBB domain from importin-alpha; the sequences VSRKRPRP and PPKKARED of the myoma T protein; the sequence POPKKKPL of human p53; the sequence SALIKKKKKMAP of mouse c-abl IV; the sequences DRLRR and PKQKKRK of the influenza virus NS1; the sequence RKLKKKIKKL of the Hepatitis virus delta antigen; the sequence REKKKFLKRR of the mouse Mx1 protein; the sequence KRKGDEVDGVDEVAKKKSKK of the human poly(ADP-ribose) polymerase; and the sequence RKCLQAGMNLEARKTKK of the steroid hormone receptors (human) glucocorticoid. In general, the one or more NLSs are of sufficient strength to drive accumulation of the CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In general, strength of nuclear localization activity may derive from the number of NLSs in the CRISPR enzyme, the particular NLS(s) used, or a combination of these factors. Detection of accumulation in the nucleus may be performed by any suitable technique. For example, a detectable marker may be fused to the CRISPR enzyme, such that location within a cell may be visualized, such as in combination with a means for detecting the location of the nucleus (e.g. a stain specific for the nucleus such as DAPI). Cell nuclei may also be isolated from cells, the contents of which may then be analyzed by any suitable process for detecting protein, such as immunohistochemistry, Western blot, or enzyme activity assay. Accumulation in the nucleus may also be determined indirectly, such as by an assay for the effect of CRISPR complex formation (e.g. assay for DNA cleavage or mutation at the target sequence, or assay for altered gene expression activity affected by CRISPR complex formation and/or CRISPR enzyme activity), as compared to a control no exposed to the CRISPR enzyme or complex, or exposed to a CRISPR enzyme lacking the one or more NLSs.

[00127] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein. Only sgRNA pairs creating 5' overhangs with less than 8bp overlap between the guide sequences (offset greater than -8 bp) were able to mediate detectable indel formation. Importantly, each guide used in these assays is able to efficiently induce indels when paired with wildtype Cas9, indicating that the relative positions of the guide pairs are the most important parameters in predicting double nicking activity. Since Cas9n and Cas9H840A nick opposite strands of DNA, substitution of Cas9n with Cas9H840A with a given sgRNA pair should have resulted in the inversion of the overhang type; but no indel formation is observed as with Cas9H840A indicating that Cas9H840A is a CRISPR enzyme substantially lacking all DNA cleavage activity (which is when the DNA cleavage activity of the mutated enzyme is about no more than 25%, 10%, 5%, 1%, 0.1%, 0.01%, or less of the DNA cleavage activity of the non-mutated form of the enzyme; whereby an example can be when the DNA cleavage activity of the mutated form is nil or negligible as compared with the non-mutated form, e.g., when no indel formation is observed as with Cas9H840A in the eukaryotic system in contrast to the biochemical or prokaryotic systems). Nonetheless, a pair of sgRNAs that will generate a 5' overhang with Cas9n should in principle generate the corresponding 3' overhang instead, and double nicking. Therefore, sgRNA pairs that lead to the generation of a 3' overhang with Cas9n can be used with another mutated Cas9 to generate a 5' overhang, and double nicking. Accordingly, in some embodiments, a recombination template is also provided. A recombination template may be a component of another vector as described herein, contained in a separate vector, or provided as a separate polynucleotide. In some embodiments, a recombination template is designed to serve as a template in homologous recombination, such as within or near a target sequence nicked or cleaved by a CRISPR enzyme as a part of a CRISPR complex. A template polynucleotide may be of any suitable length, such as about or more than about 10, 15, 20, 25, 50, 75, 100, 150, 200, 500, 1000, or more nucleotides in length. In some embodiments, the template polynucleotide is complementary to a portion of a polynucleotide comprising the target sequence. When optimally aligned, a template polynucleotide might overlap with one or more nucleotides of a target sequences (e.g. about or more than about 1, 5, 10, 15, 20, or more nucleotides). In some embodiments, when a template sequence and a polynucleotide comprising a target sequence are optimally aligned, the nearest nucleotide of the template polynucleotide is within about 1, 5, 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, 5000, 10000, or more nucleotides from the target sequence.

[00128] In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR system are introduced into a host cell such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Or, RNA(s) of the CRISPR System can be delivered to a transgenic Cas9 animal or mammal, e.g., an animal or mammal that constitutively or inducibly or conditionally expresses Cas9; or an animal or mammal that is otherwise expressing Cas9 or has cells containing Cas9, such as by way of prior administration thereto of a vector or vectors that code for and express in vivo Cas9. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. In some embodiments, a single promoter drives expression of a transcript encoding a CRISPR enzyme and one or more of the guide sequence, tracr mate sequence (optionally operably linked to the guide sequence), and a tracr sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one intron, or all in a single intron). In some embodiments, the CRISPR enzyme, guide sequence, tracr mate sequence, and tracr sequence are operably linked to and expressed from the same promoter. Delivery vehicles, vectors, particles, nanoparticles, formulations and components thereof for expression of one or more elements of a CRISPR system are as used in the foregoing documents, such as WO 2014/093622 (PCT/US2013/074667). In some embodiments, a vector comprises one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a "cloning site"). In some embodiments, one or more insertion sites (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more insertion sites) are located upstream and/or downstream of one or more sequence elements of one or more vectors. In some embodiments, a vector comprises an insertion site upstream of a tracr mate sequence, and optionally downstream of a regulatory element operably linked to the tracr mate sequence, such that following insertion of a guide sequence into the insertion site and upon expression the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell. In some embodiments, a vector comprises two or more insertion sites, each insertion site being located between two tracr mate sequences so as to allow insertion of a guide sequence at each site. In such an arrangement, the two or more guide sequences may comprise two or more copies of a single guide sequence, two or more different guide sequences, or combinations of these. When multiple different guide sequences are used, a single expression construct may be used to target CRISPR activity to multiple different, corresponding target sequences within a cell. For example, a single vector may comprise about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or more guide sequences. In some embodiments, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more such guide-sequence-containing vectors may be provided, and optionally delivered to a cell. In some embodiments, a vector comprises a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, such as a Cas protein. CRISPR enzyme or CRISPR enzyme mRNA or CRISPR guide RNA or RNA(s) can be delivered separately; and advantageously at least one of these is delivered via a nanoparticle complex. CRISPR enzyme mRNA can be delivered prior to the guide RNA to give time for CRISPR enzyme to be expressed. CRISPR enzyme mRNA might be administered 1-12 hours (preferably around 2-6 hours) prior to the administration of guide RNA. Alternatively, CRISPR enzyme mRNA and guide RNA can be administered together. Advantageously, a second booster dose of guide RNA can be administered 1-12 hours (preferably around 2-6 hours) after the initial administration of CRISPR enzyme mRNA + guide RNA. Additional administrations of CRISPR enzyme mRNA and/or guide RNA might be useful to achieve the most efficient levels of genome modification.

[00129] In one aspect, the invention provides methods for using one or more elements of a CRISPR system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utility including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene therapy, drug screening, disease diagnosis, and prognosis. An exemplary CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence. In one embodiment, this invention provides a method of cleaving a target polynucleotide. The method comprises modifying a target polynucleotide using a CRISPR complex that binds to the target polynucleotide and effect cleavage of said target polynucleotide. Typically, the CRISPR complex of the invention, when introduced into a cell, creates a break (e.g., a single or a double strand break) in the genome sequence. For example, the method can be used to cleave a disease gene in a cell. The break created by the CRISPR complex can be repaired by a repair processes such as the error prone non-homologous end joining (NHEJ) pathway or the high fidelity homology-directed repair (HDR). During these repair process, an exogenous polynucleotide template can be introduced into the genome sequence. In some methods, the HDR process is used modify genome sequence. For example, an exogenous polynucleotide template comprising a sequence to be integrated flanked by an upstream sequence and a downstream sequence is introduced into a cell. The upstream and downstream sequences share sequence similarity with either side of the site of integration in the chromosome. Where desired, a donor polynucleotide can be DNA, e.g., a DNA plasmid, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), a viral vector, a linear piece of DNA, a PCR fragment, a naked nucleic acid, or a nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. The exogenous polynucleotide template comprises a sequence to be integrated (e.g., a mutated gene). The sequence for integration may be a sequence endogenous or exogenous to the cell. Examples of a sequence to be integrated include polynucleotides encoding a protein or a non-coding RNA (e.g., a microRNA). Thus, the sequence for integration may be operably linked to an appropriate control sequence or sequences. Alternatively, the sequence to be integrated may provide a regulatory function. The upstream and downstream sequences in the exogenous polynucleotide template are selected to promote recombination between the chromosomal sequence of interest and the donor polynucleotide. The upstream sequence is a nucleic acid sequence that shares sequence similarity with the genome sequence upstream of the targeted site for integration. Similarly, the downstream sequence is a nucleic acid sequence that shares sequence similarity with the chromosomal sequence downstream of the targeted site of integration. The upstream and downstream sequences in the exogenous polynucleotide template can have 75%, 80%, 85%, 90%, 95%, or 100% sequence identity with the targeted genome sequence. Preferably, the upstream and downstream sequences in the exogenous polynucleotide template have about 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the targeted genome sequence. In some methods, the upstream and downstream sequences in the exogenous polynucleotide template have about 99% or 100% sequence identity with the targeted genome sequence. An upstream or downstream sequence may comprise from about 20 bp to about 2500 bp, for example, about 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, or 2500 bp. In some methods, the exemplary upstream or downstream sequence have about 200 bp to about 2000 bp, about 600 bp to about 1000 bp, or more particularly about 700 bp to about 1000 bp. In some methods, the exogenous polynucleotide template may further comprise a marker. Such a marker may make it easy to screen for targeted integrations. Examples of suitable markers include restriction sites, fluorescent proteins, or selectable markers. The exogenous polynucleotide template of the invention can be constructed using recombinant techniques (see, for example, Sambrook et al., 2001 and Ausubel et al., 1996). In a method for modifying a target polynucleotide by integrating an exogenous polynucleotide template, a double stranded break is introduced into the genome sequence by the CRISPR complex, the break is repaired via homologous recombination an exogenous polynucleotide template such that the template is integrated into the genome. The presence of a double-stranded break facilitates integration of the template. In other embodiments, this invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. The method comprises increasing or decreasing expression of a target polynucleotide by using a CRISPR complex that binds to the polynucleotide. In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a cell. For example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does. For example, a protein or microRNA coding sequence may be inactivated such that the protein or microRNA or pre-microRNA transcript is not produced. In some methods, a control sequence can be inactivated such that it no longer functions as a control sequence. As used herein, "control sequence" refers to any nucleic acid sequence that effects the transcription, translation, or accessibility of a nucleic acid sequence. Examples of a control sequence include, a promoter, a transcription terminator, and an enhancer are control sequences. The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Examples of target polynucleotides include a sequence associated with a signaling biochemical pathway, e.g., a signaling biochemical pathway-associated gene or polynucleotide. Examples of target polynucleotides include a disease associated gene or polynucleotide. A "disease-associated" gene or polynucleotide refers to any gene or polynucleotide which is yielding transcription or translation products at an abnormal level or in an abnormal form in cells derived from a disease-affected tissues compared with tissues or cells of a non disease control. It may be a gene that becomes expressed at an abnormally high level; it may be a gene that becomes expressed at an abnormally low level, where the altered expression correlates with the occurrence and/or progression of the disease. A disease-associated gene also refers to a gene possessing mutation(s) or genetic variation that is directly responsible or is in linkage disequilibrium with a gene(s) that is responsible for the etiology of a disease. The transcribed or translated products may be known or unknown, and may be at a normal or abnormal level. The target polynucleotide of a CRISPR complex can be

any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Without wishing to be bound by theory, it is believed that the target sequence should be associated with a PAM (protospacer adjacent motif); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence) Examples of PAM sequences are given in the examples section below, and the skilled person will be able to identify further PAM sequences for use with a given CRISPR enzyme. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.
In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.
Similar considerations and conditions apply as above for methods of modifying a target polynucleotide. In fact, these sampling, culturing and re-introduction options apply across the aspects of the present invention. In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell, which may be *in vivo, ex vivo* or *in vitro*. In some embodiments, the method comprises sampling a cell or population of cells from a human or non-human animal, and modifying the cell or cells. Culturing may occur at any stage *ex vivo*. The cell or cells may even be re-introduced into the non-human animal or plant. For re-introduced cells it is particularly preferred that the cells are stem cells.

[00130] Indeed, in any aspect of the invention, the CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence, wherein said guide sequence may be linked to a tracr mate sequence which in turn may hybridize to a tracr sequence.

[00131] The invention relates to the engineering and optimization of systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to the CRISPR-Cas system and components thereof. In advantageous embodiments, the Cas enzyme is Cas9. An advantage of the present methods is that the CRISPR system minimizes or avoids off-target binding and its resulting side effects. This is achieved using systems arranged to have a high degree of sequence specificity for the target DNA.

Self-inactivating systems

[00132] Once intended alterations have been introduced, such as by editing intended copies of a gene in the genome of a cell, continued CRISRP/Cas9 expression in that cell is no longer necessary. Indeed, sustained expression would be undesirable in certain casein case of off-target effects at unintended genomic sites, etc. Thus time-limited expression would be useful. Inducible expression offers one approach, but in addition Applicants have engineered a Self-Inactivating CRISPR-Cas9 system that relies on the use of a non-coding guide target sequence within the CRISPR vector itself. Thus, after expression begins, the CRISPR system will lead to its own destruction, but before destruction is complete it will have time to edit the genomic copies of the target gene (which, with a normal point mutation in a diploid cell, requires at most two edits). Simply, the self inactivating CRISPR-Cas system includes additional RNA (i.e., guide RNA) that targets the coding sequence for the CRISPR enzyme itself or that targets one or more non-coding guide target sequences complementary to unique sequences present in one or more of the following: (a) within the promoter driving expression of the non-coding RNA elements, (b) within the promoter driving expression of the Cas9 gene, (c) within 100bp of the ATG translational start codon in the Cas9 coding sequence, (d) within the inverted terminal repeat (iTR) of a viral delivery vector, e.g., in an AAV genome.

The Efficiency of HDR

[00133] Although the amount of genome modification in a target cell population required to create a therapeutic effect differs depending on the disease, the efficacy of most editing treatments are improved with increased editing rates. As previously noted, editing rates are controlled by the activity of DSB repair pathways and the efficiency of delivery to cells of interest. Therefore improvements to either one of these factors are likely to improve the efficacy of editing treatments.

[00134] Attempts to increase the activity rates of DSB repair pathways have largely focused on HDR, as cell cycle regulation and the challenge of delivering an HDR template with nucleases makes strategies employing this pathway less efficient than NHEJ. Cell cycle regulation has now been somewhat by-passed for slowly cycling cell types through stimulation of mitosis with pharmacologic agents *ex vivo* [Kormann, M.S., et al. Nature biotechnology 29, 154-157 (2011)]. However, truly post-mitotic cells are unlikely to be amenable to such manipulation, limiting the applicability of this strategy. Attempts have been made to completely circumvent the need for HDR through direct ligation of DNA templates containing therapeutic transgenes into targeted DSBs. Such ligation events have been observed, but the rates are too low to be useful for therapy [Ran, F.A., et al. Cell 154, 1380-1389 (2013); Orlando, S.J., et al. Nucleic acids research 38, e152 (2010)]. Likely dramatically new approaches are necessary to improve HDR efficiency and increase the therapeutic efficacy of strategies requiring precise genomic correction.

[00135] Genome editing presents tantalizing opportunities for tackling a number of intractable diseases. Nevertheless, the technology is still in its infancy and require a number of iterations to systematically optimize its efficacy, safety, and specificity. Additionally, despite the enormous excitement surrounding genome editing, strategic planning and rigorous but enabling regulatory processes are necessary to ensure successful development of this class of potentially life-changing medicine.

Delivery, including Delivery generally

[00136] A variety of nucleic acid or protein delivery methods may be used to introduce genome editing nucleases into target cells *ex vivo* or *in vivo*. Depending on the choice of delivery method, the nucleases may either be transiently or permanently expressed in the target cell. Given that nucleases may exhibit off-target cleavage activity or trigger immune responses, the delivery system should be carefully selected. For *ex vivo* applications, such as editing of hematopoietic stem cells, electroporation may be used to achieve transient nuclease expression through delivery of DNA-based nuclease expression vectors, mRNA, or protein. Both integration-competent and deficient lentiviral vectors have also been successfully used to drive nuclease expression. However, integrating lentiviral vectors may be less desirable as they drive constitutive expression and may result in more off-target activity. In addition, all three nuclease platform have also been demonstrated to be amenable to modifications so that proteins can be directly delivered into cells either through engineered cell-penetrating or chemical conjugation [Guilinger, J.P., et al. Nature methods 11, 429-435 (2014); Zuris, J.A., et al. Nature biotechnology (2014); Gaj, T., et al. Nature methods 9, 805-807 (2012)].

[00137] For *in vivo* applications, the most promising delivery systems are viral vectors, particularly adeno-associated viral (AAV) vectors, which have recently been approved for clinical use [Wirth, T., et al. Gene 525, 162-169 (2013)]. AAV comes in many serotypes and have been shown to have high delivery efficacy for a variety of tissue types including the eye, brain, liver, and muscle [Samulski, R.J. & Muzyczka, N. Annual Review of Virology 1, 427-451 (2014)]. However, the relatively small packaging capacity of AAV vectors post some challenges for nuclease delivery. Whereas ZFNs are relatively small and a dimeric ZFN pair can be packaged into a single AAV, a dimeric TALEN pair is much larger and likely need to be packaged into two separate AAV vectors. For Cas9, short orthologs may be packaged along with guide RNAs into a single AAV. So far, AAV-mediated nuclease expression has been demonstrated to be successful in several tissue types, including liver and brain [Li, H., et al. Nature 475, 217-221 (2011); Swiech, L., et al. Nature biotechnology (2014)].

[00138] Despite the potential of AAV-mediated *in vivo* nuclease expression, there are several challenges that require further development. First, AAV-mediated nuclease expression is often constitutive and it would be more desirable to be able to shut down nuclease expression after the genome editing event has successfully occurred in the target cell. Second, patients who have already been naturally exposed to AAV likely have developed immunity against specific serotypes. Therefore AAV may not be an appropriate delivery vehicle for these patients. To overcome these challenges faced by viral vectors, nanoparticle- and lipid-based *in vivo* mRNA or protein delivery systems may provide an attractive alternative [Zuris, J.A., et al. Nature biotechnology (2014); Kormann, M.S., et al. Nature biotechnology 29, 154-157 (2011)].

[00139] Through this disclosure and the knowledge in the art, CRISPR-Cas system, or components thereof or nucleic acid molecules thereof (including, for instance HDR template) or nucleic acid molecules encoding or providing components thereof may be delivered by a delivery system herein described both generally and in detail.

[00140] Vector delivery, e.g., plasmid, viral delivery: The CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can be delivered using any suitable vector, e.g., plasmid or viral vectors, such as adeno associated virus (AAV), lentivirus, adenovirus or other viral vector types, or combinations thereof. Cas9 and one or more guide RNAs can be packaged into one or more vectors, e.g., plasmid or viral vectors. In some embodiments, the vector, e.g., plasmid or viral vector is delivered to the tissue of interest by, for example, an intramuscular injection, while other times the delivery is via intravenous, transdermal, intranasal, oral, mucosal, or other delivery methods. Such delivery may be either via a single dose, or multiple doses. One skilled in the art understands that the actual dosage to be delivered herein may vary greatly depending upon a variety of factors, such as the vector choice, the target cell, organism, or tissue, the general condition of the subject to be treated, the degree of transformation/modification sought, the administration route, the administration mode, the type of transformation/modification sought, etc.

[00141] Such a dosage may further contain, for example, a carrier (water, saline, ethanol, glycerol, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, etc.), a diluent, a pharmaceutically-acceptable carrier (e.g., phosphate-buffered saline), a pharmaceutically-acceptable excipient, and/or other compounds known in the art. The dosage may further contain one or more pharmaceutically acceptable salts such as, for example, a mineral acid salt such as a hydrochloride, a hydrobromide, a phosphate, a sulfate, etc.; and the salts of organic acids such as acetates, propionates, malonates, benzoates, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, gels or gelling materials, flavorings, colorants, microspheres, polymers, suspension agents, etc. may also be present herein. In addition, one or more other conventional pharmaceutical ingredients, such as preservatives, humectants, suspending agents, surfactants, antioxidants, anticaking agents, fillers, chelating agents, coating agents, chemical stabilizers, etc. may also be present, especially if the dosage form is a reconstitutable form. Suitable exemplary ingredients include microcrystalline cellulose, carboxymethylcellulose sodium, polysorbate 80, phenylethyl alcohol, chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, parachlorophenol, gelatin, albumin and a combination thereof. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S

PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991) which is incorporated by reference herein.

[00142] In an embodiment herein the delivery is via an adenovirus, which may be at a single booster dose containing at least 1 x 10^5 particles (also referred to as particle units, pu) of adenoviral vector. In an embodiment herein, the dose preferably is at least about 1 x 10^6 particles (for example, about 1 x 10^6-1 x 10^{12} particles), more preferably at least about 1 x 10^7 particles, more preferably at least about 1 x 10^8 particles (e.g., about 1 x 10^8-1 x 10^{11} particles or about 1 x 10^8-1 x 10^{12} particles), and most preferably at least about 1 x 10^0 particles (e.g., about 1 x 10^9-1 x 10^{10} particles or about 1 x 10^9-1 x 10^{12} particles), or even at least about 1 x 10^{10} particles (e.g., about 1 x 10^{10}-1 x 10^{12} particles) of the adenoviral vector. Alternatively, the dose comprises no more than about 1 x 10^{14} particles, preferably no more than about 1 x 10^{13} particles, even more preferably no more than about 1 x 10^{12} particles, even more preferably no more than about 1 x 10^{11} particles, and most preferably no more than about 1 x 10^{10} particles (e.g., no more than about 1 x 10^9 articles). Thus, the dose may contain a single dose of adenoviral vector with, for example, about 1 x 10^6 particle units (pu), about 2 x 10^6 pu, about 4 x 10^6 pu, about 1 x 10^7 pu, about 2 x 10^7 pu, about 4 x 10^7 pu, about 1 x 10^8 pu, about 2 x 10^8 pu, about 4 x 10^8 pu, about 1 x 10^9 pu, about 2 x 10^9 pu, about 4 x 10^9 pu, about 1 x 10^{10} pu, about 2 x 10^{10} pu, about 4 x 10^{10} pu, about 1 x 10^{11} pu, about 2 x 10^{11} pu, about 4 x 10^{11} pu, about 1 x 10^{12} pu, about 2 x 10^{12} pu, or about 4 x 10^{12} pu of adenoviral vector. See, for example, the adenoviral vectors in U.S. Patent No. 8,454,972 B2 to Nabel, et. al., granted on June 4, 2013; incorporated by reference herein, and the dosages at col 29, lines 36-58 thereof. In an embodiment herein, the adenovirus is delivered via multiple doses.

[00143] In an embodiment herein, the delivery is via an AAV. A therapeutically effective dosage for in vivo delivery of the AAV to a human is believed to be in the range of from about 20 to about 50 ml of saline solution containing from about 1 x 10^{10} to about 1 x 10^{10} functional AAV/ml solution. The dosage may be adjusted to balance the therapeutic benefit against any side effects. In an embodiment herein, the AAV dose is generally in the range of concentrations of from about 1 x 10^5 to 1 x 10^{50} genomes AAV, from about 1 x 10^8 to 1 x 10^{20} genomes AAV, from about 1 x 10^{10} to about 1 x 10^{16} genomes, or about 1 x 10^{11} to about 1 x 10^{16} genomes AAV. A human dosage may be about 1 x 10^{13} genomes AAV. Such concentrations may be delivered in from about 0.001 ml to about 100 ml, about 0.05 to about 50 ml, or about 10 to about 25 ml of a carrier solution. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. See, for example, U.S. Patent No. 8,404,658 B2 to Hajjar, et al., granted on March 26, 2013, at col. 27, lines 45-60.

[00144] In an embodiment herein the delivery is via a plasmid. In such plasmid compositions, the dosage should be a sufficient amount of plasmid to elicit a response. For instance, suitable quantities of plasmid DNA in plasmid compositions can be from about 0.1 to about 2 mg, or from about 1 µg to about 10 µg per 70 kg individual. Plasmids of the invention will generally comprise (i) a promoter; (ii) a sequence encoding a CRISPR enzyme, operably linked to said promoter; (iii) a selectable marker; (iv) an origin of replication; and (v) a transcription terminator downstream of and operably linked to (ii). The plasmid can also encode the RNA components of a CRISPR complex, but one or more of these may instead be encoded on a different vector.

[00145] The doses herein are based on an average 70 kg individual. The frequency of administration is within the ambit of the medical or veterinary practitioner (e.g., physician, veterinarian), or scientist skilled in the art. It is also noted that mice used in experiments are typically about 20g and from mice experiments one can scale up to a 70 kg individual.

[00146] In some embodiments the RNA molecules of the invention are delivered in liposome or lipofectin formulations and the like and can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Pat. Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference. Delivery systems aimed specifically at the enhanced and improved delivery of siRNA into mammalian cells have been developed, (see, for example, Shen et al FEBS Let. 2003, 539:111-114; Xia et al., Nat. Biotech. 2002, 20:1006-1010; Reich et al., Mol. Vision. 2003, 9: 210-216; Sorensen et al., J. Mol. Biol. 2003, 327: 761-766; Lewis et al., Nat. Gen. 2002, 32: 107-108 and Simeoni et al., NAR 2003, 31, 11: 2717-2724) and may be applied to the present invention. siRNA has recently been successfully used for inhibition of gene expression in primates (see for example. Tolentino et al., Retina 24(4):660 which may also be applied to the present invention.

[00147] Indeed, RNA delivery is a useful method of in vivo delivery. It is possible to deliver Cas9 and gRNA (and, for instance, HR repair template) into cells using liposomes or nanoparticles. Thus delivery of the CRISPR enzyme, such as a Cas9 and/or delivery of the RNAs of the invention may be in RNA form and via microvesicles, liposomes or nanoparticles.

For example, Cas9 mRNA and gRNA can be packaged into liposomal particles for delivery in vivo. Liposomal transfection reagents such as lipofectamine from Life Technologies and other reagents on the market can effectively deliver RNA molecules into the liver.

[00148] Means of delivery of RNA also preferred include delivery of RNA via nanoparticles (Cho, S., Goldberg, M., Son, S., Xu, Q., Yang, F., Mei, Y., Bogatyrev, S., Langer, R. and Anderson, D., Lipid-like nanoparticles for small interfering RNA delivery to endothelial cells, Advanced Functional Materials, 19: 3112-3118, 2010) or exosomes (Schroeder, A., Levins, C., Cortez, C., Langer, R., and Anderson, D., Lipid-based nanotherapeutics for siRNA delivery, Journal of Internal Medicine, 267: 9-21, 2010, PMID: 20059641). Indeed, exosomes have been shown to be particularly useful in delivery siRNA, a system with some parallels to the CRISPR system. For instance, El-Andaloussi S, et al. ("Exosome-mediated delivery of siRNA in vitro and in vivo." Nat Protoc. 2012 Dec;7(12):2112-26. doi: 10.1038/nprot.2012.131. Epub 2012 Nov 15.) describe how exosomes are promising tools for drug delivery across different biological barriers and can be harnessed for delivery of siRNA in vitro and in vivo. Their approach is to generate targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. The exosomes are then purify and characterized from transfected cell supernatant, then RNA is loaded into the exosomes. Delivery or administration according to the invention can be performed with exosomes, in particular but not limited to the brain. Vitamin E (α-tocopherol) may be conjugated with CRISPR Cas and delivered to the brain along with high density lipoprotein (HDL), for example in a similar manner as was done by Uno et al. (HUMAN GENE THERAPY 22:711–719 (June 2011)) for delivering short-interfering RNA (siRNA) to the brain. Mice were infused via Osmotic minipumps (model 1007D; Alzet, Cupertino, CA) filled with phosphate-buffered saline (PBS) or free TocsiBACE or Toc-siBACE/HDL and connected with Brain Infusion Kit 3 (Alzet). A brain-infusion cannula was placed about 0.5mm posterior to the bregma at midline for infusion into the dorsal third ventricle. Uno et al. found that as little as 3 nmol of Toc-siRNA with HDL could induce a target reduction in comparable degree by the same ICV infusion method. A similar dosage of CRISPR Cas conjugated to α-tocopherol and co-administered with HDL targeted to the brain may be contemplated for humans in the present invention, for example, about 3 nmol to about 3 μmol of CRISPR Cas targeted to the brain may be contemplated. Zou et al. ((HUMAN GENE THERAPY 22:465-475 (April 2011)) describes a method of lentiviral-mediated delivery of short-hairpin RNAs targeting PKCγ for in vivo gene silencing in the spinal cord of rats. Zou et al. administered about 10 μl of a recombinant lentivirus having a titer of 1 x 109 transducing units (TU)/ml by an intrathecal catheter. A similar dosage of CRISPR Cas expressed in a lentiviral vector targeted to the brain may be contemplated for humans in the present invention, for example, about 10-50 ml of CRISPR Cas targeted to the brain in a lentivirus having a titer of 1 x 109 transducing units (TU)/ml may be contemplated.

[00149] In terms of local delivery to the brain, this can be achieved in various ways. For instance, material can be delivered intrastriatally e.g. by injection. Injection can be performed stereotactically via a craniotomy.

[00150] In some aspects, the invention provides methods comprising delivering one or more polynucleotides, such as or one or more vectors as described herein, one or more transcripts thereof, and/or one or proteins transcribed therefrom, to a host cell. In some aspects, the invention further provides cells produced by such methods, and animals comprising or produced from such cells. In some embodiments, a CRISPR enzyme in combination with (and optionally complexed with) a guide sequence is delivered to a cell. Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids in mammalian cells or target tissues.

[00151] Such methods can be used to administer nucleic acids encoding components of a CRISPR system to cells in culture, or in a host organism. Non-viral vector delivery systems include DNA plasmids, RNA (e.g. a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle, such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, Science 256:808-813 (1992); Nabel & Felgner, TIBTECH 11:211-217 (1993); Mitani & Caskey, TIBTECH 11:162-166 (1993); Dillon, TIBTECH 11:167-175 (1993); Miller, Nature 357:455-460 (1992); Van Brunt, Biotechnology 6(10):1149-1154 (1988); Vigne, Restorative Neurology and Neuroscience 8:35-36 (1995); Kremer & Perricaudet, British Medical Bulletin 51(1):31-44 (1995); Haddada et al., in Current Topics in Microbiology and Immunology Doerfler and Böhm (eds) (1995); and Yu et al., Gene Therapy 1:13-26 (1994). Methods of non-viral delivery of nucleic acids include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., U.S. Pat. Nos. 5,049,386, 4,946,787; and 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424; WO 91/16024. Delivery can be to cells (e.g. in vitro or ex vivo administration) or target tissues (e.g. in vivo administration). The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, Science 270:404-410 (1995); Blaese et al., Cancer Gene Ther. 2:291-297 (1995); Behr et al., Bioconjugate Chem. 5:382-389 (1994); Remy et al., Bioconjugate Chem. 5:647-654 (1994); Gao et al., Gene Therapy 2:710-722 (1995); Ahmad et al., Cancer Res. 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787). The use of RNA or DNA viral based systems for the delivery of nucleic acids take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro, and the modified cells may optionally be administered to patients (ex vivo). Conventional viral based systems could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues. The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher et al., J. Virol. 66:2731-2739 (1992); Johann et al., J.

Virol. 66:1635-1640 (1992); Sommnerfelt et al., Virol. 176:58-59 (1990); Wilson et al., J. Virol. 63:2374-2378 (1989); Miller et al., J. Virol. 65:2220-2224 (1991); PCT/US94/05700). In another embodiment, Cocal vesiculovirus envelope pseudotyped retroviral vector particles are contemplated (see, e.g., US Patent Publication No. 20120164118 assigned to the Fred Hutchinson Cancer Research Center). Cocal virus is in the Vesiculovirus genus, and is a causative agent of vesicular stomatitis in mammals. Cocal virus was originally isolated from mites in Trinidad (Jonkers et al., Am. J. Vet. Res. 25:236-242 (1964)), and infections have been identified in Trinidad, Brazil, and Argentina from insects, cattle, and horses. Many of the vesiculoviruses that infect mammals have been isolated from naturally infected arthropods, suggesting that they are vector-borne. Antibodies to vesiculoviruses are common among people living in rural areas where the viruses are endemic and laboratory-acquired; infections in humans usually result in influenza-like symptoms. The Cocal virus envelope glycoprotein shares 71.5% identity at the amino acid level with VSV-G Indiana, and phylogenetic comparison of the envelope gene of vesiculoviruses shows that Cocal virus is serologically distinct from, but most closely related to, VSV-G Indiana strains among the vesiculoviruses. Jonkers et al., Am. J. Vet. Res. 25:236-242 (1964) and Travassos da Rosa et al., Am. J. Tropical Med. & Hygiene 33:999-1006 (1984). The Cocal vesiculovirus envelope pseudotyped retroviral vector particles may include for example, lentiviral, alpharetroviral, betaretroviral, gammaretroviral, deltaretroviral, and epsilonretroviral vector particles that may comprise retroviral Gag, Pol, and/or one or more accessory protein(s) and a Cocal vesiculovirus envelope protein. Within certain aspects of these embodiments, the Gag, Pol, and accessory proteins are lentiviral and/or gammaretroviral. In applications where transient expression is preferred, adenoviral based systems may be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors may also be used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures (see, e.g., West et al., Virology 160:38-47 (1987); U.S. Pat. No. 4,797,368; WO 93/24641; Kotin, Human Gene Therapy 5:793-801 (1994); Muzyczka, J. Clin. Invest. 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin et al., Mol. Cell. Biol. 5:3251-3260

(1985); Tratschin, et al., Mol. Cell. Biol. 4:2072-2081 (1984); Hermonat & Muzyczka, PNAS 81:6466-6470 (1984); and Samulski et al., J. Virol. 63:03822-3828 (1989). Packaging cells are typically used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producer a cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the polynucleotide(s) to be expressed. The missing viral functions are typically supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences. The cell line may also be infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV. Accordingly, AAV is considered an ideal candidate for use as a transducing vector. Such AAV transducing vectors can comprise sufficient cis-acting functions to replicate in the presence of adenovirus or herpesvirus or poxvirus (e.g., vaccinia virus) helper functions provided in trans. Recombinant AAV (rAAV) can be used to carry exogenous genes into cells of a variety of lineages. In these vectors, the AAV cap and/or rep genes are deleted from the viral genome and replaced with a DNA segment of choice. Current AAV vectors may accommodate up to 4300 bases of inserted DNA. There are a number of ways to produce rAAV, and the invention provides rAAV and methods for preparing rAAV. For example, plasmid(s) containing or consisting essentially of the desired viral construct are transfected into AAV-infected cells. In addition, a second or additional helper plasmid is cotransfected into these cells to provide the AAV rep and/or cap genes which are obligatory for replication and packaging of the recombinant viral construct. Under these conditions, the rep and/or cap proteins of AAV act in trans to stimulate replication and packaging of the rAAV construct. Two to Three days after transfection, rAAV is harvested. Traditionally rAAV is harvested from the cells along with adenovirus. The contaminating adenovirus is then inactivated by heat treatment. In the instant invention, rAAV is advantageously harvested not from the cells themselves, but from cell supernatant. Accordingly, in an initial aspect the invention provides for preparing rAAV, and in addition to the foregoing, rAAV can be prepared by a method that comprises or consists essentially of: infecting susceptible cells with a rAAV containing exogenous DNA including DNA for expression, and helper virus (e.g., adenovirus, herpesvirus, poxvirus such as vaccinia virus) wherein the rAAV lacks functioning cap and/or rep (and the helper virus (e.g., adenovirus, herpesvirus, poxvirus such as vaccinia virus) provides the cap and/or rev function that the rAAV lacks); or infecting susceptible cells with a rAAV containing exogenous DNA including DNA for expression, wherein the recombinant lacks functioning cap and/or rep, and transfecting said cells with a plasmid supplying cap and/or rep function that the rAAV lacks; or infecting susceptible cells with a rAAV containing exogenous DNA including DNA for expression, wherein the recombinant lacks functioning cap and/or rep, wherein said cells supply cap and/or rep function that the recombinant lacks; or transfecting the susceptible cells with an AAV lacking functioning cap and/or rep and plasmids for inserting exogenous DNA into the recombinant so that the exogenous DNA is expressed by the recombinant and for supplying rep and/or cap functions whereby transfection results in an rAAV containing the exogenous DNA including DNA for expression that lacks functioning cap and/or rep. The rAAV can be from an AAV as herein described, and advantageously can be an rAAV1, rAAV2, AAV5 or rAAV having hybrid or capsid which may comprise AAV1, AAV2, AAV5 or any combination thereof. One can select the AAV of the rAAV with regard to the cells to be targeted by the rAAV; e.g., one can select AAV serotypes 1, 2, 5 or a hybrid or capsid AAV1, AAV2, AAV5 or any combination thereof for targeting brain or neuronal cells; and one can select AAV4 for targeting cardiac tissue. In addition to 293 cells, other cells that can be used in the practice of the invention and the relative infectivity of certain AAV serotypes *in vitro* as to these cells (see Grimm, D. et al, J. Virol. 82: 5887-5911 (2008)) are as follows:

Cell Line	AAV-1	AAV-2	AAV-3	AAV-4	AAV-5	AAV-6	AAV-8	AAV-9
Huh-7	13	100	2.5	0.0	0.1	10	0.7	0.0
HEK293	25	100	2.5	0.1	0.1	5	0.7	0.1
HeLa	3	100	2.0	0.1	6.7	1	0.2	0.1
HepG2	3	100	16.7	0.3	1.7	5	0.3	ND
Hep1A	20	100	0.2	1.0	0.1	1	0.2	0.0
911	17	100	11	0.2	0.1	17	0.1	ND
CHO	100	100	14	1.4	333	50	10	1.0
COS	33	100	33	3.3	5.0	14	2.0	0.5

Cell Line	AAV-1	AAV-2	AAV-3	AAV-4	AAV-5	AAV-6	AAV-8	AAV-9
MeWo	10	100	20	0.3	6.7	10	1.0	0.2
NIH3T3	10	100	2.9	2.9	0.3	10	0.3	ND
A549	14	100	20	ND	0.5	10	0.5	0.1
HT1180	20	100	10	0.1	0.3	33	0.5	0.1
Monocytes	1111	100	ND	ND	125	1429	ND	ND
Immature DC	2500	100	ND	ND	222	2857	ND	ND
Mature DC	2222	100	ND	ND	333	3333	ND	ND

[00152] The invention provides rAAV that contains or consists essentially of an exogenous nucleic acid molecule encoding a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system, e.g., a plurality of cassettes comprising or consisting a first cassette comprising or consisting essentially of a promoter, a nucleic acid molecule encoding a CRISPR-associated (Cas) protein (putative nuclease or helicase proteins), e.g., Cas9 and a terminator, and a two, or more, advantageously up to the packaging size limit of the vector, e.g., in total (including the first cassette) five, cassettes comprising or consisting essentially of a promoter, nucleic acid molecule encoding guide RNA (gRNA) and a terminator (e.g., each cassette schematically represented as Promoter-gRNA1-terminator, Promoter-gRNA2-terminator ... Promoter-gRNA(N)-terminator (where N is a number that can be inserted that is at an upper limit of the packaging size limit of the vector), or two or more individual rAAVs, each containing one or more than one cassette of a CRISPR system, e.g., a first rAAV containing the first cassette comprising or consisting essentially of a promoter, a nucleic acid molecule encoding Cas, e.g., Cas9 and a terminator, and a second rAAV containing a plurality, four, cassettes comprising or consisting essentially of a promoter, nucleic acid molecule encoding guide RNA (gRNA) and a terminator (e.g., each cassette schematically represented as Promoter-gRNA1-terminator, Promoter-gRNA2-terminator ... Promoter-gRNA(N)-terminator (where N is a number that can be inserted that is at an upper limit of the packaging size limit of the vector). As rAAV is a DNA virus, the nucleic acid molecules in the herein discussion concerning AAV or rAAV are advantageously DNA. The promoter is in some embodiments advantageously human Synapsin I promoter (hSyn). Additional methods for the delivery of nucleic acids to cells are known to those skilled in the art. See, for example, US20030087817, incorporated herein by reference. In some embodiments, a host cell is transiently or non-transiently transfected with one or more vectors described herein. In some embodiments, a cell is transfected as it naturally occurs in a subject. In some embodiments, a cell that is transfected is taken from a subject. In some embodiments, the cell is derived from cells taken from a subject, such as a cell line. A wide variety of cell lines for tissue culture are known in the art. Examples of cell lines include, but are not limited to, C8161, CCRF-CEM, MOLT, mIMCD-3, NHDF, HeLa-S3, Huh1, Huh4, Huh7, HUVEC, HASMC, HEKn, HEKa, MiaPaCell, Panc1, PC-3, TF1, CTLL-2, C1R, Rat6, CV1, RPTE, A10, T24, J82, A375, ARH-77, Calu1, SW480, SW620, SKOV3, SK-UT, CaCo2, P388D1, SEM-K2, WEHI-231, HB56, TIB55, Jurkat, J45.01, LRMB, Bcl-1, BC-3, IC21, DLD2, Raw264.7, NRK, NRK-52E, MRC5, MEF, Hep G2, HeLa B, HeLa T4, COS, COS-1, COS-6, COS-M6A, BS-C-1 monkey kidney epithelial, BALB/ 3T3 mouse embryo fibroblast, 3T3 Swiss, 3T3-L1, 132-d5 human fetal fibroblasts; 10.1 mouse fibroblasts, 293-T, 3T3, 721, 9L, A2780, A2780ADR, A2780cis, A172, A20, A253, A431, A-549, ALC, B16, B35, BCP-1 cells, BEAS-2B, bEnd.3, BHK-21, BR 293, BxPC3, C3H-10T1/2, C6/36, Cal-27, CHO, CHO-7, CHO-IR, CHO-K1, CHO-K2, CHO-T, CHO Dhfr -/-, COR-L23, COR-L23/CPR, COR-L23/5010, COR-L23/R23, COS-7, COV-434, CML T1, CMT, CT26, D17, DH82, DU145, DuCaP, EL4, EM2, EM3, EMT6/AR1, EMT6/AR10.0, FM3, H1299, H69, HB54, HB55, HCA2, HEK-293, HeLa, Hepa1c1c7, HL-60, HMEC, HT-29, Jurkat, JY cells, K562 cells, Ku812, KCL22, KG1, KYO1, LNCap, Ma-Mel 1-48, MC-38, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, MDA-MB-435, MDCK II, MDCK II, MOR/0.2R, MONO-MAC 6, MTD-1A, MyEnd, NCI-H69/CPR, NCI-H69/LX10, NCI-H69/LX20, NCI-H69/LX4, NIH-3T3, NALM-1, NW-145, OPCN / OPCT cell lines, Peer, PNT-1A / PNT 2, RenCa, RIN-5F, RMA/RMAS, Saos-2 cells, Sf-9, SkBr3, T2, T-47D, T84, THP1 cell line, U373, U87, U937, VCaP, Vero cells, WM39, WT-49, X63, YAC-1, YAR, and transgenic varieties thereof. Cell lines are available from a variety of sources known to those with skill in the art (see, e.g., the American Type Culture Collection (ATCC) (Manassus, Va.)). In some embodiments, a cell transfected with one or more vectors described herein is used to establish a new cell line comprising one or more vector-derived sequences. In some embodiments, a cell transiently transfected with the components of a CRISPR system as described herein (such as by transient transfection of one or more vectors, or transfection with RNA), and modified through the activity of a CRISPR complex, is used to establish a new cell line comprising cells containing the modification but lacking any other exogenous sequence. In some embodiments, cells transiently or non-transiently transfected with one or more vectors described herein, or cell lines derived from such cells are used in assessing one or more test compounds.

[00153] Enhancing NHEJ or HR efficiency is also helpful for delivery. It is preferred that NHEJ efficiency is enhanced by co-expressing end-processing enzymes such as Trex2 (Dumitrache et al. Genetics. 2011 August; 188(4): 787–797). It is preferred that HR efficiency is increased by transiently inhibiting NHEJ machineries such as Ku70 and Ku86. HR efficiency can also be increased by co-expressing prokaryotic or eukaryotic homologous recombination enzymes such as RecBCD, RecA.

Packaging and Promoters generally

[00154] Ways to package Cas9 coding nucleic acid molecules, e.g., DNA, into vectors, e.g., viral vectors, to mediate genome modification in vivo include:

To achieve NHEJ-mediated gene knockout:

Single virus vector:

Vector containing two or more expression cassettes:

Promoter-Cas9 coding nucleic acid molecule-terminator

Promoter-gRNA1-terminator

Promoter-gRNA2-terminator

Promoter-gRNA(N)-terminator (up to size limit of vector)

Double virus vector:

Vector 1 containing one expression cassette for driving the expression of Cas9

Promoter-Cas9 coding nucleic acid molecule-terminator

Vector 2 containing one more expression cassettes for driving the expression of one or more guideRNAs

Promoter-gRNA1-terminator

Promoter-gRNA(N)-terminator (up to size limit of vector)

To mediate homology-directed repair.

In addition to the single and double virus vector approaches described above, an additional vector is used to deliver a homology-direct repair template.

[00155] The promoter used to drive Cas9 coding nucleic acid molecule expression can include:

AAV ITR can serve as a promoter: this is advantageous for eliminating the need for an additional promoter element (which can take up space in the vector). The additional space freed up can be used to drive the expression of additional elements (gRNA, etc.). Also, ITR activity is relatively weaker, so can be used to reduce potential toxicity due to over expression of Cas9.

For ubiquitous expression, can use promoters: CMV, CAG, CBh, PGK, SV40, Ferritin heavy or light chains, etc.

For brain or other CNS expression, can use promoters: SynapsinI for all neurons, CaMKIIalpha for excitatory neurons, GAD67 or GAD65 or VGAT for GABAergic neurons, etc.

For liver expression, can use Albumin promoter.

For lung expression, can use SP-B.

For endothelial cells, can use ICAM.

For hematopoietic cells can use IFNbeta or CD45.

For Osteoblasts can use OG-2.

[00156] The promoter used to drive guide RNA can include:

Pol III promoters such as U6 or H1

Use of Pol II promoter and intronic cassettes to express gRNA

Adeno associated virus (AAV)

[00157] Cas9 and one or more guide RNA can be delivered using adeno associated virus (AAV), lentivirus, adenovirus or other plasmid or viral vector types, in particular, using formulations and doses from, for example, US Patents Nos. 8,454,972 (formulations, doses for adenovirus), 8,404,658 (formulations, doses for AAV) and 5,846,946 (formulations, doses for DNA plasmids) and from clinical trials and publications regarding the clinical trials involving lentivirus, AAV and adenovirus. For examples, for AAV, the route of administration, formulation and dose can be as in US Patent No. 8,454,972 and as in clinical trials involving AAV. For Adenovirus, the route of administration, formulation and dose can be as in US Patent No. 8,404,658 and as in clinical trials involving adenovirus. For plasmid delivery, the route of administration, formulation and dose can be as in US Patent No 5,846,946 and as in clinical studies involving plasmids. Doses may be based on or extrapolated to an average 70 kg individual (e.g. a male adult human), and can be adjusted for patients, subjects, mammals of different weight and species. Frequency of administration is within the ambit of the medical or veterinary practitioner (e.g., physician, veterinarian), depending on usual factors including the age, sex, general health, other conditions of the patient or subject and the particular condition or symptoms being addressed. The viral vectors can be injected into the tissue of interest. For cell-type specific genome modification, the expression of Cas9 can be driven by a cell-type specific promoter. For example, liver-specific expression might use the Albumin promoter and neuron-specific expression (e.g. for targeting CNS disorders) might use the Synapsin I promoter.

[00158] In terms of in vivo delivery, AAV is advantageous over other viral vectors for a couple of reasons:

Low toxicity (this may be due to the purification method not requiring ultra centrifugation of cell particles that can activate the immune response)

Low probability of causing insertional mutagenesis because it doesn't integrate into the host genome.

[00159] AAV has a packaging limit of 4.5 or 4.75 Kb. This means that Cas9 as well as a promoter and transcription terminator have to be all fit into the same viral vector. Constructs larger than 4.5 or 4.75 Kb will lead to significantly reduced virus production. SpCas9 is quite large, the gene itself is over 4.1 Kb, which makes it difficult for packing into AAV. Therefore embodiments of the invention include utilizing homologs of Cas9 that are shorter. For example:

Species	Cas9 Size
Corynebacter diphtheriae	3252
Eubacterium ventriosum	3321
Streptococcus pasteurianus	3390
Lactobacillus farciminis	3378
Sphaerochaeta globus	3537
Azospirillum B510	3504
Gluconacetobacter diazotrophicus	3150
Neisseria cinerea	3246
Roseburia intestinalis	3420
Parvibaculum lavamentivorans	3111
Staphylococcus aureus	3159
Nitratifractor salsuginis DSM 16511	3396
Campylobacter lari CF89-12	3009
Streptococcus thermophilus LMD-9	3396

[00160] These species are therefore, in general, preferred Cas9 species.

[00161] As to AAV, the AAV can be AAV1, AAV2, AAV5 or any combination thereof. One can select the AAV of the AAV with regard to the cells to be targeted; e.g., one can select AAV serotypes 1, 2, 5 or a hybrid capsid AAV1, AAV2, AAV5 or any combination thereof for targeting brain or neuronal cells; and one can select AAV4 for targeting cardiac tissue. AAV8 is useful for delivery to the liver. The herein promoters and vectors are preferred individually. A tabulation of certain AAV serotypes as to these cells (see Grimm, D. et al, J. Virol. 82: 5887-5911 (2008)) is as follows:

Cell Line	AAV-1	AAV-2	AAV-3	AAV-4	AAV-5	AAV-6	AAV-8	AAV-9
Huh-7	13	100	2.5	0.0	0.1	10	0.7	0.0
HEK293	25	100	2.5	0.1	0.1	5	0.7	0.1
HeLa	3	100	2.0	0.1	6.7	1	0.2	0.1
HepG2	3	100	16.7	0.3	1.7	5	0.3	ND
Hep1A	20	100	0.2	1.0	0.1	1	0.2	0.0
911	17	100	11	0.2	0.1	17	0.1	ND
CHO	100	100	14	1.4	333	50	10	1.0
COS	33	100	33	3.3	5.0	14	2.0	0.5
MeWo	10	100	20	0.3	6.7	10	1.0	0.2
NIH3T3	10	100	2.9	2.9	0.3	10	0.3	ND
A549	14	100	20	ND	0.5	10	0.5	0.1
HT1180	20	100	10	0.1	0.3	33	0.5	0.1
Monocytes	1111	100	ND	ND	125	1429	ND	ND
Immature DC	2500	100	ND	ND	222	2857	ND	ND
Mature DC	2222	100	ND	ND	333	3333	ND	ND

Lentivirus

[00162] Lentiviruses are complex retroviruses that have the ability to infect and express their genes in both mitotic and post-mitotic cells. The most commonly known lentivirus is the human immunodeficiency virus (HIV), which uses the envelope glycoproteins of other viruses to target a broad range of cell types.

[00163] Lentiviruses may be prepared as follows. After cloning pCasES10 (which contains a lentiviral transfer plasmid backbone), HEK293FT at low passage (p=5) were seeded in a T-75 flask to 50% confluence the day before transfection in DMEM with 10% fetal bovine serum and without antibiotics. After 20 hours, media was changed to OptiMEM (serum-free) media and transfection was done 4 hours later. Cells were transfected with 10 µg of lentiviral transfer plasmid (pCasES10) and the following packaging plasmids: 5 µg of pMD2.G (VSV-g pseudotype), and 7.5ug of psPAX2 (gag/pol/rev/tat). Transfection was done in 4mL OptiMEM with a cationic lipid delivery agent (50uL Lipofectamine 2000 and 100ul Plus reagent). After 6 hours, the media was changed to antibiotic-free DMEM with 10% fetal bovine serum. These methods use serum during cell culture, but serum-free methods are preferred.

[00164] Lentivirus may be purified as follows. Viral supernatants were harvested after 48 hours. Supernatants were first cleared of debris and filtered through a 0.45um low protein binding (PVDF) filter. They were then spun in a ultracentrifuge for 2 hours at 24,000 rpm. Viral pellets were resuspended in 50ul of DMEM overnight at 4C. They were then aliquotted and immediately frozen at -80°C.

[00165] In another embodiment, minimal non-primate lentiviral vectors based on the equine infectious anemia virus (EIAV) are also contemplated, especially for ocular gene therapy (see, e.g., Balagaan, J Gene Med 2006; 8: 275 – 285). In another embodiment, RetinoStat®, an equine infectious anemia virus-based lentiviral gene therapy vector that expresses angiostatic proteins endostatin and angiostatin that is delivered via a subretinal injection for the treatment of the web form of age-related macular degeneration is also contemplated (see, e.g., Binley et al., HUMAN GENE THERAPY 23:980–991 (September 2012)) and this vector may be modified for the CRISPR-Cas system of the present invention.

[00166] In another embodiment, self-inactivating lentiviral vectors with an siRNA targeting a common exon shared by HIV tat/rev, a nucleolar-localizing TAR decoy, and an anti–CCR5-specific hammerhead ribozyme (see, e.g., DiGiusto et al. (2010) Sci Transl Med 2:36ra43) may be used/and or adapted to the CRISPR-Cas system of the present invention. A minimum of 2.5 × 106 CD34+ cells per kilogram patient weight may be collected and prestimulated for 16 to 20 hours in X-VIVO 15 medium (Lonza) containing 2 μmol/L-glutamine, stem cell factor (100 ng/ml), Flt-3 ligand (Flt-3L) (100 ng/ml), and thrombopoietin (10 ng/ml) (CellGenix) at a density of 2 × 106 cells/ml. Prestimulated cells may be transduced with lentiviral at a multiplicity of infection of 5 for 16 to 24 hours in 75-cm2 tissue culture flasks coated with fibronectin (25 mg/cm2) (RetroNectin,Takara Bio Inc.).

[00167] Lentiviral vectors have been disclosed as in the treatment for Parkinson's Disease, see, e.g., US Patent Publication No. 20120295960 and US Patent Nos. 7303910 and 7351585. Lentiviral vectors have also been disclosed for the treatment of ocular diseases, see e.g., US Patent Publication Nos. 20060281180, 20090007284, US20110117189; US20090017543; US20070054961, US20100317109. Lentiviral vectors have also been disclosed for delivery to the brain, see, e.g., US Patent Publication Nos. US20110293571; US20110293571, US20040013648, US20070025970, US20090111106 and US Patent No. US7259015.

RNA delivery

[00168] RNA delivery: The CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can also be delivered in the form of RNA. Cas9 mRNA can be generated using in vitro transcription. For example, Cas9 mRNA can be synthesized using a PCR cassette containing the following elements: T7_promoter-kozak sequence (GCCACC)-Cas9-3' UTR from beta globin-polyA tail (a string of 120 or more adenines). The cassette can be used for transcription by T7 polymerase. Guide RNAs can also be transcribed using in vitro transcription from a cassette containing T7_promoter-GG-guide RNA sequence.

[00169] To enhance expression and reduce possible toxicity, the CRISPR enzyme-coding sequence and/or the guide RNA can be modified to include one or more modified nucleoside e.g. using pseudo-U or 5-Methyl-C.

[00170] mRNA delivery methods are especially promising for liver delivery currently.

[00171] Much clinical work on RNA delivery has focused on RNAi or antisense, but these systems can be adapted for delivery of RNA for implementing the present invention. References below to RNAi etc. should be read accordingly.

Particle delivery systems and/or formulations:

[00172] Several types of particle delivery systems and/or formulations are known to be useful in a diverse spectrum of biomedical applications. In general, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Particles are further classified according to diameter. Coarse particles cover a range between 2,500 and 10,000 nanometers. Fine particles are sized between 100 and 2,500 nanometers. Ultrafine particles, or nanoparticles, are generally between 1 and 100 nanometers in size. The basis of the 100-nm limit is the fact that novel properties that differentiate particles from the bulk material typically develop at a critical length scale of under 100 nm.

[00173] As used herein, a particle delivery system/formulation is defined as any biological delivery system/formulation which includes a particle in accordance with the present invention. A particle in accordance with the present invention is any entity having a greatest dimension (e.g. diameter) of less than 100 microns (μm). In some embodiments, inventive particles have a greatest dimension of less than 10 μm. In some embodiments, inventive particles have a greatest dimension of less than 2000 nanometers (nm). In some embodiments, inventive particles have a greatest dimension of less than 1000 nanometers (nm). In some embodiments, inventive particles have a greatest dimension of less than 900 nm, 800 nm, 700 nm, 600 nm, 500 nm, 400 nm, 300 nm, 200 nm, or 100 nm. Typically, inventive particles have a greatest dimension (e.g., diameter) of 500 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 250 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 200 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 150 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 100 nm or less. Smaller particles, e.g., having a greatest dimension of 50 nm or less are used in some embodiments of the invention. In some embodiments, inventive particles have a greatest dimension ranging between 25 nm and 200 nm.

[00174] Particle characterization (including e.g., characterizing morphology, dimension, etc.) is done using a variety of different techniques. Common techniques are electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry(MALDI-TOF), ultraviolet-visible spectroscopy, dual polarisation interferometry and nuclear magnetic resonance (NMR). Characterization (dimension measurements) may be made as to native particles (i.e., preloading) or after loading of the cargo (herein cargo refers to e.g., one or more components of CRISPR-Cas system e.g., CRISPR enzyme or mRNA or guide RNA, or any combination thereof, and may include additional carriers and/or excipients) to provide particles of an optimal size for delivery for any in vitro, ex vivo and/or in vivo application of the present invention. In certain preferred embodiments, particle dimension (e.g., diameter) characterization is based on measurements using dynamic laser scattering (DLS). Mention is made of US Patent No. 8,709,843; US Patent No. 6,007,845; US Patent No. 5,855,913; US Patent No. 5,985,309; US. Patent No. 5,543,158; and the publication by James E. Dahlman and Carmen Barnes et al. Nature Nanotechnology (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84, concerning particles, methods of making and using them and measurements thereof.

[00175] Particles delivery systems within the scope of the present invention may be provided in any form, including but not limited to solid, semi-solid, emulsion, or colloidal particles. As such any of the delivery systems described herein, including but not limited to, e.g., lipid-based systems, liposomes, micelles, microvesicles, exosomes, or gene gun may be provided as particle delivery systems within the scope of the present invention.

Nanoparticles

[00176] CRISPR enzyme mRNA and guide RNA may be delivered simultaneously using nanoparticles or lipid envelopes.

[00177] For example, Su X, Fricke J, Kavanagh DG, Irvine DJ ("In vitro and in vivo mRNA delivery using lipid-enveloped pH-responsive polymer nanoparticles" Mol Pharm. 2011 Jun 6;8(3):774-87. doi: 10.1021/mp100390w. Epub 2011 Apr 1) describes biodegradable core-shell structured nanoparticles with a poly(β-amino ester) (PBAE) core enveloped by a phospholipid bilayer shell. These were developed for in vivo mRNA delivery. The pH-responsive PBAE component was chosen to promote endosome disruption, while the lipid surface layer was selected to minimize toxicity of the polycation core. Such are, therefore, preferred for delivering RNA of the present invention.

[00178] In one embodiment, nanoparticles based on self assembling bioadhesive polymers are contemplated, which may be applied to oral delivery of peptides, intravenous delivery of peptides and nasal delivery of peptides, all to the brain. Other embodiments, such as oral absorption and ocular delivery of hydrophobic drugs are also contemplated. The molecular envelope technology involves an engineered polymer envelope which is protected and delivered to the site of the disease (see, e.g., Mazza, M. et al. ACSNano, 2013. 7(2): 1016-1026; Siew, A., et al. Mol Pharm, 2012. 9(1):14-28; Lalatsa, A., et al. J Contr Rel, 2012. 161(2):523-36; Lalatsa, A., et al., Mol Pharm, 2012. 9(6):1665-80; Lalatsa, A., et al. Mol Pharm, 2012. 9(6):1764-74; Garrett, N.L., et al. J Biophotonics, 2012. 5(5-6):458-68; Garrett, N.L., et al. J Raman Spect, 2012. 43(5):681-688; Ahmad, S., et al. J Royal Soc Interface 2010. 7:S423-33; Uchegbu, I.F. Expert Opin Drug Deliv, 2006. 3(5):629-40; Qu, X.,et al. Biomacromolecules, 2006. 7(12):3452-9 and Uchegbu, I.F., et al. Int J Pharm, 2001. 224:185-199). Doses of about 5 mg/kg are contemplated, with single or multiple doses, depending on the target tissue.

[00179] In one embodiment, nanoparticles that can deliver RNA to a cancer cell to stop tumor growth developed by Dan Anderson's lab at MIT may be used/and or adapted to the CRISPR Cas system of the present invention. In particular, the Anderson lab developed fully automated, combinatorial systems for the synthesis, purification, characterization, and formulation of new biomaterials and nanoformulations. See, e.g., Alabi et al., Proc Natl Acad Sci U S A. 2013 Aug 6;110(32):12881-6; Zhang et al., Adv Mater. 2013 Sep 6;25(33):4641-5; Jiang et al., Nano Lett. 2013 Mar 13;13(3):1059-64; Karagiannis et al., ACS Nano. 2012 Oct 23;6(10):8484-7; Whitehead et al., ACS Nano. 2012 Aug 28;6(8):6922-9 and Lee et al., Nat Nanotechnol. 2012 Jun 3;7(6):389-93.

[00180] US patent application 20110293703 relates to lipidoid compounds are also particularly useful in the administration of polynucleotides, which may be applied to deliver the CRISPR Cas system of the present invention. In one aspect, the aminoalcohol lipidoid compounds are combined with an agent to be delivered to a cell or a subject to form microparticles, nanoparticles, liposomes, or micelles. The agent to be delivered by the particles, liposomes, or micelles may be in the form of a gas, liquid, or solid, and the agent may be a polynucleotide, protein, peptide, or small molecule. The minoalcohol lipidoid compounds may be combined with other aminoalcohol lipidoid compounds, polymers (synthetic or natural), surfactants, cholesterol, carbohydrates, proteins, lipids, etc. to form the particles. These particles may then optionally be combined with a pharmaceutical excipient to form a pharmaceutical composition.

[00181] US Patent Publication No. 20110293703 also provides methods of preparing the aminoalcohol lipidoid compounds. One or more equivalents of an amine are allowed to react with one or more equivalents of an epoxide-terminated compound under suitable conditions to form an aminoalcohol lipidoid compound of the present invention. In certain embodiments, all the amino groups of the amine are fully reacted with the epoxide-terminated compound to form tertiary amines. In other embodiments, all the amino groups of the amine are not fully reacted with the epoxide-terminated compound to form tertiary amines thereby resulting in primary or secondary amines in the aminoalcohol lipidoid compound. These primary or secondary amines are left as is or may be reacted with another electrophile such as a different epoxide-terminated compound. As will be appreciated by one skilled in the art, reacting an amine with less than excess of epoxide-terminated compound will result in a plurality of different aminoalcohol lipidoid compounds with various numbers of tails. Certain amines may be fully functionalized with two epoxide-derived compound tails while other molecules will not be completely functionalized with epoxide-derived compound tails. For example, a diamine or polyamine may include one, two, three, or four epoxide-derived compound tails off the various amino moieties of the molecule resulting in primary, secondary, and tertiary amines. In certain embodiments, all the amino groups are not fully functionalized. In certain embodiments, two of the same types of epoxide-terminated compounds are used. In other embodiments, two or more different epoxide-terminated compounds are used. The synthesis of the aminoalcohol lipidoid compounds is performed with or without solvent, and the synthesis may be performed at higher temperatures ranging from 30-100 °C., preferably at approximately 50-90 °C. The prepared aminoalcohol lipidoid compounds may be optionally purified. For example, the mixture of aminoalcohol lipidoid compounds may be purified to yield an aminoalcohol lipidoid compound with a particular number of epoxide-derived compound tails. Or the mixture may be purified to yield a particular stereo- or regioisomer. The aminoalcohol lipidoid compounds may also be alkylated using an alkyl halide (e.g., methyl iodide) or other alkylating agent, and/or they may be acylated.

[00182] US Patent Publication No. 20110293703 also provides libraries of aminoalcohol lipidoid compounds prepared by the inventive methods. These aminoalcohol lipidoid compounds may be prepared and/or screened using high-throughput techniques involving liquid handlers, robots, microtiter plates, computers, etc. In certain embodiments, the aminoalcohol lipidoid compounds are screened for their ability to transfect polynucleotides or other agents (e.g., proteins, peptides, small molecules) into the cell.

[00183] US Patent Publication No. 20130302401 relates to a class of poly(beta-amino alcohols) (PBAAs) has been prepared using combinatorial polymerization. The inventive PBAAs may be used in biotechnology and biomedical applications as coatings (such as coatings of films or multilayer films for medical devices or implants), additives, materials, excipients, non-biofouling agents, micropatterning agents, and cellular encapsulation agents. When used as surface coatings, these PBAAs elicited different levels of inflammation, both in vitro and in vivo, depending on their chemical structures. The large chemical diversity of this class of materials allowed us to identify polymer coatings that inhibit macrophage activation in vitro. Furthermore, these coatings reduce the recruitment of inflammatory cells, and reduce fibrosis, following the subcutaneous implantation of carboxylated polystyrene microparticles. These polymers may be used to form polyelectrolyte complex capsules for cell encapsulation. The invention may also have many other biological applications such as antimicrobial coatings, DNA or siRNA delivery, and stem cell tissue engineering. The teachings of US Patent Publication No. 20130302401 may be applied to the CRISPR Cas system of the present invention.

[00184] In another embodiment, lipid nanoparticles (LNPs) are contemplated. An antitransthyretin small interfering RNA has been encapsulated in lipid nanoparticles and delivered to humans (see, e.g., Coelho et al., N Engl J Med 2013;369:819-29), and such a ssystem may be adapted and applied to the CRISPR Cas system of the present invention. Doses of about 0.01 to about 1 mg per kg of body weight administered intravenously are contemplated. Medications to reduce the risk of infusion-related reactions are contemplated, such as dexamethasone, acetampinophen, diphenhydramine or cetirizine, and ranitidine are contemplated. Multiple doses of about 0.3 mg per kilogram every 4 weeks for five doses are also contemplated.

[00185] LNPs have been shown to be highly effective in delivering siRNAs to the liver (see, e.g., Tabernero et al., Cancer Discovery, April 2013, Vol. 3, No. 4, pages 363-470) and are therefore contemplated for delivering RNA encoding CRISPR Cas to the liver. A dosage of about four doses of 6 mg/kg of the LNP every two weeks may be contemplated. Tabernero et al. demonstrated that tumor regression was observed after the first 2 cycles of LNPs dosed at 0.7 mg/kg, and by the end of 6 cycles the patient had achieved a partial response with complete regression of the lymph node metastasis and substantial shrinkage of the liver tumors. A complete response was obtained after 40 doses in this patient, who has remained in remission and completed treatment after receiving doses over 26 months. Two patients with RCC and extrahepatic sites of disease including kidney, lung, and lymph nodes that were progressing following prior therapy with VEGF pathway inhibitors had stable disease at all sites for approximately 8 to 12 months, and a patient with PNET and liver metastases continued on the extension study for 18 months (36 doses) with stable disease.

[00186] However, the charge of the LNP must be taken into consideration. As cationic lipids combined with negatively charged lipids to induce nonbilayer structures that facilitate intracellular delivery. Because charged LNPs are rapidly cleared from circulation following intravenous injection, ionizable cationic lipids with pKa values below 7 were developed (see, e.g., Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). Negatively charged polymers such as RNA may be loaded into LNPs at low pH values (e.g., pH 4) where the ionizable lipids display a positive charge. However, at physiological pH values, the LNPs exhibit a low surface charge compatible with longer circulation times. Four species of ionizable cationic lipids have been focused upon, namely 1,2-dilineoyl-3-dimethylammonium-propane

(DLinDAP), 1,2-dilinoleyloxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoleyloxy-keto-N,N-dimethyl-3-aminopropane (DLinKDMA), and 1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA). It has been shown that LNP siRNA systems containing these lipids exhibit remarkably different gene silencing properties in hepatocytes in vivo, with potencies varying according to the series DLinKC2-DMA>DLinKDMA>DLinDMA>>DLinDAP employing a Factor VII gene silencing model (see, e.g., Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). A dosage of 1 μg/ml of LNP or CRISPR-Cas RNA in or associated with the LNP may be contemplated, especially for a formulation containing DLinKC2-DMA.

