ENGINEERING AND OPTIMIZATION OF IMPROVED SYSTEMS, METHODS AND ENZYME COMPOSITIONS FOR SEQUENCE MANIPULATION

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

[0001] This application claims priority to US provisional patent application 61/836,101 entitled ENGINEERING AND OPTIMIZATION OF IMPROVED SYSTEMS, METHODS AND ENZYME COMPOSITIONS FOR SEQUENCE MANIPULATION filed on June 17, 2013. This application also claims priority to US provisional patent applications 61/758,468; 61/769,046; 61/802,174; 61/806,375; 61/814,263; 61/819,803 and 61/828,130 each entitled ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION, filed on January 30, 2013; February 25, 2013; March 15, 2013; March 28, 2013; April 20, 2013; May 6, 2013 and May 28, 2013, respectively. This application also claims priority to US provisional patent applications 61/736,527 and 61/748,427, both entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on December 12, 2012 and January 2, 2013, respectively. Priority is also claimed to US provisional patent applications 61/791,409 and 61/835,931 filed on March 15, 2013 and June 17, 2013 respectively.

[0002] Reference is made to US provisional patent applications 61/836,127, 61/835,936, 61/836,080, 61/836,123, and 61/835,973 each filed June 17, 2013.

[0003] 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

[0004] The present invention generally 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 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and components thereof.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0005] This invention was made with government support under the NIH Pioneer Award (1DP1MH100706) awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0006] 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, 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

[0007] The CRISPR/Cas or the CRISPR-Cas system (both terms are used interchangeably throughout this application) does not require the generation of customized proteins to target specific sequences but rather a single Cas enzyme can be programmed by a short RNA molecule to recognize a specific DNA target, in other words the Cas enzyme can be recruited to a specific DNA target using said short RNA molecule. Adding the CRISPR-Cas system to the repertoire of genome sequencing techniques and analysis methods may significantly simplify the methodology and accelerate the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. To utilize the CRISPR-Cas system effectively for genome editing without deleterious effects, it is critical to understand aspects of engineering and optimization of these genome engineering tools, which are aspects of the claimed invention.

[0008] Accordingly, there exists a pressing need for alternative and robust systems and techniques for sequence targeting with a wide array of applications. Aspects of this invention address this need and provide related advantages. An exemplary CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide, wherein the CRISPR enzyme is a Cas ortholog, e.g. a Cas9 ortholog, of a genus which includes but is not limited to *Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma* and *Campylobacter*. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[0009] 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 utilities including modifying (e.g., deleting, inserting, translocating, inactivating, activating, repressing, altering methylation, transferring specific moieties) 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 or genome editing, gene regulation, gene therapy, drug discovery, drug screening, disease diagnosis, and prognosis. In preferred aspects of the invention, the CRISPR complex comprises a Cas enzyme, preferably a Cas9 ortholog, of a genus which includes but is not limited to *Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma* and *Campylobacter*.

[0010] Aspects of the invention relate to CRISPR enzymes having optimized function. With regard to the CRISPR enzyme being a Cas enzyme, preferred embodiments of the invention relate to Cas9 orthologs having improved target specificity in a CRISPR-Cas9 system. This may be accomplished by approaches that include but are not limited to designing and preparing guide RNAs having optimal activity, selecting Cas9 enzymes of a specific length, truncating the Cas9 enzyme making it smaller in length than the corresponding wild-type Cas9 enzyme by truncating the nucleic acid molecules coding therefor and generating chimeric Cas9 enzymes wherein different parts of the enzyme are swapped or exchanged between different orthologs to arrive at

chimeric enzymes having tailored specificity. Aspects of the invention also relate to methods of improving the target specificity of a Cas9 ortholog enzyme or of designing a CRISPR-Cas9 system comprising designing or preparing guide RNAs having optimal activity and/or selecting or preparing a Cas9 ortholog enzyme having a smaller size or length than the corresponding wild-type Cas9 whereby packaging a nucleic acid coding therefor into a delivery vector is advanced as there is less coding sequence therefor in the delivery vector than for the corresponding wild-type Cas9 and/or generating chimeric Cas9 enzymes.

[0011] Also provided are uses of the present sequences, vectors, enzymes or systems, in medicine. Also provided are the same for use in gene or genome editing. Also provided is use of the same in the manufacture of a medicament for gene or genome editing, for instance treatment by gene or genome editing. Also provided are the present sequences, vectors, enzymes or systems for use in therapy.

[0012] In an additional aspect of the invention, a CRISPR enzyme, e.g. a Cas9 enzyme may comprise one or more mutations and may be used as a generic DNA binding protein with or without fusion to or being operably linked to a functional domain. The mutations may be artificially introduced mutations and may include but are not limited to one or more mutations in a catalytic domain. Examples of catalytic domains with reference to a Cas9 enzyme may include but are not limited to RuvC I, RuvC II, RuvC III and HNH domains. Preferred examples of suitable mutations are the catalytic residue(s) in the N-term RuvC I domain of Cas9 or the catalytic residue(s) in the internal HNH domain. In some embodiments, the Cas9 is (or is derived from) the *Streptococcus pyogenes* Cas9 (SpCas9). In such embodiments, preferred mutations are at any or all of positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 or corresponding positions in other Cas9 orthologs with reference to the position numbering of SpCas9 (which may be ascertained for instance by standard sequence comparison tools, e.g. ClustalW or MegAlign by Lasergene 10 suite). 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 mutations (or conservative substitutions of these mutations) at corresponding positions with reference to the position numbering of SpCas9 in other Cas9 orthologs 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 when singly mutated they provide nickase

activity and when both mutations are present the Cas9 is converted into a catalytically null mutant which is useful for generic DNA binding. Further mutations have been identified and characterized. Other aspects of the invention relate to the mutated Cas 9 enzyme being fused to or operably linked to domains which include but are not limited to a transcriptional activator, transcriptional repressor, a recombinase, a transposase, a histone remodeler, a DNA methyltransferase, a cryptochrome, a light inducible/controllable domain or a chemically inducible/controllable domain.

[0013] A further aspect of the invention provides for chimeric Cas9 proteins and methods of generating chimeric Cas9 proteins. Chimeric Cas9 proteins are proteins that comprise fragments that originate from different Cas9 orthologs. For instance, the N-terminal of a first Cas9 ortholog may be fused with the C-terminal of a second Cas9 ortholog to generate a resultant Cas9 chimeric protein. These chimeric Cas9 proteins may have a higher specificity or a higher efficiency than the original specificity or efficiency of either of the individual Cas9 enzymes from which the chimeric protein was generated. These chimeric proteins may also comprise one or more mutations or may be linked to one or more functional domains. Therefore, aspects of the invention relate to a chimeric Cas enzyme wherein the enzyme comprises one or more fragments from a first Cas ortholog and one or more fragments from a second Cas ortholog. In a embodiment of the invention the one or more fragments of the first or second Cas ortholog are from the C- or N-terminal of the first or second Cas ortholog. In a further embodiment the first or second Cas ortholog is selected from a genus belonging to the group consisting of *Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma* and *Campylobacter*.

[0014] In a further embodiment, the invention provides for methods to generate mutant components of the CRISPR complex comprising a Cas enzyme, e.g Cas9 ortholog. The mutant components may include but are not limited to mutant tracrRNA and tracr mate sequences or mutant chimeric guide sequences that allow for enhancing performance of these RNAs in cells. Use of the present composition or the enzyme in the preparation of a medicament for modification of a target sequence is also provided.

[0015] The invention in yet a further aspect provides compositions and methods related to 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,

or

(B) I. polynucleotides comprising:

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

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

II. a polynucleotide sequence encoding a CRISPR enzyme, 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 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

wherein the CRISPR enzyme is a Cas9 ortholog of a genus belonging to the group consisting of Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta,

Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma and Campylobacter.

[0016] The invention in yet a further aspect provides: (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 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, 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, wherein the CRISPR enzyme is a Cas9 ortholog of a genus belonging to the group consisting of *Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma* and *Campylobacter* 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 prokaryotic 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, wherein the CRISPR enzyme is a Cas9 ortholog of a genus belonging to the group consisting of

Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma and *Campylobacter*, and wherein: at least one of the following criteria applies.

[0017] The criteria are as follows and it will be appreciated that any number of these may apply, preferably 1 or more, preferably 2 or more, and preferably 3 or more, 4 or more, or 5 or more, or all may apply:

- the CRISPR enzyme having a specific size is selected and has a length of at least 500 amino acids, at least 800-899 amino acids, at least 900-999 amino acids, at least 1000-1099 amino acids, at least 1100-1199 amino acids, at least 1200-1299 amino acids, at least 1300-1399 amino acids, at least 1400-1499 amino acids, at least 1500-1599 amino acids, at least 1600-1699 amino acids or at least 2000 amino acids;
- and/or the CRISPR enzyme is truncated in comparison to the corresponding wild type CRISPR enzyme;
- and/or the CRISPR enzyme is a nuclease directing cleavage of both strands at the location of the target sequence, or the CRISPR enzyme is a nickase directing cleavage of one strand at the location of the target sequence;
- and/or the guide sequence comprises at least 10, at least 15 or at least 20 nucleotides;
- and/or the CRISPR enzyme is codon-optimized or codon-optimized for expression in a eukaryotic cell;
- and/or the CRISPR enzyme comprises one or more mutations;
- and/or the CRISPR enzyme comprises a chimeric CRISPR enzyme;
- and/or the CRISPR enzyme has one or more other attributes herein discussed.

[0018] In some embodiments, the CRISPR enzyme is truncated in comparison to a wild type CRISPR enzyme or the CRISPR enzyme is comprised of at least 500 amino acids, at least 800-899 amino acids, at least 900-999 amino acids, at least 1000-1099 amino acids, at least 1100-1199 amino acids, at least 1200-1299 amino acids, at least 1300-1399 amino acids, at least 1400-1499 amino acids, at least 1500-1599 amino acids, at least 1600-1699 amino acids or at least 2000 amino acids. In preferred embodiments the CRISPR enzyme is a Cas enzyme, e.g. a Cas9 ortholog.

[0019] In some embodiments, the CRISPR enzyme is a nuclease directing cleavage of both strands at the location of the target sequence, or the CRISPR enzyme is a nickase directing cleavage of one strand at the location of the target sequence. In further embodiments, the CRISPR enzyme is a catalytically null mutant that is a generic DNA binding protein. In preferred embodiments the CRISPR enzyme is a Cas enzyme, e.g. a Cas9 ortholog.

[0020] In some embodiments, the guide sequence comprises at least fifteen nucleotides. In some embodiments, the CRISPR enzyme is codon-optimized or codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme comprises one or more mutations. In some embodiments, the CRISPR enzyme comprises a chimeric CRISPR enzyme. In some embodiments, the CRISPR enzyme has one or more other attributes herein discussed. In preferred embodiments the CRISPR enzyme is a Cas enzyme, e.g. a Cas9 ortholog.

[0021] In certain embodiments, the CRISPR enzyme comprises one or more mutations. The one or more mutations may be in a particular domain of the enzyme. In a preferred embodiment, the one or more mutations may be in a catalytic domain. In a further preferred embodiment the catalytic domain is a RuvC I, RuvC II, RuvC III or HNH domain. In a more preferred embodiment, the one or more mutations is in a RuvC1 or HNH domain of the CRISPR enzyme. In a further preferred embodiment the CRISPR enzyme is a Cas enzyme, e.g. a Cas9 ortholog and the mutation may be at one or positions that include but are not limited to positions that correspond to D10A, E762A, H840A, N854A, N863A or D986A with reference to the position numbering of SpCas9 and/or is a mutation as otherwise discussed herein. In some embodiments, the CRISPR enzyme has one or more mutations in a particular domain of the enzyme, 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. The functional domain may include but is not limited to transcriptional activator, transcriptional repressor, a recombinase, a transposase, a histone remodeler, a DNA methyltransferase, a cryptochrome, a light inducible/controllable domain or a chemically inducible/controllable domain.

[0022] In some embodiments, the functional domain is the transcriptional activator domain VP64. In some embodiments, the functional domain is the transcriptional repressor domain KRAB. In some embodiments, the transcriptional repressor domain is SID, or concatemers of SID (i.e. SID4X). In some embodiments, an epigenetic modifying enzyme is provided, e.g a

histone modifying protein or an epigenetic chromatin modifying protein. In some embodiments, an activator domain is provided, which may be the P65 activator domain.

[0023] A further aspect of the invention comprehends methods of modifying two or more genomic loci of interest. In a preferred embodiment of the invention two or more genomic loci are differentially modulated by utilizing one or more CRISPR enzymes, e.g. two or more Cas9 orthologs, each ortholog being operably linked to one or more functional domain. In one aspect, the invention provides for a method of modifying two or more genomic loci in a eukaryotic cell. Therefore, aspects of the invention provide for a method of modulating the expression of two or more genomic loci of interest in an organism comprising delivering 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 first CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the first polynucleotide sequence comprises

(i) a first guide sequence capable of hybridizing to a first target sequence at a first genomic locus in a cell of the organism,

(ii) a first tracr mate sequence, and

(iii) a first tracr sequence, and

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

(i) a second guide sequence capable of hybridizing to a second target sequence at a second genomic locus in the cell of the organism,

(ii) a second tracr mate sequence, and

(iii) a second tracr sequence, and

III. a third regulatory element operably linked to an enzyme-coding sequence encoding a first CRISPR enzyme comprising at least one or more nuclear localization sequences and operably linked to a first functional domain,

IV. a fourth regulatory element operably linked to an enzyme-coding sequence encoding a second CRISPR enzyme comprising at least one or more nuclear localization sequences and operably linked to a second functional domain, wherein (i), (ii) and (iii) in I and II are arranged in a 5' to 3' orientation, wherein components I, II, III and IV are located on the same or different vectors of the system, wherein when transcribed, each tracr mate sequences hybridizes to its

corresponding tracr sequence and the first and second guide sequences direct sequence-specific binding of the first and second CRISPR complex to the first and second target sequence, 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 wherein expression of the CRISPR enzyme provides manipulation of the target sequence, wherein the first and second CRISPR enzyme each comprise two or more mutations, wherein the first and second CRISPR enzyme is a Cas9 ortholog of a genus belonging to the group consisting of *Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma* and *Campylobacter*, and wherein the first genomic locus is modulated by the activity of the first functional domain and the second genomic locus is modulated by the activity of the second functional domain. In a further embodiment the first functional domain is selected from the group consisting of a transcriptional activator, transcriptional repressor, a recombinase, a transposase, a histone remodeler, a DNA methyltransferase, a cryptochrome and a light inducible/controllable domain or a chemically inducible/controllable domain. In a further embodiment the second functional domain is selected from the group consisting of a transcriptional activator, transcriptional repressor, a recombinase, a transposase, a histone remodeler, a DNA methyltransferase, a cryptochrome and a light inducible/controllable domain or a chemically inducible/controllable domain. In preferred embodiments the first or second CRISPR enzyme is a Sutterella wadsworthensis Cas9, a Filifactor alocis Cas9, a Lactobacillus johnsonii Cas9, a Campylobacter lari Cas9, a Corynebacter diptheriae Cas9, a Parvibaculum lavamentivorans Cas9, a Mycoplasma gallisepticum Cas9, a Staphylococcus aureus subsubspecies Aureus Cas9, a Legionella pneumophila Paris Cas9, a Treponema denticola Cas9, a Staphylococcus pseudintermedius Cas9, a Neisseria cinerea Cas9.

