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Remarks:

- This application was filed on 09-07-2015 as a divisional application to the application mentioned under INID code 62.
- Claims filed after the date of filing of the application (Rule 68(4) EPC).

(54) CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS

(57) The invention provides for systems, methods, and compositions for altering expression of target gene sequences and related gene products. Provided are vectors and vector systems, some of which encode one or more components of a CRISPR complex, as well as

methods for the design and use of such vectors. Also provided are methods of directing CRISPR complex formation in eukaryotic cells and methods for utilizing the CRISPR-Cas system.

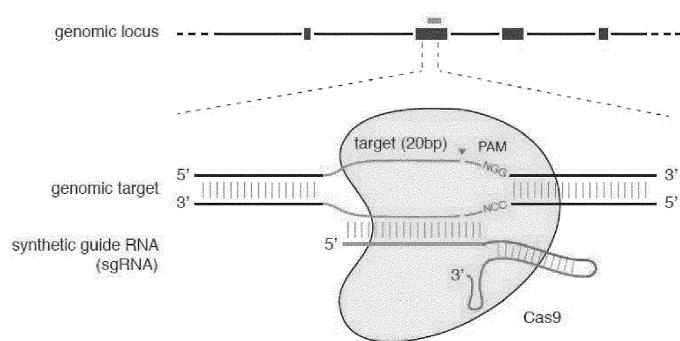


FIG. 1

Description

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

5 [0001] This application claims priority to US provisional patent application 61/842,322 and US patent application 14/054,414, each having Broad reference BI-2011/008A, entitled CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS filed on July 2, 2013 and October 15, 2013 respectively. Priority is also claimed to US provisional patent applications 61/736,527, 61/748,427, 61/791,409 and 61/835,931 having Broad reference BI-2011/008/WSGR Docket No. 44063-701.101, BI-2011/008/WSGR Docket No. 44063-701.102, Broad reference BI-2011/008/VP Docket No. 44790.02.2003 and BI-2011/008/VP Docket No. 44790.03.2003 respectively, all entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on December 12, 2012, January 2, 2013, March 15, 2013 and June 17, 2013, respectively.

10 [0002] Reference is made 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. Reference is also made to US provisional patent applications 61/835,936, 61/836,127, 61/836,101, 61/836,080 and 61/835,973 each filed June 17, 2013.

15 [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

30 [0004] The present invention generally relates to systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that may use vector systems related to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and components thereof.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

35 [0005] This invention was made with government support under NIH Pioneer Award DP1MH100706, awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

40 [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

50 [0007] There exists a pressing need for alternative and robust systems and techniques for sequence targeting with a wide array of applications. This invention addresses this need and provides related advantages. 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] In one aspect, the invention provides a method of altering or modifying expression of one or more gene products that may comprise introducing into a cell containing and expressing DNA molecules encoding the one or more gene products an engineered, non-naturally occurring CRISPR-Cas system that may comprise a Cas protein and one or more guide RNAs that target the DNA molecules, whereby the one or more guide RNAs target the genomic loci of the DNA molecules encoding the one or more gene products and the Cas protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered or modified; and, wherein the Cas protein and the guide RNA do not naturally occur together. The invention comprehends that the expression of two or more gene products may be altered or modified. The invention further comprehends that the guide RNA comprises a guide sequence fused to a tracr sequence. In a preferred embodiment the cell is a eukaryotic cell, in a more preferred embodiment the cell is a mammalian cell and in a yet more preferred embodiment the mammalian cell is a human cell. The invention also comprehends that the Cas protein may comprise one or more nuclear localization signal(s) (NLS(s)). In some embodiments, the Cas protein is a type II CRISPR system enzyme. In some embodiments, the Cas protein is a Cas9 protein. In some embodiments, the Cas9 protein is *S. pneumoniae*, *S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The protein may be a Cas9 homolog or ortholog. In some embodiments, the Cas protein is codon-optimized for expression in a eukaryotic cell. In some embodiments, the Cas protein directs cleavage of one or two strands at the location of the target sequence. In a further aspect of the invention, the expression of the gene product is decreased and the gene product is a protein. The invention comprehends that the introducing into the cell is by a delivery system that may comprise viral particles, liposomes, electroporation, microinjection or conjugation.

[0009] In another aspect the invention provides a method of altering or modifying expression of one or more gene products comprising introducing into a cell containing and expressing DNA molecules encoding the one or more gene products an engineered, non-naturally occurring vector system that may comprise one or more vectors comprising: a) a first regulatory element operably linked to one or more CRISPR-Cas system guide RNAs that hybridize with target sequences in genomic loci of the DNA molecules encoding the one or more gene products, b) a second regulatory element operably linked to a Cas protein, wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the genomic loci of the DNA molecules encoding the one or more gene products and the Cas protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered or modified; and, wherein the Cas protein and the guide RNAs do not naturally occur together. The invention comprehends that the expression of two or more gene products may be altered or modified. The invention further comprehends that the guide RNA comprises a guide sequence fused to a tracr sequence. In a preferred embodiment the cell is a eukaryotic cell, in a more preferred embodiment the cell is a mammalian cell and in a yet more preferred embodiment the mammalian cell is a human cell. The invention also comprehends that the vectors of the system may further comprise one or more NLS(s). In some embodiments, the Cas protein is a type II CRISPR system enzyme. In some embodiments, the Cas protein is a Cas9 protein. In some embodiments, the Cas9 protein is *S. pneumoniae*, *S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The protein may be a Cas9 homolog or ortholog. In some embodiments, the Cas protein is codon-optimized for expression in a eukaryotic cell. In some embodiments, the Cas protein directs cleavage of one or two strands at the location of the target sequence. In a further aspect of the invention, the expression of the gene product is decreased and the gene product is a protein. The invention comprehends that the introducing into the cell is by a delivery system that may comprise viral particles, liposomes, electroporation, microinjection or conjugation.

[0010] The invention also provides an engineered, non-naturally occurring vector system that may comprise one or more vectors comprising: a) a first regulatory element operably linked to one or more CRISPR-Cas system guide RNAs that hybridize with target sequences in genomic loci of DNA molecules encoding one or more gene products, b) a second regulatory element operably linked to a Cas protein, wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the genomic loci of the DNA molecules encoding the one or more gene products in a cell and the Cas protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered or modified; and, wherein the Cas protein and the guide RNAs do not naturally occur together. The invention comprehends that the expression of two or more gene products may be altered or modified. The invention further comprehends that the guide RNA comprises a guide sequence fused to a tracr sequence. In a preferred embodiment the cell is a eukaryotic cell, in a more preferred embodiment the cell is a mammalian cell and in a yet more preferred embodiment the mammalian cell is a human cell. The invention also comprehends that the vectors of the system may further comprise one or more NLS(s). In some embodiments, the Cas protein is a type II CRISPR system enzyme. In some embodiments, the Cas protein is a Cas9 protein. In some embodiments, the Cas9 protein is *S. pneumoniae*, *S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The protein may be a Cas9 homolog or ortholog. In some embodiments, the Cas protein is codon-optimized for expression in a eukaryotic cell. In some embodiments, the Cas protein

directs cleavage of one or two strands at the location of the target sequence. In a further aspect of the invention, the expression of the gene product is decreased and the gene product is a protein. The invention comprehends that the introducing into the cell is by a delivery system that may comprise viral particles, liposomes, electroporation, microinjection or conjugation.

5 [0011] In yet another aspect the invention provides an engineered, programmable, non-naturally occurring CRISPR-Cas system that may comprise a Cas protein and one or more guide RNAs that target the genomic loci of DNA molecules encoding one or more gene products in a cell and the Cas protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered or modified; and, wherein the Cas protein and the guide RNAs do not naturally occur together. The invention comprehends that the expression of two or more gene products may be altered or modified. The invention further comprehends that the guide RNA comprises a guide sequence fused to a tracr sequence. In a preferred embodiment the cell is a eukaryotic cell, in a more preferred embodiment the cell is a mammalian cell and in a yet more preferred embodiment the mammalian cell is a human cell. The invention also comprehends that the CRISPR-Cas system may further comprise one or more NLS(s). In some embodiments, the Cas protein is a type II CRISPR system enzyme. In some embodiments, the Cas protein is a Cas9 protein. In some embodiments, the Cas9 protein is *S. pneumoniae*, *S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The protein may be a Cas9 homolog or ortholog. In some embodiments, the Cas protein is codon-optimized for expression in a eukaryotic cell. In some embodiments, the Cas protein directs cleavage of one or two strands at the location of the target sequence. In a further aspect of the invention, the expression of the gene product is decreased and the gene product is a protein. The invention comprehends that the introducing into the cell is by a delivery system that may comprise viral particles, liposomes, electroporation, microinjection or conjugation.

10 [0012] In one aspect, the invention provides a vector system comprising one or more vectors. In some embodiments, the system comprises: (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a 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 (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence; wherein components (a) and (b) are located on the same or different vectors of the system. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the system comprises the tracr sequence under the control of a third regulatory element, such as a polymerase III promoter. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. Determining optimal alignment is within the purview of one of skill in the art. For example, there are publically and commercially available alignment algorithms and programs such as, but not limited to, ClustalW, Smith-Waterman in matlab, Bowtie, Geneious, Biopython and SeqMan. In some embodiments, the CRISPR complex comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR complex in a detectable amount in the nucleus of a eukaryotic cell. Without wishing to be bound by theory, it is believed that a nuclear localization sequence is not necessary for CRISPR complex activity in eukaryotes, but that including such sequences enhances activity of the system, especially as to targeting nucleic acid molecules in the nucleus. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae*, *S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length. In general, and throughout this specification, 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.

[0013] 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).

[0014] 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 EF1 α promoter. Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-1 (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.).

[0015] Advantageous vectors include lentiviruses and adeno-associated viruses, and types of such vectors can also be selected for targeting particular types of cells.

[0016] In one aspect, the invention provides a eukaryotic host cell comprising (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a 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/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. In some embodiments, the host cell comprises components (a) and (b). In some embodiments, component (a), component (b), or components (a) and (b) are stably integrated into a genome of the host eukaryotic cell. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the eukaryotic host cell further comprises a third regulatory element, such as a polymerase III promoter, operably linked to said tracr sequence. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments,

the CRISPR enzyme lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length. In an aspect, the invention provides a non-human eukaryotic organism; preferably a multicellular eukaryotic organism, comprising a eukaryotic host cell according to any of the described embodiments. In other aspects, the invention provides a eukaryotic organism; preferably a multicellular eukaryotic organism, comprising a eukaryotic host cell according to any of the described embodiments. The organism in some embodiments of these aspects may be an animal; for example a mammal. Also, the organism may be an arthropod such as an insect. The organism also may be a plant. Further, the organism may be a fungus.

[0017] In one aspect, the invention provides a kit comprising one or more of the components described herein. In some embodiments, the kit comprises a vector system and instructions for using the kit. In some embodiments, the vector system comprises (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a 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/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. In some embodiments, the kit comprises components (a) and (b) located on the same or different vectors of the system. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the system further comprises a third regulatory element, such as a polymerase III promoter, operably linked to said tracr sequence. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. In some embodiments, the CRISPR enzyme comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae*, *S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length.

[0018] In one aspect, the invention provides a method of modifying a target polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence. In some embodiments, the method further comprises delivering one or more vectors to said eukaryotic cell, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence. In some embodiments, said vectors are delivered to the eukaryotic cell in a subject. In some embodiments, said modifying takes place in said eukaryotic cell in a cell culture. In some embodiments, the method further comprises isolating said eukaryotic cell from a subject prior to said modifying. In some embodiments, the method further comprises returning said eukaryotic cell and/or cells derived therefrom to said subject.

[0019] 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. In some embodiments,

the method further comprises delivering one or more vectors to said eukaryotic cells, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence.

[0020] In one aspect, the invention provides a method of generating a model eukaryotic cell comprising a mutated disease gene. In some embodiments, a disease gene is any gene associated an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) introducing one or more vectors into a eukaryotic cell, wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; and (b) allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said disease gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, thereby generating a model eukaryotic cell comprising a mutated disease gene. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expression from a gene comprising the target sequence.

[0021] In one aspect, the invention provides a method for developing a biologically active agent that modulates a cell signaling event associated with a disease gene. In some embodiments, a disease gene is any gene associated an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) contacting a test compound with a model cell of any one of the described embodiments; and (b) detecting a change in a readout that is indicative of a reduction or an augmentation of a cell signaling event associated with said mutation in said disease gene, thereby developing said biologically active agent that modulates said cell signaling event associated with said disease gene.

[0022] In one aspect, the invention provides a recombinant polynucleotide comprising a guide sequence upstream of a tracr mate sequence, wherein the guide sequence when expressed directs sequence-specific binding of a CRISPR complex to a corresponding target sequence present in a eukaryotic cell. In some embodiments, the target sequence is a viral sequence present in a eukaryotic cell. In some embodiments, the target sequence is a proto-oncogene or an oncogene.

[0023] In one aspect the invention provides for a method of selecting one or more cell(s) by introducing one or more mutations in a gene in the one or more cell (s), the method comprising: introducing one or more vectors into the cell (s), wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, a tracr sequence, and an editing template; wherein the editing template comprises the one or more mutations that abolish CRISPR enzyme cleavage; allowing homologous recombination of the editing template with the target polynucleotide in the cell(s) to be selected; allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein binding of the CRISPR complex to the target polynucleotide induces cell death, thereby allowing one or more cell(s) in which one or more mutations have been introduced to be selected. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 protein. In some embodiments, the Cas9 protein is *S. pneumoniae*, *S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the enzyme directs cleavage of one or two strands at the location of the target sequence. In a preferred embodiment, the CRISPR enzyme is Cas9. In another preferred embodiment of the invention the cell to be selected may be a eukaryotic cell. Aspects of the invention allow for selection of specific cells without requiring a selection marker or a two-step process that may include a counter-selection system.

[0024] Aspects of the invention comprehend site-specific gene knockout in the endogenous genome: The present invention is advantageous over using site-specific nucleases technologies based on zinc finger and TAL effectors as it does not require elaborate design and may be used to simultaneously knockout multiple genes within the same genome. In a further aspect, the invention comprehends site-specific genome editing. The present invention is advantageous over using natural or artificial site-specific nucleases or recombinases as it may be able to introduce site-specific double strand breaks to facilitate homologous recombination at the targeted genome loci. In another aspect the invention comprehends DNA sequence-specific interference. The invention may be used to inactivate the genome of deleterious DNA-based organisms, such as microbes, viruses, or even cancerous cells, by directly introducing breaks at specific sites in the genome of these organisms. The invention provides methods and compositions for multiplexed genome engineering as the CRISPR-Cas system of the invention can be easily targeted to multiple sites in the genome through

the use of multiple sequence-specific CRISPR spacer elements or guide sequences.

[0025] Accordingly, it is an object of the invention not to 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.

[0026] 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. These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

15 BRIEF DESCRIPTION OF THE DRAWINGS

[0027] The novel features of the invention are set forth with particularity in the appended claims. A better understanding 20 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:

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

Figure 2A-F shows an exemplary CRISPR system, a possible mechanism of action, an example adaptation for expression in eukaryotic cells, and results of tests assessing nuclear localization and CRISPR activity. Figure 2C discloses SEQ ID NOS 23-24, respectively, in order of appearance. Figure 2E discloses SEQ ID NOS 25-27, respectively, in order of appearance. Figure 2F discloses SEQ ID NOS 28-32, respectively, in order of appearance.

Figure 3A-D shows results of an evaluation of SpCas9 specificity for an example target. Figure 3A discloses SEQ ID NOS 33, 26 and 34-44, respectively, in order of appearance. Figure 3C discloses SEO ID NO: 33.

Figure 4A-G shows an exemplary vector system and results for its use in directing homologous recombination in eukaryotic cells. Figure 4E discloses SEQ ID NO: 45. Figure 4F discloses SEQ ID NOS 46-47, respectively, in order of appearance. Figure 4G discloses SEQ ID NOS 48-52, respectively, in order of appearance.

Figure 5 provides a table of protospacer sequences (SEQ ID NOS 16, 15, 14, 53-58, 18, 17 and 59-63, respectively, in order of appearance) and summarizes modification efficiency results for protospacer targets designed based on exemplary *S. pyogenes*, and *S. thermophilus* CRISPR systems with corresponding PAMs against loci in human and mouse genomes. Cells were transfected with Cas9 and either pre-crRNA/tracrRNA or chimeric RNA, and analyzed 72 hours after transfection. Percent indels are calculated based on Surveyor assay results from indicated cell lines (N=3 for all protospacer targets, errors are S.E.M., N.D. indicates not detectable using the Surveyor assay, and N.T. indicates not tested in this study).