[00187] Preparation of LNPs and CRISPR Cas encapsulation may be used/and or adapted from Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). The cationic lipids 1,2-dilineoyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoleyloxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoleyloxyketo-N,N-dimethyl-3-aminopropane (DLinK-DMA), 1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA), (3-o-[2″-(methoxypolyethyleneglycol 2000) succinoyl]-1,2-dimyristoyl-sn-glycol (PEG-S-DMG), and R-3-[(ω-methoxy-poly(ethylene glycol)2000) carbamoyl]-1,2-dimyristyloxlpropyl-3-amine (PEG-C-DOMG) may be provided by Tekmira Pharmaceuticals (Vancouver, Canada) or synthesized. Cholesterol may be purchased from Sigma (St Louis, MO). The specific CRISPR Cas RNA may be encapsulated in LNPs containing DLinDAP, DLinDMA, DLinK-DMA, and DLinKC2-DMA (cationic lipid:DSPC:CHOL: PEGS-DMG or PEG-C-DOMG at 40:10:40:10 molar ratios). When required, 0.2% SP-DiOC18 (Invitrogen, Burlington, Canada) may be incorporated to assess cellular uptake, intracellular delivery, and biodistribution. Encapsulation may be performed by dissolving lipid mixtures comprised of cationic lipid:DSPC:cholesterol:PEG-c-DOMG (40:10:40:10 molar ratio) in ethanol to a final lipid concentration of 10 mmol/l. This ethanol solution of lipid may be added drop-wise to 50 mmol/l citrate, pH 4.0 to form multilamellar vesicles to produce a final concentration of 30% ethanol vol/vol. Large unilamellar vesicles may be formed following extrusion of multilamellar vesicles through two stacked 80 nm Nuclepore polycarbonate filters using the Extruder (Northern Lipids, Vancouver, Canada). Encapsulation may be achieved by adding RNA dissolved at 2 mg/ml in 50 mmol/l citrate, pH 4.0 containing 30% ethanol vol/vol drop-wise to extruded preformed large unilamellar vesicles and incubation at 31 °C for 30 minutes with constant mixing to a final

RNA/lipid weight ratio of 0.06/1 wt/wt. Removal of ethanol and neutralization of formulation buffer were performed by dialysis against phosphate-buffered saline (PBS), pH 7.4 for 16 hours using Spectra/Por 2 regenerated cellulose dialysis membranes. Nanoparticle size distribution may be determined by dynamic light scattering using a NICOMP 370 particle sizer, the vesicle/intensity modes, and Gaussian fitting (Nicomp Particle Sizing, Santa Barbara, CA). The particle size for all three LNP systems may be ~70 nm in diameter. RNA encapsulation efficiency may be determined by removal of free RNA using VivaPureD MiniH columns (Sartorius Stedim Biotech) from samples collected before and after dialysis. The encapsulated RNA may be extracted from the eluted nanoparticles and quantified at 260 nm. RNA to lipid ratio was determined by measurement of cholesterol content in vesicles using the Cholesterol E enzymatic assay from Wako Chemicals USA (Richmond, VA). In conjunction with the herein discussion of LNPs and PEG lipids, PEGylated liposomes or LNPs are likewise suitable for delivery of a CRISPR-Cas system or components thereof.

[00188] Preparation of large LNPs may be used/and or adapted from Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011. A lipid premix solution (20.4 mg/ml total lipid concentration) may be prepared in ethanol containing DLinKC2-DMA, DSPC, and cholesterol at 50:10:38.5 molar ratios. Sodium acetate may be added to the lipid premix at a molar ratio of 0.75:1 (sodium acetate:DLinKC2-DMA). The lipids may be subsequently hydrated by combining the mixture with 1.85 volumes of citrate buffer (10 mmol/l, pH 3.0) with vigorous stirring, resulting in spontaneous liposome formation in aqueous buffer containing 35% ethanol. The liposome solution may be incubated at 37 °C to allow for time-dependent increase in particle size. Aliquots may be removed at various times during incubation to investigate changes in liposome size by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). Once the desired particle size is achieved, an aqueous PEG lipid solution (stock = 10 mg/ml PEG-DMG in 35% (vol/vol) ethanol) may be added to the liposome mixture to yield a final PEG molar concentration of 3.5% of total lipid. Upon addition of PEG-lipids, the liposomes should their size, effectively quenching further growth. RNA may then be added to the empty liposomes at an RNA to total lipid ratio of approximately 1:10 (wt:wt), followed by incubation for 30 minutes at 37 °C to form loaded LNPs. The mixture may be subsequently dialyzed overnight in PBS and filtered with a 0.45-μm syringe filter.

[00189] Spherical Nucleic Acid (SNA™) constructs and other nanoparticles (particularly gold nanoparticles) are also contemplated as a means to delivery CRISPR-Cas system to intended targets. Significant data show that AuraSense Therapeutics' Spherical Nucleic Acid (SNA™) constructs, based upon nucleic acid-functionalized gold nanoparticles, are useful.

[00190] Literature that may be employed in conjunction with herein teachings include: Cutler et al., J. Am. Chem. Soc. 2011 133:9254-9257, Hao et al., Small. 2011 7:3158-3162, Zhang et al., ACS Nano. 2011 5:6962-6970, Cutler et al., J. Am. Chem. Soc. 2012 134:1376-1391, Young et al., Nano Lett. 2012 12:3867-71, Zheng et al., Proc. Natl. Acad. Sci. USA. 2012 109:11975-80, Mirkin, Nanomedicine 2012 7:635-638 Zhang et al., J. Am. Chem. Soc. 2012 134:16488-1691, Weintraub, Nature 2013 495:S14-S16, Choi et al., Proc. Natl. Acad. Sci. USA. 2013 110(19):7625-7630, Jensen et al., Sci. Transl. Med. 5, 209ra152 (2013) and Mirkin, et al., Small, 10:186-192.

[00191] Self-assembling nanoparticles with RNA may be constructed with polyethyleneimine (PEI) that is PEGylated with an Arg-Gly-Asp (RGD) peptide ligand attached at the distal end of the polyethylene glycol (PEG). This system has been used, for example, as a means to target tumor neovasculature expressing integrins and deliver siRNA inhibiting vascular endothelial growth factor receptor-2 (VEGF R2) expression and thereby achieve tumor angiogenesis (see, e.g., Schiffelers et al., Nucleic Acids Research, 2004, Vol. 32, No. 19). Nanoplexes may be prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. A dosage of about 100 to 200 mg of CRISPR Cas is envisioned for delivery in the self-assembling nanoparticles of Schiffelers et al.

[00192] The nanoplexes of Bartlett et al. (PNAS, September 25, 2007,vol. 104, no. 39) may also be applied to the present invention. The nanoplexes of Bartlett et al. are prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. The DOTA-siRNA of Bartlett et al. was synthesized as follows: 1,4,7,10-

tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(N-hydroxysuccinimide ester) (DOTA-NHSester) was ordered from Macrocyclics (Dallas, TX). The amine modified RNA sense strand with a 100-fold molar excess of DOTA-NHS-ester in carbonate buffer (pH 9) was added to a microcentrifuge tube. The contents were reacted by stirring for 4 h at room temperature. The DOTA-RNAsense conjugate was ethanol-precipitated, resuspended in water, and annealed to the unmodified antisense strand to yield DOTA-siRNA. All liquids were pretreated with Chelex-100 (Bio-Rad, Hercules, CA) to remove trace metal contaminants. Tf-targeted and nontargeted siRNA nanoparticles may be formed by using cyclodextrin-containing polycations. Typically, nanoparticles were formed in water at a charge ratio of 3 (+/-) and an siRNA concentration of 0.5 g/liter. One percent of the adamantane-PEG molecules on the surface of the targeted nanoparticles were modified with Tf (adamantane-PEG-Tf). The nanoparticles were suspended in a 5% (wt/vol) glucose carrier solution for injection.

[00193] Davis et al. (Nature, Vol 464, 15 April 2010) conducts a RNA clinical trial that uses a targeted nanoparticle-delivery system (clinical trial registration number NCT00689065). Patients with solid cancers refractory to standard-of-care therapies are administered doses of targeted nanoparticles on days 1, 3, 8 and 10 of a 21-day cycle by a 30-min intravenous infusion. The nanoparticles consist of a synthetic delivery system containing: (1) a linear, cyclodextrin-based polymer (CDP), (2) a human transferrin protein (TF) targeting ligand displayed on the exterior of the nanoparticle to engage TF receptors (TFR) on the surface of the cancer cells, (3) a hydrophilic polymer (polyethylene glycol (PEG) used to promote nanoparticle stability in biological fluids), and (4) siRNA designed to reduce the expression of the RRM2 (sequence used in the clinic was previously denoted siR2B+5). The TFR has long been known to be upregulated in malignant cells, and RRM2 is an established anti-cancer target. These nanoparticles (clinical version denoted as CALAA-01) have been shown to be well tolerated in multi-dosing studies in non-human primates. Although a single patient with chronic myeloid leukaemia has been administered siRNAby liposomal delivery, Davis et al.'s clinical trial is the initial human trial to systemically deliver siRNA with a targeted delivery system and to treat patients with solid cancer. To ascertain whether the targeted delivery system can provide effective delivery of functional siRNA to human tumours, Davis et al. investigated biopsies from three patients from three different dosing cohorts; patients A, B and C, all of whom had metastatic melanoma and received CALAA-01 doses of 18, 24 and 30 mg m-2 siRNA, respectively. Similar doses may also be contemplated for the CRISPR Cas system of the present invention. The delivery of the invention may be achieved with nanoparticles containing a linear, cyclodextrin-based polymer (CDP), a human transferrin protein (TF) targeting ligand displayed on the exterior of the nanoparticle to engage TF receptors (TFR) on the surface of the cancer cells and/or a hydrophilic polymer (for example, polyethylene glycol (PEG) used to promote nanoparticle stability in biological fluids).

[00194] In terms of this invention, it is preferred to have one or more components of CRISPR complex, e.g., CRISPR enzyme or mRNA or guide RNA or sgRNA or if present HDR template may be delivered using one or more particles or nanoparticles or lipid envelopes. Other delivery systems or vectors are may be used in conjunction with the nanoparticle aspects of the invention.

[00195] In general, a "nanoparticle" refers to any particle having a diameter of less than 1000 nm. In certain preferred embodiments, nanoparticles of the invention have a greatest dimension (e.g., diameter) of 500 nm or less. In other preferred embodiments, nanoparticles of the invention have a greatest dimension ranging between 25 nm and 200 nm. In other preferred embodiments, nanoparticles of the invention have a greatest dimension of 100 nm or less. In other preferred embodiments, nanoparticles of the invention have a greatest dimension ranging between 35 nm and 60 nm.

[00196] Nanoparticles encompassed in the present invention may be provided in different forms, e.g., as solid nanoparticles (e.g., metal such as silver, gold, iron, titanium), non-metal, lipid-based solids, polymers), suspensions of nanoparticles, or combinations thereof. Metal, dielectric, and semiconductor nanoparticles may be prepared, as well as hybrid structures (e.g., core–shell nanoparticles). Nanoparticles made of semiconducting material may also be labeled quantum dots if they are small enough (typically sub 10 nm) that quantization of electronic energy levels occurs. Such nanoscale particles are used in biomedical applications as drug carriers or imaging agents and may be adapted for similar purposes in the present invention.

[00197] Semi-solid and soft nanoparticles have been manufactured, and are within the scope of the present invention. A prototype nanoparticle of semi-solid nature is the liposome. Various types of liposome nanoparticles are currently used clinically as delivery systems for anticancer drugs and vaccines. Nanoparticles with one half hydrophilic and the other half hydrophobic are termed Janus particles and are particularly effective for stabilizing emulsions. They can self-assemble at water/oil interfaces and act as solid surfactants.

[00198] US Patent No. 8,709,843, incorporated herein by reference, provides a drug delivery system for targeted delivery of therapeutic agent-containing particles to tissues, cells, and intracellular compartments. The invention provides targeted particles comprising comprising polymer conjugated to a surfactant, hydrophilic polymer or lipid. US Patent No. 6,007,845, incorporated herein by reference, provides particles which have a core of a multiblock copolymer formed by covalently linking a multifunctional compound with one or more hydrophobic polymers and one or more hydrophilic polymers, and conatin a biologically active material. US Patent No. 5,855,913, incorporated herein by reference, provides a particulate composition having aerodynamically light particles having a tap density of less than 0.4 g/cm3 with a mean diameter of between 5 μm and 30 μm, incorporating a surfactant on the surface thereof for drug delivery to the pulmonary system. US Patent No. 5,985,309, incorporated herein by reference, provides particles incorporating a surfactant and/or a hydrophilic or hydrophobic complex of a positively or negatively charged therapeutic or diagnostic agent and a charged molecule of opposite charge for delivery to the pulmonary system. US. Patent No. 5,543,158, incorporated herein by reference, provides biodegradable injectable nanoparticles having a biodegradable solid core containing a biologically active material and poly(alkylene glycol) moieties on the surface. WO2012135025 (also published as US20120251560), incorporated herein by reference, describes conjugated polyethyleneimine (PEI) polymers and conjugated aza-macrocycles (collectively referred to as "conjugated lipomer" or "lipomers"). In certain embodiments, it can be envisioned that such methods and materials of herein-cited documents, e.g., conjugated lipomers, can be used in the context of the CRISPR-Cas system to achieve in vitro, ex vivo and in vivo genomic perturbations to modify gene expression, including modulation of protein expression.

[00199] In one embodiment, the nanoparticle may be epoxide-modified lipid–polymer, advantageously 7C1 (see, e.g., James E. Dahlman and Carmen Barnes et al. Nature Nanotechnology (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84). C71 was synthesized by reacting C15 epoxide-terminated lipids with PEI600 at a 14:1 molar ratio, and was formulated with C14PEG2000 to produce nanoparticles (diameter between 35 and 60 nm) that were stable in PBS solution for at least 40 days. An epoxide-modified lipid-polymer may be utilized to deliver the CRISPR-Cas system of the present invention to pulmonary, cardiovascular or renal cells, however, one of skill in the art may adapt the system to deliver to other target

organs. Dosage ranging from about 0.05 to about 0.6 mg/kg are envisioned. Dosages over several days or weeks are also envisioned, with a total dosage of about 2 mg/kg.

Exosomes

[00200] Exosomes are endogenous nano-vesicles that transport RNAs and proteins, and which can deliver RNA to the brain and other target organs. To reduce immunogenicity, Alvarez-Erviti et al. (2011, Nat Biotechnol 29: 341) used self-derived dendritic cells for exosome production. Targeting to the brain was achieved by engineering the dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide. Purified exosomes were loaded with exogenous RNA by electroporation. Intravenously injected RVG-targeted exosomes delivered GAPDH siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown. Pre-exposure to RVG exosomes did not attenuate knockdown, and non-specific uptake in other tissues was not observed. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of BACE1, a therapeutic target in Alzheimer's disease.

[00201] To obtain a pool of immunologically inert exosomes, Alvarez-Erviti et al. harvested bone marrow from inbred C57BL/6 mice with a homogenous major histocompatibility complex (MHC) haplotype. As immature dendritic cells produce large quantities of exosomes devoid of T-cell activators such as MHC-II and CD86, Alvarez-Erviti et al. selected for dendritic cells with granulocyte/macrophage-colony stimulating factor (GM-CSF) for 7 d. Exosomes were purified from the culture supernatant the following day using well-established ultracentrifugation protocols. The exosomes produced were physically homogenous, with a size distribution peaking at 80 nm in diameter as determined by nanoparticle tracking analysis (NTA) and electron microscopy. Alvarez-Erviti et al. obtained 6-12 μg of exosomes (measured based on protein concentration) per 106 cells.

[00202] Next, Alvarez-Erviti et al. investigated the possibility of loading modified exosomes with exogenous cargoes using electroporation protocols adapted for nanoscale applications. As electroporation for membrane particles at the nanometer scale is not well-characterized, nonspecific Cy5-labeled RNA was used for the empirical optimization of the electroporation protocol. The amount of encapsulated RNA was assayed after ultracentrifugation and lysis of exosomes. Electroporation at 400 V and 125 μF resulted in the greatest retention of RNA and was used for all subsequent experiments.

[00203] Alvarez-Erviti et al. administered 150 µg of each BACE1 siRNA encapsulated in 150 µg of RVG exosomes to normal C57BL/6 mice and compared the knockdown efficiency to four controls: untreated mice, mice injected with RVG exosomes only, mice injected with BACE1 siRNA complexed to an in vivo cationic liposome reagent and mice injected with BACE1 siRNA complexed to RVG-9R, the RVG peptide conjugated to 9 D-arginines that electrostatically binds to the siRNA. Cortical tissue samples were analyzed 3 d after administration and a significant protein knockdown (45%, $P < 0.05$, versus 62%, $P < 0.01$) in both siRNA-RVG-9R-treated and siRNARVG exosome-treated mice was observed, resulting from a significant decrease in BACE1 mRNA levels (66% [+ or -] 15%, $P < 0.001$ and 61% [+ or -] 13% respectively, $P < 0.01$). Moreover, Applicants demonstrated a significant decrease (55%, $P < 0.05$) in the total [beta]-amyloid 1-42 levels, a main component of the amyloid plaques in Alzheimer's pathology, in the RVG-exosome-treated animals. The decrease observed was greater than the β-amyloid 1-40 decrease demonstrated in normal mice after intraventricular injection of BACE1 inhibitors. Alvarez-Erviti et al. carried out 5'-rapid amplification of cDNA ends (RACE) on BACE1 cleavage product, which provided evidence of RNAi-mediated knockdown by the siRNA.

[00204] Finally, Alvarez-Erviti et al. investigated whether RNA-RVG exosomes induced immune responses in vivo by assessing IL-6, IP-10, TNFα and IFN-α serum concentrations. Following exosome treatment, nonsignificant changes in all cytokines were registered similar to siRNA-transfection reagent treatment in contrast to siRNA-RVG-9R, which potently stimulated IL-6 secretion, confirming the immunologically inert profile of the exosome treatment. Given that exosomes encapsulate only 20% of siRNA, delivery with RVG-exosome appears to be more efficient than RVG-9R delivery as comparable mRNA knockdown and greater protein knockdown was achieved with fivefold less siRNA without the corresponding level of immune stimulation. This experiment demonstrated the therapeutic potential of RVG-exosome technology, which is potentially suited for long-term silencing of genes related to neurodegenerative diseases. The exosome delivery system of Alvarez-Erviti et al. may be applied to deliver the CRISPR-Cas system of the present invention to therapeutic targets, especially neurodegenerative diseases. A dosage of about 100 to 1000 mg of CRISPR Cas encapsulated in about 100 to 1000 mg of RVG exosomes may be contemplated for the present invention.

[00205] El-Andaloussi et al. (Nature Protocols 7,2112--2126(2012)) discloses how exosomes derived from cultured cells can be harnessed for delivery of RNA in vitro and in vivo. This protocol first describes the generation of targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. Next, El-Andaloussi et al. explain how to purify and characterize exosomes from transfected cell supernatant. Next, El-Andaloussi et al. detail crucial steps for loading RNA into exosomes. Finally, El-Andaloussi et al. outline how to use exosomes to efficiently deliver RNA in vitro and in vivo in mouse brain. Examples of anticipated results in which exosome-mediated RNA delivery is evaluated by functional assays and imaging are also provided. The entire protocol takes ~3 weeks. Delivery or administration according to the invention may be performed using exosomes produced from self-derived dendritic cells. From the herein teachings, this can be employed in the practice of the invention.

[00206] In another embodiment, the plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) are contemplated. Exosomes are nano-sized vesicles (30–90nm in size) produced by many cell types, including dendritic cells (DC), B cells, T cells, mast cells, epithelial cells and tumor cells. These vesicles are formed by inward budding of late endosomes and are then released to the extracellular environment upon fusion with the plasma membrane. Because exosomes naturally carry RNA between cells, this property may be useful in gene therapy, and from this disclosure can be employed in the practice of the instant invention.

[00207] Exosomes from plasma can be prepared by centrifugation of buffy coat at 900g for 20 min to isolate the plasma followed by harvesting cell supernatants, centrifuging at 300g for 10 min to eliminate cells and at 16 500g for 30 min followed by filtration through a 0.22 mm filter. Exosomes are pelleted by ultracentrifugation at 120 000g for70 min. Chemical transfection of siRNA into exosomes is carried out according to the manufacturer's instructions in RNAi Human/Mouse Starter Kit (Quiagen, Hilden, Germany). siRNA is added to 100 ml PBS at a final concentration of 2 mmol/ml. After adding HiPerFect transfection reagent, the mixture is incubated for 10 min at RT. In order to remove the excess of micelles, the exosomes are re-isolated using aldehyde/sulfate latex beads. The chemical transfection of CRISPR Cas into exosomes may be conducted similarly to siRNA. The exosomes may be co-cultured with monocytes and lymphocytes isolated from the peripheral blood of healthy donors. Therefore, it may be contemplated that exosomes containing CRISPR Cas may be introduced to monocytes and lymphocytes of and autologously reintroduced into a human. Accordingly, delivery or administration according to the invention may be performed using plasma exosomes.

Liposomes

[00208] Delivery or administration according to the invention can be performed with liposomes. Liposomes are spherical vesicle structures composed of a uni- or multilamellar lipid bilayer surrounding internal aqueous compartments and a relatively impermeable outer lipophilic phospholipid bilayer. Liposomes have gained considerable attention as drug delivery carriers because they are biocompatible, nontoxic, can deliver both hydrophilic and lipophilic drug molecules, protect their cargo from degradation by plasma enzymes, and transport their load across biological membranes and the blood brain barrier (BBB) (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review). Liposomes can be made from several different types of lipids; however, phospholipids are most commonly used to generate liposomes as drug carriers. Although liposome formation is spontaneous when a lipid film is mixed with an aqueous solution, it can also be expedited by applying force in the form of shaking by using a homogenizer, sonicator, or an extrusion apparatus (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00209] Several other additives may be added to liposomes in order to modify their structure and properties. For instance, either cholesterol or sphingomyelin may be added to the liposomal mixture in order to help stabilize the liposomal structure and to prevent the leakage of the liposomal inner cargo. Further, liposomes are prepared from hydrogenated egg phosphatidylcholine or egg phosphatidylcholine, cholesterol, and dicetyl phosphate, and their mean vesicle sizes were adjusted to about 50 and 100 nm. (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review). A liposome formulation may be mainly comprised of natural phospholipids and lipids such as 1,2-distearoryl-sn-glycero-3-phosphatidyl choline (DSPC), sphingomyelin, egg phosphatidylcholines and monosialoganglioside. Since this formulation is made up of phospholipids only, liposomal formulations have encountered many challenges, one of the ones being the instability in plasma. Several attempts to overcome these challenges have been made, specifically in the manipulation of the lipid membrane. One of these attempts focused on the manipulation of cholesterol. Addition of cholesterol to conventional formulations reduces rapid release of the encapsulated bioactive compound into the plasma or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) increases the stability (see, e.g., Spuch and Navarro, Journal of

Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00210] In a particularly advantageous embodiment, Trojan Horse liposomes (also known as Molecular Trojan Horses) are desirable and protocols may be found at http://cshprotocols.cshlp.org/content/2010/4/pdb.prot5407.long. These particles allow delivery of a transgene to the entire brain after an intravascular injection. Without being bound by limitation, it is believed that neutral lipid particles with specific antibodies conjugated to surface allow crossing of the blood brain barrier via endocytosis. Applicant postulates utilizing Trojan Horse Liposomes to deliver the CRISPR family of nucleases to the brain via an intravascular injection, which would allow whole brain transgenic animals without the need for embryonic manipulation. About 1-5 g of DNA or RNA may be contemplated for in vivo administration in liposomes.

[00211] In another embodiment, the CRISPR Cas system or components thereof may be administered in liposomes, such as a stable nucleic-acid-lipid particle (SNALP) (see, e.g., Morrissey et al., Nature Biotechnology, Vol. 23, No. 8, August 2005). Daily intravenous injections of about 1, 3 or 5 mg/kg/day of a specific CRISPR Cas targeted in a SNALP are contemplated. The daily treatment may be over about three days and then weekly for about five weeks. In another embodiment, a specific CRISPR Cas encapsulated SNALP) administered by intravenous injection to at doses of about 1 or 2.5 mg/kg are also contemplated (see, e.g., Zimmerman et al., Nature Letters, Vol. 441, 4 May 2006). The SNALP formulation may contain the lipids 3-N-[(wmethoxypoly(ethylene glycol) 2000) carbamoyl] -1,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio (see, e.g., Zimmerman et al., Nature Letters, Vol. 441, 4 May 2006).

[00212] In another embodiment, stable nucleic-acid-lipid particles (SNALPs) have proven to be effective delivery molecules to highly vascularized HepG2-derived liver tumors but not in poorly vascularized HCT-116 derived liver tumors (see, e.g., Li, Gene Therapy (2012) 19, 775–780). The SNALP liposomes may be prepared by formulating D-Lin-DMA and PEG-C-DMA with distearoylphosphatidylcholine (DSPC), Cholesterol and siRNA using a 25:1 lipid/siRNA ratio and a 48/40/10/2 molar ratio of Cholesterol/D-Lin-DMA/DSPC/PEG-C-DMA. The resulted SNALP liposomes are about 80–100 nm in size. In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich, St Louis, MO, USA), dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA), 3-N-[(w-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxypropylamine, and cationic 1,2-dilinoleyloxy-3-N,Ndimethylaminopropane (see, e.g., Geisbert et al., Lancet 2010; 375: 1896-905). A dosage of about 2 mg/kg total CRISPR Cas per dose administered as, for example, a bolus intravenous infusion may be contemplated. In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids Inc.), PEG-cDMA, and 1,2-dilinoleyloxy-3-(N;N-dimethyl)aminopropane (DLinDMA) (see, e.g., Judge, J. Clin. Invest. 119:661-673 (2009)). Formulations used for in vivo studies may comprise a final lipid/RNA mass ratio of about 9:1.

[00213] The safety profile of RNAi nanomedicines has been reviewed by Barros and Gollob of Alnylam Pharmaceuticals (see, e.g., Advanced Drug Delivery Reviews 64 (2012) 1730–1737). The stable nucleic acid lipid particle (SNALP) is comprised of four different lipids — an ionizable lipid (DLinDMA) that is cationic at low pH, a neutral helper lipid, cholesterol, and a diffusible polyethylene glycol (PEG)-lipid. The particle is approximately 80 nm in diameter and is charge-neutral at physiologic pH. During formulation, the ionizable lipid serves to condense lipid with the anionic RNA during particle formation. When positively charged under increasingly acidic endosomal conditions, the ionizable lipid also mediates the fusion of SNALP with the endosomal membrane enabling release of RNA into the cytoplasm. The PEG-lipid stabilizes the particle and reduces aggregation during formulation, and subsequently provides a neutral hydrophilic exterior that improves pharmacokinetic properties.

[00214] To date, two clinical programs have been initiated using SNALP formulations with RNA. Tekmira Pharmaceuticals recently completed a phase I single-dose study of SNALP-ApoB in adult volunteers with elevated LDL cholesterol. ApoB is predominantly expressed in the liver and jejunum and is essential for the assembly and secretion of VLDL and LDL. Seventeen subjects received a single dose of SNALP-ApoB (dose escalation across 7 dose levels). There was no evidence of liver toxicity (anticipated as the potential dose-limiting toxicity based on preclinical studies). One (of two) subjects at the highest dose experienced flu-like symptoms consistent with immune system stimulation, and the decision was made to conclude the trial.

[00215] Alnylam Pharmaceuticals has similarly advanced ALN-TTR01, which employs the SNALP technology described above and targets hepatocyte production of both mutant and wild-type TTR to treat TTR amyloidosis (ATTR). Three ATTR syndromes have been described: familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC) — both caused by autosomal dominant mutations in TTR; and senile systemic amyloidosis (SSA) cause by wildtype TTR. A placebo-controlled, single dose-escalation phase I trial of ALN-TTR01 was recently completed in patients with ATTR. ALN-TTR01 was administered as a 15-minute IV infusion to 31 patients (23 with study drug and 8 with placebo) within a dose range of 0.01 to 1.0 mg/kg (based on siRNA). Treatment was well tolerated with no significant increases in liver function tests. Infusion-related reactions were noted in 3 of 23 patients at≥0.4 mg/kg; all responded to slowing of the infusion rate and all continued on study. Minimal and transient elevations of serum cytokines IL-6, IP-10 and IL-1ra were noted in two patients at the highest dose of 1 mg/kg (as anticipated from preclinical and NHP studies). Lowering of serum TTR, the expected pharmacodynamics effect of ALN-TTR01, was observed at 1 mg/kg.

[00216] In yet another embodiment, a SNALP may be made by solubilizing a cationic lipid, DSPC, cholesterol and PEG-lipid e.g., in ethanol, e.g., at a molar ratio of 40:10:40:10, respectively (see, Semple et al., Nature Niotechnology, Volume 28 Number 2 February 2010, pp. 172-177). The lipid mixture was added to an aqueous buffer (50 mM citrate, pH 4) with mixing to a final ethanol and lipid concentration of 30% (vol/vol) and 6.1 mg/ml, respectively, and allowed to equilibrate at 22 °C for 2 min before extrusion. The hydrated lipids were extruded through two stacked 80 nm pore-sized filters (Nuclepore) at 22 °C using a Lipex Extruder (Northern Lipids) until a vesicle diameter of 70–90 nm, as determined by dynamic light scattering analysis, was obtained. This generally required 1–3 passes. The siRNA (solubilized in a 50 mM citrate, pH 4 aqueous solution containing 30% ethanol) was added to the pre-equilibrated (35 °C) vesicles at a rate of ~5 ml/min with mixing. After a final target siRNA/lipid ratio of 0.06 (wt/wt) was reached, the mixture was incubated for a further 30 min at 35 °C to allow vesicle reorganization and encapsulation of the siRNA. The ethanol was then removed and the external buffer replaced with PBS (155 mM NaCl, 3 mM Na2HPO4, 1 mM KH2PO4, pH 7.5) by either dialysis or tangential flow diafiltration. siRNA were encapsulated in SNALP using a controlled step-wise dilution method process. The lipid constituents of KC2-SNALP were DLin-KC2-DMA (cationic lipid), dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids), synthetic cholesterol (Sigma) and PEG-C-DMA used at a molar ratio of 57.1:7.1:34.3:1.4. Upon formation of the loaded particles, SNALP were dialyzed against PBS and filter sterilized through a 0.2 μm filter before use. Mean particle sizes were 75–85 nm and 90–95% of the siRNA was encapsulated within the lipid particles. The final siRNA/lipid ratio in formulations used for in vivo testing was ~0.15 (wt/wt). LNP-siRNA systems containing Factor VII siRNA were diluted to the appropriate concentrations in sterile PBS immediately before use and the formulations were administered intravenously through the lateral tail vein in a total volume of 10 ml/kg. This method and these delivery systems may be extrapolated to the CRISPR Cas system of the present invention.

Other Lipids

[00217] Other cationic lipids, such as amino lipid 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA) may be utilized to encapsulate CRISPR Cas system or components thereof or nucleic acid molecule(s) coding therefor e.g., similar to SiRNA (see, e.g., Jayaraman, Angew. Chem. Int. Ed. 2012, 51, 8529 –8533), and hence may be employed in the practice of the invention. A preformed vesicle with the following lipid composition may be contemplated: amino lipid, distearoylphosphatidylcholine (DSPC), cholesterol and (R)-2,3-bis(octadecyloxy) propyl-1-(methoxy poly(ethylene glycol)2000)propylcarbamate (PEG-lipid) in the molar ratio 40/10/40/10, respectively, and a FVII siRNA/total lipid ratio of approximately 0.05 (w/w). To ensure a narrow particle size distribution in the range of 70–90 nm and a low polydispersity index of 0.11+0.04 (n=56), the particles may be extruded up to three times through 80 nm membranes prior to adding the CRISPR Cas RNA. Particles containing the highly potent amino lipid 16 may be used, in which the molar ratio of the four lipid components 16, DSPC, cholesterol and PEG-lipid (50/10/38.5/1.5) which may be further optimized to enhance in vivo activity.

[00218] Michael S D Kormann et al. ("Expression of therapeutic proteins after delivery of chemically modified mRNA in mice: Nature Biotechnology, Volume:29, Pages: 154–157 (2011)) describes the use of lipid envelopes to deliver RNA. Use of lipid envelopes is also preferred in the present invention.

[00219] In another embodiment, lipids may be formulated with the CRISPR Cas system of the present invention to form lipid nanoparticles (LNPs). Lipids include, but are not limited to, DLin-KC2-DMA4, C12-200 and colipids disteroylphosphatidyl choline, cholesterol, and PEG-DMG may be formulated with CRISPR Cas instead of siRNA (see, e.g., Novobrantseva, Molecular Therapy–Nucleic Acids (2012) 1, e4; doi:10.1038/mtna.2011.3) using a spontaneous vesicle formation procedure. The component molar ratio may be about 50/10/38.5/1.5 (DLin-KC2-DMA or C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG). The final lipid:siRNA weight ratio may be ~12:1 and 9:1 in the case of DLin-KC2-DMA and C12-200 lipid nanoparticles (LNPs), respectively. The formulations may have mean particle diameters of ~80 nm with >90% entrapment efficiency. A 3 mg/kg dose may be contemplated.

[00220] Tekmira has a portfolio of approximately 95 patent families, in the U.S. and abroad, that are directed to various aspects of LNPs and LNP formulations (see, e.g., U.S. Pat. Nos. 7,982,027; 7,799,565; 8,058,069; 8,283,333; 7,901,708; 7,745,651; 7,803,397; 8,101,741; 8,188,263; 7,915,399; 8,236,943 and 7,838,658 and European Pat. Nos 1766035; 1519714; 1781593 and 1664316), all of which may be used and/or adapted to the present invention.

[00221] The CRISPR Cas system or components thereof or nucleic acid molecule(s) coding therefor may be delivered encapsulated in PLGA Microspheres such as that further described in US published applications 20130252281 and 20130245107 and 20130244279 (assigned to Moderna Therapeutics) which relate to aspects of formulation of compositions comprising modified nucleic acid molecules which may encode a protein, a protein precursor, or a partially or fully processed form of the protein or a protein precursor. The formulation may have a molar ratio 50:10:38.5:1.5-3.0 (cationic lipid:fusogenic lipid:cholesterol:PEG lipid). The PEG lipid may be selected from, but is not limited to PEG-c-DOMG, PEG-DMG. The fusogenic lipid may be DSPC. See also, Schrum et al., Delivery and Formulation of Engineered Nucleic Acids, US published application 20120251618.

[00222] Nanomerics' technology addresses bioavailability challenges for a broad range of therapeutics, including low molecular weight hydrophobic drugs, peptides, and nucleic acid based therapeutics (plasmid, siRNA, miRNA). Specific administration routes for which the technology has demonstrated clear advantages include the oral route, transport across the blood-brain-barrier, delivery to solid tumours, as well as to the eye. See, e.g., Mazza et al., 2013, ACS Nano. 2013 Feb 26;7(2):1016-26; Uchegbu and Siew, 2013, J Pharm Sci. 102(2):305-10 and Lalatsa et al., 2012, J Control Release. 2012 Jul 20; 161(2):523-36.

[00223] US Patent Publication No. 20050019923 describes cationic dendrimers for delivering bioactive molecules, such as polynucleotide molecules, peptides and polypeptides and/or pharmaceutical agents, to a mammalian body. The dendrimers are suitable for targeting the delivery of the bioactive molecules to, for example, the liver, spleen, lung, kidney or heart (or even the brain). Dendrimers are synthetic 3-dimensional macromolecules that are prepared in a step-wise fashion from simple branched monomer units, the nature and functionality of which can be easily controlled and varied. Dendrimers are synthesised from the repeated addition of building blocks to a multifunctional core (divergent approach to synthesis), or towards a multifunctional core (convergent approach to synthesis) and each addition of a 3-dimensional shell of building blocks leads to the formation of a higher generation of the dendrimers. Polypropylenimine dendrimers start from a diaminobutane core to which is added twice the number of amino groups by a double Michael addition of acrylonitrile to the primary amines followed by the hydrogenation of the nitriles. This results in a doubling of the amino groups. Polypropylenimine dendrimers contain 100% protonable nitrogens and up to 64 terminal amino groups (generation 5, DAB 64). Protonable groups are usually amine groups which are able to accept protons at neutral pH. The use of dendrimers as gene delivery agents has largely focused on the use of the polyamidoamine. and phosphorous containing compounds with a mixture of amine/amide or N--P(O2)S as the conjugating units respectively with no work being reported on the use of the lower generation polypropylenimine dendrimers for gene delivery. Polypropylenimine dendrimers have also been studied as pH sensitive controlled release systems for drug delivery and for their encapsulation of guest molecules when chemically modified by peripheral amino acid groups. The cytotoxicity and interaction of polypropylenimine dendrimers with DNA as well as the transfection efficacy of DAB 64 has also been studied.

[00224] US Patent Publication No. 20050019923 is based upon the observation that, contrary to earlier reports, cationic dendrimers, such as polypropylenimine dendrimers, display suitable properties, such as specific targeting and low toxicity, for use in the targeted delivery of bioactive molecules, such as genetic material. In addition, derivatives of the cationic dendrimer also display suitable properties for the targeted delivery of bioactive molecules. See also, Bioactive Polymers, US published application 20080267903, which discloses "Various polymers, including cationic polyamine polymers and dendrimeric polymers, are shown to possess anti-proliferative activity, and may therefore be useful for treatment of disorders characterised by undesirable cellular proliferation such as neoplasms and tumours, inflammatory disorders (including autoimmune disorders), psoriasis and atherosclerosis. The polymers may be used alone as active agents, or as delivery vehicles for other therapeutic agents, such as drug molecules or nucleic acids for gene therapy. In such cases, the polymers' own intrinsic antitumour activity may complement the activity of the agent to be delivered." The disclosures of these patent publications may be employed in conjunction with herein teachings for delivery of CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor.

Supercharged proteins

[00225] Supercharged proteins are a class of engineered or naturally occurring proteins with unusually high positive or negative net theoretical charge and may be employed in delivery of CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor. Both supernegatively and superpositively charged proteins exhibit a remarkable ability to withstand thermally or chemically induced aggregation. Superpositively charged proteins are also able to penetrate mammalian cells. Associating cargo with these proteins, such as plasmid DNA, RNA, or other proteins, can enable the functional delivery of these macromolecules into mammalian cells both in vitro and in vivo. David Liu's lab reported the creation and characterization of supercharged proteins in 2007 (Lawrence et al., 2007, Journal of the American Chemical Society 129, 10110–10112).

[00226] The nonviral delivery of RNA and plasmid DNA into mammalian cells are valuable both for research and therapeutic applications (Akinc et al., 2010, Nat. Biotech. 26, 561–569). Purified +36 GFP protein (or other superpositively charged protein) is mixed with RNAs in the appropriate serum-free media and allowed to complex prior addition to cells. Inclusion of serum at this stage inhibits formation of the supercharged protein-RNA complexes and reduces the effectiveness of the treatment. The following protocol has been found to be effective for a variety of cell lines (McNaughton et al., 2009, Proc. Natl. Acad. Sci. USA 106, 6111–6116). However, pilot experiments varying the dose of protein and RNA should be performed to optimize the procedure for specific cell lines.

(1) One day before treatment, plate 1 x 105 cells per well in a 48-well plate.

(2) On the day of treatment, dilute purified +36 GFP protein in serumfree media to a final concentration 200nM. Add RNA to a final concentration of 50nM. Vortex to mix and incubate at room temperature for 10min.

(3) During incubation, aspirate media from cells and wash once with PBS.

(4) Following incubation of +36 GFP and RNA, add the protein-RNA complexes to cells.

(5) Incubate cells with complexes at 37 °C for 4h.

(6) Following incubation, aspirate the media and wash three times with 20 U/mL heparin PBS. Incubate cells with serum-containing media for a further 48h or longer depending upon the assay for activity.

(7) Analyze cells by immunoblot, qPCR, phenotypic assay, or other appropriate method.

[00227] David Liu's lab has further found +36 GFP to be an effective plasmid delivery reagent in a range of cells. As plasmid DNA is a larger cargo than siRNA, proportionately more +36 GFP protein is required to effectively complex plasmids. For effective plasmid delivery Applicants have developed a variant of +36 GFP bearing a C-terminal HA2 peptide tag, a known endosome-disrupting peptide derived from the influenza virus hemagglutinin protein. The following protocol has been effective in a variety of cells, but as above it is advised that plasmid DNA and supercharged protein doses be optimized for specific cell lines and delivery applications.

(1) One day before treatment, plate 1 x 105 per well in a 48-well plate.

(2) On the day of treatment, dilute purified þ36 GFP protein in serumfree media to a final concentration 2 mM. Add 1mg of plasmid DNA. Vortex to mix and incubate at room temperature for 10min.

(3) During incubation, aspirate media from cells and wash once with PBS.

(4) Following incubation of þ36 GFP and plasmid DNA, gently add the protein-DNA complexes to cells.

(5) Incubate cells with complexes at 37 C for 4h.

(6) Following incubation, aspirate the media and wash with PBS. Incubate cells in serum-containing media and incubate for a further 24–48h.

(7) Analyze plasmid delivery (e.g., by plasmid-driven gene expression) as appropriate.

[00228] See also, e.g., McNaughton et al., Proc. Natl. Acad. Sci. USA 106, 6111-6116 (2009); Cronican et al., ACS Chemical Biology 5, 747-752 (2010); Cronican et al., Chemistry & Biology 18, 833-838 (2011); Thompson et al., Methods in Enzymology 503, 293-319 (2012); Thompson, D.B., et al., Chemistry & Biology 19 (7), 831-843 (2012). The methods of the super charged proteins may be used and/or adapted for delivery of the CRISPR Cas system of the present invention. These systems of Dr. Lui and documents herein in inconjunction with herein teachints can be employed in the delivery of CRISPR Cas system(s) or component(s) thereof or nucleic acid molecule(s) coding therefor.

Cell Penetrating Peptides (CPPs)

[00229] In yet another embodiment, cell penetrating peptides (CPPs) are contemplated for the delivery of the CRISPR Cas system. CPPs are short peptides that facilitate cellular uptake of various molecular cargo (from nanosize particles to small chemical molecules and large fragments of DNA). The term "cargo" as used herein includes but is not limited to the group consisting of therapeutic agents, diagnostic probes, peptides, nucleic acids, antisense oligonucleotides, plasmids, proteins, nanoparticles, liposomes, chromophores, small molecules and radioactive materials. In aspects of the invention, the cargo may also comprise any component of the CRISPR Cas system or the entire functional CRISPR Cas system. Aspects of the present invention further provide methods for delivering a desired cargo into a subject comprising: (a) preparing a complex comprising the cell penetrating peptide of the present invention and a desired cargo, and (b) orally, intraarticularly, intraperitoneally, intrathecally, intrarterially, intranasally, intraparenchymally, subcutaneously, intramuscularly, intravenously, dermally, intrarectally, or topically administering the complex to a subject. The cargo is associated with the peptides either through chemical linkage via covalent bonds or through non-covalent interactions.

[00230] The function of the CPPs are to deliver the cargo into cells, a process that commonly occurs through endocytosis with the cargo delivered to the endosomes of living mammalian cells. Cell-penetrating peptides are of different sizes, amino acid sequences, and charges but all CPPs have one distinct characteristic, which is the ability to translocate the plasma membrane and facilitate the delivery of various molecular cargoes to the cytoplasm or an organelle. CPP translocation may be classified into three main entry mechanisms: direct penetration in the membrane, endocytosis-mediated entry, and translocation through the formation of a transitory structure. CPPs have found numerous applications in medicine as drug delivery agents in the treatment of different diseases including cancer and virus inhibitors, as well as contrast agents for cell labeling. Examples of the latter include acting as a carrier for GFP, MRI contrast agents, or quantum dots. CPPs hold great potential as in vitro and in vivo delivery vectors for use in research and medicine. CPPs typically have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or has sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids. These two types of structures are referred to as polycationic or amphipathic, respectively. A third class of CPPs are the hydrophobic peptides, containing only apolar residues, with low net charge or have hydrophobic amino acid groups that are crucial for cellular uptake. One of the initial CPPs discovered was the trans-activating transcriptional activator (Tat) from Human Immunodeficiency Virus 1 (HIV-1) which was found to be efficiently taken up from the surrounding media by numerous cell types in culture. Since then, the number of known CPPs has expanded considerably and small molecule synthetic analogues with more effective protein transduction properties have been generated. CPPs include but are not limited to Penetratin, Tat (48-60), Transportan, and (R-AhX-R4) (Ahx=aminohexanoyl).

[00231] US Patent 8,372,951, provides a CPP derived from eosinophil cationic protein (ECP) which exhibits highly cell-penetrating efficiency and low toxicity. Aspects of delivering the CPP with its cargo into a vertebrate subject are also provided. Further aspects of CPPs and their delivery are described in U. S. patents 8,575,305; 8;614,194 and 8,044,019. CPPs can be used to deliver the CRISPR-Cas system or components thereof. That CPPs can be employed to deliver the CRISPR-Cas system or components thereof is also provided in the manuscript "Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA", by Suresh Ramakrishna, Abu-Bonsrah Kwaku Dad, Jagadish Beloor, et al. Genome Res. 2014 Apr 2. [Epub ahead of print], incorporated by reference in its entirety, wherein it is demonstrated that treatment with CPP-conjugated recombinant Cas9 protein and CPP-complexed guide RNAs lead to endogenous gene disruptions in human cell lines. In the paper the Cas9 protein was conjugated to CPP via a thioether bond, whereas the guide RNA was complexed with CPP, forming condensed, positively charged nanoparticles. It was shown that simultaneous and sequential treatment of human cells, including embryonic stem cells, dermal fibroblasts, HEK293T cells, HeLa cells, and embryonic carcinoma cells, with the modified Cas9 and guide RNA led to efficient gene disruptions with reduced off-target mutations relative to plasmid transfections.

Implantable devices

[00232] In another embodiment, implantable devices are also contemplated for delivery of the CRISPR Cas system or component(s) thereof or nucleic acid molecule(s) coding therefor. For example, US Patent Publication 20110195123 discloses an implantable medical device which elutes a drug locally and in prolonged period is provided, including several types of such a device, the treatment modes of implementation and methods of implantation. The device comprising of polymeric substrate, such as a matrix for example, that is used as the device body, and drugs, and in some cases additional scaffolding materials, such as metals or additional polymers, and materials to enhance visibility and imaging. An implantable delivery device can be advantageous in providing release locally and over a prolonged period, where drug is released directly to the extracellular matrix (ECM) of the diseased area such as tumor, inflammation, degeneration or for symptomatic objectives, or to injured smooth muscle cells, or for prevention. One kind of drug is RNA, as disclosed above, and this system may be used/and or adapted to the CRISPR Cas system of the present invention. The modes of implantation in some embodiments are existing implantation procedures that are developed and used today for other treatments, including brachytherapy and needle biopsy. In such cases the dimensions of the new implant described in this invention are similar to the original implant. Typically a few devices are implanted during the same treatment procedure.

[00233] US Patent Publication 20110195123 provides a drug delivery implantable or insertable system, including systems applicable to a cavity such as the abdominal cavity and/or any other type of administration in which the drug delivery system is not anchored or attached, comprising a biostable and/or degradable and/or bioabsorbable polymeric substrate, which may for example optionally be a matrix. It should be noted that the term "insertion" also includes implantation. The drug delivery system is preferably implemented as a "Loder" as described in US Patent Publication 20110195123.

[00234] The polymer or plurality of polymers are biocompatible, incorporating an agent and/or plurality of agents, enabling the release of agent at a controlled rate, wherein the total volume of the polymeric substrate, such as a matrix for example, in some embodiments is optionally and preferably no greater than a maximum volume that permits a therapeutic level of the agent to be reached. As a non-limiting example, such a volume is preferably within the range of 0.1 m^3 to 1000 mm^3, as required by the volume for the agent load. The Loder may optionally be larger, for example when incorporated with a device whose size is determined by functionality, for example and without limitation, a knee joint, an intra-uterine or cervical ring and the like.

[00235] The drug delivery system (for delivering the composition) is designed in some embodiments to preferably employ degradable polymers, wherein the main release mechanism is bulk erosion; or in some embodiments, non degradable, or slowly degraded polymers are used, wherein the main release mechanism is diffusion rather than bulk erosion, so that the outer part functions as membrane, and its internal part functions as a drug reservoir, which practically is not affected by the surroundings for an extended period (for example from about a week to about a few months). Combinations of different polymers with different release mechanisms may also optionally be used. The concentration gradient at the surface is preferably maintained effectively constant during a significant period of the total drug releasing period, and therefore the diffusion rate is effectively constant (termed "zero mode" diffusion). By the term "constant" it is meant a diffusion rate that is preferably maintained above the lower threshold of therapeutic effectiveness, but which may still optionally feature an initial burst and/or may fluctuate, for example increasing and decreasing to a certain degree. The diffusion rate is preferably so maintained for a prolonged period, and it can be considered constant to a certain level to optimize the therapeutically effective period, for example the effective silencing period.

[00236] The drug delivery system optionally and preferably is designed to shield the nucleotide based therapeutic agent from degradation, whether chemical in nature or due to attack from enzymes and other factors in the body of the subject.

[00237] The drug delivery system of US Patent Publication 20110195123 is optionally associated with sensing and/or activation appliances that are operated at and/or after implantation of the device, by non and/or minimally invasive methods of activation and/or acceleration/deceleration, for example optionally including but not limited to thermal heating and cooling, laser beams, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices.

[00238] According to some embodiments of US Patent Publication 20110195123, the site for local delivery may optionally include target sites characterized by high abnormal proliferation of cells, and suppressed apoptosis, including tumors, active and or chronic inflammation and infection including autoimmune diseases states, degenerating tissue including muscle and nervous tissue, chronic pain, degenerative sites, and location of bone fractures and other wound locations for enhancement of regeneration of tissue, and injured cardiac, smooth and striated muscle.

[00239] The site for implantation of the composition, or target site, preferably features a radius, area and/or volume that is sufficiently small for targeted local delivery. For example, the target site optionally has a diameter in a range of from about 0.1 mm to about 5 cm.

[00240] The location of the target site is preferably selected for maximum therapeutic efficacy. For example, the composition of the drug delivery system (optionally with a device for implantation as described above) is optionally and preferably implanted within or in the proximity of a tumor environment, or the blood supply associated thereof.

[00241] For example the composition (optionally with the device) is optionally implanted within or in the proximity to pancreas, prostate, breast, liver, via the nipple, within the vascular system and so forth.

[00242] The target location is optionally selected from the group consisting of (as non-limiting examples only, as optionally any site within the body may be suitable for implanting a Loder): 1. brain at degenerative sites like in Parkinson or Alzheimer disease at the basal ganglia, white and gray matter; 2. spine as in the case of amyotrophic lateral sclerosis (ALS); 3. uterine cervix to prevent HPV infection; 4. active and chronic inflammatory joints; 5. dermis as in the case of psoriasis; 6. sympathetic and sensoric nervous sites for analgesic effect; 7. Intra osseous implantation; 8. acute and chronic infection sites; 9. Intra vaginal; 10. Inner ear--auditory system, labyrinth of the inner ear, vestibular system; 11. Intra tracheal; 12. Intra-cardiac; coronary, epicardiac; 13. urinary bladder; 14. biliary system; 15. parenchymal tissue including and not limited to the kidney, liver, spleen; 16. lymph nodes; 17. salivary glands; 18. dental gums; 19. Intra-articular (into joints); 20. Intra-ocular; 21. Brain tissue; 22. Brain ventricles; 23. Cavities, including abdominal cavity (for example but without limitation, for ovary cancer); 24. Intra esophageal and 25. Intra rectal.

[00243] Optionally insertion of the system (for example a device containing the composition) is associated with injection of material to the ECM at the target site and the vicinity of that site to affect local pH and/or temperature and/or other biological factors affecting the diffusion of the drug and/or drug kinetics in the ECM, of the target site and the vicinity of such a site.

[00244] Optionally, according to some embodiments, the release of said agent could be associated with sensing and/or activation appliances that are operated prior and/or at and/or after insertion, by non and/or minimally invasive and/or else methods of activation and/or acceleration/deceleration, including laser beam, radiation, thermal heating and cooling, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices, and chemical activators.

[00245] According to other embodiments of US Patent Publication 20110195123, the drug preferably comprises a RNA, for example for localized cancer cases in breast, pancreas, brain, kidney, bladder, lung, and prostate as described below. Although exemplified with RNAi, many drugs are applicable to be encapsulated in Loder, and can be used in association with this invention, as long as such drugs can be encapsulated with the Loder substrate, such as a matrix for example, and this system may be used and/or adapted to deliver the CRISPR Cas system of the present invention.

[00246] As another example of a specific application, neuro and muscular degenerative diseases develop due to abnormal gene expression. Local delivery of RNAs may have therapeutic properties for interfering with such abnormal gene expression. Local delivery of anti apoptotic, anti inflammatory and anti degenerative drugs including small drugs and macromolecules may also optionally be therapeutic. In such cases the Loder is applied for prolonged release at constant rate and/or through a dedicated device that is implanted separately. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00247] As yet another example of a specific application, psychiatric and cognitive disorders are treated with gene modifiers. Gene knockdown is a treatment option. Loders locally delivering agents to central nervous system sites are therapeutic options for psychiatric and cognitive disorders including but not limited to psychosis, bi-polar diseases, neurotic disorders and behavioral maladies. The Loders could also deliver locally drugs including small drugs and macromolecules upon implantation at specific brain sites. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00248] As another example of a specific application, silencing of innate and/or adaptive immune mediators at local sites enables the prevention of organ transplant rejection. Local delivery of RNAs and immunomodulating reagents with the Loder implanted into the transplanted organ and/or the implanted site renders local immune suppression by repelling immune cells such as CD8 activated against the transplanted organ. All of this may be used/and or adapted to the CRISPR Cas system of the present invention.

[00249] As another example of a specific application, vascular growth factors including VEGFs and angiogenin and others are essential for neovascularization. Local delivery of the factors, peptides, peptidomimetics, or suppressing their repressors is an important therapeutic modality; silencing the repressors and local delivery of the factors, peptides, macromolecules and small drugs stimulating angiogenesis with the Loder is therapeutic for peripheral, systemic and cardiac vascular disease.

[00250] The method of insertion, such as implantation, may optionally already be used for other types of tissue implantation and/or for insertions and/or for sampling tissues, optionally without modifications, or alternatively optionally only with non-major modifications in such methods. Such methods optionally include but are not limited to brachytherapy methods, biopsy, endoscopy with and/or without ultrasound, such as ERCP, stereotactic methods into the brain tissue, Laparoscopy, including implantation with a laparoscope into joints, abdominal organs, the bladder wall and body cavities.

[00251] Implantable device technology herein discussed can be employed with herein teachings and hence by this disclosure and the knowledge in the art, CRISPR-Cas system or components thereof or nucleic acid molecules thereof or encoding or providing components may be delivered via an implantable device.

Fluid delivery device methods

[00252] In another embodiment, a fluid delivery device with an array of needles (see, e.g., US Patent Publication No. 20110230839 assigned to the Fred Hutchinson Cancer Research Center) may be contemplated for delivery of CRISPR Cas to solid tissue. A device of US Patent Publication No. 20110230839 for delivery of a fluid to a solid tissue may comprise a plurality of needles arranged in an array; a plurality of reservoirs, each in fluid communication with a respective one of the plurality of needles; and a plurality of actuators operatively coupled to respective ones of the plurality of reservoirs and configured to control a fluid pressure within the reservoir. In certain embodiments each of the plurality of actuators may comprise one of a plurality of plungers, a first end of each of the plurality of plungers being received in a respective one of the plurality of reservoirs, and in certain further embodiments the plungers of the plurality of plungers are operatively coupled together at respective second ends so as to be simultaneously depressable. Certain still further embodiments may comprise a plunger driver configured to depress all of the plurality of plungers at a selectively variable rate. In other embodiments each of the plurality of actuators may comprise one of a plurality of fluid transmission lines having first and second ends, a first end of each of the plurality of fluid transmission lines being coupled to a respective one of the plurality of reservoirs. In other embodiments the device may comprise a fluid pressure source, and each of the plurality of actuators comprises a fluid coupling between the fluid pressure source and a respective one of the plurality of reservoirs. In further embodiments the fluid pressure source may comprise at least one of a compressor, a vacuum accumulator, a peristaltic pump, a master cylinder, a microfluidic pump, and a valve. In another embodiment, each of the plurality of needles may comprise a plurality of ports distributed along its length.

Patient-specific screening methods

[00253] A CRISPR-Cas system that targets nucleotide, e.g., trinucleotide repeats can be used to screen patients or patent samples for the presence of such repeats. The repeats can be the target of the RNA of the CRISPR-Cas system, and if there is binding thereto by the CRISPR-Cas system, that binding can be detected, to thereby indicate that such a repeat is present. Thus, a CRISPR-Cas system can be used to screen patients or patient samples for the presence of the repeat. The patient can then be administered suitable compound(s) to address the condition; or, can be administed a CRISPR-Cas system to bind to and cause insertion, deletion or mutation and alleviate the condition.

Bone

[00254] Oakes and Lieberman (Clin Orthop Relat Res. 2000 Oct;(379 Suppl):S101-12) discusses delivery of genes to the bone. By transferring genes into cells at a specific anatomic site, the osteoinductive properties of growth factors can be used at physiologic doses for a sustained period to facilitate a more significant healing response. The specific anatomic site, the quality of the bone, and the soft-tissue envelope, influences the selection of the target cells for regional gene therapy. Gene therapy vectors delivered to a treatment site in osteoconductive carriers have yielded promising results. Several investigators have shown exciting results using ex vivo and in vivo regional gene therapy in animal models. Such a system may be used/and or adapted to the CRISPR Cas system for delivery to the bone.

Targeted deletion, therapeutic applications

[00255] Targeted deletion of genes is preferred. Preferred are, therefore, genes involved in cholesterol biosynthesis, fatty acid biosynthesis, and other metabolic disorders, genes encoding mis-folded proteins involved in amyloid and other diseases, oncogenes leading to cellular transformation, latent viral genes, and genes leading to dominant-negative disorders, amongst other disorders. As exemplified here, Applicants prefer gene delivery of a CRISPR-Cas system to the eye (ocular), ear (auditory), epithelial, hematopoetic, or another tissue of a subject or a patient in need thereof, suffering from metabolic disorders, amyloidosis and protein-aggregation related diseases, cellular transformation arising from genetic mutations and translocations, dominant negative effects of gene mutations, latent viral infections, and other related symptoms, using either viral or nanoparticle delivery system. Therapeutic applications of the CRISPR-Cas system include hereditary ocular dieases, including but not limited to retinitis pigmentosa, achromatopsia, macular degeneration, glaucoma, etc. A list of ocular diseases is provided herein (see section entitled ocular gene therapy).

[00256] As an example, chronic infection by HIV-1 may be treated or prevented. In order to accomplish this, one may generate CRISPR-Cas guide RNAs that target the vast majority of the HIV-1 genome while taking into account HIV-1 strain variants for maximal coverage and effectiveness. One may accomplish delivery of the CRISPR-Cas system by conventional adenoviral or lentiviral-mediated infection of the host immune system. Depending on approach, host immune cells could be a) isolated, transduced with CRISPR-Cas, selected, and re-introduced in to the host or b) transduced in vivo by systemic delivery of the CRISPR-Cas system. The first approach allows for generation of a resistant immune population whereas the second is more likely to target latent viral reservoirs within the host. This is discussed in more detail in the Examples section.

[00257] In another example, US Patent Publication No. 20130171732 assigned to Sangamo BioSciences, Inc. relates to insertion of an anti-HIV transgene into the genome, methods of which may be applied to the CRISPR Cas system of the present invention. In another embodiment, the CXCR4 gene may be targeted and the TALE system of US Patent Publication No. 20100291048 assigned to Sangamo BioSciences, Inc. may be modified to the CRISPR Cas system of the present invention. The method of US Patent Publication Nos. 20130137104 and 20130122591 assigned to Sangamo BioSciences, Inc. and US Patent Publication No. 20100146651 assigned to Cellectis may be more generally applicable for transgene expression as it involves modifying a hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus for increasing the frequency of gene modification.

[00258] It is also envisaged that the present invention generates a gene knockout cell library. Each cell may have a single gene knocked out.

[00259] One may make a library of ES cells where each cell has a single gene knocked out, and the entire library of ES cells will have every single gene knocked out. This library is useful for the screening of gene function in cellular processes as well as diseases. To make this cell library, one may integrate Cas9 driven by an inducible promoter (e.g. doxycycline inducible promoter) into the ES cell. In addition, one may integrate a single guide RNA targeting a specific gene in the ES cell. To make the ES cell library, one may simply mix ES cells with a library of genes encoding guide RNAs targeting each gene in the human genome. One may first introduce a single BxB1 attB site into the AAVS1 locus of the human ES cell. Then one may use the BxB1 integrase to facilitate the integration of individual guide RNA genes into the BxB1 attB site in AAVS1 locus. To facilitate integration, each guide RNA gene may be contained on a plasmid that carries of a single attP site. This way BxB1 will recombine the attB site in the genome with the attP site on the guide RNA containing plasmid. To generate the cell library, one may take the library of cells that have single guide RNAs integrated and induce Cas9 expression. After induction, Cas9 mediates double strand break at sites specified by the guide RNA.

[00260] Chronic administration of protein therapeutics may elicit unacceptable immune responses to the specific protein. The immunogenicity of protein drugs can be ascribed to a few immunodominant helper T lymphocyte (HTL) epitopes. Reducing the MHC binding affinity of these HTL epitopes contained within these proteins can generate drugs with lower immunogenicity (Tangri S, et al. ("Rationally engineered therapeutic proteins with reduced immunogenicity" J Immunol. 2005 Mar 15;174(6):3187-96.) In the present invention, the immunogenicity of the CRISPR enzyme in particular may be reduced following the approach first set out in Tangri et al with respect to erythropoietin and subsequently developed. Accordingly, directed evolution or rational design may be used to reduce the immunogenicity of the CRISPR enzyme (for instance a Cas9) in the host species (human or other species).

[00261] Applicants have shown targeted *in vivo* cleavage using in an exemplary embodiment 3 guideRNAs of interest and are able to visualize efficient DNA cleavage *in vivo* occurring only in a small subset of cells. (*see* e.g., Example 1)In particular, this illustrates that specific targeting in higher organisms such as mammals can also be achieved. It also highlights multiplex aspect in that multiple guide sequences (i.e. separate targets) can be used simultaneously (in the sense of co-delivery). In other words, Applicants used a multiple approach, with several different sequences targeted at the same time, but independently.

Blood

[00262] The present invention also contemplates delivering the CRISPR-Cas system to the blood.

[00263] The plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) may be utilized to deliver the CRISPR Cas system to the blood. Other means of delivery or RNA are also preferred, such as via nanoparticles (Cho, S., Goldberg, M., Son, S., Xu, Q., Yang, F., Mei, Y., Bogatyrev, S., Langer, R. and Anderson, D., Lipid-like nanoparticles for small interfering RNA

[00264] The CRISPR Cas system of the present invention is also contemplated to treat hemoglobinopathies, such as thalassemias and sickle cell disease. See, e.g., International Patent Publication No. WO 2013/126794 for potential targets that may be targeted by the CRISPR Cas system of the present invention. Drakopoulou, "Review Article, The Ongoing Challenge of Hematopoietic Stem Cell-Based Gene Therapy for β-Thalassemia," Stem Cells International, Volume 2011, Article ID 987980, 10 pages, doi:10.4061/2011/987980, incorporated herein by reference along with the documents it cites, as if set out in full, discuss modifying HSCs using a lentivirus that delivers a gene for β-globin or γ-globin. In contrast to using lentivirus, with the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to β-Thalassemia using a CRISPR-Cas9 system that targets and corrects the mutation (e.g., with a suitable HDR template that delivers a coding sequence for β-globin or γ-globin, advantageously non-sickling β-globin or γ-globin); specifically, the sgRNA can target mutation that give rise to β-Thalassemia, and the HDR can provide coding for proper expression of β-globin or γ-globin. In this regard mention is made of: Cavazzana, "Outcomes of Gene Therapy for β-Thalassemia Major via Transplantation of Autologous Hematopoietic Stem Cells Transduced Ex Vivo with a Lentiviral β^{A-T87Q}-Globin Vector." tif2014.org/abstractFiles/Jean%20Antoine%20Ribeil_Abstract.pdf; Cavazzana-Calvo, "Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia", Nature 467, 318–322 (16 September 2010) doi:10.1038/nature09328; Nienhuis, "Development of Gene Therapy for Thalassemia, Cold Spring Harbor Perpsectives in Medicine, doi: 10.1101/cshperspect.a011833 (2012), LentiGlobin BB305, a lentiviral vector containing an engineered β-globin gene (βA-T87Q); and Xie et al., "Seamless gene correction of β-thalassaemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyback" Genome Research gr.173427.114 (2014) 24: 1526-1533 (Cold Spring Harbor Laboratory Press); that is the subject of Cavazzana work involving human β-thalassaemia and the subject of the Xie work, are all incorporated herein by reference, together with all documents cited therein or associated therewith. In the instant invention, the HDR template can provide for the HSC to express an engineered β-globin gene (e.g., βA-T87Q), or β-globin as in Xie. Sickle cell anemia is an autosomal recessive genetic disease in which red blood cells become sickle-shaped. It is caused by a single base substitution in the β-globin gene, which is located on the short arm of chromosome 11. As a result, valine is produced instead of glutamic acid causing the production of sickle hemoglobin (HbS). This results in the formation of a distorted shape of the erythrocytes. Due to this abnormal shape, small blood vessels can be blocked, causing serious damage to the bone, spleen and skin tissues. This may lead to episodes of pain, frequent infections, hand-foot syndrome or even multiple organ failure. The distorted erythrocytes are also more susceptible to hemolysis, which leads to serious anemia. As in the case of β-thalassaemia, sickle cell anemia can be corrected by modifying HSCs with the CRISPR/Cas9 system. The system allows the specific editing of the cell's genome by cutting its DNA and then letting it repair itself. The Cas9 protein is inserted and directed by a RNA guide to the mutated point and then it cuts the DNA at that point. Simultaneously, a healthy version of the sequence is inserted. This sequence is used by the cell's own repair system to fix the induced cut. In this way, the CRISPR/Cas9 allows the correction of the mutation in the previously obtained stem cells. With the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to sickle cell anemia using a CRISPR-Cas9 system that targets and corrects the mutation (e.g., with a suitable HDR template that delivers a coding sequence for β-globin, advantageously non-sickling β-globin); specifically, the sgRNA can target mutation that give rise to sickle cell anemia, and the HDR can provide coding for proper expression of β-globin.

[00265] US Patent Publication Nos. 20110225664, 20110091441, 20100229252, 20090271881 and 20090222937 assigned to Cellectis, relates to CREI variants , wherein at least one of the two I-CreI monomers has at least two substitutions, one in each of the two functional subdomains of the LAGLIDADG core domain situated respectively from positions 26 to 40 and 44 to 77 of I-CreI, said variant being able to cleave a DNA target sequence from the human interleukin-2 receptor gamma chain (IL2RG) gene also named common cytokine receptor gamma chain gene or gamma C gene. The target sequences identified in US Patent Publication

Nos. 20110225664, 20110091441, 20100229252, 20090271881 and 20090222937 may be utilized for the CRISPR Cas system of the present invention.