[0024] In some embodiments, the CRISPR enzyme is a type I, II or III CRISPR enzyme, preferably a type II CRISPR enzyme. This type II CRISPR enzyme may be any Cas enzyme. A 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

Staphylococcus aureus subsubspecies Aureus SaCas9. By derived, it is meant 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.

[0025] 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 those belonging to the genus *Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma* or *Campylobacter*, such as SpCas9, SaCas9, St1Cas9, St3Cas9 and so forth, wherein St is *Streptococcus thermophilus*.

[0026] 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.

[0027] Further aspects of the invention relate to improved cleavage specificity, optimized tracr sequence, optimized chimeric guide RNA, co-fold structure of tracrRNA and tracr mate sequence, stabilizing secondary structures of tracr RNA, tracrRNA with shortened region of base pairing, tracrRNA with fused RNA elements, simplified cloning and delivery, reduced toxicity and/or inducible systems. Another aspect of the invention relates to the stabilization of chimeric RNA, and/or guide sequence and or a portion thereof of CRISPR complexes wherein the CRISPR enzyme is a CRISPR ortholog, wherein the chimeric RNA, and/or guide sequence and or a portion thereof is stabilized by synthetic or chemically modified nucleotides (e.g. LNA/BNA: thiol-modification, 2'/3'-OH crosslink modification), is modified to be degradation/hydrolysis resistant and to which elements of structural stability have been added.

[0028] The invention further comprehends in certain embodiments a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest comprising delivering a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors operably encoding a composition herein discussed

for expression thereof. Preferably, the vector is a viral vector, such as a lenti- or baculo- or preferably adeno-viral/adeno-associated viral vectors, but other means of delivery are known (such as yeast systems, microvesicles, gene guns/means of attaching vectors to gold nanoparticles) and are provided.

[0029] Various means of delivery are described herein, and further discussed in this section.

[0030] 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 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 viral vectors. In some embodiments, the viral vector is delivered to the tissue of interest by, for example, an intramuscular injection, while othertimes the viral 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 chosen, 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.

[0031] 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, an adjuvant to enhance antigenicity, an immunostimulatory compound or molecule, and/or other compounds known in the art. The adjuvant herein may contain a suspension of minerals (alum, aluminum hydroxide, aluminum phosphate) on which antigen is adsorbed; or water-in-oil emulsion in which antigen solution is emulsified in oil (MF-59, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity (inhibits degradation of antigen and/or causes influx of macrophages). Adjuvants also include immunostimulatory molecules, such as cytokines, costimulatory molecules, and for example, immunostimulatory DNA or RNA molecules, such as CpG oligonucleotides. Such a dosage formulation is readily ascertainable by one skilled 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. 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.

[0032] 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 10^9 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.

[0033] 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 1×10^{10} to about 1×10^{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 1×10^{5} to 1×10^{50} genomes AAV, from about 1×10^{8} to 1×10^{20} genomes AAV, from about 1×10^{10} to about 1×10^{16} genomes, or about 1×10^{11} to about 1×10^{16} genomes AAV. A human dosage may be about 1×10^{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.

[0034] 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.

[0035] 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.

[0036] 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 might use the Synapsin I promoter.

[0037] 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.

[0038] To enhance expression and reduce toxicity, the CRISPR enzyme and/or guide RNA can be modified using pseudo-U or 5-Methyl-C.

[0039] CRISPR enzyme mRNA and guide RNA may be delivered simultaneously using nanoparticles or lipid envelopes.

[0040] 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.

[0041] Furthermore, 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) Published online 09 January 2011) describes the use of lipid envelopes to deliver RNA. Use of lipid envelopes is also preferred in the present invention.

[0042] mRNA delivery methods are especially promising for liver delivery currently.

[0043] CRISPR enzyme mRNA and guideRNA might also be delivered separately. 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 guideRNA.

[0044] 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 + guideRNA.

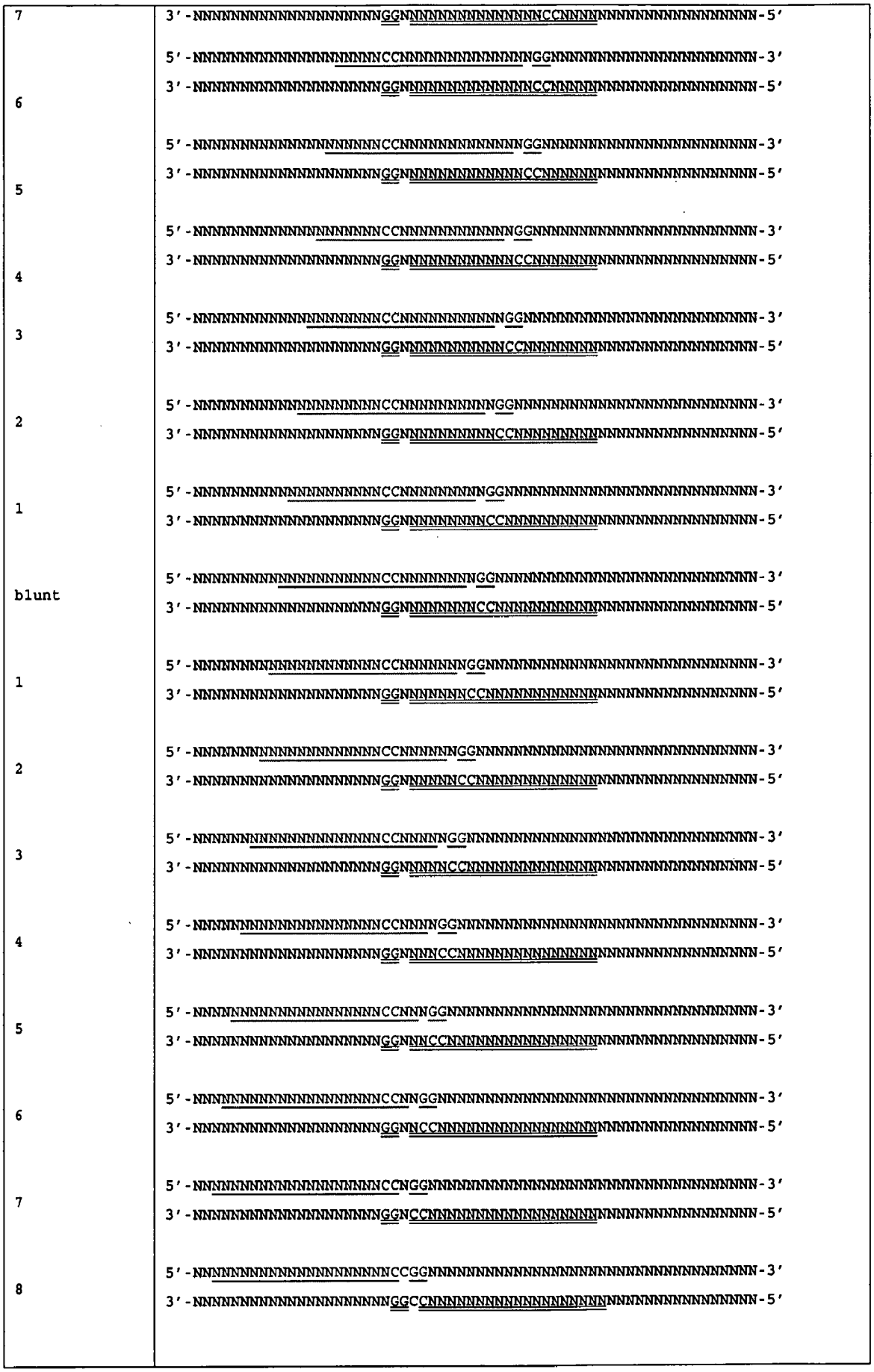
[0045] Additional administrations of CRISPR enzyme mRNA and/or guide RNA might be useful to achieve the most efficient levels of genome modification.

[0046] For minimization of toxicity and off-target effects, 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 animal model and using deep sequencing to 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.

[0047] Alternatively, to minimize the level of toxicity and off-target effects, 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 in red (single underline) and blue (double underline) respectively (these examples are based on the PAM requirement for Streptococcus pyogenes Cas9).

Overhang length (bp)	Guide RNA design (guide sequence and PAM color coded)
	5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNN-3'
14	3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNN-5'
	5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNN-3'
13	3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNN-5'
	5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNN-3'
12	3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNN-5'
	5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNN-3'
11	3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNN-5'
	5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNN-3'
10	3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNN-5'
	5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNN-3'
9	3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNN-5'
	5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNN-3'
8	3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNN-5'
	5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNN-3'


7
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN-3'
6
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN-3'
5
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN-3'
4
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN-3'
3
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN-3'
2
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN-3'
1
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNN-3'
blunt
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNN-3'
1
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNN-3'
2
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNN-3'
3
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3'
4
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3'
5
3'-NNNNNNNNNNNNNNNNNNNNGGNNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3'
6
3'-NNNNNNNNNNNNNNNNNNNNGGNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3'
7
3'-NNNNNNNNNNNNNNNNNNNNGGNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
5'-NNNNNNNNNNNNNNNNNNNNCCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3'
8
3'-NNNNNNNNNNNNNNNNNNNNGGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'

12	5'-NNNNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3' 3'-NNNNNNNNNNNNNNNNNNNNNNNNCCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
13	5'-NNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3' 3'-NNNNNNNNNNNNNNNNNNNNNNNCCNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
14	5'-NNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3' 3'-NNNNNNNNNNNNNNNNNNNNNNNCCNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
15	5'-NNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3' 3'-NNNNNNNNNNNNNNNNNNNNNNNCCNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
16	5'-NNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3' 3'-NNNNNNNNNNNNNNNNNNNNNNNCCNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'
17	5'-NNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3' 3'-NNNNNNNNNNNNNNNNNNNNNNNCCNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-5'

[0048] Further interrogation of the system has given Applicants evidence of the 5' overhang (see, e.g., Ran et al., Cell. 2013 Sep 12;154(6):1380-9 and US Provisional Patent Application Serial No. 61/871,301 filed August 28, 2013). Applicants have further identified parameters that relate to efficient cleavage by the Cas9 nickase mutant when combined with two guide RNAs and these parameters include but are not limited to the length of the 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 or 1-34 base pairs. In other 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 blunt cut or a 3' overhang. In embodiments of the invention the 3' overhang is at most 150, 100 or 25 base pairs or at least 15, 10 or 1 base pairs. In preferred embodiments the 3' overhang is 1-100 base pairs.

[0049] 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.

[0050] 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.

[0051] Since Cas9n and Cas9H840A nick opposite strands of DNA, substitution of Cas9n with Cas9H840A with a given sgRNA pair should result in the inversion of the overhang type. For example, a pair of sgRNAs that will generate a 5' overhang with Cas9n should in principle generate the corresponding 3' overhang instead. Therefore, sgRNA pairs that lead to the generation of a 3' overhang with Cas9n might be used with Cas9H840A to generate a 5' overhang. Unexpectedly, Applicants tested Cas9H840A with a set of sgRNA pairs designed to generate both 5' and 3' overhangs (offset range from –278 to +58 bp), but were unable to observe indel formation. Further work may be needed to identify the necessary design rules for sgRNA pairing to allow double nicking by Cas9H840A.

[0052] Additional delivery options for the brain include encapsulation of CRISPR enzyme and guide RNA in the form of either DNA or RNA into liposomes and conjugating to molecular Trojan horses for trans-blood brain barrier (BBB) delivery. Molecular Trojan horses have been shown to be effective for delivery of B-gal expression vectors into the brain of non-human primates. The same approach can be used to delivery vectors containing CRISPR enzyme and guide RNA. For instance, Xia CF and Boado RJ, Pardridge WM ("Antibody-mediated targeting of siRNA via the human insulin receptor using avidin-biotin technology." Mol Pharm. 2009 May-Jun;6(3):747-51. doi: 10.1021/mp800194) describes how delivery of short interfering RNA (siRNA) to cells in culture, and in vivo, is possible with combined use of a receptor-specific monoclonal antibody (mAb) and avidin-biotin technology. The authors also report that because the bond between the targeting mAb and the siRNA is stable with avidin-biotin technology, and RNAi effects at distant sites such as brain are observed in vivo following an intravenous administration of the targeted siRNA.

[0053] Zhang Y, Schlachetzki F, Pardridge WM. ("Global non-viral gene transfer to the primate brain following intravenous administration." Mol Ther. 2003 Jan;7(1):11-8.) describe

how expression plasmids encoding reporters such as luciferase were encapsulated in the interior of an "artificial virus" comprised of an 85 nm pegylated immunoliposome, which was targeted to the rhesus monkey brain in vivo with a monoclonal antibody (MAb) to the human insulin receptor (HIR). The HIRMAb enables the liposome carrying the exogenous gene to undergo transcytosis across the blood-brain barrier and endocytosis across the neuronal plasma membrane following intravenous injection. The level of luciferase gene expression in the brain was 50-fold higher in the rhesus monkey as compared to the rat. Widespread neuronal expression of the beta-galactosidase gene in primate brain was demonstrated by both histochemistry and confocal microscopy. The authors indicate that this approach makes feasible reversible adult transgenics in 24 hours. Accordingly, the use of immunoliposome is preferred. These may be used in conjuction with antibodies to target specific tissues or cell surface proteins.

[0054] 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 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, exozomes 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 purified and characterized from transfected cell supernatant, then siRNA is loaded into the exosomes.