Figure 6A-C shows a comparison of different tracrRNA transcripts for Cas9-mediated gene targeting. Figure 6A discloses SEQ ID NOS 64-65, respectively, in order of appearance.

Figure 7 shows a schematic of a surveyor nuclease assay for detection of double strand break-induced micro- insertions and -deletions.

Figure 8A-B shows exemplary bicistronic expression vectors for expression of CRISPR system elements in eukaryotic cells. Figure 8A discloses SEQ ID NOS 66-68, respectively, in order of appearance. Figure 8B discloses SEQ ID NOS 69-71, respectively, in order of appearance.

Figure 9A-C shows histograms of distances between adjacent *S. pyogenes*, SF370 locus 1 PAM (NGG) (Figure 9A) and *S. thermophilus* LMD9 locus 2 PAM (NNAGAAW) (Figure 9B) in the human genome; and distances for each PAM by chromosome (Chr) (Figure 9C).

Figure 10A-D shows an exemplary CRISPR system, an example adaptation for expression in eukaryotic cells, and results of tests assessing CRISPR activity. Figure 10B discloses SEQ ID NOS 72-73, respectively, in order of appearance. Figure 10C discloses SEQ ID NO: 74.

Figure 11A-C shows exemplary manipulations of a CRISPR system for targeting of genomic loci in mammalian cells. Figure 11A discloses SEQ ID NO: 75. Figure 11B discloses SEQ ID NOS 76-78, respectively, in order of

appearance.

Figure 12A-B shows the results of a Northern blot analysis of crRNA processing in mammalian cells. Figure 12A discloses SEQ ID NO: 79.

Figure 13A-B shows an exemplary selection of protospacers in the human *PVALB* and mouse *Th* loci. Figure 13A discloses SEQ ID NO: 80. Figure 13B discloses SEQ ID NO: 81.

Figure 14 shows example protospacer and corresponding PAM sequence targets of the *S. thermophilus* CRISPR system in the human *EMX1* locus. Figure 14 discloses SEQ ID NO: 74.

Figure 15 provides a table of sequences (SEQ ID NOS 82-93, respectively, in order of appearance) for primers and probes used for Surveyor, RFLP, genomic sequencing, and Northern blot assays.

Figure 16A-C shows exemplary manipulation of a CRISPR system with chimeric RNAs and results of SURVEYOR assays for system activity in eukaryotic cells. Figure 16A discloses SEQ ID NO: 94.

Figure 17A-B shows a graphical representation of the results of SURVEYOR assays for CRISPR system activity in eukaryotic cells.

Figure 18 shows an exemplary visualization of some *S. pyogenes*, Cas9 target sites in the human genome using the UCSC genome browser. Figure 18 discloses SEQ ID NOS 95-173, respectively, in order of appearance.

Figure 19A-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).

Figure 20A-F shows the linear 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).

Figure 21A-D shows genome editing via homologous recombination. (a) Schematic of SpCas9 nickase, with D10A mutation in the RuvC I catalytic domain. (b) Schematic representing homologous recombination (HR) at the human *EMX1* locus using either sense or antisense single stranded oligonucleotides as repair templates. Red arrow above indicates sgRNA cleavage site; PCR primers for genotyping (Tables J and K) are indicated as arrows in right panel. Figure 21C discloses SEQ ID NOS 174-176, 174, 177 and 176, respectively, in order of appearance. (c) Sequence of region modified by HR. d, SURVEYOR assay for wildtype (wt) and nickase (D10A) SpCas9-mediated indels at the *EMX1* target 1 locus (n=3). Arrows indicate positions of expected fragment sizes.

Figure 22A-B shows single vector designs for SpCas9. Figure 22A discloses SEQ ID NOS 178-180, respectively, in order of appearance. Figure 22B discloses SEQ ID NO: 181.

[0028] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[0029] 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. 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.

[0030] 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 targets 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)". An exemplary CRISPR-Cas system is illustrated in Figure 1.

[0031] 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.

[0032] 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.

[0033] 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.

[0034] "Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick 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.

[0035] 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.

[0036] "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.

[0037] As used herein, "expression" 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.

[0038] 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.

[0039] The terms "subject," "individual," and "patient" are used interchangeably herein to refer to a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets. Tissues, cells and their progeny of a biological entity obtained *in vivo* or cultured *in vitro* are also encompassed.

[0040] The terms "therapeutic agent", "therapeutic capable agent" or "treatment agent" are used interchangeably and refer to a molecule or compound that confers some beneficial effect upon administration to a subject. The beneficial effect includes enablement of diagnostic determinations; amelioration of a disease, symptom, disorder, or pathological condition; reducing or preventing the onset of a disease, symptom, disorder or condition; and generally counteracting a disease, symptom, disorder or pathological condition.

[0041] As used herein, "treatment" or "treating," or "palliating" or "ameliorating" are used interchangeably. These terms refer to an approach for obtaining beneficial or desired results including but not limited to a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant any therapeutically relevant improvement in or effect on one or more diseases, conditions, or symptoms under treatment. For prophylactic benefit, the compositions may be administered to a subject at risk of developing a particular disease, condition, or symptom, or to a subject reporting one or more of the physiological symptoms of a disease, even though the disease, condition, or symptom may not have yet been manifested.

[0042] The term "effective amount" or "therapeutically effective amount" refers to the amount of an agent that is sufficient to effect beneficial or desired results. The therapeutically effective amount may vary depending upon one or more of: the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The term also applies to a dose that will provide an image for detection by any one of the imaging methods described herein. The specific dose may vary depending on one or more of: the particular agent chosen, the dosing regimen to be followed, whether it is administered in combination with other compounds, timing of administration, the tissue to be

imaged, and the physical delivery system in which it is carried.

[0043] 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)).

[0044] Several aspects of the invention relate to vector systems comprising one or more vectors, or vectors as such. 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.

[0045] Vectors may be introduced and propagated in a prokaryote. 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.

[0046] 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).

[0047] In some embodiments, a vector is a yeast expression vector. Examples of vectors for expression in yeast *Saccharomyces cerevisiae* include pYEPSec1 (Baldari, et al., 1987. EMBO J. 6: 229-234), pMFa (Kuijana 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.).

[0048] 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).

[0049] 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.

[0050] 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).

[0051] 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*, *Methanosa-rcina*, *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*.

[0052] 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. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell. In some embodiments, the target sequence may be within an organelle of a eukaryotic cell, for example, mitochondrion or chloroplast. A sequence or template that may be used for recombination into the targeted locus comprising the target sequences is referred to as an "editing template" or "editing polynucleotide" or "editing sequence". In aspects of the invention, an exogenous template polynucleotide may be referred to as an editing template. In an aspect of the invention the recombination is homologous recombination.

[0053] 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, the tracr sequence has sufficient complementarity to a tracr mate sequence to hybridize and participate in formation of a CRISPR complex. As with the target sequence, it is believed that complete complementarity is not needed, provided there is sufficient to be functional. In some embodiments, the tracr sequence has at least 50%, 60%, 70%, 80%, 90%, 95% or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. 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. Single vector constructs for SpCas9 are illustrated in Figure 22.

[0054] 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.

[0055] 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, homologs thereof, or modified versions thereof. These enzymes are known; for example, the amino acid sequence of *S. pyogenes*, Cas9 protein may be found in the SwissProt database under accession number Q99ZW2. In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments the CRISPR enzyme is Cas9, and may be Cas9 from *S. pyogenes*, or *S. pneumoniae*. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, a vector encodes a CRISPR enzyme that is mutated to with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from *S. pyogenes* converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). Other examples of mutations that render Cas9 a nickase include, without limitation, H840A, N854A, and N863A. In aspects of the invention, nickases may be used for genome editing via homologous recombination. For example, Figure 21 shows genome editing via homologous recombination. Figure 21 (a) shows the schematic of SpCas9 nickase, with D10A mutation in the RuvC I catalytic domain. (b) Schematic representing homologous recombination (HR) at the human EMX1 locus using either sense or antisense single stranded oligonucleotides as repair templates. (c) Sequence of region modified by HR. d, SURVEYOR assay for wildtype (wt) and nickase (D10A) SpCas9-mediated indels at the EMX1 target 1 locus (n=3). Arrows indicate positions of expected fragment sizes.

[0056] In some embodiments, a Cas9 nickase may be used in combination with guide sequence(s), e.g., two guide sequences, which target respectively sense and antisense strands of the DNA target. This combination allows both strands to be nicked and used to induce NHEJ. Applicants have demonstrated (data not shown) the efficacy of two nickase targets (i.e., sgRNAs targeted at the same location but to different strands of DNA) in inducing mutagenic NHEJ. A single nickase (Cas9-D10A with a single sgRNA) is unable to induce NHEJ and create indels but Applicants have shown that double nickase (Cas9-D10A and two sgRNAs targeted to different strands at the same location) can do so in human embryonic stem cells (hESCs). The efficiency is about 50% of nuclease (i.e., regular Cas9 without D10 mutation)

in hESCs.

[0057] As a further example, two or more catalytic domains of Cas9 (RuvC I, RuvC II, and RuvC III) may be mutated to produce a mutated Cas9 substantially lacking all DNA cleavage activity. In some embodiments, a D10A mutation is combined with one or more of H840A, N854A, or N863A mutations to produce a Cas9 enzyme substantially lacking all

5 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. Other mutations may be useful; where the Cas9 or other CRISPR enzyme is from a species other than *S. pyogenes*, mutations in corresponding amino acids may be made to achieve similar effects.

[0058] In some embodiments, an enzyme coding sequence encoding a CRISPR enzyme is codon optimized for 10 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 primate. 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

15 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,

20 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", 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.

25 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.

[0059] In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target 30 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, ELAND (Illumina, San Diego, CA), SOAP

35 (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,

40 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

45 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.

[0060] A guide sequence may be selected to target any target sequence. In some embodiments, the target sequence 50 is a sequence within a genome of a cell. Exemplary target sequences include those that are unique in the target genome.

For example, for the *S. pyogenes*, Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMNNNNNNNNNNXGG where NNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes*, Cas9 target site of the form MMMMMMMNNNNNNNNNNXGG where NNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. For the *S. thermophilus* CRISPR1 Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMNNNNNNNNNNXXAGAAW (SEQ ID NO: 1) where NNNNNNNNNNNXXAGAAW (SEQ ID NO: 2) (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. thermophilus* CRISPR1 Cas9 target site of the form MMMMMMMNNNNNNNNNNXXAGAAW (SEQ ID NO: 3) where

NNNNNNNNNNXXAGAAW (SEQ ID NO: 4) (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. For the *S. pyogenes*, Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMNNNNNNNNNNXGGXG where NNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes*, Cas9 target site of the form MMMMMMMNNNNNNNNNNXGGXG where NNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. In each of these sequences "M" may be A, G, T, or C, and need not be considered in identifying a sequence as unique.

[0061] In some embodiments, a guide sequence is selected to reduce the degree of secondary structure within the guide sequence. Secondary structure 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). Further algorithms may be found in U.S. application Serial No. 61/836,080 (attorney docket 44790.11.2022; Broad Reference BI-2013/004A); incorporated herein by reference.

[0062] 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. Example illustrations of optimal alignment between a tracr sequence and a tracr mate sequence are provided in Figures 10B and 11B. 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. Preferred loop forming sequences for use in hairpin structures are four nucleotides in length, and most preferably have the sequence GAAA. However, longer or shorter loop sequences may be used, as may alternative sequences. The sequences preferably include a nucleotide triplet (for example, AAA), and an additional nucleotide (for example C or G). Examples of loop forming sequences include CAAA and AAAG. In 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 some embodiments, the single transcript further includes a transcription termination sequence; preferably this is a polyT sequence, for example six T nucleotides. An example illustration of such a hairpin structure is provided in the lower portion of Figure 11B, where 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) NNNNNNNNNNNNNNNNNNNNgttttgtactctcaagatttaGAAAtaaatctgcagaagctacaaaagataaggctt catggcggaaatcaacaccctgtcatttatggcagggtttcggttatttaaTTTTTT (SEQ ID NO: 5); (2) NNNNNNNNNNNNNNNNNNNNgttttgtactctcaGAAAtgcagaagctacaaaagataaggcttcatggcggaaatca acaccctgtcatttatggcagggtttcggttatttaaTTTTTT (SEQ ID NO: 6); (3) NNNNNNNNNNNNNNNNNNNNgttttgtactctcaGAAAtgcagaagctacaaaagataaggcttcatggcggaaatca acaccctgtcatttatggcagggtttcggttatttaaTTTTTT (SEQ ID NO: 7); (4) NNNNNNNNNNNNNNNNNNgttttagagctaGAAAtagcaagttaaaataaggctgtccgttatcaactgaaaa agtggccaccgagtcggcTTTTTT (SEQ ID NO: 8); (5) NNNNNNNNNNNNNNNNNNgttttagagctaGAAATAGcaagttaaaataaggctgtccgttatcaactgaaatggcTTTTTT (SEQ ID NO: 9); and (6) NNNNNNNNNNNNNNNNNNgttttagagctaGAAATAGcaagttaaaataaggctgtccgttatcaTTTTTT (SEQ ID NO: 10). 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 (such as illustrated in the top portion of Figure 11B).

[0063] 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.

[0064] In an aspect of the invention, a reporter gene which includes but is 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), may be introduced into a cell to encode a gene product which serves as a marker by which to measure the alteration or modification of expression of the gene product. In a further embodiment of the invention, the DNA molecule encoding the gene product may be introduced into the cell via a vector. In a preferred embodiment of the invention the gene product is luciferase. In a further embodiment of the invention the expression of the gene product is decreased.

[0065] 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 more proteins transcribed therefrom, to a host cell. The invention serves as a basic platform for enabling targeted modification of DNA-based genomes. It can interface with many delivery systems, including but not limited to viral, liposome, electroporation, microinjection and conjugation. In some aspects, the invention further provides cells produced by such methods, and organisms (such as animals, plants, or fungi) 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 Bohm (eds) (1995); and Yu et al., *Gene Therapy* 1:13-26 (1994).

[0066] Methods of non-viral delivery of nucleic acids include lipofection, nucleofection, 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).

[0067] 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).

[0068] 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.

[0069] The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher et al., *J. Virol.* 66:2731-2739 (1992); Johann et al., *J. Virol.* 66:1635-1640 (1992); Sommnerfelt et al., *Virol.* 176:58-59 (1990); Wilson et al., *J. Virol.* 63:2374-2378 (1989); Miller et al., *J. Virol.* 65:2220-2224 (1991); PCT/US94/05700). In applications where transient expression is preferred, adenoviral based systems may be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors may also be used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures (see, e.g., West et al., *Virology* 160:38-47 (1987); U.S. Pat. No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin et al., *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, et al., *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *PNAS* 81:6466-6470 (1984); and Samulski et al., *J. Virol.* 63:03822-3828 (1989).

[0070] 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 producing a cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the polynucleotide(s) to be expressed. The missing viral functions are typically supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences. The cell line may also be infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV. 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.

[0071] 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.

[0072] Aspects of the invention relate to the generation of isogenic lines of mammalian cells for the study of genetic variations in disease. A further aspect of the invention relates to the generation of genetically-modified animal models, either transgenic or viral-mediated delivery. The invention also comprehends genome modification of microbes, cells, plants, animals or synthetic organisms for the generation of biomedically, agriculturally, and industrially useful products. In yet another aspect, the invention comprehends gene therapy. The invention may be used as a biological research tool, for understanding the genome, e.g. gene knockout studies. The invention relates to many other methods and compositions that depend on the basic ability of editing and rewriting the DNA content of genomes, as well as targeted inactivation of DNA-based organisms. The invention also may be used as therapeutic for targeting specific strains of bacterial infections, viral infection, etc.

[0073] 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. In certain embodiments, the organism or subject is a plant. In certain embodiments, the organism or subject or plant is algae. 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. Transgenic animals are also provided, as are transgenic plants, especially crops and algae. The transgenic animal or plant may be useful in applications outside of providing a disease model. These may include food or feed production through expression of, for instance, higher protein, carbohydrate, nutrient or vitamins levels than would normally be seen in the wildtype. In this regard, transgenic plants, especially pulses and tubers, and animals, especially mammals such as livestock (cows, sheep, goats and pigs), but also poultry and edible insects, are preferred.