[00266] Severe Combined Immune Deficiency (SCID) results from a defect in lymphocytes T maturation, always associated with a functional defect in lymphocytes B (Cavazzana-Calvo et al., Annu. Rev. Med., 2005, 56, 585-602; Fischer et al., Immunol. Rev., 2005, 203, 98-109). Overall incidence is estimated to 1 in 75 000 births. Patients with untreated SCID are subject to multiple opportunist micro-organism infections, and do generally not live beyond one year. SCID can be treated by allogenic hematopoietic stem cell transfer, from a familial donor. Histocompatibility with the donor can vary widely. In the case of Adenosine Deaminase (ADA) deficiency, one of the SCID forms, patients can be treated by injection of recombinant Adenosine Deaminase enzyme.

[00267] Since the ADA gene has been shown to be mutated in SCID patients (Giblett et al., Lancet, 1972, 2, 1067-1069), several other genes involved in SCID have been identified (Cavazzana-Calvo et al., Annu. Rev. Med., 2005, 56, 585-602; Fischer et al., Immunol. Rev., 2005, 203, 98-109). There are four major causes for SCID: (i) the most frequent form of SCID, SCID-X1 (X-linked SCID or X-SCID), is caused by mutation in the IL2RG gene, resulting in the absence of mature T lymphocytes and NK cells. IL2RG encodes the gamma C protein (Noguchi, et al., Cell, 1993, 73, 147-157), a common component of at least five interleukin receptor complexes. These receptors activate several targets through the JAK3 kinase (Macchi et al., Nature, 1995, 377, 65-68), which inactivation results in the same syndrome as gamma C inactivation; (ii) mutation in the ADA gene results in a defect in purine metabolism that is lethal for lymphocyte precursors, which in turn results in the quasi absence of B, T and NK cells; (iii) V(D)J recombination is an essential step in the maturation of immunoglobulins and T lymphocytes receptors (TCRs). Mutations in Recombination Activating Gene 1 and 2 (RAG1 and RAG2) and Artemis, three genes involved in this process, result in the absence of mature T and B lymphocytes; and (iv) Mutations in other genes such as CD45, involved in T cell specific signaling have also been reported, although they represent a minority of cases (Cavazzana-Calvo et al., Annu. Rev. Med., 2005, 56, 585-602; Fischer et al., Immunol. Rev., 2005, 203, 98-109).

[00268] Since when their genetic bases have been identified, the different SCID forms have become a paradigm for gene therapy approaches (Fischer et al., Immunol. Rev., 2005, 203, 98-109) for two major reasons. An ex vivo treatment is envisioned. Hematopoietic Stem Cells

(HSCs) can be recovered from bone marrow, and keep their pluripotent properties for a few cell divisions. Therefore, they can be treated in vitro, and then reinjected into the patient, where they repopulate the bone marrow. Since the maturation of lymphocytes is impaired in SCID patients, corrected cells have a selective advantage. Therefore, a small number of corrected cells can restore a functional immune system. This hypothesis has been validated in other systems by (i) the partial restoration of immune functions associated with the reversion of mutations in SCID patients (Hirschhorn et al., Nat. Genet., 1996, 13, 290-295; Stephan et al., N. Engl. J. Med., 1996, 335, 1563-1567; Bousso et al., Proc. Natl., Acad. Sci. USA, 2000, 97, 274-278; Wada et al., Proc. Natl. Acad. Sci. USA, 2001, 98, 8697-8702; Nishikomori et al., Blood, 2004, 103, 4565-4572), (ii) the correction of SCID-X1 deficiencies in vitro in hematopoietic cells (Candotti et al., Blood, 1996, 87, 3097-3102; Cavazzana-Calvo et al., Blood, 1996, Blood, 88, 3901-3909; Taylor et al., Blood, 1996, 87, 3103-3107; Hacein-Bey et al., Blood, 1998, 92, 4090-4097), (iii) the correction of SCID-X1 (Soudais et al., Blood, 2000, 95, 3071-3077; Tsai et al., Blood, 2002, 100, 72-79), JAK-3 (Bunting et al., Nat. Med., 1998, 4, 58-64; Bunting et al., Hum. Gene Ther., 2000, 11, 2353-2364) and RAG2 (Yates et al., Blood, 2002, 100, 3942-3949) deficiencies in vivo in animal models and (iv) by the result of gene therapy clinical trials (Cavazzana-Calvo et al., Science, 2000, 288, 669-672; Aiuti et al., Nat. Med., 2002; 8, 423-425; Gaspar et al., Lancet, 2004, 364, 2181-2187). From this disclosure, one can use a CRISPR-Cas9 system that targets and one or more of the mutations associated with SCID, for instance a CRISPR-Cas9 system having sgRNA(s) and HDR template(s) that respectively targets mutation of *IL2RG* that give rise to SCID and provide corrective expression of the gamma C protein.

[00269] US Patent Publication No. 20110182867 assigned to the Children's Medical Center Corporation and the President and Fellows of Harvard College relates to methods and uses of modulating fetal hemoglobin expression (HbF) in a hematopoietic progenitor cells via inhibitors of BCL11A expression or activity, such as RNAi and antibodies. The targets disclosed in US Patent Publication No. 20110182867, such as BCL11A, may be targeted by the CRISPR Cas system of the present invention for modulating fetal hemoglobin expression. See also Bauer et al. (Science 11 October 2013: Vol. 342 no. 6155 pp. 253-257) and Xu et al. (Science 18 November 2011: Vol. 334 no. 6058 pp. 993-996) for additional BCL11A targets.

Ears

[00270] The present invention also contemplates delivering the CRISPR-Cas system to one or both ears.

[00271] Researchers are looking into whether gene therapy could be used to aid current deafness treatments—namely, cochlear implants. Deafness is often caused by lost or damaged hair cells that cannot relay signals to auditory neurons. In such cases, cochlear implants may be used to respond to sound and transmit electrical signals to the nerve cells. But these neurons often degenerate and retract from the cochlea as fewer growth factors are released by impaired hair cells.

[00272] US patent application 20120328580 describes injection of a pharmaceutical composition into the ear (e.g., auricular administration), such as into the luminae of the cochlea (e.g., the Scala media, Sc vestibulae, and Sc tympani), e.g., using a syringe, e.g., a single-dose syringe. For example, one or more of the compounds described herein can be administered by intratympanic injection (e.g., into the middle ear), and/or injections into the outer, middle, and/or inner ear. Such methods are routinely used in the art, for example, for the administration of steroids and antibiotics into human ears. Injection can be, for example, through the round window of the ear or through the cochlear capsule. Other inner ear administration methods are known in the art (see, e.g., Salt and Plontke, Drug Discovery Today, 10:1299-1306, 2005).

[00273] In another mode of administration, the pharmaceutical composition can be administered in situ, via a catheter or pump. A catheter or pump can, for example, direct a pharmaceutical composition into the cochlear luminae or the round window of the ear and/or the lumen of the colon. Exemplary drug delivery apparatus and methods suitable for administering one or more of the compounds described herein into an ear, e.g., a human ear, are described by McKenna et al., (U.S. Publication No. 2006/0030837) and Jacobsen et al., (U.S. Pat. No. 7,206,639). In some embodiments, a catheter or pump can be positioned, e.g., in the ear (e.g., the outer, middle, and/or inner ear) of a patient during a surgical procedure. In some embodiments, a catheter or pump can be positioned, e.g., in the ear (e.g., the outer, middle, and/or inner ear) of a patient without the need for a surgical procedure.

[00274] Alternatively or in addition, one or more of the compounds described herein can be administered in combination with a mechanical device such as a cochlear implant or a hearing aid, which is worn in the outer ear. An exemplary cochlear implant that is suitable for use with the present invention is described by Edge et al., (U.S. Publication No. 2007/0093878).

[00275] In some embodiments, the modes of administration described above may be combined in any order and can be simultaneous or interspersed.

[00276] Alternatively or in addition, the present invention may be administered according to any of the Food and Drug Administration approved methods, for example, as described in CDER Data Standards Manual, version number 004 (which is available at fda.give/cder/dsm/DRG/drg00301.htm).

[00277] In general, the cell therapy methods described in US patent application 20120328580 can be used to promote complete or partial differentiation of a cell to or towards a mature cell type of the inner ear (e.g., a hair cell) in vitro. Cells resulting from such methods can then be transplanted or implanted into a patient in need of such treatment. The cell culture methods required to practice these methods, including methods for identifying and selecting suitable cell types, methods for promoting complete or partial differentiation of selected cells, methods for identifying complete or partially differentiated cell types, and methods for implanting complete or partially differentiated cells are described below.

[00278] Cells suitable for use in the present invention include, but are not limited to, cells that are capable of differentiating completely or partially into a mature cell of the inner ear, e.g., a hair cell (e.g., an inner and/or outer hair cell), when contacted, e.g., in vitro, with one or more of the compounds described herein. Exemplary cells that are capable of differentiating into a hair cell include, but are not limited to stem cells (e.g., inner ear stem cells, adult stem cells, bone marrow derived stem cells, embryonic stem cells, mesenchymal stem cells, skin stem cells, iPS cells, and fat derived stem cells), progenitor cells (e.g., inner ear progenitor cells), support cells (e.g., Deiters' cells, pillar cells, inner phalangeal cells, tectal cells and Hensen's cells), and/or germ cells. The use of stem cells for the replacement of inner ear sensory cells is described in Li et al., (U.S. Publication No. 2005/0287127) and Li et al., (U.S. patent Ser. No. 11/953,797). The use of bone marrow derived stem cells for the replacement of inner ear sensory cells is described in Edge et al., PCT/US2007/084654. iPS cells are described, e.g., at Takahashi et al., Cell, Volume 131, Issue 5, Pages 861-872 (2007); Takahashi and Yamanaka, Cell 126, 663-76 (2006); Okita et al., Nature 448, 260-262 (2007); Yu, J. et al., Science 318(5858):1917-1920 (2007); Nakagawa et al., Nat. Biotechnol. 26:101-106 (2008); and Zaehres and Scholer, Cell 131(5):834-835 (2007).

[00279] Such suitable cells can be identified by analyzing (e.g., qualitatively or quantitatively) the presence of one or more tissue specific genes. For example, gene expression can be detected by detecting the protein product of one or more tissue-specific genes. Protein detection techniques involve staining proteins (e.g., using cell extracts or whole cells) using antibodies against the appropriate antigen. In this case, the appropriate antigen is the protein product of the tissue-specific gene expression. Although, in principle, a first antibody (i.e., the antibody that binds the antigen) can be labeled, it is more common (and improves the visualization) to use a second antibody directed against the first (e.g., an anti-IgG). This second antibody is conjugated either with fluorochromes, or appropriate enzymes for colorimetric reactions, or gold beads (for electron microscopy), or with the biotin-avidin system, so that the location of the primary antibody, and thus the antigen, can be recognized.

[00280] The CRISPR Cas molecules of the present invention may be delivered to the ear by direct application of pharmaceutical composition to the outer ear, with compositions modified from US Published application, 20110142917. In some embodiments the pharmaceutical composition is applied to the ear canal. Delivery to the ear may also be refered to as aural or otic delivery.

[00281] In some embodiments the RNA molecules of the invention are delivered in liposome or lipofectin formulations and the like and can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Pat. Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

[00282] Delivery systems aimed specifically at the enhanced and improved delivery of siRNA into mammalian cells have been developed, (see, for example, Shen et al FEBS Let. 2003, 539:111-114; Xia et al., Nat. Biotech. 2002, 20:1006-1010; Reich et al., Mol. Vision. 2003, 9: 210-216; Sorensen et al., J. Mol. Biol. 2003, 327: 761-766; Lewis et al., Nat. Gen. 2002, 32: 107-108 and Simeoni et al., NAR 2003, 31, 11: 2717-2724) and may be applied to the present invention. siRNA has recently been successfully used for inhibition of gene expression in primates (see for example. Tolentino et al., Retina 24(4):660 which may also be applied to the present invention.

[00283] Qi et al. discloses methods for efficient siRNA transfection to the inner ear through the intact round window by a novel proteidic delivery technology which may be applied to the CRISPR Cas system of the present invention (see, e.g., Qi et al., Gene Therapy (2013), 1-9). In

particular, a TAT double stranded RNA-binding domains (TAT-DRBDs), which can transfect Cy3-labeled siRNA into cells of the inner ear, including the inner and outer hair cells, crista ampullaris, macula utriculi and macula sacculi, through intact round-window permeation was successful for delivering double stranded siRNAs in vivo for treating various inner ear ailments and preservation of hearing function. About 40 µl of 10mM RNA may be contemplated as the dosage for administration to the ear.

[00284] According to Rejali et al. (Hear Res. 2007 Jun;228(1-2):180-7), cochlear implant function can be improved by good preservation of the spiral ganglion neurons, which are the target of electrical stimulation by the implant and brain derived neurotrophic factor (BDNF) has previously been shown to enhance spiral ganglion survival in experimentally deafened ears. Rejali et al. tested a modified design of the cochlear implant electrode that includes a coating of fibroblast cells transduced by a viral vector with a BDNF gene insert. To accomplish this type of ex vivo gene transfer, Rejali et al. transduced guinea pig fibroblasts with an adenovirus with a BDNF gene cassette insert, and determined that these cells secreted BDNF and then attached BDNF-secreting cells to the cochlear implant electrode via an agarose gel, and implanted the electrode in the scala tympani. Rejali et al. determined that the BDNF expressing electrodes were able to preserve significantly more spiral ganglion neurons in the basal turns of the cochlea after 48 days of implantation when compared to control electrodes and demonstrated the feasibility of combining cochlear implant therapy with ex vivo gene transfer for enhancing spiral ganglion neuron survival. Such a system may be applied to the CRISPR Cas system of the present invention for delivery to the ear.

[00285] Mukherjea et al. (Antioxidants & Redox Signaling, Volume 13, Number 5, 2010) document that knockdown of NOX3 using short interfering (si) RNA abrogated cisplatin ototoxicity, as evidenced by protection of OHCs from damage and reduced threshold shifts in auditory brainstem responses (ABRs). Different doses of siNOX3 (0.3, 0.6, and 0.9 µg) were administered to rats and NOX3 expression was evaluated by real time RT-PCR. The lowest dose of NOX3 siRNA used (0.3 µg) did not show any inhibition of NOX3 mRNA when compared to transtympanic administration of scrambled siRNA or untreated cochleae. However, administration of the higher doses of NOX3 siRNA (0.6 and 0.9 µg) reduced NOX3 expression compared to control scrambled siRNA. Such a system may be applied to the CRISPR Cas system of the present invention for transtympanic administration with a dosage of about 2 mg to about 4 mg of CRISPR Cas for administration to a human.

[00286] Jung et al. (Molecular Therapy, vol. 21 no. 4, 834–841 apr. 2013) demonstrate that Hes5 levels in the utricle decreased after the application of siRNA and that the number of hair cells in these utricles was significantly larger than following control treatment. The data suggest that siRNA technology may be useful for inducing repair and regeneration in the inner ear and that the Notch signaling pathway is a potentially useful target for specific gene expression inhibition. Jung et al. injected 8 µg of Hes5 siRNA in 2 µl volume, prepared by adding sterile normal saline to the lyophilized siRNA to a vestibular epithelium of the ear. Such a system may be applied to the CRISPR Cas system of the present invention for administration to the vestibular epithelium of the ear with a dosage of about 1 to about 30 mg of CRISPR Cas for administration to a human.

[00287] Pinyon J. L. et al. (Sci. Transl Med. 2014 Apr 23;6(233):233ra54. doi: 10.1126/scitranslmed.3008177. Close-field electroporation gene delivery using the cochlear implant electrode array enhances the bionic ear.) reported their studies in guinea pigs which showed that neurotrophin gene therapy integrated into the cochlear implant improved its performance by stimulating spiral ganglion neurite regeneration. The authors used the cochlear implant electrode array for novel "close-field" electroporation to transduce mesenchymal cells lining the cochlear perilymphatic canals with a naked complementary DNA gene construct driving expression of brain-derived neurotrophic factor (BDNF) and a green fluorescent protein (GFP) reporter. The focusing of electric fields by particular cochlear implant electrode configurations led to surprisingly efficient gene delivery to adjacent mesenchymal cells. The resulting BDNF expression stimulated regeneration of spiral ganglion neurites, which had atrophied 2 weeks after ototoxic treatment, in a bilateral sensorineural deafness model. In this model, delivery of a control GFP-only vector failed to restore neuron structure, with atrophied neurons indistinguishable from unimplanted cochleae. With BDNF therapy, the regenerated spiral ganglion neurites extended close to the cochlear implant electrodes, with localized ectopic branching. This neural remodeling enabled bipolar stimulation via the cochlear implant array, with low stimulus thresholds and expanded dynamic range of the cochlear nerve, determined via electrically evoked auditory brainstem responses. This development may broadly improve neural interfaces and extend molecular medicine applications.

[00288] Atkinson P. J. et al. (PLoS One 2014 Jul 18;9(7):e102077. doi: 10.1371/journal.pone.0102077. eCollection 2014. Hair cell regeneration after ATOH1 gene therapy in the cochlea of profoundly deaf adult guinea pigs.) reported the results of a study aimed to promote the regeneration of sensory hair cells in the mature cochlea and their reconnection with auditory neurons through the introduction of ATOH1, a transcription factor known to be necessary for hair cell development, and the introduction of neurotrophic factors. Adenoviral vectors containing ATOH1 alone, or with neurotrophin-3 and brain derived neurotrophic factor were injected into the lower basal scala media of guinea pig cochleae four days post ototoxic deafening. Guinea pigs treated with ATOH1 gene therapy, alone, had a significantly greater number of cells expressing hair cell markers compared to the contralateral non-treated cochlea when examined 3 weeks post-treatment. This increase, however, did not result in a commensurate improvement in hearing thresholds, nor was there an increase in synaptic ribbons, as measured by CtBP2 puncta after ATOH1 treatment alone, or when combined with neurotrophins. However, hair cell formation and synaptogenesis after co-treatment with ATOH1 and neurotrophic factors remain inconclusive as viral transduction was reduced due to the halving of viral titres when the samples were combined. The authors concluded that collectively, these data suggests that, whilst ATOH1 alone can drive non-sensory cells towards an immature sensory hair cell phenotype in the mature cochlea, this does not result in functional improvements after aminoglycoside-induced deafness.

Deafness and Hearing Impairments Gene Therapy Description

[00289] One major cause of bearing and balance impairments is the loss of hair cell within the human cochleae. The loss can be due to noise, ototoxic damage, etc. Unfortunately, there is no evidence to support that new hair cells can be produced spontaneously in adult mammals including humans, and there is no method of reliably stimulating hair cell regeneration in mammalian

Cochleae after birth

[00290] Nonetheless, recent reports have shown that overexpression of certain gene such as human ATOH1 can induce the production of new hair cells. ATOH1 is a basic helix-loop-helix (bHLH) transcription factor that has been shown to be a key regulator in hair cell regeneration. Thus, the modulation of ATOH1 expression in vivo via epigenetic engineering using CRISPR-Cas9 system can be used as a potential therapeutic approach for human deafness or other types of hearing impairments. The major challenges for development of effective gene therapy for this type of hearing diseases are: (a) Lack of easily designable genome engineering: This is addressed by the CRISPR-Cas9 technology. In particular, the ability to create non-cleaving mutant version of the Cas9 protein, dCas9, that is capable of binding to target DNA sequence but not introducing any DNA damage or modification. (b) Low efficiency of in vivo delivery: This is improved by the small Cas9, SaCas9 from Staphylococcus aureus, which can be readily packaged into a single AAV vector to express both the dCas9 protein, fusion effector, and corresponding chimeric guide RNA(s). (c) Low efficiency of epigenetic modulation due to necessity to apply multiple guides to manipulate a single gene and thus the requirement of co-delivery of multiple viral vectors.

[00291] Applicants have solved the multiple-guide issue by the optimization of dCas9 and the fusion effector, chimeric guide RNA design. In particular, multiple MS2 binding sites could be engineered into the chimeric RNA backbone through tandem insertion. In this way, the epigenetic engineering is carried out by the tri-component complex consists of dCas9, the modified chimeric guide RNA, and fusion efforts. The fusion effectors harbor the MS2 protein and the epigenetic modifiers such as VP64, p65, KRAB, SID, or SID4X domains. Importantly, other RNA-protein interactions can be explored as well in the same manner. Additionally, the multiple-vector issue is also improved by the introduction of small SaCas9, that reduced the number of viral vectors required to perform the experiments from (2 or 3 vectors in total) to just (1 or 2 vectors in total).

[00292] Applicants genome engineering system using SaCas9 could be effective packaged into AAV or Ad viruses, and in particular can be used to modify endogenous epigenetic state in mammalian cells in vivo, thereby modulate the expression level of disease-relevant gene or genomic loci to execute therapeutic effects. The components of the system in a single-vector design is shown on Figure 24, which shows design of dCas9-based epigenetic modulation system (3 components of the system, dSaCas9, fusion effector, and sgRNA are shown). This system can be combined with delivery method based on Adeno-associated virus (AAV), Adeno viruses (Ad) or other delivery vehicles to modify the epigenetic state of cells in vivo.

[00293] An non-limiting example for use of dSaCas9 to stimulate ATOH1 expression to treat deafness or hearing impairments is provided herein at Example 6, and Figures 24 and 25.

Eyes

[00294] The present invention also contemplates delivering the CRISPR-Cas system to one or both eyes.

[00295] In yet another aspect of the invention, the CRISPR-Cas system may be used to correct ocular defects that arise from several genetic mutations further described in Genetic Diseases of the Eye, Second Edition, edited by Elias I. Traboulsi, Oxford University Press, 2012.

[00296] For administration to the eye, lentiviral vectors, in particular equine infectious anemia viruses (EIAV) are particularly preferred.

[00297] In another embodiment, minimal non-primate lentiviral vectors based on the equine infectious anemia virus (EIAV) are also contemplated, especially for ocular gene therapy (see, e.g., Balagaan, J Gene Med 2006; 8: 275 – 285, Published online 21 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.845). The vectors are contemplated to have cytomegalovirus (CMV) promoter driving expression of the target gene. Intracameral, subretinal, intraocular and intravitreal injections are all contemplated (see, e.g., Balagaan, J Gene Med 2006; 8: 275 – 285, Published online 21 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.845). Intraocular injections may be performed with the aid of an operating microscope. For subretinal and intravitreal injections, eyes may be prolapsed by gentle digital pressure and fundi visualised using a contact lens system consisting of a drop of a coupling medium solution on the cornea covered with a glass microscope slide coverslip. For subretinal injections, the tip of a 10-mm 34-gauge needle, mounted on a 5-μl Hamilton syringe may be advanced under direct visualisation through the superior equatorial sclera tangentially towards the posterior pole until the aperture of the needle was visible in the subretinal space. Then, 2 μl of vector suspension may be injected to produce a superior bullous retinal detachment, thus confirming subretinal vector administration. This approach creates a self-sealing sclerotomy allowing the vector suspension to be retained in the subretinal space until it is absorbed by the RPE, usually within 48 h of the procedure. This procedure may be repeated in the inferior hemisphere to produce an inferior retinal detachment. This technique results in the exposure of approximately 70% of neurosensory retina and RPE to the vector suspension. For intravitreal injections, the needle tip may be advanced through the sclera 1 mm posterior to the corneoscleral limbus and 2 μl of vector suspension injected into the vitreous cavity. For intracameral injections, the needle tip may be advanced through a corneoscleral limbal paracentesis, directed towards the central cornea, and 2 μl of vector

suspension may be injected. For intracameral injections, the needle tip may be advanced through a corneoscleral limbal paracentesis, directed towards the central cornea, and 2 µl of vector suspension may be injected. These vectors may be injected at titres of either $1.0–1.4 \times 10^{10}$ or $1.0–1.4 \times 10^{9}$ transducing units (TU)/ml.

[00298] In another embodiment, RetinoStat®, an equine infectious anemia virus-based lentiviral gene therapy vector that expresses angiostatic proteins endostain and angiostatin that is delivered via a subretinal injection for the treatment of the web form of age-related macular degeneration is also contemplated (see, e.g., Binley et al., HUMAN GENE THERAPY 23:980–991 (September 2012)). Such a vector may be modified for the CRISPR-Cas system of the present invention. Each eye may be treated with either RetinoStat® at a dose of 1.1×10^{5} transducing units per eye (TU/eye) in a total volume of 100 µl.

[00299] In another embodiment, an E1-, partial E3-, E4-deleted adenoviral vector may be contemplated for delivery to the eye. Twenty-eight patients with advanced neovascular age-related macular degeneration (AMD) were given a single intravitreous injection of an E1-, partial E3-, E4-deleted adenoviral vector expressing human pigment ep- ithelium-derived factor (AdPEDF.ll) (see, e.g., Campochiaro et al., Human Gene Therapy 17:167-176 (February 2006)). Doses ranging from 10^{6} to $10^{9.5}$ particle units (PU) were investigated and there were no serious adverse events related to AdPEDF.ll and no dose-limiting toxicities (see, e.g., Campochiaro et al., Human Gene Therapy 17:167-176 (February 2006)). Adenoviral vector-mediated ocular gene transfer appears to be a viable approach for the treatment of ocular disorders and could be applied to the CRISPR Cas system.

[00300] In another embodiment, the sd-rxRNA® system of RXi Pharmaceuticals may be used/and or adapted for delivering CRISPR Cas to the eye. In this system, a single intravitreal administration of 3 µg of sd-rxRNA results in sequence-specific reduction of PPIB mRNA levels for 14 days. The the sd-rxRNA® system may be applied to the CRISPR Cas system of the present invention, contemplating a dose of about 3 to 20 mg of CRISPR administered to a human.

[00301] Millington-Ward et al. (Molecular Therapy, vol. 19 no. 4, 642–649 apr. 2011) describes adeno-associated virus (AAV) vectors to deliver an RNA interference (RNAi)-based rhodopsin suppressor and a codon-modified rhodopsin replacement gene resistant to suppression due to nucleotide alterations at degenerate positions over the RNAi target site. An injection of either 6.0×10^8 vp or 1.8×10^{10} vp AAV were subretinally injected into the eyes by Millington-Ward et al. The AAV vectors of Millington-Ward et al. may be applied to the CRISPR Cas system of the present invention, contemplating a dose of about 2×10^{11} to about 6×10^{13} vp administered to a human.

[00302] Dalkara et al. (Sci Transl Med 5, 189ra76 (2013)) also relates to in vivo directed evolution to fashion an AAV vector that delivers wild-type versions of defective genes throughout the retina after noninjurious injection into the eyes' vitreous humor. Dalkara describes a a 7mer peptide display library and an AAV library constructed by DNA shuffling of cap genes from AAV1, 2, 4, 5, 6, 8, and 9. The rcAAV libraries and rAAV vectors expressing GFP under a CAG or Rho promoter were packaged and and deoxyribonuclease-resistant genomic titers were obtained through quantitative PCR. The libraries were pooled, and two rounds of evolution were performed, each consisting of initial library diversification followed by three in vivo selection steps. In each such step, P30 rho-GFP mice were intravitreally injected with 2 ml of iodixanol-purified, phosphate-buffered saline (PBS)–dialyzed library with a genomic titer of about 1×10^{12} vg/ml. The AAV vectors of Dalkara et al. may be applied to the CRISPR Cas system of the present invention, contemplating a dose of about 1×10^{15} to about 1×10^{16} vg/ml administered to a human.

[00303] In another embodiment, the rhodopsin gene may be targeted for the treatment of retinitis pigmentosa (RP), wherein the system of US Patent Publication No. 20120204282 assigned to Sangamo BioSciences, Inc. may be modified in accordance of the CRISPR Cas system of the present invention.

[00304] In another embodiment, the methods of US Patent Publication No. 20130183282 assigned to Cellectis, which is directed to methods of cleaving a target sequence from the human rhodopsin gene, may also be modified to the CRISPR Cas system of the present invention.

[00305] US Patent Publication No. 20130202678 assigned to Academia Sinica relates to methods for treating retinopathies and sight-threatening ophthalmologic disorders relating to delivering of the Puf-A gene (which is expressed in retinal ganglion and pigmented cells of eye tissues and displays a unique anti-apoptotic activity) to the sub-retinal or intravitreal space in the eye. In particular, desirable targets are zgc:193933, prdm1a, spata2, tex10, rbb4, ddx3, zp2.2, Blimp-1 and HtrA2, all of which may be targeted by the CRISPR Cas system of the present invention.

[00306] Wu (Cell Stem Cell,13:659–62, 2013) designed a guide RNA that led Cas9 to a single base pair mutation that causes cataracts in mice, where it induced DNA cleavage. Then using either the other wild-type allele or oligos given to the zygotes repair mechanisms corrected the sequence of the broken allele and corrected the cataract-causing genetic defect in mutant mouse.

[00307] US Patent Publication No. 20120159653, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with macular degeration (MD). Macular degeneration (MD) is the primary cause of visual impairment in the elderly, but is also a hallmark symptom of childhood diseases such as Stargardt disease, Sorsby fundus, and fatal childhood neurodegenerative diseases, with an age of onset as young as infancy. Macular degeneration results in a loss of vision in the center of the visual field (the macula) because of damage to the retina. Currently existing animal models do not recapitulate major hallmarks of the disease as it is observed in humans. The available animal models comprising mutant genes encoding proteins associated with MD also produce highly variable phenotypes, making translations to human disease and therapy development problematic.

[00308] One aspect of US Patent Publication No. 20120159653 relates to editing of any chromosomal sequences that encode proteins associated with MD which may be applied to the CRISPR Cas system of the present invention. The proteins associated with MD are typically selected based on an experimental association of the protein associated with MD to an MD disorder. For example, the production rate or circulating concentration of a protein associated with MD may be elevated or depressed in a population having an MD disorder relative to a population lacking the MD disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the proteins associated with MD may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00309] By way of non-limiting example, proteins associated with MD include but are not limited to the following proteins: (ABCA4) ATP-binding cassette, sub-family A (ABC1), member 4 ACHM1 achromatopsia (rod monochromacy) 1 ApoE Apolipoprotein E (ApoE) C1QTNF5 (CTRP5) C1q and tumor necrosis factor related protein 5 (C1QTNF5) C2

Complement component 2 (C2) C3 Complement components (C3) CCL2 Chemokine (C-C motif) Ligand 2 (CCL2) CCR2 Chemokine (C-C motif) receptor 2 (CCR2) CD36 Cluster of Differentiation 36 CFB Complement factor B CFH Complement factor CFH H CFHR1 complement factor H-related 1 CFHR3 complement factor H-related 3 CNGB3 cyclic nucleotide gated channel beta 3 CP ceruloplasmin (CP) CRP C reactive protein (CRP) CST3 cystatin C or cystatin 3 (CST3) CTSD Cathepsin D (CTSD) CX3CR1 chemokine (C-X3-C motif) receptor 1 ELOVL4 Elongation of very long chain fatty acids 4 ERCC6 excision repair cross-complementing rodent repair deficiency, complementation group 6 FBLN5 Fibulin-5 FBLN5 Fibulin 5 FBLN6 Fibulin 6 FSCN2 fascin (FSCN2) HMCN1 Hemicentrin 1 HMCN1 hemicentin 1 HTRA1 HtrA serine peptidase 1 (HTRA1) HTRA1 HtrA serine peptidase 1 IL-6 Interleukin 6 IL-8 Interleukin 8 LOC387715 Hypothetical protein PLEKHA1 Pleckstrin homology domain-containing family A member 1 (PLEKHA1) PROM1 Prominin 1(PROM1 or CD133) PRPH2 Peripherin-2 RPGR retinitis pigmentosa GTPase regulator SERPING1 serpin peptidase inhibitor, clade G, member 1 (C1- inhibitor) TCOF1 Treacle TIMP3 Metalloproteinase inhibitor 3 (TIMP3) TLR3 Toll-like receptor 3.

[00310] The identity of the protein associated with MD whose chromosomal sequence is edited can and will vary. In preferred embodiments, the proteins associated with MD whose chromosomal sequence is edited may be the ATP-binding cassette, sub-family A (ABC1) member 4 protein (ABCA4) encoded by the ABCR gene, the apolipoprotein E protein (APOE) encoded by the APOE gene, the chemokine (C-C motif) Ligand 2 protein (CCL2) encoded by the CCL2 gene, the chemokine (C-C motif) receptor 2 protein (CCR2) encoded by the CCR2 gene, the ceruloplasmin protein (CP) encoded by the CP gene, the cathepsin D protein (CTSD) encoded by the CTSD gene, or the metalloproteinase inhibitor 3 protein (TIMP3) encoded by the TIMP3 gene. In an exemplary embodiment, the genetically modified animal is a rat, and the edited chromosomal sequence encoding the protein associated with MD may be: (ABCA4) ATP-binding cassette, NM_000350 sub-family A (ABC1), member 4 APOE Apolipoprotein E NM_138828 (APOE) CCL2 Chemokine (C-C NM_031530 motif) Ligand 2 (CCL2) CCR2 Chemokine (C-C NM_021866 motif) receptor 2 (CCR2) CP ceruloplasmin (CP) NM_012532 CTSD Cathepsin D (CTSD) NM_134334 TIMP3 Metalloproteinase NM_012886 inhibitor 3 (TIMP3) The animal or cell may comprise 1, 2, 3, 4, 5, 6, 7 or more disrupted chromosomal sequences encoding a protein associated with MD and zero, 1, 2, 3, 4, 5, 6, 7 or more chromosomally integrated sequences encoding the disrupted protein associated with MD.

[00311] The edited or integrated chromosomal sequence may be modified to encode an altered protein associated with MD. Several mutations in MD-related chromosomal sequences have been associated with MD. Non-limiting examples of mutations in chromosomal sequences associated with MD include those that may cause MD including in the ABCR protein, E471K (i.e. glutamate at position 471 is changed to lysine), R1129L (i.e. arginine at position 1129 is changed to leucine), T1428M (i.e. threonine at position 1428 is changed to methionine), R1517S (i.e. arginine at position 1517 is changed to serine), I1562T (i.e. isoleucine at position 1562 is changed to threonine), and G1578R (i.e. glycine at position 1578 is changed to arginine); in the CCR2 protein, V64I (i.e. valine at position 192 is changed to isoleucine); in CP protein, G969B (i.e. glycine at position 969 is changed to asparagine or aspartate); in TIMP3 protein, S156C (i.e. serine at position 156 is changed to cysteine), G166C (i.e. glycine at position 166 is changed to cysteine), G167C (i.e. glycine at position 167 is changed to cysteine), Y168C (i.e. tyrosine at position 168 is changed to cysteine), S170C (i.e. serine at position 170 is changed to cysteine), Y172C (i.e. tyrosine at position 172 is changed to cysteine) and S181C (i.e. serine at position 181 is changed to cysteine). Other associations of genetic variants in MD-associated genes and disease are known in the art.

Ocular Disease Gene Therapy

[00312] There are many types of hereditary retina disease that have been mapped extensively for their genetic basis and thus provide good avenue for employing genome engineering technology to develop effective gene therapy to treat these conditions in human patients. Based on the feasibility, a list of ocular diseases is shown on the table below with annotations on their genetics and mode of inheritance.

Disease list and annotations

Ocular diseases	Causal genomic loci in human genome	Mode of inheritance
Stargardt Disease and Retinal Degeneration	ABCA4 & ELOVL4	Autosomal Recessive and Autosomal Dominant
Achromatopsia	CNGA3(exon 8) & CNGB3(Exon 10)	Autosomal Recessive
	CNGA3, CNGB3, CNNM4, GNAT2, KCNV2, NBAS, OPN1LW, PDE6C, PDE6H & RPGR	Autosomal Recessive & X-linked

Bardet-Biedl Syndrome	ARL6, BBS1, BBS2, BBS4, BBS5, BBS7, BBS9, BBS10, BBS12, CEP290, INPP5E, LZTFL1, MKS1, MKKS, SDCCAG8, TRIM32 & TTC8	Autosomal Recessive
Best Disease	BEST1	Autosomal Dominant
Blue Cone Monochromacy	OPNL1W	X-Linked
Choroideremia	CHM	X-Linked
Cone-Rod Dystrophy	CRX, GUCA1A(Leu151Phe) & GUCY2D (Exon 13)	Autosomal Dominant
	ADAM9, AIPL1, C21ORF2, C8ORF37, CACNA1F, CACNA2D4, CDHR1, CERKL, CNGB3, CNNM4, CRX, GUCA1A, GUCY2D, KCNV2, PDE6C, PDE6H, PITPNM3, PROM1, PRPH2, RAP28, RAX2, RDH5, RIMS1, RPGR, RPGRIP1 & UNC119	Autosomal Dominant, Autosomal Recessive & X-Linked
Congenital Stationary Night Blindness	CACNA1F, GRM6, PDE6B & TRPM1	Autosomal Dominant, Autosomal Recessive & X-Linked
	CABP4, CACNA1F, GNAT1, GPR179, GRK1, GRM6, LRIT3, NYX, PDE6B, RDH5, RHO, SAG, SLC24A1, TRPM1	
Corneal Dystrophy-Stromal	TGFBI (Exons 4 & 11-14)	Autosomal Dominant
Enhanced S-Cone Syndrome	NR2E3 (Exons 2-8)	Autosomal Recessive
Juvenile Open Angle Glaucoma or Primary Open Angle Glaucoma	MYOC	Autosomal Dominant
Juvenile X-Linked Retinoschisis	RS1	X-Linked
Leber Congenital Amaurosis (LCA)	AIPL1, CEP290, CRB1, CRX, GUCY2D, IQCB1, LCA5, LRAT, NMNAT1, RD3, RDH12, RPE65, RPGRIP1, SPATA7, TULP1	Autosomal Recessive
Malattia Leventinese	EFEMP1 (Arg345Trp mutation)	Autosomal Dominant
Norrie Disease or X-Linked Familial Exudative Vitreoretinopathy (XL-FEVR)	NDP	X-Linked
Pattern Dystrophy	RDS	Autosomal Dominant
Retinitis Pigmentosa	C1QTNF5,IMPDH1, NR2E3, PRPF3, PRPF31, PRPF8, RDH12, RDS, RHO, RP1, RP9, SNRNP200, TOPORS	Autosomal Dominant
	ABCA4, CC2D2A, CERKL, CLRN1, CNGA1, CRB1, DHDDS, EYS, FAM161A, FLVCR1, IDH3B, IMPG2, LRAT, MAK, NR2E3, NRL, PDE6A, PDE6B, PDE6G, PROM1, RBP3, RDH12, RGR, RLBP1, RPE65, SAG, TTPA, TULP1, USH2A, ZNF513	Autosomal Recessive
	RP2, RPGR	X-Linked
Sorsby Dystrophy	TIMP3 (Exons 1 & 5)	Autosomal Dominant

Usher Syndrome	ABHD12, CDH23, CIB2, CLRN1, DFNB31, GPR98, HARS, MYO7A, PCDH15, USH1C, USH1G & USH2A	Autosomal Recessive
Aniridia	PAX6	Autosomal Dominant
Dominant Optic Atrophy	OPA1	Autosomal Dominant

[00313] The major challenges for development of effective gene therapy for ocular diseases are: (a) lack of easily designable genome engineering; (b) low efficiency of *in vivo* delivery; and (c) low efficiency of HDR due to co-delivery of multiple viral vectors. Applicants have shown that the CRISPR-Cas9 technology effectively addresses and provides solutions to these challenges. Applicants have shown that the challenges of low efficiency of *in vivo* delivery and low efficiency of HDR and co-delivery is solved by the small Cas9, SaCas9 from *Staphylococcus aureus*, which can be readily packaged into a single Adeno-associated virus (AAV) vector to express both the Cas9 protein and its corresponding sgRNA(s). Further, Applicants have shown that introduction of small SaCas9, has reduced the number of viral vectors required to perform homology-directed repair (HDR) from 3 vectors to 2 vectors.

[00314] Modifying endogenous genome sequence in mammalian cells in vivo using SaCas9-based CRISPR-Cas genome engineering system:

Applicants have shown that a genome engineering system using SaCas9 can be effectively packaged into AAV, and in particular can be used to modify endogenous genome sequence in mammalian cells *in vivo*.

The basic features of Applicants' SaCas9 system is shown in Figure 15. This system can be combined with delivery methods based on Adeno-associated virus (AAV) to edit post-mitotic cells *in vivo* and is effective for a number of cell types in human retina when combined with specific delivery vehicles. In the case of retina disease therapy, two delivery routes are employed: intravitreal AAV injection, where AAV is injected in the vitreous humor of the eye, can be used to targets retinal ganglion cells and Muller glial cells, or to systain long-term expression of the transgene within ocular cells. On the other hand, subretinal AAV injection, where a small amount of fluid is injected underneath the retina, efficiently targets photoreceptors and retinal pigment epithelium (RPE) cells.

[00315] AAV serotype 2 and 8 (AAV2 and AAV8) are the most effective serotypes that can be used for the delivery using the intravitral route for ganglion and Muller cells. AAV serotype 1, 2, 5, 7, 8, DJ can be used to deliver transgene into the photoreceptor and RPE cells via the subretinal injection procedure. The detailed model for gene therapy using this system is shown in Figure 16.

[00316] It will be readily appreciated that *in vivo* therapeutic genome engineering approach described herein, illustrated in Figure 16, and exemplified in Examples 2-5, can be employed to correct disease-causing mutations or other types of genomic abnormalities in the ocular system. The protocol can be summarized into the following steps: (i) *in vitro* target and HDR template validation; (ii) virus production; (iii) virus purification; and (iv) ocular injection.

In vivo therapeutic genome engineering for retinitis pigmentosa

[00317] Retinitis Pigmentosa (RP) is a hereditary ocular disorder that can lead to vision impairement and in some cases blindness. It is a type of degenerative eye disease that is often caused by missense mutations in genes involved in the function or regulation of photoreceptors cells or retinal pigment epithelium (RPE) cells in human eyes. RP is one of the most common forms of inherited retinal degeneration.

[00318] A key gene in the molecular genetics of RP is the rhodopsin gene (RHO). RHO gene encodes a principal protein of photoreceptor outer segments. Studies show that mutations in this gene are responsible for approximately 25% of autosomal dominant forms of RP.

[00319] One example of RHO mutation that causes RP is nucleotide substitution at codon 23, CCC to CAC, which encoding the amino acid substitution of histidine for phenylalanine at position 23 of the RHO gene, or RHO (P23H). The P23H mutation is one of the most common causes of autosomal dominant retinitis pigmentosa (Figure 18A). The phenotype in heterozygous patient is predominantly retinopathy and progressive retinal degeneration. Patients homozygous for this disease exhibit a more severe phenotype. It has been observed that glycosylation of the mutant P23H protein is severely diminished. In general, patients may experience defective light to dark, dark to light adaptation or nyctalopia, as the result of the degeneration of the peripheral visual field. Central vision loss is also observed in some cases. RP can be non-syndromic, or syndromic with deafness, ataxia, etc.

List of other RP disease mutations/genomic abnormalities

Disease	Mutated Genes	Mode of inheritance
Retinitis Pigmentosa	C1QTNF5,IMPDH1, NR2E3, PRPF3, PRPF31, PRPF8, RDH12, RDS, RHO, RP1, RP9, SNRNP200, TOPORS	Autosomal Dominant

Disease	Mutated Genes	Mode of inheritance
	ABCA4, CC2D2A, CERKL, CLRN1, CNGA1, CRB1, DHDDS, EYS, FAM161A, FLVCR1, IDH3B, IMPG2, LRAT, MAK, NR2E3, NRL, PDE6A, PDE6B, PDE6G, PROM1, RBP3, RDH12, RGR, RLBP1, RPE65, SAG, TTPA, TULP1, USH2A, ZNF513	Autosomal Recessive
	RP2, RPGR	X-Linked

[00320] As noted above, it will be readily appreciated that Applicants' exemplary *in vivo* therapeutic genome engineering approach described herein and shown in Figure 5 can be employed to correct disease-causing mutations or other types of genomic abnormalities in the ocular system. Applicants' genome engineering approach for retinitis pigmentosa gene therapy targeting the RHO gene is discussed in Examples 2 and 3 herein.

In vivo therapeutic genome engineering for Achromatopsia

[00321] Achromatopsia (ACHM) is a medical condition that is described by the inability of the patient to perceive color, maintain normal visual acuity at high light levels (i.e. exterior daylight). Although it can refer to acquired conditions, it typically refers to the autosomal recessive congenital color vision condition. The condition can also manifest as an incomplete form, defined as dyschromatopsia. The estimated occurrence is around 1 in 33,000 people in the general population.

[00322] There are five major symptoms that are associated with ACHM, namely achromatopia, amblyopia, hemeralopia, nystagmus, and iris operating abnormalities.

[00323] The key gene in the molecular genetics of ACHM is the cone cell cyclic nucleotide-gated ion channel genes CNGA3, CNGB3, and transducing gene GNAT2. Mutations in these genes will lead to malfunction of the retinal phototransduction pathway. Specifically, this type of congenital ACHM is thought to result from the inability of cone cells to properly respond to light input by hyperpolarizing. Achromatopsia caused by CNGA3 mutation is categorized as ACHM2, CNGB3 mutation as ACHM3, and GNAT2 as ACHM4. These are the major types of ACHM, while some minority of cases are caused by mutation of gene PDE6C and other genes, called ACHM5.

[00324] Applicants' genome engineering approach for ACHM gene therapy targeting the CNGA3 and CNGB3 mutations is described herein in Example 4.

[00325] For CNGA3 (ACHM2), there are four major mutations, arg277 to cys (R277C), arg283 to trp (R283W), arg436 to trp (R435W), and phe547 to leu (F547L). These disease-causing mutations accounted for 41.8% of all the detected mutations (from the report in Wissinger et al. 2001, Am. J. Hum. Genet. http://www.ncbi.nlm.nih.gov/pubmed/11536077). Here Applicants select the first and second mutation (R277C and R283W) as example. Because their close proximity, these two mutations can be corrected with the same strategy, constructs, viral vector sets, and procedure.

[00326] For CNGB3 (ACHM3), the 1148delC mutation is a prevalent form of disease-causing mutation and has been reported to account for 75% of patients (Wiszniewski et al. 2007, http://www.ncbi.nlm.nih.gov/pubmed/17265047). And the correction of this mutation through Cas9-mediated genome engineering approach with a HR template vector will be able to rescue the disease phenotype.

List of other Achromatopsia disease mutations/genomic abnormalities

Disease	Mutated Genes	Mode of inheritance
Achromatopsia	CNGA3, CNGB3, CNNM4, GNAT2, KCNV2, NBAS, OPN1LW, PDE6C, PDE6H & RPGR	Autosomal Recessive

In vivo therapeutic genome engineering for Age related Macular Degeneration

[00327] Age-related macular degeneration (AMD or ARMD) is a medical condition that usually affects older adults and results in a loss of vision in the center of the visual field because of damage to the retina. It occurs in "dry" and "wet" forms. It is a major cause of blindness and visual impairment in adults aged with 50.

[00328] There are two types of ARMD, the wet and the dry forms. The wet or exudative form of ARMD is characterized by angiogenesis from the choroid behind the retina. The new vessels are fragile and can result in blood and protein leakage below the macula. Bleeding, leaking, and scarring from these blood vessels eventually cause irreversible damage to the photoreceptors and thus rapid vision loss if left untreated. The retina can become detached because of the growing blood vessels.

[00329] In the dry or nonexudative form, cellular debris called drusen accumulates between the retina and the choroid, and this affect the vision of the patients and can ultimately cause retina detachment as well.

Molecular target for treating Age-related Macular Degeneration

[00330] The most relevant form of ARMD that can potentially be treated with gene therapy is the 'wet' form of ARMD. In this case, the proliferation of abnormal blood vessels in the retina is stimulated by vascular endothelial growth factor (VEGF), or the genomic locus VEGFA in human genome. Hence, methods that can repress VEGF expression or inhibit its activity can stop, or in some cases, reverse the growth of blood vessels. This is a promising molecular approach to treat this type of ARMD effectively in human patients.

[00331] An exemplary non-limiting genome engineering approach for gene therapy for treating age-realted macular degeneration is exemplified herein at Example 5 and Figure 23A-B.

Heart

[00332] The present invention also contemplates delivering the CRISPR-Cas system to the heart. For the heart, a myocardium tropic adena-associated virus (AAVM) is preferred, in particular AAVM41 which showed preferential gene transfer in the heart (see, e.g., Lin-Yanga et al., PNAS, March 10, 2009, vol. 106, no. 10). Administration may be systemic or local. A dosage of about 1-10 x 10^{14} vector genomes are contemplated for systemic administration. See also, e.g., Eulalio et al. (2012) Nature 492: 376 and Somasuntharam et al. (2013) Biomaterials 34: 7790.

[00333] For example, US Patent Publication No. 20110023139, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with cardiovascular disease. Cardiovascular diseases generally include high blood pressure, heart attacks, heart failure, and stroke and TIA. Any chromosomal sequence involved in cardiovascular disease or the protein encoded by any chromosomal sequence involved in cardiovascular disease may be utilized in the methods described in this disclosure. The cardiovascular-related proteins are typically selected based on an experimental association of the cardiovascular-related protein to the development of cardiovascular disease. For example, the production rate or circulating concentration of a cardiovascular-related protein may be elevated or depressed in a population having a cardiovascular disorder relative to a population lacking the cardiovascular disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay

(ELISA), and mass spectrometry. Alternatively, the cardiovascular-related proteins may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00334] By way of example, the chromosomal sequence may comprise, but is not limited to, IL1B (interleukin 1, beta), XDH (xanthine dehydrogenase), TP53 (tumor protein p53), PTGIS (prostaglandin 12 (prostacyclin) synthase), MB (myoglobin), IL4 (interleukin 4), ANGPT1 (angiopoietin 1), ABCG8 (ATP-binding cassette, sub-family G (WHITE), member 8), CTSK (cathepsin K), PTGIR (prostaglandin 12 (prostacyclin) receptor (IP)), KCNJ11 (potassium inwardly-rectifying channel, subfamily J, member 11), INS (insulin), CRP (C-reactive protein, pentraxin-related), PDGFRB (platelet-derived growth factor receptor, beta polypeptide), CCNA2 (cyclin A2), PDGFB (platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)), KCNJ5 (potassium inwardly-rectifying channel, subfamily J, member 5), KCNN3 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3), CAPN10 (calpain 10), PTGES (prostaglandin E synthase), ADRA2B (adrenergic, alpha-2B-, receptor), ABCG5 (ATP-binding cassette, sub-family G (WHITE), member 5), PRDX2 (peroxiredoxin 2), CAPN5 (calpain 5), PARP14 (poly (ADP-ribose) polymerase family, member 14), MEX3C (mex-3 homolog C (C. elegans)), ACE angiotensin I converting enzyme (peptidyl-dipeptidase A) 1), TNF (tumor necrosis factor (TNF superfamily, member 2)), IL6 (interleukin 6 (interferon, beta 2)), STN (statin), SERPINE1 (serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1), ALB (albumin), ADIPOQ (adiponectin, C1Q and collagen domain containing), APOB (apolipoprotein B (including Ag(x) antigen)), APOE (apolipoprotein E), LEP (leptin), MTHFR (5,10-methylenetetrahydrofolate reductase (NADPH)), APOA1 (apolipoprotein A-I), EDN1 (endothelin 1), NPPB (natriuretic peptide precursor B), NOS3 (nitric oxide synthase 3 (endothelial cell)), PPARG (peroxisome proliferator-activated receptor gamma), PLAT (plasminogen activator, tissue), PTGS2 (prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)), CETP (cholesteryl ester transfer protein, plasma), AGTR1 (angiotensin II receptor, type 1), HMGCR (3-hydroxy-3-methylglutaryl-Coenzyme A reductase), IGF1 (insulin-like growth factor 1 (somatomedin C)), SELE (selectin E), REN (renin), PPARA (peroxisome proliferator-activated receptor alpha), PON1 (paraoxonase 1), KNG1 (kininogen 1), CCL2 (chemokine (C-C motif) ligand 2), LPL (lipoprotein lipase), VWF (von Willebrand factor), F2 (coagulation factor II (thrombin)), ICAM1 (intercellular adhesion molecule 1), TGFB1 (transforming growth factor, beta 1), NPPA (natriuretic peptide precursor A), IL10 (interleukin 10), EPO (erythropoietin), SOD1 (superoxide dismutase 1, soluble), VCAM1 (vascular cell adhesion molecule 1), IFNG (interferon, gamma), LPA (lipoprotein, Lp(a)), MPO (myeloperoxidase), ESR1 (estrogen receptor 1), MAPK1 (mitogen-activated protein kinase 1), HP (haptoglobin), F3 (coagulation factor III (thromboplastin, tissue factor)), CST3 (cystatin C), COG2 (component of oligomeric golgi complex 2), MMP9 (matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)), SERPINC1 (serpin peptidase inhibitor, clade C (antithrombin), member 1), F8 (coagulation factor VIII, procoagulant component), HMOX1 (heme oxygenase (decycling) 1), APOC3 (apolipoprotein C-III), IL8 (interleukin 8), PROK1 (prokineticin 1), CBS (cystathionine-beta-synthase), NOS2 (nitric oxide synthase 2, inducible), TLR4 (toll-like receptor 4), SELP (selectin P (granule membrane protein 140 kDa, antigen CD62)), ABCA1 (ATP-binding cassette, sub-family A (ABC1), member 1), AGT (angiotensinogen (serpin peptidase inhibitor, clade A, member 8)), LDLR (low density lipoprotein receptor), GPT (glutamic-pyruvate transaminase (alanine aminotransferase)), VEGFA (vascular endothelial growth factor A), NR3C2 (nuclear receptor subfamily 3, group C, member 2), IL18 (interleukin 18 (interferon-gamma-inducing factor)), NOS1 (nitric oxide synthase 1 (neuronal)), NR3C1 (nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)), FGB (fibrinogen beta chain), HGF (hepatocyte growth factor (hepapoietin A; scatter factor)), IL1A (interleukin 1, alpha), RETN (resistin), AKT1 (v-akt murine thymoma viral oncogene homolog 1), LIPC (lipase, hepatic), HSPD1 (heat shock 60 kDa protein 1 (chaperonin)), MAPK14 (mitogen-activated protein kinase 14), SPP1 (secreted phosphoprotein 1), ITGB3 (integrin, beta 3 (platelet glycoprotein 111a, antigen CD61)), CAT (catalase), UTS2 (urotensin 2), THBD (thrombomodulin), F10 (coagulation factor X), CP (ceruloplasmin (ferroxidase)), TNFRSF11B (tumor necrosis factor receptor superfamily, member 11b), EDNRA (endothelin receptor type A), EGFR (epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)), MMP2 (matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)), PLG (plasminogen), NPY (neuropeptide Y), RHOD (ras homolog gene family, member D), MAPK8 (mitogen-activated protein kinase 8), MYC (v-myc myelocytomatosis viral oncogene homolog (avian)), FN1 (fibronectin 1), CMA1 (chymase 1, mast cell), PLAU (plasminogen activator, urokinase), GNB3 (guanine nucleotide binding protein (G protein), beta polypeptide 3), ADRB2 (adrenergic, beta-2-, receptor, surface), APOA5 (apolipoprotein A-V), SOD2 (superoxide dismutase 2, mitochondrial), F5 (coagulation factor V (proaccelerin, labile factor)), VDR (vitamin D (1,25-dihydroxyvitamin D3) receptor), ALOX5 (arachidonate 5-lipoxygenase), HLA-DRB1 (major histocompatibility complex, class II, DR beta 1), PARP1 (poly (ADP-ribose) polymerase 1), CD40LG (CD40 ligand), PON2 (paraoxonase 2), AGER (advanced glycosylation end product-specific receptor), IRS1 (insulin receptor substrate 1), PTGS1 (prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)), ECE1 (endothelin converting enzyme 1), F7 (coagulation factor VII (serum prothrombin conversion accelerator)), URN (interleukin 1 receptor antagonist), EPHX2 (epoxide hydrolase 2, cytoplasmic), IGFBP1 (insulin-like growth factor binding protein 1), MAPK10 (mitogen-activated protein kinase 10), FAS (Fas (TNF receptor superfamily, member 6)), ABCB1 (ATP-binding cassette, sub-family B (MDR/TAP), member 1), JUN (jun oncogene), IGFBP3 (insulin-like growth factor binding protein 3), CD14 (CD14 molecule), PDE5A (phosphodiesterase 5A, cGMP-specific), AGTR2 (angiotensin II receptor, type 2), CD40 (CD40 molecule, TNF receptor superfamily member 5), LCAT (lecithin-cholesterol acyltransferase), CCR5 (chemokine (C-C motif) receptor 5), MMP1 (matrix metallopeptidase 1 (interstitial collagenase)), TIMP1 (TIMP metallopeptidase inhibitor 1), ADM (adrenomedullin), DYT10 (dystonia 10), STAT3 (signal transducer and activator of transcription 3 (acute-phase response factor)), MMP3 (matrix metallopeptidase 3 (stromelysin 1, progelatinase)), ELN (elastin), USF1 (upstream transcription factor 1), CFH (complement factor H), HSPA4 (heat shock 70 kDa protein 4), MMP12 (matrix metallopeptidase 12 (macrophage elastase)), MME (membrane metallo-endopeptidase), F2R (coagulation factor II (thrombin) receptor), SELL (selectin L), CTSB (cathepsin B), ANXA5 (annexin A5), ADRB1 (adrenergic, beta-1-, receptor), CYBA (cytochrome b-245, alpha polypeptide), FGA (fibrinogen alpha chain), GGT1 (gamma-glutamyltransferase 1), LIPG (lipase, endothelial), HIF1A (hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)), CXCR4 (chemokine (C-X-C motif) receptor 4), PROC (protein C (inactivator of coagulation factors Va and VIIIa)), SCARB1 (scavenger receptor class B, member 1), CD79A (CD79a molecule, immunoglobulin-associated alpha), PLTP (phospholipid transfer protein), ADD1 (adducin 1 (alpha)), FGG (fibrinogen gamma chain), SAA1 (serum amyloid A1), KCNH2 (potassium voltage-gated channel, subfamily H (eag-related), member 2), DPP4 (dipeptidyl-peptidase 4), G6PD (glucose-6-phosphate dehydrogenase), NPR1 (natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)), VTN (vitronectin), KIAA0101 (KIAA0101), FOS (FBJ murine osteosarcoma viral oncogene homolog), TLR2 (toll-like receptor 2), PPIG (peptidylprolyl isomerase G (cyclophilin G)), IL1R1 (interleukin 1 receptor, type I), AR (androgen receptor), CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1), SERPINA1 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1), MTR (5-methyltetrahydrofolate-homocysteine methyltransferase), RBP4 (retinol binding protein 4, plasma), APOA4 (apolipoprotein A-IV), CDKN2A (cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)), FGF2 (fibroblast growth factor 2 (basic)), EDNRB (endothelin receptor type B), ITGA2 (integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)), CABIN1 (calcineurin binding protein 1), SHBG (sex hormone-binding globulin), HMGB1 (high-mobility group box 1), HSP90B2P (heat shock protein 90 kDa beta (Grp94), member 2 (pseudogene)), CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4), GJA1 (gap junction protein, alpha 1, 43 kDa), CAV1 (caveolin 1, caveolae protein, 22 kDa), ESR2 (estrogen receptor 2 (ER beta)), LTA (lymphotoxin alpha (TNF superfamily, member 1)), GDF15 (growth differentiation factor 15), BDNF (brain-derived neurotrophic factor), CYP2D6 (cytochrome P450, family 2, subfamily D, polypeptide 6), NGF (nerve growth factor (beta polypeptide)), SP1 (Sp1 transcription factor), TGIF1 (TGFB-induced factor homeobox 1), SRC (v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)), EGF (epidermal growth factor (beta-urogastrone)), PIK3CG (phosphoinositide-3-kinase, catalytic, gamma polypeptide), HLA-A (major histocompatibility complex, class I, A), KCNQ1 (potassium voltage-gated channel, KQT-like subfamily, member 1), CNR1 (cannabinoid receptor 1 (brain)), FBN1 (fibrillin 1), CHKA (choline kinase alpha), BEST1 (bestrophin 1), APP (amyloid beta (A4) precursor protein), CTNNB1 (catenin (cadherin-associated protein), beta 1, 88 kDa), IL2 (interleukin 2), CD36 (CD36 molecule (thrombospondin receptor)), PRKAB1 (protein kinase, AMP-activated, beta 1 non-catalytic subunit), TPO (thyroid peroxidase), ALDH7A1 (aldehyde dehydrogenase 7 family, member A1), CX3CR1 (chemokine (C-X3-C motif) receptor 1), TH (tyrosine hydroxylase), F9 (coagulation factor IX), GH1 (growth hormone 1), TF (transferrin), HFE (hemochromatosis), IL17A (interleukin 17A), PTEN (phosphatase and tensin homolog), GSTM1 (glutathione S-transferase mu 1), DMD (dystrophin), GATA4 (GATA binding protein