[0055] One aspect of manipulation of a target sequence also refers to 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 or reducing 3D folding.

[0056] A vector may mean not only a viral or yeast system (for instance, where the nucleic acids of interest may be operably linked to and under the control of (in terms of expression, such as to ultimately provide a processed RNA) a promoter, but also direct delivery of nucleic acids into a host cell.

[0057] The invention also comprehends, in certain embodiments, 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 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 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 a vector system comprising one or more vectors comprising operably encoding a composition herein discussed for expression thereof, wherein the target sequence is manipulated by the composition when expressed.

[0058] In certain embodiments of the herein methods, the methods can include inducing expression, which can be inducing expression of the CRISPR enzyme and/or inducing expression of the guide, tracr or tracr mate sequences. In certain embodiments of the herein methods, the organism or subject is a eukaryote or a non-human eukaryote. In certain embodiments of the herein methods, the organism or subject is a plant. In certain embodiments of the herein methods, the organism or subject is a mammal or a non-human mammal. In certain embodiments of the herein methods, the organism or subject is algae.

[0059] While in herein methods the vector may be a viral vector and this is advantageously an AAV, other viral vectors as herein discussed can be employed. For example, baculoviruses may be used for expression in insect cells. These insect cells may, in turn be useful for producing large quantities of further vectors, such as AAV vectors adapted for delivery of the present invention.

[0060] Also envisaged is a method of delivering the present CRISPR enzyme comprising delivering to a cell mRNA encoding the CRISPR enzyme. It will be appreciated that the CRISPR enzyme is truncated, is of a specific size as decribed herein, is a nuclease or nickase or generic DNA binding protein, is codon-optimized, comprises one or more mutations, and/or comprises a chimeric CRISPR enzyme, or the other options as herein discussed.

[0061] Also envisaged is a method of preparing an vector for delivery of the compositions or the present CRISPR enzymes of the invention and for use in the present methods.

[0062] AAV viral vectors are preferred. Thus, in a further aspect, there is provided a method of preparing an AAV viral vector, comprising transfecting plasmid(s) containing or consisting essentially of nucleic acid molecule(s) coding for the AAV into AAV-infected cells, and supplying AAV rep and/or cap obligatory for replication and packaging of the AAV. In this regard, it will be appreciated that the CRISPR enzyme is truncated, comprised of less than one thousand amino acids or less than four thousand amino acids, is a nuclease or nickase, is codon-optimized comprises one or more mutations, and/or comprises a chimeric CRISPR enzyme, as herein discussed. In some embodiments the AAV rep and/or cap obligatory for replication and packaging of the AAV are supplied by transfecting the cells with helper plasmid(s) or helper virus(es). In some embodiments the helper virus is a poxvirus, adenovirus, herpesvirus or baculovirus. In some embodiments the poxvirus is a vaccinia virus. In some embodiments the cells are mammalian cells. And in some embodiments the cells are insect cells and the helper virus is baculovirus.

[0063] The invention further comprehends in certain embodiments a modified CRISPR enzyme. Differences from the wild type CRISPR enzyme can comprise: the modified CRISPR enzyme is truncated in comparison to a wild type CRISPR enzyme, or the CRISPR enzyme is of a specific size, e.g. at least 500 amino acids, at least 800-899 amino acids, at least 900-999 amino acids, at least 1000-1099 amino acids, at least 1100-1199 amino acids, at least 1200-1299 amino acids, at least 1300-1399 amino acids, at least 1400-1499 amino acids, at least 1500-1599 amino acids, at least 1600-1699 amino acids or at least 2000 amino acids; and/or the CRISPR enzyme is a nuclease directing cleavage of both strands at the location of the target sequence, or the CRISPR enzyme is a nickase directing cleavage of one strand at the location of the target sequence, or the CRISPR enzyme is a catalytic null mutant that functions as a DNA binding protein; and/or the CRISPR enzyme is codon-optimized or codon-optimized for expression in a eukaryotic cell, and/or the CRISPR enzyme comprises one or more mutations, and/or the CRISPR enzyme comprises a chimeric CRISPR enzyme and/or the CRISPR enzyme has one or more other attributes herein discussed. Accordingly, in certain embodiments, the CRISPR enzyme comprises one or more mutations in catalytic residues such as spCa9 D10A, E762A, H840A, N854A, N863A or D986A or those corresponding to them in other Cas enzymes (as

described herein) with reference to the position numbering of SpCas9, and/or has one or more mutations is in a RuvC I, RuvC II, RuvC III, HNH or other domain described herein of the CRISPR enzyme and/or the CRISPR enzyme has one or more mutations in a catalytic domain, wherein when transcribed, a tracr mate sequence hybridizes to a tracr sequence and a guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence, and wherein the enzyme further comprises a functional domain. The functional domain may include but is not limited to transcriptional activator, transcriptional repressor, a recombinase, a transposase, a histone remodeler, a DNA methyltransferase, a cryptochrome, a light inducible/controllable domain or a chemically inducible/controllable domain. The CRISPR enzyme in certain embodiments can have the functional domain be a transcriptional activator domain, e.g., VP64. In some embodiments, a transcription repressor domains is KRAB. In some embodiments, a transcription repressor domain is SID, or concatemers of SID (i.e. SID4X). In some embodiments, an epigenetic modifying enzyme is provided.

[0064] In preferred embodiments the CRSIPR enzyme is a Cas enzyme, e.g. a Cas9 ortholog.

[0065] Aspects of the invention also comprehend identifying novel orthologs of CRISPR enzymes. Methods of identifying novel orthologs of CRISPR enzymes may involve identifying tracr sequences in genomes of interest. Identification of tracr sequences may relate to the following steps: Search for the direct repeats or tracr mate sequences in a database to identify a CRISPR region comprising a CRISPR enzyme, e.g. Figure 18. Search for homologous sequences in the CRISPR region flanking the CRISPR enzyme in both the sense and antisense directions. Look for transcriptional terminators and secondary structures. Identify any sequence that is not a direct repeat or a tracr mate sequence but has more than 50% identity to the direct repeat ot tracr mate sequence as a potential tracr sequence. Take the potential tracr sequence and analyze for transcriptional terminator sequences associated therewith.

[0066] The invention comprehends in certain embodiments use of a composition as described herein or a CRISPR enzyme in medicine. The invention further comprehends in certain embodiments a composition or CRISPR enzyme of the invention used in a method of the invention. The invention also comprehends, in certain embodiments, use of a composition or a CRISPR enzyme of the invention in, preferably *ex vivo,* gene or genome editing or, preferably an *ex vivo,* gene or genome editing method. The invention accordingly also comprehends in certain embodiments use of a composition according or a CRISPR enzyme of the invention in the

manufacture of a medicament for *ex vivo* gene or genome editing or for use in a method as herein discussed.

[0067] In addition, the invention in certain embodiments comprehends a composition or a CRISPR enzyme of the invention, wherein the target sequence is flanked or followed, at its 3' end, by a PAM suitable for the CRISPR enzyme, typically a Cas and in particular a Cas9. This PAM sequence is specific for each Cas9 but may be readily determined by methods described herein.

[0068] For example, a suitable PAM is 5'-NRG or 5'-NNGRR for SpCas9 or SaCas9 enzymes (or derived enzymes), respectively. It will be appreciated that reference made herein to *Staphylococcus aureus* preferably includes *Staphylococcus aureus* subspecies *Aureus*.

[0069] 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.

[0070] 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.

[0071] These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0072] 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:

[0073] **Figure 1** shows a schematic of RNA-guided Cas9 nuclease. The Cas9 nuclease from Streptococcus pyogenes is targeted to genomic DNA by a synthetic guide RNA (sgRNA) consisting of a 20-nt guide sequence and a scaffold. The guide sequence base-pairs with the DNA target, directly upstream of a requisite 5'-NGG protospacer adjacent motif (PAM), and Cas9 mediates a double-stranded break (DSB) ~3 bp upstream of the PAM (indicated by triangle).

[0074] **Figure 2A-F** shows an exemplary CRISPR system and a possible mechanism of action (A), an example adaptation for expression in eukaryotic cells, and results of tests assessing nuclear localization and CRISPR activity (B-F).

[0075] **Figure 3** shows a schematic representation of AAV in vivo delivery plasmids utilizing inverted terminal repeats (ITRs) sequences and guide chimeric RNAs to preferably aid delivery by AAV or AAV-associated systems.

[0076] **Figure 4A-D** shows a circular depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[0077] **Figure 5 A-F** shows a liner depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[0078] **Figure 6** shows a graph representing the length distribution of Cas9 orthologs.

[0079] **Figure 7** shows a representation of the sequence logos of the PAMs of the Cas9 orthologs as reverse complements.

[0080] **Figure 8 A-J** shows 18 chimeric RNA structures that preserved the sequence and secondary structures of the tracr mate:tracr sequence duplex while shortening the region of base-pairing and fusing the two RNA elements through an artificial loop.

[0081] **Figure 9 A-O** shows a list of the human codon optimized Cas9 sequences to pair with the chimeric guide RNAs provided in Figures 8 A-J.

[0082] **Figure 10 A-M** shows sequences where the mutation points are located within the SpCas9 gene.

[0083] **Figure 11** shows Type II CRISPR loci in different organisms.

[0084] **Figure 12** shows guide RNA sequences corresponding to CRISPR loci in different organisms.

[0085] **Figure 13 A-II** shows a table listing Cas9 orthologs and their corresponding PAM sequences.

[0086] **Figure 14** shows that the PAM for Staphylococcus aureus subspecies Aureus Cas9 is NNGRR.

[0087] **Figure 15A-D** shows single and multiple vector designs for SaCas9.

[0088] **Figrue 16** shows a vector design and gel images for Cas9 orthologs and respective sgRNAs being used to cleave two candidate targets present in a pUC19-based library.

[0089] **Figure 17** shows in vitro cleavage by SpCas9, St3Cas9, Sp_St3 chimera and St3_Sp chimera. The PAMs for St3Cas9 and St3_Sp chimeric Cas9 are NGG.

[0090] **Figure 18** shows an image of the CRISPRs web server.

[0091] **Figure 19 A-L** shows a multiple sequence alignment for 12 Cas9 orthologs. Two catalytic residues are highlighted. The first residue highlighted is the catalytic Asp residue in the RuvCI domain, and the second residue highlighted is the catalytic His residue in the HNH domain. Mutation of one or the other residue into Ala can convert Cas9 into a nickase. Mutation of both residues converts Cas9 into a catalytically null mutant – useful for generic DNA binding.

[0092] **Figure 20** shows a western blot showing that Cas9 orthologs are expressed in HEK 293FT cells; DNA plasmids encoding Cas9 orthologs are transfected into HEK 293FT cells and cell lysates are harvested for Western blot. No DNA is transfected for Cas9 ortholog #8.

[0093] **Figure 21** shows in vitro cleavage of candidate targets on pUC19 plasmid by 10 Cas9 orthologs. Consensus PAMs are predicted by sequence logos (Figure 7), based on which candidate targets on pUC19 are chosen. 7 of 9 new Cas9 orthologs tested have successfully cleaved at least one pUC19 target. SpCas9 can also cleave NGA in vitro.

[0094] **Figure 22** shows a Representative Surveyor Gel showing genomic cleavage by SaCas9.

[0095] **Figure 23** shows Genome Cleavage Efficiency of PAM Sequences (All targets).

[0096] **Figure 24** shows Genome Cleavage Efficiency of PAM Sequences (Cleaved targets)

[0097] **Figure 25** shows Genome Cleavage Efficiency of PAM Sequences (All targets, discard low-efficiency and orphan targets).

[0098] **Figure 26** shows Genome Cleavage Efficiency of PAM Sequences (Cleaved targets, discard low-efficiency and orphan targets).

[0099] **Figure 27** shows a Sequence Logo for Working Cleaved Spacers & PAMs (New endogenous genome test showing that T is not required).

[00100] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[00101] 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 CRISPR enzyme is a Cas enzyme, e.g. a Cas9 ortholog.

[00102] The invention uses nucleic acids to bind target DNA sequences. This is advantageous as nucleic acids are much easier and cheaper to produce than, for example, peptides, 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.

[00103] 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.

[00104] In aspects of the invention the terms "chimeric RNA", "chimeric guide RNA", "guide RNA", "single guide RNA" and "synthetic guide RNA" are used interchangeably and refer to the polynucleotide sequence comprising the guide sequence, the tracr sequence and the tracr mate sequence. The term "guide sequence" refers to the about 20bp sequence within the guide RNA that specifies the target site and may be used interchangeably with the terms "guide" or "spacer". The term "tracr mate sequence" may also be used interchangeably with the term "direct repeat(s)".

[00105] 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.

[00106] 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.

[00107] 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.

[00108] "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.

[00109] 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 (Tm). The Tm 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 Tm . 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 Tm . Highly permissive (very low stringency) washing conditions may be as low as 50° C below the Tm , 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.

[00110] "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.

[00111] 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.

[00112] 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.

[00113] 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.

[00114] 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.

[00115] 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.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.

[00116] % 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.

[00117] 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.

[00118] 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.

[00119] 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 that 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).

[00120] 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.

[00121] Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASISTM (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.

[00122] 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

[00123] 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), pyridylalanine, thienylalanine, naphthylalanine and phenylglycine.

[00124] 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.

[00125] 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)).

[00126] In one aspect, the invention provides for vectors that are used in the engineering and optimization of CRISPR-Cas systems.

[00127] As 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). 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. Further discussion of vectors is provided herein.

[00128] 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.

[00129] Aspects and embodiments of the invention relate to bicistronic vectors for chimeric RNA and Cas9 or a Cas9 ortholog. In some embodiments, the Cas9 is driven by the CBh promoter. In some embodiments, the chimeric RNA is driven by a U6 promoter. Preferably, the CBh and U6 are used together in the sense that the Cas9 is driven by the CBh promoter and the chimeric RNA is driven by a U6 promoter. In some embodiments, the chimeric guide RNA consists of a 20bp guide sequence (Ns) joined to the tracr sequence (running from the first "U" of the lower strand to the end of the transcript), which is truncated at various positions as indicated. The guide and tracr sequences are preferably separated by the tracr-mate sequence. A preferred example of a tracr-mate sequence is GUUUUAGAGCUA. This is preferably followed by a loop sequence. The loop is preferably GAAA, but it is not limited to this sequence or indeed to being only 4bp in length. Indeeed, 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. Throughout this application, chimeric RNA may also be called single guide, or synthetic guide RNA (sgRNA).