[0074] Transgenic algae or other plants such as rape may be particularly useful in the production of vegetable oils or biofuels such as alcohols (especially methanol and ethanol), for instance. These may be engineered to express or overexpress high levels of oil or alcohols for use in the oil or biofuel industries.

[0075] 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).

[0076] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell. 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.

[0077] 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.

[0078] 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.

[0079] In plants, pathogens are often host-specific. For example, *Fusarium oxysporum* f. sp. *lycopersici* causes tomato wilt but attacks only tomato, and *F. oxysporum* f. *dianthii* *Puccinia graminis* f. sp. *tritici* attacks only wheat. Plants have existing and induced defenses to resist most pathogens. Mutations and recombination events across plant generations lead to genetic variability that gives rise to susceptibility, especially as pathogens reproduce with more frequency than plants. In plants there can be non-host resistance, e.g., the host and pathogen are incompatible. There can also be Horizontal Resistance, e.g., partial resistance against all races of a pathogen, typically controlled by many genes and Vertical Resistance, e.g., complete resistance to some races of a pathogen but not to other races, typically controlled by a few genes. In a Gene-for-Gene level, plants and pathogens evolve together, and the genetic changes in one balance changes in other. Accordingly, using Natural Variability, breeders combine most useful genes for Yield, Quality, Uniformity, Hardiness, Resistance. The sources of resistance genes include native or foreign Varieties, Heirloom Varieties, Wild

Plant Relatives, and Induced Mutations, e.g., treating plant material with mutagenic agents. Using the present invention, plant breeders are provided with a new tool to induce mutations. Accordingly, one skilled in the art can analyze the genome of sources of resistance genes, and in Varieties having desired characteristics or traits employ the present invention to induce the rise of resistance genes, with more precision than previous mutagenic agents and hence accelerate and improve plant breeding programs.

[0080] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. In some embodiments, the kit comprises a vector system and instructions for using the kit. In some embodiments, the vector system comprises (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting a guide sequence upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a 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/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. Elements may be provided individually or in combinations, and may be provided in any suitable container, such as a vial, a bottle, or a tube. In some embodiments, the kit includes instructions in one or more languages, for example in more than one language.

[0081] 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.

[0082] 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.

[0083] The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Without wishing to be bound by theory, it is believed that the target sequence should be associated with a PAM (protospacer adjacent motif); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence) Examples of PAM sequences are given in the examples section below, and the skilled person will be able to identify further PAM sequences for use with a given CRISPR enzyme.

[0084] 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. 44063-701.101 and 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.

[0085] 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.

[0086] 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.

[0087] 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.

[0088] 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 December 12, 2012 and 61/748,427 filed 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 Degeneration	Abcr; Ccl2; Cc2; cp (ceruloplasmin); Timp3; cathepsinD; 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 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 (Crb-BP - global instability); VLDLR (Alzheimer's); Atxn7; Atxn10
Fragile X Syndrome	FMR2; FXR1; FXR2; mGLUR5
Secretase Related Disorders	APH-1 (alpha and beta); Presenilin (Psen1); nicastrin (Ncstn); PEN-2
Others	Nos1; Parp1; Nat1; Nat2
Prion - related disorders	Prp
ALS	SOD1; ALS2; STEX; FUS; TARDBP; VEGF (VEGF-a;

(continued)

DISEASE/DISORDERS	GENE(S)
	VEGF-b; VEGF-c)
Drug addiction	Prkce (alcohol); Drd2; Drd4; ABAT (alcohol); GRIA2;
	Grm5; Grin1; Htrlb; 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	α-Synuclein; DJ-1; LRRK2; Parkin; PINK1

Table B:

5	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); Leukocyte 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).
10	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).
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5	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).
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15	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).
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30	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, DMA1, SCG3, SGCA, ADL, DAG2, LGMD2D, DMA2, 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).
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5	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 (Friedreich's Ataxia), ATX3 (Machado- Joseph's Dx), ATXN1 and ATXN2 (spinocerebellar ataxias), DMPK (myotonic dystrophy), Atrophin-1 and Atn1 (DRPLA Dx), CBP (Crb-BP - global instability), VLDLR (Alzheimer's), Atxn7, Atxn10).
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25	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, TGFB1, 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).
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40	Epilepsy, myoclonic, Lafora type, 254780	EPM2A, MELF, EPM2
45	Epilepsy, myoclonic, Lafora type, 254780	NHLRC1, EPM2A, EPM2B
50	Duchenne muscular dystrophy, 310200 (3)	DMD, BMD
55	AIDS, delayed/rapid progression to (3)	KIR3DL1, NKAT3, NKB1, AMB11,
		KIR3DS1

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5	AIDS, rapid progression to, 609423 (3)	IFNG
	AIDS, resistance to (3)	CXCL12, SDF1
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15	Alpha 1-Antitrypsin Deficiency	SERPINA1 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1]; SERPINA2 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 2]; SERPINA3 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3]; SERPINA5 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5]; SERPINA6 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6]; SERPINA7 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7];" AND "SERPINA6 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6)

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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; PXX; RAF1; FYN; DYRK1A; ITGB1; MAP2K2; PAK4; PIK3R1; STAT3; PPP2R5C; MAP2K1; PAK3; ITGB3; ESR1; ITGA2; MYC; TTK; CSNK1A1; CRKL; BRAF; ATF4; PRKCA; SRF; STAT1; SGK
Glucocorticoid Receptor	RAC1; TAF4B; EP300; SMAD2; TRAF6; PCAF; ELK1;

(continued)

CELLULAR FUNCTION	GENES
5	Signaling 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
10	Axonal Guidance Signaling PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; ADAM 12; 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; ADAM 17; AKT1; PIK3R1; GLI1; WNT5A; ADAM10; MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2; EPHA8; CRKL; RND1; GSK3B; AKT3; PRKCA
15	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
20	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;
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(continued)

CELLULAR FUNCTION	GENES
	PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; ITGB7;
	PPP1CC; PNX; VIL2; RAF1; GSN; DYRK1A; ITGB1;
	MAP2K2; PAK4; PIP5K1A; PIK3R1; MAP2K1; PAK3;
	ITGB3; CDC42; APC; ITGA2; TTK; CSNK1A1; CRKL;
5	BRAF; VAV3; SGK
Huntington's Disease	PRKCE; IGF1; EP300; RCOR1; PRKCZ; HDAC4; TGM2;
	MAPK1; CAPNS1; AKT2; EGFR; NCOR2; SP1; CAPN2;
10	PIK3CA; HDAC5; CREB1; PRKCI; HSPA5; REST;
	GNAQ; PIK3CB; PIK3C3; MAPK8; IGF1R; PRKD1;
15	GNB2L1; BCL2L1; CAPN1; MAPK3; CASP8; HDAC2;
	HDAC7A; PRKCD; HDAC11; MAPK9; HDAC9; PIK3C2A;
20	HDAC3; TP53; CASP9; CREBBP; AKT1; PIK3R1;
	PDPK1; CASP1; APAF1; FRAP1; CASP2; JUN; BAX;
	ATF4; AKT3; PRKCA; CLTC; SGK; HDAC6; CASP3
25	Apoptosis Signaling
	PRKCE; ROCK1; BID; IRAK1; PRKAA2; EIF2AK2; BAK1;
	BIRC4; GRK6; MAPK1; CAPNS1; PLK1; AKT2; IKBKB;
	CAPN2; CDK8; FAS; NFKB2; BCL2; MAP3K14; MAPK8;
30	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;
35	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;
40	MAP3K14; PIK3CB; PIK3C3; MAPK8; BCL2L1; ABL1;
	MAPK3; ETS1; KRAS; MAPK13; RELA; PTPN6; MAPK9;
	EGR1; PIK3C2A; BTK; MAPK14; RAF1; IKBKG; RELB;
45	MAP3K7; MAP2K2; AKT1; PIK3R1; CHUK; MAP2K1;
	NFKB1; CDC42; GSK3A; FRAP1; BCL6; BCL10; JUN;
	GSK3B; ATF4; AKT3; VAV3; RPS6KB1
Leukocyte Extravasation	ACTN4; CD44; PRKCE; ITGAM; ROCK1; CXCR4; CYBA;
50	Signalng
	RAC1; RAP1A; PRKCZ; ROCK2; RAC2; PTPN11;
	MMP14; PIK3CA; PRKCI; PTK2; PIK3CB; CXCL12;
	PIK3C3; MAPK8; PRKD1; ABL1; MAPK10; CYBB;
55	MAPK13; RHOA; PRKCD; MAPK9; SRC; PIK3C2A; BTK;
	MAPK14; NOX1; PNX; VIL2; VASP; ITGB1; MAP2K2;
	CTNND1; PIK3R1; CTNNB1; CLDN1; CDC42; F11R; ITK;

(continued)

CELLULAR FUNCTION	GENES
	CRKL; VAV3; CTTN; PRKCA; MMP1; MMP9
5 Integrin Signaling	ACTN4; ITGAM; ROCK1; ITGA5; RAC1; PTEN; RAP1A;
	TLN1; ARHGEF7; MAPK1; RAC2; CAPNS1; AKT2;
10	CAPN2; PIK3CA; PTK2; PIK3CB; PIK3C3; MAPK8;
	CAV1; CAPN1; ABL1; MAPK3; ITGA1; KRAS; RHOA;
	SRC; PIK3C2A; ITGB7; PPP1CC; ILK; PXN; VASP;
15	RAF1; FYN; ITGB1; MAP2K2; PAK4; AKT1; PIK3R1;
	TNK2; MAP2K1; PAK3; ITGB3; CDC42; RND3; ITGA2;
	CRKL; BRAF; GSK3B; AKT3
20 Acute Phase Response	IRAK1; SOD2; MYD88; TRAF6; ELK1; MAPK1; PTPN11;
Signalizing	AKT2; IKBKB; PIK3CA; FOS; NFKB2; MAP3K14;
25	PIK3CB; MAPK8; RIPK1; MAPK3; IL6ST; KRAS;
	MAPK13; IL6R; RELA; SOCS1; MAPK9; FTL; NR3C1;
	TRAF2; SERPINE1; MAPK14; TNF; RAF1; PDK1;
30	IKBKG; RELB; MAP3K7; MAP2K2; AKT1; JAK2; PIK3R1;
	CHUK; STAT3; MAP2K1; NFKB1; FRAP1; CEBPB; JUN;
	AKT3; IL1R1; IL6
35 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;
40	RAF1; IKBKG; CASP9; CDKN1A; ITGB1; MAP2K2;
	AKT1; PIK3R1; CHUK; PDGFRA; PDPK1; MAP2K1;
	NFKB1; ITGB3; CDC42; CCND1; GSK3A; ITGA2;
	GSK3B; AKT3; FOXO1; CASP3; RPS6KB1
45 p53 Signaling	PTEN; EP300; BBC3; PCAF; FASN; BRCA1; GADD45A;
	BIRC5; AKT2; PIK3CA; CHEK1; TP53INP1; BCL2;
	PIK3CB; PIK3C3; MAPK8; THBS1; ATR; BCL2L1; E2F1;
50	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;
55	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;

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CELLULAR FUNCTION	GENES
	CDKN1A; NCOA2; APAF1; NFKB1; CCND1; ATM; ESR1;
	CDKN2A; MYC; JUN; ESR2; BAX; IL6; CYP1B1;
	HSP90AA1
Xenobiotic Metabolism	PRKCE; EP300; PRKCZ; RXRA; MAPK1; NQO1;
10 Signaling	NCOR2; PIK3CA; ARNT; PRKCI; NFKB2; CAMK2A;
	PIK3CB; PPP2R1A; PIK3C3; MAPK8; PRKD1;
	ALDH1A1; MAPK3; NRIP1; KRAS; MAPK13; PRKCD;
15	GSTP1; MAPK9; NOS2A; ABCB1; AHR; PPP2CA; FTL;
	NFE2L2; PIK3C2A; PPARGC1A; MAPK14; TNF; RAF1;
	CREBBP; MAP2K2; PIK3R1; PPP2R5C; MAP2K1;
	NFKB1; KEAP1; PRKCA; EIF2AK3; IL6; CYP1B1;
20	HSP90AA1
SAPK/JNK Signaling	PRKCE; IRAK1; PRKAA2; EIF2AK2; RAC1; ELK1;
	GRK6; MAPK1; GADD45A; RAC2; PLK1; AKT2; PIK3CA;
25	FADD; CDK8; PIK3CB; PIK3C3; MAPK8; RIPK1;
	GNB2L1; IRS1; MAPK3; MAPK10; DAXX; KRAS;
	PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A;
30	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;
35	RXRA; MAPK1; SMAD3; GNAS; IKBKB; NCOR2;
	ABCA1; GNAQ; NFKB2; MAP3K14; STAT5B; MAPK8;
	IRS1; MAPK3; KRAS; RELA; PRKAA1; PPARGC1A;
40	NCOA3; MAPK14; INSR; RAF1; IKBKG; RELB; MAP3K7;
	CREBBP; MAP2K2; JAK2; CHUK; MAP2K1; NFKB1;
	TGFBR1; SMAD4; JUN; IL1R1; PRKCA; IL6; HSP90AA1;
	ADIPOQ
45 NF-KB Signaling	IRAK1; EIF2AK2; EP300; INS; MYD88; PRKCZ; TRAF6;
	TBK1; AKT2; EGFR; IKBKB; PIK3CA; BTRC; NFKB2;
	MAP3K14; PIK3CB; PIK3C3; MAPK8; RIPK1; HDAC2;
50	KRAS; RELA; PIK3C2A; TRAF2; TLR4; PDGFRB; TNF;
	INSR; LCK; IKBKG; RELB; MAP3K7; CREBBP; AKT1;
	PIK3R1; CHUK; PDGFRA; NFKB1; TLR2; BCL10;
	GSK3B; AKT3; TNFAIP3; IL1R1
55 Neuregulin Signaling	ERBB4; PRKCE; ITGAM; ITGA5; PTEN; PRKCZ; ELK1;
	MAPK1; PTPN11; AKT2; EGFR; ERBB2; PRKCI;
	CDKN1B; STAT5B; PRKD1; MAPK3; ITGA1; KRAS;

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CELLULAR FUNCTION	GENES
	PRKCD; STAT5A; SRC; ITGB7; RAF1; ITGB1; MAP2K2;
	ADAM17; AKT1; PIK3R1; PDPK1; MAP2K1; ITGB3;
	EREG; FRAP1; PSEN1; ITGA2; MYC; NRG1; CRKL;
	AKT3; PRKCA; HSP90AA1; RPS6KB1
5 Wnt & Beta catenin	CD44; EP300; LRP6; DVL3; CSNK1E; GJA1; SMO;
10 Signaling	AKT2; PIN1; CDH1; BTRC; GNAQ; MARK2; PPP2R1A;
	WNT11; SRC; DKK1; PPP2CA; SOX6; SFRP2; ILK;
15	LEF1; SOX9; TP53; MAP3K7; CREBBP; TCF7L2; AKT1;
	PPP2R5C; WNT5A; LRP5; CTNNB1; TGFBR1; CCND1;
	GSK3A; DVL1; APC; CDKN2A; MYC; CSNK1A1; GSK3B;
	AKT3; SOX2
20 Insulin Receptor Signaling	PTEN; INS; EIF4E; PTPN1; PRKCZ; MAPK1; TSC1;
	PTPN11; AKT2; CBL; PIK3CA; PRKCI; PIK3CB; PIK3C3;
	MAPK8; IRS1; MAPK3; TSC2; KRAS; EIF4EBP1;
25	SLC2A4; PIK3C2A; PPP1CC; INSR; RAF1; FYN;
	MAP2K2; JAK1; AKT1; JAK2; PIK3R1; PDPK1; MAP2K1;
	GSK3A; FRAP1; CRKL; GSK3B; AKT3; FOXO1; SGK;
	RPS6KB1
30 IL-6 Signaling	HSPB1; TRAF6; MAPKAPK2; ELK1; MAPK1; PTPN11;
	IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK3;
	MAPK10; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1;
35	MAPK9; ABCB1; TRAF2; MAPK14; TNF; RAF1; IKBKG;
	RELB; MAP3K7; MAP2K2; IL8; JAK2; CHUK; STAT3;
	MAP2K1; NFKB1; CEBPB; JUN; IL1R1; SRF; IL6
40 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;
45	CHUK; NR1H2; TJP2; NFKB1; ESR1; SREBF1; FGFR4;
	JUN; IL1R1; PRKCA; IL6
IGF-1 Signaling	IGF1; PRKCZ; ELK1; MAPK1; PTPN11; NEDD4; AKT2;
50	PIK3CA; PRKCI; PTK2; FOS; PIK3CB; PIK3C3; MAPK8;
	IGF1R; IRS1; MAPK3; IGFBP7; KRAS; PIK3C2A;
	YWHAZ; PNX; RAF1; CASP9; MAP2K2; AKT1; PIK3R1;
55	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;