4), F13A1 (coagulation factor XIII, A1 polypeptide), TTR (transthyretin), FABP4 (fatty acid binding protein 4, adipocyte), PON3 (paraoxonase 3), APOC1 (apolipoprotein C-I), INSR (insulin receptor), TNFRSF1B (tumor necrosis factor receptor superfamily, member 1B), HTR2A (5-hydroxytryptamine (serotonin) receptor 2A), CSF3 (colony stimulating factor 3 (granulocyte)), CYP2C9 (cytochrome P450, family 2, subfamily C, polypeptide 9), TXN (thioredoxin), CYP11B2 (cytochrome P450, family 11, subfamily B, polypeptide 2), PTH (parathyroid hormone), CSF2 (colony stimulating factor 2 (granulocyte-macrophage)), KDR (kinase insert domain receptor (a type III receptor tyrosine kinase)), PLA2G2A (phospholipase A2, group IIA (platelets, synovial fluid)), B2M (beta-2-microglobulin), THBS1 (thrombospondin 1), GCG (glucagon), RHOA (ras homolog gene family, member A), ALDH2 (aldehyde dehydrogenase 2 family (mitochondrial)), TCF7L2 (transcription factor 7-like 2 (T-cell specific, HMG-box)), BDKRB2 (bradykinin receptor B2), NFE2L2 (nuclear factor (erythroid-derived 2)-like 2), NOTCH1 (Notch homolog 1, translocation-associated (Drosophila)), UGT1A1 (UDP glucuronosyltransferase 1 family, polypeptide A1), IFNA1 (interferon, alpha 1), PPARD (peroxisome proliferator-activated receptor delta), SIRT1 (sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae)), GNRH1 (gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)), PAPPA (pregnancy-associated plasma protein A, pappalysin 1), ARR3 (arrestin 3, retinal (X-arrestin)), NPPC (natriuretic peptide precursor C), AHSP (alpha hemoglobin stabilizing protein), PTK2 (PTK2 protein tyrosine kinase 2), IL13 (interleukin 13), MTOR (mechanistic target of rapamycin (serine/threonine kinase)), ITGB2 (integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)), GSTT1 (glutathione S-transferase theta 1), IL6ST (interleukin 6 signal transducer (gp130, oncostatin M receptor)), CPB2 (carboxypeptidase B2 (plasma)), CYP1A2 (cytochrome P450, family 1, subfamily A, polypeptide 2), HNF4A (hepatocyte nuclear factor 4, alpha), SLC6A4 (solute carrier family 6 (neurotransmitter transporter, serotonin), member 4), PLA2G6 (phospholipase A2, group VI (cytosolic, calcium-independent)), TNFSF11 (tumor necrosis factor (ligand) superfamily, member 11), SLC8A1 (solute carrier family 8 (sodium/calcium exchanger), member 1), F2RL1 (coagulation factor II (thrombin) receptor-like 1), AKR1A1 (aldo-keto reductase family 1, member A1 (aldehyde reductase)), ALDH9A1 (aldehyde dehydrogenase 9 family, member A1), BGLAP (bone gamma-carboxyglutamate (gla) protein), MTTP (microsomal triglyceride transfer protein), MTRR (5-methyltetrahydrofolate-homocysteine methyltransferase reductase), SULT1A3 (sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3), RAGE (renal tumor antigen), C4B (complement component 4B (Chido blood group), P2RY12 (purinergic receptor P2Y, G-protein coupled, 12), RNLS (renalase, FAD-dependent amine oxidase), CREB1 (cAMP responsive element binding protein 1), POMC (proopiomelanocortin), RAC1 (ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)), LMNA (lamin NC), CD59 (CD59 molecule, complement regulatory protein), SCN5A (sodium channel, voltage-gated, type V, alpha subunit), CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1), MIF (macrophage migration inhibitory factor (glycosylation-inhibiting factor)), MMP13 (matrix metallopeptidase 13 (collagenase 3)), TIMP2 (TIMP metallopeptidase inhibitor 2), CYP19A1 (cytochrome P450, family 19, subfamily A, polypeptide 1), CYP21A2 (cytochrome P450, family 21, subfamily A, polypeptide 2), PTPN22 (protein tyrosine phosphatase, non-receptor type 22 (lymphoid)), MYH14 (myosin, heavy chain 14, non-muscle), MBL2 (mannose-binding lectin (protein C) 2, soluble (opsonic defect)), SELPLG (selectin P ligand), AOC3 (amine oxidase, copper containing 3 (vascular adhesion protein 1)), CTSL1 (cathepsin L1), PCNA (proliferating cell nuclear antigen), IGF2 (insulin-like growth factor 2 (somatomedin A)), ITGB1 (integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)), CAST (calpastatin), CXCL12 (chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)), IGHE (immunoglobulin heavy constant epsilon), KCNE1 (potassium voltage-gated channel, Isk-related family, member 1), TFRC (transferrin receptor (p90, CD71)), COL1A1 (collagen, type I, alpha 1), COL1A2 (collagen, type I, alpha 2), IL2RB (interleukin 2 receptor, beta), PLA2G10 (phospholipase A2, group X), ANGPT2 (angiopoietin 2), PROCR (protein C receptor, endothelial (EPCR)), NOX4 (NADPH oxidase 4), HAMP (hepcidin antimicrobial peptide), PTPN11 (protein tyrosine phosphatase, non-receptor type 11), SLC2A1 (solute carrier family 2 (facilitated glucose transporter), member 1), IL2RA (interleukin 2 receptor, alpha), CCL5 (chemokine (C-C motif) ligand 5), IRF1 (interferon regulatory factor 1), CFLAR (CASP8 and FADD-like apoptosis regulator), CALCA (calcitonin-related polypeptide alpha), EIF4E (eukaryotic translation initiation factor 4E), GSTP1 (glutathione S-transferase pi 1), JAK2 (Janus kinase 2), CYP3A5 (cytochrome P450, family 3, subfamily A, polypeptide 5), HSPG2 (heparan sulfate proteoglycan 2), CCL3 (chemokine (C-C motif) ligand 3), MYD88 (myeloid differentiation primary response gene (88)), VIP (vasoactive intestinal peptide), SOAT1 (sterol O-acyltransferase 1), ADRBK1 (adrenergic, beta, receptor kinase 1), NR4A2 (nuclear receptor subfamily 4, group A, member 2), MMP8 (matrix metallopeptidase 8 (neutrophil collagenase)), NPR2 (natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)), GCH1 (GTP cyclohydrolase 1), EPRS (glutamyl-prolyl-tRNA synthetase), PPARGC1A (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha), F12 (coagulation factor XII (Hageman factor)), PECAM1 (platelet/endothelial cell adhesion molecule), CCL4 (chemokine (C-C motif) ligand 4), SERPINA3 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3), CASR (calcium-sensing receptor), GJA5 (gap junction protein, alpha 5, 40 kDa), FABP2 (fatty acid binding protein 2, intestinal), TTF2 (transcription termination factor, RNA polymerase II), PROS1 (protein S (alpha)), CTF1 (cardiotrophin 1), SGCB (sarcoglycan, beta (43 kDa dystrophin-associated glycoprotein)), YME1L1 (YME1-like 1 (S. cerevisiae)), CAMP (cathelicidin antimicrobial peptide), ZC3H12A (zinc finger CCCH-type containing 12A), AKR1B1 (aldo-keto reductase family 1, member B1 (aldose reductase)), DES (desmin), MMP7 (matrix metallopeptidase 7 (matrilysin, uterine)), AHR (aryl hydrocarbon receptor), CSF1 (colony stimulating factor 1 (macrophage)), HDAC9 (histone deacetylase 9), CTGF (connective tissue growth factor), KCNMA1 (potassium large conductance calcium-activated channel, subfamily M, alpha member 1), UGT1A (UDP glucuronosyltransferase 1 family, polypeptide A complex locus), PRKCA (protein kinase C, alpha), COMT (catechol-.beta.-methyltransferase), S100B (S100 calcium binding protein B), EGR1 (early growth response 1), PRL (prolactin), IL15 (interleukin 15), DRD4 (dopamine receptor D4), CAMK2G (calcium/calmodulin-dependent protein kinase II gamma), SLC22A2 (solute carrier family 22 (organic cation transporter), member 2), CCL11 (chemokine (C-C motif) ligand 11), PGF (B321 placental growth factor), THPO (thrombopoietin), GP6 (glycoprotein VI (platelet)), TACR1 (tachykinin receptor 1), NTS (neurotensin), HNF1A (HNF1 homeobox A), SST (somatostatin), KCND1 (potassium voltage-gated channel, Shal-related subfamily, member 1), LOC646627 (phospholipase inhibitor), TBXAS1 (thromboxane A synthase 1 (platelet)), CYP2J2 (cytochrome P450, family 2, subfamily J, polypeptide 2), TBXA2R (thromboxane A2 receptor), ADH1C (alcohol dehydrogenase 1C (class I), gamma polypeptide), ALOX12 (arachidonate 12-lipoxygenase), AHSG (alpha-2-HS-glycoprotein), BHMT (betaine-homocysteine methyltransferase), GJA4 (gap junction protein, alpha 4, 37 kDa), SLC25A4 (solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4), ACLY (ATP citrate lyase), ALOX5AP (arachidonate 5-lipoxygenase-activating protein), NUMA1 (nuclear mitotic apparatus protein 1), CYP27B1 (cytochrome P450, family 27, subfamily B, polypeptide 1), CYSLTR2 (cysteinyl leukotriene receptor 2), SOD3 (superoxide dismutase 3, extracellular), LTC4S (leukotriene C4 synthase), UCN (urocortin), GHRL (ghrelin/obestatin prepropeptide), APOC2 (apolipoprotein C-II), CLEC4A (C-type lectin domain family 4, member A), KBTBD10 (kelch repeat and BTB (POZ) domain containing 10), TNC (tenascin C), TYMS (thymidylate synthetase), SHCl (SHC (Src homology 2 domain containing) transforming protein 1), LRP1 (low density lipoprotein receptor-related protein 1), SOCS3 (suppressor of cytokine signaling 3), ADH1B (alcohol dehydrogenase 1B (class I), beta polypeptide), KLK3 (kallikrein-related peptidase 3), HSD11B1 (hydroxysteroid (11-beta) dehydrogenase 1), VKORC1 (vitamin K epoxide reductase complex, subunit 1), SERPINB2 (serpin peptidase inhibitor, clade B (ovalbumin), member 2), TNS1 (tensin 1), RNF19A (ring finger protein 19A), EPOR (erythropoietin receptor), ITGAM (integrin, alpha M (complement component 3 receptor 3 subunit)), PITX2 (paired-like homeodomain 2), MAPK7 (mitogen-activated protein kinase 7), FCGR3A (Fc fragment of IgG, low affinity 111a, receptor (CD16a)), LEPR (leptin receptor), ENG (endoglin), GPX1 (glutathione peroxidase 1), GOT2 (glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)), HRH1 (histamine receptor H1), NR112 (nuclear receptor subfamily 1, group I, member 2), CRH (corticotropin releasing hormone), HTR1A (5-hydroxytryptamine (serotonin) receptor 1A), VDAC1 (voltage-dependent anion channel 1), HPSE (heparanase), SFTPD (surfactant protein D), TAP2 (transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)), RNF123 (ring finger protein 123), PTK2B (PTK2B protein tyrosine kinase 2 beta), NTRK2 (neurotrophic tyrosine kinase, receptor, type 2), IL6R (interleukin 6 receptor), ACHE (acetylcholinesterase (Yt blood group)), GLP1R (glucagon-like peptide 1 receptor), GHR (growth hormone receptor), GSR (glutathione reductase), NQO1 (NAD(P)H dehydrogenase, quinone 1), NR5A1 (nuclear receptor subfamily 5, group A, member 1), GJB2 (gap junction protein, beta 2, 26 kDa), SLC9A1 (solute carrier family 9 (sodium/hydrogen exchanger), member 1), MAOA (monoamine oxidase A), PCSK9 (proprotein convertase subtilisin/kexin type 9), FCGR2A (Fc fragment of IgG, low affinity IIa, receptor (CD32)), SERPINF1 (serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1), EDN3 (endothelin 3), DHFR (dihydrofolate reductase), GAS6 (growth arrest-specific 6), SMPD1 (sphingomyelin phosphodiesterase 1, acid lysosomal), UCP2 (uncoupling protein 2 (mitochondrial, proton carrier)), TFAP2A (transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)), C4BPA (complement component 4 binding protein, alpha), SERPINF2 (serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2), TYMP (thymidine phosphorylase), ALPP (alkaline phosphatase, placental (Regan isozyme)), CXCR2 (chemokine (C-X-C motif) receptor 2), SLC39A3 (solute carrier family 39 (zinc transporter), member 3), ABCG2 (ATP-binding cassette, sub-family G (WHITE), member 2), ADA (adenosine deaminase), JAK3 (Janus kinase 3), HSPA1A (heat shock 70 kDa protein 1A), FASN (fatty acid synthase), FGF1 (fibroblast growth factor 1 (acidic)), F11 (coagulation factor XI), ATP7A (ATPase, Cu++ transporting, alpha polypeptide), CR1 (complement component (3b/4b) receptor 1 (Knops blood group)), GFAP (glial fibrillary acidic protein), ROCK1 (Rho-associated, coiled-coil containing protein kinase 1), MECP2 (methyl CpG binding protein 2 (Rett syndrome)), MYLK (myosin light chain kinase), BCHE (butyrylcholinesterase), LIPE (lipase, hormone-sensitive), PRDX5 (peroxiredoxin 5), ADORA1 (adenosine A1 receptor), WRN (Werner syndrome, RecQ helicase-like), CXCR3 (chemokine (C-X-C motif) receptor 3), CD81 (CD81 molecule), SMAD7 (SMAD family member 7), LAMC2 (laminin, gamma 2), MAP3K5 (mitogen-activated protein kinase kinase kinase 5), CHGA (chromogranin A (parathyroid secretory protein 1)), IAPP (islet amyloid polypeptide), RHO (rhodopsin), ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1), PTHLH (parathyroid hormone-like hormone), NRG1 (neuregulin 1), VEGFC (vascular endothelial growth factor C), ENPEP (glutamyl aminopeptidase (aminopeptidase A)), CEBPB (CCAAT/enhancer binding protein (C/EBP), beta), NAGLU (N-acetylglucosaminidase, alpha-), F2RL3 (coagulation factor II (thrombin) receptor-like 3), CX3CL1 (chemokine (C-X3-C motif) ligand 1), BDKRB1 (bradykinin receptor B1), ADAMTS13 (ADAM metallopeptidase with thrombospondin type 1 motif, 13), ELANE (elastase, neutrophil expressed), ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase 2), CISH (cytokine inducible SH2-containing protein), GAST (gastrin), MYOC (myocilin, trabecular meshwork inducible glucocorticoid response), ATP1A2 (ATPase, Na+/K+ transporting, alpha 2 polypeptide), NF1 (neurofibromin 1), GJB1 (gap junction protein, beta 1, 32 kDa), MEF2A (myocyte enhancer factor 2A), VCL (vinculin), BMPR2 (bone morphogenetic protein receptor, type II (serine/threonine kinase)), TUBB (tubulin, beta), CDC42 (cell division cycle 42 (GTP binding protein, 25 kDa)), KRT18 (keratin 18), HSF1 (heat shock transcription factor 1), MYB (v-myb myeloblastosis viral oncogene homolog (avian)), PRKAA2 (protein kinase, AMP-activated, alpha 2 catalytic subunit), ROCK2 (Rho-associated, coiled-coil containing protein kinase 2), TFPI (tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)), PRKG1 (protein kinase, cGMP-dependent, type I), BMP2 (bone morphogenetic protein 2), CTNND1 (catenin (cadherin-associated protein), delta 1), CTH (cystathionase (cystathionine gamma-lyase)), CTSS (cathepsin S), VAV2 (vav 2 guanine nucleotide exchange factor), NPY2R (neuropeptide Y receptor Y2), IGFBP2 (insulin-like growth factor binding protein 2, 36 kDa), CD28 (CD28 molecule), GSTA1 (glutathione S-transferase alpha 1), PPIA (peptidylprolyl isomerase A (cyclophilin A)), APOH (apolipoprotein H (beta-2-glycoprotein I)), S100A8 (S100 calcium binding protein A8), IL11 (interleukin 11), ALOX15 (arachidonate 15-lipoxygenase), FBLN1 (fibulin 1), NR1H3 (nuclear receptor subfamily 1, group H, member 3), SCD (stearoyl-CoA desaturase (delta-9-desaturase)), GIP (gastric inhibitory polypeptide), CHGB (chromogranin B (secretogranin 1)), PRKCB (protein kinase C, beta), SRD5A1 (steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)), HSD11B2 (hydroxysteroid (11-beta) dehydrogenase 2), CALCRL (calcitonin receptor-like), GALNT2 (UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)), ANGPTL4 (angiopoietin-like 4), KCNN4 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4), PIK3C2A (phosphoinositide-3-kinase, class 2, alpha polypeptide), HBEGF (heparin-binding EGF-like growth factor), CYP7A1 (cytochrome P450, family 7, subfamily A, polypeptide 1), HLA-DRB5 (major histocompatibility complex, class II, DR beta 5), BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3), GCKR (glucokinase (hexokinase 4) regulator), S100A12 (S100 calcium binding protein A12), PADI4 (peptidyl arginine deiminase, type IV), HSPA14 (heat shock 70 kDa protein 14), CXCR1 (chemokine (C-X-C motif) receptor 1), H19 (H19, imprinted maternally expressed transcript (non-protein coding)), KRTAP19-3 (keratin associated protein 19-3), IDDM2 (insulin-dependent diabetes mellitus 2), RAC2 (ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)), RYR1 (ryanodine receptor 1 (skeletal)), CLOCK (clock homolog (mouse)), NGFR (nerve growth factor receptor (TNFR superfamily, member 16)), DBH (dopamine beta-hydroxylase (dopamine beta-monooxygenase)), CHRNA4 (cholinergic receptor, nicotinic, alpha 4), CACNA1C (calcium channel, voltage-dependent, L type, alpha 1C subunit), PRKAG2 (protein kinase, AMP-activated, gamma 2 non-catalytic subunit), CHAT (choline acetyltransferase), PTGDS (prostaglandin D2 synthase 21 kDa (brain)), NR1H2 (nuclear receptor subfamily 1, group H, member 2), TEK (TEK tyrosine kinase, endothelial), VEGFB (vascular endothelial growth factor B), MEF2C (myocyte enhancer factor 2C), MAPKAPK2 (mitogen-activated protein kinase-activated protein kinase 2), TNFRSF11A (tumor necrosis factor receptor superfamily, member 11a, NFKB activator), HSPA9 (heat shock 70 kDa protein 9 (mortalin)), CYSLTR1 (cysteinyl leukotriene receptor 1), MAT1A (methionine adenosyltransferase I, alpha), OPRL1 (opiate receptor-like 1), IMPA1 (inositol(myo)-1(or 4)-monophosphatase 1), CLCN2 (chloride channel 2), DLD (dihydrolipoamide dehydrogenase), PSMA6 (proteasome (prosome, macropain) subunit, alpha type, 6), PSMB8 (proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)), CHI3L1 (chitinase 3-like 1 (cartilage glycoprotein-39)), ALDH1B1 (aldehyde dehydrogenase 1 family, member B1), PARP2 (poly (ADP-ribose) polymerase 2), STAR (steroidogenic acute regulatory protein), LBP (lipopolysaccharide binding protein), ABCC6 (ATP-binding cassette, sub-family C(CFTR/MRP), member 6), RGS2 (regulator of G-protein signaling 2, 24 kDa), EFNB2 (ephrin-B2), GJB6 (gap junction protein, beta 6, 30 kDa), APOA2 (apolipoprotein A-II), AMPD1 (adenosine monophosphate deaminase 1), DYSF (dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)), FDFT1 (farnesyl-diphosphate farnesyltransferase 1), EDN2 (endothelin 2), CCR6 (chemokine (C-C motif) receptor 6), GJB3 (gap junction protein, beta 3, 31 kDa), IL1RL1 (interleukin 1 receptor-like 1), ENTPD1 (ectonucleoside triphosphate diphosphohydrolase 1), BBS4 (Bardet-Biedl syndrome 4), CELSR2 (cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)), F11R (F11 receptor), RAPGEF3 (Rap guanine nucleotide exchange factor (GEF) 3), HYAL1 (hyaluronoglucosaminidase 1), ZNF259 (zinc finger protein 259), ATOX1 (ATX1 antioxidant protein 1 homolog (yeast)), ATF6 (activating transcription factor 6), KHK (ketohexokinase (fructokinase)), SAT1 (spermidine/spermine N1-acetyltransferase 1), GGH (gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)), TIMP4 (TIMP metallopeptidase inhibitor 4), SLC4A4 (solute carrier family 4, sodium bicarbonate cotransporter, member 4), PDE2A (phosphodiesterase 2A, cGMP-stimulated), PDE3B (phosphodiesterase 3B, cGMP-inhibited), FADS1 (fatty acid desaturase 1), FADS2 (fatty acid desaturase 2), TMSB4X (thymosin beta 4, X-linked), TXNIP (thioredoxin interacting protein), LIMS1 (LIM and senescent cell antigen-like domains 1), RHOB (ras homolog gene family, member B), LY96 (lymphocyte antigen 96), FOXO1 (forkhead box O1), PNPLA2 (patatin-like phospholipase domain containing 2), TRH (thyrotropin-releasing hormone), GJC1 (gap junction protein, gamma 1, 45 kDa), SLC17A5 (solute carrier family 17 (anion/sugar transporter), member 5), FTO (fat mass and obesity associated), GJD2 (gap junction protein, delta 2, 36 kDa), PSRC1 (proline/serine-rich coiled-coil 1), CASP12 (caspase 12 (gene/pseudogene)), GPBAR1 (G protein-coupled bile acid receptor 1), PXK (PX domain containing serine/threonine kinase), IL33 (interleukin 33), TRIB1 (tribbles homolog 1 (Drosophila)), PBX4 (pre-B-cell leukemia homeobox 4), NUPR1 (nuclear protein, transcriptional regulator, 1), 15-Sep(15 kDa selenoprotein), CILP2 (cartilage intermediate layer protein 2), TERC (telomerase RNA component), GGT2 (gamma-glutamyltransferase 2), MT-CO1 (mitochondrially encoded cytochrome c oxidase I), and UOX (urate oxidase, pseudogene).

[00335] In an additional embodiment, the chromosomal sequence may further be selected from Pon1 (paraoxonase 1), LDLR (LDL receptor), ApoE (Apolipoprotein E), Apo B-100 (Apolipoprotein B-100), ApoA (Apolipoprotein(a)), ApoA1 (Apolipoprotein A1), CBS (Cystathione B-synthase), Glycoprotein IIb/IIb, MTHRF (5,10-methylenetetrahydrofolate reductase (NADPH), and combinations thereof. In one iteration, the chromosomal sequences and proteins encoded by chromosomal sequences involved in cardiovascular disease may be chosen from Cacna1C, Sod1, Pten, Ppar(alpha), Apo E, Leptin, and combinations thereof.

Lungs

[00336] The present invention also contemplates delivering the CRISPR-Cas system to one or both lungs.

[00337] Although AAV-2-based vectors were originally proposed for CFTR delivery to CF airways, other serotypes such as AAV-1, AAV-5, AAV-6, and AAV-9 exhibit improved gene transfer efficiency in a variety of models of the lung epithelium (see, e.g., Li et al., Molecular Therapy, vol. 17 no. 12, 2067-2077 Dec 2009). AAV-1 was demonstrated to be ~100-fold more efficient than AAV-2 and AAV-5 at transducing human airway epithelial cells in vitro,5 although AAV-1 transduced murine tracheal airway epithelia in vivo with an efficiency equal to that of AAV-5. Other studies have shown that AAV-5 is 50-fold more efficient than AAV-2 at gene delivery to human airway epithelium (HAE) in vitro and significantly more efficient in the mouse lung airway epithelium in vivo. AAV-6 has also been shown to be more efficient than AAV-2 in human airway epithelial cells in vitro and murine airways in vivo.8 The more recent isolate, AAV-9, was shown to display greater gene transfer efficiency than AAV-5 in murine nasal and alveolar epithelia in vivo with gene expression detected for over 9 months suggesting

AAV may enable long-term gene expression in vivo, a desirable property for a CFTR gene delivery vector. Furthermore, it was demonstrated that AAV-9 could be readministered to the murine lung with no loss of CFTR expression and minimal immune consequences. CF and non-CF HAE cultures may be inoculated on the apical surface with 100 μl of AAV vectors for hours (see, e.g., Li et al., Molecular Therapy, vol. 17 no. 12, 2067-2077 Dec 2009). The MOI may vary from 1×10^3 to 4×10^5 vector genomes/cell, depending on virus concentration and purposes of the experiments. The above cited vectors are contemplated for the delivery and/or administration of the invention.

[00338] Zamora et al. (Am J Respir Crit Care Med Vol 183. pp 531–538, 2011) reported an example of the application of an RNA interference therapeutic to the treatment of human infectious disease and also a randomized trial of an antiviral drug in respiratory syncytial virus (RSV)-infected lung transplant recipients. Zamora et al. performed a randomized, double-blind, placebocontrolled trial in LTX recipients with RSV respiratory tract infection. Patients were permitted to receive standard of care for RSV. Aerosolized ALN-RSV01 (0.6 mg/kg) or placebo was administered daily for 3 days. This study demonstrates that an RNAi therapeutic targeting RSV can be safely administered to LTX recipients with RSV infection. Three daily doses of ALN-RSV01 did not result in any exacerbation of respiratory tract symptoms or impairment of lung function and did not exhibit any systemic proinflammatory effects, such as induction of cytokines or CRP. Pharmacokinetics showed only low, transient systemic exposure after inhalation, consistent with preclinical animal data showing that ALN-RSV01, administered intravenously or by inhalation, is rapidly cleared from the circulation through exonucleasemediated digestion and renal excretion. The method of Zamora et al. may be applied to the CRISPR Cas system of the present invention and an aerosolized CRISPR Cas, for example with a dosage of 0.6 mg/kg, may be contemplated for the present invention.

[00339] CFTRdelta508 chimeric guide RNA has been used for gene transfer or gene delivery of a CRISPR-Cas system in airways of subject or a patient in need thereof, suffering from cystic fibrosis or from cystic fibrosis (CF) related symptoms, using adeno-associated virus (AAV) particles. This repair strategy exemplifies use for Cystic Fibrosis delta F508 mutation. This type of strategy should apply across all organisms. With particular reference to CF, suitable patients may include: Human, non-primate human, canine, feline, bovine, equine and other domestic animals. Applicants utilized a CRISPR-Cas system comprising a Cas9 enzyme to target deltaF508 or other CFTR-inducing mutations.

[00340] The treated subjects in this instance receive pharmaceutically effective amount of aerosolized AAV vector system per lung endobronchially delivered while spontaneously breathing. As such, aerosolized delivery is preferred for AAV delivery in general. An adenovirus or an AAV particle may be used for delivery. Suitable gene constructs, each operably linked to one or more regulatory sequences, may be cloned into the delivery vector. In this instance, the following constructs are provided as examples: Cbh or EF1a promoter for Cas9, U6 or H1 promoter for chimeric guide RNA),: A preferred arrangement is to use a CFTRdelta508 targeting chimeric guide, a repair template for deltaF508 mutation and a codon optimized Cas9 enzyme (preferred Cas9s are those with nuclease or nickase activity) with optionally one or more nuclear localization signal or sequence(s) (NLS(s)), e.g., two (2) NLSs. Constructs without NLS are also envisaged.

[00341] In order to identify the Cas9 target site, Applicants analyzed the human CFTR genomic locus and identified the Cas9 target site. Preferably, in general and in this CF case, the PAM may contain a NGG or a NNAGAAW motif.

[00342] Accordingly, in the case of CF, the present method comprises manipulation of a target sequence in a genomic locus of interest comprising delivering a non-naturally occurring or engineered composition comprising a viral vector system comprising one or more viral vectors operably encoding a composition for expression thereof, wherein the composition comprises:

a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the CF target sequence in a suitable mammalian cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence. In respect of CF, preferred target DNA sequences comprise the CFTRdelta508 mutation. A preferred PAM is described above. A preferred CRISPR enzyme is any Cas. Alternatives to CF include any genetic disorder and examples of these are well known. Another preferred method or use of the invention is for correcting defects in the EMP2A and EMP2B genes that have been identified to be associated with Lafora disease.

[00343] In some embodiments, a "guide sequence" may be distinct from "guide RNA". A guide sequence may refer to an approx. 20bp sequence, within the guide RNA, that specifies the target site. In some embodiments, the Cas9 is (or is derived from) SpCas9. In such embodiments, preferred mutations are at any or all or positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 or corresponding positions in other Cas9s (which may be ascertained for instance by standard sequence comparison tools. In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. The same (or conservative substitutions of these mutations) at corresponding positions in other Cas9s are also preferred. Particularly preferred are D10 and H840 in SpCas9. However, in other Cas9s, residues corresponding to SpCas9 D10 and H840 are also preferred. These are advantageous as they provide nickase activity. Such mutations may be applied to all aspects of the present invention, not only treatment of CF. Schwank et al. (Cell Stem Cell, 13:653–58, 2013) used CRISPR/Cas9 to correct a defect associated with cystic fibrosis in human stem cells. The team's target was the gene for an ion channel, cystic fibrosis transmembrane conductor receptor (CFTR). A deletion in CFTR causes the protein to misfold in cystic fibrosis patients. Using cultured intestinal stem cells developed from cell samples from two children with cystic fibrosis, Schwank et al. were able to correct the defect using CRISPR along with a donor plasmid containing the reparative sequence to be inserted. The researchers then grew the cells into intestinal "organoids," or miniature guts, and showed that they functioned normally. In this case, about half of clonal organoids underwent the proper genetic correction.

Muscles

[00344] The present invention also contemplates delivering the CRISPR-Cas system to muscle(s).

[00345] Bortolanza et al. (Molecular Therapy vol. 19 no. 11, 2055–2064 Nov. 2011) shows that systemic delivery of RNA interference expression cassettes in the FRG1 mouse, after the onset of facioscapulohumeral muscular dystrophy (FSHD), led to a dose-dependent long-term FRG1 knockdown without signs of toxicity. Bortolanza et al. found that a single intravenous injection of 5×10^{12} vg of rAAV6-sh1FRG1 rescues muscle histopathology and muscle function of FRG1 mice. In detail, 200 μl containing 2×10^{12} or 5×10^{12} vg of vector in physiological solution were injected into the tail vein using a 25-gauge Terumo syringe. The method of Bortolanza et al. may be applied to an AAV expressing CRISPR Cas and injected into humans at a dosage of about 2×10^{15} or 2×10^{16} vg of vector.

[00346] Dumonceaux et al. (Molecular Therapy vol. 18 no. 5, 881–887 May 2010) inhibit the myostatin pathway using the technique of RNA interference directed against the myostatin receptor AcvRIIb mRNA (sh-AcvRIIb). The restoration of a quasi-dystrophin was mediated by the vectorized U7 exon-skipping technique (U7-DYS). Adeno-associated vectors carrying either the sh-AcvrIIb construct alone, the U7-DYS construct alone, or a combination of both constructs were injected in the tibialis anterior (TA) muscle of dystrophic mdx mice. The injections were performed with 10^{11} AAV viral genomes. The method of Dumonceaux et al. may be applied to an AAV expressing CRISPR Cas and injected into humans, for example, at a dosage of about 10^{14} to about 10^{15} vg of vector.

[00347] Kinouchi et al. (Gene Therapy (2008) 15, 1126–1130) report the effectiveness of in vivo siRNA delivery into skeletal muscles of normal or diseased mice through nanoparticle formation of chemically unmodified siRNAs with atelocollagen (ATCOL). ATCOL-mediated local application of siRNA targeting myostatin, a negative regulator of skeletal muscle growth, in mouse skeletal muscles or intravenously, caused a marked increase in the muscle mass within a few weeks after application. These results imply that ATCOL-mediated application of siRNAs is a powerful tool for future therapeutic use for diseases including muscular atrophy. Mst-siRNAs (final concentration, 10 mM) were mixed with ATCOL (final concentration for local administration, 0.5%) (AteloGene, Kohken, Tokyo, Japan) according to the manufacturer's instructions. After anesthesia of mice (20-week-old male C57BL/6) by Nembutal (25 mg/kg, i.p.), the Mst-siRNA/ATCOL complex was injected into the masseter and biceps femoris muscles. The method of Kinouchi et al. may be applied to CRISPR Cas and injected into a human, for example, at a dosage of about 500 to 1000 ml of a 40 μM solution into the muscle.

[00348] Hagstrom et al. (Molecular Therapy Vol. 10, No. 2, August 2004) describe an intravascular, nonviral methodology that enables efficient and repeatable delivery of nucleic acids to muscle cells (myofibers) throughout the limb muscles of mammals. The procedure involves the injection of naked plasmid DNA or siRNA into a distal vein of a limb that is transiently isolated by a tourniquet or blood pressure cuff. Nucleic acid delivery to myofibers is facilitated by its rapid injection in sufficient volume to enable extravasation of the nucleic acid solution into muscle tissue. High levels of transgene expression in skeletal muscle were achieved in both small and large animals with minimal toxicity. Evidence of siRNA delivery to limb muscle was also obtained. For plasmid DNA intravenous injection into a rhesus monkey, a threeway stopcock was connected to two syringe pumps (Model PHD 2000; Harvard Instruments), each loaded with a single syringe. Five minutes after a papaverine injection, pDNA (15.5 to 25.7 mg in 40 –100 ml saline) was injected at a rate of 1.7 or 2.0 ml/s. This could be scaled up for plasmid DNA expressing CRISPR Cas of the present invention with an injection of about 300 to 500 mg in 800 to 2000 ml saline for a human. For adenoviral vector injections into a rat, 2×10^9 infectious particles were injected in 3 ml of normal saline solution (NSS). This could be scaled up for an adenoviral vector expressing CRISPR Cas of the present invention with an injection of about 1×10^{13} infectious particles were injected in 10 liters of NSS for a human. For siRNA, a rat was injected into the great saphenous vein with 12.5 μg of a siRNA and a primate was injected injected into the great saphenous vein with 750 μg of a siRNA. This could be scaled up for a CRISPR Cas of the present invention, for example, with an injection of about 15 to about 50 mg into the great saphenous vein of a human.

Skin

[00349] The present invention also contemplates delivering the CRISPR-Cas system to the skin. Hickerson et al. (Molecular Therapy—Nucleic Acids (2013) 2, e129) relates to a motorized microneedle array skin delivery device for delivering self-delivery (sd)-siRNA to human and murine skin. The primary challenge to translating siRNA-based skin therapeutics to the clinic is the development of effective delivery systems. Substantial effort has been invested in a variety of skin delivery technologies with limited success. In a clinical study in which skin was treated with siRNA, the exquisite pain associated with the hypodermic needle injection precluded enrollment of additional patients in the trial, highlighting the need for improved, more "patient-friendly" (i.e., little or no pain) delivery approaches. Microneedles represent an efficient way to deliver large charged cargos including siRNAs across the primary barrier, the stratum corneum, and are generally regarded as less painful than conventional hypodermic needles. Motorized "stamp type" microneedle devices, including the motorized microneedle array (MMNA) device used by Hickerson et al., have been shown to be safe in hairless mice studies and cause little or no pain as evidenced by (i) widespread use in the cosmetic industry and (ii) limited testing in which nearly all volunteers found use of the device to be much less painful than a flushot, suggesting siRNA delivery using this device will result in much less pain than was experienced in the previous clinical trial using hypodermic needle injections. The MMNA device (marketed as Triple-M or Tri-M by Bomtech Electronic Co, Seoul, South Korea) was adapted for delivery of siRNA to mouse and human skin. sd-siRNA solution (up to 300 μl of 0.1 mg/ml RNA) was introduced into the chamber of the disposable Tri-M needle cartridge (Bomtech), which was set to a depth of 0.1 mm. For treating human skin, deidentified skin (obtained immediately following surgical procedures) was manually stretched and pinned to a cork platform before treatment. All intradermal injections were performed using an insulin syringe with a 28-gauge 0.5-inch needle. The MMNA device and method of Hickerson et al. could be used and/or adapted to deliver the CRISPR Cas of the present invention, for example, at a dosage of up to 300 μl of 0.1 mg/ml CRISPR Cas to the skin.

[00350] Leachman et al. (Molecular Therapy, vol. 18 no. 2, 442–446 Feb. 2010) relates to a phase Ib clinical trial for treatment of a rare skin disorder pachyonychia congenita (PC), an autosomal dominant syndrome that includes a disabling plantar keratoderma, utilizing the first short-interfering RNA (siRNA)-based therapeutic for skin. This siRNA, called TD101, specifically and potently targets the keratin 6a (K6a) N171K mutant mRNA without affecting wild-type K6a mRNA. The dose-escalation schedule is presented below:

Week	Dose no.	Days	Volume (ml)	Concentration of TD101 (mg/ml)	Total dose TD101 (mg)
1	1–2	1–7	0.1	1.0	0.10
2	3–4	8–14	0.25	1.0	0.25
3	5–6	15–21	0.50	1.0	0.50
4	7–8	22–28	1.0	1.0	1.0
5	9–10	29–35	1.5	1.0	1.5
6	11–12	36–42	2.0	1.0	2.0
7	13–14	43–49	2.0	1.5	3.0
8	15–16	50–56	2.0	2.0	4.0
9	17–18	57–63	2.0	2.5	5.0
10	19–20	64–70	2.0	3.0	6.0
11	21–22	71–77	2.0	3.5	7.0
12	23–24	78–84	2.0	4.0	8.0
13	25–26	85–91	2.0	4.5	9.0
14	27–28	92–98	2.0	5.0	10.0
15	29–30	99–105	2.0	6.0	12.0
16	31–32	106–112	2.0	7.0	14.0
17	33	113–119	2.0	8.5	17.0

[00351] Initially, 0.1 ml of a 1.0 mg/ml solution of TD101 or vehicle alone (Dulbecco's phosphate-buffered saline without calcium or magnesium) was administered to symmetric calluses. Six rising dose-volumes were completed without an adverse reaction to the increases: 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 ml of a 1.0 mg/ml solution of TD101 solution per injection. As the highest planned volume (2.0 ml) was well tolerated, the concentration of TD101 was then increased each week from 1 mg/ml up to a final concentration of 8.5 mg/ml. Similar dosages are contemplated for the administration of a CRISPR Cas that specifically and potently targets the keratin 6a (K6a) N171K mutant mRNA.

[00352] Zheng et al. (PNAS, July 24, 2012, vol. 109, no. 30, 11975–11980) show that spherical nucleic acid nanoparticle conjugates (SNA-NCs), gold cores surrounded by a dense shell of highly oriented, covalently immobilized siRNA, freely penetrate almost 100% of keratinocytes in vitro, mouse skin, and human epidermis within hours after application. Zheng et al. demonstrated that a single application of 25 nM epidermal growth factor receptor (EGFR) SNA-NCs for 60 h demonstrate effective gene knockdown in human skin. A similar dosage may be contemplated for CRISPR Cas immobilized in SNA-NCs for administration to the skin.

Nucleic acids, amino acids and proteins, Regulatory sequences, Vectors, etc

[00353] Nucleic acids, amino acids and proteins: The invention uses nucleic acids to bind target DNA sequences. This is advantageous as nucleic acids are much easier and cheaper to produce than proteins, and the specificity can be varied according to the length of the stretch where homology is sought. Complex 3-D positioning of multiple fingers, for example is not required. The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. The term also encompasses nucleic-acid-like structures with synthetic backbones, see, e.g., Eckstein, 1991; Baserga et al., 1992; Milligan, 1993; WO 97/03211; WO 96/39154; Mata, 1997; Strauss-Soukup, 1997; and Samstag, 1996. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. As used herein the term "wild type" is a term of the art understood by skilled persons and means the typical form of an organism, strain, gene or characteristic as it occurs in nature as distinguished from mutant or variant forms. A "wild type" can be a base line. As used herein the term "variant" should be taken to mean the exhibition of qualities that have a pattern that deviates from what occurs in nature. The terms "non-naturally occurring" or "engineered" are used interchangeably and indicate the involvement of the hand of man. The terms, when referring to nucleic acid molecules or polypeptides mean that the nucleic acid molecule or the polypeptide is at least substantially free from at least one other component with which they are naturally associated in nature and as found in nature. "Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick base pairing or other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100%

complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. "Substantially complementary" as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions. As used herein, "stringent conditions" for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent, and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), Laboratory Techniques In Biochemistry And Molecular Biology-Hybridization With Nucleic Acid Probes Part I, Second Chapter "Overview of principles of hybridization and the strategy of nucleic acid probe assay", Elsevier, N.Y. Where reference is made to a polynucleotide sequence, then complementary or partially complementary sequences are also envisaged. These are preferably capable of hybridising to the reference sequence under highly stringent conditions. Generally, in order to maximize the hybridization rate, relatively low-stringency hybridization conditions are selected: about 20 to 25° C lower than the thermal melting point (T_m). The T_m is the temperature at which 50% of specific target sequence hybridizes to a perfectly complementary probe in solution at a defined ionic strength and pH. Generally, in order to require at least about 85% nucleotide complementarity of hybridized sequences, highly stringent washing conditions are selected to be about 5 to 15° C lower than the T_m . In order to require at least about 70% nucleotide complementarity of hybridized sequences, moderately-stringent washing conditions are selected to be about 15 to 30° C lower than the T_m . Highly permissive (very low stringency) washing conditions may be as low as 50° C below the T_m , allowing a high level of mis-matching between hybridized sequences. Those skilled in the art will recognize that other physical and chemical parameters in the hybridization and wash stages can also be altered to affect the outcome of a detectable hybridization signal from a specific level of homology between target and probe sequences. Preferred highly stringent conditions comprise incubation in 50% formamide, 5×SSC, and 1% SDS at 42° C, or incubation in 5×SSC and 1% SDS at 65° C, with wash in 0.2×SSC and 0.1% SDS at 65° C. "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the "complement" of the given sequence. As used herein, the term "genomic locus" or "locus" (plural loci) is the specific location of a gene or DNA sequence on a chromosome. A "gene" refers to stretches of DNA or RNA that encode a polypeptide or an RNA chain that has functional role to play in an organism and hence is the molecular unit of heredity in living organisms. For the purpose of this invention it may be considered that genes include 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. As used herein, "expression of a genomic locus" or "gene expression" is the process by which information from a gene is used in the synthesis of a functional gene product. The products of gene expression are often proteins, but in non-protein coding genes such as rRNA genes or tRNA genes, the product is functional RNA. The process of gene expression is used by all known life - eukaryotes (including multicellular organisms), prokaryotes (bacteria and archaea) and viruses to generate functional products to survive. As used herein "expression" of a gene or nucleic acid encompasses not only cellular gene expression, but also the transcription and translation of nucleic acid(s) in cloning systems and in any other context. As used herein, "expression" also refers to the process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as "gene product." If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell. The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term "amino acid" includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. As used herein, the term "domain" or "protein domain" refers to a part of a protein sequence that may exist and function independently of the rest of the protein chain. As described in aspects of the invention, sequence identity is related to sequence homology. Homology comparisons may be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs may calculate percent (%) homology between two or more sequences and may also calculate the sequence identity shared by two or more amino acid or nucleic acid sequences. In some preferred embodiments, the capping region of the dTALEs described herein have sequences that are at least 95% identical or share identity to the capping region amino acid sequences provided herein. Sequence homologies may be generated by any of a number of computer programs known in the art, for example BLAST or FASTA, etc. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984, Nucleic Acids Research 12:387). Examples of other software than may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 ibid – Chapter 18), FASTA (Atschul et al., 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999 ibid, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program. Percentage (%) sequence homology may be calculated over contiguous sequences, i.e., one sequence is aligned with the other sequence and each amino acid or nucleotide in one sequence is directly compared with the corresponding amino acid or nucleotide in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues. Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion may cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without unduly penalizing the overall homology or identity score. This is achieved by inserting "gaps" in the sequence alignment to try to maximize local homology or identity. However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - may achieve a higher score than one with many gaps. "Affinity gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties may, of course, produce optimized alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example, when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension. Calculation of maximum % homology therefore first requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux et al., 1984 *Nuc. Acids Research* 12 p387). Examples of other software than may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 *Short Protocols in Molecular Biology*, 4th Ed. – Chapter 18), FASTA (Altschul et al., 1990 *J. Mol. Biol.* 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999, *Short Protocols in Molecular Biology*, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequences (see *FEMS Microbiol Lett.* 1999 174(2): 247-50; *FEMS Microbiol Lett.* 1999 177(1): 187-8 and the website of the National Center for Biotechnology information at the website of the National Institutes for Health). Although the final % homology may be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pair-wise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table, if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62. Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244). Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result. The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in amino acid properties (such as polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues) and it is therefore useful to group amino acids together in functional groups. Amino acids may be grouped together based on the properties of their side chains alone. However, it is more useful to include mutation data as well. The sets of amino acids thus derived are likely to be conserved for structural reasons. These sets may be described in the form of a Venn diagram (Livingstone C.D. and Barton G.J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" *Comput. Appl. Biosci.* 9: 745-756) (Taylor W.R. (1986) "The classification of amino acid conservation" *J. Theor. Biol.* 119; 205-218). Conservative substitutions may be made, for example according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

Set		Sub-set	
Hydrophobic	F W Y H K M I L V A G C	Aromatic	F W Y H
		Aliphatic	I L V
Polar	W Y H K R E D C S T N Q	Charged	H K R E D
		Positively charged	H K R
		Negatively charged	E D
Small	V C A G S P T N D	Tiny	A G S

[00354] Embodiments of the invention include sequences (both polynucleotide or polypeptide) which may comprise homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue or nucleotide, with an alternative residue or nucleotide) that may occur i.e., like-for-like substitution in the case of amino acids such as basic for basic, acidic for acidic, polar for polar, etc. Non-homologous substitution may also occur i.e., from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine. Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, which involves the presence of one or more amino acid residues in peptoid form, may be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., *PNAS* (1992) 89(20), 9367-9371 and Horwell DC, *Trends Biotechnol.* (1995) 13(4), 132-134.

[00355] For purpose of this invention, amplification means any method employing a primer and a polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA polymerases such as

TaqGold™, T7 DNA polymerase, Klenow fragment of E.coli DNA polymerase, and reverse transcriptase. A preferred amplification method is PCR.

[00356] In certain aspects the invention involves vectors. A used herein, a "vector" is a tool that allows or facilitates the transfer of an entity from one environment to another. It is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a vector is capable of replication when associated with the proper control elements. In general, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses (AAVs)). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as "expression vectors." Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[00357] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). With regards to recombination and cloning methods, mention is made of U.S. patent application 10/815,730, published September 2, 2004 as US 2004-0171156 A1, the contents of which are herein incorporated by reference in their entirety.

[00358] Aspects of the invention relate to bicistronic vectors for chimeric RNA and Cas9. Bicistronic expression vectors for chimeric RNA and Cas9 are preferred. In general and particularly in this embodiment Cas9 is preferably driven by the CBh promoter. The chimeric RNA may preferably be driven by a Pol III promoter, such as a U6 promoter. Ideally the two are combined. The chimeric guide RNA typically consists of a 20bp guide sequence (Ns) and this may be joined to the tracr sequence (running from the first "U" of the lower strand to the end of the transcript). The tracr sequence may be truncated at various positions as indicated. The guide and tracr sequences are separated by the tracr-mate sequence, which may be GUUUUAGAGCUA. This may be followed by the loop sequence GAAA as shown. Both of these are preferred examples. Applicants have demonstrated Cas9-mediated indels at the human *EMX1* and *PVALB* loci by SURVEYOR assays. ChiRNAs are indicated by their "+n" designation, and crRNA refers to a hybrid RNA where guide and tracr sequences are expressed as separate transcripts. Throughout this application, chimeric RNA may also be called single guide, or synthetic guide RNA (sgRNA). The loop is preferably GAAA, but it is not limited to this sequence or indeed to being only 4bp in length. Indeed, preferred loop forming sequences for use in hairpin structures are four nucleotides in length, and most preferably have the sequence GAAA. However, longer or shorter loop sequences may be used, as may alternative sequences. The sequences preferably include a nucleotide triplet (for example, AAA), and an additional nucleotide (for example C or G). Examples of loop forming sequences include CAAA and AAAG. In practicing any of the methods disclosed herein, a suitable vector can be introduced to a cell or an embryo via one or more methods known in the art, including without limitation, microinjection, electroporation, sonoporation, biolistics, calcium phosphate-mediated transfection, cationic transfection, liposome transfection, dendrimer transfection, heat shock transfection, nucleofection transfection, magnetofection, lipofection, impalefection, optical transfection, proprietary agent-enhanced uptake of nucleic acids, and delivery via liposomes, immunoliposomes, virosomes, or artificial virions. In some methods, the vector is introduced into an embryo by microinjection. The vector or vectors may be microinjected into the nucleus or the cytoplasm of the embryo. In some methods, the vector or vectors may be introduced into a cell by nucleofection.

[00359] The term "regulatory element" is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g. transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g. liver, pancreas), or particular cell types (e.g. lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector comprises one or more pol III promoter (e.g. 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (e.g. 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g. 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter. Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit β-globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., clustered regularly interspersed short palindromic repeats (CRISPR) transcripts, proteins, enzymes, mutant forms thereof, fusion proteins thereof, etc.). With regards to regulatory sequences, mention is made of U.S. patent application 10/491,026, the contents of which are incorporated by reference herein in their entirety. With regards to promoters, mention is made of PCT publication WO 2011/028929 and U.S. application 12/511,940, the contents of which are incorporated by reference herein in their entirety.

[00360] Vectors can be designed for expression of CRISPR transcripts (e.g. nucleic acid transcripts, proteins, or enzymes) in prokaryotic or eukaryotic cells. For example, CRISPR transcripts can be expressed in bacterial cells such as Escherichia coli, insect cells (using baculovirus expression vectors), yeast cells, or mammalian cells. Suitable host cells are discussed further in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

[00361] Vectors may be introduced and propagated in a prokaryote or prokaryotic cell. In some embodiments, a prokaryote is used to amplify copies of a vector to be introduced into a eukaryotic cell or as an intermediate vector in the production of a vector to be introduced into a eukaryotic cell (e.g. amplifying a plasmid as part of a viral vector packaging system). In some embodiments, a prokaryote is used to amplify copies of a vector and express one or more nucleic acids, such as to provide a source of one or more proteins for delivery to a host cell or host organism. Expression of proteins in prokaryotes is most often carried out in Escherichia coli with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, such as to the amino terminus of the recombinant protein. Such fusion vectors may serve one or more purposes, such as: (i) to increase expression of recombinant protein; (ii) to increase the solubility of the recombinant protein; and (iii) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Example fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson, 1988. *Gene* 67: 31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) that fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Examples of suitable inducible non-fusion E. coli expression vectors include pTrc (Amrann et al., (1988) Gene 69:301-315) and pET 11d (Studier et al., GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990) 60-89). In some embodiments, a vector is a yeast expression vector. Examples of vectors for expression in yeast Saccharomyces cerivisae include pYepSec1 (Baldari, et al., 1987. EMBO J. 6: 229-234), pMFa (Kuijan and Herskowitz, 1982. Cell 30: 933-943), pJRY88 (Schultz et al., 1987. Gene 54: 113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and picZ (InVitrogen Corp, San Diego, Calif.). In some embodiments, a vector drives protein expression in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., SF9 cells) include the pAc series (Smith, et al., 1983. Mol. Cell. Biol. 3: 2156-2165) and the pVL series (Lucklow and Summers, 1989. Virology 170: 31-39).

[00362] In some embodiments, a vector is capable of driving expression of one or more sequences in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, 1987. Nature 329: 840) and pMT2PC (Kaufman, et al., 1987. EMBO J. 6: 187-195). When used in mammalian cells, the expression vector's control functions are typically provided by one or more regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, simian virus 40, and others disclosed herein and known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells see, e.g., Chapters 16 and 17 of Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

[00363] In some embodiments, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, et al., 1987. *Genes Dev.* 1: 268-277), lymphoid-specific promoters (Calame and Eaton, 1988. *Adv. Immunol.* 43: 235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989. *EMBO J.* 8: 729-733) and immunoglobulins (Baneiji, et al., 1983. *Cell* 33: 729-740; Queen and Baltimore, 1983. *Cell* 33: 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle, 1989. *Proc. Natl. Acad. Sci. USA* 86: 5473-5477), pancreas-specific promoters (Edlund, et al., 1985. *Science* 230: 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, e.g., the murine hox promoters (Kessel and Gruss, 1990. *Science* 249: 374-379) and the α-fetoprotein promoter (Campes and Tilghman, 1989. *Genes Dev.* 3: 537-546). With regards to these prokaryotic and eukaryotic vectors, mention is made of U.S. Patent 6,750,059, the contents of which are incorporated by reference herein in their entirety. Other embodiments of the invention may relate to the use of viral vectors, with regards to which mention is made of U.S. Patent application 13/092,085, the contents of which are incorporated by reference herein in their entirety. Tissue-specific regulatory elements are known in the art and in this regard, mention is made of U.S. Patent 7,776,321, the contents of which are incorporated by reference herein in their entirety. In some embodiments, a regulatory element is operably linked to one or more elements of a CRISPR system so as to drive expression of the one or more elements of the CRISPR system. In general, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats), also known as SPIDRs (SPacer Interspersed Direct Repeats), constitute a family of DNA loci that are usually specific to a particular bacterial species. The CRISPR locus comprises a distinct class of interspersed short sequence repeats (SSRs) that were recognized in E. coli (Ishino et al., J. Bacteriol., 169:5429-5433 [1987]; and Nakata et al., J. Bacteriol., 171:3553-3556 [1989]), and associated genes. Similar interspersed SSRs have been identified in Haloferax mediterranei, Streptococcus pyogenes, Anabaena, and Mycobacterium tuberculosis (See, Groenen et al., Mol. Microbiol., 10:1057-1065 [1993]; Hoe et al., Emerg. Infect. Dis., 5:254-263 [1999]; Masepohl et al., Biochim. Biophys. Acta 1307:26-30 [1996]; and Mojica et al., Mol. Microbiol., 17:85-93 [1995]). The CRISPR loci typically differ from other SSRs by the structure of the repeats, which have been termed short regularly spaced repeats (SRSRs) (Janssen et al., OMICS J. Integ. Biol., 6:23-33 [2002]; and Mojica et al., Mol. Microbiol., 36:244-246 [2000]). In general, the repeats are short elements that occur in clusters that are regularly spaced by unique intervening sequences with a substantially constant length (Mojica et al., [2000], supra). Although the repeat sequences are highly conserved between strains, the number of interspersed repeats and the sequences of the spacer regions typically differ from strain to strain (van Embden et al., J. Bacteriol., 182:2393-2401 [2000]). CRISPR loci have been identified in more than 40 prokaryotes (See e.g., Jansen et al., Mol. Microbiol., 43:1565-1575 [2002]; and Mojica et al., [2005]) including, but not limited to Aeropyrum, Pyrobaculum, Sulfolobus, Archaeoglobus, Halocarcula, Methanobacterium, Methanococcus, Methanosarcina, Methanopyrus, Pyrococcus, Picrophilus, Thermoplasma, Corynebacterium, Mycobacterium, Streptomyces, Aquifex, Porphyromonas, Chlorobium, Thermus, Bacillus, Listeria, Staphylococcus, Clostridium, Thermoanaerobacter, Mycoplasma, Fusobacterium, Azarcus, Chromobacterium, Neisseria, Nitrosomonas, Desulfovibrio, Geobacter, Myxococcus, Campylobacter, Wolinella, Acinetobacter, Erwinia, Escherichia, Legionella, Methylococcus, Pasteurella, Photobacterium, Salmonella, Xanthomonas, Yersinia, Treponema, and Thermotoga.

[00364] In some embodiments, the CRISPR enzyme is part of a fusion protein comprising one or more heterologous protein domains (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more domains in addition to the CRISPR enzyme). A CRISPR enzyme fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains. Examples of protein domains that may be fused to a CRISPR enzyme include, without limitation, epitope tags, reporter gene sequences, and protein domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporter genes include, but are not limited to, glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta-galactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and autofluorescent proteins including blue fluorescent protein (BFP). A CRISPR enzyme may be fused to a gene sequence encoding a protein or a fragment of a protein that bind DNA molecules or bind other cellular molecules, including but not limited to maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. Additional domains that may form part of a fusion protein comprising a CRISPR enzyme are described in

US20110059502, incorporated herein by reference. In some embodiments, a tagged CRISPR enzyme is used to identify the location of a target sequence.

[00365] In some embodiments, a CRISPR enzyme may form a component of an inducible system. The inducible nature of the system would allow for spatiotemporal control of gene editing or gene expression using a form of energy. The form of energy may include but is not limited to electromagnetic radiation, sound energy, chemical energy and thermal energy. Examples of inducible system include tetracycline inducible promoters (Tet-On or Tet-Off), small molecule two-hybrid transcription activations systems (FKBP, ABA, etc), or light inducible systems (Phytochrome, LOV domains, or cryptochrome).In one embodiment, the CRISPR enzyme may be a part of a Light Inducible Transcriptional Effector (LITE) to direct changes in transcriptional activity in a sequence-specific manner. The components of a light may include a CRISPR enzyme, a light-responsive cytochrome heterodimer (e.g. from Arabidopsis thaliana), and a transcriptional activation/repression domain. Further examples of inducible DNA binding proteins and methods for their use are provided in US 61/736465 and US 61/721,283, which is hereby incorporated by reference in its entirety.

[00366] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

Recombination template (e.g., HDR template)

[00367] In some embodiments, a recombination template is also provided. A recombination template may be a component of another vector as described herein, contained in a separate vector, or provided as a separate polynucleotide. In some embodiments, a recombination template is designed to serve as a template in homologous recombination, such as within or near a target sequence nicked or cleaved by a CRISPR enzyme as a part of a CRISPR complex. A template polynucleotide may be of any suitable length, such as about or more than about 10, 15, 20, 25, 50, 75, 100, 150, 200, 500, 1000, or more nucleotides in length. In some embodiments, the template polynucleotide is complementary to a portion of a polynucleotide comprising the target sequence. When optimally aligned, a template polynucleotide might overlap with one or more nucleotides of a target sequences (e.g. about or more than about 1, 5, 10, 15, 20, or more nucleotides). In some embodiments, when a template sequence and a polynucleotide comprising a target sequence are optimally aligned, the nearest nucleotide of the template polynucleotide is within about 1, 5, 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, 5000, 10000, or more nucleotides from the target sequence.

Modifying a target

[00368] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell, which may be *in vivo, ex vivo* or *in vitro*. In some embodiments, the method comprises sampling a cell or population of cells from a human or non-human animal, or a plant, and modifying the cell or cells. Culturing may occur at any stage *ex vivo*. The cell or cells may even be re-introduced into the non-human animal or plant. For re-introduced cells it is particularly preferred that the cells are stem cells.

[00369] In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[00370] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. Similar considerations and conditions apply as above for methods of modifying a target polynucleotide. In fact, these sampling, culturing and re-introduction options apply across the aspects of the present invention.

[00371] Indeed, in any aspect of the invention, the CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence, wherein said guide sequence may be linked to a tracr mate sequence which in turn may hybridize to a tracr sequence. Similar considerations and conditions apply as above for methods of modifying a target polynucleotide.

Kits

[00372] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. Elements may be provided individually or in combinations, and may be provided in any suitable container, such as a vial, a bottle, or a tube. In some embodiments, the kit includes instructions in one or more languages, for example in more than one language.

[00373] In some embodiments, a kit comprises one or more reagents for use in a process utilizing one or more of the elements described herein. Reagents may be provided in any suitable container. For example, a kit may provide one or more reaction or storage buffers. Reagents may be provided in a form that is usable in a particular assay, or in a form that requires addition of one or more other components before use (e.g. in concentrate or lyophilized form). A buffer can be any buffer, including but not limited to a sodium carbonate buffer, a sodium bicarbonate buffer, a borate buffer, a Tris buffer, a MOPS buffer, a HEPES buffer, and combinations thereof. In some embodiments, the buffer is alkaline. In some embodiments, the buffer has a pH from about 7 to about 10. In some embodiments, the kit comprises one or more oligonucleotides corresponding to a guide sequence for insertion into a vector so as to operably link the guide sequence and a regulatory element. In some embodiments, the kit comprises a homologous recombination template polynucleotide. In some embodiments, the kit comprises one or more of the vectors and/or one or more of the polynucleotides described herein. The kit may advantageously allows to provide all elements of the systems of the invention.

Disease-associated genes and polynucleotides

[00374] Examples of disease-associated genes and polynucleotides that can be targeted in the practice of the invention are listed in Tables A and B. Disease specific information is available from McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web. Examples of signaling biochemical pathway-associated genes and polynucleotides are listed in Table C. Mutations in these genes and pathways can result in production of improper proteins or proteins in improper amounts which affect function. Further examples of genes, diseases and proteins are hereby incorporated by reference from US Provisional application 61/736,527 filed December 12, 2012. Such genes, proteins and pathways may be the target polynucleotide of a CRISPR complex.

Table A

DISEASE/DISORDERS	GENE(S)
Neoplasia	PTEN; ATM; ATR; EGFR; ERBB2; ERBB3; ERBB4;
	Notch1; Notch2; Notch3; Notch4; AKT; AKT2; AKT3; HIF;
	HIF1a; HIF3a; Met; HRG; Bcl2; PPAR alpha; PPAR
	gamma; WT1 (Wilms Tumor); FGF Receptor Family
	members (5 members: 1, 2, 3, 4, 5); CDKN2a; APC; RB
	(retinoblastoma); MEN1; VHL; BRCA1; BRCA2; AR
	(Androgen Receptor); TSG101; IGF; IGF Receptor; Igf1 (4
	variants); Igf2 (3 variants); Igf 1 Receptor; Igf 2 Receptor;
	Bax; Bcl2; caspases family (9 members:
	1, 2, 3, 4, 6, 7, 8, 9, 12); Kras; Apc
Age-related Macular	Abcr; Ccl2; Cc2; cp (ceruloplasmin); Timp3; cathepsinD;
Degeneration	Vldlr; Ccr2
Schizophrenia	Neuregulin1 (Nrg1); Erb4 (receptor for Neuregulin);
	Complexin1 (Cplx1); Tph1 Tryptophan hydroxylase; Tph2
	Tryptophan hydroxylase 2; Neurexin 1; GSK3; GSK3a;
	GSK3b
Disorders	5-HTT (Slc6a4); COMT; DRD (Drd1a); SLC6A3; DAOA;
	DTNBP1; Dao (Dao1)
Trinucleotide Repeat	HTT (Huntington's Dx); SBMA/SMAX1/AR (Kennedy's
Disorders	Dx); FXN/X25 (Friedrich's Ataxia); ATX3 (Machado-
	Joseph's Dx); ATXN1 and ATXN2 (spinocerebellar
	ataxias); DMPK (myotonic dystrophy); Atrophin-1 and Atn1
	(DRPLA Dx); CBP (Creb-BP - global instability); VLDLR
	(Alzheimer's); Atxn7; Atxn10
Fragile X Syndrome	FMR2; FXR1; FXR2; mGLUR5
Secretase Related	APH-1 (alpha and beta); Presenilin (Psen1); nicastrin
Disorders	(Ncstn); PEN-2
Others	Nos1; Parp1; Nat1; Nat2
Prion - related disorders	Prp
ALS	SOD1; ALS2; STEX; FUS; TARDBP; VEGF (VEGF-a;
	VEGF-b; VEGF-c)
Drug addiction	Prkce (alcohol); Drd2; Drd4; ABAT (alcohol); GRIA2;
	Grm5; Grin1; Htr1b; Grin2a; Drd3; Pdyn; Gria1 (alcohol)
Autism	Mecp2; BZRAP1; MDGA2; Sema5A; Neurexin 1; Fragile X

DISEASE/DISORDERS	GENE(S)
	(FMR2 (AFF2); FXR1; FXR2; Mglur5)
Alzheimer's Disease	E1; CHIP; UCH; UBB; Tau; LRP; PICALM; Clusterin; PS1;
	SORL1; CR1; Vldlr; Uba1; Uba3; CHIP28 (Aqp1,
	Aquaporin 1); Uchl1; Uchl3; APP
Inflammation	IL-10; IL-1 (IL-1a; IL-1b); IL-13; IL-17 (IL-17a (CTLA8); IL-
	17b; IL-17c; IL-17d; IL-17f); II-23; Cx3cr1; ptpn22; TNFa;
	NOD2/CARD15 for IBD; IL-6; IL-12 (IL-12a; IL-12b);
	CTLA4; Cx3cl1
Parkinson's Disease	x-Synuclein; DJ-1; LRRK2; Parkin; PINK1

Table B:

Blood and coagulation diseases and disorders	Anemia (CDAN1, CDA1, RPS19, DBA, PKLR, PK1, NT5C3, UMPH1, PSN1, RHAG, RH50A, NRAMP2, SPTB, ALAS2, ANH1, ASB, ABCB7, ABC7, ASAT); Bare lymphocyte syndrome (TAPBP, TPSN, TAP2, ABCB3, PSF2, RING11, MHC2TA, C2TA, RFX5, RFXAP, RFX5), Bleeding disorders (TBXA2R, P2RX1, P2X1); Factor H and factor H-like 1 (HF1, CFH, HUS); Factor V and factor VIII (MCFD2); Factor VII deficiency (F7); Factor X deficiency (F10); Factor XI deficiency (F11); Factor XII deficiency (F12, HAF); Factor XIIIA deficiency (F13A1, F13A); Factor XIIIB deficiency (F13B); Fanconi anemia (FANCA, FACA, FA1, FA, FAA, FAAP95, FAAP90, FLJ34064, FANCB, FANCC, FACC, BRCA2, FANCD1, FANCD2, FANCD, FACD, FAD, FANCE, FACE, FANCF, XRCC9, FANCG, BRIP1, BACH1, FANCJ, PHF9, FANCL, FANCM, KIAA1596); Hemophagocytic lymphohistiocytosis disorders (PRF1, HPLH2, UNC13D, MUNC13-4, HPLH3, HLH3, FHL3); Hemophilia A (F8, F8C, HEMA); Hemophilia B (F9, HEMB), Hemorrhagic disorders (PI, ATT, F5); Leukocyde deficiencies and disorders (ITGB2, CD18, LCAMB, LAD, EIF2B1, EIF2BA, EIF2B2, EIF2B3, EIF2B5, LVWM, CACH, CLE, EIF2B4); Sickle cell anemia (HBB); Thalassemia (HBA2, HBB, HBD, LCRB, HBA1).
Cell dysregulation and oncology diseases and disorders	B-cell non-Hodgkin lymphoma (BCL7A, BCL7); Leukemia (TAL1, TCL5, SCL, TAL2, FLT3, NBS1, NBS, ZNFN1A1, IK1, LYF1, HOXD4, HOX4B, BCR, CML, PHL, ALL, ARNT, KRAS2, RASK2, GMPS, AF10, ARHGEF12, LARG, KIAA0382, CALM, CLTH, CEBPA, CEBP, CHIC2, BTL, FLT3, KIT, PBT, LPP, NPM1, NUP214, D9S46E, CAN, CAIN, RUNX1, CBFA2, AML1, WHSC1L1, NSD3, FLT3, AF1Q, NPM1, NUMA1, ZNF145, PLZF, PML, MYL, STAT5B, AF10, CALM, CLTH, ARL11, ARLTS1, P2RX7, P2X7, BCR, CML, PHL, ALL, GRAF, NF1, VRNF, WSS, NFNS, PTPN11, PTP2C,

	SHP2, NS1, BCL2, CCND1, PRAD1, BCL1, TCRA, GATA1, GF1, ERYF1, NFE1, ABL1, NQO1, DIA4, NMOR1, NUP214, D9S46E, CAN, CAIN).
Inflammation and immune related diseases and disorders	AIDS (KIR3DL1, NKAT3, NKB1, AMB11, KIR3DS1, IFNG, CXCL12, SDF1); Autoimmune lymphoproliferative syndrome (TNFRSF6, APT1, FAS, CD95, ALPS1A); Combined immunodeficiency, (IL2RG, SCIDX1, SCIDX, IMD4); HIV-1 (CCL5, SCYA5, D17S136E, TCP228), HIV susceptibility or infection (IL10, CSIF, CMKBR2, CCR2, CMKBR5, CCCKR5 (CCR5)); Immunodeficiencies (CD3E, CD3G, AICDA, AID, HIGM2, TNFRSF5, CD40, UNG, DGU, HIGM4, TNFSF5, CD40LG, HIGM1, IGM, FOXP3, IPEX, AIID, XPID, PIDX, TNFRSF14B, TACI); Inflammation (IL-10, IL-1 (IL-1a, IL-1b), IL-13, IL-17 (IL-17a (CTLA8), IL-17b, IL-17c, IL-17d, IL-17f), II-23, Cx3cr1, ptpn22, TNFa, NOD2/CARD15 for IBD, IL-6, IL-12 (IL-12a, IL-12b), CTLA4, Cx3cl1); Severe combined immunodeficiencies (SCIDs)(JAK3, JAKL, DCLRE1C, ARTEMIS, SCIDA, RAG1, RAG2, ADA, PTPRC, CD45, LCA, IL7R, CD3D, T3D, IL2RG, SCIDX1, SCIDX, IMD4).
Metabolic, liver, kidney and protein diseases and disorders	Amyloid neuropathy (TTR, PALB); Amyloidosis (APOA1, APP, AAA, CVAP, AD1, GSN, FGA, LYZ, TTR, PALB); Cirrhosis (KRT18, KRT8, CIRH1A, NAIC, TEX292, KIAA1988); Cystic fibrosis (CFTR, ABCC7, CF, MRP7); Glycogen storage diseases (SLC2A2, GLUT2, G6PC, G6PT, G6PT1, GAA, LAMP2, LAMPB, AGL, GDE, GBE1, GYS2, PYGL, PFKM); Hepatic adenoma, 142330 (TCF1, HNF1A, MODY3), Hepatic failure, early onset, and neurologic disorder (SCOD1, SCO1), Hepatic lipase deficiency (LIPC), Hepatoblastoma, cancer and carcinomas (CTNNB1, PDGFRL, PDGRL, PRLTS, AXIN1, AXIN, CTNNB1, TP53, P53, LFS1, IGF2R, MPRI, MET, CASP8, MCH5; Medullary cystic kidney disease (UMOD, HNFJ, FJHN, MCKD2, ADMCKD2); Phenylketonuria (PAH, PKU1, QDPR, DHPR, PTS); Polycystic kidney and hepatic disease (FCYT, PKHD1, ARPKD, PKD1, PKD2, PKD4, PKDTS, PRKCSH, G19P1, PCLD, SEC63).
Muscular / Skeletal diseases and disorders	Becker muscular dystrophy (DMD, BMD, MYF6), Duchenne Muscular Dystrophy (DMD, BMD); Emery-Dreifuss muscular dystrophy (LMNA, LMN1, EMD2, FPLD, CMD1A, HGPS, LGMD1B, LMNA, LMN1, EMD2, FPLD, CMD1A); Facioscapulohumeral muscular dystrophy (FSHMD1A, FSHD1A); Muscular dystrophy (FKRP, MDC1C, LGMD2I, LAMA2, LAMM, LARGE, KIAA0609, MDC1D, FCMD, TTID, MYOT, CAPN3, CANP3, DYSF, LGMD2B, SGCG, LGMD2C, DMDA1, SCG3, SGCA, ADL, DAG2, LGMD2D, DMDA2,

	SGCB, LGMD2E, SGCD, SGD, LGMD2F, CMD1L, TCAP, LGMD2G, CMD1N, TRIM32, HT2A, LGMD2H, FKRP, MDC1C, LGMD2I, TTN, CMD1G, TMD, LGMD2J, POMT1, CAV3, LGMD1C, SEPN1, SELN, RSMD1, PLEC1, PLTN, EBS1); Osteopetrosis (LRP5, BMND1, LRP7, LR3, OPPG, VBCH2, CLCN7, CLC7, OPTA2, OSTM1, GL, TCIRG1, TIRC7, OC116, OPTB1); Muscular atrophy (VAPB, VAPC, ALS8, SMN1, SMA1, SMA2, SMA3, SMA4, BSCL2, SPG17, GARS, SMAD1, CMT2D, HEXB, IGHMBP2, SMUBP2, CATF1, SMARD1).
Neurological and neuronal diseases and disorders	ALS (SOD1, ALS2, STEX, FUS, TARDBP, VEGF (VEGF-a, VEGF-b, VEGF-c); Alzheimer disease (APP, AAA, CVAP, AD1, APOE, AD2, PSEN2, AD4, STM2, APBB2, FE65L1, NOS3, PLAU, URK, ACE, DCP1, ACE1, MPO, PACIP1, PAXIP1L, PTIP, A2M, BLMH, BMH, PSEN1, AD3); Autism (Mecp2, BZRAP1, MDGA2, Sema5A, Neurexin 1, GLO1, MECP2, RTT, PPMX, MRX16, MRX79, NLGN3, NLGN4, KIAA1260, AUTSX2); Fragile X Syndrome (FMR2, FXR1, FXR2, mGLUR5); Huntington's disease and disease like disorders (HD, IT15, PRNP, PRIP, JPH3, JP3, HDL2, TBP, SCA17); Parkinson disease (NR4A2, NURR1, NOT, TINUR, SNCAIP, TBP, SCA17, SNCA, NACP, PARK1, PARK4, DJ1, PARK7, LRRK2, PARK8, PINK1, PARK6, UCHL1, PARK5, SNCA, NACP, PARK1, PARK4, PRKN, PARK2, PDJ, DBH, NDUFV2); Rett syndrome (MECP2, RTT, PPMX, MRX16, MRX79, CDKL5, STK9, MECP2, RTT, PPMX, MRX16, MRX79, x-Synuclein, DJ-1); Schizophrenia (Neuregulin1 (Nrg1), Erb4 (receptor for Neuregulin), Complexin1 (Cplx1), Tph1 Tryptophan hydroxylase, Tph2, Tryptophan hydroxylase 2, Neurexin 1, GSK3, GSK3a, GSK3b, 5-HTT (Slc6a4), COMT, DRD (Drd1a), SLC6A3, DAOA, DTNBP1, Dao (Dao1)); Secretase Related Disorders (APH-1 (alpha and beta), Presenilin (Psen1), nicastrin, (Ncstn), PEN-2, Nos1, Parp1, Nat1, Nat2); Trinucleotide Repeat Disorders (HTT (Huntington's Dx), SBMA/SMAX1/AR (Kennedy's Dx), FXN/X25 (Friedrich's Ataxia), ATX3 (Machado- Joseph's Dx), ATXN1 and ATXN2 (spinocerebellar ataxias), DMPK (myotonic dystrophy), Atrophin-1 and Atn1 (DRPLA Dx), CBP (Creb-BP - global instability), VLDLR (Alzheimer's), Atxn7, Atxn10).
Occular diseases and disorders	Age-related macular degeneration (Abcr, Ccl2, Cc2, cp (ceruloplasmin), Timp3, cathepsinD, Vldlr, Ccr2); Cataract (CRYAA, CRYA1, CRYBB2, CRYB2, PITX3, BFSP2, CP49, CP47, CRYAA, CRYA1, PAX6, AN2, MGDA, CRYBA1, CRYB1, CRYGC, CRYG3, CCL, LIM2, MP19, CRYGD, CRYG4, BFSP2, CP49, CP47, HSF4, CTM, HSF4, CTM, MIP,

	AQP0, CRYAB, CRYA2, CTPP2, CRYBB1, CRYGD, CRYG4, CRYBB2, CRYB2, CRYGC, CRYG3, CCL, CRYAA, CRYA1, GJA8, CX50, CAE1, GJA3, CX46, CZP3, CAE3, CCM1, CAM, KRIT1); Corneal clouding and dystrophy (APOA1, TGFBI, CSD2, CDGG1, CSD, BIGH3, CDG2, TACSTD2, TROP2, M1S1, VSX1, RINX, PPCD, PPD, KTCN, COL8A2, FECD, PPCD2, PIP5K3, CFD); Cornea plana congenital (KERA, CNA2); Glaucoma (MYOC, TIGR, GLC1A, JOAG, GPOA, OPTN, GLC1E, FIP2, HYPL, NRP, CYP1B1, GLC3A, OPA1, NTG, NPG, CYP1B1, GLC3A); Leber congenital amaurosis (CRB1, RP12, CRX, CORD2, CRD, RPGRIP1, LCA6, CORD9, RPE65, RP20, AIPL1, LCA4, GUCY2D, GUC2D, LCA1, CORD6, RDH12, LCA3); Macular dystrophy (ELOVL4, ADMD, STGD2, STGD3, RDS, RP7, PRPH2, PRPH, AVMD, AOFMD, VMD2).