[00130] For instance, as described in specific detail in Example 2, chimeric guide RNAs may be designed as shown in Figure 8. The CRISPR loci in some of these families is depicted in Figure 11. The corresponding guide RNA sequences are shown in Figure 12. We analyzed the genomic DNA sequence within ~2kb of the Cas9 proteins and identified direct repeats ranging from 35bp to 50bp, with intervening spacers ranging from 29bp to 35bp. Based on the direct repeat sequence, we searched for tracrRNA candidate sequences with the following criteria: outside the crRNA array but containing high degree of homology to direct repeats (as required for direct repeat:tracrRNA base-pairing; custom computational analysis), outside the coding regions of the protein components, containing Rho-independent transcriptional termination signals ~60bp-120bp downstream from region of homology from with direct repeats, and co-folding with direct repeat to form a duplex, followed by two or more hairpin structures in the distal end of tracrRNA sequence. Based on these prediction criteria, we selected an initial set of 18 Cas9 proteins and their uniquely associated direct repeats and tracrRNAs distributed across all five Cas9 families. Applicants further generated a set of 18 chimeric RNA structures that preserved the sequence and secondary structures of the native direct repeat:tracrRNA duplex while shortening the region of base-pairing and fusing the two RNA elements through an artificial loop (Figures 6 A-J).

[00131] 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.

[00132] 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.

[00133] 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.

[00134] 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).

[00135] In some embodiments, a vector is a yeast expression vector. Examples of vectors for expression in yeast Saccharomyces cerevisae 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.).

[00136] 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).

[00137] 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.

[00138] 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.

[00139] 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.

[00140] In general, "CRISPR system" 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 other sequences and transcripts from a CRISPR locus. In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type III 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 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.

[00141] 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. 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 NGG /NRG (for example, as discussed elsewhere, a suitable PAM is 5'-NRG or 5'-NNGRR for SpCas9 or SaCas9 enzymes (or derived enzymes), respectively) 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 defense in bacteria and archae, Mole Cell 2010, January 15; 37(1): 7.

[00142] The type II CRISPR locus from Streptooccus pyogenes SF370 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 (Figure 2A). 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 (Figure 2A). Figure 2B demonstrates the nuclear localization of the codon optimized Cas9. To promote precise transcriptional initiation, the RNA polymerase III-based U6 promoter was selected to drive the expression of tracrRNA (Figure 2C). Similarly, a U6 promoter-based construct was developed to express a pre-crRNA array consisting of a single spacer flanked by two direct repeats (DRs, also encompassed by the term "tracr-mate sequences"; Figure 2C). The initial spacer was designed to target a 33-base-pair (bp) target site (30-bp protospacer plus a 3-bp CRISPR motif (PAM) sequence satisfying the NGG recognition motif of Cas9) in the human EMX1 locus (Figure 2C), a key gene in the development of the cerebral cortex.

[00143] Figure 16 shows Cas9 orthologs and respective sgRNAs are used to cleave two candidate targets present in a pUC19-based library. Target 1 is followed by a randomized PAM containing 7 degenerate bases (5'-NNNNNNN-3'), and target 1', which contains the same target sequence as target 1, is followed by a fixed PAM (5'-TGGAGAAT-3'). The sgRNA of each Cas9 ortholog contains the guide sequence against target 1 or target 1'. Gel images show successful cleavage by 20 Cas9 orthologs, indicating that these sgRNA designs are functional with their respective Cas9 enzymes.

[00144] In some embodiments, direct repeats or tracr mate sequences are either downloaded from the CRISPRs database or identified in silico by searching for repetitive motifs that are 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.

[00145] In some embodiments candidate tracrRNA are subsequently predicted by 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. In some embodiments, chimeric synthetic guide RNAs (sgRNAs) designs incorporate at least 8 bp of duplex structure between the direct repeat and tracrRNA.

[00146] Several aspects of the CRISPR system can be further improved to increase the efficiency and versatility of CRISPR targeting. Optimal Cas9 activity may depend on the availability of free Mg2+ at levels higher than that present in the mammalian nucleus (see e.g. Jinek et al., 2012, Science, 337:816), and the preference for an NGG/NRG motif immediately downstream of the protospacer restricts the ability to target on average every 12-bp in the human genome.

[00147] 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. 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. 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.

[00148] 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.

[00149] 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. 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. 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 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 in SpCas9. As discusssed herein, corresponding postions may be conserved in other Cas9s, i.e. in Cas9 orthologs from or derived from other bacterial species, with reference to the position numbering of SpCas9. (Figure 19) shows a multiple sequence alignment of 12 Cas9 orthologs and indicates the conserved catalytic Asp residue in the RuvC I domain and the conserved catalytic His residue in the HNH domain. Mutation of one or the other residue into Ala may convert the Cas9 ortholog into a nickase. Mutation of both residues may convert the Cas9 ortholog into a catalytically null mutant – useful for generic DNA binding. 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 ortholog 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 less than about 25%, 10%, 5%, 1%, 0.1%, 0.01%, or lower with respect to its non-mutated form.

[00150] An aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of SpCas9 was engineered to convert the nuclease into a nickase (see e.g. Sapranauskas et al., 2011, Nucleic Acis Research, 39: 9275; Gasiunas et al., 2012, Proc. Natl. Acad. Sci. USA, 109:E2579), such that nicked genomic DNA undergoes the high-fidelity homology-directed repair (HDR). Applicants used SURVEYOR assay to confirm that SpCas9n does not generate indels at the EMX1 protospacer target. It was seen that co-expression of EMX1-targeting chimeric crRNA (having the tracrRNA component as well) with SpCas9 produced indels in the target site,

whereas co-expression with SpCas9n did not (n=3). Moreover, sequencing of 327 amplicons did not detect any indels induced by SpCas9n. The same locus was selected to test CRISPR-mediated HR by co-transfecting HEK 293FT cells with the chimeric RNA targeting EMX1, hSpCas9 or hSpCas9n, as well as a HR template to introduce a pair of restriction sites (HindIII and NheI) near the protospacer. SpCas9 and SpCas9n indeed catalyzed integration of the HR template into the EMX1 locus. PCR amplification of the target region followed by restriction digest with HindIII revealed cleavage products corresponding to expected fragment sizes, with SpCas9 and SpCas9n mediating similar levels of HR efficiencies. Applicants further verified HR using Sanger sequencing of genomic amplicons and demonstrated the utility of CRISPR for facilitating targeted gene insertion in the mammalian genome. Given the 14-bp (12-bp from the spacer and 2-bp from the PAM) target specificity of the wild type SpCas9, the availability of a nickase can significantly reduce the likelihood of off-target modifications, since single strand breaks are not substrates for the error-prone NHEJ pathway. Figure 10A-M provides a scheme indicating positions of mutations in SpCas9 and 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.

[00151] 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 Francisciella novicida type II CRISPR locus), and the conserved Asp residue 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.

[00152] 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, mouse, rat, rabbit, dog, or non-human mammal including non-human 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.

[00153] 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/ (visited Jul. 9, 2002), 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.

[00154] 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. one or more NLS at the amino-terminus and 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.

[00155] 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.

[00156] 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. 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.

[00157] Multiplexed Nickase: Aspects of optimization and the teachings of Cas9 detailed in this application may also be used to generate Cas9 nickases. Cas9 nickases may be advantageously used in combination with pairs of guide RNAs to generate DNA double strand breaks with defined overhangs. When two pairs of guide RNAs are used, it is possible to excise an intervening DNA fragment. If an exogenous piece of DNA is cleaved by the two pairs of

guide RNAs to generate compatible overhangs with the genomic DNA, then the exogenous DNA fragment may be ligated into the genomic DNA to replace the excised fragment. For example, this may be used to remove trinucleotide repeat expansion in the huntingtin (HTT) gene to treat Huntington's Disease.

[00158] Cas9 and its chimeric guide RNA, or combination of tracrRNA and crRNA, can be delivered either as DNA or RNA. Delivery of Cas9 and guide RNA both as RNA (normal or containing base or backbone modifications) molecules can be used to reduce the amount of time that Cas9 protein persist in the cell. This may reduce the level of off-target cleavage activity in the target cell. Since delivery of Cas9 as mRNA takes time to be translated into protein, it might be advantageous to deliver the guide RNA several hours following the delivery of Cas9 mRNA, to maximize the level of guide RNA available for interaction with Cas9 protein.

[00159] In situations where guide RNA amount is limiting, it may be desirable to introduce Cas9 as mRNA and guide RNA in the form of a DNA expression cassette with a promoter driving the expression of the guide RNA. This way the amount of guide RNA available will be amplified via transcription.

[00160] A variety of delivery systems can be introduced to introduce Cas9 (DNA or RNA) and guide RNA (DNA or RNA) into the host cell. These include the use of liposomes, viral vectors, electroporation, nanoparticles , nanowires (Shalek et al., Nano Letters, 2012), exosomes. Molecular trojan horses liposomes (Pardridge et al., Cold Spring Harb Protoc; 2010; doi:10.1101/pdb.prot5407) may be used to deliver Cas9 and guide RNA across the blood brain barrier.

[00161] Discussion of guide RNAs for orthologs: In some embodiments, a guide sequence is selected to reduce the degree secondary structure within the guide RNA. 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 RNA 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). A method of optimizing the guide RNA of a Cas9 ortholog comprises breaking up polyU tracts in the guide RNA. PolyU tracts that may be broken up may comprise a series of 4, 5, 6, 7, 8, 9 or 10 Us.

[00162] 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) NNNNNNNNNNNNNNNNNNNgttttgtactctcaagatttaGAAAtaaatcttgcagaagctacaaagataaggctt catgccgaaatcaacaccctgtcattttatggcagggtgttttcgttatttaaTTTTTT; (2)

NNNNNNNNNNNNNNNNNNNgttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccgaaatca acacccctgtcatttatggcagggtgttttcgttatttaaTTTTTT; (3)

NNNNNNNNNNNNNNNNNNNgttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccgaaatca acacccctgtcatttatggcagggtgtTTTTTT; (4)

NNNNNNNNNNNNNNNNNNNgttttagagctaGAAAtagcaagttaaaataaggctagtccgttatcaacttgaaaa agtggcaccgagtcggtgcTTTTTT; (5)

NNNNNNNNNNNNNNNNNNNgttttagagctaGAAATAGcaagttaaaataaggctagtccgttatcaacttgaa aaagtgTTTTTTT; and (6)

NNNNNNNNNNNNNNNNNNNgttttagagctagAAATAGcaagttaaaataaggctagtccgttatcaTTTTT TTT. 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.

[00163] Discussion of tracr mates for orthologs: 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.

[00164] 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.

[00165] 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 cryptochorome). 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.

[00166] 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. 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).

[00167] 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).

[00168] 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).

[00169] 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.

[00170] 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).

[00171] 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.

[00172] 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).

[00173] 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 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. 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.

[00174] 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.

[00175] 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.

[00176] 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, AAV8 for targeting of liver tissue.

[00177] 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
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

[00178] 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).

[00179] Two ways to package Cas9 coding nucleic acid molecules, e.g., DNA, into viral vectors to mediate genome modification in vivo are preferred:

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)

[00180] 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.

[00181] 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 toxicity due to over expression of Cas9.

[00182] For ubiquitous expression, can use promoters: CMV, CAG, CBh, PGK, SV40, Ferritin heavy or light chains, etc.

[00183] For brain 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

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

[00184] 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 or 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 above promoters and vectors are preferred individually.

[00185] 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.

[00186] 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.

[00187] In some embodiments, one or more vectors described herein are used to produce a non-human transgenic animal or transgenic plant. In some embodiments, the transgenic animal is a mammal, such as a mouse, rat, or rabbit. Methods for producing transgenic plants and animals are known in the art, and generally begin with a method of cell transfection, such as described herein.

[00188] With recent advances in crop genomics, the ability to use CRISPR-Cas systems to perform efficient and cost effective gene editing and manipulation will allow the rapid selection and comparison of single and and multiplexed genetic manipulations to transform such genomes for improved production and enhanced traits. In this regard reference is made to US patents and

publications: US Patent No. 6,603,061 - Agrobacterium-Mediated Plant Transformation Method; US Patent No. 7,868,149 - Plant Genome Sequences and Uses Thereof and US 2009/0100536 - Transgenic Plants with Enhanced Agronomic Traits, all the contents and disclosure of each of which are herein incorporated by reference in their entirety. In the practice of the invention, the contents and disclosure of Morrell et al "Crop genomics:advances and applications" Nat Rev Genet. 2011 Dec 29;13(2):85-96 are also herein incorporated by reference in their entirety. In an advantageous embodiment of the invention, the CRSIPR/Cas9 system is used to engineer microalgae (Example 7). Accordingly, reference herein to animal cells may also apply, mutatis mutandis, to plant cells unless otherwise apparent.

[00189] 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 plant (including micro-algae), 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 (including micro-algae). For re-introduced cells it is particularly preferred that the cells are stem cells.

[00190] 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.

[00191] 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 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.

[00192] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. Elements may provide individually or in combinations, and may 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.

[00193] 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.

[00194] 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.

[00195] 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.

[00196] 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 processes, an exogenous polynucleotide template can be introduced into the genome sequence. In some methods, the HDR process is used to modify a 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.

[00197] 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.

[00198] 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.

[00199] 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.

[00200] 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 has about 200 bp to about 2000 bp, about 600 bp to about 1000 bp, or more particularly about 700 bp to about 1000 bp.

[00201] 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).

[00202] In an exemplary 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.

[00203] 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.

[00204] 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 is not produced.

[00205] 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.

[00206] The inactivated target sequence may include a deletion mutation (i.e., deletion of one or more nucleotides), an insertion mutation (i.e., insertion of one or more nucleotides), or a nonsense mutation (i.e., substitution of a single nucleotide for another nucleotide such that a stop codon is introduced). In some methods, the inactivation of a target sequence results in "knock-out" of the target sequence.

[00207] An altered expression of one or more genome sequences associated with a signaling biochemical pathway can be determined by assaying for a difference in the mRNA levels of the corresponding genes between the test model cell and a control cell, when they are contacted with a candidate agent. Alternatively, the differential expression of the sequences associated with a signaling biochemical pathway is determined by detecting a difference in the level of the encoded polypeptide or gene product.