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CELLULAR FUNCTION	GENES
	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
5 Hepatic Fibrosis/Hepatic	EDN1; IGF1; KDR; FLT1; SMAD2; FGFR1; MET; PGF;
10 Stellate Cell Activation	SMAD3; EGFR; FAS; CSF1; NFKB2; BCL2; MYH9;
	IGF1R; IL6R; RELA; TLR4; PDGFRB; TNF; RELB; IL8;
15	PDGFRA; NFKB1; TGFBR1; SMAD4; VEGFA; BAX;
	IL1R1; CCL2; HGF; MMP1; STAT1; IL6; CTGF; MMP9
20 PPAR Signaling	EP300; INS; TRAF6; PPARA; RXRA; MAPK1; IKBKB;
	NCOR2; FOS; NFKB2; MAP3K14; STAT5B; MAPK3;
25	NRIP1; KRAS; PPARG; RELA; STAT5A; TRAF2;
	PPARGC1A; PDGFRB; TNF; INSR; RAF1; IKBKG;
	RELB; MAP3K7; CREBBP; MAP2K2; CHUK; PDGFRA;
	MAP2K1; NFKB1; JUN; IL1R1; HSP90AA1
30 Fc Epsilon RI Signaling	PRKCE; RAC1; PRKCZ; LYN; MAPK1; RAC2; PTPN11;
	AKT2; PIK3CA; SYK; PRKCI; PIK3CB; PIK3C3; MAPK8;
35	PRKD1; MAPK3; MAPK10; KRAS; MAPK13; PRKCD;
	MAPK9; PIK3C2A; BTK; MAPK14; TNF; RAF1; FYN;
	MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; AKT3;
	VAV3; PRKCA
40 G-Protein Coupled	PRKCE; RAP1A; RGS16; MAPK1; GNAS; AKT2; IKBKB;
45 Receptor Signaling	PIK3CA; CREB1; GNAQ; NFKB2; CAMK2A; PIK3CB;
	PIK3C3; MAPK3; KRAS; RELA; SRC; PIK3C2A; RAF1;
	IKBKG; RELB; FYN; MAP2K2; AKT1; PIK3R1; CHUK;
50	PDPK1; STAT3; MAP2K1; NFKB1; BRAF; ATF4; AKT3;
	PRKCA
Inositol Phosphate	PRKCE; IRAK1; PRKAA2; EIF2AK2; PTEN; GRK6;
55 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;
55	PIK3R1; PDGFRA; STAT3; SPHK1; MAP2K1; MYC;
	JUN; CRKL; PRKCA; SRF; STAT1; SPHK2
VEGF Signaling	ACTN4; ROCK1; KDR; FLT1; ROCK2; MAPK1; PGF;

(continued)

CELLULAR FUNCTION	GENES
5	AKT2; PIK3CA; ARNT; PTK2; BCL2; PIK3CB; PIK3C3;
	BCL2L1; MAPK3; KRAS; HIF1A; NOS3; PIK3C2A; PNX;
	RAF1; MAP2K2; ELAVL1; AKT1; PIK3R1; MAP2K1; SFN;
	VEGFA; AKT3; FOXO1; PRKCA
10	Natural Killer Cell Signaling
	PRKCE; RAC1; PRKCZ; MAPK1; RAC2; PTPN11;
	KIR2DL3; AKT2; PIK3CA; SYK; PRKCI; PIK3CB;
	PIK3C3; PRKD1; MAPK3; KRAS; PRKCD; PTPN6;
15	
	PIK3C2A; LCK; RAF1; FYN; MAP2K2; PAK4; AKT1;
	PIK3R1; MAP2K1; PAK3; AKT3; VAV3; PRKCA
20	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;
25	
	E2F4; ATM; RBL2; SMAD4; CDKN2A; MYC; NRG1;
	GSK3B; RBL1; HDAC6
30	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;
35	
	MAP2K2; PIK3R1; CHUK; MAP2K1; NFKB1; ITK; BCL10;
	JUN; VAV3
40	Death Receptor Signaling
	CRADD; HSPB1; BID; BIRC4; TBK1; IKBKB; FADD;
	FAS; NFKB2; BCL2; MAP3K14; MAPK8; RIPK1; CASP8;
	DAXX; TNFRSF10B; RELA; TRAF2; TNF; IKBKG; RELB;
45	
	CASP9; CHUK; APAF1; NFKB1; CASP2; BIRC2; CASP3;
	BIRC3
50	FGF Signaling
	RAC1; FGFR1; MET; MAPKAPK2; MAPK1; PTPN11;
	AKT2; PIK3CA; CREB1; PIK3CB; PIK3C3; MAPK8;
	MAPK3; MAPK13; PTPN6; PIK3C2A; MAPK14; RAF1;
55	
	AKT1; PIK3R1; STAT3; MAP2K1; FGFR4; CRKL; ATF4;
	AKT3; PRKCA; HGF
60	GM-CSF Signaling
	LYN; ELK1; MAPK1; PTPN11; AKT2; PIK3CA; CAMK2A;
	STAT5B; PIK3CB; PIK3C3; GNB2L1; BCL2L1; MAPK3;
	ETS1; KRAS; RUNX1; PIM1; PIK3C2A; RAF1; MAP2K2;
65	
	AKT1; JAK2; PIK3R1; STAT3; MAP2K1; CCND1; AKT3;
	STAT1
70	Amyotrophic Lateral
	BID; IGF1; RAC1; BIRC4; PGF; CAPNS1; CAPN2;
	Sclerosis Signaling
	PIK3CA; BCL2; PIK3CB; PIK3C3; BCL2L1; CAPN1;
75	
	PIK3C2A; TP53; CASP9; PIK3R1; RAB5A; CASP1;
	APAF1; VEGFA; BIRC2; BAX; AKT3; CASP3; BIRC3

(continued)

CELLULAR FUNCTION	GENES
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; PLK1; AKT2; CDK8; MAPK8; MAPK3; PRKCD; PRKAA1; PBEF1; MAPK9; CDK2; PIM1; DYRK1A; MAP2K2; MAP2K1; PAK3; NT5E; TTK; CSNK1A1; BRAF; SGK
Metabolism	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
Chemokine 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
IL-2 Signaling	PRKCE; IGF1; PRKCZ; PRDX6; LYN; MAPK1; GNAS; PRKCI; GNAQ; PPP2R1A; IGF1R; PRKD1; MAPK3; KRAS; GRN; PRKCD; NOS3; NOS2A; PPP2CA; YWHAZ; RAF1; MAP2K2; PPP2R5C; MAP2K1; PRKCA
Synaptic Long Term Depression	TAF4B; EP300; CARM1; PCAF; MAPK1; NCOR2; SMARCA4; MAPK3; NRIP1; KRAS; SRC; NR3C1; HDAC3; PPARC1A; RBM9; NCOA3; RAF1; CREBBP; MAP2K2; NCOA2; MAP2K1; PRKDC; ESR1; ESR2
Estrogen Receptor Signaling	TRAF6; SMURF1; BIRC4; BRCA1; UCHL1; NEDD4; CBL; UBE2I; BTRC; HSPA5; USP7; USP10; FBXW7; USP9X; STUB1; USP22; B2M; BIRC2; PARK2; USP8; USP1; VHL; HSP90AA1; BIRC3
Protein Ubiquitination Pathway	TRAF6; CCR1; ELK1; IKBKB; SP1; FOS; NFKB2; MAP3K14; MAPK8; MAPK13; RELA; MAPK14; TNF; IKBKG; RELB; MAP3K7; JAK1; CHUK; STAT3; NFKB1; JUN; IL1R1; IL6
IL-10 Signaling	PRKCE; EP300; PRKCZ; RXRA; GADD45A; HES1; NCOR2; SP1; PRKCI; CDKN1B; PRKD1; PRKCD; RUNX2; KLF4; YY1; NCOA3; CDKN1A; NCOA2; SPP1; LRP5; CEBPB; FOXO1; PRKCA
VDR/RXR Activation	EP300; SMAD2; SMURF1; MAPK1; SMAD3; SMAD1; TGF-beta Signaling

(continued)

CELLULAR FUNCTION	GENES
	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; DDT3; 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; CREB 1; 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

(continued)

CELLULAR FUNCTION	GENES
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; ADAM 17; NOTCH2; PSEN1; NOTCH3; NOTCH 1; DLL4
Endoplasmic Reticulum	HSPA5; MAPK8; XBP1; TRAF2; ATF6; CASP9; ATF4; 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; PPP2R5C
Signaling	HK2; GCK; GPI; ALDH1A1; PKM2; LDHA; HK1
Glycolysis/Gluconeogenesis	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

(continued)

CELLULAR FUNCTION	GENES
5 Pathway	
Starch and Sucrose	UCHL1; HK2; GCK; GPI; HK1
Metabolism	
10 Aminosugars Metabolism	NQO1; HK2; GCK; HK1
Arachidonic Acid	PRDX6; GRN; YWHAZ; CYP1B1
Metabolism	
15 Circadian Rhythm Signaling	CSNK1E; CREB1; ATF4; NR1D1
Coagulation System	BDKRB1; F2R; SERPINE1; F3
15 Dopamine Receptor	PPP2R1A; PPP2CA; PPP1CC; PPP2R5C
Signaling	
20 Glutathione Metabolism	IDH2; GSTP1; ANPEP; IDH1
Glycerolipid Metabolism	ALDH1A1; GPAM; SPHK1; SPHK2
Linoleic Acid Metabolism	PRDX6; GRN; YWHAZ; CYP1B1
25 Methionine Metabolism	DNMT1; DNMT3B; AHCY; DNMT3A
Pyruvate Metabolism	GLO1; ALDH1A1; PKM2; LDHA
Arginine and Proline	ALDH1A1; NOS3; NOS2A
Metabolism	
30 Eicosanoid Signaling	PRDX6; GRN; YWHAZ
Fructose and Mannose	HK2; GCK; HK1
Metabolism	
35 Galactose Metabolism	HK2; GCK; HK1
Stilbene, Coumarine and	PRDX6; PRDX1; TYR
Lignin Biosynthesis	
Antigen Presentation	CALR; B2M
40 Pathway	
Biosynthesis of Steroids	NQO1; DHCR7
Butanoate Metabolism	ALDH1A1; NLGN1
Citrate Cycle	IDH2; IDH1
45 Fatty Acid Metabolism	ALDH1A1; CYP1B1
Glycerophospholipid	PRDX6; CHKA
Metabolism	
50 Histidine Metabolism	PRMT5; ALDH1A1
Inositol Metabolism	ERO1L; APEX1
Metabolism of Xenobiotics	GSTP1; CYP1B1
by Cytochrome p450	
55 Methane Metabolism	PRDX6; PRDX1
Phenylalanine Metabolism	PRDX6; PRDX1
Propanoate Metabolism	ALDH1A1; LDHA

(continued)

	CELLULAR FUNCTION	GENES
5	Selenoamino Acid	PRMT5; AHCY
	Metabolism	
	Sphingolipid Metabolism	SPHK1; SPHK2
10	Aminophosphonate	PRMT5
	Metabolism	
	Androgen and Estrogen	PRMT5
	Metabolism	
15	Ascorbate and Aldarate	ALDH1A1
	Metabolism	
	Bile Acid Biosynthesis	ALDH1A1
20	Cysteine Metabolism	LDHA
	Fatty Acid Biosynthesis	FASN
	Glutamate Receptor	GNB2L1
	Signaling	
25	NRF2-mediated Oxidative	PRDX1
	Stress Response	
	Pentose Phosphate	GPI
30	Pathway	
	Pentose and Glucuronate	UCHL1
	Interconversions	
	Retinol Metabolism	ALDH1A1
35	Riboflavin Metabolism	TYR
	Tyrosine Metabolism	PRMT5, TYR
	Ubiquinone Biosynthesis	PRMT5
40	Valine, Leucine and	ALDH1A1
	Isoleucine Degradation	
	Glycine, Serine and	CHKA
	Threonine Metabolism	
45	Lysine Degradation	ALDH1A1
	Pain/Taste	TRPM5; TRPA1
	Pain	TRPM7; TRPC5; TRPC6; TRPC1; Cnr1; cnr2; Grk2;
50		Trpa1; Pomp; 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;
55		Wnt2b; Wnt3a; Wnt4; Wnt5a; Wnt6; Wnt7b; Wnt8b;
		Wnt9a; Wnt9b; Wnt10a; Wnt10b; Wnt16); beta-catenin;
		Dkk-1; Frizzled related proteins; Otx-2; Gbx2; FGF-8;

(continued)

CELLULAR FUNCTION	GENES
	Reelin; Dab1; unc-86 (Pou4f1 or Brn3a); Numb; Reln

5 [0089] 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 10 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.

15 [0090] 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 20 adolescence. A few cases of the disease may be caused by mutations in genes yet to be identified. The disease causes seizures, muscle spasms, difficulty walking, dementia, and eventually death. There is currently no therapy that has proven effective against disease progression. Other genetic abnormalities associated with epilepsy may also be targeted by the CRISPR-Cas system and the underlying genetics is further described in Genetics of Epilepsy and Genetic Epilepsies, edited by Giuliano Avanzini, Jeffrey L. Noebels, Mariani Foundation Paediatric Neurology:20; 2009.

25 [0091] 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.

30 [0092] 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.

35 [0093] In some embodiments, the condition may be neoplasia. In some embodiments, where the condition is neoplasia, the genes to be targeted are any of those listed in Table A (in this case PTEN and so forth). In some embodiments, the condition may be Age-related Macular Degeneration. In some embodiments, the condition may be a Schizophrenic Disorder. In some embodiments, the condition may be a Trinucleotide Repeat Disorder. In some embodiments, the condition may be Fragile X Syndrome. In some embodiments, the condition may be a Secretase Related Disorder. In some 40 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.

45 [0094] 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.

[0095] Examples of addiction-related proteins may include ABAT for example.

50 [0096] 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.

[0097] 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.

55 [0098] 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.

[0099] 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.

[0100] 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.

[0101] Examples of proteins associated Schizophrenia may include NRG1, ErbB4, CPLX1, TPH1, TPH2, NRXN1, GSK3A, BDNF, DISC1, GSK3B, and combinations thereof.

[0102] 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.

[0103] 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.

[0104] 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.

[0105] 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.

[0106] 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.

[0107] 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.

[0108] 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.

[0109] 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.

[0110] Examples of neurodevelopmental-associated sequences include A2BP1 [ataxin 2-binding protein 1], AADAT [aminoacidate 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.

[0111] 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 Chondrodyplasia 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.

[0112] 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

[0113] 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.

20 *Example 1: CRISPR Complex Activity in the Nucleus of a Eukaryotic Cell*

[0114] An example type II CRISPR system is the type II CRISPR locus from *Streptococcus pyogenes* SF370, which contains a cluster of four genes Cas9, Cas1, Cas2, and Csn1, as well as two non-coding RNA elements, tracrRNA and a characteristic array of repetitive sequences (direct repeats) interspaced by short stretches of non-repetitive sequences (spacers, about 30bp each). In this system, targeted DNA double-strand break (DSB) is generated in four sequential steps (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). This example describes an example process for adapting this RNA-programmable nuclease system to direct CRISPR complex activity in the nuclei of eukaryotic cells.

35 *Cell culture and transfection*

[0115] Human embryonic kidney (HEK) cell line HEK 293FT (Life Technologies) was maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2mM GlutaMAX (Life Technologies), 100U/mL penicillin, and 100 μ g/mL streptomycin at 37°C with 5% CO₂ incubation. Mouse neuro2A (N2A) cell line (ATCC) was maintained with DMEM supplemented with 5% fetal bovine serum (HyClone), 2mM GlutaMAX (Life Technologies), 100U/mL penicillin, and 100 μ g/mL streptomycin at 37°C with 5% CO₂.