Table C:

CELLULAR FUNCTION	GENES
PI3K/AKT Signaling	PRKCE; ITGAM; ITGA5; IRAK1; PRKAA2; EIF2AK2;
	PTEN; EIF4E; PRKCZ; GRK6; MAPK1; TSC1; PLK1;
	AKT2; IKBKB; PIK3CA; CDK8; CDKN1B; NFKB2; BCL2;
	PIK3CB; PPP2R1A; MAPK8; BCL2L1; MAPK3; TSC2;
	ITGA1; KRAS; EIF4EBP1; RELA; PRKCD; NOS3;
	PRKAA1; MAPK9; CDK2; PPP2CA; PIM1; ITGB7;
	YWHAZ; ILK; TP53; RAF1; IKBKG; RELB; DYRK1A;
	CDKN1A; ITGB1; MAP2K2; JAK1; AKT1; JAK2; PIK3R1;
	CHUK; PDPK1; PPP2R5C; CTNNB1; MAP2K1; NFKB1;
	PAK3; ITGB3; CCND1; GSK3A; FRAP1; SFN; ITGA2;
	TTK; CSNK1A1; BRAF; GSK3B; AKT3; FOXO1; SGK;
	HSP90AA1; RPS6KB1
ERK/MAPK Signaling	PRKCE; ITGAM; ITGA5; HSPB1; IRAK1; PRKAA2;
	EIF2AK2; RAC1; RAP1A; TLN1; EIF4E; ELK1; GRK6;
	MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8; CREB1;
	PRKCI; PTK2; FOS; RPS6KA4; PIK3CB; PPP2R1A;
	PIK3C3; MAPK8; MAPK3; ITGA1; ETS1; KRAS; MYCN;
	EIF4EBP1; PPARG; PRKCD; PRKAA1; MAPK9; SRC;
	CDK2; PPP2CA; PIM1; PIK3C2A; ITGB7; YWHAZ;
	PPP1CC; KSR1; PXN; RAF1; FYN; DYRK1A; ITGB1;
	MAP2K2; PAK4; PIK3R1; STAT3; PPP2R5C; MAP2K1;
	PAK3; ITGB3; ESR1; ITGA2; MYC; TTK; CSNK1A1;
	CRKL; BRAF; ATF4; PRKCA; SRF; STAT1; SGK
Glucocorticoid Receptor	RAC1; TAF4B; EP300; SMAD2; TRAF6; PCAF; ELK1;
Signaling	MAPK1; SMAD3; AKT2; IKBKB; NCOR2; UBE2I;

CELLULAR FUNCTION	GENES
	PIK3CA; CREB1; FOS; HSPA5; NFKB2; BCL2;
	MAP3K14; STAT5B; PIK3CB; PIK3C3; MAPK8; BCL2L1;
	MAPK3; TSC22D3; MAPK10; NRIP1; KRAS; MAPK13;
	RELA; STAT5A; MAPK9; NOS2A; PBX1; NR3C1;
	PIK3C2A; CDKN1C; TRAF2; SERPINE1; NCOA3;
	MAPK14; TNF; RAF1; IKBKG; MAP3K7; CREBBP;
	CDKN1A; MAP2K2; JAK1; IL8; NCOA2; AKT1; JAK2;
	PIK3R1; CHUK; STAT3; MAP2K1; NFKB1; TGFBR1;
	ESR1; SMAD4; CEBPB; JUN; AR; AKT3; CCL2; MMP1;
	STAT1; IL6; HSP90AA1
Axonal Guidance Signaling	PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; ADAM12;
	IGF1; RAC1; RAP1A; EIF4E; PRKCZ; NRP1; NTRK2;
	ARHGEF7; SMO; ROCK2; MAPK1; PGF; RAC2;
	PTPN11; GNAS; AKT2; PIK3CA; ERBB2; PRKCI; PTK2;
	CFL1; GNAQ; PIK3CB; CXCL12; PIK3C3; WNT11;
	PRKD1; GNB2L1; ABL1; MAPK3; ITGA1; KRAS; RHOA;
	PRKCD; PIK3C2A; ITGB7; GLI2; PXN; VASP; RAF1;
	FYN; ITGB1; MAP2K2; PAK4; ADAM17; AKT1; PIK3R1;
	GLI1; WNT5A; ADAM10; MAP2K1; PAK3; ITGB3;
	CDC42; VEGFA; ITGA2; EPHA8; CRKL; RND1; GSK3B;
	AKT3; PRKCA
Ephrin Receptor Signaling	PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; IRAK1;
	PRKAA2; EIF2AK2; RAC1; RAP1A; GRK6; ROCK2;
	MAPK1; PGF; RAC2; PTPN11; GNAS; PLK1; AKT2;
	DOK1; CDK8; CREB1; PTK2; CFL1; GNAQ; MAP3K14;
	CXCL12; MAPK8; GNB2L1; ABL1; MAPK3; ITGA1;
	KRAS; RHOA; PRKCD; PRKAA1; MAPK9; SRC; CDK2;
	PIM1; ITGB7; PXN; RAF1; FYN; DYRK1A; ITGB1;
	MAP2K2; PAK4; AKT1; JAK2; STAT3; ADAM10;
	MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2;
	EPHA8; TTK; CSNK1A1; CRKL; BRAF; PTPN13; ATF4;
	AKT3; SGK
Actin Cytoskeleton	ACTN4; PRKCE; ITGAM; ROCK1; ITGA5; IRAK1;
Signaling	PRKAA2; EIF2AK2; RAC1; INS; ARHGEF7; GRK6;
	ROCK2; MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8;
	PTK2; CFL1; PIK3CB; MYH9; DIAPH1; PIK3C3; MAPK8;
	F2R; MAPK3; SLC9A1; ITGA1; KRAS; RHOA; PRKCD;
	PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; ITGB7;
	PPP1CC; PXN; VIL2; RAF1; GSN; DYRK1A; ITGB1;
	MAP2K2; PAK4; PIP5K1A; PIK3R1; MAP2K1; PAK3;
	ITGB3; CDC42; APC; ITGA2; TTK; CSNK1A1; CRKL;
	BRAF; VAV3; SGK
Huntington's Disease	PRKCE; IGF1; EP300; RCOR1; PRKCZ; HDAC4; TGM2;

CELLULAR FUNCTION	GENES
Signaling	MAPK1; CAPNS1; AKT2; EGFR; NCOR2; SP1; CAPN2;
	PIK3CA; HDAC5; CREB1; PRKCI; HSPA5; REST;
	GNAQ; PIK3CB; PIK3C3; MAPK8; IGF1R; PRKD1;
	GNB2L1; BCL2L1; CAPN1; MAPK3; CASP8; HDAC2;
	HDAC7A; PRKCD; HDAC11; MAPK9; HDAC9; PIK3C2A;
	HDAC3; TP53; CASP9; CREBBP; AKT1; PIK3R1;
	PDPK1; CASP1; APAF1; FRAP1; CASP2; JUN; BAX;
	ATF4; AKT3; PRKCA; CLTC; SGK; HDAC6; CASP3
Apoptosis Signaling	PRKCE; ROCK1; BID; IRAK1; PRKAA2; EIF2AK2; BAK1;
	BIRC4; GRK6; MAPK1; CAPNS1; PLK1; AKT2; IKBKB;
	CAPN2; CDK8; FAS; NFKB2; BCL2; MAP3K14; MAPK8;
	BCL2L1; CAPN1; MAPK3; CASP8; KRAS; RELA;
	PRKCD; PRKAA1; MAPK9; CDK2; PIM1; TP53; TNF;
	RAF1; IKBKG; RELB; CASP9; DYRK1A; MAP2K2;
	CHUK; APAF1; MAP2K1; NFKB1; PAK3; LMNA; CASP2;
	BIRC2; TTK; CSNK1A1; BRAF; BAX; PRKCA; SGK;
	CASP3; BIRC3; PARP1
B Cell Receptor Signaling	RAC1; PTEN; LYN; ELK1; MAPK1; RAC2; PTPN11;
	AKT2; IKBKB; PIK3CA; CREB1; SYK; NFKB2; CAMK2A;
	MAP3K14; PIK3CB; PIK3C3; MAPK8; BCL2L1; ABL1;
	MAPK3; ETS1; KRAS; MAPK13; RELA; PTPN6; MAPK9;
	EGR1; PIK3C2A; BTK; MAPK14; RAF1; IKBKG; RELB;
	MAP3K7; MAP2K2; AKT1; PIK3R1; CHUK; MAP2K1;
	NFKB1; CDC42; GSK3A; FRAP1; BCL6; BCL10; JUN;
	GSK3B; ATF4; AKT3; VAV3; RPS6KB1
Leukocyte Extravasation	ACTN4; CD44; PRKCE; ITGAM; ROCK1; CXCR4; CYBA;
Signaling	RAC1; RAP1A; PRKCZ; ROCK2; RAC2; PTPN11;
	MMP14; PIK3CA; PRKCI; PTK2; PIK3CB; CXCL12;
	PIK3C3; MAPK8; PRKD1; ABL1; MAPK10; CYBB;
	MAPK13; RHOA; PRKCD; MAPK9; SRC; PIK3C2A; BTK;
	MAPK14; NOX1; PXN; VIL2; VASP; ITGB1; MAP2K2;
	CTNND1; PIK3R1; CTNNB1; CLDN1; CDC42; F11R; ITK;
	CRKL; VAV3; CTTN; PRKCA; MMP1; MMP9
Integrin Signaling	ACTN4; ITGAM; ROCK1; ITGA5; RAC1; PTEN; RAP1A;
	TLN1; ARHGEF7; MAPK1; RAC2; CAPNS1; AKT2;
	CAPN2; PIK3CA; PTK2; PIK3CB; PIK3C3; MAPK8;
	CAV1; CAPN1; ABL1; MAPK3; ITGA1; KRAS; RHOA;
	SRC; PIK3C2A; ITGB7; PPP1CC; ILK; PXN; VASP;
	RAF1; FYN; ITGB1; MAP2K2; PAK4; AKT1; PIK3R1;
	TNK2; MAP2K1; PAK3; ITGB3; CDC42; RND3; ITGA2;
	CRKL; BRAF; GSK3B; AKT3
Acute Phase Response	IRAK1; SOD2; MYD88; TRAF6; ELK1; MAPK1; PTPN11;
Signaling	AKT2; IKBKB; PIK3CA; FOS; NFKB2; MAP3K14;

CELLULAR FUNCTION	GENES
	PIK3CB; MAPK8; RIPK1; MAPK3; IL6ST; KRAS;
	MAPK13; IL6R; RELA; SOCS1; MAPK9; FTL; NR3C1;
	TRAF2; SERPINE1; MAPK14; TNF; RAF1; PDK1;
	IKBKG; RELB; MAP3K7; MAP2K2; AKT1; JAK2; PIK3R1;
	CHUK; STAT3; MAP2K1; NFKB1; FRAP1; CEBPB; JUN;
	AKT3; IL1R1; IL6
PTEN Signaling	ITGAM; ITGA5; RAC1; PTEN; PRKCZ; BCL2L11;
	MAPK1; RAC2; AKT2; EGFR; IKBKB; CBL; PIK3CA;
	CDKN1B; PTK2; NFKB2; BCL2; PIK3CB; BCL2L1;
	MAPK3; ITGA1; KRAS; ITGB7; ILK; PDGFRB; INSR;
	RAF1; IKBKG; CASP9; CDKN1A; ITGB1; MAP2K2;
	AKT1; PIK3R1; CHUK; PDGFRA; PDPK1; MAP2K1;
	NFKB1; ITGB3; CDC42; CCND1; GSK3A; ITGA2;
	GSK3B; AKT3; FOXO1; CASP3; RPS6KB1
p53 Signaling	PTEN; EP300; BBC3; PCAF; FASN; BRCA1; GADD45A;
	BIRC5; AKT2; PIK3CA; CHEK1; TP53INP1; BCL2;
	PIK3CB; PIK3C3; MAPK8; THBS1; ATR; BCL2L1; E2F1;
	PMAIP1; CHEK2; TNFRSF10B; TP73; RB1; HDAC9;
	CDK2; PIK3C2A; MAPK14; TP53; LRDD; CDKN1A;
	HIPK2; AKT1; PIK3R1; RRM2B; APAF1; CTNNB1;
	SIRT1; CCND1; PRKDC; ATM; SFN; CDKN2A; JUN;
	SNAI2; GSK3B; BAX; AKT3
Aryl Hydrocarbon Receptor	HSPB1; EP300; FASN; TGM2; RXRA; MAPK1; NQO1;
Signaling	NCOR2; SP1; ARNT; CDKN1B; FOS; CHEK1;
	SMARCA4; NFKB2; MAPK8; ALDH1A1; ATR; E2F1;
	MAPK3; NRIP1; CHEK2; RELA; TP73; GSTP1; RB1;
	SRC; CDK2; AHR; NFE2L2; NCOA3; TP53; TNF;
	CDKN1A; NCOA2; APAF1; NFKB1; CCND1; ATM; ESR1;
	CDKN2A; MYC; JUN; ESR2; BAX; IL6; CYP1B1;
	HSP90AA1
Xenobiotic Metabolism	PRKCE; EP300; PRKCZ; RXRA; MAPK1; NQO1;
Signaling	NCOR2; PIK3CA; ARNT; PRKCI; NFKB2; CAMK2A;
	PIK3CB; PPP2R1A; PIK3C3; MAPK8; PRKD1;
	ALDH1A1; MAPK3; NRIP1; KRAS; MAPK13; PRKCD;
	GSTP1; MAPK9; NOS2A; ABCB1; AHR; PPP2CA; FTL;
	NFE2L2; PIK3C2A; PPARGC1A; MAPK14; TNF; RAF1;
	CREBBP; MAP2K2; PIK3R1; PPP2R5C; MAP2K1;
	NFKB1; KEAP1; PRKCA; EIF2AK3; IL6; CYP1B1;
	HSP90AA1
SAPK/JNK Signaling	PRKCE; IRAK1; PRKAA2; EIF2AK2; RAC1; ELK1;
	GRK6; MAPK1; GADD45A; RAC2; PLK1; AKT2; PIK3CA;
	FADD; CDK8; PIK3CB; PIK3C3; MAPK8; RIPK1;
	GNB2L1; IRS1; MAPK3; MAPK10; DAXX; KRAS;

CELLULAR FUNCTION	GENES
	PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A;
	TRAF2; TP53; LCK; MAP3K7; DYRK1A; MAP2K2;
	PIK3R1; MAP2K1; PAK3; CDC42; JUN; TTK; CSNK1A1;
	CRKL; BRAF; SGK
PPAr/RXR Signaling	PRKAA2; EP300; INS; SMAD2; TRAF6; PPARA; FASN;
	RXRA; MAPK1; SMAD3; GNAS; IKBKB; NCOR2;
	ABCA1; GNAQ; NFKB2; MAP3K14; STAT5B; MAPK8;
	IRS1; MAPK3; KRAS; RELA; PRKAA1; PPARGC1A;
	NCOA3; MAPK14; INSR; RAF1; IKBKG; RELB; MAP3K7;
	CREBBP; MAP2K2; JAK2; CHUK; MAP2K1; NFKB1;
	TGFBR1; SMAD4; JUN; IL1R1; PRKCA; IL6; HSP90AA1;
	ADIPOQ
NF-KB Signaling	IRAK1; EIF2AK2; EP300; INS; MYD88; PRKCZ; TRAF6;
	TBK1; AKT2; EGFR; IKBKB; PIK3CA; BTRC; NFKB2;
	MAP3K14; PIK3CB; PIK3C3; MAPK8; RIPK1; HDAC2;
	KRAS; RELA; PIK3C2A; TRAF2; TLR4; PDGFRB; TNF;
	INSR; LCK; IKBKG; RELB; MAP3K7; CREBBP; AKT1;
	PIK3R1; CHUK; PDGFRA; NFKB1; TLR2; BCL10;
	GSK3B; AKT3; TNFAIP3; IL1R1
Neuregulin Signaling	ERBB4; PRKCE; ITGAM; ITGA5; PTEN; PRKCZ; ELK1;
	MAPK1; PTPN11; AKT2; EGFR; ERBB2; PRKCI;
	CDKN1B; STAT5B; PRKD1; MAPK3; ITGA1; KRAS;
	PRKCD; STAT5A; SRC; ITGB7; RAF1; ITGB1; MAP2K2;
	ADAM17; AKT1; PIK3R1; PDPK1; MAP2K1; ITGB3;
	EREG; FRAP1; PSEN1; ITGA2; MYC; NRG1; CRKL;
	AKT3; PRKCA; HSP90AA1; RPS6KB1
Wnt & Beta catenin	CD44; EP300; LRP6; DVL3; CSNK1E; GJA1; SMO;
Signaling	AKT2; PIN1; CDH1; BTRC; GNAQ; MARK2; PPP2R1A;
	WNT11; SRC; DKK1; PPP2CA; SOX6; SFRP2; ILK;
	LEF1; SOX9; TP53; MAP3K7; CREBBP; TCF7L2; AKT1;
	PPP2R5C; WNT5A; LRP5; CTNNB1; TGFBR1; CCND1;
	GSK3A; DVL1; APC; CDKN2A; MYC; CSNK1A1; GSK3B;
	AKT3; SOX2
Insulin Receptor Signaling	PTEN; INS; EIF4E; PTPN1; PRKCZ; MAPK1; TSC1;
	PTPN11; AKT2; CBL; PIK3CA; PRKCI; PIK3CB; PIK3C3;
	MAPK8; IRS1; MAPK3; TSC2; KRAS; EIF4EBP1;
	SLC2A4; PIK3C2A; PPP1CC; INSR; RAF1; FYN;
	MAP2K2; JAK1; AKT1; JAK2; PIK3R1; PDPK1; MAP2K1;
	GSK3A; FRAP1; CRKL; GSK3B; AKT3; FOXO1; SGK;
	RPS6KB1
IL-6 Signaling	HSPB1; TRAF6; MAPKAPK2; ELK1; MAPK1; PTPN11;
	IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK3;
	MAPK10; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1;

CELLULAR FUNCTION	GENES
	MAPK9; ABCB1; TRAF2; MAPK14; TNF; RAF1; IKBKG;
	RELB; MAP3K7; MAP2K2; IL8; JAK2; CHUK; STAT3;
	MAP2K1; NFKB1; CEBPB; JUN; IL1R1; SRF; IL6
Hepatic Cholestasis	PRKCE; IRAK1; INS; MYD88; PRKCZ; TRAF6; PPARA;
	RXRA; IKBKB; PRKCI; NFKB2; MAP3K14; MAPK8;
	PRKD1; MAPK10; RELA; PRKCD; MAPK9; ABCB1;
	TRAF2; TLR4; TNF; INSR; IKBKG; RELB; MAP3K7; IL8;
	CHUK; NR1H2; TJP2; NFKB1; ESR1; SREBF1; FGFR4;
	JUN; IL1R1; PRKCA; IL6
IGF-1 Signaling	IGF1; PRKCZ; ELK1; MAPK1; PTPN11; NEDD4; AKT2;
	PIK3CA; PRKCI; PTK2; FOS; PIK3CB; PIK3C3; MAPK8;
	IGF1R; IRS1; MAPK3; IGFBP7; KRAS; PIK3C2A;
	YWHAZ; PXN; RAF1; CASP9; MAP2K2; AKT1; PIK3R1;
	PDPK1; MAP2K1; IGFBP2; SFN; JUN; CYR61; AKT3;
	FOXO1; SRF; CTGF; RPS6KB1
NRF2-mediated Oxidative	PRKCE; EP300; SOD2; PRKCZ; MAPK1; SQSTM1;
Stress Response	NQO1; PIK3CA; PRKCI; FOS; PIK3CB; PIK3C3; MAPK8;
	PRKD1; MAPK3; KRAS; PRKCD; GSTP1; MAPK9; FTL;
	NFE2L2; PIK3C2A; MAPK14; RAF1; MAP3K7; CREBBP;
	MAP2K2; AKT1; PIK3R1; MAP2K1; PPIB; JUN; KEAP1;
	GSK3B; ATF4; PRKCA; EIF2AK3; HSP90AA1
Hepatic Fibrosis/Hepatic	EDN1; IGF1; KDR; FLT1; SMAD2; FGFR1; MET; PGF;
Stellate Cell Activation	SMAD3; EGFR; FAS; CSF1; NFKB2; BCL2; MYH9;
	IGF1R; IL6R; RELA; TLR4; PDGFRB; TNF; RELB; IL8;
	PDGFRA; NFKB1; TGFBR1; SMAD4; VEGFA; BAX;
	IL1R1; CCL2; HGF; MMP1; STAT1; IL6; CTGF; MMP9
PPAR Signaling	EP300; INS; TRAF6; PPARA; RXRA; MAPK1; IKBKB;
	NCOR2; FOS; NFKB2; MAP3K14; STAT5B; MAPK3;
	NRIP1; KRAS; PPARG; RELA; STAT5A; TRAF2;
	PPARGC1A; PDGFRB; TNF; INSR; RAF1; IKBKG;
	RELB; MAP3K7; CREBBP; MAP2K2; CHUK; PDGFRA;
	MAP2K1; NFKB1; JUN; IL1R1; HSP90AA1
Fc Epsilon RI Signaling	PRKCE; RAC1; PRKCZ; LYN; MAPK1; RAC2; PTPN11;
	AKT2; PIK3CA; SYK; PRKCI; PIK3CB; PIK3C3; MAPK8;
	PRKD1; MAPK3; MAPK10; KRAS; MAPK13; PRKCD;
	MAPK9; PIK3C2A; BTK; MAPK14; TNF; RAF1; FYN;
	MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; AKT3;
	VAV3; PRKCA
G-Protein Coupled	PRKCE; RAP1A; RGS16; MAPK1; GNAS; AKT2; IKBKB;
Receptor Signaling	PIK3CA; CREB1; GNAQ; NFKB2; CAMK2A; PIK3CB;
	PIK3C3; MAPK3; KRAS; RELA; SRC; PIK3C2A; RAF1;
	IKBKG; RELB; FYN; MAP2K2; AKT1; PIK3R1; CHUK;
	PDPK1; STAT3; MAP2K1; NFKB1; BRAF; ATF4; AKT3;

CELLULAR FUNCTION	GENES
	PRKCA
Inositol Phosphate	PRKCE; IRAK1; PRKAA2; EIF2AK2; PTEN; GRK6;
Metabolism	MAPK1; PLK1; AKT2; PIK3CA; CDK8; PIK3CB; PIK3C3;
	MAPK8; MAPK3; PRKCD; PRKAA1; MAPK9; CDK2;
	PIM1; PIK3C2A; DYRK1A; MAP2K2; PIP5K1A; PIK3R1;
	MAP2K1; PAK3; ATM; TTK; CSNK1A1; BRAF; SGK
PDGF Signaling	EIF2AK2; ELK1; ABL2; MAPK1; PIK3CA; FOS; PIK3CB;
	PIK3C3; MAPK8; CAV1; ABL1; MAPK3; KRAS; SRC;
	PIK3C2A; PDGFRB; RAF1; MAP2K2; JAK1; JAK2;
	PIK3R1; PDGFRA; STAT3; SPHK1; MAP2K1; MYC;
	JUN; CRKL; PRKCA; SRF; STAT1; SPHK2
VEGF Signaling	ACTN4; ROCK1; KDR; FLT1; ROCK2; MAPK1; PGF;
	AKT2; PIK3CA; ARNT; PTK2; BCL2; PIK3CB; PIK3C3;
	BCL2L1; MAPK3; KRAS; HIF1A; NOS3; PIK3C2A; PXN;
	RAF1; MAP2K2; ELAVL1; AKT1; PIK3R1; MAP2K1; SFN;
	VEGFA; AKT3; FOXO1; PRKCA
Natural Killer Cell Signaling	PRKCE; RAC1; PRKCZ; MAPK1; RAC2; PTPN11;
	KIR2DL3; AKT2; PIK3CA; SYK; PRKCI; PIK3CB;
	PIK3C3; PRKD1; MAPK3; KRAS; PRKCD; PTPN6;
	PIK3C2A; LCK; RAF1; FYN; MAP2K2; PAK4; AKT1;
	PIK3R1; MAP2K1; PAK3; AKT3; VAV3; PRKCA
Cell Cycle: G1/S	HDAC4; SMAD3; SUV39H1; HDAC5; CDKN1B; BTRC;
Checkpoint Regulation	ATR; ABL1; E2F1; HDAC2; HDAC7A; RB1; HDAC11;
	HDAC9; CDK2; E2F2; HDAC3; TP53; CDKN1A; CCND1;
	E2F4; ATM; RBL2; SMAD4; CDKN2A; MYC; NRG1;
	GSK3B; RBL1; HDAC6
T Cell Receptor Signaling	RAC1; ELK1; MAPK1; IKBKB; CBL; PIK3CA; FOS;
	NFKB2; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS;
	RELA; PIK3C2A; BTK; LCK; RAF1; IKBKG; RELB; FYN;
	MAP2K2; PIK3R1; CHUK; MAP2K1; NFKB1; ITK; BCL10;
	JUN; VAV3
Death Receptor Signaling	CRADD; HSPB1; BID; BIRC4; TBK1; IKBKB; FADD;
	FAS; NFKB2; BCL2; MAP3K14; MAPK8; RIPK1; CASP8;
	DAXX; TNFRSF10B; RELA; TRAF2; TNF; IKBKG; RELB;
	CASP9; CHUK; APAF1; NFKB1; CASP2; BIRC2; CASP3;
	BIRC3
FGF Signaling	RAC1; FGFR1; MET; MAPKAPK2; MAPK1; PTPN11;
	AKT2; PIK3CA; CREB1; PIK3CB; PIK3C3; MAPK8;
	MAPK3; MAPK13; PTPN6; PIK3C2A; MAPK14; RAF1;
	AKT1; PIK3R1; STAT3; MAP2K1; FGFR4; CRKL; ATF4;
	AKT3; PRKCA; HGF
GM-CSF Signaling	LYN; ELK1; MAPK1; PTPN11; AKT2; PIK3CA; CAMK2A;

CELLULAR FUNCTION	GENES
	STAT5B; PIK3CB; PIK3C3; GNB2L1; BCL2L1; MAPK3;
	ETS1; KRAS; RUNX1; PIM1; PIK3C2A; RAF1; MAP2K2;
	AKT1; JAK2; PIK3R1; STAT3; MAP2K1; CCND1; AKT3;
	STAT1
Amyotrophic Lateral	BID; IGF1; RAC1; BIRC4; PGF; CAPNS1; CAPN2;
Sclerosis Signaling	PIK3CA; BCL2; PIK3CB; PIK3C3; BCL2L1; CAPN1;
	PIK3C2A; TP53; CASP9; PIK3R1; RAB5A; CASP1;
	APAF1; VEGFA; BIRC2; BAX; AKT3; CASP3; BIRC3
JAK/Stat Signaling	PTPN1; MAPK1; PTPN11; AKT2; PIK3CA; STAT5B;
	PIK3CB; PIK3C3; MAPK3; KRAS; SOCS1; STAT5A;
	PTPN6; PIK3C2A; RAF1; CDKN1A; MAP2K2; JAK1;
	AKT1; JAK2; PIK3R1; STAT3; MAP2K1; FRAP1; AKT3;
	STAT1
Nicotinate and Nicotinamide	PRKCE; IRAK1; PRKAA2; EIF2AK2; GRK6; MAPK1;
Metabolism	PLK1; AKT2; CDK8; MAPK8; MAPK3; PRKCD; PRKAA1;
	PBEF1; MAPK9; CDK2; PIM1; DYRK1A; MAP2K2;
	MAP2K1; PAK3; NT5E; TTK; CSNK1A1; BRAF; SGK
Chemokine Signaling	CXCR4; ROCK2; MAPK1; PTK2; FOS; CFL1; GNAQ;
	CAMK2A; CXCL12; MAPK8; MAPK3; KRAS; MAPK13;
	RHOA; CCR3; SRC; PPP1CC; MAPK14; NOX1; RAF1;
	MAP2K2; MAP2K1; JUN; CCL2; PRKCA
IL-2 Signaling	ELK1; MAPK1; PTPN11; AKT2; PIK3CA; SYK; FOS;
	STAT5B; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS;
	SOCS1; STAT5A; PIK3C2A; LCK; RAF1; MAP2K2;
	JAK1; AKT1; PIK3R1; MAP2K1; JUN; AKT3
Synaptic Long Term	PRKCE; IGF1; PRKCZ; PRDX6; LYN; MAPK1; GNAS;
Depression	PRKCI; GNAQ; PPP2R1A; IGF1R; PRKD1; MAPK3;
	KRAS; GRN; PRKCD; NOS3; NOS2A; PPP2CA;
	YWHAZ; RAF1; MAP2K2; PPP2R5C; MAP2K1; PRKCA
Estrogen Receptor	TAF4B; EP300; CARM1; PCAF; MAPK1; NCOR2;
Signaling	SMARCA4; MAPK3; NRIP1; KRAS; SRC; NR3C1;
	HDAC3; PPARGC1A; RBM9; NCOA3; RAF1; CREBBP;
	MAP2K2; NCOA2; MAP2K1; PRKDC; ESR1; ESR2
Protein Ubiquitination	TRAF6; SMURF1; BIRC4; BRCA1; UCHL1; NEDD4;
Pathway	CBL; UBE2I; BTRC; HSPA5; USP7; USP10; FBXW7;
	USP9X; STUB1; USP22; B2M; BIRC2; PARK2; USP8;
	USP1; VHL; HSP90AA1; BIRC3
IL-10 Signaling	TRAF6; CCR1; ELK1; IKBKB; SP1; FOS; NFKB2;
	MAP3K14; MAPK8; MAPK13; RELA; MAPK14; TNF;
	IKBKG; RELB; MAP3K7; JAK1; CHUK; STAT3; NFKB1;
	JUN; IL1R1; IL6
VDR/RXR Activation	PRKCE; EP300; PRKCZ; RXRA; GADD45A; HES1;

CELLULAR FUNCTION	GENES
	NCOR2; SP1; PRKCI; CDKN1B; PRKD1; PRKCD;
	RUNX2; KLF4; YY1; NCOA3; CDKN1A; NCOA2; SPP1;
	LRP5; CEBPB; FOXO1; PRKCA
TGF-beta Signaling	EP300; SMAD2; SMURF1; MAPK1; SMAD3; SMAD1;
	FOS; MAPK8; MAPK3; KRAS; MAPK9; RUNX2;
	SERPINE1; RAF1; MAP3K7; CREBBP; MAP2K2;
	MAP2K1; TGFBR1; SMAD4; JUN; SMAD5
Toll-like Receptor Signaling	IRAK1; EIF2AK2; MYD88; TRAF6; PPARA; ELK1;
	IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK13;
	RELA; TLR4; MAPK14; IKBKG; RELB; MAP3K7; CHUK;
	NFKB1; TLR2; JUN
p38 MAPK Signaling	HSPB1; IRAK1; TRAF6; MAPKAPK2; ELK1; FADD; FAS;
	CREB1; DDIT3; RPS6KA4; DAXX; MAPK13; TRAF2;
	MAPK14; TNF; MAP3K7; TGFBR1; MYC; ATF4; IL1R1;
	SRF; STAT1
Neurotrophin/TRK Signaling	NTRK2; MAPK1; PTPN11; PIK3CA; CREB1; FOS;
	PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; PIK3C2A;
	RAF1; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1;
	CDC42; JUN; ATF4
FXR/RXR Activation	INS; PPARA; FASN; RXRA; AKT2; SDC1; MAPK8;
	APOB; MAPK10; PPARG; MTTP; MAPK9; PPARGC1A;
	TNF; CREBBP; AKT1; SREBF1; FGFR4; AKT3; FOXO1
Synaptic Long Term	PRKCE; RAP1A; EP300; PRKCZ; MAPK1; CREB1;
Potentiation	PRKCI; GNAQ; CAMK2A; PRKD1; MAPK3; KRAS;
	PRKCD; PPP1CC; RAF1; CREBBP; MAP2K2; MAP2K1;
	ATF4; PRKCA
Calcium Signaling	RAP1A; EP300; HDAC4; MAPK1; HDAC5; CREB1;
	CAMK2A; MYH9; MAPK3; HDAC2; HDAC7A; HDAC11;
	HDAC9; HDAC3; CREBBP; CALR; CAMKK2; ATF4;
	HDAC6
EGF Signaling	ELK1; MAPK1; EGFR; PIK3CA; FOS; PIK3CB; PIK3C3;
	MAPK8; MAPK3; PIK3C2A; RAF1; JAK1; PIK3R1;
	STAT3; MAP2K1; JUN; PRKCA; SRF; STAT1
Hypoxia Signaling in the	EDN1; PTEN; EP300; NQO1; UBE2I; CREB1; ARNT;
Cardiovascular System	HIF1A; SLC2A4; NOS3; TP53; LDHA; AKT1; ATM;
	VEGFA; JUN; ATF4; VHL; HSP90AA1
LPS/IL-1 Mediated Inhibition	IRAK1; MYD88; TRAF6; PPARA; RXRA; ABCA1;
of RXR Function	MAPK8; ALDH1A1; GSTP1; MAPK9; ABCB1; TRAF2;
	TLR4; TNF; MAP3K7; NR1H2; SREBF1; JUN; IL1R1
LXR/RXR Activation	FASN; RXRA; NCOR2; ABCA1; NFKB2; IRF3; RELA;

CELLULAR FUNCTION	GENES
	NOS2A; TLR4; TNF; RELB; LDLR; NR1H2; NFKB1;
	SREBF1; IL1R1; CCL2; IL6; MMP9
Amyloid Processing	PRKCE; CSNK1E; MAPK1; CAPNS1; AKT2; CAPN2;
	CAPN1; MAPK3; MAPK13; MAPT; MAPK14; AKT1;
	PSEN1; CSNK1A1; GSK3B; AKT3; APP
IL-4 Signaling	AKT2; PIK3CA; PIK3CB; PIK3C3; IRS1; KRAS; SOCS1;
	PTPN6; NR3C1; PIK3C2A; JAK1; AKT1; JAK2; PIK3R1;
	FRAP1; AKT3; RPS6KB1
Cell Cycle: G2/M DNA	EP300; PCAF; BRCA1; GADD45A; PLK1; BTRC;
Damage Checkpoint	CHEK1; ATR; CHEK2; YWHAZ; TP53; CDKN1A;
Regulation	PRKDC; ATM; SFN; CDKN2A
Nitric Oxide Signaling in the	KDR; FLT1; PGF; AKT2; PIK3CA; PIK3CB; PIK3C3;
Cardiovascular System	CAV1; PRKCD; NOS3; PIK3C2A; AKT1; PIK3R1;
	VEGFA; AKT3; HSP90AA1
Purine Metabolism	NME2; SMARCA4; MYH9; RRM2; ADAR; EIF2AK4;
	PKM2; ENTPD1; RAD51; RRM2B; TJP2; RAD51C;
	NT5E; POLD1; NME1
cAMP-mediated Signaling	RAP1A; MAPK1; GNAS; CREB1; CAMK2A; MAPK3;
	SRC; RAF1; MAP2K2; STAT3; MAP2K1; BRAF; ATF4
Mitochondrial Dysfunction	SOD2; MAPK8; CASP8; MAPK10; MAPK9; CASP9;
	PARK7; PSEN1; PARK2; APP; CASP3
Notch Signaling	HES1; JAG1; NUMB; NOTCH4; ADAM17; NOTCH2;
	PSEN1; NOTCH3; NOTCH1; DLL4
Endoplasmic Reticulum	HSPA5; MAPK8; XBP1; TRAF2; ATF6; CASP9; ATF4;
Stress Pathway	EIF2AK3; CASP3
Pyrimidine Metabolism	NME2; AICDA; RRM2; EIF2AK4; ENTPD1; RRM2B;
	NT5E; POLD1; NME1
Parkinson's Signaling	UCHL1; MAPK8; MAPK13; MAPK14; CASP9; PARK7;
	PARK2; CASP3
Cardiac & Beta Adrenergic	GNAS; GNAQ; PPP2R1A; GNB2L1; PPP2CA; PPP1CC;
Signaling	PPP2R5C
Glycolysis/Gluconeogenesis	HK2; GCK; GPI; ALDH1A1; PKM2; LDHA; HK1
Interferon Signaling	IRF1; SOCS1; JAK1; JAK2; IFITM1; STAT1; IFIT3
Sonic Hedgehog Signaling	ARRB2; SMO; GLI2; DYRK1A; GLI1; GSK3B; DYRK1B
Glycerophospholipid	PLD1; GRN; GPAM; YWHAZ; SPHK1; SPHK2
Metabolism	
Phospholipid Degradation	PRDX6; PLD1; GRN; YWHAZ; SPHK1; SPHK2
Tryptophan Metabolism	SIAH2; PRMT5; NEDD4; ALDH1A1; CYP1B1; SIAH1
Lysine Degradation	SUV39H1; EHMT2; NSD1; SETD7; PPP2R5C
Nucleotide Excision Repair	ERCC5; ERCC4; XPA; XPC; ERCC1
Pathway	
Starch and Sucrose	UCHL1; HK2; GCK; GPI; HK1

CELLULAR FUNCTION	GENES
Metabolism	
Aminosugars Metabolism	NQO1; HK2; GCK; HK1
Arachidonic Acid	PRDX6; GRN; YWHAZ; CYP1B1
Metabolism	
Circadian Rhythm Signaling	CSNK1E; CREB1; ATF4; NR1D1
Coagulation System	BDKRB1; F2R; SERPINE1; F3
Dopamine Receptor	PPP2R1A; PPP2CA; PPP1CC; PPP2R5C
Signaling	
Glutathione Metabolism	IDH2; GSTP1; ANPEP; IDH1
Glycerolipid Metabolism	ALDH1A1; GPAM; SPHK1; SPHK2
Linoleic Acid Metabolism	PRDX6; GRN; YWHAZ; CYP1B1
Methionine Metabolism	DNMT1; DNMT3B; AHCY; DNMT3A
Pyruvate Metabolism	GLO1; ALDH1A1; PKM2; LDHA
Arginine and Proline	ALDH1A1; NOS3; NOS2A
Metabolism	
Eicosanoid Signaling	PRDX6; GRN; YWHAZ
Fructose and Mannose	HK2; GCK; HK1
Metabolism	
Galactose Metabolism	HK2; GCK; HK1
Stilbene, Coumarine and	PRDX6; PRDX1; TYR
Lignin Biosynthesis	
Antigen Presentation	CALR; B2M
Pathway	
Biosynthesis of Steroids	NQO1; DHCR7
Butanoate Metabolism	ALDH1A1; NLGN1
Citrate Cycle	IDH2; IDH1
Fatty Acid Metabolism	ALDH1A1; CYP1B1
Glycerophospholipid	PRDX6; CHKA
Metabolism	
Histidine Metabolism	PRMT5; ALDH1A1
Inositol Metabolism	ERO1L; APEX1
Metabolism of Xenobiotics	GSTP1; CYP1B1
by Cytochrome p450	
Methane Metabolism	PRDX6; PRDX1
Phenylalanine Metabolism	PRDX6; PRDX1
Propanoate Metabolism	ALDH1A1; LDHA
Selenoamino Acid	PRMT5; AHCY
Metabolism	
Sphingolipid Metabolism	SPHK1; SPHK2
Aminophosphonate	PRMT5
Metabolism	
Androgen and Estrogen	PRMT5

CELLULAR FUNCTION	GENES
Metabolism	
Ascorbate and Aldarate	ALDH1A1
Metabolism	
Bile Acid Biosynthesis	ALDH1A1
Cysteine Metabolism	LDHA
Fatty Acid Biosynthesis	FASN
Glutamate Receptor	GNB2L1
Signaling	
NRF2-mediated Oxidative	PRDX1
Stress Response	
Pentose Phosphate	GPI
Pathway	
Pentose and Glucuronate	UCHL1
Interconversions	
Retinol Metabolism	ALDH1A1
Riboflavin Metabolism	TYR
Tyrosine Metabolism	PRMT5, TYR
Ubiquinone Biosynthesis	PRMT5
Valine, Leucine and	ALDH1A1
Isoleucine Degradation	
Glycine, Serine and	CHKA
Threonine Metabolism	
Lysine Degradation	ALDH1A1
Pain/Taste	TRPM5; TRPA1
Pain	TRPM7; TRPC5; TRPC6; TRPC1; Cnr1; cnr2; Grk2;
	Trpa1; Pomc; Cgrp; Crf; Pka; Era; Nr2b; TRPM5; Prkaca;
	Prkacb; Prkar1a; Prkar2a
Mitochondrial Function	AIF; CytC; SMAC (Diablo); Aifm-1; Aifm-2
Developmental Neurology	BMP-4; Chordin (Chrd); Noggin (Nog); WNT (Wnt2;
	Wnt2b; Wnt3a; Wnt4; Wnt5a; Wnt6; Wnt7b; Wnt8b;
	Wnt9a; Wnt9b; Wnt10a; Wnt10b; Wnt16); beta-catenin;
	Dkk-1; Frizzled related proteins; Otx-2; Gbx2; FGF-8;
	Reelin; Dab1; unc-86 (Pou4f1 or Brn3a); Numb; Reln

[00375] Embodiments of the invention also relate to methods and compositions related to knocking out genes, amplifying genes and repairing particular mutations associated with DNA repeat instability and neurological disorders (Robert D. Wells, Tetsuo Ashizawa, Genetic Instabilities and Neurological Diseases, Second Edition, Academic Press, Oct 13, 2011 – Medical). Specific aspects of tandem repeat sequences have been found to be responsible for more than twenty human diseases (New insights into repeat instability: role of RNA•DNA hybrids. McIvor EI, Polak U, Napierala M. RNA Biol. 2010 Sep-Oct;7(5):551-8). The CRISPR-Cas system may be harnessed to correct these defects of genomic instability.

[00376] A further aspect of the invention relates to utilizing the CRISPR-Cas system for correcting defects in the EMP2A and EMP2B genes that have been identified to be associated with Lafora disease. Lafora disease is an autosomal recessive condition which is characterized by progressive myoclonus epilepsy which may start as epileptic seizures in adolescence. A few cases of the disease may be caused by mutations in genes yet to be identified. The disease causes seizures, muscle spasms, difficulty walking, dementia, and eventually death. There is currently no therapy that has proven effective against disease progression. Other genetic abnormalities associated with epilepsy may also be targeted by the CRISPR-Cas system and the underlying genetics is further described in Genetics of Epilepsy and Genetic Epilepsies, edited by Giuliano Avanzini, Jeffrey L. Noebels, Mariani Foundation Paediatric Neurology:20; 2009).

[00377] The methods of US Patent Publication No. 20110158957 assigned to Sangamo BioSciences, Inc. involved in inactivating T cell receptor (TCR) genes may also be modified to the CRISPR Cas system of the present invention. In another example, the methods of US Patent Publication No. 20100311124 assigned to Sangamo BioSciences, Inc. and US Patent Publication No. 20110225664 assigned to Cellectis, which are both involved in inactivating glutamine synthetase gene expression genes may also be modified to the CRISPR Cas system of the present invention.

[00378] Several further aspects of the invention relate to correcting defects associated with a wide range of genetic diseases which are further described on the website of the National Institutes of Health under the topic subsection Genetic Disorders (website at health.nih.gov/topic/GeneticDisorders). The genetic brain diseases may include but are not limited to Adrenoleukodystrophy, Agenesis of the Corpus Callosum, Aicardi Syndrome, Alpers' Disease, Alzheimer's Disease, Barth Syndrome, Batten Disease, CADASIL, Cerebellar Degeneration, Fabry's Disease, Gerstmann-Straussler-Scheinker Disease, Huntington's Disease and other Triplet Repeat Disorders, Leigh's Disease, Lesch-Nyhan Syndrome, Menkes Disease, Mitochondrial Myopathies and NINDS Colpocephaly. These diseases are further described on the website of the National Institutes of Health under the subsection Genetic Brain Disorders.

[00379] In Cartier, "MINI-SYMPOSIUM: X-Linked Adrenoleukodystrophypa, Hematopoietic Stem Cell Transplantation and Hematopoietic Stem Cell Gene Therapy in X-

Linked Adrenoleukodystrophy," Brain Pathology 20 (2010) 857–862, incorporated herein by reference along with the documents it cites, as if set out in full, there is recognition that allogeneic hematopoietic stem cell transplantation (HSCT) was utilized to deliver normal lysosomal enzyme to the brain of a patient with Hurler's disease, and a discussion of HSC gene therapy to treat ALD. In two patients, peripheral CD34+cells were collected after granulocyte-colony stimulating factor (G-CSF) mobilization and transduced with an myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer binding site substituted (MND)-ALD lentiviral vector. CD34+ cells from the patients were transduced with the MND-ALD vector during 16 h in the presence of cytokines at low concentrations. Transduced CD34+ cells were frozen after transduction to perform on 5% of cells various safety tests that included in particular three replication-competent lentivirus (RCL) assays. Transduction efficacy of CD34+ cells ranged from 35% to 50% with a mean number of lentiviral integrated copy between 0.65 and 0.70. After the thawing of transduced CD34+ cells, the patients were reinfused with more than 4.10^6 transduced CD34+ cells/kg following full myeloablation with busulfan and cyclophos-phamide. The patient's HSCs were ablated to favor engraftment of the gene-corrected HSCs. Hematological recovery occurred between days 13 and 15 for the two patients. Nearly complete immunological recovery occurred at 12 months for the first patient, and at 9 months for the second patient. In contrast to using lentivirus, with the knowledge in the art and the teachings in this disclosure, the skilled person can correct HSCs as to ALD using a CRISPR-Cas9 system that targets and corrects the mutation (e.g., with a suitable HDR template); specifically, the sgRNA can target mutations in *ABCD1*, a gene located on the X chromosome that codes for ALD, a peroxisomal membrane transporter protein, and the HDR can provide coding for proper expression of the protein. From this disclosure an sgRNA that targets the mutation and a Cas9 protein can be contacted with an hematopoetic stem cell, and an HDR template introduced, for correction of the mutation for expression of peroxisomal membrane transporter protein.

[00380] In some embodiments, the condition may be neoplasia. In some embodiments, where the condition is neoplasia, the genes to be targeted are any of those listed in Table A (in this case PTEN and so forth). In some embodiments, the condition may be Age-related Macular Degeneration. In some embodiments, the condition may be a Schizophrenic Disorder. In some embodiments, the condition may be a Trinucleotide Repeat Disorder. In some embodiments, the condition may be Fragile X Syndrome. In some embodiments, the condition may be a Secretase

Related Disorder. In some embodiments, the condition may be a Prion - related disorder. In some embodiments, the condition may be ALS. In some embodiments, the condition may be a drug addiction. In some embodiments, the condition may be Autism. In some embodiments, the condition may be Alzheimer's Disease. In some embodiments, the condition may be inflammation. In some embodiments, the condition may be Parkinson's Disease.

[00381] For example, US Patent Publication No. 20110023145, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with autism spectrum disorders (ASD). Autism spectrum disorders (ASDs) are a group of disorders characterized by qualitative impairment in social interaction and communication, and restricted repetitive and stereotyped patterns of behavior, interests, and activities. The three disorders, autism, Asperger syndrome (AS) and pervasive developmental disorder-not otherwise specified (PDD-NOS) are a continuum of the same disorder with varying degrees of severity, associated intellectual functioning and medical conditions. ASDs are predominantly genetically determined disorders with a heritability of around 90%.

[00382] US Patent Publication No. 20110023145 comprises editing of any chromosomal sequences that encode proteins associated with ASD which may be applied to the CRISPR Cas system of the present invention. The proteins associated with ASD are typically selected based on an experimental association of the protein associated with ASD to an incidence or indication of an ASD. For example, the production rate or circulating concentration of a protein associated with ASD may be elevated or depressed in a population having an ASD relative to a population lacking the ASD. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the proteins associated with ASD may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00383] Non limiting examples of disease states or disorders that may be associated with proteins associated with ASD include autism, Asperger syndrome (AS), pervasive developmental disorder-not otherwise specified (PDD-NOS), Rett's syndrome, tuberous sclerosis, phenylketonuria, Smith-Lemli-Opitz syndrome and fragile X syndrome. By way of non-limiting example, proteins associated with ASD include but are not limited to the following proteins: ATP10C aminophospholipid- MET MET receptor transporting ATPase tyrosine kinase (ATP10C) BZRAP1 MGLUR5 (GRM5) Metabotropic glutamate receptor 5 (MGLUR5) CDH10 Cadherin-10 MGLUR6 (GRM6) Metabotropic glutamate receptor 6 (MGLUR6) CDH9 Cadherin-9 NLGN1 Neuroligin-1 CNTN4 Contactin-4 NLGN2 Neuroligin-2 CNTNAP2 Contactin-associated SEMA5A Neuroligin-3 protein-like 2 (CNTNAP2) DHCR7 7-dehydrocholesterol NLGN4X Neuroligin-4 X- reductase (DHCR7) linked DOC2A Double C2-like domain- NLGN4Y Neuroligin-4 Y- containing protein alpha linked DPP6 Dipeptidyl NLGN5 Neuroligin-5 aminopeptidase-like protein 6 EN2 engrailed 2 (EN2) NRCAM Neuronal cell adhesion molecule (NRCAM) MDGA2 fragile X mental retardation NRXN1 Neurexin-1 1 (MDGA2) FMR2 (AFF2) AF4/FMR2 family member 2 OR4M2 Olfactory receptor (AFF2) 4M2 FOXP2 Forkhead box protein P2 OR4N4 Olfactory receptor (FOXP2) 4N4 FXR1 Fragile X mental OXTR oxytocin receptor retardation, autosomal (OXTR) homolog 1 (FXR1) FXR2 Fragile X mental PAH phenylalanine retardation, autosomal hydroxylase (PAH) homolog 2 (FXR2) GABRA1 Gamma-aminobutyric acid PTEN Phosphatase and receptor subunit alpha-1 tensin homologue (GABRA1) (PTEN) GABRA5 GABAA (.gamma.-aminobutyric PTPRZ1 Receptor-type acid) receptor alpha 5 tyrosine-protein subunit (GABRA5) phosphatase zeta (PTPRZ1) GABRB1 Gamma-aminobutyric acid RELN Reelin receptor subunit beta-1 (GABRB1) GABRB3 GABAA (.gamma.-aminobutyric RPL10 60S ribosomal acid) receptor .beta.3 subunit protein L10 (GABRB3) GABRG1 Gamma-aminobutyric acid SEMA5A Semaphorin-5A receptor subunit gamma-1 (SEMA5A) (GABRG1) HIRIP3 HIRA-interacting protein 3 SEZ6L2 seizure related 6 homolog (mouse)- like 2 HOXA1 Homeobox protein Hox-A1 SHANK3 SH3 and multiple (HOXA1) ankyrin repeat domains 3 (SHANK3) IL6 Interleukin-6 SHBZRAP1 SH3 and multiple ankyrin repeat domains 3 (SHBZRAP1) LAMB1 Laminin subunit beta-1 SLC6A4 Serotonin (LAMB1) transporter (SERT) MAPK3 Mitogen-activated protein TAS2R1 Taste receptor kinase 3 type 2 member 1 TAS2R1 MAZ Myc-associated zinc finger TSC1 Tuberous sclerosis protein protein 1 MDGA2 MAM domain containing TSC2 Tuberous sclerosis glycosylphosphatidylinositol protein 2 anchor 2 (MDGA2) MECP2 Methyl CpG binding UBE3A Ubiquitin protein protein 2 (MECP2) ligase E3A (UBE3A) MECP2 methyl CpG binding WNT2 Wingless-type protein 2 (MECP2) MMTV integration site family, member 2 (WNT2)

[00384] The identity of the protein associated with ASD whose chromosomal sequence is edited can and will vary. In preferred embodiments, the proteins associated with ASD whose chromosomal sequence is edited may be the benzodiazapine receptor (peripheral) associated protein 1 (BZRAP1) encoded by the BZRAP1 gene, the AF4/FMR2 family member 2 protein (AFF2) encoded by the AFF2 gene (also termed MFR2), the fragile X mental retardation autosomal homolog 1 protein (FXR1) encoded by the FXR1 gene, the fragile X mental retardation autosomal homolog 2 protein (FXR2) encoded by the FXR2 gene, the MAM domain containing glycosylphosphatidylinositol anchor 2 protein (MDGA2) encoded by the MDGA2 gene, the methyl CpG binding protein 2 (MECP2) encoded by the MECP2 gene, the metabotropic glutamate receptor 5 (MGLUR5) encoded by the MGLUR5-1 gene (also termed GRM5), the neurexin 1 protein encoded by the NRXN1 gene, or the semaphorin-5A protein (SEMA5A) encoded by the SEMA5A gene. In an exemplary embodiment, the genetically modified animal is a rat, and the edited chromosomal sequence encoding the protein associated with ASD is as listed below: BZRAP1 benzodiazapine receptor XM_002727789, (peripheral) associated XM_213427, protein 1 (BZRAP1) XM_002724533, XM_001081125 AFF2 (FMR2) AF4/FMR2 family member 2 XM_219832, (AFF2) XM_001054673 FXR1 Fragile X mental NM_001012179 retardation, autosomal homolog 1 (FXR1) FXR2 Fragile X mental NM_001100647 retardation, autosomal homolog 2 (FXR2) MDGA2 MAM domain containing NM_199269 glycosylphosphatidylinositol anchor 2 (MDGA2) MECP2 Methyl CpG binding NM_022673 protein 2 (MECP2) MGLUR5 Metabotropic glutamate NM_017012 (GRM5) receptor 5 (MGLUR5) NRXN1 Neurexin-1 NM_021767 SEMA5A Semaphorin-5A (SEMA5A) NM_001107659

[00385] Exemplary animals or cells may comprise one, two, three, four, five, six, seven, eight, or nine or more inactivated chromosomal sequences encoding a protein associated with ASD, and zero, one, two, three, four, five, six, seven, eight, nine or more chromosomally integrated sequences encoding proteins associated with ASD. The edited or integrated chromosomal sequence may be modified to encode an altered protein associated with ASD. Non-limiting examples of mutations in proteins associated with ASD include the L18Q mutation in neurexin 1 where the leucine at position 18 is replaced with a glutamine, the R451C mutation in neuroligin 3 where the arginine at position 451 is replaced with a cysteine, the R87W mutation in neuroligin 4 where the arginine at position 87 is replaced with a tryptophan, and the I425V mutation in serotonin transporter where the isoleucine at position 425 is replaced with a valine. A number of other mutations and chromosomal rearrangements in ASD-related chromosomal sequences have been associated with ASD and are known in the art. See, for example, Freitag et al. (2010) Eur. Child. Adolesc. Psychiatry 19:169-178, and Bucan et al. (2009) PLoS Genetics 5: e1000536, the disclosure of which is incorporated by reference herein in its entirety. Examples of proteins associated with Parkinson's disease include but are not limited to α-synuclein, DJ-1, LRRK2, PINK1, Parkin, UCHL1, Synphilin-1, and NURR1. Examples of addiction-related proteins may include ABAT for example. Examples of inflammation-related proteins may include the monocyte chemoattractant protein-1 (MCP1) encoded by the Ccr2 gene, the C-C chemokine receptor type 5 (CCR5) encoded by the Ccr5 gene, the IgG receptor IIB (FCGR2b, also termed CD32) encoded by the Fcgr2b gene, or the Fc epsilon R1g (FCER1g) protein encoded by the Fcer1g gene, for example. Examples of cardiovascular diseases associated proteins may include IL1B (interleukin 1, beta), XDH (xanthine dehydrogenase), TP53 (tumor protein p53), PTGIS (prostaglandin I2 (prostacyclin) synthase), MB (myoglobin), IL4 (interleukin 4), ANGPT1 (angiopoietin 1), ABCG8 (ATP-binding cassette, sub-family G (WHITE), member 8), or CTSK (cathepsin K), for example. For example, US Patent Publication No. 20110023153, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with Alzheimer's Disease. Once modified cells and animals may be further tested using known methods to study the effects of the targeted mutations on the development and/or progression of AD using measures commonly used in the study of AD – such as, without limitation, learning and memory, anxiety, depression, addiction, and sensory motor functions as well as assays that measure behavioral, functional, pathological, metaboloic and biochemical function.

[00386] The present invention comprises editing of any chromosomal sequences that encode proteins associated with AD. The AD-related proteins are typically selected based on an experimental association of the AD-related protein to an AD disorder. For example, the production rate or circulating concentration of an AD-related protein may be elevated or depressed in a population having an AD disorder relative to a population lacking the AD disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the AD-related proteins may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR). Examples of Alzheimer's disease associated proteins may include the very low density lipoprotein receptor protein (VLDLR) encoded by the VLDLR gene, the ubiquitin-like modifier activating enzyme 1 (UBA1) encoded by the UBA1 gene, or the NEDD8-activating enzyme E1 catalytic subunit protein (UBE1C) encoded by the UBA3 gene, for example. By way of non-limiting example, proteins associated with AD include but are not limited to the proteins listed as follows: Chromosomal Sequence Encoded Protein ALAS2 Delta-aminolevulinate synthase 2 (ALAS2) ABCA1 ATP-binding cassette transporter (ABCA1) ACE Angiotensin I-converting enzyme (ACE) APOE Apolipoprotein E precursor (APOE) APP amyloid precursor protein (APP) AQP1 aquaporin 1 protein (AQP1) BIN1 Myc box-dependent-interacting protein 1 or bridging integrator 1 protein (BIN1) BDNF brain-derived neurotrophic factor (BDNF) BTNL8 Butyrophilin-like protein 8 (BTNL8) C1ORF49 chromosome 1 open reading frame 49 CDH4 Cadherin-4 CHRNB2 Neuronal acetylcholine receptor subunit beta-2 CKLFSF2 CKLF-like MARVEL transmembrane domain- containing protein 2 (CKLFSF2) CLEC4E C-type lectin domain family 4, member e (CLEC4E) CLU clusterin protein (also known as apoplipoprotein J) CR1 Erythrocyte complement receptor 1 (CR1, also known as CD35, C3b/C4b receptor and immune adherence receptor) CR1L Erythrocyte complement receptor 1 (CR1L) CSF3R granulocyte colony-stimulating factor 3 receptor (CSF3R) CST3 Cystatin C or cystatin 3 CYP2C Cytochrome P450 2C DAPK1 Death-associated protein kinase 1 (DAPK1) ESR1 Estrogen receptor 1 FCAR Fc fragment of IgA receptor (FCAR, also known as CD89) FCGR3B Fc fragment of IgG, low affinity IIIb, receptor (FCGR3B or CD16b) FFA2 Free fatty acid receptor 2 (FFA2) FGA Fibrinogen (Factor I) GAB2 GRB2-associated-binding protein 2 (GAB2) GAB2 GRB2-associated-binding protein 2 (GAB2) GALP Galanin-like peptide GAPDHS Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic (GAPDHS) GMPB GMBP HP Haptoglobin (HP) HTR7 5-hydroxytryptamine (serotonin) receptor 7 (adenylate cyclase-coupled) IDE Insulin degrading enzyme IF127 IF127 IFI6 Interferon, alpha-inducible protein 6 (IFI6) IFIT2 Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2) IL1RN interleukin-1 receptor antagonist (IL-1RA) IL8RA Interleukin 8 receptor, alpha (IL8RA or CD181) IL8RB Interleukin 8 receptor, beta (IL8RB) JAG1 Jagged 1 (JAG1) KCNJ15 Potassium inwardly-rectifying channel, subfamily J, member 15 (KCNJ15) LRP6 Low-density lipoprotein receptor-related protein 6 (LRP6) MAPT microtubule-associated protein tau (MAPT) MARK4 MAP/microtubule affinity-regulating kinase 4 (MARK4) MPHOSPH1 M-phase phosphoprotein 1 MTHFR 5,10-methylenetetrahydrofolate reductase MX2 Interferon-induced GTP-binding protein Mx2 NBN Nibrin, also known as NBN NCSTN Nicastrin NIACR2 Niacin receptor 2 (NIACR2, also known as GPR109B) NMNAT3 nicotinamide nucleotide adenylyltransferase 3 NTM Neurotrimin (or HNT) ORM1 Orosmucoid 1 (ORM1) or Alpha-1-acid glycoprotein 1 P2RY13 P2Y purinoceptor 13 (P2RY13) PBEF1 Nicotinamide phosphoribosyltransferase (NAmPRTase or Nampt) also known as pre-B-cell colony-enhancing factor 1 (PBEF1) or visfatin PCK1 Phosphoenolpyruvate carboxykinase PICALM phosphatidylinositol binding clathrin assembly protein (PICALM) PLAU Urokinase-type plasminogen activator (PLAU) PLXNC1 Plexin C1 (PLXNC1) PRNP Prion protein PSEN1 presenilin 1 protein (PSEN1) PSEN2 presenilin 2 protein (PSEN2) PTPRA protein tyrosine phosphatase receptor type A protein (PTPRA) RALGPS2 Ral GEF with PH domain and SH3 binding motif 2 (RALGPS2) RGSL2 regulator of G-protein signaling like 2 (RGSL2) SELENBP1 Selenium binding protein 1 (SELNBP1) SLC25A37 Mitoferrin-1 SORL1 sortilin-related receptor L(DLR class) A repeats-containing protein (SORL1) TF Transferrin TFAM Mitochondrial transcription factor A TNF Tumor necrosis factor TNFRSF10C Tumor necrosis factor receptor superfamily member 10C (TNFRSF10C) TNFSF10 Tumor necrosis factor receptor superfamily, (TRAIL) member 10a (TNFSF10) UBA1 ubiquitin-like modifier activating enzyme 1 (UBA1) UBA3 NEDD8-activating enzyme E1 catalytic subunit protein (UBE1C) UBB ubiquitin B protein (UBB) UBQLN1 Ubiquilin-1 UCHL1 ubiquitin carboxyl-terminal esterase L1 protein (UCHL1) UCHL3 ubiquitin carboxyl-terminal hydrolase isozyme L3 protein (UCHL3) VLDLR very low density lipoprotein receptor protein (VLDLR). In exemplary embodiments, the proteins associated with AD whose chromosomal sequence is edited may be the very low density lipoprotein receptor protein (VLDLR) encoded by the VLDLR gene, the ubiquitin-like modifier activating enzyme 1 (UBA1) encoded by the UBA1 gene, the NEDD8-activating enzyme E1 catalytic subunit protein (UBE1C) encoded by the UBA3 gene, the aquaporin 1 protein (AQP1) encoded by the AQP1 gene, the ubiquitin carboxyl-terminal esterase L1 protein (UCHL1) encoded by the UCHL1 gene, the ubiquitin carboxyl-terminal hydrolase isozyme L3 protein (UCHL3) encoded by the UCHL3 gene, the ubiquitin B protein (UBB) encoded by the UBB gene, the microtubule-associated protein tau (MAPT) encoded by the MAPT gene, the protein tyrosine phosphatase receptor type A protein (PTPRA) encoded by the PTPRA gene, the phosphatidylinositol binding clathrin assembly protein (PICALM) encoded by the PICALM gene, the clusterin protein (also known as apoplipoprotein J) encoded by the CLU gene, the presenilin 1 protein encoded by the PSEN1 gene, the presenilin 2 protein encoded by the PSEN2 gene, the sortilin-related receptor L(DLR class) A repeats-containing protein (SORL1) protein encoded by the SORL1 gene, the amyloid precursor protein (APP) encoded by the APP gene, the Apolipoprotein E precursor (APOE) encoded by the APOE gene, or the brain-derived neurotrophic factor (BDNF) encoded by the BDNF gene. In an exemplary embodiment, the genetically modified animal is a rat, and the edited chromosomal sequence encoding the protein associated with AD is as as follows: APP amyloid precursor protein (APP) NM_019288 AQP1 aquaporin 1 protein (AQP1) NM_012778 BDNF Brain-derived neurotrophic factor NM_012513 CLU clusterin protein (also known as NM_053021 apoplipoprotein J) MAPT microtubule-associated protein NM_017212 tau (MAPT) PICALM phosphatidylinositol binding NM_053554 clathrin assembly protein (PICALM) PSEN1 presenilin 1 protein (PSEN1) NM_019163 PSEN2 presenilin 2 protein (PSEN2) NM_031087 PTPRA protein tyrosine phosphatase NM_012763 receptor type A protein (PTPRA) SORL1 sortilin-related receptor L(DLR NM_053519, class) A repeats-containing XM_001065506, protein (SORL1) XM_217115 UBA1 ubiquitin-like modifier activating NM_001014080 enzyme 1 (UBA1) UBA3 NEDD8-activating enzyme E1 NM_057205 catalytic subunit protein (UBE1C) UBB ubiquitin B protein (UBB) NM_138895 UCHL1 ubiquitin carboxyl-terminal NM_017237 esterase L1 protein (UCHL1) UCHL3 ubiquitin carboxyl-terminal NM_001110165 hydrolase isozyme L3 protein (UCHL3) VLDLR very low density lipoprotein NM_013155 receptor protein (VLDLR). The animal or cell may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9,10, 11, 12, 13, 14, 15 or more disrupted chromosomal sequences encoding a protein associated with AD and zero, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more chromosomally integrated sequences encoding a protein associated with AD. The edited or integrated chromosomal sequence may be modified to encode an altered protein associated with AD. A number of mutations in AD-related chromosomal sequences have been associated with AD. For instance, the V7171 (i.e. valine at position 717 is changed to isoleucine) missense mutation in APP causes familial AD. Multiple mutations in the presenilin-1 protein, such as H163R (i.e. histidine at position 163 is changed to arginine), A246E (i.e. alanine at position 246 is changed to glutamate), L286V (i.e. leucine at position 286 is changed to valine) and C410Y (i.e. cysteine at position 410 is changed to tyrosine) cause familial Alzheimer's type 3. Mutations in the presenilin-2 protein, such as N141 I (i.e. asparagine at position 141 is changed to isoleucine), M239V (i.e. methionine at position 239 is changed to valine), and D439A (i.e. aspartate at position 439 is changed to alanine) cause familial Alzheimer's type 4. Other associations of genetic variants in AD-associated genes and disease are known in the art. See, for example, Waring et al. (2008) Arch. Neurol. 65:329-334, the disclosure of which is incorporated by reference herein in its entirety.

[00387] Examples of proteins associated Autism Spectrum Disorder may include the benzodiazapine receptor (peripheral) associated protein 1 (BZRAP1) encoded by the BZRAP1 gene, the AF4/FMR2 family member 2 protein (AFF2) encoded by the AFF2 gene (also termed MFR2), the fragile X mental retardation autosomal homolog 1 protein (FXR1) encoded by the FXR1 gene, or the fragile X mental retardation autosomal homolog 2 protein (FXR2) encoded by the FXR2 gene, for example.

[00388] Examples of proteins associated Macular Degeneration may include the ATP-binding cassette, sub-family A (ABC1) member 4 protein (ABCA4) encoded by the ABCR gene, the apolipoprotein E protein (APOE) encoded by the APOE gene, or the chemokine (C-C motif) Ligand 2 protein (CCL2) encoded by the CCL2 gene, for example.

[00389] Examples of proteins associated Schizophrenia may include NRG1, ErbB4, CPLX1, TPH1, TPH2, NRXN1, GSK3A, BDNF, DISC1, GSK3B, and combinations thereof.

[00390] Examples of proteins involved in tumor suppression may include ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), EGFR (epidermal growth factor receptor), ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2), ERBB3 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 3), ERBB4 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 4), Notch 1, Notch2, Notch 3, or Notch 4, for example.