[00208] To assay for an agent-induced alteration in the level of mRNA transcripts or corresponding polynucleotides, nucleic acid contained in a sample is first extracted according to standard methods in the art. For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook et al. (1989), or extracted by nucleic-acid-binding resins following the accompanying instructions provided by the manufacturers. The mRNA contained in the extracted nucleic acid sample is then detected by amplification procedures or conventional hybridization assays (e.g. Northern blot analysis) according to methods widely known in the art or based on the methods exemplified herein.

[00209] 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. In particular, the isolated RNA can be subjected to a reverse transcription assay that is coupled with a quantitative polymerase chain reaction (RT-PCR) in order to quantify the expression level of a sequence associated with a signaling biochemical pathway.

[00210] Detection of the gene expression level can be conducted in real time in an amplification assay. In one aspect, the amplified products can be directly visualized with fluorescent DNA-binding agents including but not limited to DNA intercalators and DNA groove binders. Because the amount of the intercalators incorporated into the double-stranded DNA

molecules is typically proportional to the amount of the amplified DNA products, one can conveniently determine the amount of the amplified products by quantifying the fluorescence of the intercalated dye using conventional optical systems in the art. DNA-binding dye suitable for this application include SYBR green, SYBR blue, DAPI, propidium iodine, Hoeste, SYBR gold, ethidium bromide, acridines, proflavine, acridine orange, acriflavine, fluorcoumanin, ellipticine, daunomycin, chloroquine, distamycin D, chromomycin, homidium, mithramycin, ruthenium polypyridyls, anthramycin, and the like.

[00211] In another aspect, other fluorescent labels such as sequence specific probes can be employed in the amplification reaction to facilitate the detection and quantification of the amplified products. Probe-based quantitative amplification relies on the sequence-specific detection of a desired amplified product. It utilizes fluorescent, target-specific probes (e.g., TaqMan® probes) resulting in increased specificity and sensitivity. Methods for performing probe-based quantitative amplification are well established in the art and are taught in U.S. Patent No. 5,210,015.

[00212] In yet another aspect, conventional hybridization assays using hybridization probes that share sequence homology with sequences associated with a signaling biochemical pathway can be performed. Typically, probes are allowed to form stable complexes with the sequences associated with a signaling biochemical pathway contained within the biological sample derived from the test subject in a hybridization reaction. It will be appreciated by one of skill in the art that where antisense is used as the probe nucleic acid, the target polynucleotides provided in the sample are chosen to be complementary to sequences of the antisense nucleic acids. Conversely, where the nucleotide probe is a sense nucleic acid, the target polynucleotide is selected to be complementary to sequences of the sense nucleic acid.

[00213] Hybridization can be performed under conditions of various stringency, for instance as described herein. Suitable hybridization conditions for the practice of the present invention are such that the recognition interaction between the probe and sequences associated with a signaling biochemical pathway is both sufficiently specific and sufficiently stable. Conditions that increase the stringency of a hybridization reaction are widely known and published in the art. See, for example, (Sambrook, et al., (1989); Nonradioactive In Situ Hybridization Application Manual, Boehringer Mannheim, second edition). The hybridization assay can be formed using probes immobilized on any solid support, including but are not limited to

nitrocellulose, glass, silicon, and a variety of gene arrays. A preferred hybridization assay is conducted on high-density gene chips as described in U.S. Patent No. 5,445,934.

[00214] For a convenient detection of the probe-target complexes formed during the hybridization assay, the nucleotide probes are conjugated to a detectable label. Detectable labels suitable for use in the present invention include any composition detectable by photochemical, biochemical, spectroscopic, immunochemical, electrical, optical or chemical means. A wide variety of appropriate detectable labels are known in the art, which include fluorescent or chemiluminescent labels, radioactive isotope labels, enzymatic or other ligands. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as digoxigenin, ß-galactosidase, urease, alkaline phosphatase or peroxidase, avidin/biotin complex.

[00215] The detection methods used to detect or quantify the hybridization intensity will typically depend upon the label selected above. For example, radiolabels may be detected using photographic film or a phosphoimager. Fluorescent markers may be detected and quantified using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and measuring the reaction product produced by the action of the enzyme on the substrate; and finally colorimetric labels are detected by simply visualizing the colored label.

[00216] An agent-induced change in expression of sequences associated with a signaling biochemical pathway can also be determined by examining the corresponding gene products. Determining the protein level typically involves a) contacting the protein contained in a biological sample with an agent that specifically bind to a protein associated with a signaling biochemical pathway; and (b) identifying any agent:protein complex so formed. In one aspect of this embodiment, the agent that specifically binds a protein associated with a signaling biochemical pathway is an antibody, preferably a monoclonal antibody.

[00217] The reaction is performed by contacting the agent with a sample of the proteins associated with a signaling biochemical pathway derived from the test samples under conditions that will allow a complex to form between the agent and the proteins associated with a signaling biochemical pathway. The formation of the complex can be detected directly or indirectly according to standard procedures in the art. In the direct detection method, the agents are supplied with a detectable label and unreacted agents may be removed from the complex; the amount of remaining label thereby indicating the amount of complex formed. For such method,

it is preferable to select labels that remain attached to the agents even during stringent washing conditions. It is preferable that the label does not interfere with the binding reaction. In the alternative, an indirect detection procedure may use an agent that contains a label introduced either chemically or enzymatically. A desirable label generally does not interfere with binding or the stability of the resulting agent:polypeptide complex. However, the label is typically designed to be accessible to an antibody for an effective binding and hence generating a detectable signal.

[00218] A wide variety of labels suitable for detecting protein levels are known in the art. Non-limiting examples include radioisotopes, enzymes, colloidal metals, fluorescent compounds, bioluminescent compounds, and chemiluminescent compounds.

[00219] The amount of agent:polypeptide complexes formed during the binding reaction can be quantified by standard quantitative assays. As illustrated above, the formation of agent:polypeptide complex can be measured directly by the amount of label remained at the site of binding. In an alternative, the protein associated with a signaling biochemical pathway is tested for its ability to compete with a labeled analog for binding sites on the specific agent. In this competitive assay, the amount of label captured is inversely proportional to the amount of protein sequences associated with a signaling biochemical pathway present in a test sample.

[00220] A number of techniques for protein analysis based on the general principles outlined above are available in the art. They include but are not limited to radioimmunoassays, ELISA (enzyme linked immunoradiometric assays), "sandwich" immunoassays, immunoradiometric assays, in situ immunoassays (using e.g., colloidal gold, enzyme or radioisotope labels), western blot analysis, immunoprecipitation assays, immunofluorescent assays, and SDS-PAGE.

[00221] Antibodies that specifically recognize or bind to proteins associated with a signaling biochemical pathway are preferable for conducting the aforementioned protein analyses. Where desired, antibodies that recognize a specific type of post-translational modifications (e.g., signaling biochemical pathway inducible modifications) can be used. Post-translational modifications include but are not limited to glycosylation, lipidation, acetylation, and phosphorylation. These antibodies may be purchased from commercial vendors. For example, anti-phosphotyrosine antibodies that specifically recognize tyrosine-phosphorylated proteins are available from a number of vendors including Invitrogen and Perkin Elmer. Anti-phosphotyrosine antibodies are particularly useful in detecting proteins that are differentially

phosphorylated on their tyrosine residues in response to an ER stress. Such proteins include but are not limited to eukaryotic translation initiation factor 2 alpha (eIF-2α). Alternatively, these antibodies can be generated using conventional polyclonal or monoclonal antibody technologies by immunizing a host animal or an antibody-producing cell with a target protein that exhibits the desired post-translational modification.

[00222] In practicing the subject method, it may be desirable to discern the expression pattern of an protein associated with a signaling biochemical pathway in different bodily tissue, in different cell types, and/or in different subcellular structures. These studies can be performed with the use of tissue-specific, cell-specific or subcellular structure specific antibodies capable of binding to protein markers that are preferentially expressed in certain tissues, cell types, or subcellular structures.

[00223] An altered expression of a gene associated with a signaling biochemical pathway can also be determined by examining a change in activity of the gene product relative to a control cell. The assay for an agent-induced change in the activity of a protein associated with a signaling biochemical pathway will dependent on the biological activity and/or the signal transduction pathway that is under investigation. For example, where the protein is a kinase, a change in its ability to phosphorylate the downstream substrate(s) can be determined by a variety of assays known in the art. Representative assays include but are not limited to immunoblotting and immunoprecipitation with antibodies such as anti-phosphotyrosine antibodies that recognize phosphorylated proteins. In addition, kinase activity can be detected by high throughput chemiluminescent assays such as AlphaScreen™ (available from Perkin Elmer) and eTag™ assay (Chan-Hui, et al. (2003) Clinical Immunology 111: 162-174).

[00224] Where the protein associated with a signaling biochemical pathway is part of a signaling cascade leading to a fluctuation of intracellular pH condition, pH sensitive molecules such as fluorescent pH dyes can be used as the reporter molecules. In another example where the protein associated with a signaling biochemical pathway is an ion channel, fluctuations in membrane potential and/or intracellular ion concentration can be monitored. A number of commercial kits and high-throughput devices are particularly suited for a rapid and robust screening for modulators of ion channels. Representative instruments include FLIPRTM (Molecular Devices, Inc.) and VIPR (Aurora Biosciences). These instruments are capable of

detecting reactions in over 1000 sample wells of a microplate simultaneously, and providing real-time measurement and functional data within a second or even a minisecond.

[00225] 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.

[00226] 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).

[00227] 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.

[00228] 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).

[00229] The target polynucleotide of a CRISPR complex may include a number of disease-associated genes and polynucleotides as well as signaling biochemical pathway-associated genes and polynucleotides as listed in US provisional patent applications 61/736,527 and 61/748,427 having Broad reference BI-2011/008/WSGR Docket No. 44063-701.101and BI-2011/008/WSGR Docket No. 44063-701.102 respectively, both entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on December 12, 2012 and January 2, 2013, respectively, the contents of all of which are herein incorporated by reference in their entirety.

[00230] 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.

[00231] Examples of disease-associated genes and polynucleotides are 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.

[00232] Examples of disease-associated genes and polynucleotides 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.

[00233] 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 applications 61/736,527 filed on December 12, 2012 and 61/748,427 filed on January 2, 2013. 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	Aber; 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
	(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); Il-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), Il-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 Signaling	RAC1; TAF4B; EP300; SMAD2; TRAF6; PCAF; ELK1; MAPK1; SMAD3; AKT2; IKBKB; NCOR2; UBE2I;
	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;
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 Signaling	ACTN4; CD44; PRKCE; ITGAM; ROCK1; CXCR4; CYBA;
	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 Signaling	IRAK1; SOD2; MYD88; TRAF6; ELK1; MAPK1; PTPN11;
	AKT2; IKBKB; PIK3CA; FOS; NFKB2; MAP3K14;
	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;
	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;
	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;
	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;
	STAT5B; PIK3CB; PIK3C3; GNB2L1; BCL2L1; MAPK3;
	ETS1; KRAS; RUNX1; PIM1; PIK3C2A; RAF1; MAP2K2;
	AKT1; JAK2; PIK3R1; STAT3; MAP2K1; CCND1; AKT3;
	STAT1
Amyotrophic Lateral Sclerosis Signaling	BID; IGF1; RAC1; BIRC4; PGF; CAPNS1; CAPN2;
	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;
	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;
	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
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 Signaling	PPP2R1A; PPP2CA; PPP1CC; PPP2R5C
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 Metabolism	ALDH1A1; NOS3; NOS2A
Eicosanoid Signaling	PRDX6; GRN; YWHAZ
Fructose and Mannose Metabolism	HK2; GCK; HK1
Galactose Metabolism	HK2; GCK; HK1
Stilbene, Coumarine and Lignin Biosynthesis	PRDX6; PRDX1; TYR
Antigen Presentation Pathway	CALR; B2M
Biosynthesis of Steroids	NQO1; DHCR7
Butanoate Metabolism	ALDH1A1; NLGN1
Citrate Cycle	IDH2; IDH1
Fatty Acid Metabolism	ALDH1A1; CYP1B1
Glycerophospholipid Metabolism	PRDX6; CHKA
Histidine Metabolism	PRMT5; ALDH1A1
Inositol Metabolism	ERO1L; APEX1
Metabolism of Xenobiotics by Cytochrome p450	GSTP1; CYP1B1
Methane Metabolism	PRDX6; PRDX1
Phenylalanine Metabolism	PRDX6; PRDX1
Propanoate Metabolism	ALDH1A1; LDHA
Selenoamino Acid Metabolism	PRMT5; AHCY
Sphingolipid Metabolism	SPHK1; SPHK2
Aminophosphonate Metabolism	PRMT5
Androgen and Estrogen Metabolism	PRMT5
Ascorbate and Aldarate Metabolism	ALDH1A1
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

[00234] 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.

[00235] 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 sytem 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).

[00236] 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.

[00237] 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.

[00238] 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 asn 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.

[00239] 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.

[00240] Examples of addiction-related proteins may include ABAT for example.

[00241] 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.

[00242] 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.

[00243] 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.

[00244] 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.

[00245] 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.

[00246] Examples of proteins associated Schizophrenia may include NRG1, ErbB4, CPLX1, TPH1, TPH2, NRXN1, GSK3A, BDNF, DISC1, GSK3B, and combinations thereof.

[00247] 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.

[00248] 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.

[00249] 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.

[00250] 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.

[00251] 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.

[00252] 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.

[00253] 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.

[00254] 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.

[00255] 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.

[00256] 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.

[00257] 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 above and examples of genes currently associated with those conditions are also provided there. However, the genes exemplified are not exhaustive.

EXAMPLES

[00258] 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: Improvement of the Cas9 system for in vivo application

[00259] Applicants conducted a Metagenomic search for a Cas9 with small molecular weight. Most Cas9 orthologs are fairly large. Many known Cas9 orthologs are large and contain more than 1300 amino acids. For example the SpCas9 is around 1368aa long, which is too large to be easily packaged into viral vectors for delivery. A graph representing the length distribution of Cas9 homologs is shown in Figure 6: The graph is generated from sequences deposited in GenBank. Some of the sequences may have been mis-annotated and therefore the exact

frequency for each length may not necessarily be accurate. Nevertheless it provides a glimpse at distribution of Cas9 proteins and suggest that there are shorter Cas9 homologs.