[0116] HEK 293FT or N2A cells were seeded into 24-well plates (Corning) one day prior to transfection at a density of 200,000 cells per well. Cells were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer's recommended protocol. For each well of a 24-well plate a total of 800ng of plasmids were used.

45 *Surveyor assay and sequencing analysis for genome modification*

[0117] HEK 293FT or N2A cells were transfected with plasmid DNA as described above. After transfection, the cells were incubated at 37°C for 72 hours before genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA extraction kit (Epicentre) following the manufacturer's protocol. Briefly, cells were resuspended in QuickExtract solution and incubated at 65°C for 15 minutes and 98°C for 10 minutes. Extracted genomic DNA was immediately processed or stored at -20°C.

[0118] The genomic region surrounding a CRISPR target site for each gene was PCR amplified, and products were purified using QiaQuick Spin Column (Qiagen) following manufacturer's protocol. A total of 400ng of the purified PCR products were mixed with 2 μ l 10X Taq polymerase PCR buffer (Enzymatics) and ultrapure water to a final volume of 20 μ l, and subjected to a re-annealing process to enable heteroduplex formation: 95°C for 10min, 95°C to 85°C ramping at - 2°C/s, 85°C to 25°C at - 0.25°C/s, and 25°C hold for 1 minute. After re-annealing, products were treated with Surveyor nuclease and Surveyor enhancer S (Transgenomics) following the manufacturer's recommended protocol, and analyzed on 4-20% Novex TBE poly-acrylamide gels (Life Technologies). Gels were stained with SYBR Gold DNA stain (Life

Technologies) for 30 minutes and imaged with a Gel Doc gel imaging system (Bio-rad). Quantification was based on relative band intensities, as a measure of the fraction of cleaved DNA. Figure 7 provides a schematic illustration of this Surveyor assay.

[0119] Restriction fragment length polymorphism assay for detection of homologous recombination.

[0120] HEK 293FT and N2A cells were transfected with plasmid DNA, and incubated at 37°C for 72 hours before genomic DNA extraction as described above. The target genomic region was PCR amplified using primers outside the homology arms of the homologous recombination (HR) template. PCR products were separated on a 1% agarose gel and extracted with MinElute GelExtraction Kit (Qiagen). Purified products were digested with HindIII (Fermentas) and analyzed on a 6% Novex TBE poly-acrylamide gel (Life Technologies).

10 RNA secondary structure prediction and analysis

[0121] RNA secondary structure prediction was performed using 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).

15 RNA purification

[0122] HEK 293FT cells were maintained and transfected as stated above. Cells were harvested by trypsinization followed by washing in phosphate buffered saline (PBS). Total cell RNA was extracted with TRI reagent (Sigma) following manufacturer's protocol. Extracted total RNA was quantified using Naonodrop (Thermo Scientific) and normalized to same concentration.

20 *Northern blot analysis of crRNA and tracrRNA Expression in mammalian cells*

[0123] RNAs were mixed with equal volumes of 2X loading buffer (Ambion), heated to 95°C for 5 min, chilled on ice for 1 min, and then loaded onto 8% denaturing polyacrylamide gels (SequaGel, National Diagnostics) after pre-running the gel for at least 30 minutes. The samples were electrophoresed for 1.5 hours at 40W limit. Afterwards, the RNA was transferred to Hybond N+ membrane (GE Healthcare) at 300 mA in a semi-dry transfer apparatus (Bio-rad) at room temperature for 1.5 hours. The RNA was crosslinked to the membrane using autocrosslink button on Stratagene UV Crosslinker the Stratalinker (Stratagene). The membrane was pre-hybridized in ULTRAhyb-Oligo Hybridization Buffer (Ambion) for 30 min with rotation at 42°C, and probes were then added and hybridized overnight. Probes were ordered from IDT and labeled with [γ -³²P] ATP (Perkin Elmer) with T4 polynucleotide kinase (New England Biolabs). The membrane was washed once with pre-warmed (42°C) 2xSSC, 0.5% SDS for 1 min followed by two 30 minute washes at 42°C. The membrane was exposed to a phosphor screen for one hour or overnight at room temperature and then scanned with a phosphorimager (Typhoon).

25 *Bacterial CRISPR system construction and evaluation*

[0124] CRISPR locus elements, including tracrRNA, Cas9, and leader were PCR amplified from *Streptococcus pyogenes* SF370 genomic DNA with flanking homology arms for Gibson Assembly. Two *Bsal* type IIS sites were introduced in between two direct repeats to facilitate easy insertion of spacers (Figure 8). PCR products were cloned into EcoRV-digested pACYC184 downstream of the tet promoter using Gibson Assembly Master Mix (NEB). Other endogenous CRISPR system elements were omitted, with the exception of the last 50bp of Csn2. Oligos (Integrated DNA Technology) encoding spacers with complimentary overhangs were cloned into the *Bsal*-digested vector pDC000 (NEB) and then ligated with T7 ligase (Enzymatics) to generate pCRISPR plasmids. Challenge plasmids containing spacers with PAM

[0125] expression in mammalian cells (expression constructs illustrated in Figure 6A, with functionality as determined by results of the Surveyor assay shown in Figure 6B). Transcription start sites are marked as +1, and transcription terminator and the sequence probed by northern blot are also indicated. Expression of processed tracrRNA was also confirmed by Northern blot. Figure 6C shows results of a Northern blot analysis of total RNA extracted from 293FT cells transfected with U6 expression constructs carrying long or short tracrRNA, as well as SpCas9 and DR-EMX1(1)-DR. Left and right panels are from 293FT cells transfected without or with SpRNase III, respectively. U6 indicate loading control blotted with a probe targeting human U6 snRNA. Transfection of the short tracrRNA expression construct led to abundant levels of the processed form of tracrRNA (~75bp). Very low amounts of long tracrRNA are detected on the Northern blot.

[0126] 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.

[0127] To test whether heterologous expression of the CRISPR system (SpCas9, SpRNase III, tracrRNA, and pre-crRNA) in mammalian cells can achieve targeted cleavage of mammalian chromosomes, HEK 293FT cells were transfected with combinations of CRISPR components. Since DSBs in mammalian nuclei are partially repaired by the non-homologous end joining (NHEJ) pathway, which leads to the formation of indels, the Surveyor assay was used to detect potential cleavage activity at the target *EMX1* locus (Figure 7) (see e.g. Guschin *et al.*, 2010, *Methods Mol Biol* 649: 247). Co-transfection of all four CRISPR components was able to induce up to 5.0% cleavage in the protospacer (see Figure 2D). Co-transfection of all CRISPR components minus SpRNase III also induced up to 4.7% indel in the protospacer, suggesting that there may be endogenous mammalian RNases that are capable of assisting with crRNA maturation, such as for example the related Dicer and Drosha enzymes. Removing any of the remaining three components abolished the genome cleavage activity of the CRISPR system (Figure 2D). Sanger sequencing of amplicons containing the target locus verified the cleavage activity: in 43 sequenced clones, 5 mutated alleles (11.6%) were found. Similar experiments using a variety of guide sequences produced indel percentages as high as 29% (see Figures 3-6, 10, and 11). These results define a three-component system for efficient CRISPR-mediated genome modification in mammalian cells. To optimize the cleavage efficiency, Applicants also tested whether different isoforms of tracrRNA affected the cleavage efficiency and found that, in this example system, only the short (89-bp) transcript form was able to mediate cleavage of the human *EMX1* genomic locus (Figure 6B).

[0128] Figure 12 provides an additional Northern blot analysis of crRNA processing in mammalian cells. Figure 12A illustrates a schematic showing the expression vector for a single spacer flanked by two direct repeats (DR-EMX1(1)-DR). The 30bp spacer targeting the human *EMX1* locus protospacer 1 (see Figure 6) and the direct repeat sequences are shown in the sequence beneath Figure 12A. The line indicates the region whose reverse-complement sequence was used to generate Northern blot probes for *EMX1*(1) crRNA detection. Figure 12B shows a Northern blot analysis of total RNA extracted from 293FT cells transfected with U6 expression constructs carrying DR-EMX1(1)-DR. Left and right panels are from 293FT cells transfected without or with SpRNase III respectively. DR-EMX1(1)-DR was processed into mature crRNAs only in the presence of SpCas9 and short tracrRNA and was not dependent on the presence of SpRNase III. The mature crRNA detected from transfected 293FT total RNA is ~33bp and is shorter than the 39-42bp mature crRNA from *S. pyogenes*. These results demonstrate that a CRISPR system can be transplanted into eukaryotic cells and reprogrammed to facilitate cleavage of endogenous mammalian target polynucleotides.

[0129] Figure 2 illustrates the bacterial CRISPR system described in this example. Figure 2A illustrates a schematic showing the CRISPR locus 1 from *Streptococcus pyogenes* SF370 and a proposed mechanism of CRISPR-mediated DNA cleavage by this system. Mature crRNA processed from the direct repeat-spacer array directs Cas9 to genomic targets consisting of complimentary protospacers and a protospacer-adjacent motif (PAM). Upon target-spacer base pairing, Cas9 mediates a double-strand break in the target DNA. Figure 2B illustrates engineering of *S. pyogenes* Cas9 (SpCas9) and RNase III (SpRNase III) with nuclear localization signals (NLSS) to enable import into the mammalian nucleus. Figure 2C illustrates mammalian expression of SpCas9 and SpRNase III driven by the constitutive EF1 α promoter and tracrRNA and pre-crRNA array (DR-Spacer-DR) driven by the RNA Pol3 promoter U6 to promote precise transcription initiation and termination. A protospacer from the human *EMX1* locus with a satisfactory PAM sequence is used as the spacer in the pre-crRNA array. Figure 2D illustrates surveyor nuclease assay for SpCas9-mediated minor insertions and deletions. SpCas9 was expressed with and without SpRNase III, tracrRNA, and a pre-crRNA array carrying the *EMX1*-target spacer. Figure 2E illustrates a schematic representation of base pairing between target locus and *EMX1*-targeting crRNA, as well as an example chromatogram showing a micro deletion adjacent to the SpCas9 cleavage site. Figure 2F illustrates mutated alleles identified from sequencing analysis of 43 clonal amplicons showing a variety of micro insertions and deletions. Dashes indicate deleted bases, and non-aligned or mismatched bases indicate insertions or mutations. Scale bar = 10 μ m.

[0130] To further simplify the three-component system, a chimeric crRNA-tracrRNA hybrid design was adapted, where a mature crRNA (comprising a guide sequence) may be fused to a partial tracrRNA via a stem-loop to mimic the natural crRNA:tracrRNA duplex. To increase co-delivery efficiency, a bicistronic expression vector was created to drive co-expression of a chimeric RNA and SpCas9 in transfected cells. In parallel, the bicistronic vectors were used to express a pre-crRNA (DR-guide sequence-DR) with SpCas9, to induce processing into crRNA with a separately expressed tracrRNA (compare Figure 11B top and bottom). Figure 8 provides schematic illustrations of bicistronic expression vectors for pre-crRNA array (Figure 8A) or chimeric crRNA (represented by the short line downstream of the guide sequence insertion site and upstream of the EF1 α promoter in Figure 8B) with hSpCas9, showing location of various elements and the point of guide sequence insertion. The expanded sequence around the location of the guide sequence insertion site in Figure 8B also shows a partial DR sequence (GTTTTAGAGCTA) (SEQ ID NO: 11) and a partial tracrRNA sequence (TAGCAAGTAAAATAAGGCTAGTCCGTTTT) (SEQ ID NO: 12). Guide sequences can be inserted between BbsI sites using annealed oligonucleotides. Sequence design for the oligonucleotides are shown below the schematic

illustrations in Figure 8, with appropriate ligation adapters indicated. WPRE represents the Woodchuck hepatitis virus post-transcriptional regulatory element. The efficiency of chimeric RNA-mediated cleavage was tested by targeting the same *EMX1* locus described above. Using both Surveyor assay and Sanger sequencing of amplicons, Applicants confirmed that the chimeric RNA design facilitates cleavage of human *EMX1* locus with approximately a 4.7% modification rate (Figure 3).

[0131] Generalizability of CRISPR-mediated cleavage in eukaryotic cells was tested by targeting additional genomic loci in both human and mouse cells by designing chimeric RNA targeting multiple sites in the human *EMX1* and *PVALB*, as well as the mouse *Th* loci. Figure 13 illustrates the selection of some additional targeted protospacers in human *PVALB* (Figure 13A) and mouse *Th* (Figure 13B) loci. Schematics of the gene loci and the location of three protospacers within the last exon of each are provided. The underlined sequences include 30bp of protospacer sequence and 3bp at the 3' end corresponding to the PAM sequences. Protospacers on the sense and anti-sense strands are indicated above and below the DNA sequences, respectively. A modification rate of 6.3% and 0.75% was achieved for the human *PVALB* and mouse *Th* loci respectively, demonstrating the broad applicability of the CRISPR system in modifying different loci across multiple organisms (Figure 5). While cleavage was only detected with one out of three spacers for each locus using the chimeric constructs, all target sequences were cleaved with efficiency of indel production reaching 27% when using the co-expressed pre-crRNA arrangement (Figures 6 and 13).

[0132] Figure 11 provides a further illustration that SpCas9 can be reprogrammed to target multiple genomic loci in mammalian cells. Figure 11A provides a schematic of the human *EMX1* locus showing the location of five protospacers, indicated by the underlined sequences. Figure 11B provides a schematic of the pre-crRNA/trcrRNA complex showing hybridization between the direct repeat region of the pre-crRNA and trcrRNA (top), and a schematic of a chimeric RNA design comprising a 20bp guide sequence, and tracr mate and tracr sequences consisting of partial direct repeat and trcrRNA sequences hybridized in a hairpin structure (bottom). Results of a Surveyor assay comparing the efficacy of Cas9-mediated cleavage at five protospacers in the human *EMX1* locus is illustrated in Figure 11C. Each protospacer is targeted using either processed pre-crRNA/trcrRNA complex (crRNA) or chimeric RNA (chiRNA).

[0133] Since the secondary structure of RNA can be crucial for intermolecular interactions, a structure prediction algorithm based on minimum free energy and Boltzmann-weighted structure ensemble was used to compare the putative secondary structure of all guide sequences used in the genome targeting experiment (see e.g. Gruber et al., 2008, Nucleic Acids Research, 36: W70). Analysis revealed that in most cases, the effective guide sequences in the chimeric crRNA context were substantially free of secondary structure motifs, whereas the ineffective guide sequences were more likely to form internal secondary structures that could prevent base pairing with the target protospacer DNA. It is thus possible that variability in the spacer secondary structure might impact the efficiency of CRISPR-mediated interference when using a chimeric crRNA.

[0134] Further vector designs for SpCas9 are shown in Figure 22, which illustrates single expression vectors incorporating a U6 promoter linked to an insertion site for a guide oligo, and a Cbh promoter linked to SpCas9 coding sequence. The vector shown in Figure 22b includes a tracrRNA coding sequence linked to an H1 promoter.

[0135] In the bacterial assay, all spacers facilitated efficient CRISPR interference (Figure 3C). These results suggest that there may be additional factors affecting the efficiency of CRISPR activity in mammalian cells.

[0136] To investigate the specificity of CRISPR-mediated cleavage, the effect of single-nucleotide mutations in the guide sequence on protospacer cleavage in the mammalian genome was analyzed using a series of *EMX1*-targeting chimeric crRNAs with single point mutations (Figure 3A). Figure 3B illustrates results of a Surveyor nuclease assay comparing the cleavage efficiency of Cas9 when paired with different mutant chimeric RNAs. Single-base mismatch up to 12-bp 5' of the PAM substantially abrogated genomic cleavage by SpCas9, whereas spacers with mutations at farther upstream positions retained activity against the original protospacer target (Figure 3B). In addition to the PAM, SpCas9 has single-base specificity within the last 12-bp of the spacer. Furthermore, CRISPR is able to mediate genomic cleavage as efficiently as a pair of TALE nucleases (TALEN) targeting the same *EMX1* protospacer. Figure 3C provides a schematic showing the design of TALENs targeting *EMX1*, and Figure 3D shows a Surveyor gel comparing the efficiency of TALEN and Cas9 (n=3).