[00391] Examples of proteins associated with a secretase disorder may include PSENEN (presenilin enhancer 2 homolog (C. elegans)), CTSB (cathepsin B), PSEN1 (presenilin 1), APP (amyloid beta (A4) precursor protein), APH1B (anterior pharynx defective 1 homolog B (C. elegans)), PSEN2 (presenilin 2 (Alzheimer disease 4)), or BACE1 (beta-site APP-cleaving enzyme 1), for example. For example, US Patent Publication No. 20110023146, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with secretase-associated disorders. Secretases are essential for processing pre-proteins into their biologically active forms. Defects in various components of the secretase pathways contribute to many disorders, particularly those with hallmark amyloidogenesis or amyloid plaques, such as Alzheimer's disease (AD).

[00392] A secretase disorder and the proteins associated with these disorders are a diverse set of proteins that effect susceptibility for numerous disorders, the presence of the disorder, the severity of the disorder, or any combination thereof. The present disclosure comprises editing of any chromosomal sequences that encode proteins associated with a secretase disorder. The proteins associated with a secretase disorder are typically selected based on an experimental association of the secretase--related proteins with the development of a secretase disorder. For example, the production rate or circulating concentration of a protein associated with a secretase disorder may be elevated or depressed in a population with a secretase disorder relative to a population without a secretase disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the protein associated with a secretase disorder may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR). By way of non-limiting example, proteins associated with a secretase disorder include PSENEN (presenilin enhancer 2 homolog (C. elegans)), CTSB (cathepsin B), PSEN1 (presenilin 1), APP (amyloid beta (A4) precursor protein), APH1B (anterior pharynx defective 1 homolog B (C. elegans)), PSEN2 (presenilin 2 (Alzheimer disease 4)), BACE1 (beta-site APP-cleaving enzyme 1), ITM2B (integral membrane protein 2B), CTSD (cathepsin D), NOTCH1 (Notch homolog 1, translocation-associated (Drosophila)), TNF (tumor necrosis factor (TNF superfamily, member 2)), INS (insulin), DYT10 (dystonia 10), ADAM17 (ADAM metallopeptidase domain 17), APOE (apolipoprotein E), ACE (angiotensin I converting enzyme (peptidyl-dipeptidase A) 1), STN (statin), TP53 (tumor protein p53), IL6 (interleukin 6 (interferon, beta 2)), NGFR (nerve growth factor receptor (TNFR superfamily, member 16)), IL1B (interleukin 1, beta), ACHE (acetylcholinesterase (Yt blood group)), CTNNB1 (catenin (cadherin-associated protein), beta 1, 88kDa), IGF1 (insulin-like growth factor 1 (somatomedin C)), IFNG (interferon, gamma), NRG1 (neuregulin 1), CASP3 (caspase 3, apoptosis-related cysteine peptidase), MAPK1 (mitogen-activated protein kinase 1), CDH1 (cadherin 1, type 1, E-cadherin (epithelial)), APBB1 (amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)), HMGCR (3-hydroxy-3-methylglutaryl-Coenzyme A reductase), CREB1 (cAMP responsive element binding protein 1), PTGS2 (prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)), HES1 (hairy and enhancer of split 1, (Drosophila)), CAT (catalase), TGFB1 (transforming growth factor, beta 1), ENO2 (enolase 2 (gamma, neuronal)), ERBB4 (v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)), TRAPPC10 (trafficking protein particle complex 10), MAOB (monoamine oxidase B), NGF (nerve growth factor (beta polypeptide)), MMP12 (matrix metallopeptidase 12 (macrophage elastase)), JAG1 (jagged 1 (Alagille syndrome)), CD40LG (CD40 ligand), PPARG (peroxisome proliferator-activated receptor gamma), FGF2 (fibroblast growth factor 2 (basic)), IL3 (interleukin 3 (colony-stimulating factor, multiple)), LRP1 (low density lipoprotein receptor-related protein 1), NOTCH4 (Notch homolog 4 (Drosophila)), MAPK8 (mitogen-activated protein kinase 8), PREP (prolyl endopeptidase), NOTCH3 (Notch homolog 3 (Drosophila)), PRNP (prion protein), CTSG (cathepsin G), EGF (epidermal growth factor (beta-urogastrone)), REN (renin), CD44 (CD44 molecule (Indian blood group)), SELP (selectin P (granule membrane protein 140 kDa, antigen CD62)), GHR (growth hormone receptor), ADCYAP1 (adenylate cyclase activating polypeptide 1 (pituitary)), INSR (insulin receptor), GFAP (glial fibrillary acidic protein), MMP3 (matrix metallopeptidase 3 (stromelysin 1, progelatinase)), MAPK10 (mitogen-activated protein kinase 10), SP1 (Sp1 transcription factor), MYC (v-myc myelocytomatosis viral oncogene homolog (avian)), CTSE (cathepsin E), PPARA (peroxisome proliferator-activated receptor alpha), JUN (jun oncogene), TIMP1 (TIMP metallopeptidase inhibitor 1), IL5 (interleukin 5 (colony-stimulating factor, eosinophil)), IL1A (interleukin 1, alpha), MMP9 (matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)), HTR4 (5-hydroxytryptamine (serotonin) receptor 4), HSPG2 (heparan sulfate proteoglycan 2), KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), CYCS (cytochrome c, somatic), SMG1 (SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)), IL1R1 (interleukin 1 receptor, type I), PROK1 (prokineticin 1), MAPK3 (mitogen-activated protein kinase 3), NTRK1 (neurotrophic tyrosine kinase, receptor, type 1), IL13 (interleukin 13), MME (membrane metallo-endopeptidase), TKT (transketolase), CXCR2 (chemokine (C-X-C motif) receptor 2), IGF1R (insulin-like growth factor 1 receptor), RARA (retinoic acid receptor, alpha), CREBBP (CREB binding protein), PTGS1 (prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)), GALT (galactose-1-phosphate uridylyltransferase), CHRM1 (cholinergic receptor, muscarinic 1), ATXN1 (ataxin 1), PAWR (PRKC, apoptosis, WT1, regulator), NOTCH2 (Notch homolog 2 (Drosophila)), M6PR (mannose-6-phosphate receptor (cation dependent)), CYP46A1 (cytochrome P450, family 46, subfamily A, polypeptide 1), CSNK1 D (casein kinase 1, delta), MAPK14 (mitogen-activated protein kinase 14), PRG2 (proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein)), PRKCA (protein kinase C, alpha), L1 CAM (L1 cell adhesion molecule), CD40 (CD40 molecule, TNF receptor superfamily member 5), NR1I2 (nuclear receptor subfamily 1, group I, member 2), JAG2 (jagged 2), CTNND1 (catenin (cadherin-associated protein), delta 1), CDH2 (cadherin 2, type 1, N-cadherin (neuronal)), CMA1 (chymase 1, mast cell), SORT1 (sortilin 1), DLK1 (delta-like 1 homolog (Drosophila)), THEM4 (thioesterase superfamily member 4), JUP (junction plakoglobin), CD46 (CD46 molecule, complement regulatory protein), CCL11 (chemokine (C-C motif) ligand 11), CAV3 (caveolin 3), RNASE3 (ribonuclease, RNase A family, 3 (eosinophil cationic protein)), HSPA8 (heat shock 70kDa protein 8), CASP9 (caspase 9, apoptosis-related cysteine peptidase), CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4), CCR3 (chemokine (C-C motif) receptor 3), TFAP2A (transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)), SCP2 (sterol carrier protein 2), CDK4 (cyclin-dependent kinase 4), HIF1A (hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)), TCF7L2 (transcription factor 7-like 2 (T-cell specific, HMG-box)), IL1R2 (interleukin 1 receptor, type II), B3GALTL (beta 1,3-galactosyltransferase-like), MDM2 (Mdm2 p53 binding protein homolog (mouse)), RELA (v-rel reticuloendotheliosis viral oncogene homolog A (avian)), CASP7 (caspase 7, apoptosis-related cysteine peptidase), IDE (insulin-degrading enzyme), FABP4 (fatty acid binding protein 4, adipocyte), CASK (calcium/calmodulin-dependent serine protein kinase (MAGUK family)), ADCYAP1R1 (adenylate cyclase activating polypeptide 1 (pituitary) receptor type I), ATF4 (activating transcription factor 4 (tax-responsive enhancer element B67)), PDGFA (platelet-derived growth factor alpha polypeptide), C21 or f33 (chromosome 21 open reading frame 33), SCG5 (secretogranin V (7B2 protein)), RNF123 (ring finger protein 123), NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)), CAV1 (caveolin 1, caveolae protein, 22 kDa), MMP7 (matrix metallopeptidase 7 (matrilysin, uterine)), TGFA (transforming growth factor, alpha), RXRA (retinoid X receptor, alpha), STX1A (syntaxin 1A (brain)), PSMC4 (proteasome (prosome, macropain) 26S subunit, ATPase, 4), P2RY2 (purinergic receptor P2Y, G-protein coupled, 2), TNFRSF21 (tumor necrosis factor receptor superfamily, member 21), DLG1 (discs, large homolog 1 (Drosophila)), NUMBL (numb homolog (Drosophila)-like), SPN (sialophorin), PLSCR1 (phospholipid scramblase 1), UBQLN2 (ubiquilin 2), UBQLN1 (ubiquilin 1), PCSK7 (proprotein convertase subtilisin/kexin type 7), SPON1 (spondin 1, extracellular matrix protein), SILV (silver homolog (mouse)), QPCT (glutaminyl-peptide cyclotransferase), HESS (hairy and enhancer of split 5 (Drosophila)), GCC1 (GRIP and coiled-coil domain containing 1), and any combination thereof. The genetically modified animal or cell may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more disrupted chromosomal sequences encoding a protein associated with a secretase disorder and zero, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more chromosomally integrated sequences encoding a disrupted protein associated with a secretase disorder.

[00393] Examples of proteins associated with Amyotrophic Lateral Sclerosis may include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof. For example, US Patent Publication No. 20110023144, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with amyotrophyic lateral sclerosis (ALS) disease. ALS is characterized by the gradual steady degeneration of certain nerve cells in the brain cortex, brain stem, and spinal cord involved in voluntary movement.

[00394] Motor neuron disorders and the proteins associated with these disorders are a diverse set of proteins that effect susceptibility for developing a motor neuron disorder, the presence of the motor neuron disorder, the severity of the motor neuron disorder or any combination thereof. The present disclosure comprises editing of any chromosomal sequences that encode proteins associated with ALS disease, a specific motor neuron disorder. The proteins associated with ALS are typically selected based on an experimental association of ALS--related proteins to ALS. For example, the production rate or circulating concentration of a protein associated with ALS may be elevated or depressed in a population with ALS relative to a population without ALS. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the proteins associated with ALS may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR). By way of non-limiting example, proteins associated with ALS include but are not limited to the following proteins: SOD1 superoxide dismutase 1, ALS3 amyotrophic lateral soluble sclerosis 3 SETX senataxin ALS5 amyotrophic lateral sclerosis 5 FUS fused in sarcoma ALS7 amyotrophic lateral sclerosis 7 ALS2 amyotrophic lateral DPP6 Dipeptidyl-peptidase 6 sclerosis 2 NEFH neurofilament, heavy PTGS1 prostaglandin- polypeptide endoperoxide synthase 1 SLC1A2 solute carrier family 1 TNFRSF10B tumor necrosis factor (glial high affinity receptor superfamily, glutamate transporter), member 10b member 2 PRPH peripherin HSP90AA1 heat shock protein 90 kDa alpha (cytosolic), class A member 1 GRIA2 glutamate receptor, IFNG interferon, gamma ionotropic, AMPA 2 S100B S100 calcium binding FGF2 fibroblast growth factor 2 protein B AOX1 aldehyde oxidase 1 CS citrate synthase TARDBP TAR DNA binding protein TXN thioredoxin RAPH1 Ras association MAP3K5 mitogen-activated protein (RalGDS/AF-6) and kinase 5 pleckstrin homology domains 1 NBEAL1 neurobeachin-like 1 GPX1 glutathione peroxidase 1 ICA1L islet cell autoantigen RAC1 ras-related C3 botulinum 1.69 kDa-like toxin substrate 1 MAPT microtubule-associated ITPR2 inositol 1,4,5- protein tau triphosphate receptor, type 2 ALS2CR4 amyotrophic lateral GLS glutaminase sclerosis 2 (juvenile) chromosome region, candidate 4 ALS2CR8 amyotrophic lateral CNTFR ciliary neurotrophic factor sclerosis 2 (juvenile) receptor chromosome region, candidate 8 ALS2CR11 amyotrophic lateral FOLH1 folate hydrolase 1 sclerosis 2 (juvenile) chromosome region, candidate 11 FAM117B family with sequence P4HB prolyl 4-hydroxylase, similarity 117, member B beta polypeptide CNTF ciliary neurotrophic factor SQSTM1 sequestosome 1 STRADB STE20-related kinase NAIP NLR family, apoptosis adaptor beta inhibitory protein YWHAQ tyrosine 3- SLC33A1 solute carrier family 33 monooxygenase/tryptoph (acetyl-CoA transporter), an 5-monooxygenase member 1 activation protein, theta polypeptide TRAK2 trafficking protein, FIG. 4 FIG. 4 homolog, SAC1 kinesin binding 2 lipid phosphatase domain containing NIF3L1 NIF3 NGG1 interacting INA internexin neuronal factor 3-like 1 intermediate filament protein, alpha PARD3B par-3 partitioning COX8A cytochrome c oxidase defective 3 homolog B subunit VIIIA CDK15 cyclin-dependent kinase HECW1 HECT, C2 and WW 15 domain containing E3 ubiquitin protein ligase 1 NOS1 nitric oxide synthase 1 MET met proto-oncogene SOD2 superoxide dismutase 2, HSPB1 heat shock 27 kDa mitochondrial protein 1 NEFL neurofilament, light CTSB cathepsin B polypeptide ANG angiogenin, HSPA8 heat shock 70 kDa ribonuclease, RNase A protein 8 family, 5 VAPB VAMP (vesicle- ESR1 estrogen receptor 1 associated membrane protein)-associated protein B and C SNCA synuclein, alpha HGF hepatocyte growth factor CAT catalase ACTB actin, beta NEFM neurofilament, medium TH tyrosine hydroxylase polypeptide BCL2 B-cell CLL/lymphoma 2 FAS Fas (TNF receptor superfamily, member 6) CASP3 caspase 3, apoptosis- CLU clusterin related cysteine peptidase SMN1 survival of motor neuron G6PD glucose-6-phosphate 1, telomeric dehydrogenase BAX BCL2-associated X HSF1 heat shock transcription protein factor 1 RNF19A ring finger protein 19A JUN jun oncogene ALS2CR12 amyotrophic lateral HSPA5 heat shock 70 kDa sclerosis 2 (juvenile) protein 5 chromosome region, candidate 12 MAPK14 mitogen-activated protein IL10 interleukin 10 kinase 14 APEX1 APEX nuclease TXNRD1 thioredoxin reductase 1 (multifunctional DNA repair enzyme) 1 NOS2 nitric oxide synthase 2, TIMP1 TIMP metallopeptidase inducible inhibitor 1 CASP9 caspase 9, apoptosis- XIAP X-linked inhibitor of related cysteine apoptosis peptidase GLG1 golgi glycoprotein 1 EPO erythropoietin VEGFA vascular endothelial ELN elastin growth factor A GDNF glial cell derived NFE2L2 nuclear factor (erythroid- neurotrophic factor derived 2)-like 2 SLC6A3 solute carrier family 6 HSPA4 heat shock 70 kDa (neurotransmitter protein 4 transporter, dopamine), member 3 APOE apolipoprotein E PSMB8 proteasome (prosome, macropain) subunit, beta type, 8 DCTN1 dynactin 1 TIMP3 TIMP metallopeptidase inhibitor 3 KIFAP3 kinesin-associated SLC1A1 solute carrier family 1 protein 3 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1 SMN2 survival of motor neuron CCNC cyclin C 2, centromeric MPP4 membrane protein, STUB1 STIP1 homology and U- palmitoylated 4 box containing protein 1 ALS2 amyloid beta (A4) PRDX6 peroxiredoxin 6 precursor protein SYP synaptophysin CABIN1 calcineurin binding protein 1 CASP1 caspase 1, apoptosis- GART phosphoribosylglycinami related cysteine de formyltransferase, peptidase phosphoribosylglycinami de synthetase, phosphoribosylaminoimi dazole synthetase CDK5 cyclin-dependent kinase 5 ATXN3 ataxin 3 RTN4 reticulon 4 C1QB complement component 1, q subcomponent, B chain VEGFC nerve growth factor HTT huntingtin receptor PARK7

Parkinson disease 7 XDH xanthine dehydrogenase GFAP glial fibrillary acidic MAP2 microtubule-associated protein protein 2 CYCS cytochrome c, somatic FCGR3B Fc fragment of IgG, low affinity IIIb, CCS copper chaperone for UBL5 ubiquitin-like 5 superoxide dismutase MMP9 matrix metallopeptidase SLC18A3 solute carrier family 18 9 ((vesicular acetylcholine), member 3 TRPM7 transient receptor HSPB2 heat shock 27 kDa potential cation channel, protein 2 subfamily M, member 7 AKT1 v-akt murine thymoma DERL1 Der1-like domain family, viral oncogene homolog 1 member 1 CCL2 chemokine (C--C motif) NGRN neugrin, neurite ligand 2 outgrowth associated GSR glutathione reductase TPPP3 tubulin polymerization- promoting protein family member 3 APAF1 apoptotic peptidase BTBD10 BTB (POZ) domain activating factor 1 containing 10 GLUD1 glutamate CXCR4 chemokine (C--X--C motif) dehydrogenase 1 receptor 4 SLC1A3 solute carrier family 1 FLT1 fms-related tyrosine (glial high affinity glutamate transporter), member 3 kinase 1 PON1 paraoxonase 1 AR androgen receptor LIF leukemia inhibitory factor ERBB3 v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 LGALS1 lectin, galactoside- CD44 CD44 molecule binding, soluble, 1 TP53 tumor protein p53 TLR3 toll-like receptor 3 GRIA1 glutamate receptor, GAPDH glyceraldehyde-3- ionotropic, AMPA 1 phosphate dehydrogenase GRIK1 glutamate receptor, DES desmin ionotropic, kainate 1 CHAT choline acetyltransferase FLT4 fms-related tyrosine kinase 4 CHMP2B chromatin modifying BAG1 BCL2-associated protein 2B athanogene MT3 metallothionein 3 CHRNA4 cholinergic receptor, nicotinic, alpha 4 GSS glutathione synthetase BAK1 BCL2-antagonist/killer 1 KDR kinase insert domain GSTP1 glutathione S-transferase receptor (a type III pi 1 receptor tyrosine kinase) OGG1 8-oxoguanine DNA IL6 interleukin 6 (interferon, glycosylase beta 2). The animal or cell may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more disrupted chromosomal sequences encoding a protein associated with ALS and zero, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more chromosomally integrated sequences encoding the disrupted protein associated with ALS. Preferred proteins associated with ALS include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof.

[00395] Examples of proteins associated with prion diseases may include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof.

[00396] Examples of proteins related to neurodegenerative conditions in prion disorders may include A2M (Alpha-2-Macroglobulin), AATF (Apoptosis antagonizing transcription factor), ACPP (Acid phosphatase prostate), ACTA2 (Actin alpha 2 smooth muscle aorta), ADAM22 (ADAM metallopeptidase domain), ADORA3 (Adenosine A3 receptor), or ADRA1D (Alpha-1D adrenergic receptor for Alpha-1D adrenoreceptor), for example.

[00397] Examples of proteins associated with Immunodeficiency may include A2M [alpha-2-macroglobulin]; AANAT [arylalkylamine N-acetyltransferase]; ABCA1 [ATP-binding cassette, sub-family A (ABC1), member 1]; ABCA2 [ATP-binding cassette, sub-family A (ABC1), member 2]; or ABCA3 [ATP-binding cassette, sub-family A (ABC1), member 3]; for example.

[00398] Examples of proteins associated with Trinucleotide Repeat Disorders include AR (androgen receptor), FMR1 (fragile X mental retardation 1), HTT (huntingtin), or DMPK (dystrophia myotonica-protein kinase), FXN (frataxin), ATXN2 (ataxin 2), for example. Examples of proteins associated with Neurotransmission Disorders include SST (somatostatin), NOS1 (nitric oxide synthase 1 (neuronal)), ADRA2A (adrenergic, alpha-2A-, receptor), ADRA2C (adrenergic, alpha-2C-, receptor), TACR1 (tachykinin receptor 1), or HTR2c (5-hydroxytryptamine (serotonin) receptor 2C), for example. Examples of neurodevelopmental-associated sequences include A2BP1 [ataxin 2-binding protein 1], AADAT [aminoadipate aminotransferase], AANAT [arylalkylamine N-acetyltransferase], ABAT [4-aminobutyrate aminotransferase], ABCA1 [ATP-binding cassette, sub-family A (ABC1), member 1], or ABCA13 [ATP-binding cassette, sub-family A (ABC1), member 13], for example. Further examples of preferred conditions treatable with the present system include may be selected from: Aicardi-Goutières Syndrome; Alexander Disease; Allan-Herndon-Dudley Syndrome; POLG-Related Disorders; Alpha-Mannosidosis (Type II and III); Alström Syndrome; Angelman; Syndrome; Ataxia-Telangiectasia; Neuronal Ceroid-Lipofuscinoses; Beta-Thalassemia; Bilateral Optic Atrophy and (Infantile) Optic Atrophy Type 1; Retinoblastoma (bilateral); Canavan Disease; Cerebrooculofacioskeletal Syndrome 1 [COFS1]; Cerebrotendinous Xanthomatosis; Cornelia de Lange Syndrome; MAPT-Related Disorders; Genetic Prion Diseases; Dravet Syndrome; Early-Onset Familial Alzheimer Disease; Friedreich Ataxia [FRDA]; Fryns Syndrome; Fucosidosis; Fukuyama Congenital Muscular Dystrophy; Galactosialidosis; Gaucher

Disease; Organic Acidemias; Hemophagocytic Lymphohistiocytosis; Hutchinson-Gilford Progeria Syndrome; Mucolipidosis II; Infantile Free Sialic Acid Storage Disease; PLA2G6-Associated Neurodegeneration; Jervell and Lange-Nielsen Syndrome; Junctional Epidermolysis Bullosa; Huntington Disease; Krabbe Disease (Infantile); Mitochondrial DNA-Associated Leigh Syndrome and NARP; Lesch-Nyhan Syndrome; LIS1-Associated Lissencephaly; Lowe Syndrome; Maple Syrup Urine Disease; MECP2 Duplication Syndrome; ATP7A-Related Copper Transport Disorders; LAMA2-Related Muscular Dystrophy; Arylsulfatase A Deficiency; Mucopolysaccharidosis Types I, II or III; Peroxisome Biogenesis Disorders, Zellweger Syndrome Spectrum; Neurodegeneration with Brain Iron Accumulation Disorders; Acid Sphingomyelinase Deficiency; Niemann-Pick Disease Type C; Glycine Encephalopathy; ARX-Related Disorders; Urea Cycle Disorders; COL1A1/2-Related Osteogenesis Imperfecta; Mitochondrial DNA Deletion Syndromes; PLP1-Related Disorders; Perry Syndrome; Phelan-McDermid Syndrome; Glycogen Storage Disease Type II (Pompe Disease) (Infantile); MAPT-Related Disorders; MECP2-Related Disorders; Rhizomelic Chondrodysplasia Punctata Type 1; Roberts Syndrome; Sandhoff Disease; Schindler Disease - Type 1; Adenosine Deaminase Deficiency; Smith-Lemli-Opitz Syndrome; Spinal Muscular Atrophy; Infantile-Onset Spinocerebellar Ataxia; Hexosaminidase A Deficiency; Thanatophoric Dysplasia Type 1; Collagen Type VI-Related Disorders; Usher Syndrome Type I; Congenital Muscular Dystrophy; Wolf-Hirschhorn Syndrome; Lysosomal Acid Lipase Deficiency; and Xeroderma Pigmentosum.

[00399] As will be apparent, it is envisaged that the present system can be used to target any polynucleotide sequence of interest. Some examples of conditions or diseases that might be usefully treated using the present system are included in the Tables herein and examples of genes currently associated with those conditions are also provided there. However, the genes exemplified are not exhaustive.

EXAMPLES

[00400] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1: In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9

[00401] The publication by Swiech, L. et al. entitled "In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9." Nat Biotechnol. 2014 Oct 19. doi: 10.1038/nbt.3055. [Epub ahead of print] is incoroporated herein by reference. This work presents the following main points:

- First demonstration of successful AAV-mediated Cas9 delivery in vivo as well as efficient genome modification in post-mitotic neurons;
- Development of a nuclear tagging technique which enables easy isolation of neuronal nuclei from Cas9 and sgRNA-expressing cells;
- Demonstration of application toward RNAseq analysis of neuronal transcriptome;
- Integration of electrophysiological studies with Cas9-mediated genome perturbation; and
- And demonstration of multiplex targeting and the ability to study gene function on rodent behavior using Cas9-mediated genome editing.

[00402] Transgenic animal models carrying disease-associated mutations are enormously useful for the study of neurological disorders, helping to elucidate the genetic and pathophysiological mechanism of disease. However, generation of animal models that carry single or multiple genetic modifications is particularly labor intensive and requires time-consuming breeding over many generations. Therefore, to facilitate the rapid dissection of gene function in normal and disease-related brain processes Applicants need ability to precisely and efficiently manipulate the genome of neurons in vivo. The CRISPR-associated endonuclease Cas9 from Streptococcus pyogenes (SpCas9) has been shown to mediate precise and efficient genome cleavage of single and multiple genes in replicating eukaryotic cells, resulting in frame shifting insertion/deletion (indel) mutations. Here, Applicants integrate Cas9-mediated genome perturbation with biochemical, sequencing, electrophysiological, and behavioral readouts to study the function of individual as wells as groups of genes in neural processes and their roles in brain disorders in vivo.

Discussion

[00403] Adeno-associated viral (AAV) vectors are commonly used to deliver recombinant genes into the mouse brain. The main limitation of the AAV system is its small packaging size, capped at approximately 4.5 kb without ITRs, which limits the amount of genetic material that can be packaged into a single vector. Since the size of the SpCas9 is already 4.2 kb, leaving less than 0.3 kb for other genetic elements within a single AAV vector, Applicants designed a dual-vector system that packages SpCas9 (AAV-SpCas9) and sgRNA expression cassettes (AAV-SpGuide) on two separate viral vectors (Figure 1). While designing the AAV-SpCas9 vector, Applicants compared various short neuron-specific promoters as well as poly adenylation signals to optimize SpCas9 expression. For Applicants' final design Applicants chose the mouse Mecp2 promoter (235 bp, pMecp2) and a minimal polyadenylation signal (48 bp, spA) based on their ability to achieve sufficient levels of SpCas9 expression in cultured primary mouse cortical neurons (Figure 5c). To facilitate immunofluorescence identification of SpCas9-expressing neurons, Applicants tagged SpCas9 with a HA-epitope tag. For the AAV-SpGuide vector, Applicants packaged an U6-sgRNA expression cassette as well as the green fluorescent protein (GFP)-fused with the KASH nuclear trans-membrane domain9 driven by the human Synapsin I promoter (Figure 1a). The GFP-KASH fusion protein directs GFP to the outer nuclear membrane (Figure 5c,d) and enables fluorescence-based identification and purification of intact neuronal nuclei transduced by AAV-SpGuide.

[00404] To test the delivery efficacy of Applicants' dual-vector delivery system, Applicants first transduced cultured primary mouse cortical neurons in vitro and observed robust expression by AAV-SpCas9 and AAV-SpGuide (Figure 5c), with greater than 80% co-transduction efficiency (Figure 5e). Importantly, compared with un-transduced neurons, expression of SpCas9 did not adversely affect the morphology and survival rate of transduced neurons (Figure 5c,f).

[00405] Having established an efficient delivery system, Applicants next sought to test SpCas9-mediated genome editing in mouse primary neurons. Whereas SpCas9 has been used to achieve efficient genome modifications in a variety of dividing cell types, it is unclear whether SpCas9 can be used to efficiently achieve genome editing in post-mitotic neurons. For Applicants' initial test Applicants targeted the Mecp2 gene, which plays a principal role in Rett syndrome, a type of autism spectrum disorder. MeCP2 protein is ubiquitously expressed in neurons throughout the brain but nearly absent in glial cells and its deficiency has been shown to be associated with severe morphological and electrophysiological phenotypes in neurons, and both are believed to contribute to the neurological symptoms observed in patients with Rett syndrome. To target Mecp2, Applicants first designed several sgRNAs targeting exon 3 of the mouse Mecp2 gene (Figure 6a) and evaluated their efficacy using Neuro-2a cells. The most efficient sgRNA was identified using the SURVEYOR nuclease assay (Figure 6b). Applicants chose the most effective sgRNA (Mecp2 target 5) for subsequent in vitro and in vivo Mecp2 targeting experiments.

[00406] To assess the editing efficiency of Applicants' dual-vector system in neurons, Applicants transduced primary mouse cortical neurons at 7 days in vitro (7 DIV, Figure 7a) and measured indel rate using the SURVEYOR nuclease assay 7 days post transduction (Figure 7b). Of note, neuron culture co-transduced with AAV-SpCas9 and AAV-SpGuide targeting Mecp2 showed up to 80% reduction in MeCP2 protein levels compared to control neurons (Figure 7c,d). One possible explanation for the observed discrepancy between relatively low indel frequency (~14%) and robust protein depletion (~80%) could be that mere binding by SpCas9 at the target site may interfere with transcription, which has been shown in E. coli. Applicants investigated this possibility using a mutant of SpCas9 with both RuvC and HNH catalytic domains inactivated (D10A and H840A, dSpCas9). Co-expression of dSpCas9 and Mecp2-targeting sgRNA did not reduce MeCP2 protein levels (Figure 7a,d), suggesting that the observed decrease of MeCP2 level in presence of active SpCas9 is due to occurrence of modification in the Mecp2 locus. Another possible explanation for the discrepancy between the low level of detected indel and high level of protein depletion may be due to underestimation of the true indel rate by the SURVEYOR nuclease assay – the detection accuracy of SURVEYOR has been previously shown to be sensitive to the indel sequence composition

[00407] MeCP2 loss-of-function has been previously shown to be associated with dendritic tree abnormalities and spine morphogenesis defects in neurons. These phenotypes of MeCP2 deprivation have also been reproduced in neurons differentiated from MeCP-KO iPS cells. Therefore, Applicants investigated whether SpCas9-mediated MeCP2-depletion in neurons can similarly recapitulate morphological phenotypes of Rett syndrome. Indeed, neurons co-expressing SpCas9 and Mecp2-targeting sgRNA exhibited altered dendritic tree morphology and spine density when compared with control neurons (Figure 8). These results demonstrate that SpCas9 can be used to facilitate the study of gene functions in cellular assays by enabling targeted knockout in post-mitotic neurons.

[00408] Given the complexity of the nervous system, which consists of intricate networks of heterogeneous cell types, being able to efficiently edit the genome of neurons in vivo would enable direct testing of gene function in relevant cell types embedded in native contexts. Consequently, Applicants stereotactically injected a mixture (1:1 ratio) of high titer AAV-SpCas9 and AAV-SpGuide into the hippocampal dentate gyrus in adult mice. Applicants observed high co-transduction efficiency of both vectors (over 80%) in hippocampal granule cells at 4 weeks after viral injection (Figure 1b,c) resulting in genomic modifications of the Mecp2 locus. (Figure 1d). Using SURVEYOR nuclease assay Applicants detected ~13% indel frequency in brain punches obtained from injected brain regions (Figure 1e). Similar to Applicants' finding in cultured primary neurons, SpCas9-mediated cutting of the Mecp2 locus efficiently decreased MeCP2 protein levels by over 60% (Fig. 1f). Additionally the number of MeCP2-positive nuclei in the dentate gyrus decreased by over 75% when injected with AAV-SpCas9 and AAV-SpGuide compared to AAV-SpCas9 alone (Figure 1g-h). These results suggest that SpCas9 can be used to directly perturb specific genes within intact biological contexts.

[00409] Targeted genomic perturbations can be coupled with quantitative readouts to provide insights into the biological function of specific genomic elements. To facilitate analysis of AAV-SpCas9 and AAV-SpGuide transduced cells, Applicants developed a method to purify GFP-KASH labeled nuclei using fluorescent activated cell sorting (FACS) (Figure 2a). Sorted nuclei can be directly used to purify nuclear DNA and RNA for downstream biochemical or sequencing analysis. Using sanger sequencing, Applicants found that 13 out of 14 single GFP-positive nuclei contained an indel mutation at the sgRNA target site.

[00410] In addition to genomic DNA sequencing, purified GFP-positive nuclei can also be used for RNAseq analysis to study transcriptional consequences of MeCP2 depletion (Figure 2b and Figure 9). To test the effect of Mecp2 knockout on transcription of neurons from the dentate gyrus, Applicants prepared RNAseq libraries using FACS purified GFP^+ nuclei from animals receiving AAV-SpCas9 as well as either a control sgRNA that has been designed to target bacterial lacZ gene and not the mouse genome, or a Mecp2-targeting sgRNA. All sgRNAs have been optimized to minimize their off-target score (CRISPR Design Tool: http://tools.genome-engineering.org). Applicants were able to find differentially expressed genes (Figure 2b) between control and Mecp2 sgRNA expressing nuclei ($p<0.01$). Applicants identified several interesting candidates among genes that were down-regulated in Mecp2 sgRNA expressing nuclei: Hpca, Olfm1, and Ncdn, which have been previously reported to play important roles in learning behaviors; and Cplx2, which has been shown to be involved in synaptic vesicle release and related to neuronal firing rate. These results demonstrate that the combination of SpCas9-mediated genome perturbation and population level RNAseq analysis provides a way to characterize transcriptional regulations in neurons and suggest genes that may be important to specific neuronal functions or disease processes.

[00411] SpCas9-mediated in vivo genome editing in the brain can also be coupled with electrophysiological recording to study the effect of genomic perturbation on specific cell types or circuit components. To study the functional effect of MeCP2 depletion on neuronal physiology Applicants stereotactically co-delivered AAV-SpCas9 and AAV-SpGuide targeting Mecp2 into the superficial layer of the primary visual cortex (V1) of male mice. V1 was chosen since the superficial layer cortical excitatory neurons are more accessible to two-photon imaging and two-photon guided targeted recording. Two weeks after SpCas9 delivery, mice were subjected to two-photon guided juxtacellular recordings (Figure 3) to compare the electrophysiological response of KASH-GFP$^+$ neurons and GFP$^-$ neighboring neurons in layer 2/3 of mouse V1 (Figure 3a-c). Applicants measured neuronal responses to 18 drifting gratings in 20-degree increments and calculated evoked firing rate (FR) and orientation selectivity index (OSI) of cells by vector averaging the response. Both FR and OSI were significantly reduced for excitatory GFP$^+$, MeCP2 knockout neurons, compared to neighboring GFP$^-$ excitatory neurons (Figure 3d-e). In comparison, control sgRNA expression together with SpCas9 did not have any effect on FR and OSI when compared with neighboring uninfected neurons (Figure 3d-e). These results show that SpCas9 mediated depletion of MeCP2 in adult V1 cortical neurons alters the visual response properties of excitatory neurons in vivo within two weeks and further demonstrate the versatility of SpCas9 in facilitating targeted gene knockout in the mammalian brain in vivo, for studying genes functions and dissection of neuronal circuits.

[00412] One key advantage of the SpCas9 system is its ability to facilitate multiplex genome editing. Introducing stable knockouts of multiple genes in the brain of living animals will have potentially far-reaching applications, such as causal interrogation of multigenic mechanisms in physiological and neuropathological conditions. To test the possibility of multiplex genome editing in the brain Applicants designed a multiplex sgRNA expression vector consisting of three sgRNAs in tandem, along with GFP-KASH for nuclei labeling (Figure 4a). Applicants chose sgRNAs targeting the DNA methyltransferases gene family (DNMTs), which consists of Dnmt1, Dnmt3a and Dnmt3b. Dnmt1 and 3a are highly expressed in the adult brain and it was previously shown that DNMT activity alters DNA methylation and both Dnmt3a and Dnmt1 are required for synaptic plasticity and learning and memory formation. Applicants designed individual sgRNAs against Dnmt3a and Dnmt1 with high modification efficiency. To avoid any potential compensatory effects by Dnmt3b Applicants decided also to additionally target this gene even though it is expressed mainly during neurodevelopment [27]. Applicants finally selected individual sgRNAs for high simultaneous DNA cleavage for all three targeted genes (Figure 4b and Figure 10).

[00413] To test the efficacy of multiplex genome editing in vivo, Applicants stereotactically delivered a mixture of high titer AAV-SpCas9 and AAV-SpGuide into the dorsal and ventral dentate gyrus of male adult mice. After 4 weeks, hippocampi were dissected and targeted cell nuclei were sorted via FACS. Applicants detected ~19% (Dnmt3a), 18% (Dnmt1) and 4% (Dnmt3b) indel frequency in the sorted nuclei population using SURVEYOR nuclease assay (Figure 4c) and sequencing (Figure 11). Targeting multiple loci raises the question about the effective rate of multiple-knockouts in individual cells. By using single nuclei sorting combined with targeted sequencing, Applicants quantified simultaneous targeting of multiple DNMT loci in individual neuronal nuclei (Fig. 4d). Of neuronal nuclei carrying modification in at least one Dnmt locus, more than 70% of nuclei contained indels in both Dnmt3a and Dnmt1 (~40% contained indels at all 3 loci, and ~30% at both Dnmt3a and Dnmt1 loci). These results are in agreement with Dnmt3a and Dnmt1 protein depletion levels in the dentate gyrus (Figure 4e). Due to the low expression of Dnmt3b in the adult brain, Applicants were not able to detect Dnmt3b protein.

[00414] Recent studies with SpCas9 have shown that, although each base within the 20-nt sgRNA sequence contributes to overall specificity, genomic loci that partially match the sgRNA can result in off-target double strand brakes and indel formations. To assess the rate of off-target modifications, Applicants computationally identified a list of highly similar genomic target sites[2] and quantified the rate of modifications using targeted deep sequencing. Indel analysis of the top predicted off-target loci revealed a 0-1.6% rate of indel formations demonstrating that SpCas9 modification is specific (Supplementary Table 1). To increase the specificity of SpCas9-mediated genome editing in vivo, future studies may use off-targeting minimization strategies such as double nicking and truncated sgRNAs.

[00415] Knockdown of Dnmt3a and Dnmt1 have been previous shown to impact hippocampus-dependent memory formation[27]. Consequently, Applicants performed contextual fear-conditioning behavior tests to investigate the effect of SpCas9-mediated triple knockout (Dnmt3a, Dnmt1 and Dnmt3b) on memory acquisition and consolidation. While Applicants did not observe any differences between control and triple knockout mice in the memory acquisition phase, knockout mice showed impaired memory consolidation when tested under trained context conditions (Figure 4f). This effect was abolished when mice were tested in the altered context. Applicants' results demonstrate that CRIPSR-Cas9-mediated knockout of DNMT family members in dentate gyrus neurons is sufficient to probe the function of genes in behavioral tasks.

[00416] Applicants' results demonstrate that AAV-mediated in vivo delivery of SpCas9 and sgRNA provides a rapid and powerful technology for achieving precise genomic perturbations within intact neural circuits. Whereas SpCas9 has been broadly used to engineer dividing cells, Applicants demonstrate that SpCas9 can also be used to engineer the genome of postmitotic neurons with high efficiency via NHEJ-mediated indel generation. SpCas9-mediated genomic perturbations can be combined with biochemical, sequencing, electrophysiological, and behavioral analysis to study the function of the targeted genomic element. Applicants demonstrated that SpCas9-mediated targeting of single or multiple genes can recapitulate morphological, electrophysiological, and behavioral phenotypes observed using classical, more time-consuming genetic mouse models. The current study employed the Streptococcus pyogenes Cas9, which not only necessitates the use of two AAV vectors but also limits the size of promoter elements can be used to achieve cell type-specific targeting. Given the diversity of Cas9 orthologues, with some being substantially shorter than SpCas9, it should be possible to engineer single AAV vectors expressing both Cas9 and sgRNA, as described herein.

Methods

DNA constructs

[00417] For SpCas9 targets selection and generation of single guide RNA (sgRNA), the 20-nt target sequences were selected to precede a 5′-NGG PAM sequence. To minimize off-targeting effects, the CRIPSR design tool was used (http://tools.genome-engineering.org). sgRNA was PCR amplified using U6 promoter as a template with forward primer: 5'-cgcacgcgtaattcgaacgctgacgtcatc-3' and reverse primer containing the sgRNA with 20-nt DNA target site (Bold): 5'-cacacgcgtAAAAAgcaccgactcggtgccactttttcaagttgataacg

gactagccttattttaacttgctaTTTCtagctctaaaacNNNNNNNNNNNNNNNNNNNNCGGTGTTTCGTCCTTTCCAC-3'. (SEQ ID NO:) Control sgRNA sequence was designed to target lacZ gene from E. coli:

target sequence: TGCGAATACGCCCACGCGATGGG (SEQ ID NO:)

[00418] EGFP-KASH construct was a generous gift from Prof. Worman (Columbia University, NYC) and was used as PCR template for cloning the coding cassette into AAV backbone under the human Synapsin promoter (hSyn). Next, U6-Mecp2sgRNA coding sequence was introduced using MluI site. For the multiplex gene targeting strategy, individual sgRNAs were PCR amplified as described above. All three sgRNAs were ligated with PCR amplified hSyn-GFP-KASH-bGHpA cassette (see Figure. 1A) by using the Golden Gate cloning strategy. After PCR amplification, the Golden Gate ligation product containing 3 sgRNAs and hSyn-GFP-KASH-bGH pA was cloned into AAV backbone. All obtained constructs were sequenced verified. In order to find the optimal promoter sequence to drive SpCas9 expression in neurons Applicants tested: hSyn1, mouse truncated Mecp2 (pMecp2), and truncated rat Map1b (pMap1b) promoter sequences[2] (see Figure 5a). Following primers were used to amplify promoter regions:

hSyn_F: 5'-GTGTCTAGACTGCAGAGGGCCCTG-3'; (SEQ ID NO:)

hSyn_R: 5'-GTGTCGTGCCTGAGAGCGCAGTCGAGAA-3'; (SEQ ID NO:)

Mecp2_F 5'-gagaagcttAGCTGAATGGGGTCCGCCTC-3'; (SEQ ID NO:)

Mecp2_R 5'-ctcaccggtGCGCGCAACCGATGCCGGGACC-3'; (SEQ ID NO:)

Map1b-283/-58_F 5'-gagaagcttGGCGAAATGATTTGCTGCAGATG-3'; (SEQ ID NO:)

Map1b-283/-58_R 5'-ctcaccggtGCGCGCGTCGCCTCCCCCTCCGC-3'. (SEQ ID NO:)

[00419] Another truncation of rat map1b promoter was assembled with the following oligos:

5'-agcttCGCGCCGGGAGGAGGGGGACGCAGTGGCGGAGCGGAGACAGCACCTTCGGAGATAATCCTTTCTCCTGCCGCAGAGCAGAGGAGCGGCGGGAGAGGAACACTTCTCCCAGGCTTTAGCAGAGCCGGa-3' and

5'-ccggtCCGGCTCTGCTAAAGCCTGGGAGAAGTGTTCCTCTCCCGCCGCTCCTCTGCTCTGCGGCAGGAGAAAGGATTATCTCCGAAGGTGCTGTCTCCGCTCCGCCCACTGCGTCCCCCCTCCTCCCGGCGCGa-3'. (SEQ ID NO:)

[00420] Short synthetic polyadenylation signal (spA)[3] was assembled using following oligos:

5'-aattcAATAAAAGATCTTTATTTTCATTAGATCTGTGTGTTGGTTTTTTG TGTgc-3' (SEQ ID NO:) and

5'-ggccgcACACAAAAAACCAACACACAGATCTAATGAAAATAAAGAT CTTTTATTg-3'. (SEQ ID NO:)

[00421] SpCas9 and its D10A mutant version (dSpCas9) were described previously[4, 5]. Plasmid encoding red fluorescent protein (mCherry) under control of EF1α promoter was used for neuron transfection with Lipofectamine®2000 (Life Technologies).

Cell line cultures and transfection

[00422] Neuro-2a (N2a) cells were grown in DMEM containing 5% fetal bovine serum (BSA). For HEK293FT cells DMEM containing 10% fetal bovine serum (FBS) was used. Cells were maintained at 37°C in 5% CO_2 atmosphere. Cells were transfected using Lipofectamine®2000 or Polyethylenimine (PEI) "MAX" reagent (Polysciences), according to manufacturer's protocols.

Production of concentrated AAV vectors

[00423] High titer AAV1/2 particles were produced using AAV1 and AAV2 serotype plasmids at equal ratios and pDF6 helper plasmid and purified on heparin affinity column[6]. Titering of viral particles was done by qPCR. High titer AAV1 particles were produced by the UNC Vector Core Services (University of North Carolina at Chapel Hill). Low titer AAV1 particles in DMEM were produced as described previously[7]. Briefly, HEK293FT cells were transfected with transgene plasmid, pAAV1 serotype plasmid and pDF6 helper plasmid using PEI "MAX". Culture medium was collected after 48 h and filtered through a 0.45 µm PVDF filter (Millipore).

Primary cortical neuron culture

[00424] Animals used to obtain neurons for tissue cultures were sacrificed according to the protocol approved by the MIT Committee on Animal Care (MIT CAC). Primary cultures were prepared from embryonic day 16 mouse brains[8]. Embryos of either sex were used. Cells were plated on poly-D-lysine (PDL) coated 24-well plates (BD Biosciences) or laminin/PDL coated coverslips (VWR). Cultures were grown at 37°C and 5% CO_2 in Neurobasal medium, supplemented with B27, Glutamax (Life Technologies) and penicillin/streptomycin mix. For AAV transduction, cortical neurons in 500 µl Neurobasal culture medium were incubated at 7 DIV with 300 µl (double infection at 1:1 ratio) AAV1-containing conditioned medium from HEK293FT cells[7]. One week after transduction neurons have been harvested for downstream

processing or fixed in 4% paraformaldehyde for immunofluorescent stainings or morphology analysis.

[00425] For visualization of neuronal morphology, cells at DIV7 were transfected with EF1α-mCherry expression vector using Lipofectamine®2000 (Life Technologies) for one week as previously described[9]. For measurement of total dendrite length, all dendrites of individual neurons were traced using ImageJ software. Quantification of the number of primary dendrites, dendritic tips and the Sholl analysis[10] were performed on images acquired with fluorescent microscope at a 40x objective (Zeiss AxioCam Ax10 microscope, Axiocam MRm camera). For dendrites number, ends of all non-axonal protrusions longer than 10 μm were counted. For Sholl analysis, concentric circles with 5 μm step in diameter were automatically drawn around the cell body, and the number of dendrites crossing each circle was counted using ImageJ software with a Sholl plug-in.

Stereotactic injection of AAV1/2 into the mouse brain

[00426] The MIT CAC approved all animal procedures described here. Adult (12-16 weeks old) male C57BL/6N mice were anaesthetized by intraperitoneal (i.p.) injection of 100 mg/kg Ketamine and 10 mg/kg Xylazine. Pre-emptive analgesia was given (Buprenex, 1 mg/kg, i.p.). Craniotomy was performed according to approved procedures and 1 μl of 1:1 AAV mixture (1x1013 Vg/ml of sMecp2-SpCas9; 6x1012 Vg/ml of DNMT 3xsgRNA; 3-5x1012 Vg/ml of hSyn-GFP-KASH) was injected into: dorsal dentate gyrus (anterior/posterior: -1.7; mediolateral: 0.6; dorsal/ventral: -2.15) and/or ventral dentate gyrus (anterior/posterior: -3.52; mediolateral: 2.65; dorsal/ventral: -3). For in vivo electrophysiology recordings experiments (Fig. 3) virus injection coordinates were 3 mm lateral (from Bregma) and 1 mm anterior from the posterior suture. The skull was thinned using a dremel drill with occasional cooling with saline, and the remaining dura was punctured using a glass micropipette filled with the virus suspended in mineral oil. Several injections (3-4) were made at neighboring sites, at a depth of 200-250 μm. A volume of 150-200 nl of virus mixture was injected at 75 nl/min rate at each site. After each injection, the pipette was held in place for 3-5 minutes prior to retraction to prevent leakage. The incision was sutured and proper post-operative analgesics (Meloxicam, 1-2 mg/kg) were administered for three days following surgery.

In vivo two-photon guided targeted loose patch recordings

[00427] Two weeks after virus injection, mice were used for electrophysiology experiments. Mice were anesthetized with 2% isoflurane and maintained using 0.8% isoflurane. The skin was excised, cleaned with sugi and a metal head plate was attached to the skull using glue and dental acrylic, and a 2 mm x 2 mm craniotomy was performed over the primary visual cortex (V1). The exposed area was then covered with a thin layer of 1.5% agarose in artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 0.01 mM EDTA, 10 mM HEPES, 10 mM glucose; pH 7.4). Animal body temperature was maintained during experiment 37.5°C with a heating blanket. Borosilicate pipettes (WPI) were pulled using a Sutter P-2000 laser puller (Sutter Instruments). Tip diameter was around 1 μm while the resistance was between 3-5 MΩ. Recordings were made using custom software (Network Prism, Sur lab), written in Matlab (MathWorks), controlling a MultiClamp 700B amplifier (Axon). A glass pipette electrode was inserted into the brain at an angle of 20-35^0 and an Ag/AgCl ground electrode pellet (Warner Instruments) was positioned in the same solution as the brain and the objective. For fluorescent visualization, pipettes were filled with Alexa Fluor 594 (Molecular Probes). The pipette was first targeted to the injection site using a 10x lens, and then targeted to individual GFP+ cells using a 25x lens via simultaneous two-photon imaging at 770 nm. Cell proximity was detected through deflections in resistance observed in voltage clamp during a rapidly time-varying 5 mV command voltage pulse. Once resistance had increased by 5-10 MΩ, the amplifier was switched to current clamp, and spikes were recorded with zero injected current, under a Bessel filter of 4 KHz and an AC filter of 300 Hz. Virus injected brains were perfused post hoc and immunohistochemistry was performed.

Visual stimulation and data analysis from in vivo two-photon guided targeted loose patch recordings

[00428] To assess the orientation selectivity and tuning of genome-edited neurons, Applicants presented oriented gratings using custom software written in Matlab PsychToolbox-3. Gratings were optimized for cellular responsiveness and were presented by stepping the orientation from 0-360 degrees in steps of 20 degrees, with each grating presentation being preceded for 4 seconds "off" followed by 4 seconds "on", for a total presentation duration of 144 seconds.

[00429] Data was acquired directly into Matlab and saved as .mat files. Spike detection was performed via analysis routines that used manually defined thresholds followed by spike shape template matching for further verification. Every spike was tagged and displayed on screen in a graphical user interface whereupon it was manually reviewed for false positives and negatives by the experimenter. Spike times in response to every stimulus were then grouped into "on" or "off" periods based on their timing relative to visual stimulation, and "on" spikes for each stimulus were decremented by the number of "off" spikes observed during an equal time period. For orientation experiments, # spikes per stimulus = (# spikes "on") - (# spikes "off") because "on" and "off" periods were the same duration. For every cell of interest, the methods were used to collect responses for each oriented stimulus (0 to 360 degrees, in steps of 20 degrees). These responses were then turned into a "tuning curve" of orientation vs. response for each trial. Orientation Selectivity Index (OSI) was computed by taking the vector average for the preferred orientation according to the formulae as follows:

$$OSI = \frac{\sqrt{(\sum_i R(\theta_i)\sin(2\theta_i))^2 + (\sum_i R(\theta_i)\cos(2\theta_i))^2}}{\sum_i R(\theta_i)}$$

Tissue preparation and purification of cell nuclei

[00430] Total hippocampus or dentate gyrus was quickly dissected in ice cold DPBS (Life Sciences) and shock frozen on dry ice. For cell nuclei purification, tissue was gently homogenized in 2 ml ice-cold homogenization buffer (HB) (320 mM Sucrose, 5 mM CaCl, 3 mM $Mg(Ac)_2$, 10 mM Tris pH7.8, 0.1 mM EDTA, 0.1 % NP40, 0.1 mM PMSF, 1 mM beta-mercaptoethanol) using 2 ml Dounce homogenizer (Sigma); 25 times with pestle A, followed by 25 times with pestle B. Next, 3 ml of HB was added up to 5 ml total and kept on ice for 5 min. For gradient centrifugation, 5 ml of 50% OptiPrep™ density gradient medium (Sigma) containing 5 mM CaCl, 3 mM $Mg(Ac)_2$, 10 mM Tris pH 7.8, 0.1 mM PMSF, 1 mM beta-mercaptoethanol was added and mixed. The lysate was gently loaded on the top of 10 ml 29% iso-osmolar OptiPrep™ solution in a conical 30 ml centrifuge tube (Beckman Coulter, SW28 rotor). Samples were centrifuged at 10,100 xg (7,500 rpm) for 30 min at 4°C. The supernatant was removed and the nuclei pellet was gently resuspended in 65 mM beta-glycerophosphate (pH 7.0), 2 mM $MgCl_2$, 25 mM KCl, 340 mM sucrose and 5% glycerol. Number and quality of purified nuclei was controlled using bright field microscopy.

Cell nuclei sorting

[00431] Purified GFP-positive (GFP^+) and negative (GFP^-) intact nuclei were co-labeled with Vybrant® DyeCycle™ Ruby Stain (1:500, Life Technologies) and sorted using BD FACSAria III (Koch Institute Flow Cytometry Core, MIT). GFP^+ and GFP^- nuclei were collected in 1.5 ml

Eppendorf tubes coated with 1% BSA and containing 400 µl of resuspension buffer (65 mM beta-glycerophosphate pH 7.0, 2 mM $MgCl_2$, 25 mM KCl, 340 mM sucrose and 5% glycerol). After sorting, all samples were kept on ice and centrifuged at 10,000 xg for 20 min at 4°C. Nuclei pellets were stored at -80°C or were directly used for downstream processing.

Genomic DNA extraction and SURVEYOR™ assay

[00432] For functional testing of sgRNA, 50-70% confluent N2a cells were co-transfected with a single PCR amplified sgRNA and SpCas9 vector. Cells transfected with SpCas9 only served as negative control. Cells were harvested 48 h after transfection, and DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. To isolate genomic DNA from AAV1 transduced primary neurons, DNeasy Blood & Tissue Kit was used 7 days post AAV transduction, according to the manufacturer's instruction. Sorted nuclei or dissected tissues were lysed in lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 10 mM EDTA, 0.5 mM SDS, Proteinase K (PK, 1mg/ml) and RNAse A) at 55°C for 30 min. Next, chloroform-phenol extraction was performed followed by DNA precipitation with ethanol, according to standard procedures. DNA was finally resuspended in TE Buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) and used for downstream analysis. Functional testing of individual sgRNAs was performed by SURVEYOR™ nuclease assay (Transgenomics) using PCR primers listed in Supplementary Table 2. Band intensity quantification was performed as described herein

RNA library preparation and sequencing

[00433] Two weeks after bilateral viral delivery of SpCas9 with guide targeting Mecp2 (4 animals) or SpCas9 with gRNA targeting lacZ (4 animals), the dentate gyrus was quickly dissected in ice cold DPBS (Life Sciences) and transferred immediately to RNA-later solution (Ambion). After 24 hours in 4°C the tissue was moved to -80°C. Populations of 100 targeted neuronal nuclei were FACS sorted into 10 µl TCL buffer supplemented with 1% 2-mercaptoethanol (Qiagen). After centrifuging, samples were frozen immediately at -80°C. The RNA was purified by AMPure RNAcleanXP SPRI beads (Beckman Coulter Genomics) following the manufactures' instructions, and washed three times with 80% ethanol, omitting the final elution. The beads with captured RNA were air-dried and processed immediately for cDNA synthesis. Samples with no nuclei were used as negative controls. Three population samples were used for each animal, total of 24 population sample, in cDNA library preparations following the SMART-seq2 protocol only replacing the reverse transcriptase enzyme with 0.1 ul of Maxima H Minus enzyme (200 U/ul, Thermo Scientific), and scaling down the PCR reaction to a volume of 25 ul. The tagmentation reaction and final PCR amplification were done using the Nextera XT DNA Sample preparation kit (Illumina), with the following modifications. All reaction volumes were scaled down by a factor of 4, and the libraries were pooled after the PCR amplification step by taking 2.5 ul of each sample. The pooled libraries were cleaned and size-selected using two rounds of 0.7 volume of AMPure XP SPRI bead cleanup (Beckman Coulter Genomics). Samples were loaded on a High-Sensitivity DNA chip (Agilent) to check the quality of the library, while quantification was done with Qubit High-Sensitivity DNA kit (Invitrogen). The pooled libraries were diluted to a final concentration of 4 nM and 12 pmol and were sequenced using Illumina Miseq with 75 bp paired end reads.

RNA libraries data analysis

[00434] Bowtie2 index was created based on the mouse mm9 UCSC genome and known Gene transcriptome[13], and paired-end reads were aligned directly to this index using Bowtie2 with command line options -q --phred33-quals -n 2 -e 99999999 -l 25 -I 1 -X 1000 -a -m 200 --p 4 --chunkmbs 512. Next, RSEM v1.27 was run with default parameters on the alignments created by Bowtie2 to estimate expression levels. RSEM's gene level expression estimates (tau) were multiplied by 1,000,000 to obtain transcript per million (TPM) estimates for each gene, and TPM estimates were transformed to log-space by taking log2(TPM+1). Genes were considered detected if their transformed expression level equal to or above 2 (in log2(TPM+1) scale). A library is filtered out if it has less than 8000 genes detected. Based on this criterion, 4 libraries were filtered and excluded from the downstream analysis. To find differentially expressed genes between control animals and Mecp2 sgRNA expressing animals, Student's t-test (Matlab V2013b) and cross validation was used in 20 random permutation runs, where in each run one library from each animal was randomly chosen to exclude (this results in a total of 12 libraries used in the t-test each time). The t-test was run on all genes that have mean expression level above 0.9 quantile (usually around 5 log2(TPM+1)) for each sample. Then, genes that were significant ($p<0.01$) in more than one thirds of the permutation runs were chosen. The log2(TPM+1) expression levels of these genes across samples were clustered using hierarchical clustering (Matlab V2013b).

Immunofluorescent staining

[00435] Cell culture: For immunofluorescent staining of primary neurons, cells were fixed 7 days after viral delivery with 4% paraformaldehyd (PFA) for 20 min at RT. After washing 3 times with PBS, cells were blocked with 5% normal goat serum (NGS) (Life Technologies), 5% donkey serum (DS) (Sigma) and 0.1% Triton-X100 (Sigma) in PBS for 30 min at RT. Cells were incubated with primary antibodies in 2.5% NGS, 2.5% DS and 0.1% Triton-X100 for 1 hour at RT or overnight at 4°C. After washing 3 times with PBST, cells were incubated with secondary antibodies for 1 hour at RT. Finally, coverslips were mounted using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories) and imaged using an Zeiss AxioCam Ax10 microscope and an Axiocam MRm camera. Images were processed using the Zen 2012 software (Zeiss). Quantifications were performed by using ImageJ software 1.48 h and Neuron detector plugin. Mice were sacrified 4 weeks after viral delivery by a lethal dose of Ketamine/Xylazine and transcardially perfused with PBS followed by PFA. Fixed tissue was sectioned using vibratome (Leica, VT1000S). Next, 30 μm sections were boiled for 2 min in sodium citrate buffer (10 mM tri-sodium citrate dehydrate, 0.05% Tween20, pH 6.0) and cool down at RT for 20 min. Sections were blocked with 4% normal goat serum (NGS) in TBST (137 mM NaCl, 20 mM Tris pH 7.6, 0.2% Tween-20) for 1 hour. Paraffin sections were cut using a microtom (Leica RM2125 RTS) to 8 μm, and stained as described previously. Sections were incubated with primary antibodies diluted in TBST with 4% NGS overnight at 4°C. After 3 washes in TBST, samples were incubated with secondary antibodies. After washing with TBST 3 times, sections were mounted using VECTASHIELD HardSet Mounting Medium with DAPI and visualized with confocal microscope (Zeiss LSM 710, Ax10 ImagerZ2, Zen 2012 Software). Following primary antibodies were used: rabbit anti-Dnmt3a (Santa Cruz, 1:100); rabbit anti-MeCP2 (Millipore, 1:200); mouse anti-NeuN (Millipore, 1:50-1:400); chicken anti-GFAP (Abcam, 1:400); mouse anti-Map2 (Sigma, 1:500); chicken anti-GFP (Aves labs, 1:200-1:400); mouse anti-HA (Cell Signaling, 1:100). Secondary antibodies: AlexaFluor®488, 568 or 633 (Life Technologies, 1:500-1:1,000).

Quantification of LIVE/DEAD® assay

[00436] Control and transduced primary neurons were stained using the LIVE/DEAD® assay (Life technologies) according to the manufacturer's instruction. To avoid interference with the GFP-signal from GFP-KASH expression, cells were stained for DEAD (ethidium homodimer) and DAPI (all cells) only. Stained cells were imaged using fluorescence microscopy and DEAD,

GFP and DAPI positive cells were counted by using ImageJ 1.48h software and Neuron detector plugin.

Western blot analysis

[00437] Transduced primary cortical neurons (24 well, 7 days after viral delivery) and transduced tissue samples (4 weeks after viral delivery) were lysed in 50 μL of ice-cold RIPA buffer (Cell Signaling) containing 0.1% SDS and proteases inhibitors (Roche, Sigma). Cell lysates were sonicated for 5 min in a Bioruptor sonicater (Diagenode) and protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Inc.). Protein lysats were dissolved in SDS-PAGE sample buffer, separated under reducing conditions on 4-15% Tris-HCl gels (Bio-Rad) and analyzed by Western blotting using primary antibodies: rabbit anti-Dnmt3a (Santa Cruz, 1:500), mouse anti-Dnmt1 (Novus Biologicals, 1:800), rabbit anti-Mecp2 (Millipore, 1:400), rabbit anti-Tubulin (Cell Signaling, 1:10,000) followed by secondary anti-mouse and anti-rabbbit HRP antibodies (Sigma-Aldrich, 1:10,000). GAPDH was directly visualized with rabbit HRP coupled anti-GAPDH antibody (Cell Signaling, 1:10,000). Tubulin or GAPDH served as loading control. Blots were imaged with ChemiDoc™ MP system with ImageLab 4.1 software (BioRad), and quantified using ImageJ software 1.48h.

Delay Contextual Fear Conditioning (DCFC)

[00438] 8 weeks after bilateral SpCas9/DNMT 3xsgRNA delivery into the dorsal and ventral dentate gyrus of 12 weeks old C57BL/6N male mice, animals were habituated to the experimentor and the behavior room for 7 days. SpCas9/GFP-KASH injected littermates served as controls. At day 1 of DCFC, mouse cages were placed into an islolated anterroom to prevent mice from auditory cues before and after testing. Indivdual mice were placed into the FC chamber (Med Associates Inc.) and a 12 min habituation period was performed. After habituation the mice were placed back to their homecages. The next day (training day) individual mice were placed into the chamber and were allowed to habituate for 4 min. After another 20 sec (pre-tone) interval, the tone (auditory cue) at a level of 85 dB, 2.8 kHz was presented for 20 sec follwed by 18 sec delay interval before the foot-shock was presented (0.5 mA, 2 sec). After the foot-shock, 40 sec interval (post-tone/shock) preceded a next identical trial starting with the 20 sec pre-tone period. The training trial was repeated 6 times before the mice were placed back to their homecages. At day 3 (testing day), mice were first placed in the conditioning context chamber for 3 min. Next, mice underwent 4x 100 sec testing trials starting with a 20 sec interval followed by 20 sec tone and a 60 sec post-tone interval. Finally, mice were placed in an altered context-conditioning chamber (flat floor vs. grid, tetrameric vs. heptameric chamber, vanillin scent) and the testing trial was repeated. Freezing behavior was recorded and analysis was performed blind off-line manually and confirmed with Noldus EthoVision XT software (Noldus Information Technology).

Deep sequencing analysis and indel detection

[00439] CRISPR Design Tool (http://crispr.mit.edu/) was used to find potential off-targets for DNMT family genes, targeted by CRISPR-SpCas9 in the brain. Targeted cell nuclei from dentate gyrus were FACS sorted 12 weeks after viral delivery and genomic DNA was purified as described above. For each gene of interest, the genomic region flanking the CRISPR target site was amplified by a fusion PCR method to attach the Illumina P5 adapters as well as unique sample-specific barcodes to the target amplicons (for on- and off-target primers see Supplementary Table 3). Barcoded and purified DNA samples were quantified by Qubit 2.0 Fluorometer (Life Technologies) and pooled in an equimolar ratio. Sequencing libraries were then sequenced with the Illumina MiSeq Personal Sequencer (Life Technologies), with read length 300 bp. The MiSeq reads were analyzed as described previously in[15]. Briefly, reads were filtered by Phred quality (Q score) and aligned using a Smith-Waterman algorithm to the genomic region 50 nucleotides upstream and downstream of the target site. Indels were estimated in the aligned region from 5 nucleotides upstream to 5 nucleotides downstream of the target site (a total of 30 bp). Negative controls for each sample were used to estimate the inclusion or exclusion of indels as putative cutting events. Applicants computed a maximum-likelihood estimator (MLE) for the fraction of reads having target-regions with true-indels, using the per-target-region-per-read error rate from the data of the negative control sample. The MLE scores and cutting rates for each target are listed in Supplementary Table 1.

Statistical analysis

[00440] All experiments were performed with a minimum of two independent biological replicates. Statistics were performed with Prism6 (GraphPa*d)* using Student's two tailed t-test.