[00260] Through computational analysis, Applicants found that in the bacterial strain Campylobacter, there are two Cas9 proteins with less than 1000 amino acids. The sequence for one Cas9 from *Campylobacter jejuni* is presented below. At this length, CjCas9 can be easily packaged into AAV, lentiviruses, Adenoviruses, and other viral vectors for robust delivery into primary cells and in vivo animal models. In a preferred embodiment of the invention, the Cas9 protein from *S. aureus* is used.

[00261] Campylobacter jejuni Cas9 (CjCas9)

[00262] MARILAFDIGISSIGWAFSENDELKDCGVRIFTKVENPKTGESLALPRRLARSA
RKRLARRKARLNHLKHLIANEFKLNYEDYQSFDESLAKAYKGSLISPYELRFRALNELLS
KQDFARVILHIAKRRGYDDIKNSDDKEKGAILKAIKQNEEKLANYQSVGEYLYKEYFQK
FKENSKEFTNVRNKKESYERCIAQSFLKDELKLIFKKQREFGFSFSKKFEEEVLSVAFYKR
ALKDFSHLVGNCSFFTDEKRAPKNSPLAFMFVALTRIINLLNNLKNTEGILYTKDDLNAL
LNEVLKNGTLTYKQTKKLLGLSDDYEFKGEKGTYFIEFKKYKEFIKALGEHNLSQDDLN
EIAKDITLIKDEIKLKKALAKYDLNQNQIDSLSKLEFKDHLNISFKALKLVTPLMLEGKKY
DEACNELNLKVAINEDKKDFLPAFNETYYKDEVTNPVVLRAIKEYRKVLNALLKKYGK
VHKINIELAREVGKNHSQRAKIEKEQNENYKAKKDAELECEKLGLKINSKNILKLRLFKE
QKEFCAYSGEKIKISDLQDEKMLEIDHIYPYSRSFDDSYMNKVLVFTKQNQEKLNQTPFE
AFGNDSAKWQKIEVLAKNLPTKKQKRILDKNYKDKEQKNFKDRNLNDTRYIARLVLNY
TKDYLDFLPLSDDENTKLNDTQKGSKVHVEAKSGMLTSALRHTWGFSAKDRNNHLHH
AIDAVIIAYANNSIVKAFSDFKKEQESNSAELYAKKISELDYKNKRKFFEPFSGFRQKVLD
KIDEIFVSKPERKKPSGALHEETFRKEEEFYQSYGGKEGVLKALELGKIRKVNGKIVKNG
DMFRVDIFKHKKTNKFYAVPIYTMDFALKVLPNKAVARSKKGEIKDWILMDENYEFCF
SLYKDSLILIQTKDMQEPEFVYYNAFTSSTVSLIVSKHDNKFETLSKNQKILFKNANEKE
VIAKSIGIQNLKVFEKYIVSALGEVTKAEFRQREDFKK.

[00263] The putative tracrRNA element for this CjCas9 is:

TATAATCTCATAAGAAATTTAAAAAGGGACTAAAATAAAGAGTTTGCGGGACTCTG
CGGGGTTACAATCCCCTAAAACCGCTTTTAAAATT

[00264] The Direct Repeat sequence is:

GTTTTAGTCCCTTTTTAAATTTCTTTATGGTAAAAT

[00265] An example of a chimeric guideRNA for CjCas9 is:
NNNNNNNNNNNNNNNNNNNNGUUUUAGUCCCGAAAGGGACUAAAAUAAAGAGUU
UGCGGGACUCUGCGGGGUUACAAUCCCCUAAAACCGCUUUU

Example 2: Cas9 diversity and chimeric RNAs

[00266] The wild-type CRISPR-Cas system is an adaptive immune mechanism against invading exogenous DNA employed by diverse species across bacteria and archaea. The type II CRISPR-Cas system consists of a set of genes encoding proteins responsible for the "acquisition" of foreign DNA into the CRISPR locus, as well as a set of genes encoding the "execution" of the DNA cleavage mechanism; these include the DNA nuclease (Cas9), a non-coding transactivating cr-RNA (tracrRNA), and an array of foreign DNA-derived spacers flanked by direct repeats (crRNAs). Upon maturation by Cas9, the tracRNA and crRNA duplex guide the Cas9 nuclease to a target DNA sequence specified by the spacer guide sequences, and mediates double-stranded breaks in the DNA near a short sequence motif in the target DNA that is required for cleavage and specific to each CRISPR-Cas system. The type II CRISPR-Cas systems are found throughout the bacterial kingdom and highly diverse in Cas9 protein sequence and size, tracrRNA and crRNA direct repeat sequence, genome organization of these elements, and the motif requirement for target cleavage. One species may have multiple distinct CRISPR-Cas systems.

[00267] Applicants evaluated 207 putative Cas9s from bacterial species identified based on sequence homology to known Cas9s and structures orthologous to known subdomains, including the HNH endonuclease domain and the RuvC endonuclease domains [information from the Eugene Koonin and Kira Makarova]. Phylogenetic analysis based on the protein sequence conservation of this set revealed five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids) (Figures 4 and 5A-F).

[00268] In some embodiments, the tracr mate sequences or the direct repeats are either downloaded from the CRISPRs database or identified in silico by searching for repetitive motifs that are 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 candidate tracrRNA are subsequently predicted by

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

[00269] The list of the human codon optimized Cas9 ortholog sequences to pair with the chimeric RNAs provided in Figures 8 A-J is provided in Figures 9 A-O. Applicants have also shown that the Cas9 orthologs can cleave their targets in in vitro cleavage assays (Figure 16). The CRISPR loci in some of these families is depicted in Figure 11. The corresponding guide RNA sequences are shown in Figure 12. Applicants systematically analyzed the genomic DNA sequence within ~2kb of the Cas9 proteins using custom computational analysis code and identified direct repeats ranging from 35bp to 50bp, with intervening spacers ranging from 29bp to 35bp. Based on the direct repeat sequence, Applicants computationally searched for tracrRNA candidate sequences with the following criteria: outside the crRNA array but containing high degree of homology to direct repeats (as required for direct repeat:tracrRNA base-pairing; custom computational analysis), outside the coding regions of the protein components, containing Rho-independent transcriptional termination signals ~60bp-120bp downstream from region of homology from with direct repeats, and co-folding with direct repeat to form a duplex, followed by two or more hairpin structures in the distal end of tracrRNA sequence. Based on these prediction criteria, Applicants selected an initial set of 18 Cas9 proteins and their uniquely associated direct repeats and tracrRNAs distributed across all five Cas9 families. Applicants further generated a set of 18 chimeric RNA structures that preserved the sequence and secondary structures of the native direct repeat:tracrRNA duplex while shortening the region of base-pairing and fusing the two RNA elements through an artificial loop (Figures 8A-J).

Example 3: Cas9 orthologs

[00270] Applicants have generated codon optimized Cas9 orthologs to advance expression in eukaryotic cells.

[00271] Example of a human codon optimized sequence (i.e. being optimized for expression in humans) sequence : SaCas9 : is provided below

ACCGGTGCCACCATGTACCCATACGATGTTCCAGATTACGCTTCGCCGAAGAAAAAGCGCAA
GGTCGAAGCGTCCATGAAAAGGAACTACATTCTGGGGCTGGACATCGGGATTACAAGCGTG
GGGTATGGGATTATTGACTATGAAACAAGGGACGTGATCGACGCAGGCGTCAGACTGTTCA
AGGAGGCCAACGTGGAAAACAATGAGGGACGGAGAAGCAAGAGGGGAGCCAGGCGCCTGA
AACGACGGAGAAGGCACAGAATCCAGAGGGTGAAGAAACTGCTGTTCGATTACAACCTGCT
GACCGACCATTCTGAGCTGAGTGGAATTAATCCTTATGAAGCCAGGGTGAAAGGCCTGAGTC
AGAAGCTGTCAGAGGAAGAGTTTTCCGCAGCTCTGCTGCACCTGGCTAAGCGCCGAGGAGT
GCATAACGTCAATGAGGTGGAAGAGGACACCGGCAACGAGCTGTCTACAAAGGAACAGATC
TCACGCAATAGCAAAGCTCTGGAAGAGAAGTATGTCGCAGAGCTGCAGCTGGAACGGCTGA
AGAAAGATGGCGAGGTGAGAGGGTCAATTAATAGGTTCAAGACAAGCGACTACGTCAAAGA
AGCCAAGCAGCTGCTGAAAGTGCAGAAGGCTTACCACCAGCTGGATCAGAGCTTCATCGAT
ACTTATATCGACCTGCTGGAGACTCGGAGAACCTACTATGAGGGACCAGGAGAAGGGAGCC
CCTTCGGATGGAAAGACATCAAGGAATGGTACGAGATGCTGATGGGACATTGCACCTATTTT
CCAGAAGAGCTGAGAAGCGTCAAGTACGCTTATAACGCAGATCTGTACAACGCCCTGAATG
ACCTGAACAACCTGGTCATCACCAGGGATGAAAACGAGAAACTGGAATACTATGAGAAGTT
CCAGATCATCGAAAACGTGTTTAAGCAGAAGAAAAAGCCTACACTGAAACAGATTGCTAAG
GAGATCCTGGTCAACGAAGAGGACATCAAGGGCTACCGGGTGACAAGCACTGGAAAACCAG
AGTTCACCAATCTGAAAGTGTATCACGATATTAAGGACATCACAGCACGGAAAGAAATCAT
TGAGAACGCCGAACTGCTGGATCAGATTGCTAAGATCCTGACTATCTACCAGAGCTCCGAGG
ACATCCAGGAAGAGCTGACTAACCTGAACAGCGAGCTGACCCAGGAAGAGATCGAACAGAT
TAGTAATCTGAAGGGGTACACCGGAACACACAACCTGTCCCTGAAAGCTATCAATCTGATTC
TGGATGAGCTGTGGCATACAAACGACAATCAGATTGCAATCTTTAACCGGCTGAAGCTGGTC
CCAAAAAAGGTGGACCTGAGTCAGCAGAAAGAGATCCCAACCACACTGGTGGACGATTTCA
TTCTGTCACCCGTGGTCAAGCGGAGCTTCATCCAGAGCATCAAAGTGATCAACGCCATCATC
AAGAAGTACGGCCTGCCCAATGATATCATTATCGAGCTGGCTAGGGAGAAGAACAGCAAGG
ACGCACAGAAGATGATCAATGAGATGCAGAAACGAAACCGGCAGACCAATGAACGCATTG
AAGAGATTATCCGAACTACCGGGAAAGAGAACGCAAAGTACCTGATTGAAAAAATCAAGCT
GCACGATATGCAGGAGGGAAAGTGTCTGTATTCTCTGGAGGCCATCCCCCTGGAGGACCTGC
TGAACAATCCATTCAACTACGAGGTCGATCATATTATCCCCAGAAGCGTGTCCTTCGACAAT
TCCTTTAACAACAAGGTGCTGGTCAAGCAGGAAGAGAACTCTAAAAAGGGCAATAGGACTC
CTTTCCAGTACCTGTCTAGTTCAGATTCCAAGATCTCTTACGAAACCTTTAAAAAGCACATTC
TGAATCTGGCCAAAGGAAAGGGCCGCATCAGCAAGACCAAAAAGGAGTACCTGCTGGAAG

AGCGGGACATCAACAGATTCTCCGTCCAGAAGGATTTTATTAACCGGAATCTGGTGGACACA
AGATACGCTACTCGCGGCCTGATGAATCTGCTGCGATCCTATTTCCGGGTGAACAATCTGGA
TGTGAAAGTCAAGTCCATCAACGGCGGGTTCACATCTTTTCTGAGGCGCAAATGGAAGTTTA
AAAAGGAGCGCAACAAAGGGTACAAGCACCATGCCGAAGATGCTCTGATTATCGCAAATGC
CGACTTCATCTTTAAGGAGTGGAAAAAGCTGGACAAAGCCAAGAAAGTGATGGAGAACCAG
ATGTTCGAAGAGAAGCAGGCCGAATCTATGCCCGAAATCGAGACAGAACAGGAGTACAAGG
AGATTTTCATCACTCCTCACCAGATCAAGCATATCAAGGATTTCAAGGACTACAAGTACTCT
CACCGGGTGGATAAAAAGCCCAACAGAGAGCTGATCAATGACACCCTGTATAGTACAAGAA
AAGACGATAAGGGGAATACCCTGATTGTGAACAATCTGAACGGACTGTACGACAAAGATAA
TGACAAGCTGAAAAAGCTGATCAACAAAAGTCCCGAGAAGCTGCTGATGTACCACCATGAT
CCTCAGACATATCAGAAACTGAAGCTGATTATGGAGCAGTACGGCGACGAGAAGAACCCAC
TGTATAAGTACTATGAAGAGACTGGGAACTACCTGACCAAGTATAGCAAAAAGGATAATGG
CCCCGTGATCAAGAAGATCAAGTACTATGGGAACAAGCTGAATGCCCATCTGGACATCACA
GACGATTACCCTAACAGTCGCAACAAGGTGGTCAAGCTGTCACTGAAGCCATACAGATTCG
ATGTCTATCTGGACAACGGCGTGTATAAATTTGTGACTGTCAAGAATCTGGATGTCATCAAA
AAGGAGAACTACTATGAAGTGAATAGCAAGTGCTACGAAGAGGCTAAAAAGCTGAAAAAG
ATTAGCAACCAGGCAGAGTTCATCGCCTCCTTTTACAACAACGACCTGATTAAGATCAATGG
CGAACTGTATAGGGTCATCGGGGTGAACAATGATCTGCTGAACCGCATTGAAGTGAATATG
ATTGACATCACTTACCGAGAGTATCTGGAAAACATGAATGATAAGCGCCCCCCTCGAATTAT
CAAAACAATTGCCTCTAAGACTCAGAGTATCAAAAAGTACTCAACCGACATTCTGGGAAAC
CTGTATGAGGTGAAGAGCAAAAAGCACCCTCAGATTATCAAAAAGGGCTAAGAATTC

[00272] Applicants analyzed Cas9 orthologs to identify the relevant PAM sequences and the corresponding chimeric guide RNA as indicated in Figure 13A-II. This expanded set of PAMs provides broader targeting across the genome and also significantly increases the number of unique target sites and provides potential for identifying novel Cas9s with increased levels of specificity in the genome. Applicants determined the PAM for Staphylococcus aureus subspecies Aureus Cas9 to be NNGRR (Figure 14). Staphylococcus aureus subspecies Aureus Cas9 is also known as SaCas9. Figure 15 a-d provides SaCas9 single or multiple vector designs.