[0137] Having established a set of components for achieving CRISPR-mediated gene editing in mammalian cells through the error-prone NHEJ mechanism, the ability of CRISPR to stimulate homologous recombination (HR), a high fidelity gene repair pathway for making precise edits in the genome, was tested. The wild type SpCas9 is able to mediate site-specific DSBs, which can be repaired through both NHEJ and HR. In addition, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of SpCas9 was engineered to convert the nuclease into a nickase (SpCas9n; illustrated in Figure 4A) (see e.g. Sapranausaks et al., 2011, Nucleic Acids 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). Surveyor assay confirmed that SpCas9n does not generate indels at the *EMX1* protospacer target. As illustrated in Figure 4B, co-expression of *EMX1*-targeting chimeric crRNA 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 (*Hind*III and *Nhe*I) near the protospacer. Figure 4C provides a schematic illustration of the HR strategy, with relative locations of recombination points and primer annealing sequences (arrows). 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 *Hind*III revealed cleavage products corresponding to expected fragment sizes (arrows in restriction fragment length polymorphism gel analysis shown in Figure 4D), with SpCas9 and SpCas9n mediating similar levels of HR efficiencies. Applicants further verified HR using Sanger sequencing of genomic amplicons (Figure 4E). These results demonstrate 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 nuclease can significantly reduce the likelihood of off-target modifications, since single strand breaks are not substrates for the error-prone NHEJ pathway.

[0138] Expression constructs mimicking the natural architecture of CRISPR loci with arrayed spacers (Figure 2A) were constructed to test the possibility of multiplexed sequence targeting. Using a single CRISPR array encoding a pair of *EMX1*- and *PVALB*-targeting spacers, efficient cleavage at both loci was detected (Figure 4F, showing both a schematic 15 design of the crRNA array and a Surveyor blot showing efficient mediation of cleavage). Targeted deletion of larger genomic regions through concurrent DSBs using spacers against two targets within *EMX1* spaced by 119bp was also tested, and a 1.6% deletion efficacy (3 out of 182 amplicons; Figure 4G) was detected. This demonstrates that the CRISPR system can mediate multiplexed editing within a single genome.

20 *Example 2: CRISPR system modifications and alternatives*

[0139] The ability to use RNA to program sequence-specific DNA cleavage defines a new class of genome engineering tools for a variety of research and industrial applications. 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 25 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 motif immediately downstream of the protospacer restricts the ability to target on average every 12-bp in the human genome (Figure 9, evaluating both plus and minus strands of human chromosomal sequences). Some of these constraints can be overcome by exploring the diversity of CRISPR loci across the microbial metagenome 30 (see e.g. Makarova et al., 2011, *Nat Rev Microbiol*, 9:467). Other CRISPR loci may be transplanted into the mammalian cellular milieu by a process similar to that described in Example 1. For example, Figure 10 illustrates adaptation of the Type II CRISPR system from CRISPR 1 of *Streptococcus thermophilus* LMD-9 for heterologous expression in mammalian cells to achieve CRISPR-mediated genome editing. Figure 10A provides a Schematic illustration of CRISPR 1 from *S. thermophilus* LMD-9. Figure 10B illustrates the design of an expression system for the *S. thermophilus* CRISPR system. Human codon-optimized *hStCas9* is expressed using a constitutive EF1 α promoter. Mature versions of tracrRNA and 35 crRNA are expressed using the U6 promoter to promote precise transcription initiation. Sequences from the mature crRNA and tracrRNA are illustrated. A single base indicated by the lower case "a" in the crRNA sequence is used to remove the polyU sequence, which serves as a RNA polIII transcriptional terminator. Figure 10C provides a schematic showing guide sequences targeting the human *EMX1* locus. Figure 10D shows the results of hStCas9-mediated cleavage in the target locus using the Surveyor assay. RNA guide spacers 1 and 2 induced 14% and 6.4%, respectively. Statistical 40 analysis of cleavage activity across biological replica at these two protospacer sites is also provided in Figure 5. Figure 14 provides a schematic of additional protospacer and corresponding PAM sequence targets of the *S. thermophilus* CRISPR system in the human *EMX1* locus. Two protospacer sequences are highlighted and their corresponding PAM sequences satisfying NNAGAAW motif are indicated by underlining 3' with respect to the corresponding highlighted sequence. Both protospacers target the anti-sense strand.

45 *Example 3: Sample target sequence selection algorithm*

[0140] A software program is designed to identify candidate CRISPR target sequences on both strands of an input DNA sequence based on desired guide sequence length and a CRISPR motif sequence (PAM) for a specified CRISPR enzyme. For example, target sites for Cas9 from *S. pyogenes*, with PAM sequences NGG, may be identified by searching for 5'-N_x-NGG-3' both on the input sequence and on the reverse-complement of the input. Likewise, target sites for Cas9 of *S. thermophilus* CRISPR1, with PAM sequence NNAGAAW, may be identified by searching for 5'-N_x-NNAGAAW-3' both on the input sequence and on the reverse-complement of the input. Likewise, target sites for Cas9 of *S. thermophilus* CRISPR3, with PAM sequence NGGNG, may be identified by searching for 5'-N_x-NGGNG-3' both on the input sequence and on the reverse-complement of the input. The value "x" in N_x may be fixed by the program or specified by the user, such as 20.

[0141] Since multiple occurrences in the genome of the DNA target site may lead to nonspecific genome editing, after identifying all potential sites, the program filters out sequences based on the number of times they appear in the relevant

reference genome. For those CRISPR enzymes for which sequence specificity is determined by a 'seed' sequence, such as the 11-12bp 5' from the PAM sequence, including the PAM sequence itself, the filtering step may be based on the seed sequence. Thus, to avoid editing at additional genomic loci, results are filtered based on the number of occurrences of the seed:PAM sequence in the relevant genome. The user may be allowed to choose the length of the seed sequence. The user may also be allowed to specify the number of occurrences of the seed:PAM sequence in a genome for purposes of passing the filter. The default is to screen for unique sequences. Filtration level is altered by changing both the length of the seed sequence and the number of occurrences of the sequence in the genome. The program may in addition or alternatively provide the sequence of a guide sequence complementary to the reported target sequence(s) by providing the reverse complement of the identified target sequence(s). An example visualization of some target sites in the human genome is provided in Figure 18.

[0142] Further details of methods and algorithms to optimize sequence selection can be found in U.S. application Serial No. 61/064,798 (Attorney docket 44790.11.2022; Broad Reference BI-2012/084); incorporated herein by reference.

Example 4: Evaluation of multiple chimeric crRNA-tracrRNA hybrids

[0143] This example describes results obtained for chimeric RNAs (chiRNAs; comprising a guide sequence, a tracr mate sequence, and a tracr sequence in a single transcript) having tracr sequences that incorporate different lengths of wild-type tracrRNA sequence. Figure 16a illustrates a schematic of a bicistronic expression vector for chimeric RNA and Cas9. Cas9 is driven by the CBh promoter and the chimeric RNA is driven by a U6 promoter. 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 separated by the tracr-mate sequence GUUUUAGAGCUA (SEQ ID NO: 13) followed by the loop sequence GAAA. Results of SURVEYOR assays for Cas9-mediated indels at the human *EMX1* and *PVALB* loci are illustrated in Figure 16b and 16c, respectively. Arrows indicate the expected SURVEYOR fragments. ChiRNAs are indicated by their "+n" designation, and crRNA refers to a hybrid RNA where guide and tracr sequences are expressed as separate transcripts. Quantification of these results, performed in triplicate, are illustrated by histogram in Figures 17a and 17b, corresponding to Figures 16b and 16c, respectively ("N.D." indicates no indels detected). Protospacer IDs and their corresponding genomic target, protospacer sequence, PAM sequence, and strand location are provided in Table D. Guide sequences were designed to be complementary to the entire protospacer sequence in the case of separate transcripts in the hybrid system, or only to the underlined portion in the case of chimeric RNAs.

Table D:

protospacer ID	genomic target	protospacer sequence (5' to 3')	PAM	SEQ ID NO:	strand
1	<i>EMX1</i>	GGACATCGAT <u>GTCACCTCCAATGACTAGGG</u>	TGG	14	+
2	<i>EMX1</i>	CATTGGAGGT <u>GACATCGATGTCCTCCCCAT</u>	TGG	15	-
3	<i>EMX1</i>	GGAAGGGCCT <u>GAGTCGAGCAGAAGAAGAA</u>	GGG	16	+
4	<i>PVALB</i>	GGTGGCGAG <u>AGGGGCCGAGATTGGGTGTC</u>	AGG	17	+
5	<i>PVALB</i>	ATGCAGGAGGG <u>TGGCGAGAGGGGCCGAGAT</u>	TGG	18	+

[0144] Further details to optimize guide sequences can be found in U.S. application Serial No. 61/836,127 (Attorney docket 44790.08.2022; Broad Reference BI-2013/004G); incorporated herein by reference.

[0145] Initially, three sites within the *EMX1* locus in human HEK 293FT cells were targeted. Genome modification efficiency of each chiRNA was assessed using the SURVEYOR nuclease assay, which detects mutations resulting from DNA double-strand breaks (DSBs) and their subsequent repair by the non-homologous end joining (NHEJ) DNA damage repair pathway. Constructs designated chiRNA(+n) indicate that up to the +n nucleotide of wild-type tracrRNA is included in the chimeric RNA construct, with values of 48, 54, 67, and 85 used for n. Chimeric RNAs containing longer fragments of wild-type tracrRNA (chiRNA(+67) and chiRNA(+85)) mediated DNA cleavage at all three *EMX1* target sites, with chiRNA(+85) in particular demonstrating significantly higher levels of DNA cleavage than the corresponding crRNA/tracrRNA hybrids that expressed guide and tracr sequences in separate transcripts (Figures 16b and 17a). Two sites in the *PVALB* locus that yielded no detectable cleavage using the hybrid system (guide sequence and tracr sequence expressed as separate transcripts) were also targeted using chiRNAs. chiRNA(+67) and chiRNA(+85) were able to mediate significant cleavage at the two *PVALB* protospacers (Figures 16c and 17b).

For all five targets in the EMX1 and PVALB loci, a consistent increase in genome modification efficiency with increasing tracr sequence length was observed. Without wishing to be bound by any theory, the secondary structure formed by the 3' end of the tracrRNA may play a role in enhancing the rate of CRISPR complex formation.

5 *Example 5: Cas9 diversity*

[0146] The CRISPR-Cas system is an adaptive immune mechanism against invading exogenous DNA employed by diverse species across bacteria and archaea. The type II CRISPR-Cas9 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 crRNA (tracrRNA), and an array of foreign DNA-derived spacers flanked by direct repeats (crRNAs). Upon maturation by Cas9, the tracrRNA 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.

[0147] 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) (see Figures 19 and 20A-F).

[0148] Further details of Cas9s and mutations of the Cas9 enzyme to convert into a nickase or DNA binding protein and use of same with altered functionality can be found in U.S. application Serial Nos 61/836,101 and 61/835,936 (Attorney docket 44790.09.2022 and 4790.07.2022 and Broad Reference BI-2013/004E and BI-2013/004F respectively) incorporated herein by reference.

Example 6: Cas9 orthologs

[0149] Applicants analyzed Cas9 orthologs to identify the relevant PAM sequences and the corresponding chimeric guide RNA. Having an 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.

[0150] The specificity of Cas9 orthologs can be evaluated by testing the ability of each Cas9 to tolerate mismatches between the guide RNA and its DNA target. For example, the specificity of SpCas9 has been characterized by testing the effect of mutations in the guide RNA on cleavage efficiency. Libraries of guide RNAs were made with single or multiple mismatches between the guide sequence and the target DNA. Based on these findings, target sites for SpCas9 can be selected based on the following guidelines:

[0151] To maximize SpCas9 specificity for editing a particular gene, one should choose a target site within the locus of interest such that potential 'off-target' genomic sequences abide by the following four constraints: First and foremost, they should not be followed by a PAM with either 5'-NGG or NAG sequences. Second, their global sequence similarity to the target sequence should be minimized. Third, a maximal number of mismatches should lie within the PAM-proximal region of the off-target site. Finally, a maximal number of mismatches should be consecutive or spaced less than four bases apart.

[0152] Similar methods can be used to evaluate the specificity of other Cas9 orthologs and to establish criteria for the selection of specific target sites within the genomes of target species. As mentioned previously 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) (see Figures 19 and 20A-F). Further details on Cas orthologs can be found in U.S. application Serial Nos 61/836,101 and 61/835,936 (Attorney docket 44790.09.2022 and 4790.07.2022 and Broad Reference BI-2013/004E and BI-2013/004F respectively) incorporated herein by reference.

Example 7: Engineering of Plants (Micro-algae) using Cas9 to target and manipulate plant genes

Methods of delivering Cas9

55 **[0153]**

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.

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.

5 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.

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

10 [0154] Sequence for a cassette driving the expression of Cas9 under the control of beta-2 tubulin promoter, followed by the 3' UTR of Cop1.

15 TCTTTCTTGCCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGA
 CGGCTTCCCGCGCTGCATGCAACACCCGATGATGCTTCGACCCCCCGAAGCTCCTTC
 GGGGCTGCATGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTAAATAGCCAG
 20 GCCCCCCGATTGCAAAGACATTATAGCGAGCTACCAAAGCCATTCAAACACCTAG
 ATCACTACCACTTCTACACAGGCCACTCGAGCTTGTATCGCACTCCGCTAAGGGGG
 CGCCTCTCCTCTCGTTCAAGTCACAACCCGCAAACATGTACCCATACGATGTTCCA
 25 GATTACGCTTCGCCGAAGAAAAAGCGCAAGGTCGAAGCGTCCGACAAGAAGTACAG
 CATCGGCCTGGACATCGGCACCAACTCTGTGGCTGGCCGTGATCACCAGCAG
 ACAAGGTGCCAGCAAGAAATTCAAGGTGCTGGCAACACCGACCGAACAGGCCAC
 30 AAGAAGAACCTGATCGGAGGCCCTGCTTGCACAGCGGCAAACAGCCGAGGCCAC
 CCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGC
 TATCTGCAAGAGATCTCAGCAACGAGATGGCCAAGGTGGACGACAGCTCTCCAC
 35 AGACTGGAAGAGTCCTCCTGGTGGAAAGAGGATAAGAAGACGACGAGCGGACCCCAT
 CTTCGGCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCACCATCTACC
 ACCTGAGAAAGAAACTGGTGGACAGCACCGACAAGGCCACCTGCGGCTGATCTAT
 40 CTGGCCCTGGCCCACATGATCAAGTTCCGGGCCACTCCTGATCGAGGGCGACCTG
 AACCCGACAACAGCGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAA
 CCAGCTTCTGAGGAAAACCCATCAACGCCAGCGCGTGGACGCCAAGGCCATCC
 45 TGTCTGCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTGATCGCCAGCTGCC
 GGCGAGAAGAAGAATGCCCTGTCGGCAACCTGATTGCCCTGAGCCTGGCCTGAC
 CCCCAACTCAAGAGCAACTCGACCTGGCCAGGATGCCAAACTGCAGCTGAGCA
 50 AGGACACCTACGACGACGACCTGGACAACCTGCTGGCCCAGATCGCGACCAGTAC
 GCCGACCTGTTCTGGCCGCCAAGAACCTGTCGACGCCATCCTGCTGAGCGACATC
 CTGAGAGTGAACACCGAGATACCCAAGGCCCCCTGAGCGCCTATGATCAAGAG