Supplementary Table 1. Off-target analysis for DNMTs targeting

	Gene	GI	Potential off-target	MLE (%)	SEM	SEQ

			sequences			ID NO:
	Abca1	NM_013454	GGAGCTGGAGCTGTTCACGTTGG	0.0000	0.00	
	Mctp1	NM_030174	CGGGCAGCAGATGTTCGCGTAGG	0.0806	0.08	
	Exd2	NM_133798	AGGGCTTGAGATGTTCGGGCTGG	0.0612	0.06	
	Pik3r6	NM_001004435	CCGGCTGGGGCTGTCCTCGCTAG	0.0000	0.00	
	Sobp	NM_175407	CGGGGTGCAGCTGCTCACGCCAG	0.0000	0.00	
Dnmt1	**Vac14**	NM_146216	CTGGCGGGAGCTGGTCGCGTGAG	0.0083	0.00	
	Efemp2	NM_021474	TGAGCATGGGCCGCTGGCGGTGG	0.0050	0.01	
	Bmpr1b	NM_001277217	ATGGCATAGGCCGCTGACAGAGG	0.0117	0.01	
	Syce1	NM_001143765	TTGGCATGGTGAGCTGGCGGGGG	0.0067	0.00	
	Atp8b3	NM_026094	TGGGCAGGGGTCTCTGAGGGCAG	0.0067	0.01	
Dnmt3a	**Rdh11**	NM_021557	TTGGCATGGGTCTCTTACCAAGG	0.0017	0.00	
	Hecw2	NM_001001883	ACATGGTTCCAGTGGGTATGTAG	0.0000	0.00	
	Plekhg3	NM_153804	GGAGGTGGGCAGCGGGTATGTAG	0.0954	0.01	
	Cdc25b	NM_001111075	AGAAGGTCCCCGCGGGCATGGAG	0.2421	0.12	
	Top1mt	NM_028404	GGAGGGAACCAGCCGGTATGGGG	0.0167	0.01	
	Sesn2	NM_144907	AGAGAGTGGCAGTGGGTAAGCAG	0.0000	0.00	
	Ncan	NM_007789	AGAGGTGGCCAGCGGGCAGGAAG	0.0017	0.00	
Dnmt3b	**Nacad**	NM_001081652	TGAGGGGGCCAGCTGGGATGCAG	1.6254	0.76	

Supplementary Table 2. PCR primers used in the SURVEYOR assay

Gene	Forward primer sequence (5'-3')	SEQ ID NO:	Reverse primer sequence (5'-3')	SEQ ID NO:
Mecp2	GGTCTCATGTGTGGCACTCA		TGTCCAACCTTCAGGCAAGG	
Dnmt3a	ATCCCTCCTCAGAGGGTCAGC		TACCTCATGCACAGCTAGCACC	
Dnmt1	TTCGGGCATAGCATGGTCTTCC		GTTCTATTTCAGAGGGCTGATCCC	
Dnmt3b	GTTCTGAGCCGCACAGTTTGG		GGATAAGAAGGGACAATACAGG	

Supplementary Table 3. Primers used for on- and off-target genomic loci amplification

Gene	Forward primer sequence (5'-3')	SEQ ID NO:	Reverse primer sequence (5'-3')	SEQ ID NO:
Dnmt1	GCCGGGGTCTC GTTCAGAGCT		CTACCGCCTGCGGA CATGGT	
Dnmt3a	CCTGTCTCTCTGT CCTAGGGCTCC		CCGTTTGCTGATGTAGTA GGGGTCC	
Dnmt3b	CCCACAGGAAA CAATGAAGGGAGAC		CATCCTTCGTGTCT GAGGACTGGTC	
Abca1	CCCTGACACCAGC TGTTCAGCAC		CTCTGGGTGAC CACACACGATGC	
Mctp1	GAGCAGGCAGA GCCGAGCAAG		GGAGAGCGTCC GCCAGGAG	
Exd2	GGGTCTTGTTGTG AGTAGGGTGTG		GAAGCTCTCTTAA CTACTGTTC	

Pik3r6	CCTGGAATACTAT TTCCACGCCG		CAGGCCCTAGCAGCG AGCAG	
Sobp	GCAGCACACTCCA CCCTCACAT		GGAAGGGGCTTTCC TCCGAGC	
Vac14	CGGCGTCACG TGACCTGAGTAAC		GCTCCGACCCTGCT CTCCCA	
Efemp2	GTGTCTGCCTC GCTCTGCTGC		CCTGTTCATCAGGCTC GTAGCCC	
Bmpr1b	CTATCTGAAATCC ACCACCTTAGACGC		CGATTGCTGGCTTGC CTTGAG	
Syce1	GCCTGAGGGGG CCAGAGGT		GGTTCGCGTCCGCC CGCGTGAT	
Atp8b3	GGGACTCC CCGGGTGGTG		GAGAGGTGGTC CTGTCGCCTATG	
Rdh11	GACCCTGTGTTT CAAGTCTCTCTG		CCCAGCAGGTCACA GCTGACATC	
Hecw2	GGCCATCCAGTAC ATTCAATACG		AGCACAGTATGTATTC TATAAAATAATACGAC	
Plekhg3	GCAGAAGCCGT GACTCACAGCA		GTGGGAGGGGACAG AGACCATG	
Cdc25b	CTTGTGCTTG TGATTCTGTCCTTACTGC		CCTTACCTGTTCCTCT TCCTTATCCAGC	
Top1mt	CGAGAAGTC		ATACCCAGTCCAC	

	GATGCAGACACTTCAA		ATCCCTGCC	
Sesn2	GCTGAAGACTGGC GAGCACAGCT		CCTCTGCATCTCCCTCAGGAAGT ATT	
Ncan	GACCTGAATGTTG TGGCTGAGAGTCC		GCCTCCTGTC CCCAGGTCCC	
Nacad	CCCTCACGTTCC TGTCCAGCAA		CACTAGGCTT GGGCTGCCCTCT	

Example 2: In vivo Retina Disease Model (Mouse model for Retinitis Pigmentosa)

[00441] Applicants demonstrate the *in vivo* effectiveness of a genome engineering approach using Cas9 when packaged into AAV, and have successfully used it to modify endogenous genome sequence in mammalian cells. Applicants use this system to demonstrate genetic engineering potential in neurons, which represents one key group of post-mitotic cells in human body. Applicants' studies underscore the therapeutic potential of this *in vivo* genome engineering of somatic tissue through the correction of mutations associated with human disease Retinitis Pigmentosa in the mouse model that bears mutation corresponding to the similar genetic defects found in human patients. The mouse strain used for Applicants' studies is C57BL/6 strain B6.129S6(Cg)-Rhotm1.1Kpal/J. This mouse strain was chosen because these mice carry a nucleotide substitution at codon 23 in the mouse rhodopsin (Rho) gene, CCC to CAC. This codon encodes the amino acid substitution of histidine for phenylalanine at position 23, P23H. The P23H mutation is one of the most common causes of autosomal dominant retinitis pigmentosa. The genomic location of the Rho gene is on mouse chromosome 6: 115,931,927-115,938,829. Applicants observed that mice that are homozygous for the targeted mutation are viable and fertile. Both the mutant and wildtype gene product (mRNA) is detected by cDNA sequencing chromatogram. The phenotype in heterozygous mice mimics the retinopathy and progressive retinal degeneration observed in patients with autosomal dominant retinitis pigmentosa caused by the P23H mutation. By 35 days of age, heterozygotes have a shorter rod outer segment when compared to controls. At post natal day 63, heterozygotes have fewer rod nuclei (half the number observed in wildtype mice), and decreased length of the rod outer segment. Homozygous mice exhibit a more severe phenotype with thinner outer nuclear layer, severe photoreceptor degeneration by post natal day 23 and loss of almost all photoreceptor cells by post natal day 63. Glycosylation of the mutant P23H protein is severely diminished.

AAV delivery of Cas9 system to the retina neurons in the mouse strain B6.129S6(Cg)-Rhotm1.1Kpal/J:

[00442] Applicants chose to target the gene Rho that is critical for the normal function of retina neurons, and induce homologous recombination on the target to correct the related disease-relevant mutations P23H using Adeno-associated virus (AAV) delivery of cas9 genome engineering tools and a recombination template. Applicants designed three targets, P23H mutation site labeled in orange with the single nucleotide mutation C-A labeled in red. (*see* Fig. 18A). This approach demonstrates that the correction of the genetic mutation in this mouse model can rescue the disease-associated phenotypes and further demonstrates the feasibility of performing genome modification in neurons of adult animals. Further, the study provides information to assess the level of homologous recombination that could be induced in neuronal cell types for genetic engineering purposes, using the retina neurons in this strain as a model system. The experimental set up is as shown in the table below:

Group purpose	Injection Routes	Number of groups	Mice per group	Total
Neg. ctrl. – saline injection	IV	1 group, 3 time points	5	15
Control – non-targeting vector	IV	1 group, 3 time points	5	15
Targeting vector	IV	3 time points X 3 conditions	5	45

[00443] In total, 25 mice are used to set up breeder pairs so in total = 100 mice for each strain are used for this study.

Retina injection of AAV:

[00444] Subretinal injection is used as the delivery route. Mice receive subretinal injection of 0.5-1 microliters of saline solution (0.9% sodium chloride) warmed to body temperature and containing up to 1E12 viral particles. These mice are more than 6 weeks old. The animal is kept warm using a heating pad and/or lamp and is monitored. Animals are monitored daily for clinical signs of procedure related complications. Up to two injection per mouse are administered.

Tissue collection:

[00445] After 1 to 4 weeks after injections of materials, mice are sacrificed by the CO_2 inhalation method prior to tissue sampling from mice. Tissues are analyzed for evidence of successful gene transfection, genome alterations, or toxicity.

Example 3: In vivo therapeutic genome engineering approach for Retinitis Pigmentosa

Guide selection and in vitro validation

[00446] First, guides for SaCas9 that target the gene RHO in human genome are designed as shown below. It has been selected based on the locus of disease-causing mutation P23H. The design was generated through computational algorithm to maximize the potential of introducing on-target cleavage that is closest to the disease mutation site to facilitate highest efficiency and specificity of therapeutic gene correction. Guides were also screened for their DNaseI hyper-sensitivity (HS) assay results to maximize the accessibility of the genomic region corresponding to the target guide sequence, thereby increasing the expected efficiency of the guides following delivery in vivo. More than one guides are selected and then screened using Surveyor assay *in vitro* in cultured human cells (HEK 293FT) to measure their efficiency for inducing indel formation at target genomic locus. The guide with highest efficiency *in vitro* is then selected for virus production and downstream experiment in vivo. Figure 18A-B shows the guide design for RHO locus, and *in vitro* guide screening results using the Surveyor assay.

Homologous recombination template design and validation

[00447] Secondly, to induce homologous recombination on the target to correct the related disease-relevant mutations P23H using homology-directed repair, a HR vector is synthesized based on the wild type (normal) genomic sequence of the unaffected human individual. This vector bears the AAV packaging signal, and the normal version of genomic sequence with up to 5kb total homology arms: two homology arms, left and right, are on each side of the sequence that sandwich the target mutation site P23H in the middle, as shown below. This vector is validated *in vitro* by co-transfecting at 1:1, 1:3, 1:5 ratio with the corresponding vector encoding the SaCas9 system with the best guide as measured in previous section, then the homologous recombination (HR) efficiency at target P23H locus is measured using restriction fragment length polymorphism assay (RFLP) assay and thus validate the optimal condition for the HDR procedure.

[00448] Figure 19 shows an RHO HR AAV vector. The specific sequence for the part responsible for serving as homologous recombination template is listed below.

>Rho HR AAV Vector Template Region

ACCAGAAAGTCTCTAGCTGTCCAGAGGACATAGCACAGAGGCCCATGGTCCCTATTTCAAACC
CAGGCCACCAGACTGAGCTGGGACCTTGGGACAGACAAGTCATGCAGAAGTTAGGGGACCTTC
TCCTCCCTTTTCCTGGATCCTGAGTACCTCTCCTCCCTGACCTCAGGCTTCCTCCTAGTGTCA
CCTTGGCCCCTCTTAGAAGCCAATTAGGCCCTCAGTTTCTGCAGCGGGGATTAATATGATTAT
GAACACCCCCAATCTCCCAGATGCTGATTCAGCCAGGAGCTTAGGAGGGGGAGGTCACTTTAT
AAGGGTCTGGGGGGGTCAGAACCCAGAGTCATCCAGCTGGAGCCCTGAGTGGCTGAGCTCAGG
CCTTCGCAGCATTCTTGGGTGGGAGCAGCCACGGGTCAGCCACAAGGGCCACAGCCATGAATG
GCACAGAAGGCCCTAACTTCTACGTGCCCTTCTCCAATGCGACGGGTGTGGTACGCAGCCCCT
TCGAGTACCCACAGTACTACCTGGCTGAGCCATGGCAGTTCTCCATGCTGGCCGCCTACATGT
TTCTGCTGATCGTGCTGGGCTTCCCCATCAACTTCCTCACGCTCTACGTCACCGTCCAGCACA
AGAAGCTGCGCACGCCTCTCAACTACATCCTGCTCAACCTAGCCGTGGCTGACCTCTTCATGG
TCCTAGGTGGCTTCACCAGCACCCTCTACACCTCTCTGCATGGATACTTCGTCTTCGGGCCCA
CAGGATGCAATTTGGAGGGCTTCTTTGCCACCCTGGGCGGTATGAGCCGGGTGTGGGTGGGGT
GTGCAGGAGCCCGGGAGCATGGAGGGGTCTGGGAGAGTCCCGGGCTTGGCGGTGGTGGCTGAG
AGGCCTTCTCCCTTCTCCTGTCCTGTCAATGTTATCCAAAGCCCTCATATATTCAGTCAACAA
ACACCATTCATGGTGATAGCCGGGCTGCTGTTTGTGCAGGGCTGGCACTGAACACTGCCTTGA
TCTTATTTGGAGCAATATGCGCTTGTCTAATTTCACAGCAAGAAAACTGAGCTGAGGCTCAAA
GAAGTCAAGCGCCCTGCTGGGGCGTCACACAGGGACGGGTGCAGAGTTGAGTTGGAAGCCCGC
ATCTATCTCGGGCCATGTTTGCAGCACCAAGCCTCTGTTTCCCTTGGAGCAGCTGTGCTGAGT
CAGACCCAGGCTGGGCACTGAGGGAGAGCTGGGCAAGCCAGACCCCTCCTCTCTGGGGGCCCA
AGCTCAGGGTGGGAAGTGGATTTTCCATTCTCCAGTCATTGGGTCTTCCCTGTGCTGGGCAAT
GGGCTCGGTCCCCTCTGGCATCCTCTGCCTCCCCTCTCAGCCCCTGTCCTCAGGTGCCCCTCC
AGCCTCCCTGCCGCGTTCCAAGTCTCCTGGTGTTGAGAACCGCAAGCAGCCGCTCTGAAGCAG
TTCCTTTTTGCTTTAGAATAATGTCTTGCATTTAACAGGAAAACAGATGGGGTGCTGCAGGGA
TAACAGATCCCACTTAACAGAGAGGAAAACTGAGGCAGGGAGAGGGGAAGAGACTCATTTAGG
GATGTGGCCAGGCAGCAACAAGAGCCTAGGTCTCCTGGCTGTGATCCAGGAATATCTCTGCTG
AGATGCAGGAGGAGACGCTAGAAGCAGCCATTGCAAAGCTGGGTGACGGGGAGAGCTTACCGC
CAGCCACAAGCGTCTCTCTGCCAGCCTTGCCCTGTCTCCCCCATGTCCAGGCTGCTGCCTCGG
TCCCATTCTCAGGGAATCTCTGGCCATTGTTGGGTGTTTGTTGCATTCAATAATCACAGATCA
CTCAGTTCTGGCCAGAAGGTGGGTGTGCCACTTACGGGTGGTTGTTCTCTGCAGGGTCAGTCC
CAGTTTACAAATATTGTCCCTTTCACTGTTAGGAATGTCCCAGTTTGGTTGATTAACTATATG
GCCACTCTCCCTATGGAACTTCATGGGGTGGTGAGCAGGACAGATGTCTGAATTCCATCATTT
CCTTCTTCTTCCTCTGGGCAAAACATTGCACATTGCTTCATGGCTCCTAGGAGAGGCCCCCAC
ATGTCCGGGTTATTTCATTTCCCGAGAAGGGAGAGGGAGGAAGGACTGCCAATTCTGGGTTTC
CACCACCTCTGCATTCCTTCCCAACAAGGAACTCTGCCCCACATTAGGATGCATTCTTCTGCT
AAACACACACACACACACACACACACACAACACACACACACACACACACACACACACACACAC
AAAACTCCCTACCGGGTTCCCAGTTCAATCCTGACCCCCTGATCTGATTCGTGTCCCTTATGG
GCCCAGAGCGCTAAGCAAATAACTTCCCCCATTCCCTGGAATTTCTTTGCCCAGCTCTCCTCA
GCGTGTGGTCCCTCTGCCCCTTCCCCCTCCTCCCAGCACCAAGCTCTCTCCTTCCCCAAGGCC
TCCTCAAATCCCTCTCCCACTCCTGGTTGCCTTCCTAGCTACCCTC

AAV delivery of Cas9 genome engineering tools and a recombination template

[00449] Finally, for employing the Adeno-associated virus (AAV) delivery of cas9 genome engineering tools and a recombination template in vivo, both viruses are produced with serotype

AAV1, AAV2, AAV5, AAV7, AAV8 (all are effective), purified by gradient ultracentrifugation or chromatography methods. The virus particles are then injected using the optimal condition as determined in previous section through the subretinal route for delivery to the photoreceptor and RPE cells.

Detailed injection protocol

[00450] Retina injection of AAV: subretinal injection is performed by injection of up to 1 milliliter of saline solution (0.9% sodium chloride) containing different dose of AAV viral particles warmed to body temperature. The two different AAV vector, one for SaCas9 and one for HR template aree mixed at different ratios (e.g. SaCas9 vs. HR template = 1:1, 1:3, or 1:5) to determine the optimal condition. The dose used for the injection can be as low as 1.5 x 10E10 vector genomes total, or up to 1.5 x 10E11 vector genomes total. The higher dose gives better gene therapy effectiveness but may lead to higher chance of complications due to immune response to the viral vector injection. Patients are monitored daily for clinical signs of procedure related complications. This procedure essentially follows guidelines provided in the following publications: Maguire A. M. *et al*. *N Engl. J. Med.* 2008 May 22;358(21):2240-8. doi: 10.1056/NEJMoa0802315. Epub 2008 Apr 27. Safety and efficacy of gene transfer for Leber's congenital amaurosis. Simonelli F. *et al*. *Mol. Ther.* 2010 Mar;18(3):643-50. doi: 10.1038/mt.2009.277. Epub 2009 Dec 1. Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Maguire A. M. *et al*. Lancet. 2009 Nov 7;374(9701):1597-605. doi: 10.1016/S0140-6736(09)61836-5. Epub 2009 Oct 23. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial.

Post injection procedures

[00451] Post injection, the patient is monitored for any immunological response or adverse effect. After at least 4 weeks, restriction fragment length polymorphism assay (RFLP) is used to assess the level of homologous recombination that could be induced in ocular cell types. And the relief or recovery of RP phenotype is then also evaluated.

Example 4: In vivo therapeutic genome engineering for Achromatopsia

Guide selection and in vitro validation

[00452] First, guides for SaCas9 that target the gene CNGA3 and CNGB3 in human genome are designed as shown below. It has been selected based on the locus of disease-causing mutation, i.e. R277C and R283W for CNGA3 and 1148delC for CNGB3. The design was generated through computational algorithm to maximize the potential of introducing on-target cleavage that is closest to the disease mutation site to facilitate highest efficiency and specificity of therapeutic gene correction. Guides were also screened for their DNaseI hyper-sensitivity (HS) assay results to maximize the accessibility of the genomic region corresponding to the target guide sequence, thereby increasing the expected efficiency of the guides following delivery in vivo. More than one guides are selected so that the most efficient guides can be tested in vitro via Surveyor assay. The guide with highest efficiency in vitro is then selected for virus production and downstream experiment *in vivo*. Figure 20A-B shows guide selction for CNGA3 and CNGB3.

Homologous recombination template design and validation

[00453] Second, to induce homologous recombination on the target to correct the related disease-relevant mutations using homology-directed repair, a HR vector need to be synthesized based on the wild type (normal) genomic sequence of the CNGA3 and CNGB3 genes from unaffected human individual, respectively.

[00454] Figures 21 and 22 show maps of the CNGA3 HR AAV vector and CNGB3 HR AAV vector, respectively. These vectors bear the AAV packaging signal, and the normal version of genomic sequence with up to 5 kb total homology arms: two homology arms, left and right, are on each side of the sequence that sandwich the target mutation site in the middle, as shown below. This vector is validated in vitro by co-transfecting at 1:1, 1:3, 1:5 ratio with the corresponding vector encoding the SaCas9 system with the best guide as measured in previous section, then the homologous recombination (HR) efficiency at target locus is measured using restriction fragment length polymorphism assay (RFLP) assay and thus validate the optimal condition for the HDR procedure.

[00455] The specific sequence for the part responsible for serving as homologous recombination template is listed below.

>CNGA3 HR AAV Vector Template Region

CTGCTGCCTGCTCTGTCCCCTTTAAGTATTGACATCCTCAAAACCCTCTTTGGAAAAAGCACA
GGCCACAGATCTTACTGTGACTTGTGTTTCTTTCTCCTAGGTGTACCTTCAACCTTGATAAAA
ATAAACCTCTAAATCAATTGAGATCTGCCTCCGTCACTTTTTTTTTTTCAAAGACTCAGAGTC
TCACTCTGTTGCCCAGGCTGGAGTGTAGTGGTGCGATCTTGGCTCATTGCACCCTCCACCTCC
TGGGTTCAAGTGGTTCTCGTGCCTCAGCTTCCTGAGTAGCTGGGATTACAGGGGTGCACCACC

ACATCTGGCTAATTTTTGTATTTTTAGTAGAGACAGGGTTTCACCATGTTGCCCAGGCTGGTC
TCAAACCCTTGACCTCAGGTGATCCACCCGCCTCGGCCTCTCAAAGTACTGGGATTATAGGCA
TGAGCCACGGCACCCGGCCCTCTGTCACTTTTTGATTTACAACATGTATCTCTAATTTTAAAG
GATCCTTTTTTAAAATATGTATATAATTTCCATTTATCTTTTAAAATTTAATAATCATTCTTT
GTTATCATGTAATACCCAATTTATATTTAAATTTACTCAATCAACCTATGTTTTAAAAAAATT
CAATAGAATAGATTAGAACCTCATAGAATAGAAAATATCAGAGTGCATTTCCTGTAGTAATGG
TAAGTGTTGTTTTTGAAATCATTTCTATTATATATGTATCACTGCATACTGTGTAGCCGTGAG
GTAAAATATGTTTCTTTGTACTATGGTCAAAAAAAGTCAGCCTCTGTGATGCCCAATGACCTC
CATCTTCTTCTTTAGGTTTTCTCGAGCAAGGCTTAATGGTCAGTGATACCAACAGGCTGTGGC
AGCATTACAAGACGACCACGCAGTTCAAGCTGGATGTGTTGTCCCTGGTCCCCACCGACCTGG
CTTACTTAAAGGTGGGCACAAACTACCCAGAAGTGAGGTTCAACCGCCTACTGAAGTTTTCCC
GGCTCTTTGAATTCTTTGACCGCACAGAGACAAGGACCAACTACCCCAATATGTTCAGGATTG
GGAACTTGGTCTTGTACATTCTCATCATCATCCACTGGAATGCCTGCATCTACTTTGCCATTT
CCAAGTTCATTGGTTTTGGGACAGACTCCTGGGTCTACCCAAACATCTCAATCCCAGAGCATG
GGCGCCTCTCCAGGAAGTACATTTACAGTCTCTACTGGTCCACCTTGACCCTTACCACCATTG
GTGAGACCCCACCCCCCGTGAAAGATGAGGAGTATCTCTTTGTGGTCGTAGACTTCTTGGTGG
GTGTTCTGATTTTTGCCACCATTGTGGGCAATGTGGGCTCCATGATCTCGAATATGAATGCCT
CACGGGCAGAGTTCCAGGCCAAGATTGATTCCATCAAGCAGTACATGCAGTTCCGCAAGGTCA
CCAAGGACTTGGAGACGCGGGTTATCCGGTGGTTTGACTACCTGTGGGCCAACAAGAAGACGG
TGGATGAGAAGGAGGTGCTCAAGAGCCTCCCAGACAAGCTGAAGGCTGAGATCGCCATCAACG
TGCACCTGGACACGCTGAAGAAGGTTCGCATCTTCCAGGACTGTGAGGCAGGGCTGCTGGTGG
AGCTGGTGCTGAAGCTGCGACCCACTGTGTTCAGCCCTGGGGATTATATCTGCAAGAAGGGAG
ATATTGGGAAGGAGATGTACATCATCAACGAGGGCAAGCTGGCCGTGGTGGCTGATGATGGGG
TCACCCAGTTCGTGGTCCTCAGCGATGGCAGCTACTTCGGGGAGATCAGCATTCTGAACATCA
AGGGGAGCAAGTCGGGGAACCGCAGGACGGCCAACATCCGCAGCATTGGCTACTCAGACCTGT
TCTGCCTCTCAAAGGACGATCTCATGGAGGCCCTCACCGAGTACCCCGAAGCCAAGAAGGCCC
TGGAGGAGAAAGGACGGCAGATCCTGATGAAAGACAACCTGATCGATGAGGAGCTGGCCAGGG
CGGGCGCGGACCCCAAGGACCTTGAGGAGAAAGTGGAGCAGCTGGGGTCCTCCCTGGACACCC
TGCAGACCAGGTTTGCACGCCTCCTGGCTGAGTACAACGCCACCCAGATGAAGATGAAGCAGC
GTCTCAGCCAACTGGAAAGCCAGGTGAAGGGTGGTGGGGACAAGCCCCTGGCTGATGGGGAAG
TTCCCGGGGATGCTACAAAAACAGAGGACAAACAACAGTGAAAATGCAGCATCTGTCTCCTGC
TTCACAGGGTCGACTGTCAGGGTGACCGTATGTGGCCGCAGCTGTGTGGCATGGAACTTGGTC
AGGGTTGAATTCCAGCTCTACTCACCCTTTGAAAGCTGTGTGACTGCCTGAGAGAACCTGTTT
CTTCACCTAAAAAATGGGACTTTTTGTCTCAGTCCCAGTGAAGTGCCAGGTTTGATTGTGAAG
TCCGCATGAAACACTGCACCAGGCAGGGCTTTGCAAAGTGCAA

>CNGB3 HR AAV Vector Template Region

ACTTTGAGGCAATTTTACTGTAGCTGGTATTTTAGTCAATTTTTAGATAAATTAGTTGTTTAT
ATCAAAGTAAATAATTCACATTCTAAAGGGAATTATTTATTTAGTAAATTTTCTGGAAATTGA
GTGTCTGTGTGTGTGTGTTTCCCAATCAGTGGTCCTTCTGACTTTAAATTCTTTAAAATCGGT
TCTGGTTGTTATAATCCCTTATACATATCCAACTCACTCTAGGTAGTATGTAATTTTGTAAGT
TATTTTCCCTCTCTTTGCTCTATCCTATAATTGCTCTCCATCCCAAGGCTGCAGTGAGTTGCC
CTTCAAAGTAATGCTGGGACCTGCTTTTTTTCCAGTTTGGACATTGCCTTATTATATGTTCAA
TGTCATTTCACTGGAGCAGAAAGTTAGTGAAGTCAACTTTATGCCAGGTCTTTGTATTTTACC
AAAAGGAAATTTCACTATTAAATAACCCAGTTGCCATTTCTGAGTCCTGATTCTACTGTTCTA
AATTTTTCAAGTGATCTTTTTTTATTTCTGGGACACTTGCATACCTAATTGTCAAGTTTAATT

TATGATCCTCGTTACTCTCTAAGTGTTTAATTGAGTTAGTGGTTATAGCTGACTCATAAACCC
ATAAAACCCTTCACTGGTAAACTAATTAGCCACTGCACCTGCCCTTTAAATAATTAACATTGT
TCATTACTAACAATCGGCATCGGAGTTATTAAAAGTTACCTTACTGCTCAGTTGTCTAGAGGC
TTTCAAACTTTTTTGATCATGATCCAGAGTAAAAAATGCATTTCACAGGCCAACTCAGGATAC
ACACACACACACACACACTCCCCTACTATCTATCAGACTCTGATATTTTCTATTCTATTATTC
TCTATCTTCTTTCATTTAAAAATGTATTGACTTACTAAAGAGGTTTTTCAGCTTAAAAATTTT
TATTTAGACCAATTCATGGGGTACTCATGCAATTTTGTTACATGTATACAATGCATAGTGATC
AAGTCAGGGTGTTTAGGGTGTTTATTACTCGAGTACAATACATGTTTTGAAACTATAGTCACC
CTACTCTGTTGCAAACGTTGAATATATTCTTACTGTATGTTTGTATCCTTTAATCCACTTTTC
TTTATGCGCTCCTCTCCCCACCACTCACCCTTCCCAGTCTCTGTTATCTTTCCACTCTCTGTC
TCTATGTGATCAAAATTTTTAGCTCCCACATATATGTGAGAACGTGTGATATTTATCGTTTTG
GGTCTGGCTTATTTCACTAAGATAATGACCTTCAGTCCCATCCATTTTGGTGCAAATGACATG
ATTTTATCCTTTTTTATGGCCAAATAGCATTTACCAGCCATTGAATGGGTTATGACAGCTTCA
AAAACACTGGCTCTCATAAATTCATACAATGAAACAGAATGTTAAAAATAATCAATAAAGGTT
TCTTTCAAAATCAGAACTTACTCGTTTCCTTCCCCATCATACACCCATCTAGTAGTGCCAATT
CCTTCATAGTTTGAAGCCCAGTAATAAACACAGGCATTAATGTGCAGAATAAACAGCAAGTAT
CCAGTTGTTCGAATAACTCTGTCAGAGAGAATAGATGCAAAGTAAGATTCATGTTGTTTCTGA
AATACAGCCTATTTTAACATTTTCTTTTCCTTAAAGTCACCAGCGAACCCCTTGCTTTGGATT
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GCAATGTCTTGACTGGGATTGACAGAGTGTAAAGAAAAAAAAAAAGAAAAAGAAAACTTCCTCT
CTTTTCACAGATGTTGTGAGTCAACTCCGTGAAAGACATGCCTCAAAGGTCACTTCTTCAGTT
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TTTTCTGTACTTGTTTTGACAGAGGCAGATAATAAGTCCTATTTTCTTAGTCCAGTATTCTAA
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TCCTATATTTTATATAGCCAAAGCTGAAATTATATCCTTTTTTTGAAGAGGGGGGTCATATCC
CTGCCAAATTCCGTCTAAAATGTTGTACCATTGCTTTTTCCCCTTCCCCCAAGTATACTGAGT
TATACTTTACCTGTAGATATATGCTTTGTCCATTATAGACTCTAGGTGATGATTAAATTCAAA
AAATGAAGTGTACTATATAGAAAAGCAAAAGAAATCCAAAAGCATGTTAGTCTTAAATATATA
TATTTAAATAAAACTATATGAAATAGATTTTATTACTGAAAAT

AAV delivery of Cas9 genome engineering tools and a recombination template

[00456] Finally for employing the Adeno-associated virus (AAV) delivery of cas9 genome engineering tools and a recombination template in vivo, both viruses are produced with serotype AAV1, AAV2, AAV5, AAV7, AAV8 (all are effective), purified by gradient ultracentrifugation or chromatography methods. The virus particles are then injected using the optimal condition as determined in previous section through the subretinal route for delivery to the photoreceptor and RPE cells.

Detailed injection protocol

[00457] Retina injection of AAV: subretinal injection is performed by injection of up to 1 milliliter of saline solution (0.9% sodium chloride) containing different dose of AAV viral particles warmed to body temperature. The two different AAV vector, one for SaCas9 and one for HR template are mixed at different ratios (*e.g.* SaCas9 vs. HR template = 1:1, 1:3, or 1:5) to determine the optimal condition. The dose used for the injection can be as low as 1.5 x 10E10 vector genomes total, or up to 1.5 x 10E11 vector genomes total. The higher dose may give better gene therapy effectiveness but might lead to higher chance of complications due to immune response to the viral vector injection. Patients are monitored daily for clinical signs of procedure related complications.

Post injection procedures

[00458] Post injection, the patient is monitored for any immunological response or adverse effect. After at least 4 weeks, restriction fragment length polymorphism assay (RFLP) is be used to assess the level of homologous recombination that could be induced in ocular cell types. And the relief or recovery of Achromatopsia phenotype is then also evaluated.

Example 5: In vivo therapeutic genome engineering approach for age-related macular degeneration.

Guide selection and in vitro validation

[00459] First, guides for SaCas9 that target the genomic locus VEGFA in human genome are designed as shown below. It has been selected within the first exon of the gene, but other parts of the gene can also be targeted as well so that a wide range of target region can be screened to determine the most effective guide design. All the designs were generated through computational algorithm to maximize the potential of introducing on-target cleavage that is closest to the transcriptional start site and located within the common region of different expressed transcripts in retina. This will facilitate highest efficiency and specificity of therapeutic gene correction. Guides were also screened for their DNaseI hyper-sensitivity (HS) assay results to maximize the accessibility of the genomic region corresponding to the target guide sequence, thereby increasing the expected efficiency of the guides following delivery in vivo. More than one guides are selected so that the most efficient guides can be tested in vitro. Figure 23A-B shows guide seletion for VEGFA.

[00460] To validate that the designed guides can effective repress the expression of VEGFA gene in human cells and finding the optimal guides and conditions. The closed AAV vector bearing the designed guides are delivered in vitro into human cells (e.g. HEK293). The cells were harvested 72-96 hours post delivery. RNAs and proteins are extracted from the cells. The mRNA level of VEGF is measured via qRT-PCR method or other RNA measurement method, whereas protein level of VEGF is also measured via ELISA or other protein measurement method. The most critical criterion is the VEGF protein level because this is directly relevant for the clinical effectiveness of the system. The guide with highest efficiency in vitro to lower the expression level of VEGF is then selected for virus production and downstream experiment in vivo.

[00461] Finally for employing the Adeno-associated virus (AAV) delivery of cas9 genome engineering tools to disrupt VEGF expression in vivo, viruses are produced with serotype AAV2 and AAV8 (both are effective, other AAV serotype such as AAV1, AAV5, AAV7, AAV9, or AAV-DJ might be used but will be less potent). The viral particles are all purified by gradient ultracentrifugation or chromatography methods.

[00462] The AAV virus particles are then injected using the optimal condition as determined in previous section through the intravitreal route, where AAV is injected in the vitreous humor of the eye. This procedure is less invasive and can sustain persistent expression of the construct to obtain sufficient disruption of VEGF expression to induce therapeutic corrective effects.

Detailed injection protocol

[00463] Retina injection of AAV: intravitreal injection is performed by injection of typically 100 microliter (or up to 1 milliliter but not recommended) of saline solution (0.9% sodium chloride) containing different dose of AAV viral particles warmed to body temperature. The dose used for the injection can be as low as 1 x 10E8 vector genomes in total, or up to 1 x 10E11 vector genomes in total. The use of different dosage allows the determination of optimal dose.

[00464] The higher dose will give better gene therapy effectiveness but might lead to higher chance of complications due to immune response to the viral vector injection. Patients will be monitored daily for clinical signs of procedure related complications.

[00465] Reference for human injection parameter is available at Clinical Trials (dot) Gov - the US Government website for information on clinical trials, having a web address of: clinicaltrials.gov/ct2/show/NCT01024998.

Post injection procedures

[00466] Post injection, the patient are monitored for any immunological response or adverse effect. After at least 4 weeks, ELISA or other protein measurement methods can be employed to assess the level of VEGF in the retina. And the relief or recovery of ARMD phenotype is then evaluated.

[00467] Key safety measures to be followed in the patients include the maximum tolerated dose of a single uniocular intravitreal injection, number of treatment emergent adverse events, and the thickness of the retina post injection.

Example 6: dSaCas9 to stimulate ATOH1 expression to treat deafness or hearing impairments.

Guide selection and in vitro validation

[00468] First, guides for SaCas9 that target the gene ATOH1 in human genome are designed as shown below. It has been selected based on the optimal parameters to maximize the efficiency of epigenetic modulation on target gene expression. Specifically, the design was generated through computational algorithm to maximize the potential of introducing on-target binding to facilitate highest efficiency and specificity of therapeutic gene therapy. Guides were also screened for their DNaseI hyper-sensitivity (HS) assay results to maximize the accessibility of the genomic region corresponding to the target guide sequence, thereby increasing the expected efficiency of the guides following delivery in vivo. Moreover, transcription factor sites that potentially might interfere with the binding were also considered during the selection of the guides. More than one guides are selected and then screened in vitro in cultured human cells (HEK 293FT) to measure their efficiency for inducing ATOH1 gene expression. The guide with highest efficiency in vitro is then selected for downstream experiment and therapeutic intervention in vivo. The specific design of guides for ATOH1 is shown at Figure 25A-C.

Guide screening and validation

[00469] To validate that the designed guides can effective induce the expression of ATOH1 gene in human cells and finding the optimal guides and conditions, in vitro screening and verification is performed with human cell lines. The system, which contains dSaCas9, fusion effector, and optimal guide RNAs, is delivered in vitro into human cells (e.g. HEK293) (Figure 24). The cells were harvested 72-96 hours post delivery. RNAs and proteins are extracted from the cells. The mRNA level of ATOH1 is measured via qRT-PCR method or other RNA measurement methods, whereas protein level of ATOH1 is measured via ELISA or other protein measurement methods. The most critical criteria is the ATOH1 protein level because this is directly relevant for the clinical effectiveness of the system. The guide with highest efficiency in vitro to stimulate the expression of ATOH1 is then selected for in vivo delivery and therapeutic demonstration.

[00470] AAV or Ad viral particles or other delivery vehicle packaging the entire system are then produced, and injected using the optimal conditions as determined experimentally. The most critical parameter to optimize is the amount (i.e. dosage) of the delivery vehicle used for each individual injection.

[00471] The higher dose will give better gene therapy effectiveness but might lead to higher chance of complications due to immune response to the vehicle injection or the off-target effects of the system. Patients are monitored daily for clinical signs of procedure related complications.

Post injection procedures

[00472] Post injection, the patient are monitored for any immunological response or adverse effect. Each week post injection, ELISA or other protein measurement methods can be employed to assess the level of ATOH1 stimulation. New hair cell growth in the Cochleae can be visualized through biopsy and imaging methods. And the relief or recovery of deafness and hearing impairments is then evaluated through hearing tests on the human patients.

[00473] Key safety measures to be followed in the patients include the maximum tolerated dose of a single injection into the human Cochleae, number of treatment emergent adverse events post injection.

* * *

[00474] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention.

WHAT IS CLAIMED IS:

1. A method of modeling a disease associated with a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus comprising delivering a non-naturally occurring or engineered composition comprising :

(A) - I. a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a guide sequence capable of hybridizing to the target sequence,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a polynucleotide sequence encoding Cas9, optionally comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding Cas9 is DNA or RNA,

or

(B) I. polynucleotides comprising:

(a) a guide sequence capable of hybridizing to the target sequence, and

(b) at least one or more tracr mate sequences,

II. a polynucleotide sequence encoding Cas9, and

III. a polynucleotide sequence comprising a tracr sequence,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and the polynucleotide sequence encoding Cas9 is DNA or RNA.

2. The method of claim 1, wherein the Cas9 is SaCas9.

3. The method of claim 1 or 2, wherein the polynucleotides encoding the sequence encoding Cas9, the guide sequence, tracr mate sequence or tracr sequence is/are RNA and are delivered via liposomes, nanoparticles, cell penetrating peptides, exosomes, microvesicles, or a gene-gun.

4. The method of any of claims 1 to 3, wherein the polynucleotides are comprised within a vector system comprising one or more vectors.

5. A method of modeling a disease associated with a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus comprising delivering a non-naturally occurring or engineered composition comprising a viral vector system comprising one or more viral vectors operably encoding a composition for expression thereof, wherein the composition comprises:

(A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the target sequence,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, optionally comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

or

(B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to the target sequence, and

(b) at least one or more tracr mate sequences,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence; (2) the tracr mate sequence that is hybridized to the tracr sequence; and wherein the Cas9 is preferably SaCas9.

6. The method of claim 5, wherein one or more of the viral vectors are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

7. A method of treating or inhibiting a condition or a disease caused by one or more mutations in a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus in a target sequence in a subject or a non-human subject in need thereof comprising modifying the subject or a non-human subject by manipulation of the target sequence and wherein the condition or disease is susceptible to treatment or inhibition by manipulation of the target sequence comprising providing treatment comprising:

delivering a non-naturally occurring or engineered composition comprising an AAV or lentivirus vector system, comprising one or more AAV or lentivirus vectors operably encoding a composition for expression thereof, wherein the target sequence is manipulated by the composition when expressed, wherein the composition comprises:

(A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9 comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the arget sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

or

(B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to an target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, wherein the CRISPR complex comprises Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and wherein the Cas9 is preferably SaCas9.

8. The method of any preceding claim, wherein the method is carried out *in vitro*, *ex vivo* or *in vivo*.

9. The method of any preceding claim including inducing expression.

10. The method of claim 1 wherein the condition or disease is an ocular disease.

11. The method of claim 10 where the ocular disease is retinitis pigmentosa or achromatopsia.

12. The method of any of claims 4 to 8 wherein the viral vector is an AAV or lentiviral vector.

13. A method of delivering the Cas9 of any preceding claim, comprising delivering to a cell mRNA encoding the Cas9.

14. The method of any one of claims 1 to 13, wherein the polynucleotide or sequence encoding the Cas9 is delivered to the cell by delivering mRNA encoding the Cas9 to the cell.

15. A method of preparing the AAV or lentivirus vector of claim 7 comprising transfecting plasmid(s) containing or consisting essentially of nucleic acid molecule(s) coding for the AAV or lentivirus into AAV-infected or lentivirus-infected cells, and supplying AAV AAV or lentivirus rep and/or cap and/or helper nucleic acid molecules obligatory for replication and packaging of the AAV or lentivirus.

16. A method of preparing an AAV or lentivirus vector for use in the method of claim 7, comprising transfecting plasmid(s) containing or consisting essentially of nucleic acid molecule(s) coding for the AAV or lentivirus into AAV-infected or lentivirus-infected cells, and supplying AAV AAV or lentivirus rep and/or cap and/or helper nucleic acid molecules obligatory for replication and packaging of the AAV or lentivirus.

17. The method of claim 15 or 16 wherein the AAV or lentivirus rep and/or cap obligatory for replication and packaging of the AAV or lentivirus are supplied by transfecting the cells with helper plasmid(s) or helper virus(es).

18. The method of claim 17 wherein the helper virus is a poxvirus, adenovirus, lentivirus, herpesvirus or baculovirus.

19. The method of claim 18 wherein the poxvirus is a vaccinia virus.

20. The method of any of claims 15 to 20 wherein the cells are mammalian cells.

21. The method of any of claims 15 to 20 wherein the cells are insect cells and the helper virus (where present) is baculovirus.

22. The method of any preceeding claim wherein the Cas9 is a wild type, truncated or a chimeric Cas9.

23. A composition as defined in any of claims 1-22 for use in medicine or in therapy.

24. A composition as defined in any of claims 1-22 for use in a method of modeling a disease associated with a genetic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genetic locus.

25. Use of a composition as defined in any of claims 1-24 in *ex vivo or in vivo* gene or genome editing.

26. Use of a composition as defined in any of claims 1-24 in the manufacture of a medicament for *in vitro*, *ex vivo or in vivo* gene or genome editing or for use in a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus associated with a disease or in a method of treating or inhibiting a condition or diesease caused by one or more mutations in a genomic locus in a eukaryotic organism or a non-human organism.

27. A composition comprising:

(A) - I. a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a polynucleotide sequence encoding Cas9, optionally comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding SaCas9 is DNA or RNA,

or

(B) I. polynucleotides comprising:

(a) a guide sequence capable of hybridizing to an target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a polynucleotide sequence encoding Cas9, and

III. a polynucleotide sequence comprising a tracr sequence,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, (2) the tracr mate sequence that is hybridized to the tracr sequence, and the polynucleotide sequence encoding Cas9 is DNA or RNA; and the Cas9 is preferably SaCas9.

for use in medicine or therapy; or for use in a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus associated with a disease or disorder; or for use in a method of treating or inhibiting a condition caused by one or more mutations in a genetic locus associated with a disease in a eukaryotic organism or a non-human organism.; or for use in *in vitro, ex vivo or in vivo* gene or genome editing.

28. The composition of claim 27, wherein the polynucleotides are comprised within a vector system comprising one or more vectors.

29. The method, use or composition of any of the preceding claims, wherein the CRISPR-Cas system RNA is a chimeric RNA (chiRNA).

30. The method, use or composition of any of the preceding claims, wherein the CRISPR-Cas system is a multiplexed SaCas9 enzyme system further comprising multiple chimeras and/or multiple multiguide sequences and a single tracr sequence.

31. The method, use or composition according any of the preceding claims, wherein theCas9 is a nuclease directing cleavage of both strands at the location of the target sequence.

32. The method, use or composition according to any of the preceding claims, wherein the Cas9 comprises one or more mutations.

33. The method, use or composition according to claim 32, wherein the Cas9 comprises one or more mutations D10A, E762A, H840A, N854A, N863A or D986A.

34. The method, use or composition according to claim 32 wherein the one or more mutations is in a RuvC1 domain of the Cas9.

35. The method, use or composition according to claim 30, wherein the Cas9 is a nickase directing cleavage at the location of the target sequence.

36. The method, use or composition according to claim 35, wherein the nickase is a double nickase.

37. The method, use or composition according to any preceding claim further comprising at least two or more NLS.

38. The method, use or composition according to any preceding claim, wherein the SaCas9 has one or more mutations in a catalytic domain, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the enzyme further comprises a functional domain.

39. The method, use or composition according to claim 38, wherein the functional domain is a transcriptional activation domain.

40. The method, use or composition according to claim 39, wherein the transcriptional activation domain is VP64.

41. A therapeutic genome editing method for treating or inhibiting a condition or a disease caused by one or more mutations in a genomic locus in a eukaryotic organism or a non-human organism comprising manipulation of a target sequence within a coding, non-coding or regulatory element of said genomic locus in a target sequence in a subject or a non-human subject in need thereof comprising modifying the subject or a non-human subject by manipulation of the target sequence and wherein the condition or disease is susceptible to treatment or inhibition by manipulation of the target sequence comprising providing treatment comprising:

delivering a non-naturally occurring or engineered composition comprising an AAV or lentivirus vector system, comprising one or more AAV or lentivirus vectors operably encoding a composition for expression thereof, wherein the target sequence is manipulated by the composition when expressed, wherein the composition comprises:

(A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9 comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the Cas9 complexed with (1) the guide sequence that is hybridized to the arget sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

or

(B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(a) a guide sequence capable of hybridizing to an target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding Cas9, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises Cas9 complexed with (1) the guide sequence that is hybridized to the target sequence, (2) the tracr mate sequence that is hybridized to the tracr; and the Cas9 is preferably SaCas9.

42. The method of claim 41, wherein the condition or disease is retinitis pigentosa or achromatopsia.

43. The method of claim 41, wherein the AAV is AAV1, AAV2, AAV5, AAV7, AAV8, AAV DJ or any combination thereof.

44. A method of individualized or personalized treatment of a genetic disease in a subject in need of such treatment comprising:

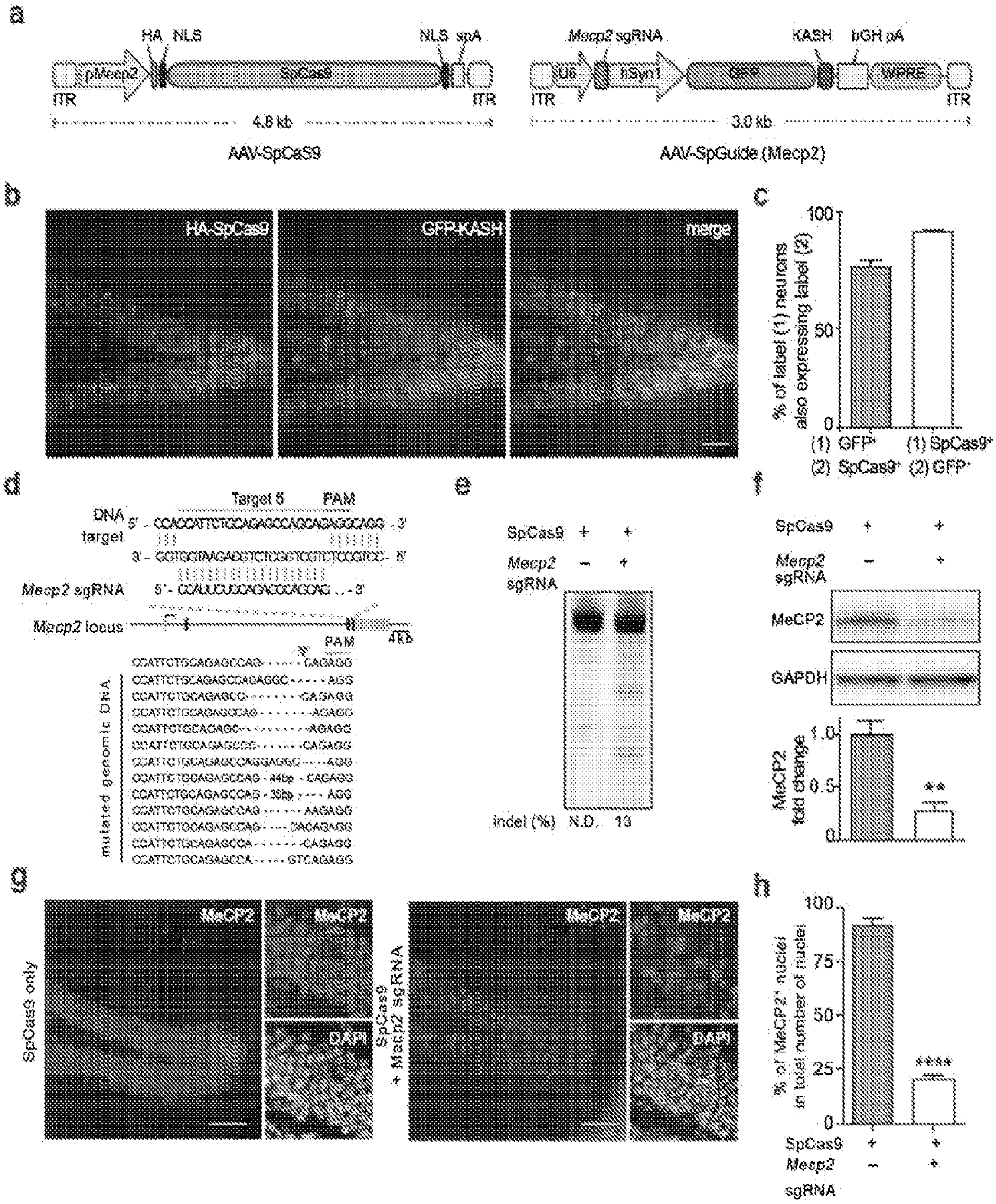
(a) introducing multiple mutations *ex vivo* in a tissue, organ or a cell line comprising SaCas9-expressing eukaryotic cell(s), or *in vivo* in a transgenic non-human mammal having cells that express SaCas9, comprising delivering to cell(s) of the tissue, organ, cell or mammal the vector as herein-discussed, wherein the specific mutations or precise sequence substitutions are or have been correlated to the genetic disease;

(b) testing treatment(s) for the genetic disease on the cells to which the vector has been delivered that have the specific mutations or precise sequence substitutions correlated to the genetic disease; and

(c) treating the subject based on results from the testing of treatment(s) of step (b).

45. The method of claim 44, wherein the genetic disease is an ocular disease.

46. The method of claim 45, wherein the ocular disease is retinitis pigmentosa or achromatopsia.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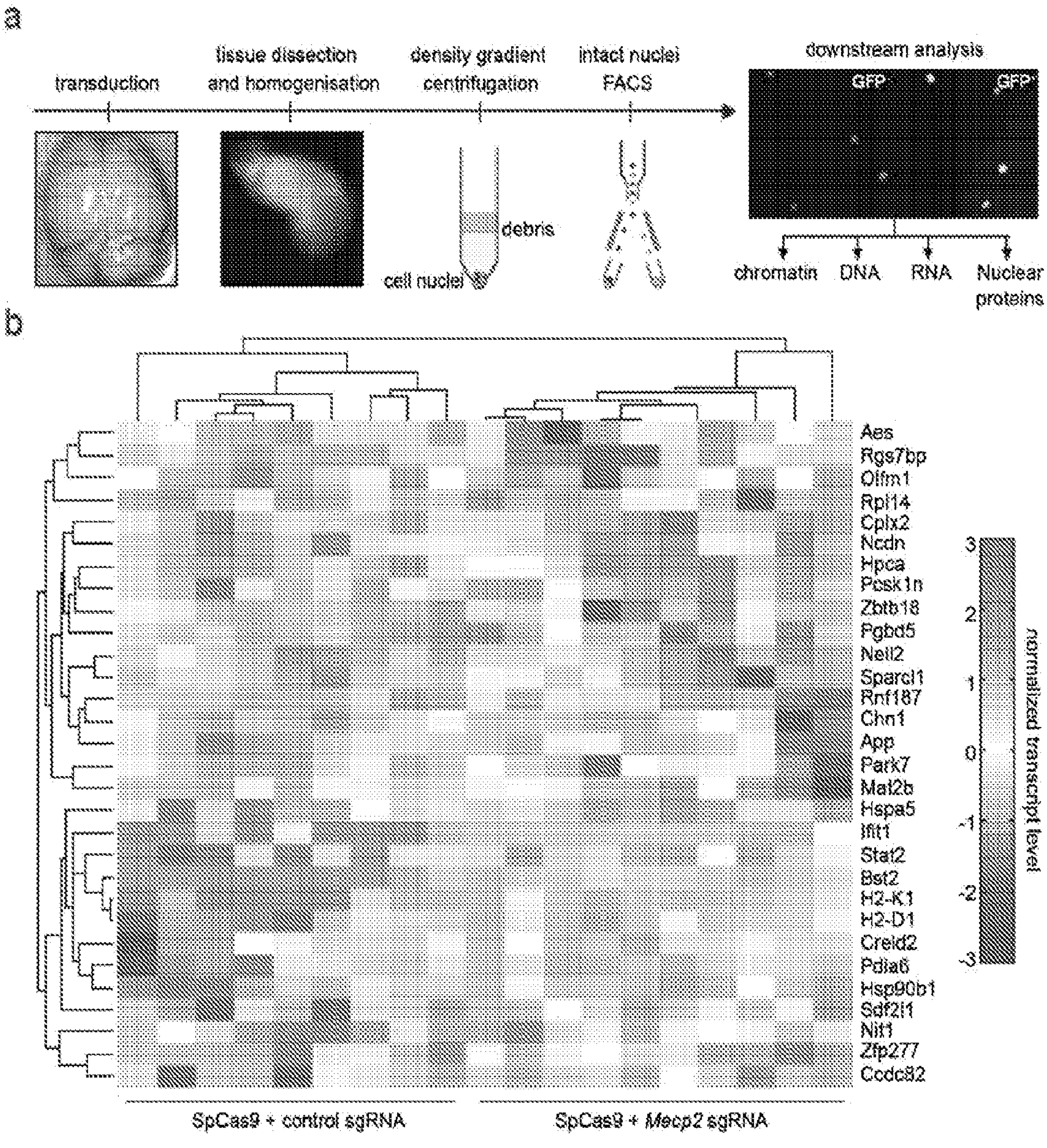
HA NLS
NLS spA
pMecp2
SpCas9
ITR
ITR
4.8 kb
AAV-SpCas9
Mecp2 sgRNA
KASH bGH pA
U6
hSyn1
GFP
WPRE
3.0 kb
AAV-SpGuide (Mecp2)
b
HA-SpCas9
GFP-KASH
merge
c
% of label (1) neurons also expressing label (2)
(1) GFP+ (2) SpCas9+
(1) SpCas9+ (2) GFP+
d
Target 5
PAM
DNA target
Mecp2 sgRNA
Mecp2 locus
mutated genomic DNA
e
SpCas9
Mecp2 sgRNA
indel (%) N.D. 13
f
SpCas9
Mecp2 sgRNA
MeCP2
GAPDH
MeCP2 fold change
g
MeCP2
DAPI
SpCas9 only
SpCas9 + Mecp2 sgRNA
h
% of MeCP2+ nuclei in total number of nuclei
SpCas9
Mecp2 sgRNA

FIG. 1A-H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transduction
tissue dissection and homogenisation
density gradient centrifugation
intact nuclei FACS
downstream analysis
GFP
GFP
debris
cell nuclei
chromatin
DNA
RNA
Nuclear proteins
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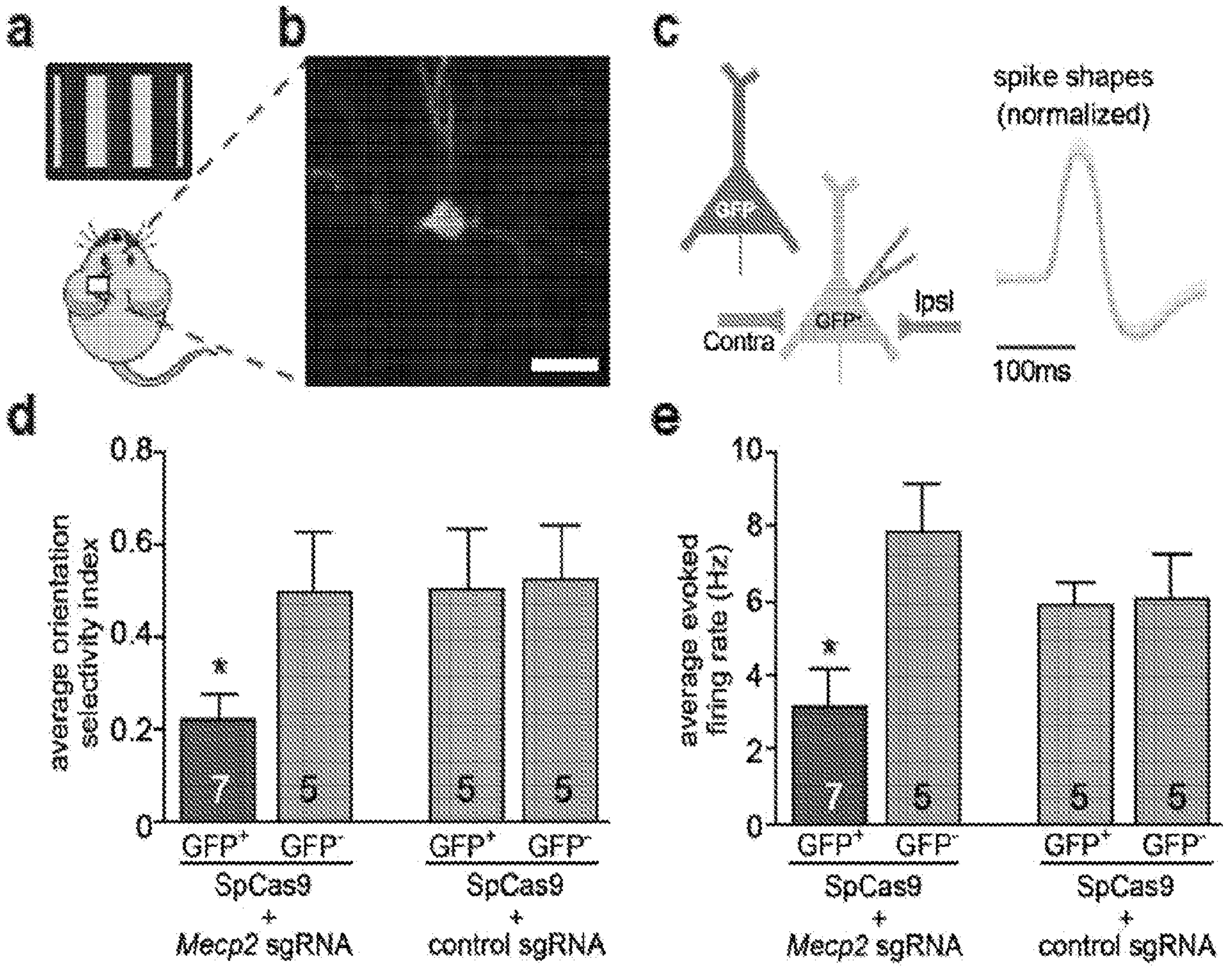
Aes
Rgs7bp
Olfm1
Rpl14
Cplx2
Ncdn
Hpca
Pcsk1n
Zbtb18
Pgbd5
Nell2
Sparcl1
Rnf187
Chn1
App
Park7
Mat2b
Hspa5
Ifit1
Stat2
Bst2
H2-K1
H2-D1
Creld2
Pdia6
Hsp90b1
Sdf2l1
Nit1
Zfp277
Ccdc82
3
2
1
0
-1
-2
-3
normalized transcript level
SpCas9 + control sgRNA
SpCas9 + *Mecp2* sgRNA

FIG. 2A-B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b
c
spike shapes
(normalized)
GFP⁻
GFP⁺
Contra
Ipsi
100ms
d
average orientation selectivity index
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0.6
0.4
0.2
0
*
7
5
5
5
GFP⁺ GFP⁻
SpCas9 + Mecp2 sgRNA
GFP⁺ GFP⁻
SpCas9 + control sgRNA
e
average evoked firing rate (Hz)
10
8
6
4
2
0
*
7
5
5
5
GFP⁺ GFP⁻
SpCas9 + Mecp2 sgRNA
GFP⁺ GFP⁻
SpCas9 + control sgRNA

FIG. 3A-E

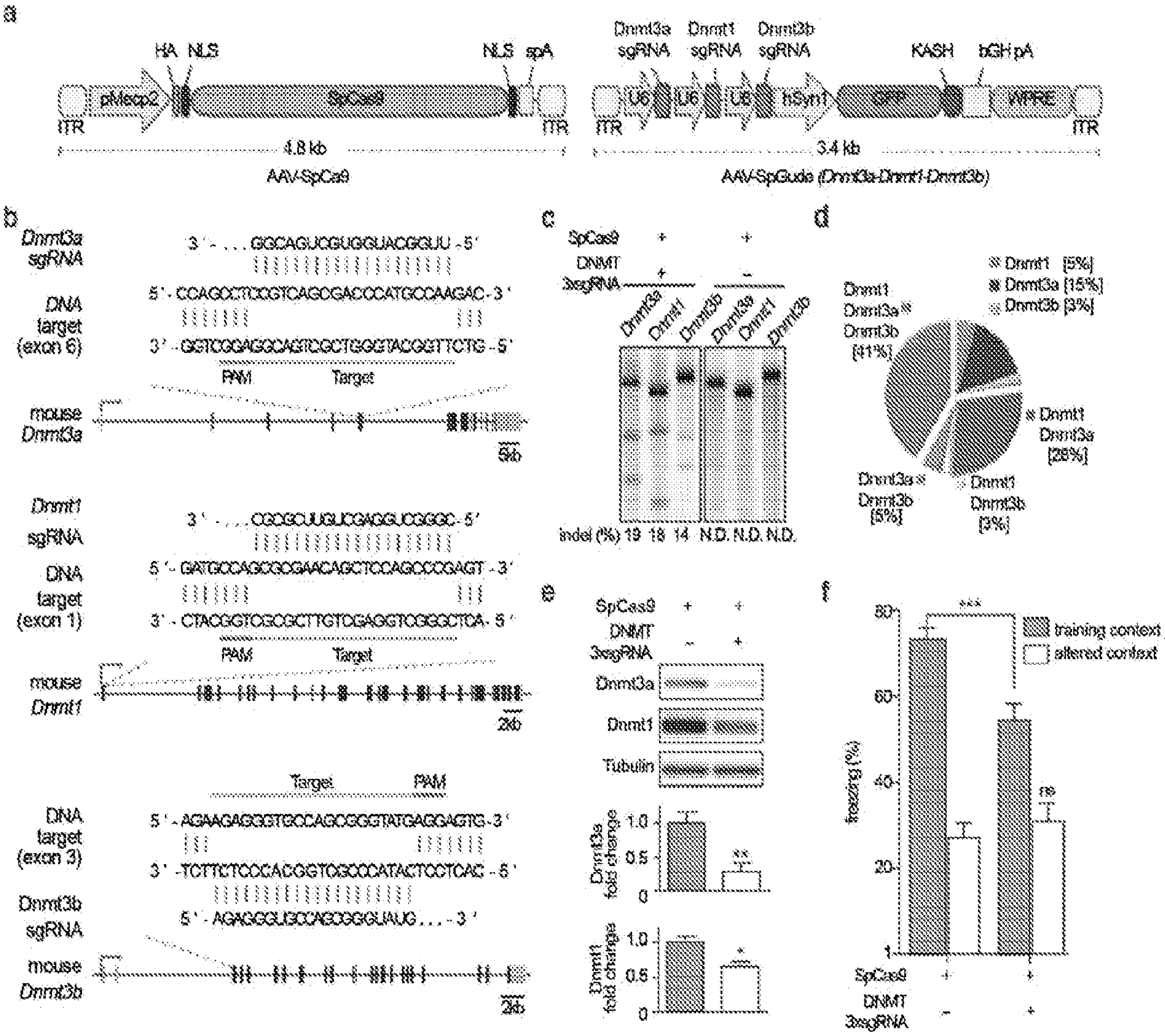


FIG. 4A-F

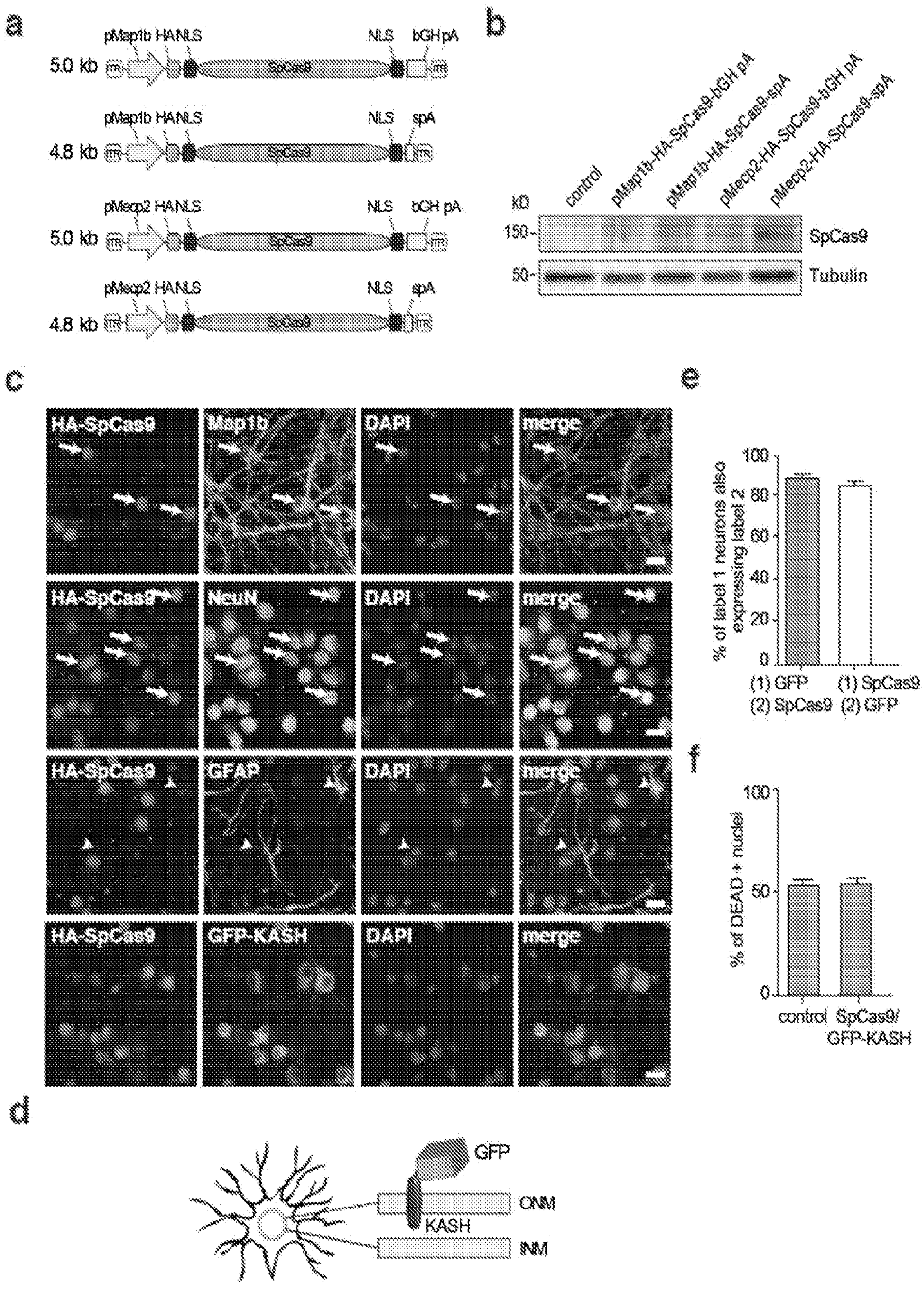

a
pMap1b HA NLS
NLS bGH pA
5.0 kb
pMap1b HA NLS
NLS spA
4.8 kb
pMecp2 HA NLS
NLS bGH pA
5.0 kb
pMecp2 HA NLS
NLS spA
4.8 kb
b
control
pMap1b-HA-SpCas9-bGH pA
pMap1b-HA-SpCas9-spA
pMecp2-HA-SpCas9-bGH pA
pMecp2-HA-SpCas9-spA
kD
150
SpCas9
50
Tubulin
c
HA-SpCas9
Map1b
DAPI
merge
HA-SpCas9
NeuN
DAPI
merge
HA-SpCas9
GFAP
DAPI
merge
HA-SpCas9
GFP-KASH
DAPI
merge
e
% of label 1 neurons also expressing label 2
100
80
60
40
20
0
(1) GFP
(2) SpCas9
(1) SpCas9
(2) GFP
f
% of DEAD + nuclei
100
50
0
control
SpCas9/
GFP-KASH
d
GFP
ONM
KASH
INM