[00273] Figure 7 show sequence logos for putative PAMs as indicated by reverse complements. Cas9 orthologs and their respective sgRNAs were used to cleave a library of targets bearing a randomized PAM (7-bp sequence immediately 3' of the target sequence). Cleaved products were isolated and deep-sequenced to yield 7-bp candidate sequences that were

permissive to cleavage for each Cas9 ortholog. For each Cas9 ortholog, consensus PAMs were determined by aligning all 7-bp candidate sequences. (Figures 7 and 21).

[00274] Further work examining the thermodynamics and *in vivo* stability of sgRNA-DNA duplexes will likely yield additional predictive power for off-target activity, while exploration of SpCas9 mutants and orthologs may yield novel variants with improved specificity. The specificity of Cas9 orthologs can be further evaluated by testing the ability of each Cas9 to tolerate mismatches between the guide RNA and its DNA target.

Example 4: Cas9 mutations

[00275] In this example, Applicants show that the following mutations can convert SpCas9 into a nicking enzyme: D10A, E762A, H840A, N854A, N863A, D986A.

[00276] Applicants provide sequences showing where the mutation points are located within the SpCas9 gene (Figure 10A-M). Applicants also show that the nickases are still able to mediate homologous recombination. Furthermore, SpCas9 with these mutations (individually) reduce the level of double strand break. Cas9 orthologs all share the general organization of 3-4 RuvC domains and a HNH domain (Figure 19). 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.

[00277] 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 Francisella novicida type II CRISPR locus), and the conserved Asp residue 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.

Example 5: Cas9 functional optimization

[00278] For enhanced function or to develop new functions, Applicants generate chimeric Cas9 proteins by combining fragments from different Cas9 orthologs.

[00279] For instance, Applicants fused the N-term of St1Cas9 (fragment from this protein is in bold) with C-term of SpCas9 (fragment from this protein is underlined).

[00280] >St1(N)Sp(C)Cas9

MSDLVLGLDIGIGSVGVGILNKVTGEIIHKNSRIFPAAQAENNLVRRTNRQGR
RLARRKKHRRVRLNRLFEESGLITDFTKISINLNPYQLRVKGLTDELSNEELF
IALKNMVKHRGISYLDDASDDGNSSVGDYAQIVKENSKQLETKTPGQIQLER
YQTYGQLRGDFTVEKDGKKHRLINVFPTSAYRSEALRILQTQQEFNPQITDE
FINRYLEILTGKRKYYHGPGNEKSRTDYGRYRTSGETLDNIFGILIGKCTFYP
DEFRAAKASYTAQEFNLLNDLNNLTVPTETKKLSKEQKNQIINYVKNEKAM
GPAKLFKYIAKLLSCDVADIKGYRIDKSGKAEIHTFEAYRKMKTLETLDIEQ
MDRETLDKLAYVLTLNTEREGIQEALEHEFADGSFSQKQVDELVQFRKANS
SIFGKGWHNFSVKLMMELIPELYETSEEQMTILTRLGKQKTTSSSNKTKYID
EKLLTEEIYNPVVAKSVRQAIKIVNAAIKEYGDFDNIVIEMARENQITQKGQK
NSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDI
NRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYW
RQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSR
MNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAV
VGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFF
KTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQ
TGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSK
KLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKR
MLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHY
LDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPA
AFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD

>Sp(N)St1(C)Cas9

MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDS
GETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEED
KKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRR
LENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLD
NLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDL
TLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTE
ELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKIL
TFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFD
KNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLF
KTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLD
NEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRL
SRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQG
DSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARE**TNEDD**
EKKAIQKIQKANKDEKDAAMLKAANQYNGKAELPHSVFHGHKQLATKIRL
WHQQGERCLYTGKTISIHDLINNSNQFEVDHILPLSITFDDSLANKVLVYATA
NQEKGQRTPYQALDSMDDAWSFRELKAFVRESKTLSNKKKEYLLTEEDISK
FDVRKKFIERNLVDTRYASRVVLNALQEHFRAHKIDTKVSVVRGQFTSQLR
RHWGIEKTRDTYHHHAVDALIIAASSQLNLWKKQKNTLVSYSEDQLLDIET
GELISDDEYKESVFKAPYQHFVDTLKSKEFEDSILFSYQVDSKFNRKISDATIY
ATRQAKVGKDKADETYVLGKIKDIYTQDGYDAFMKIYKKDKSKFLMYRHD
PQTFEKVIEPILENYPNKQINEKGKEVPCNPFLKYKEEHGYIRKYSKKGNGP

EIKSLKYYDSKLGNHIDITPKDSNNKVVLQSVSPWRADVYFNKTTGKYEILG LKYADLQFEKGTGTYKISQEKYNDIKKKEGVDSDSEFKFTLYKNDLLLVKD TETKEQQLFRFLSRTMPKQKHYVELKPYDKQKFEGGEALIKVLGNVANSG QCKKGLGKSNISIYKVRTDVLGNQHIIKNEGDKPKLDF

[00281] Applicants have also generated Sp_St3 chimeric proteins and have shown *in vitro* cleavage by SpCas9, St3Cas9, Sp_St3 chimera and St3_Sp chimera (Figure 17).

[00282] The benefit of making chimeric Cas9 include:

a. reduce toxicity
b. improve expression in eukaryotic cells
c. enhance specifity
d. reduce molecular weight of protein, make protein smaller by combining the smallest domains from different Cas9 homologs.
e. Altering the PAM sequence requirement

Example 6: Cas9 delivery in vivo using AAV particles or vectors
In vivo delivery – AAV method

[00283] AAV is advantagenous 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 does not integrate into the host genome.

[00284] While certain current AAV vectors may accommodate up to 4300 bases of inserted DNA, as an upper limit or a packaging limit, AAV can have of 4.5 or 4.75 KB inserted DNA. This means that DNA encoding a Cas9 enzyme 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 4.1kb, which makes it difficult for packing into AAV. Therefore embdiments of the invention include utilizing orthologs of Cas9 that are shorter. For example:

Species	Cas9 Size
Corynebacter diphtheria	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

[00285] Figure 3 provides schematic representations of AAV vectors which may be used in methods and compositions of the invention. Packaging is discussed above.

Example 7: Engineering of Microalgae using Cas9

[00286] Methods of delivering Cas9

[00287] Method 1: Applicants deliver Cas9 and guide RNA using a vector that expresses Cas9 under the control of a constitutive promoter such as Hsp70A-Rbc S2 or Beta2-tubulin.

[00288] Method 2: Applicants deliver Cas9 and T7 polymerase using vectors that expresses Cas9 and T7 polymerase under the control of a constitutive promoter such as Hsp70A-Rbc S2 or Beta2-tubulin. Guide RNA will be delivered using a vector containing T7 promoter driving the guide RNA.

[00289] Method 3: Applicants deliver Cas9 mRNA and in vitro transcribed guide RNA to algae cells. RNA can be in vitro transcribed. Cas9 mRNA will consist of the coding region for Cas9 as well as 3'UTR from Cop1 to ensure stabilization of the Cas9 mRNA.

[00290] For Homologous recombination, Applicants provide an additional homology directed repair template.

[00291] Sequence for a cassette driving the expression of Cas9 under the control of beta-2 tubulin promoter, followed by the 3' UTR of Cop1.

TCTTTCTTGCGCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGACGGCTTC
CCGGCGCTGCATGCAACACCGATGATGCTTCGACCCCCCGAAGCTCCTTCGGGGCTG
CATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCG
ATTGCAAAGACATTATAGCGAGCTACCAAAGCCATATTCAAACACCTAGATCACTAC
CACTTCTACACAGGCCACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTT
CCTCTTCGTTTCAGTCACAACCCGCAAACATGTACCCATACGATGTTCCAGATTACG

CTTCGCCGAAGAAAAAGCGCAAGGTCGAAGCGTCCGACAAGAAGTACAGCATCGGC
CTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTGATCACCGACGAGTACAAGGT
GCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGGCACAGCATCAAGAAG
AACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAGGCCACCCGGCT
GAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGCTATCTG
CAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGACT
GGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCG
GCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCTACCACCTG
AGAAAGAAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGC
CCTGGCCCACATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCC
CGACAACAGCGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGC
TGTTCGAGGAAAACCCCATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCT
GCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGA
GAAGAAGAATGGCCTGTTCGGCAACCTGATTGCCCTGAGCCTGGGCCTGACCCCCA
ACTTCAAGAGCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGAGCAAGGAC
ACCTACGACGACGACCTGGACAACCTGCTGGCCCAGATCGGCGACCAGTACGCCGA
CCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGCTGAGCGACATCCTGAG
AGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCTATGATCAAGAGATACG
ACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGTGCGGCAGCAGCTGCCT
GAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCCGGCTACAT
TGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTGGAAA
AGATGGACGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGG
AAGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGGAGAGCT
GCACGCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGGACAACCGGG
AAAAGATCGAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCA
GGGGAAACAGCAGATTCGCCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCC
CTGGAACTTCGAGGAAGTGGTGGACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGC
GGATGACCAACTTCGATAAGAACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGC
CTGCTGTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATACGTGACC
GAGGGAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAGGCCATCGTGG
ACCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAGGACTAC

TTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATCTCCGGCGTGGAAGATCGGTTC
AACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATTATCAAGGACAAGGACTT
CCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTGCTGACCCTGACAC
TGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCCCACCTGTTC
GACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGCAGGC
TGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACAATCCTG
GATTTCCTGAAGTCCGACGGCTTCGCCAACAGAAACTTCATGCAGCTGATCCACGAC
GACAGCCTGACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGCCAGGGCGA
TAGCCTGCACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGAAGGGCA
TCCTGCAGACAGTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAG
CCCGAGAACATCGTGATCGAAATGGCCAGAGAGAACCAGACCACCCAGAAGGGAC
AGAAGAACAGCCGCGAGAGAATGAAGCGGATCGAAGAGGGCATCAAAGAGCTGGG
CAGCCAGATCCTGAAAGAACACCCCGTGGAAAACACCCAGCTGCAGAACGAGAAG
CTGTACCTGTACTACCTGCAGAATGGGCGGGATATGTACGTGGACCAGGAACTGGA
CATCAACCGGCTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAGCTTTCTGAA
GGACGACTCCATCGACAACAAGGTGCTGACCAGAAGCGACAAGAACCGGGGCAAG
AGCGACAACGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAGAACTACTGGCGGCA
GCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTGACCAAGGCCG
AGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAGCTGGTG
GAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCGGATGAACAC
TAAGTACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCCTGAAGT
CCAAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCGAGATCA
ACAACTACCACCACGCCCACGACGCCTACCTGAACGCCGTCGTGGGAACCGCCCTG
ATCAAAAAGTACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTA
CGACGTGCGGAAGATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCTACCGCC
AAGTACTTCTTCTACAGCAACATCATGAACTTTTTCAAGACCGAGATTACCCTGGCC
AACGGCGAGATCCGGAAGCGGCCTCTGATCGAGACAAACGGCGAAACCGGGGAGA
TCGTGTGGGATAAGGGCCGGGATTTTGCCACCGTGCGGAAAGTGCTGAGCATGCCC
CAAGTGAATATCGTGAAAAAGACCGAGGTGCAGACAGGCGGCTTCAGCAAAGAGTC
TATCCTGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTGGGACC
CTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGTGCTGGTGGTGG

CCAAAGTGGAAAAGGGCAAGTCCAAGAAACTGAAGAGTGTGAAAGAGCTGCTGGG
GATCACCATCATGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGACTTTCTGGAAG
CCAAGGGCTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCTAAGTACTCC
CTGTTCGAGCTGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAACTGCA
GAAGGGAAACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGGCCA
GCCACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAACAGCTGTTT
GTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGTTCTC
CAAGAGAGTGATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACAACA
AGCACCGGGATAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACC
CTGACCAATCTGGGAGCCCCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGG
AAGAGGTACACCAGCACCAAAGAGGTGCTGGACGCCACCCTGATCCACCAGAGCAT
CACCGGCCTGTACGAGACACGGATCGACCTGTCTCAGCTGGGAGGCGACAGCCCCA
AGAAGAAGAGAAAGGTGGAGGCCAGCTAAGGATCCGGCAAGACTGGCCCCGCTTG
GCAACGCAACAGTGAGCCCCTCCCTAGTGTGTTTGGGGATGTGACTATGTATTCGTG
TGTTGGCCAACGGGTCAACCCGAACAGATTGATACCCGCCTTGGCATTTCCTGTCAG
AATGTAACGTCAGTTGATGGTACT

[0100] Sequence for a cassette driving the expression of T7 polymerase under the control of beta-2 tubulin promoter, followed by the 3' UTR of Cop1:

TCTTTCTTGCGCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGACGGCTTC
CCGGCGCTGCATGCAACACCGATGATGCTTCGACCCCCCGAAGCTCCTTCGGGGCTG
CATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCG
ATTGCAAAGACATTATAGCGAGCTACCAAAGCCATATTCAAACACCTAGATCACTAC
CACTTCTACACAGGCCACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTT
CCTCTTCGTTTCAGTCACAACCCGCAAACatgcctaagaagaagaggaaggttaacacgattaacatcgctaag
aacgacttctctgacatcgaactggctgctatcccgttcaacactctggctgaccattacggtgagcgtttagctcgcgaacagttggcccttg
agcatgagtcttacgagatgggtgaagcacgcttccgcaagatgtttgagcgtcaacttaaagctggtgaggttgcggataacgctgccgcc
aagcctctcatcactaccctactccctaagatgattgcacgcatcaacgactggtttgaggaagtgaaagctaagcgcggcaagcgcccga
cagccttccagttcctgcaagaaatcaagccggaagccgtagcgtacatcaccattaagaccactctggcttgcctaaccagtgctgacaat
acaaccgttcaggctgtagcaagcgcaatcggtcgggccattgaggacgaggctcgcttcggtcgtatccgtgaccttgaagctaagcact
tcaagaaaaacgttgaggaacaactcaacaagcgcgtagggcacgtctacaagaaagcatttatgcaagttgtcgaggctgacatgctctct
aagggtctactcggtggcgaggcgtggtcttcgtggcataaggaagactctattcatgtaggagtacgctgcatcgagatgctcattgagtca

accggaatggttagcttacaccgccaaaatgctggcgtagtaggtcaagactctgagactatcgaactcgcacctgaatacgctgaggctat
cgcaacccgtgcaggtgcgctggctggcatctctccgatgttccaaccttgcgtagttcctcctaagccgtggactggcattactggtggtgg
ctattgggctaacggtcgtcgtcctctggcgctggtgcgtactcacagtaagaaagcactgatgcgctacgaagacgtttacatgcctgaggt
gtacaaagcgattaacattgcgcaaaacaccgcatggaaaatcaacaagaaagtcctagcggtcgccaacgtaatcaccaagtggaagca
ttgtccggtcgaggacatccctgcgattgagcgtgaagaactcccgatgaaaccggaagacatcgacatgaatcctgaggctctcaccgcg
tggaaacgtgctgccgctgctgtgtaccgcaaggacaaggctcgcaagtctcgccgtatcagccttgagttcatgcttgagcaagccaataa
gtttgctaaccataaggccatctggttcccttacaacatggactggcgcggtcgtgtttacgctgtgtcaatgttcaacccgcaaggtaacgat
atgaccaaaggactgcttacgctggcgaaaggtaaaccaatcggtaaggaaggttactactggctgaaaatccacggtgcaaactgtgcg
ggtgtcgacaaggttccgttccctgagcgcatcaagttcattgaggaaaaccacgagaacatcatggcttgcgctaagtctccactggagaa
cacttggtgggctgagcaagattctccgttctgcttccttgcgttctgctttgagtacgctggggtacagcaccacggcctgagctataactgct
ccctttccgctggcgtttgacgggtcttgctctggcatccagcacttctccgcgatgctccgagatgaggtaggtggtcgcgcggttaacttgc
ttcctagtgaaaccgttcaggacatctacgggattgttgctaagaaagtcaacgagattctacaagcagacgcaatcaatgggaccgataac
gaagtagttaccgtgaccgatgagaacactggtgaaatctctgagaaagtcaagctgggcactaaggcactggctggtcaatggctggctt
acggtgttactcgcagtgtgactaagcgttcagtcatgacgctggcttacgggtccaaagagttcggcttccgtcaacaagtgctggaagata
ccattcagccagctattgattccggcaagggtctgatgttcactcagccgaatcaggctgctggatacatggctaagctgatttgggaatctgt
gagcgtgacggtggtagctgcggttgaagcaatgaactggcttaagtctgctgctaagctgctggctgctgaggtcaaagataagaagact
ggagagattcttcgcaagcgttgcgctgtgcattgggtaactcctgatggtttccctgtgtggcaggaatacaagaagcctattcagacgcgc
ttgaacctgatgttcctcggtcagttccgcttacagcctaccattaacaccaacaaagatagcgagattgatgcacacaaacaggagtctggt
atcgctcctaactttgtacacagccaagacggtagccaccttcgtaagactgtagtgtgggcacacgagaagtacggaatcgaatcttttgca
ctgattcacgactccttcggtacgattccggctgacgctgcgaacctgttcaaagcagtgcgcgaaactatggttgacacatatgagtcttgtg
atgtactggctgatttctacgaccagttcgctgaccagttgcacgagtctcaattggacaaaatgccagcacttccggctaaaggtaacttgaa
cctccgtgacatcttagagtcggacttcgcgttcgcgtaaGGATCCGGCAAGACTGGCCCCGCTTGGCAACG
CAACAGTGAGCCCCTCCCTAGTGTGTTTGGGGATGTGACTATGTATTCGTGTGTTGG
CCAACGGGTCAACCCGAACAGATTGATACCCGCCTTGGCATTTCCTGTCAGAATGTA
ACGTCAGTTGATGGTACT

[00292] Sequence of guide RNA driven by the T7 promoter (T7 promoter, Ns represent targeting sequence):

gaaat**TAATACGACTCACTATA**NNNNNNNNNNNNNNNNNNgttttagagctaGAAAtagcaa
gttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttttt

[00293] Gene delivery:

[00294] Chlamydomonas reinhardtii strain CC-124 and CC-125 from the Chlamydomonas Resource Center will be used for electroporation. Electroporation protocol follows standard recommended protocol from the GeneArt Chlamydomonas Engineering kit (website information at tools.invitrogen.com/content/sfs/manuals/geneart_chlamy_kits_man.pdf).

[00295] Also, Applicants generate a line of Chlamydomonas reinhardtii that expresses Cas9 constitutively. This can be done by using pChlamy1 (linearized using PvuI) and selecting for hygromycin resistant colonies. Sequence for pChlamy1 containing Cas9 is below. In this way to achieve gene knockout one simply needs to deliver RNA for the guideRNA. For homologous recombination Applicants deliver guideRNA as well as a linearized homologous recombination template.

[00296] pChlamy1-Cas9:

TGCGGTATTTCACACCGCATCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCC
TATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGATTATCAAAA
AGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGT
ATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATC
TCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAA
CTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAC
CCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGA
GCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCG
GGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGC
TACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCC
CAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCC
TTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTT
ATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGA
CTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCT
CTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTG
CTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTG
AGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTT
TCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGG
AATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTG
AAGCATTTATCAGGGTTATTGTCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTC

CACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTT
CTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGT
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TAGTGTGTTTGGGGATGTGACTATGTATTCGTGTGTTGGCCAACGGGTCAACCCGAA
CAGATTGATACCCGCCTTGGCATTTCCTGTCAGAATGTAACGTCAGTTGATGGTACT

[00297] For all modified Chlamydomonas reinhardtii cells, Applicants use PCR, SURVEYOR nuclease assay, and DNA sequencing to verify successful modification.

Example 8: SaCas9 and PAM recognition for in vivo applications

[00298] The project started as Applicants wanted to further explore the diversity of the type II CRISPR/Cas system following the identification of *Streptococcus pyogenes* (Sp) and *Streptococcus thermophiles* (St) CRISPR/Cas system as a functional genome engineering tool in mammalian cells.

[00299] By defining new functional type II CRISPR/Cas systems for application in mammalian cells, Applicants will potentially be able to find:

[00300] (1) CRISPR/Cas system with higher efficiency and/or specificity

[00301] (2) CRISPR/Cas system with different Protospacer Adjacent Motif (PAM) that allows the targeting of broader range of genomic loci

[00302] (3) CRISPR/Cas system with smaller size so Applicants could deliver them in vivo in a single vector with mammalian viral delivery system such as adeno-associated virus (AAV) vectors that has a packaging size limit (the current Sp or St system exceed this limit of 4.7kb)

[00303] and other desirable traits.

[00304] **Identification and Design of Sa CRISPR/Cas System for in vivo application.** Applicants tested a new type II CRISPR/Cas system in *Staphylococcus aureus* (Sa) that works in vitro in dsDNA cleavage assay and identified a putative PAM of NNGRRT. The components of this system are a Cas9 protein from Sa, a guide CRISPR RNA with direct repeats (DR) from Sa that will form a functional guide RNA complex with tracrRNA from Sa. This three-component system is similar to all other type II CRISPR/Cas systems. Hence, Applicants designed a two-component system, where Applicants fused the Sa tracrRNA to the Sa guide CRISPR RNA via a short stem-loop to form a chimeric guide RNA, exactly as Applicants did with the *Streptococcus*

pyogenes (Sp) CRISPR/Cas system. This chimeric guide RNA was able to support cleavage of dsDNA in vitro. Therefore, Applicants decided to clone the full two-component system: cas9 and the chimeric guide RNA, into an AAV vector to test its functionality in living organisms.

[00305] Applicants chose the AAV system because it is a non-integrating, ssDNA-based, non-immunogenic mammalian virus that has broad-spectrum of tropism in different tissues/organs depending on the serotype that has been shown to be safe for in vivo application and also support long-term expression of transgene in living organisms.

[00306] Design of the initial AAV vector has (1) CMV promoter driving SaCas9 protein with a single NLS and a HA epitope tag. (2) human U6 promoter driving the chimeric RNA. These are placed in between two Inverted Terminal Repeats (ITRs) from the most-well studied AAV serotype 2 that serve as the viral packaging signal.

[00307] The PAM sequence test on endogenous mammalian genome is as follows: SaCas9 target spacers were selected across multiple genes to cover different potential PAM sequences. Different spacers were cloned into U6-sgRNA (single-guide RNA) expression dsDNA cassette U6-sgRNA expression dsDNA cassette were co-transfected into mammalian cells lines (293FT for human targets, N2a and Hepa for mouse targets). 72 hours following transfection, all genomic DNA were extracted and subjected to surveyor nuclease assay. Run through TBE Page Gel to detect genomic cleavage. Quantify genomic DNA cleavage efficiency and plot.

[00308] Summary of Genome Cleavage Efficiency and other Statistics on All Tested Targets

3nt Core PAM Sequence	Targets Count	Cleavaged Targets Count	Percentage of Cleaved Targets (%)	Cumulative Coverage Score (%)	Average Score (%) Cleaved (%)
GAAA	1	1	100.0	5.4	65.0
GAAC	2	2	100.0	6.1	55.0
GAAG	8	8	100.0	47.1	65.0
GAAT	9	8	88.9	138.4	66.1
GAGA	3	3	100.0	17.5	63.3
GAGC	8	8	100.0	44.2	60.0
GAGG	12	12	100.0	93.3	58.8
GAGT	44	20	45.5	434.9	56.9
GGAA	2	2	100.0	4.7	50.0
GGAC	3	2	66.7	39.9	60.0
GGAG	12	9	75.0	36.9	52.6
GGAT	20	10	50.0	196.2	52.0
GGGA	7	5	71.4	39.1	53.6
GGGC	11	9	81.8	79.3	65.5
GGGG	8	5	62.5	53.3	70.0
GGGT	45	18	40.0	402.3	58.2
Grand Total	196	120	61.2	1618.6	58.4

[00309] Summary of Genome Cleavage Efficiency and other Statistics on All Tested Targets (cleaned up)

Subset PAM Sequence	Target Count	Cleaved Target Count	Percentage of Cleaved Targets (%)	Cumulative Cleavage Efficiency (%)	Average Cleavage Efficiency (%)	Average Sanger GC Content (%)
GAAA	1	1	100.0	5.4	5.4	65.0
GAAC	2	2	100.0	6.1	3.0	55.0
GAAG	9	9	100.0	47.1	5.9	65.0
GAAT	4	4	100.0	68.4	17.1	65.0
GAGA	2	2	100.0	12.5	6.3	67.5
GAGC	5	5	100.0	39.2	7.8	61.0
GAGG	11	11	100.0	88.3	8.0	58.2
GAGT	13	10	76.9	199.0	15.3	58.2
GGAA	2	2	100.0	4.7	2.3	60.0
GGAC	3	2	66.7	39.9	13.3	60.0
GGAG	12	9	75.0	36.9	3.1	59.6
GGAT	13	9	69.2	161.2	12.4	58.8
GGGA	7	5	71.4	39.1	5.6	63.6
GGGC	11	9	81.8	79.3	8.4	65.5
GGGG	9	5	62.5	53.3	8.7	73.0
GGGT	14	8	57.1	182.8	13.9	54.6
Grand Total	116	92	79.3	1053.6	9.1	60.5

[00310] Results from the PAM test are shown in Figs. 22-27. A comprehensive test of over 100 targets identified that the PAM for SaCas9 could be described as NNGRR (but not the NNGRRT as indicated earlier).

[00311] PAM Test Summary: (1) NNGRR for general SaCas9 PAM - helpful for design new targets, (2) Testing double-nickase with new targets, (3) NNGRG may be a more potent PAM.

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[00313] 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.

CLAIMS

1. A CRISPR-Cas system chimeric RNA (chiRNA) comprising: (a) a guide sequence capable of hybridizing to a target sequence, (b) a tracr mate sequence capable of hybridizing to a tracr sequence and (c) a tracr sequence, in which the chiRNA is capable of forming a CRISPR-Cas complex with a *Staphylococcus aureus* Cas9 (SaCas9) protein and directing sequence-specific binding of the CRISPR-Cas complex to a target sequence adjacent to a protospacer adjacent motif (PAM) in a eukaryotic cell.

2. A CRISPR-Cas system chiRNA according to Claim 1, in which the PAM comprises NNGRR.

3. A CRISPR-Cas system chiRNA according to Claim 1 or 2, in which the tracr sequence is at least 30 nucleotides in length.

4. A CRISPR-Cas system chiRNA according to Claim 1, 2 or 3, in which the tracr sequence is at least 40 nucleotides in length.

5. A CRISPR-Cas system chiRNA according to any preceding claim, in which the tracr sequence is at least 50 nucleotides in length.

6. A CRISPR-Cas system chiRNA according to any preceding claim, in which the guide sequence is 10-30 nucleotides in length.

7. A CRISPR-Cas system chiRNA according to any preceding claim, in which the guide sequence is at least 20 nucleotides in length.

8. A CRISPR-Cas system chiRNA according to any preceding claim, in which the guide sequence is 20, 21, 22, 23 or 24 nucleotides in length.

9. A CRISPR-Cas system chiRNA according to Claim 1, in which the chiRNA comprises the following sequence:
NNNNNNNNNNNNNNNNNNNGUUUUAGUACUCUGGAAACAGAAUCUACUAAA
ACAAGGCAAAAUGCCGUGUUUAUCUCGUCAACUUGUUGGCGAGAUUUUUUU.

10. A vector comprising a polynucleotide sequence encoding a CRISPR-Cas system chiRNA according to any preceding claim.

11. A vector according to Claim 10, in which the vector encodes two or more CRISPR-Cas system chiRNAs.

12. A vector according to Claim 10 or 11, further comprising a polynucleotide sequence encoding a SaCas9 protein which is codon-optimized for expression in a eukaryotic cell.

13. A vector according to Claim 10, 11 or 12, which is a viral vector.

14. A vector according to Claim 13, which is an adeno-associated virus (AAV) vector.

15. A composition comprising a CRISPR-Cas system chiRNA according to any of Claims 1 to 9 and a SaCas9 protein.

16. A composition comprising a CRISPR-Cas system chiRNA according to any of Claims 1 to 9 and an mRNA encoding a SaCas9 protein.

17. A composition according to Claim 15 or 16, in which the CRISPR-Cas system chiRNA and the SaCas9 protein or mRNA is comprised in a nanoparticle, liposome, exosome or microvesicle.

18. A eukaryotic cell comprising a CRISPR-Cas system chiRNA according to any of Claims 1 to 9, a vector according to any of Claims 10 to 14 or a composition according to Claim 15, 16 or 17.