55

ATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGTCGGCAGCAGC
 TGCCTGAGAAGTACAAAGAGATTTCTCGACCAGAGCAAGAACGGCTACGCCGGC
 5 TACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCT
 GGAAAAGATGGACGGCACCGAGGAACCTGCTCGTAAGCTGAACAGAGAGGACCTG
 CTGCGGAAGCAGCGGACCTCGACAACGGCAGCATTCCCCACCAGATCCACCTGGG
 10 AGAGCTGCACGCCATTCTCGGGCGCAGGAAGATTTACCCATTCTGAAGGACAA
 CCGGGAAAAGATCGAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCT
 GGCCAGGGAAACAGCAGATTGCCTGGATGACCAGAAAGAGCGAGGAAACCATC
 15 ACCCCCTGGAACCTCGAGGAAGTGGTGGACAAGGGCGCTCCGCCAGAGCTTCAT
 CGAGCGGATGACCAACTCGATAAGAACCTGCCAACGAGAAGGTGCTGCCAACGC
 ACAGCCTGCTGTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATAC
 20 GTGACCGAGGGATGAGAAAGCCCGCTCCTGAGCGCGAGCAGAAAAAGGCCA
 TCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAG
 GACTACTCAAGAAAATCGAGTGCTCGACTCCGTGAAATCTCCGGCGTGGAAAGAT
 25 CGGTTCAACGCCTCCCTGGCACATACCACGATCTGCTGAAAATTATCAAGGACAA
 GGACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTGTGACCC
 30 TGACACTGTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCCAC
 CTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGCGGAGATAACCGGCTGGGG
 CAGGCTGAGCCGGAAGCTGATCAACGGCATCCGGACAAGCAGTCCGGCAAGACA
 35 ATCCTGGATTCTGAAGTCCGACGGCTCGCCAACAGAAAACCTCATGCAGCTGATC
 CACGACGACAGCCTGACCTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGCCA
 GGGCGATAGCCTGCACGAGCACATTCCAATCTGGCCGGCAGCCCCGCCATTAAGA
 40 AGGGCATCCTGCAGACAGTGAAGGTGGACGAGCTCGTAAAGTGATGGGCCGG
 CACAAGCCCGAGAACATCGTATCGAAATGCCAGAGAGAACCCAGACCACCCAGA
 AGGGACAGAACAGCCGCGAGAGAACATGAGCGGATCGAACAGAGGGCATCAAAGA
 45 GCTGGCAGCCAGATCCTGAAAGAACACCCCCGTGGAAAACACCCAGCTGCAGAACG
 AGAAGCTGTACCTGTACTACCTGCAGAATGGCGGGATATGTACGTGGACCAGGAA
 CTGGACATCAACCGGCTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAGCTT
 50 CTGAAGGACGACTCCATCGACAACAAGGTGCTGACCAGAACGACAAGAACCGGG
 GCAAGAGCGACAACGTGCCCTCCGAAGAGGTGCTGAAGAAGATGAAGAAACTACTGG
 CGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTGACCAA
 55

GGCGAGAGAGGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAG
 CTGGTGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCGGAT
 5 GAACACTAAGTACGACGAGAATGACAAGCTGATCCGGAAAGTGAAGTGATCACCC
 TGAAGTCCAAGCTGGTGTCCGATTCCGAAGGATTCCAGTTTACAAAGTGCACG
 AGATCAACAACTACCACCGCCCACGACGCCTACCTGAACGCCGTGGAAACC
 10 GCCCTGATCAAAAAGTACCCCTAACGCTGGAAAGCGAGTTCGTACGGCGACTACAA
 GGTGTACGACGTGCGGAAGATGATGCCAACAGAGCGAGCAGGAAATCGGCAAGGCT
 ACCGCCAACAGTACTTCTTACAGCAACATCATGAACCTTTCAAGACCGAGATTACC
 15 CTGGCCAACGGCGAGATCCGGAAAGCGGCCTCTGATCGAGACAAACGGCGAAACCGG
 GGAGATCGTGTGGATAAGGGCCGGATTGCCCCGTGCGAAAGTGCTGAGCA
 TGCCCCAAGTGAATATCGTAAAAAGACCGAGGTGCAGACAGGCGCTTCAGCAA
 20 GAGTCTATCCTGCCAACAGAGAACAGCGATAAGCTGATGCCAGAAAGAAGGACTG
 GGACCCCTAACAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGTGCTGGT
 GGTGGCCAAGTGGAAAAGGGCAAGTCCAAGAAACTGAAGAGTGAAAGAGCTG
 25 CTGGGGATCACCACATGGAAAGAACAGCTTCGAGAAGAACATCCATCGACTTCT
 GGAAGCCAAGGGTACAAAGAACAGTAAAAAGGACCTGATCATCAAGCTGCCTAAGT
 30 ACTCCCTGTCGAGCTGGAAAACGGCCGGAAAGAGAACATGCTGGCCTCTGCCGGCGAA
 CTGCAGAACGGAAACGAACGGCCCTGCCCTCCAAATATGTGAACCTCCTGTACCTG
 GCCAGCCACTATGAGAACAGCTGAAGGGCTCCCCGAGGATAATGAGCAGAACAGCT
 35 GTTTGTGGAACAGCACAAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGT
 TCTCCAAGAGAGTGATCCTGGCCGACGCTAACATCTGGACAAAGTGCTGTCCGCCTACA
 ACAAGCACCGGGATAAGCCCACATCAGAGAGCAGGCCGAGAACATCATCCACCTGTT
 40 ACCCTGACCAATCTGGAGCCCTGCCGCTTCAAGTACTTGACACCACCATCGAC
 CGGAAGAGGTACACCAGCACCAAAGAGGTGCTGGACGCCACCCGATCCACCAAGAG
 CATCACCGGCCTGTACGAGAACACGGATCGACCTGTCTCAGCTGGAGGCGACAGCC
 45 CCAAGAACAGAACAGGAAAGGTGGAGGCCAGCTAACGGATCCGGCAAGACTGGCCCCGC
 TTGGCAACGCAACAGTGAGCCCTCCCTAGTGTGTTGGGATGTGACTATGTATTG
 GTGTGTTGGCCAACGGGTCAACCCGAACAGATTGATAACCGCCTGGCATTCTGT
 50 CAGAATGTAACGTCAGTTGATGGTACT (SEQ ID NO: 19)

[0155] 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:

TCTTTCTTGCCTATGACACTCCAGCAAAAGGTAGGGCGGGCTGCGAGA
 CGGCTTCCCAGCGCTGCATGCAACACCGATGATGCTCGACCCCCGAAGCTCCTC
 5 GGGGCTGCATGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTAAATAGCCAG
 GCCCCCCGATTGCAAAGACATTATAGCGAGCTACCAAAGCCATTCAAACACCTAG
 ATCACTACCACTTCTACACAGGCCACTCGAGCTGTGATCGCACTCCGCTAAGGGGG
 10 CGCCTCTCCTCTCGTTCAAGTCACAACCCGCAAACatgcctaagaagaagaggttaacacgatt
 aacatcgctaagaacgacttctgacatcgaactggctgctatccgttcaacactctggctgaccattacggtgagcggttagctcgaa
 15 cagttggcccttggcatgagtcttacgagatgggtgaagcacgcttccgcaagatgttggctgcaactaaagctggtaggtcgat
 aacgctgccccaagcctctcatcactaccctactccctaagatgattgcacgcatcaacgactgggttaggaagtgaaagctacg
 gcaagcgccccacagcctccagttctgcaagaaatcaagccgaagccgtacgtacatcaccattaagaccactctggcttgc
 20 taac cagtgtgacaataacaaccgttccaggctgttagcaagcgaatcggtcgccattgaggacgaggctcgctcgatccgt
 gacatgctcttaagggtactcgggtcgaggcgtggcttgcataaggaagactctattcatgttaggatcgctgc
 25 atgctcattgagtcaaccggaatggtagttacaccgcaaaatgctggcttaggtcaagactctgagactatcgac
 atacgctgaggctatcgcaaccctgcagggtgcgtggctggcatctccgatgttccaacccgttagtccctctaagccgt
 30 gggacttacatgggtgttggctattggctaacgggtcgtggctggcatctccgatgttccaacccgttagtccctcta
 accaagtggaaacgttccggcgaggacatccctgcgttgcattggctgttgcattggatcgacatcgacat
 35 gggctctaccgcgtggaaacgtgtccgtcgaggacatccctgcgttgcattggatcgacatcgacat
 tttagcaagccaataagttgtcaaccataaggccatctgggtcccttacaacatggactggcggtcgtagttac
 40 ctgcgttcaatgttcaacccgttgcgtggctggcatctccgatccctgcgttgcattggatcgacatcgacat
 ctggcttgcgttgcgtggctggcatctccgatccctgcgttgcattggatcgacatcgacat
 45 ctggcttgcgttgcgtggctggcatctccgatccctgcgttgcattggatcgacatcgacat
 ggatcgacatcgacatcgacatcgacatcgacatcgacatcgacat
 50 ctggatcgacatcgacatcgacatcgacatcgacatcgacatcgacatcgacat
 ctggatcgacatcgacatcgacatcgacatcgacatcgacat
 55 ctggatcgacatcgacatcgacatcgacatcgacatcgacatcgacatcgacat

5 caaacaggagtctggtacgctctaacttgtacacagccaagacggtagccacccgtaaagactgttagtgtggcacacgagaagtac
 ggaatcgaatttgcactgattcacgactcctcgtacgattccggctgacgctgcgaacctgtcaaaggcagtgcgcgaaactatgg
 10 acacatatgagtcttgtatgtactggctgattctacgaccagttcgtgaccagttcaattggacaaaatgccagacttcc
 ggctaaaggtaactgaacctccgtgacatcttagagtcggacttcgcgtcgtaaGGATCCGGCAAGACTGGCCCC
 GCTTGGCAACGCAACAGTGAGCCCCTCCCTAGTGTGTTGGGATGTGACTATGTAT
 15 TCGTGTGTTGGCCAACGGTCAACCCGAACAGATTGATAACCGCCTGGCATTTCCT
 GTCAGAATGTAACGTCAGTTGATGGTACT (SEQ ID NO: 20)

15 [0156] Sequence of guide RNA driven by the T7 promoter (T7 promoter, Ns represent targeting sequence):

20 gaaatTAATACGACTCACTATNNNNNNNNNNNNNNNNNNNgttttagagctaGA
 AAtagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggcgtttttt (SEQ ID NO: 21)

20 Gene delivery:

25 [0157] *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.

30 [0158] Also, Applicants generate a line of *Chlamydomonas reinhardtii* that expresses Cas9 constitutively. This can be done by using pChlamy1 (linearized using Pvul) 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.

30 pChlamy1-Cas9:

[0159]

35 TCGGGTATTCACACCGCATCAGGTGGCACTTTGGGGAAATGTGCGCG
 GAACCCCTATTGTTATTTCTAAATACATTCAAATATGTATCCGCTCATGAGATT
 40 ATCAAAAAGGATCTTCACCTAGATCCTTAAATTAAAAATGAAGTTAAATCAAT
 CTAAAGTATATGAGTAAACTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGC
 ACCTATCTCAGCGATCTGTCTATTCGTTATCCATAGTTGCCTGACTCCCCGTCGTG
 45 TAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCG
 CGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAAACCAAGCCAGCCGGAAG
 GGCGGAGCGCAGAAGTGGCCTGCAACTTATCCGCTCCATCCAGTCTATTAAATTG
 50 TTGCCGGGAAGCTAGAGTAAGTAGTCGCCAGTTAATAGTTGCGAACGTTGTC

CATTGCTACAGGCATCGTGGTGCACGCTCGTCTGGTATGGCTTCATTAGCTCC
 GGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGCAAAAAAGCGGTT
 5 AGCTCCTCGGTCTCCGATCGTGTAGAAGTAAGTTGGCCGCAGTGTATCACTC
 ATGGTTATGGCAGCACTGCATAATTCTTACTGTCATGCCATCCGTAAGATGCTTT
 CTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCCGCACCGA
 10 GTTGCTCTGCCCGCGTCAATACGGATAATACCGGCCACATAGCAGAACTTAA
 AAGTGCTCATCATTGAAAACGTTCTCGGGCGAAAACCTCTCAAGGATCTTACCGC
 15 TGTTGAGATCCAGTTGATGTAACCCACTCGTGCACCCAACTGATCTCAGCATT
 TACTTCACCAGCGTTCTGGGTGAGAAAAACAGGAAGGCAAAATGCCGAAAAAA
 AGGAAATAAGGGCGACACGAAATGTTGAATACTCATACTCTCCTTTCAATATT
 ATTGAAGCATTATCAGGGTATTGTCTCATGACCAAAATCCCTAACGTGAGTTTC
 20 GTTCCACTGAGCGTCAGACCCGTAGAAAAGATCAAAGGATCTTCTGAGATCCTT
 TTTCTGCGCGTAATCTGCTGCTGCAAACAAAAACCCACCGCTACCAGCGGTGGT
 TTGTTGCCGGATCAAGAGCTACCAACTCTTCCGAAGGTAACTGGCTTCAGCAG
 25 AGCGCAGATAACAAATACTGTTCTCTAGTGTAGCCGTAGTTAGGCCACCACTCAA
 GAACTCTGTAGCACCGCCTACATACCTCGCTTGCTAACCTGTTACCAAGTGGCTGTT
 30 GCCAGTGGCGATAAGTCGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGAT
 AAGGCGCAGCGGTCGGCTGAACGGGGGTTCGTGCACACAGCCCAGCTGGAGCG
 AACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGC
 35 TTCCCGAAGGGAGAAAGGCGGACAGGTATCCGTAAGCGGCAGGGTCGGAACAGG
 AGAGCGCACGAGGGAGCTCCAGGGGAAACGCCCTGGTATCTTATAGCCTGTCG
 GGTTGCCACCTCTGACTTGAGCGTCGATTGTGATGCTCGTCAGGGGGCGGA
 40 GCCTATGGAAAAACGCCAGCAACGCCCTTTACGGTCTGGCCTTGCTGGC
 CTTTGCTCACATGTTCTTCTCGTTACCCCTGATTCTGTGGATAACCGTATTACC
 GCCTTGAGTGAGCTGATACCGCTGCCGAGCCGAACGACCGAGCGCAGCGAGTC
 45 AGTGAGCGAGGAAGCGGTGCTGAGGCTGACATGATTGGTGCATGTTGTATGA
 AGCTACAGGACTGATTGGCGGGCTATGAGGGGGGGAAAGCTCTGGAAGGGCCGC
 GATGGGGCGCGCGCGTCCAGAAGGCCATACGGCCCTGGCGCACCCATCCG
 50 GTATAAAAGCCCGCACCCGAACGGTGACCTCCACTTCAGCGACAAACGAGCAC
 TTATACATACCGGACTATTCTGCCGCTATACATAACCACCTCAGCTAGCTTAAGATCC
 CATCAAGCTTGCATGCCGGCGCCAGAAGGAGCGCAGCCAAACCAGGATGATGT
 55

TTGATGGGTATTGAGCACTGCAACCTTATCCGAAGCCCCCTGGCCCACAAAG
 GCTAGGCGCCAATGCAAGCAGTCGATGCAGCCCCCTGGAGCGGTGCCCTCTGAT
 5 AAACCGGCCAGGGGGCTATGTTCTTACTTTTACAAGAGAAGTCACTCAACATC
 TTAAAATGCCAGGTGAGTCGACGAGCAAGCCGGCGATCAGGCAGCGTGCTTGC
 AGATTGACTTGCACGCCGCATTGTGTCGACGAAGGCTTTGGCTCCTCTGCGCT
 10 GTCTCAAGCAGCATCTAACCCCTCGTCGCCGTTCCATTGCAGGAGATTGAGGTA
 CCATGTACCCATACGATGTTCCAGATTACGCTTCGCCGAAGAAAAAGCGCAAGGTC
 GAAGCGTCCGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGG
 15 CTGGGCCGTGATCACCGACGAGTACAAGGTGCCAGCAAGAAATTCAAGGTGCTGG
 GCAACACCGACCGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTGAC
 AGCGCGAAACAGCCAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACA
 20 CCAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGCAACGAGATGGCC
 AAGGTGGACGACAGCTTCTCACAGACTGGAAGAGTCCTCCTGGTGGAAAGAGGA
 TAAGAACGACGAGCGGCACCCATCTCGGCAACATCGTGGACGAGGTGGCTTACCC
 25 ACGAGAAGTACCCACCACCATCTACCACCTGAGAAAGAAACTGGTGGACAGCACCAC
 AAGGCCGACCTCGGGCTGATCTATCTGCCCTGGCCCACATGATCAAGTCCGGGGC
 CACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAGCGACGTGGACAAGCTGTT
 30 CATCCAGCTGGTGCAGACCTACAACCAGCTGTCAGGAAAACCCCATCAACGCCA
 GCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGACGGCTG
 35 GAAAATCTGATCGCCCAGCTGCCCGCGAGAAGAAGAATGGCCTGTCGGCAACCT
 GATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGACAACCTGC
 GGATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGACGACCTGGACAACCTGC
 40 TGGCCCAGATCGCGACCAGTACGCCGACCTGTTCTGCCGCCAAGAACCTGTCCG
 ACGCCATCCTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCC
 CTGAGCGCCTCTATGATCAAGAGATACGACGAGCACCACCGAGCTGACCTGCT
 45 GAAAGCTCTCGTGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTCTCGACC
 AGAGCAAGAACGGCTACGCCGGCTACATTGACGGCGAGCCAGCCAGGAAGAGTTC
 TACAAGTTCATCAAGCCCACCTGGAAAAGATGGACGGCACCGAGGAACCTGCTCGT
 50 GAAGCTGAACAGAGAGGGACCTGCTGCCAAGCAGCGGACCTCGACAACGGCAGC
 ATCCCCCACCAGATCCACCTGGAGAGCTGCACGCCATTCTGCCGGCAGGAAGA
 TTTTACCCATTCTGAAGGACAACCGGGAAAAGATCGAGAAGATCCTGACCTTCCG
 55

CATCCCCTACTACGTGGGCCCTCTGGCCAGGGAAACAGCAGATTGCCCTGGATGA
 CCAGAAAGAGCGAGGAAACCATCACCCCTGGAACCTCGAGGAAGTGGTGGACAA
 5 GGGCGCTTCCGCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAGAACCTGC
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 AACGAGCTGACCAAAGTGAATACGTGACCGAGGGATGAGAAAGCCGCCTCCT
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[0160] For all modified *Chlamydomonas reinhardtii* cells, Applicants use PCR, SURVEYOR nuclease assay, and DNA sequencing to verify successful modification.