FIG. 5A-F

a

	Mecp2 target sequence	PAM
Target 1	CTGGGAGAGGGAGCCCCTCC	AGG
Target 2	AAAGGTGGGAGACACCTCCT	TGG
Target 3	TCCAACCTTCAGGCAAGGTG	GGG
Target 4	AGGAAGTCTGGCCGATCTGC	TGG
Target 5	CCATTCTGCAGAGCCAGCAG	AGG
Target 6	CTCTGAGGCCCTGGAGATCC	TGG

b

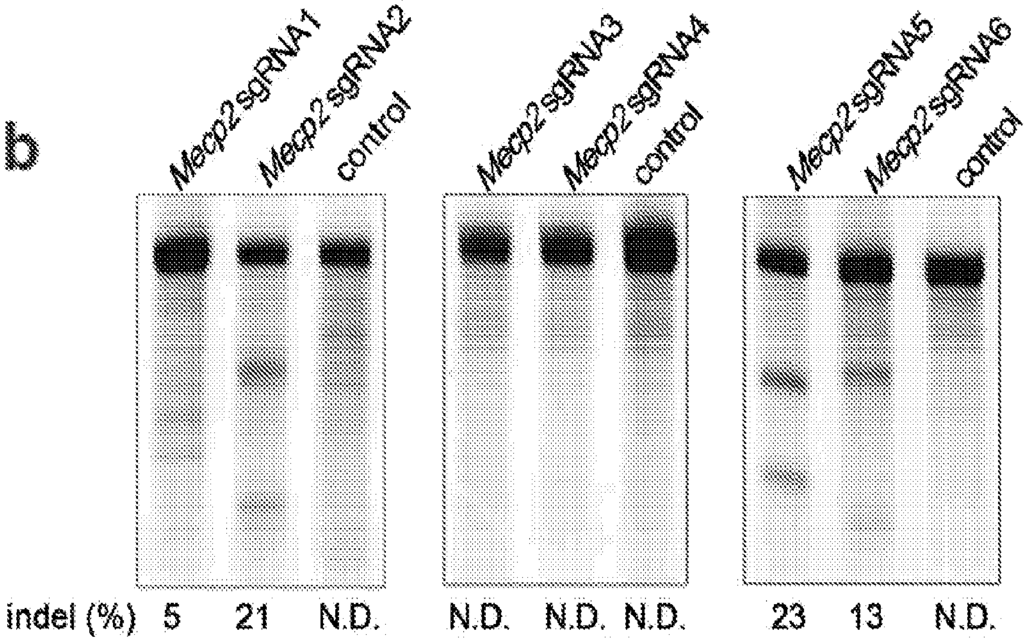


FIG. 6A-B

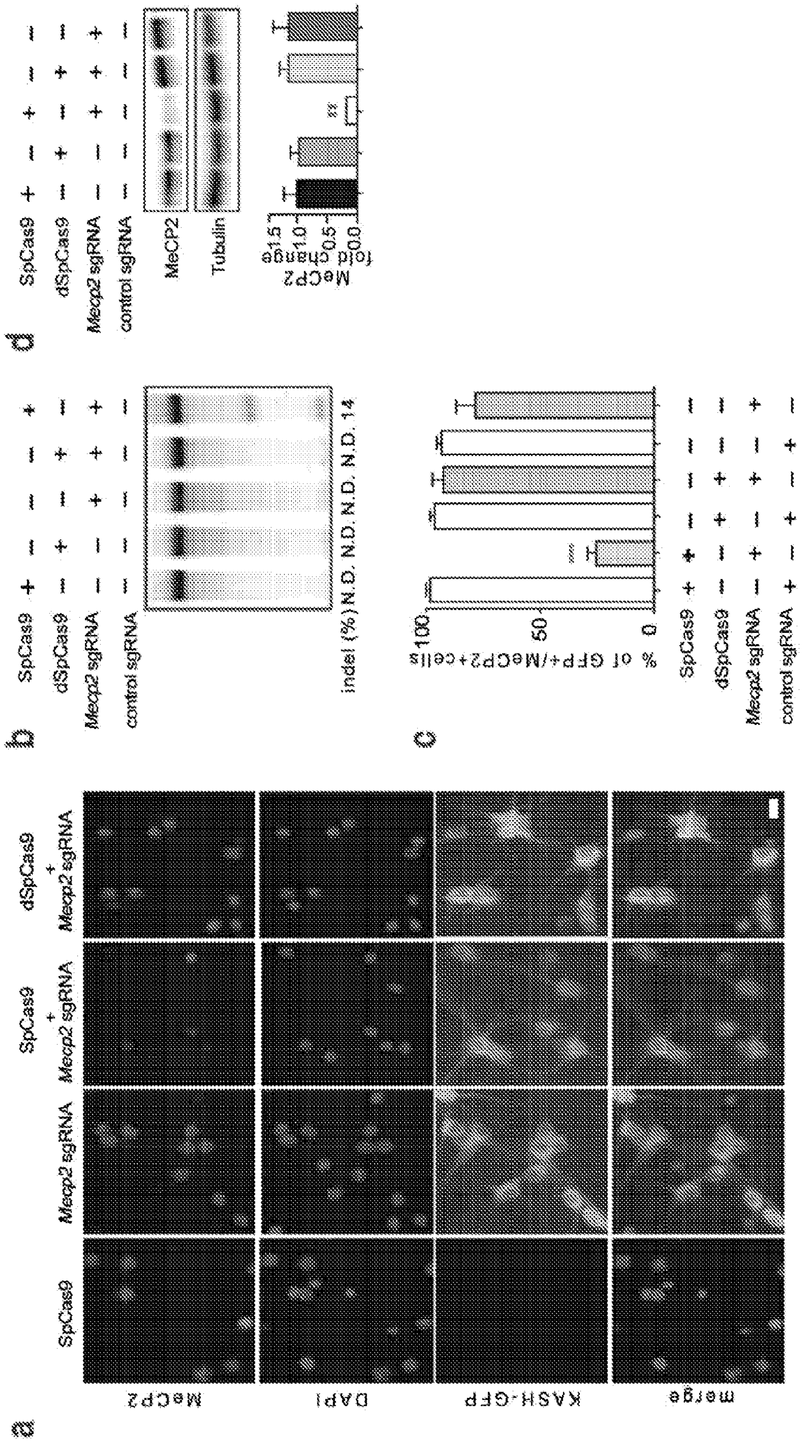

a
SpCas9
Mecp2 sgRNA
SpCas9 + Mecp2 sgRNA
dSpCas9 + Mecp2 sgRNA
MeCP2
DAPI
KASH-GFP
merge
b
SpCas9
dSpCas9
Mecp2 sgRNA
control sgRNA
indel (%) N.D. N.D. N.D. N.D. 14
c
% of GFP+/MeCP2+cells
100
50
0
d
MeCP2
Tubulin
MeCP2 fold change
1.5
1.0
0.5
0.0

FIG. 7A-D

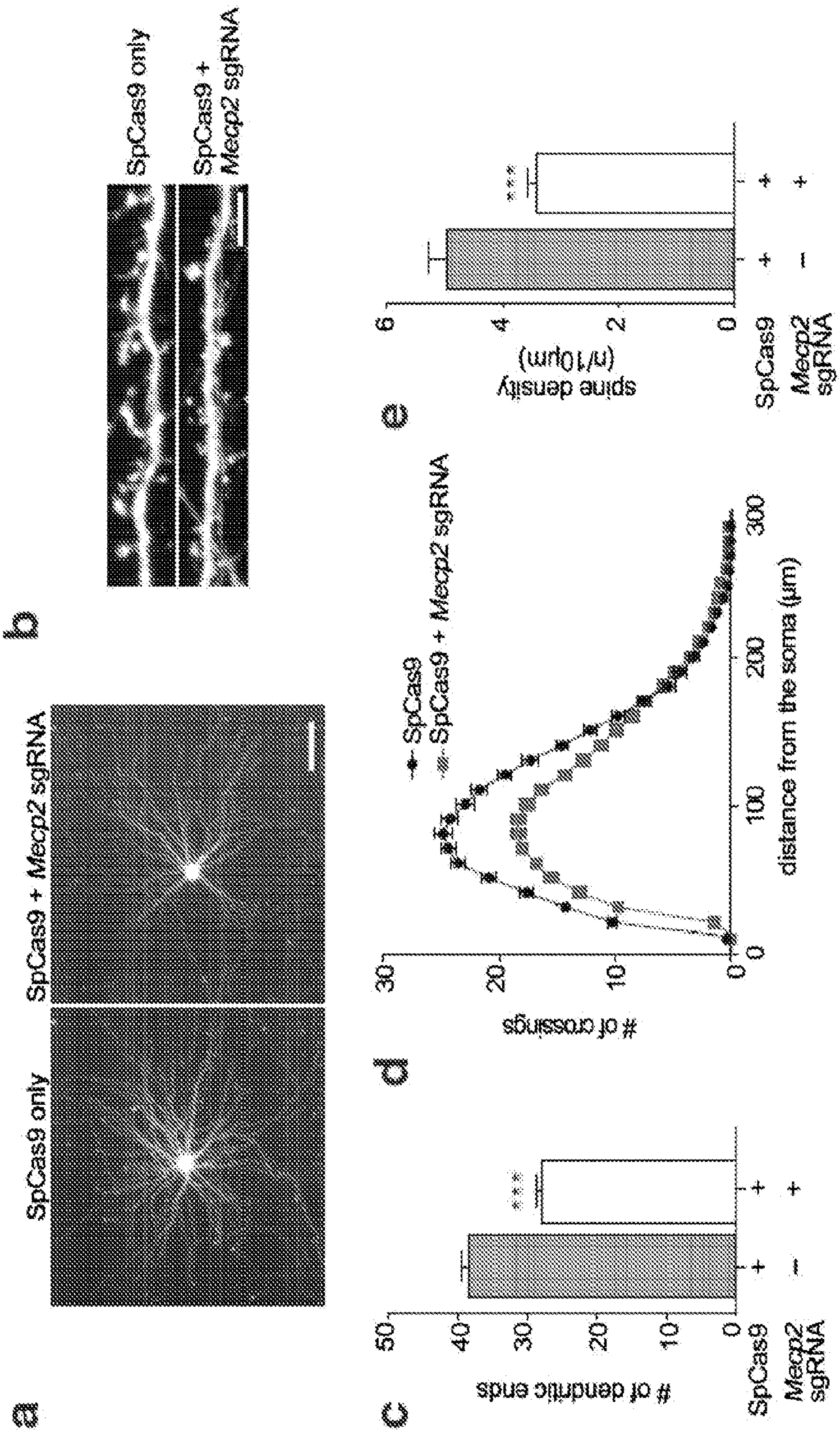
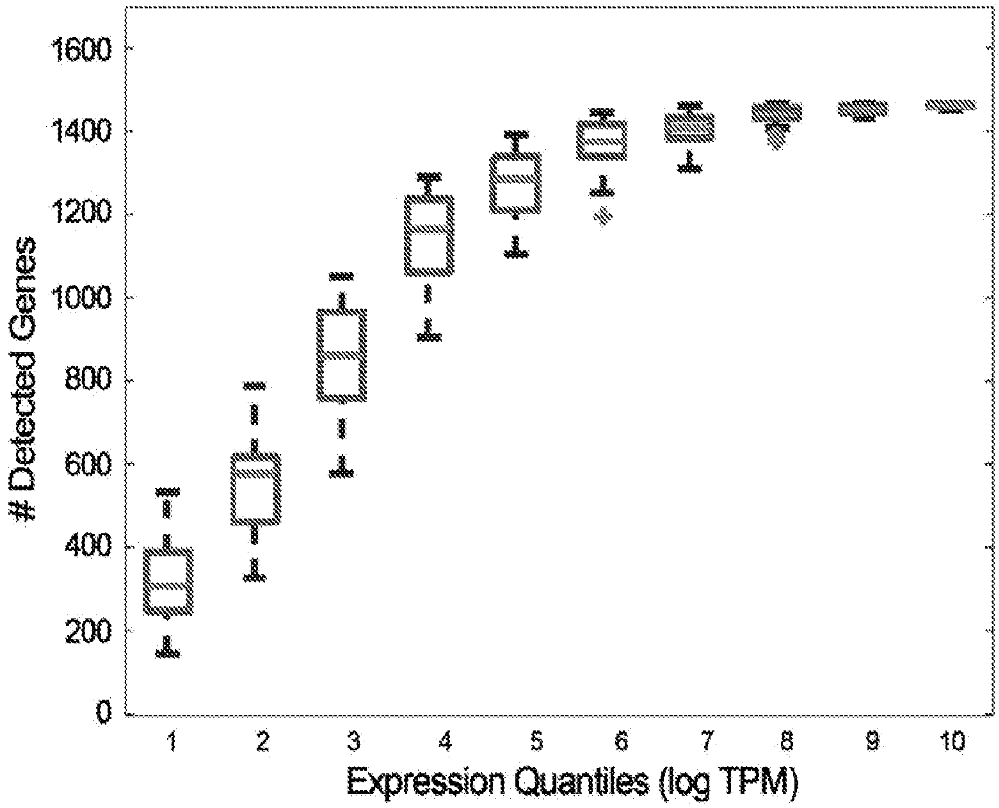

a
SpCas9 only
SpCas9 + Mecp2 sgRNA
b
SpCas9 only
SpCas9 + Mecp2 sgRNA
c
# of dendritic ends
50
40
30
20
10
0
SpCas9
Mecp2 sgRNA
+
−
+
+
***
d
# of crossings
30
20
10
0
0
100
200
300
distance from the soma (μm)
SpCas9
SpCas9 + Mecp2 sgRNA
e
spine density (n/10μm)
6
4
2
0
SpCas9
Mecp2 sgRNA
+
−
+
+
***

FIG. 8A-E



1600
1400
1200
1000
800
600
400
200
0
Detected Genes
1
2
3
4
5
6
7
8
9
10
Expression Quantiles (log TPM)

FIG. 9

a

target	target sequence	PAM
Dnmt3a	TTGGCATGGGTCGCTGACGG	AGG
Dnmt1	CGGGCTGGAGCTGTTCGCGC	TGG
Dnmt3b	AGAGGGTGCCAGCGGGTATG	AGG

b

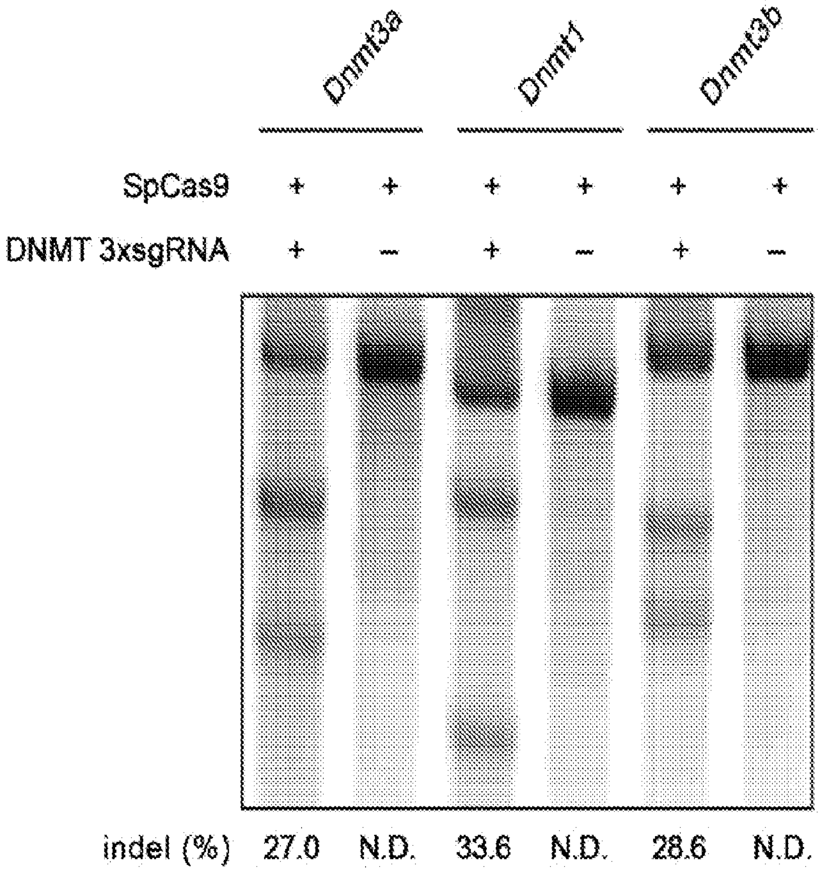


FIG. 10A-B

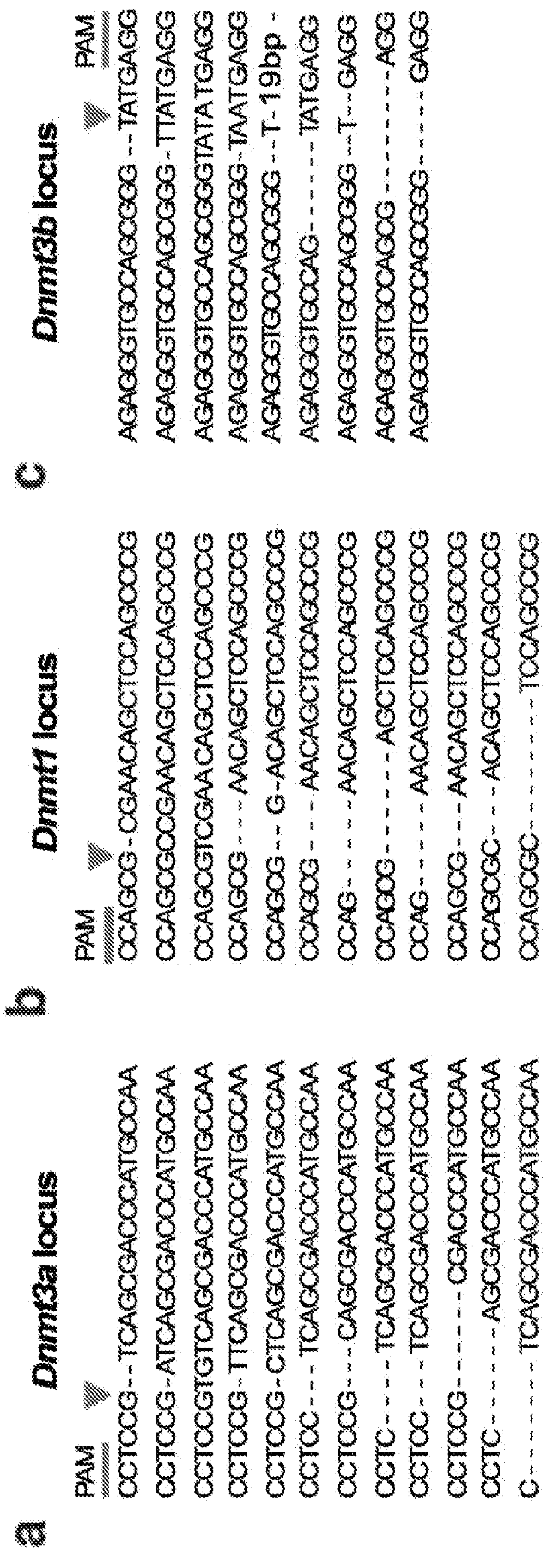

a
Dnmt3a locus
PAM
CCTCCG--TCAGCGACCCATGCCAA
CCTCCG-ATCAGCGACCCATGCCAA
CCTCCGTGTCAGCGACCCATGCCAA
CCTCCG-TTCAGCGACCCATGCCAA
CCTCCG-CTCAGCGACCCATGCCAA
CCTCC---TCAGCGACCCATGCCAA
CCTCCG----CAGCGACCCATGCCAA
CCTC-----TCAGCGACCCATGCCAA
CCTCC----TCAGCGACCCATGCCAA
CCTCCG------CGACCCATGCCAA
CCTC-------AGCGACCCATGCCAA
C---------TCAGCGACCCATGCCAA
b
Dnmt1 locus
PAM
CCAGCG-CGAACAGCTCCAGCCCG
CCAGCGCCGAACAGCTCCAGCCCG
CCAGCGTCGAACAGCTCCAGCCCG
CCAGCG----AACAGCTCCAGCCCG
CCAGCG---G-ACAGCTCCAGCCCG
CCAGCG----AACAGCTCCAGCCCG
CCAG-------AACAGCTCCAGCCCG
CCAGCG--------AGCTCCAGCCCG
CCAG------AACAGCTCCAGCCCG
CCAGCG----AACAGCTCCAGCCCG
CCAGCGC----ACAGCTCCAGCCCG
CCAGCGC---------TCCAGCCCG
c
Dnmt3b locus
PAM
AGAGGGTGCCAGCGGG---TATGAGG
AGAGGGTGCCAGCGGG-TTATGAGG
AGAGGGTGCCAGCGGGTATATGAGG
AGAGGGTGCCAGCGGG-TAATGAGG
AGAGGGTGCCAGCGGG---T-19bp-
AGAGGGTGCCAG-------TATGAGG
AGAGGGTGCCAGCGGG---T--GAGG
AGAGGGTGCCAGCG----------AGG
AGAGGGTGCCAGCGGG------GAGG

FIG. 11A-C

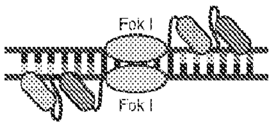
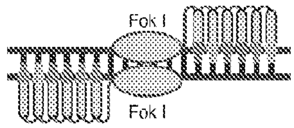
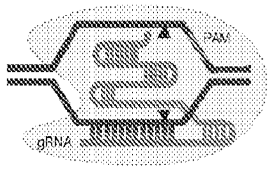
	Zinc Finger Nucleases	TALE Nucleases	CRISPR-Cas9
			
Recognition site	Typically 9 to 18 bp per ZFP, 18 to 36 bp per ZFN pair	Typically 14 to 20 bp per TALE, 28 to 40bp per TALEN	22bp (20bp guide sequence + 2bp PAM sequence); up to 44 bp for double nicking
Specificty	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated
Targeting constraints	Difficult to target non-G-rich sequences	5' targeted base must be a T	Targeted sequence must precede a PAM
Ease of engineering	Difficult, may require substantial protein engineering	Moderate, requires complex molecular cloning methods	Easily re-targeted using standard cloning procedures and oligo synthesis
Immunogenicity	Likely low, as ZFs are based on human transcription factor protein scaffold	Unknown, protein derived from the bacteria *Xanthomonas sp.*	Unknown, protein derived from various bacterial species
Ease of *ex vivo* delivery	Relatively easy through methods such as electroporation and lentiviral transduction	Relatively easy through methods such as electroporation and lentiviral transduction	Relatively easy through methods such as electroporation and lentiviral transduction
Ease of *in vivo* delivery	Relatively easy due to small size of ZFN expression casettes, allows use in a variety of viral vectors	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events	Moderate: The commonly used Cas9 from *S. pyogenes* may be large enough to impose delivery problems, but other, smaller orthologoues exist
Ease of multiplexing	Low	Low	High

FIG. 12

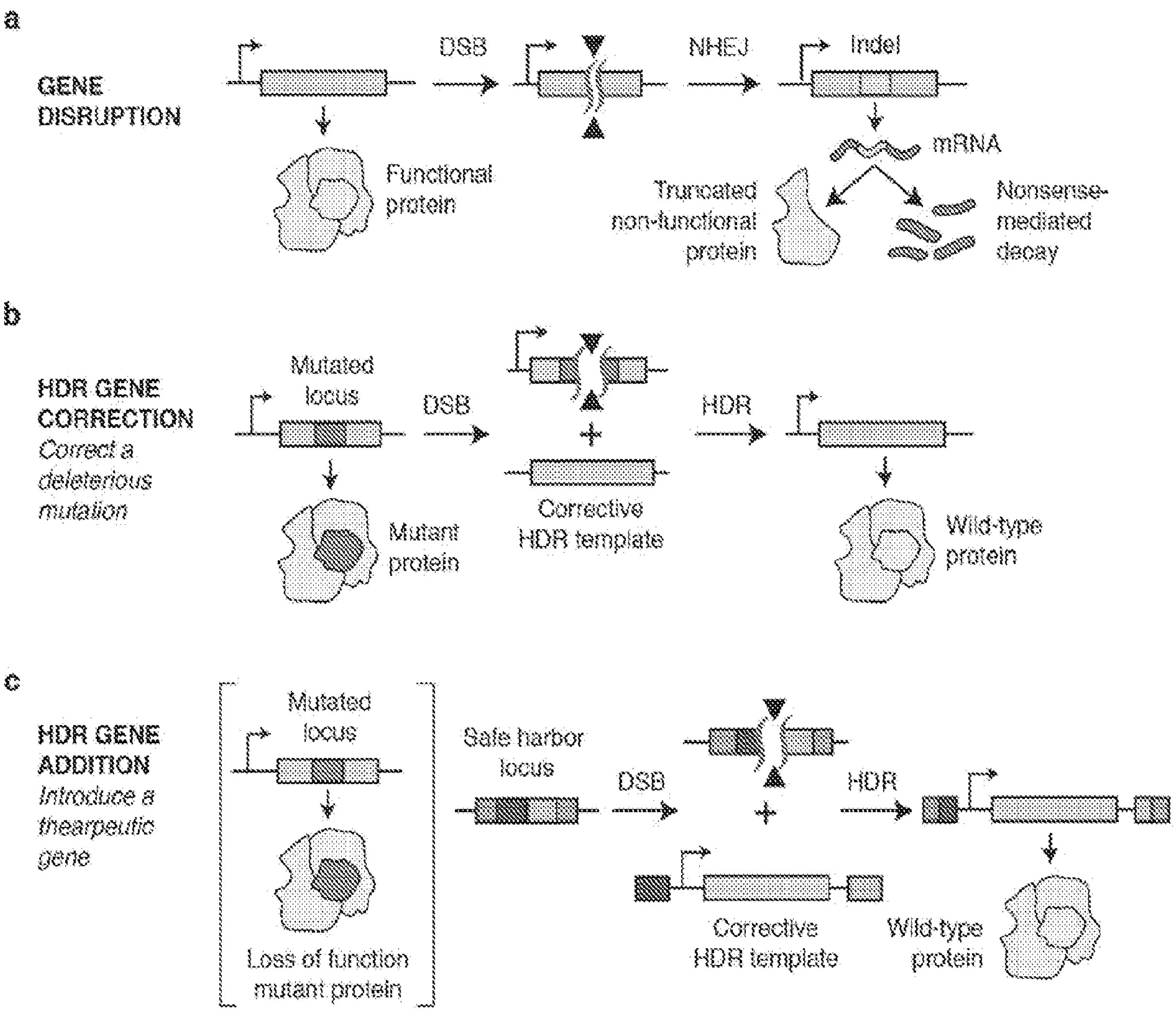

a
GENE DISRUPTION
DSB
NHEJ
Indel
Functional protein
mRNA
Truncated non-functional protein
Nonsense-mediated decay
b
HDR GENE CORRECTION
Correct a deleterious mutation
Mutated locus
DSB
HDR
Mutant protein
Corrective HDR template
Wild-type protein
c
HDR GENE ADDITION
Introduce a thearpeutic gene
Mutated locus
Loss of function mutant protein
Safe harbor locus
DSB
HDR
Corrective HDR template
Wild-type protein

FIG. 13A-C

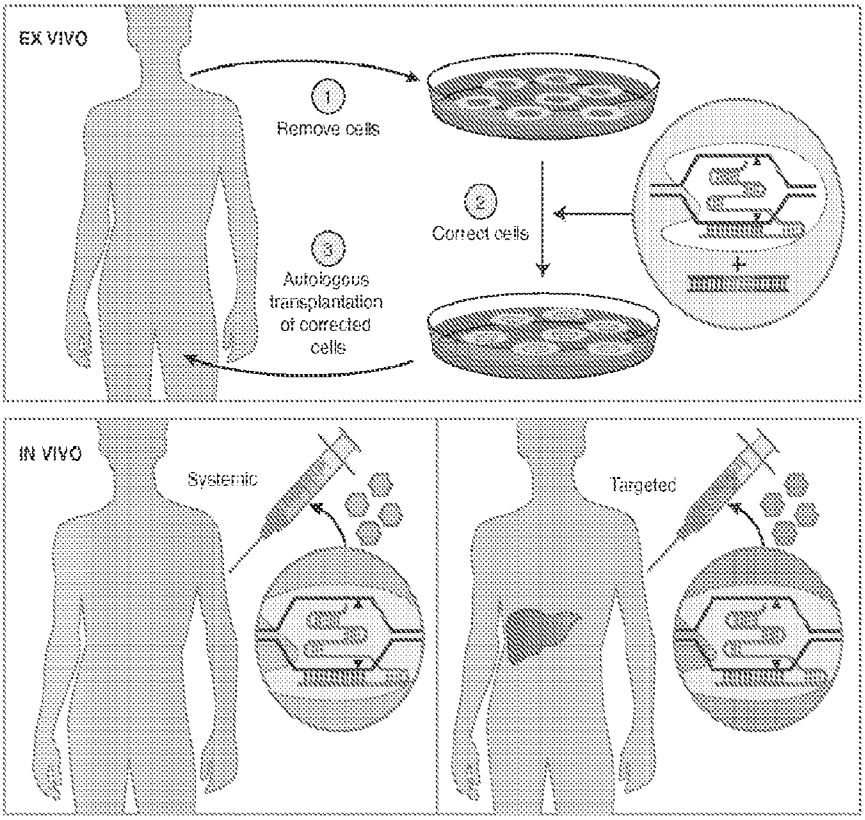
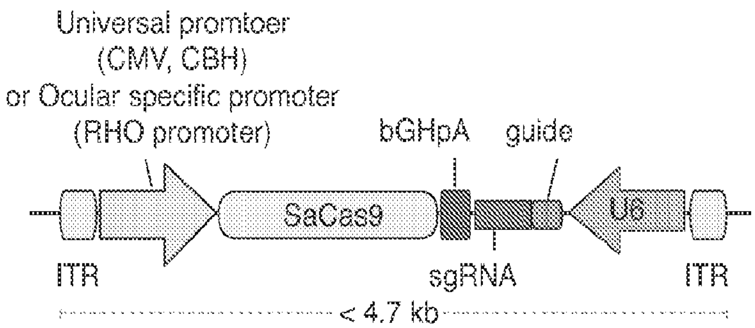

EX VIVO
1
Remove cells
2
Correct cells
3
Autologous transplantation of corrected cells
IN VIVO
Systemic
Targeted

FIG. 14



CMV: cytomegalovirus promoter
CBH: CMV Beta-actin Hybrid promoter
RHO: Rhodopsin promoter

FIG. 15

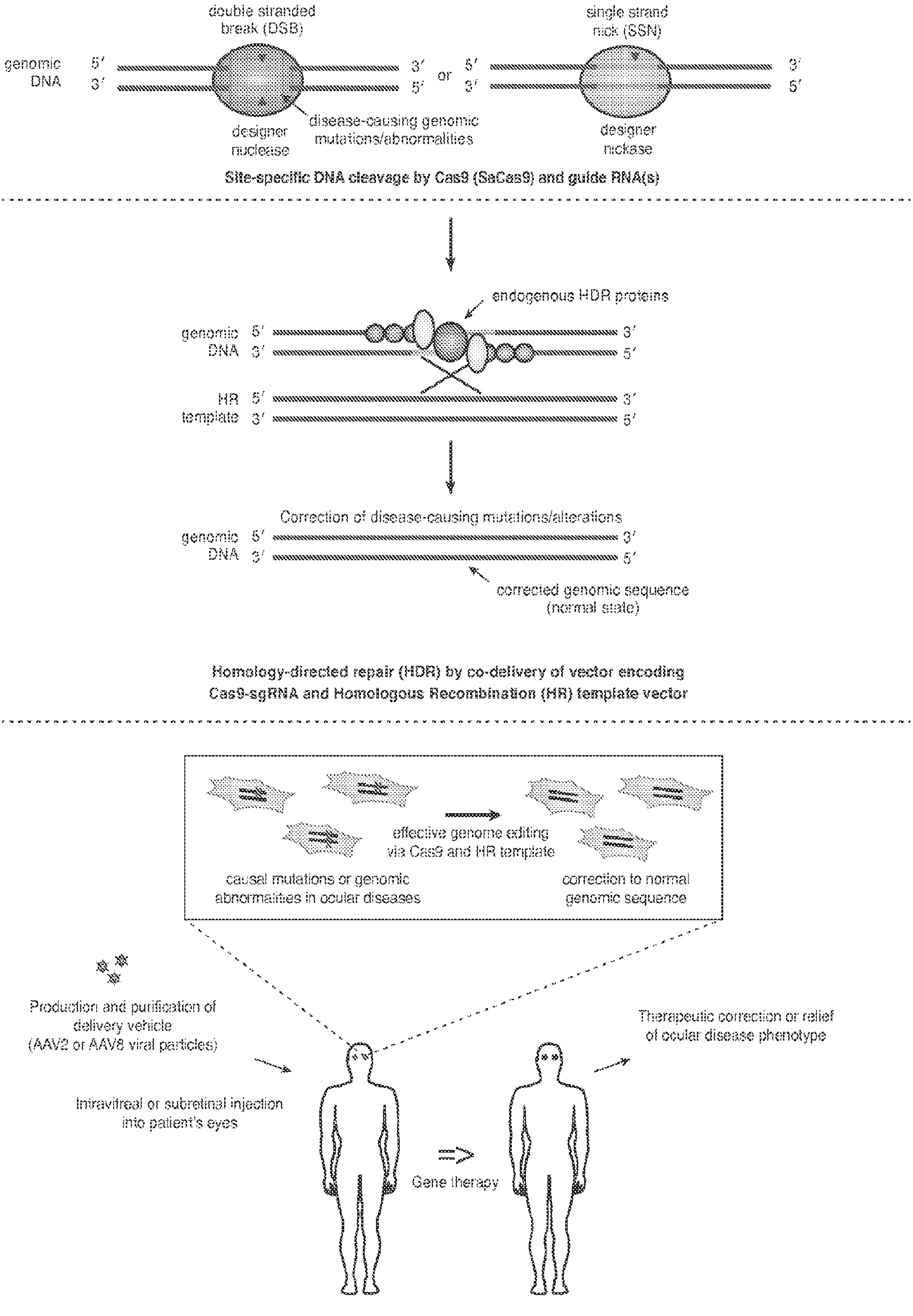

double stranded
break (DSB)
genomic
DNA
5′
3′
3′
5′
or
5′
3′
3′
5′
single strand
nick (SSN)
designer
nuclease
disease-causing genomic
mutations/abnormalities
designer
nickase
Site-specific DNA cleavage by Cas9 (SaCas9) and guide RNA(s)
endogenous HDR proteins
genomic
DNA
HR
template
Correction of disease-causing mutations/alterations
corrected genomic sequence
(normal state)
Homology-directed repair (HDR) by co-delivery of vector encoding
Cas9-sgRNA and Homologous Recombination (HR) template vector
effective genome editing
via Cas9 and HR template
causal mutations or genomic
abnormalities in ocular diseases
correction to normal
genomic sequence
Production and purification of
delivery vehicle
(AAV2 or AAV8 viral particles)
Intravitreal or subretinal injection
into patient's eyes
Gene therapy
Therapeutic correction or relief
of ocular disease phenotype

FIG. 16

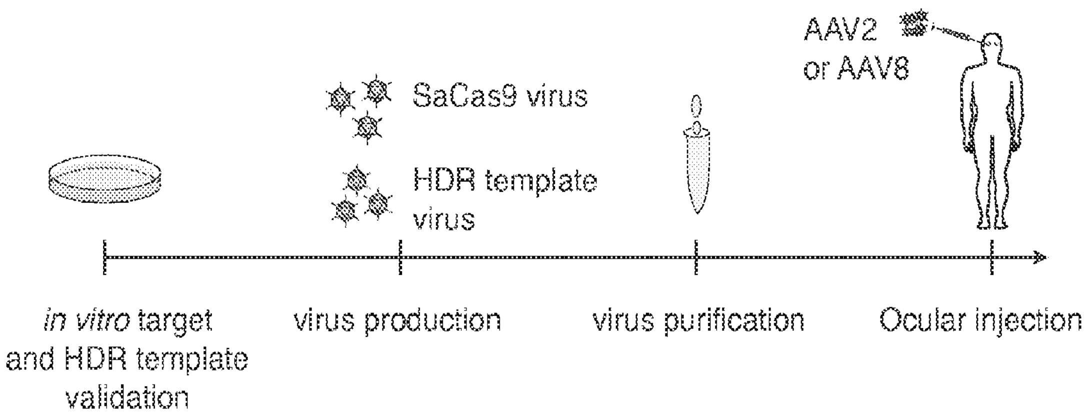

AAV2
or AAV8
SaCas9 virus
HDR template
virus
in vitro target
and HDR template
validation
virus production
virus purification
Ocular injection

FIG. 17

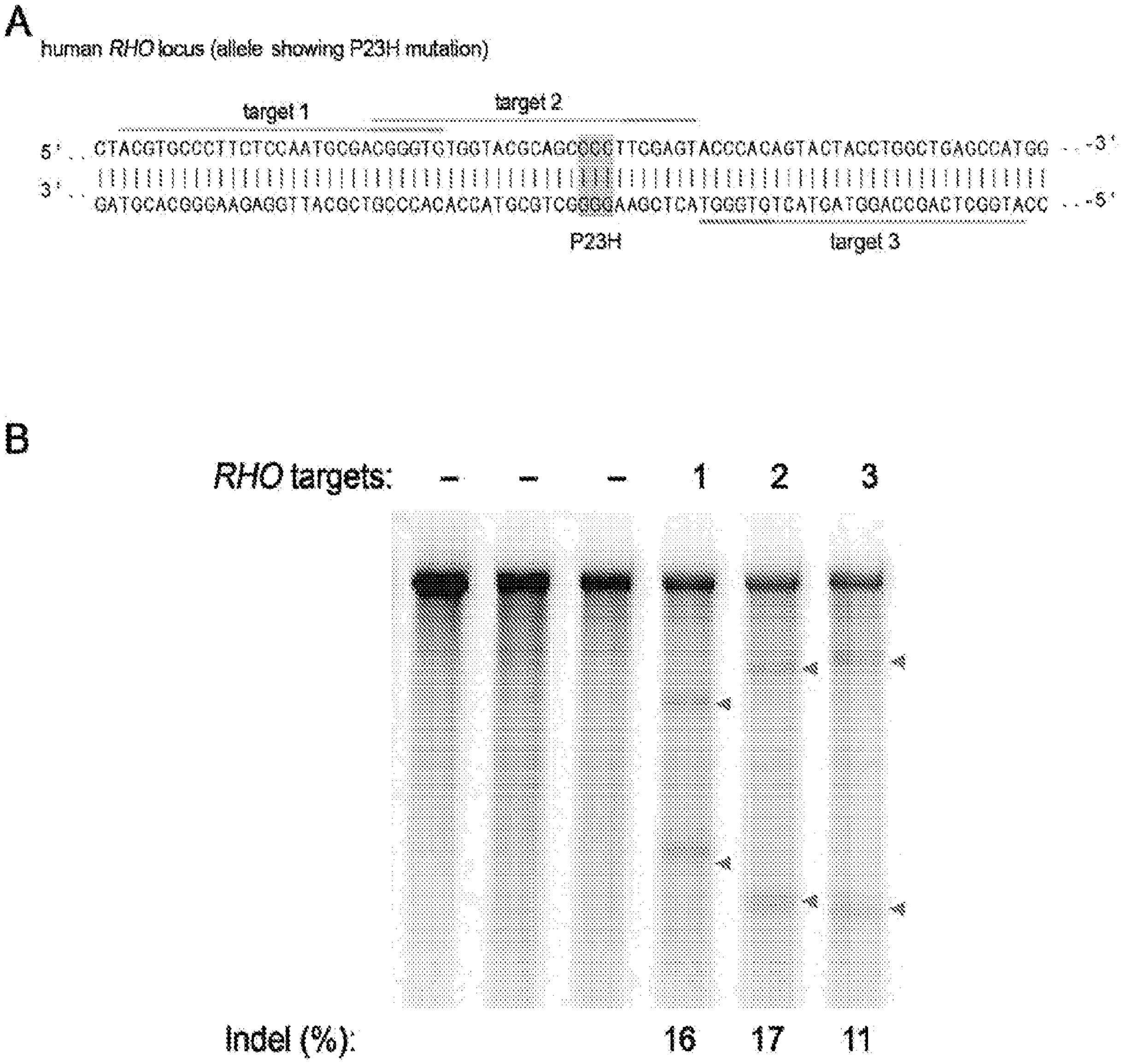

A
human RHO locus (allele showing P23H mutation)
target 1
target 2
P23H
target 3
B
RHO targets: – – – 1 2 3
Indel (%): 16 17 11

FIG. 18A-B

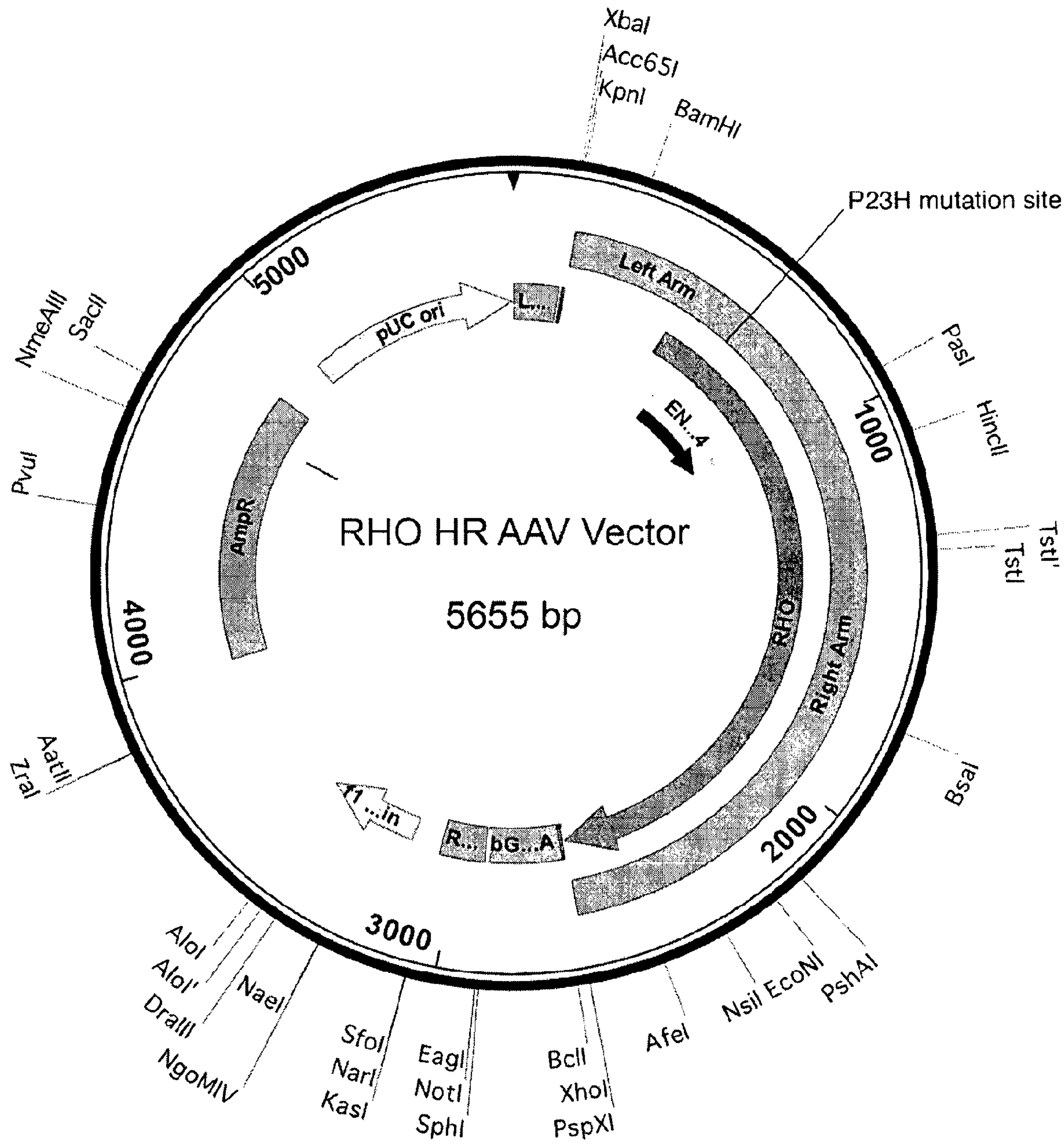

RHO HR AAV Vector
5655 bp
XbaI
Acc65I
KpnI
BamHI
P23H mutation site
PasI
HincII
TstI'
TstI
BsaI
PshAI
NsiI EcoNI
AfeI
BclI
XhoI
PspXI
EagI
NotI
SphI
SfoI
NarI
KasI
AloI
AloI'
DraIII
NaeI
NgoMIV
AatII
ZraI
PvuI
NmeAIII
SacII
Left Arm
Right Arm
RHO
EN...4
pUC ori
L...
AmpR
f1 ...in
R...
bG...A
5000
1000
2000
3000
4000

FIG. 19

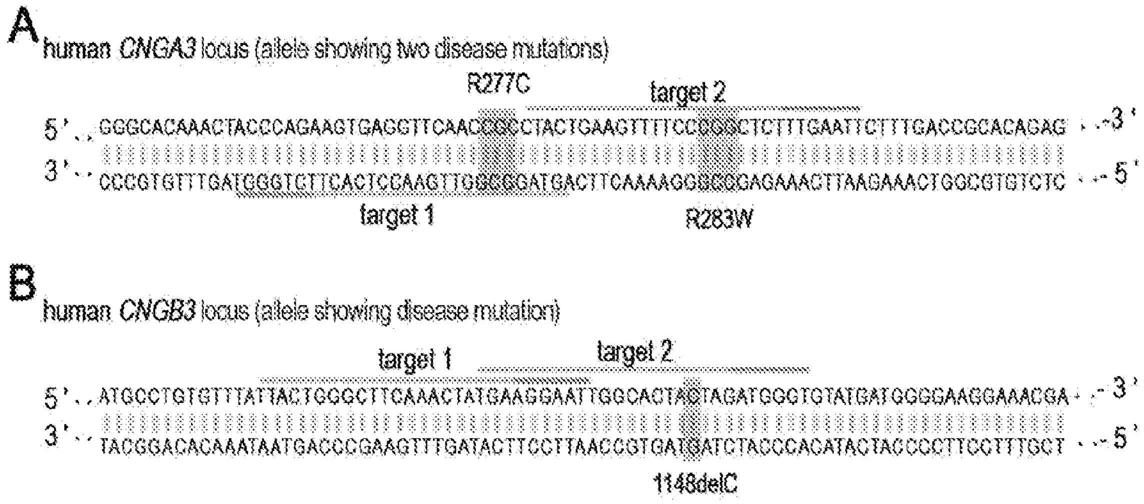

A
human CNGA3 locus (allele showing two disease mutations)
R277C
target 2
target 1
R283W
B
human CNGB3 locus (allele showing disease mutation)
target 1
target 2
1148delC

FIG. 20A-B

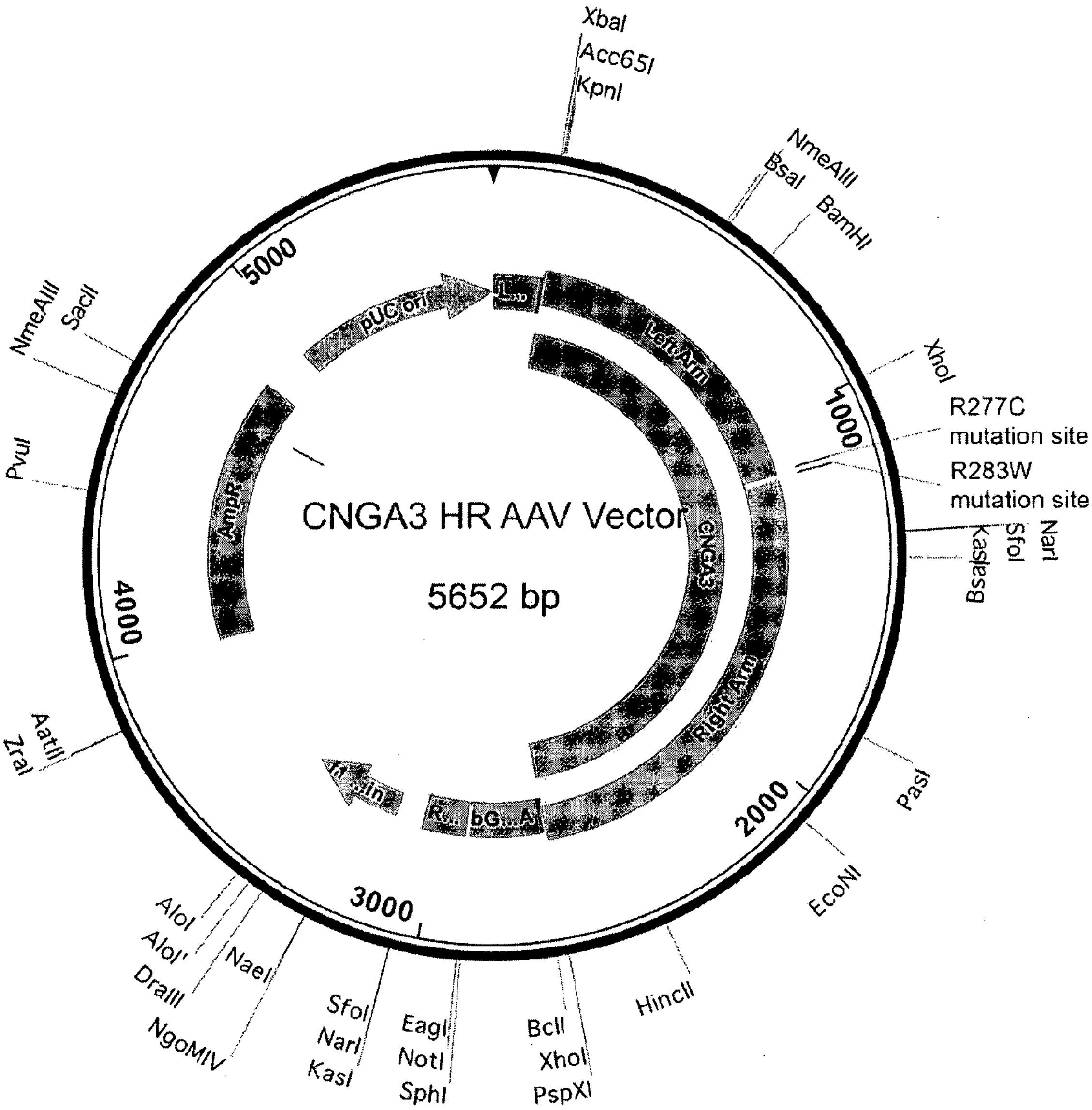

CNGA3 HR AAV Vector
5652 bp
XbaI
Acc65I
KpnI
NmeAIII
BsaI
BamHI
XhoI
R277C mutation site
R283W mutation site
NarI
SfoI
KasI
BsaI
PasI
EcoNI
HincII
BclI
XhoI
PspXI
EagI
NotI
SphI
SfoI
NarI
KasI
AloI
AloI'
DraIII
NaeI
NgoMIV
AatII
ZraI
PvuI
NmeAIII
SacII
pUC ori
Left Arm
CNGA3
Right Arm
AmpR
bG...A
1000
2000
3000
4000
5000

FIG. 21

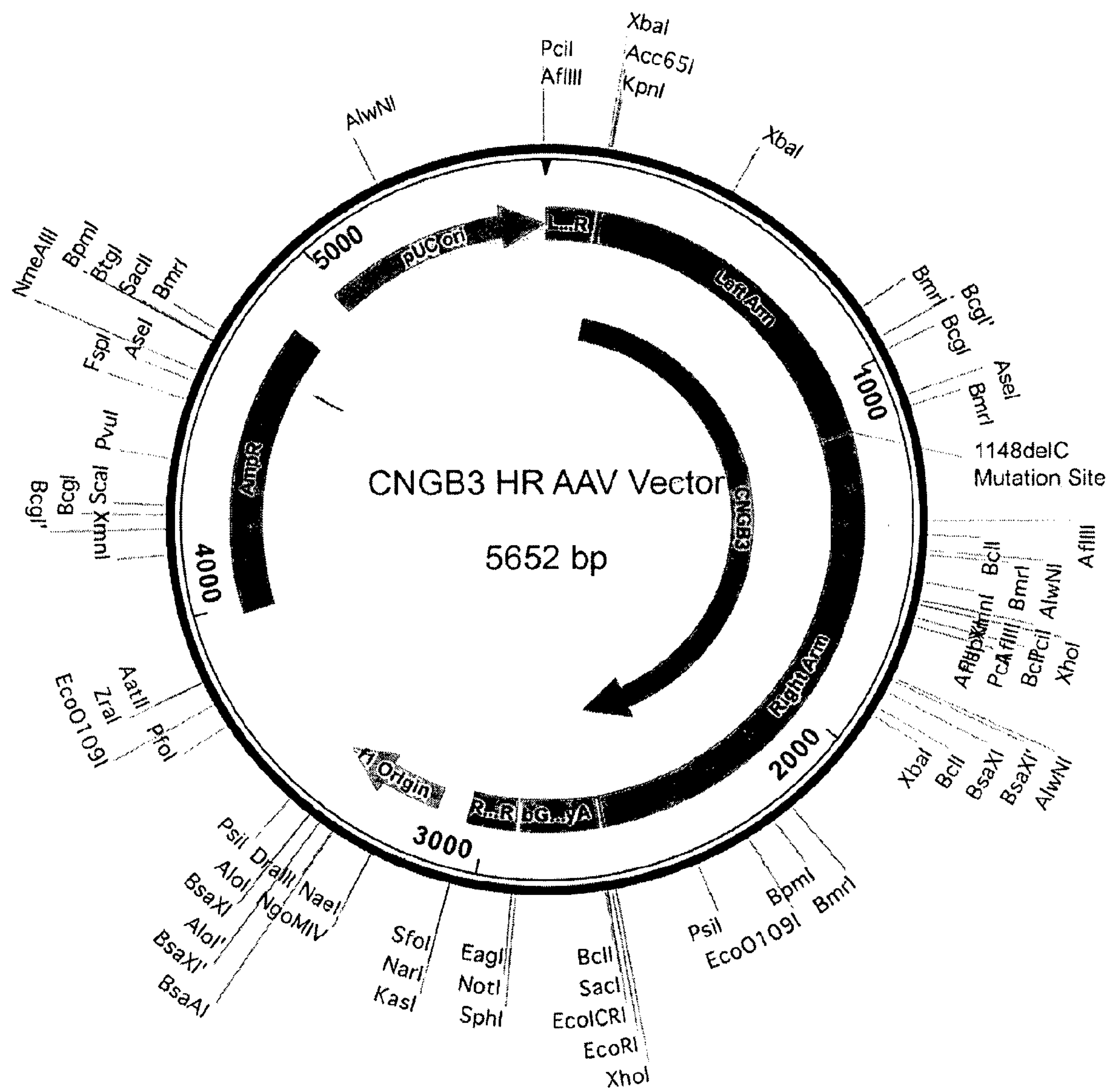

CNGB3 HR AAV Vector
5652 bp
pUC ori
L...R
Left Arm
CNGB3
Right Arm
bG...yA
R...R
f1 Origin
AmpR
1000
2000
3000
4000
5000
1148delC
Mutation Site
PciI
AflIII
XbaI
Acc65I
KpnI
AlwNI
BmrI
BcgI'
BcgI
AseI
XhoI
BclI
BsaXI
BsaXI'
PsiI
BpmI
EcoO109I
SacI
EcoICRI
EcoRI
EagI
NotI
SphI
SfoI
NarI
KasI
NaeI
NgoMIV
DraIII
AloI
AloI'
BsaAI
PfoI
ZraI
AatII
XmnI
ScaI
PvuI
FspI
NmeAIII
BtgI
SacII

FIG. 22

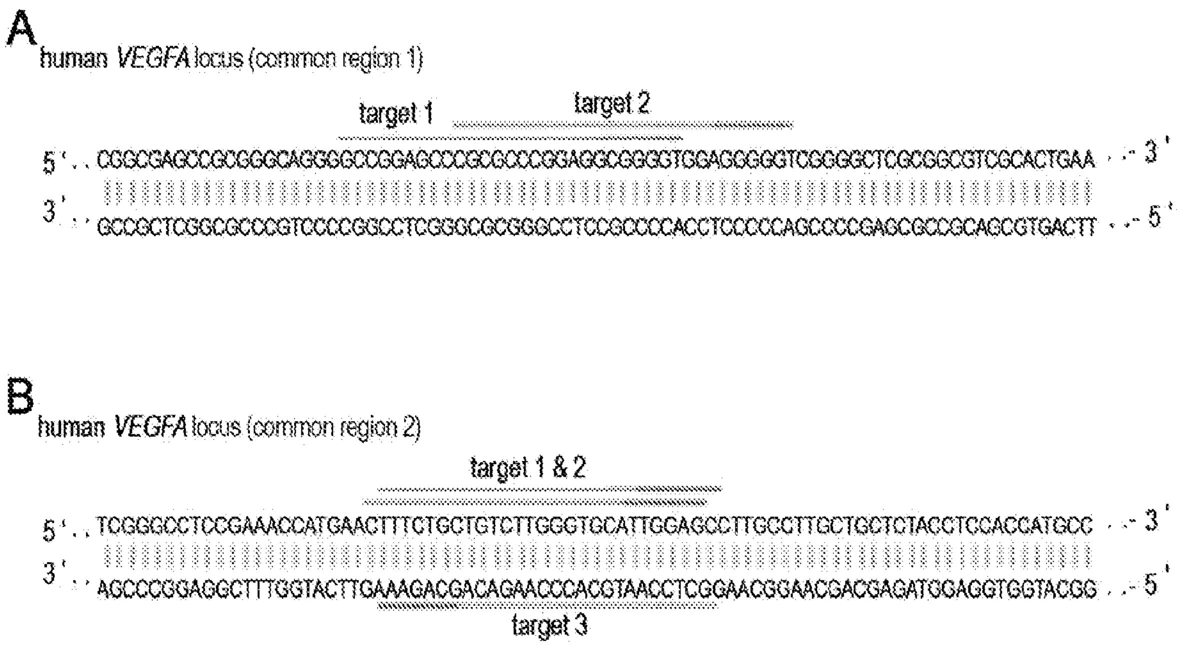
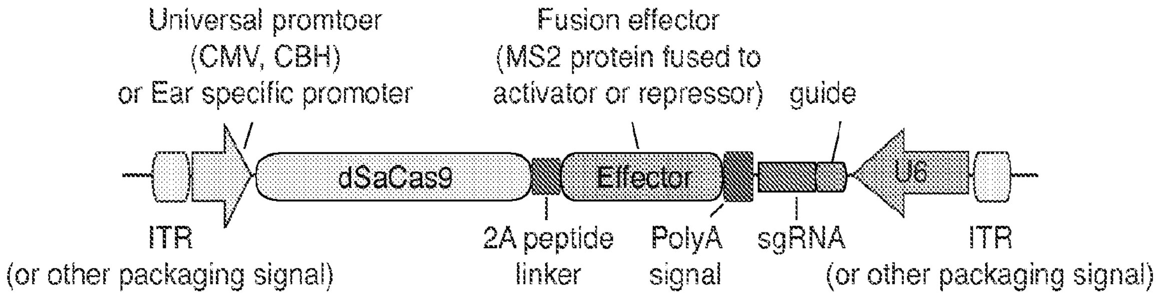

A
human VEGFA locus (common region 1)
target 1
target 2
5'.. CGGCGAGCCGCGGGCAGGGGCCGGAGCCCGCGCCCGGAGGCGGGGTGGAGGGGGTCGGGGCTCGCGGCGTCGCACTGAA ..3'
3'.. GCCGCTCGGCGCCCGTCCCCGGCCTCGGGCGCGGGCCTCCGCCCCACCTCCCCCAGCCCCGAGCGCCGCAGCGTGACTT ..5'
B
human VEGFA locus (common region 2)
target 1 & 2
5'.. TCGGGCCTCCGAAACCATGAACTTTCTGCTGTCTTGGGTGCATTGGAGCCTTGCCTTGCTGCTCTACCTCCACCATGCC ..3'
3'.. AGCCCGGAGGCTTTGGTACTTGAAAGACGACAGAACCCACGTAACCTCGGAACGGAACGACGAGATGGAGGTGGTACGG ..5'
target 3

FIG. 23A-B



CMV: cytomegalovirus promoter
CBH: CMV Beta-actin Hybrid promoter

FIG. 24

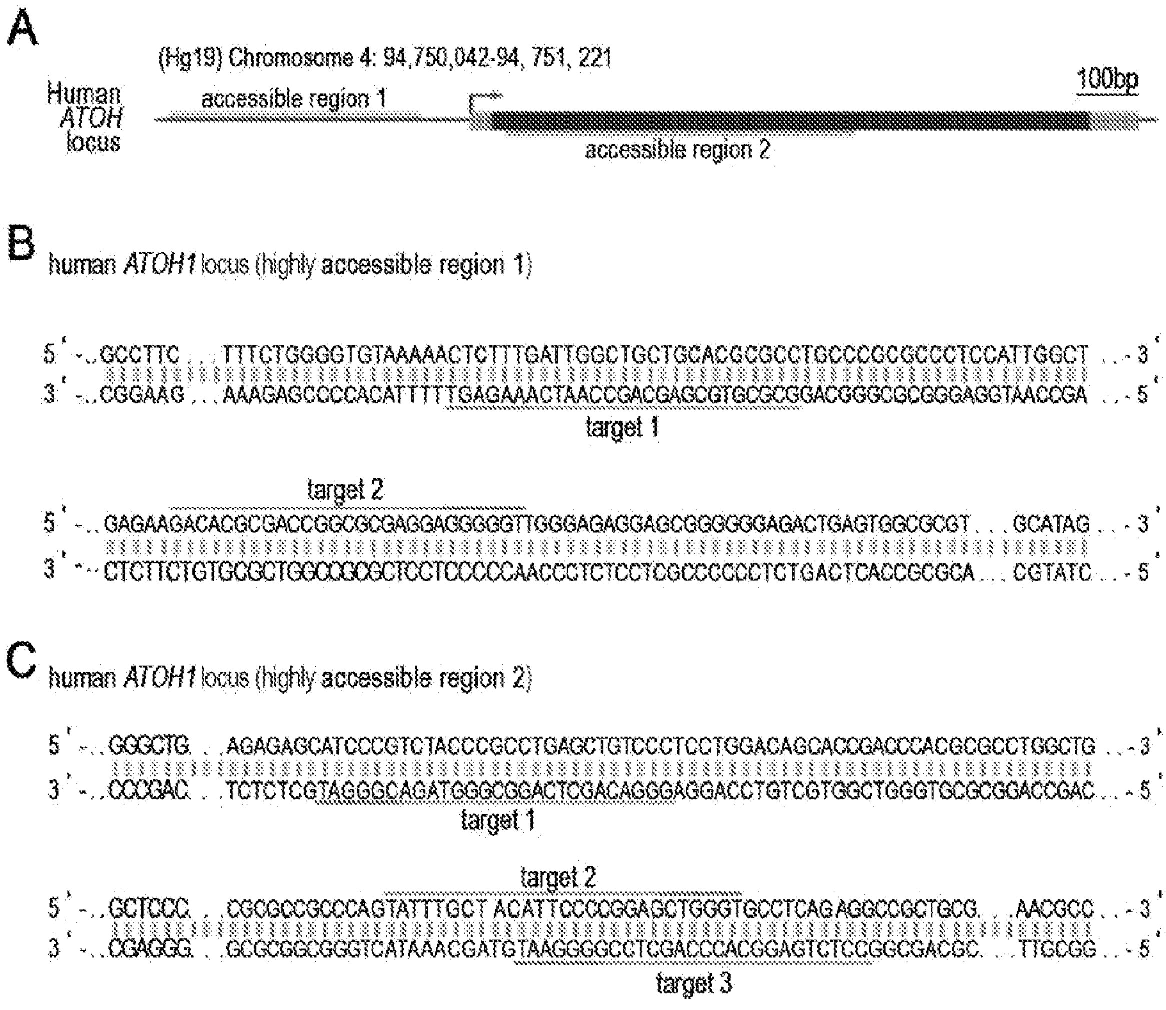

A
(Hg19) Chromosome 4: 94,750,042-94, 751, 221
Human
ATOH
locus
accessible region 1
100bp
accessible region 2
B
human ATOH1 locus (highly accessible region 1)
target 1
target 2
C
human ATOH1 locus (highly accessible region 2)
target 1
target 2
target 3

FIG. 25A-C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/070127

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K48/00 C12N15/10
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)
A61K 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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YANG HUI ET AL: "One-Step Generation of Mice Carrying Reporter and Conditional Alleles by CRISPR/Cas-Mediated Genome Engineering", CELL, vol. 154, no. 6, 12 September 2013 (2013-09-12), pages 1370-1379, XP028716273, ISSN: 0092-8674, DOI: 10.1016/J.CELL.2013.08.022	1,3,4, 13,14, 24,25,27
Y	page 1373, column 1, paragraph 2 - column 2, paragraph 2; table 2 page 1378, column 1, paragraph 2 -----	2,30

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 March 2015	15/04/2015

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

3

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG HAOYI ET AL: "One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering", CELL, CELL PRESS, US, vol. 153, no. 4, 2 May 2013 (2013-05-02), pages 910-918, XP028538358, ISSN: 0092-8674, DOI: 10.1016/J.CELL.2013.04.025	1,3,4, 13,14, 24,25,27
Y	abstract page 911, column 1, paragraph 2 - column 2; tables 2,3 page 916, column 1, paragraphs 2,3	2,30
X	WOONG Y HWANG ET AL: "Efficient genome editing in zebrafish using a CRISPR-Cas system", NATURE BIOTECHNOLOGY, vol. 31, no. 3, 29 January 2013 (2013-01-29), pages 227-229, XP055086625, ISSN: 1087-0156, DOI: 10.1038/nbt.2501 page 227, column 1, paragraph 2 - column 2, paragraph 2; figure 1	13,14
X	LUKE A. GILBERT ET AL: "CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes", CELL, vol. 154, no. 2, 1 July 2013 (2013-07-01), pages 442-451, XP055115843, ISSN: 0092-8674, DOI: 10.1016/j.cell.2013.06.044	15-22
Y	abstract page 443, column 1, paragraph 3 - column 2, paragraph 3; figure 1	29,39,40
X	A. MALINA ET AL: "Repurposing CRISPR/Cas9 for in situ functional assays", GENES & DEVELOPMENT, vol. 27, no. 23, 1 December 2013 (2013-12-01), pages 2602-2614, XP055177303, ISSN: 0890-9369, DOI: 10.1101/gad.227132.113	1,3-12, 24,25, 27,28, 41,42
Y	abstract page 2603, column 2, paragraph 2; figures 1,3 page 2608, column 1, paragraph 1	2,29-38, 44-46

-/--

3

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	N. MANJUNATH ET AL: "Newer Gene Editing Technologies toward HIV Gene Therapy", VIRUSES, vol. 5, no. 11, 14 November 2013 (2013-11-14), pages 2748-2766, XP055110267, DOI: 10.3390/v5112748 page 2757 - page 2759 -----	23,26
Y	PRASHANT MALI ET AL: "CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering", NATURE BIOTECHNOLOGY, vol. 31, no. 9, 1 August 2013 (2013-08-01), pages 833-838, XP055153963, ISSN: 1087-0156, DOI: 10.1038/nbt.2675 figure 1 abstract -----	31-40
Y	Z. HOU ET AL: "Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 110, no. 39, 12 August 2013 (2013-08-12), pages 15644-15649, XP055118866, ISSN: 0027-8424, DOI: 10.1073/pnas.1313587110 abstract page 15649, column 1, paragraph 1; figure 2 -----	44-46
Y	DATABASE UniProt 31 October 2012 (2012-10-31), "CRISPR-associated andonuclease Cas9, Staphylococcus aureus", XP002737561, Database accession no. J7RUA5 the whole document -----	2,30, 44-46

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International application No
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