[0161] 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. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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[0162]

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[0163] Aspects of the invention may also be defined by the following numbered clauses.

25 1. A method of altering expression of one or more gene products comprising introducing into a cell containing and expressing DNA molecules encoding the one or more gene products an engineered, non-naturally occurring CRISPR-Cas system comprising a Cas protein and one or more guide RNAs that target the DNA molecules, whereby the one or more guide RNAs target the genomic loci of the DNA molecules encoding the one or more gene products and the Cas protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered; and, wherein the Cas protein and the guide RNA do not naturally occur together.

30 2. A method of altering expression of one or more gene products comprising introducing into a cell containing and expressing DNA molecules encoding the one or more gene products an engineered, non-naturally occurring vector system comprising one or more vectors comprising:

35 a) a first regulatory element operably linked to one or more CRISPR-Cas system guide RNAs that hybridize with target sequences in genomic loci of the DNA molecules encoding the one or more gene products,

40 b) a second regulatory element operably linked to a Cas protein, wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the genomic loci of the DNA molecules encoding the one or more gene products and the Cas protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered; and, wherein the Cas protein and the guide RNAs do not naturally occur together.

45 3. The method of clause 1 or clause 2, wherein the expression of two or more gene products is altered.

50 4. The method of any preceding clause, wherein the vectors or the Cas protein of the system further comprise one or more NLS(s).

55 5. The method of any preceding clause, wherein the guide RNAs comprise a guide sequence fused to a tracr sequence.

6. The method of any preceding clause, wherein the cell is a eukaryotic cell.

7. The method of clause 6, wherein the eukaryotic cell is a mammalian cell.

8. The method of clause 7, wherein the mammalian cell is a human cell.
9. The method of any preceding clause, wherein the Cas protein is a Cas9 protein.
- 5 10. The method of any preceding clause, wherein the Cas protein is codon optimized for expression in a eukaryotic cell.
11. The method of any preceding clause, wherein the expression of the gene product is decreased.
- 10 12. The method of any preceding clause, wherein the gene product is a protein.
13. The method of any preceding clause, wherein the introducing into the cell is by a delivery system comprising viral particles, liposomes, electroporation, microinjection or conjugation.
- 15 14. An engineered, non-naturally occurring vector system comprising one or more vectors comprising:
 - a) a first regulatory element operably linked to one or more CRISPR-Cas system guide RNAs that hybridize with target sequences in genomic loci of DNA molecules encoding one or more gene products,
 - 20 b) a second regulatory element operably linked to a Cas protein,

wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the genomic loci of the DNA molecules encoding the one or more gene products in a cell and the Cas protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered; and, wherein the Cas protein and the guide RNAs do not naturally occur together.

- 30 15. An engineered, programmable, non-naturally occurring CRISPR-Cas system comprising a Cas protein and one or more guide RNAs that target the genomic loci of DNA molecules encoding one or more gene products in a cell and the Cas protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered; and, wherein the Cas protein and the guide RNAs do not naturally occur together.
- 35 16. The system of clause 14 or 15, wherein the expression of two or more gene products is altered.
- 17. The system of any of clauses 14 to 16, wherein the system further comprises one or more NLS(s).
- 40 18. The system of any of clauses 14 to 17, wherein the guide RNAs comprise a guide sequence fused to a tracr sequence.
- 19. The system of any of clauses 14 to 18, wherein the cell is a eukaryotic cell.
- 20. The system of clause 19, wherein the eukaryotic cell is a mammalian cell.
- 45 21. The system of clause 20, wherein the mammalian cell is a human cell.
- 22. The system of any of clauses 14 to 21, wherein the CRISPR-Cas system is a Type II CRISPR-Cas system.
- 50 23. The system of any of clauses 14 to 22, wherein the Cas protein is a Cas9 protein.
- 24. The system of any of clauses 14 to 23, wherein the Cas protein is codon optimized for expression in a eukaryotic cell.
- 55 25. The system of any of clauses 14 to 24, wherein the expression of the gene product is decreased.
- 26. The system of any of clauses 14 to 25, wherein the gene product is a protein.
- 27. The system of any of clauses 14 to 26, wherein the system is introduced into the cell by a delivery system

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comprising viral particles, liposomes, electroporation, microinjection or conjugation.

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40	<211> 50						
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25 <210> 29
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<213> Homo sapiens

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30 <210> 30
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35 <400> 30
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<400> 32
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55 <210> 33
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20 <210> 39
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45  <400> 45
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50  <400> 51
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55  <210> 52
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	<400> 60 aatgcatagg gtaccaccca caggtgccag ggg	33
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	<211> 37	
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      tttttt      186

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35 cauuuuuauugg caggguguuu ucguuuauuu a	91	
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45 tacctccctcc	70	
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55 cattgccacg aagcaggcca atggggagga catcgatgtc acctccaatg actagggtgg	120	

gc

122

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54

40 **Claims**

1. A Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) vector system comprising one or more vectors comprising:
 - a) a first regulatory element operably linked to one or more nucleotide sequences encoding one or more engineered CRISPR-Cas system guide RNAs, wherein the guide RNA hybridizes with a target sequence in a polynucleotide in a eukaryotic cell, and the guide RNA comprises a guide sequence, a tracr sequence, and a tracr mate sequence, wherein the tracr sequence comprises 30 or more nucleotides in length;
 - b) a second regulatory element operably linked to a nucleotide sequence encoding Type II Cas9 protein; wherein components (a) and (b) are located on the same or different vectors of the system, whereby transcription of the guide RNA and expression of the Cas9 protein *in vivo* in a eukaryotic cell promotes formation of a CRISPR complex at the target sequence, wherein the target sequence is associated with a PAM (protospacer adjacent motif) sequence recognized by the CRISPR complex, whereby formation of the CRISPR complex at the target sequence modifies the polynucleotide; and, wherein the Cas9 protein and the one or more guide RNAs do not naturally occur together.
2. A system according to claim 1 wherein the tracr sequence comprises 40 or more nucleotides in length.

3. A system according to claim 1 wherein the tracr sequence comprises 50 or more nucleotides in length.
4. A system according to claim 1 wherein the tracr sequence comprises 67 nucleotides in length.
5. A system according to claim 1 wherein the tracr sequence comprises 75 nucleotides in length,
6. A system according to claim 1 wherein the tracr sequence comprises 89 nucleotides in length
7. A system according to any one of claims 1 to 6 wherein the Cas9 protein comprises a nuclear localization signal (NLS).
8. A system according to any one of claims 1 to 7, wherein the Cas9 protein comprises one or more mutations in a catalytic domain, such that the mutated Cas9 protein lacks the ability to cleave one strand of the polynucleotide, and is a nickase.
9. The system according to any one of claims 1 to 8, wherein the one or more vectors comprise one or more viral vectors.
10. The system of claim 9, wherein the one or more viral vectors comprise one or more retroviral, lentiviral, adenoviral, adeno-associated or herpes simplex viral vectors.
11. The system according to any one of claims 1 to 10 wherein the Cas9 protein comprises one or more mutations in the RuvC I, RuvC II or RuvC III catalytic domains.
12. The system according to any one of claims 1 to 10 wherein the Cas9 protein comprises a mutation selected from the group consisting of D10A, H840A, N854A and N863A with reference to the position numbering of a *Streptococcus pyogenes* Cas9 (SpCas9) protein.
13. The system of any preceding claim, wherein the guide RNA is a chimeric RNA comprising the guide sequence, the tracr sequence, and a tracr mate sequence.
14. The system of claim 13, wherein hybridization between the tracr sequence and the tracr mate sequence produces a transcript having a secondary structure.
15. The system of claim 14, wherein the secondary structure comprises a hairpin.
16. The system of any preceding claim, wherein the eukaryotic cell is a mammalian cell.
17. The system of claim 16 wherein the mammalian cell is a human cell.
18. The system of any preceding claim, wherein the nucleotide sequence encoding the Cas9 protein is codon optimized for expression in a eukaryotic cell.
19. The system of any preceding claim, wherein a repair template is inserted into the cleaved polynucleotide.
20. The system of any preceding claim, wherein (a) and (b) are on the same vector.
21. The system of any one of claims 1 to 19 wherein (a) and (b) are on different vectors.
22. The system of any one of claims 1 to 7, 9, 10, 13 to 21 wherein the Cas9 protein is a nuclease.
23. A method of altering a target sequence in a polynucleotide in a eukaryotic cell, comprising introducing into the eukaryotic cell the system according to any one of claims 1 to 22, wherein the guide RNA hybridizes with the target sequence and the method is not a method for treatment of the human or animal body by therapy and is not a process for modifying the germ line genetic identity of human beings.
24. A method of altering expression of at least one gene product of a polynucleotide of a eukaryotic cell, comprising introducing the system according to any one of claims 1 to 22 into the eukaryotic cell, wherein the polynucleotide contains a target sequence and the guide RNA hybridizes with the target sequence and the method is not a method for treatment of the human or animal body by therapy and is not a process for modifying the germ line genetic identity

of human beings.

25. Use of the system of any of claims 1 to 22:

5 a) for site-specific gene knockout;
 b) for site-specific genome editing;
 c) for DNA sequence-specific interference; or
 d) for multiplexed genome engineering;

10 provided the use does not comprise a process for modifying the germ line genetic identity of human beings; and
 wherein the use i) is in vitro or ex vivo; or ii) is not a method for treatment of the human or animal body by therapy.

15 26. The use of claim 25 wherein the use further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide.

20 27. Use of the system of any of claims 1 to 22 in the production of a non-human transgenic animal or transgenic plant, provided that the use is not a method for treatment of the animal body by therapy.

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55

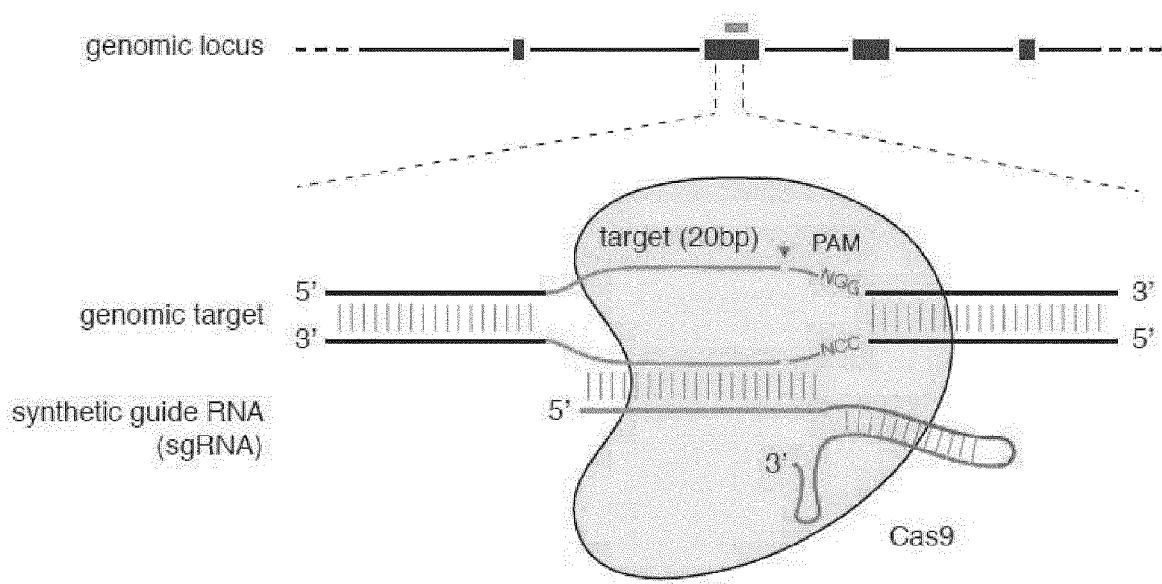


FIG. 1

A

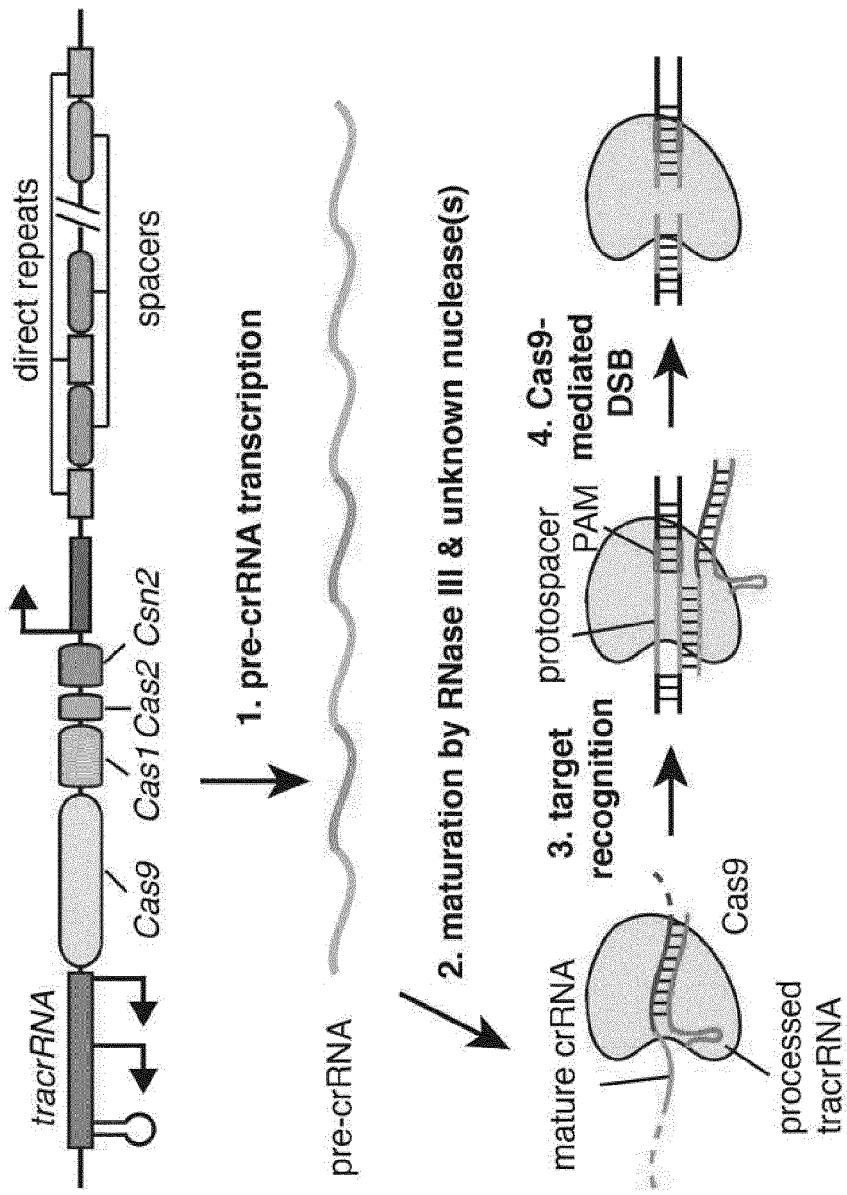
Streptococcus pyogenes SF370 CRISPR locus 1

FIG. 2A

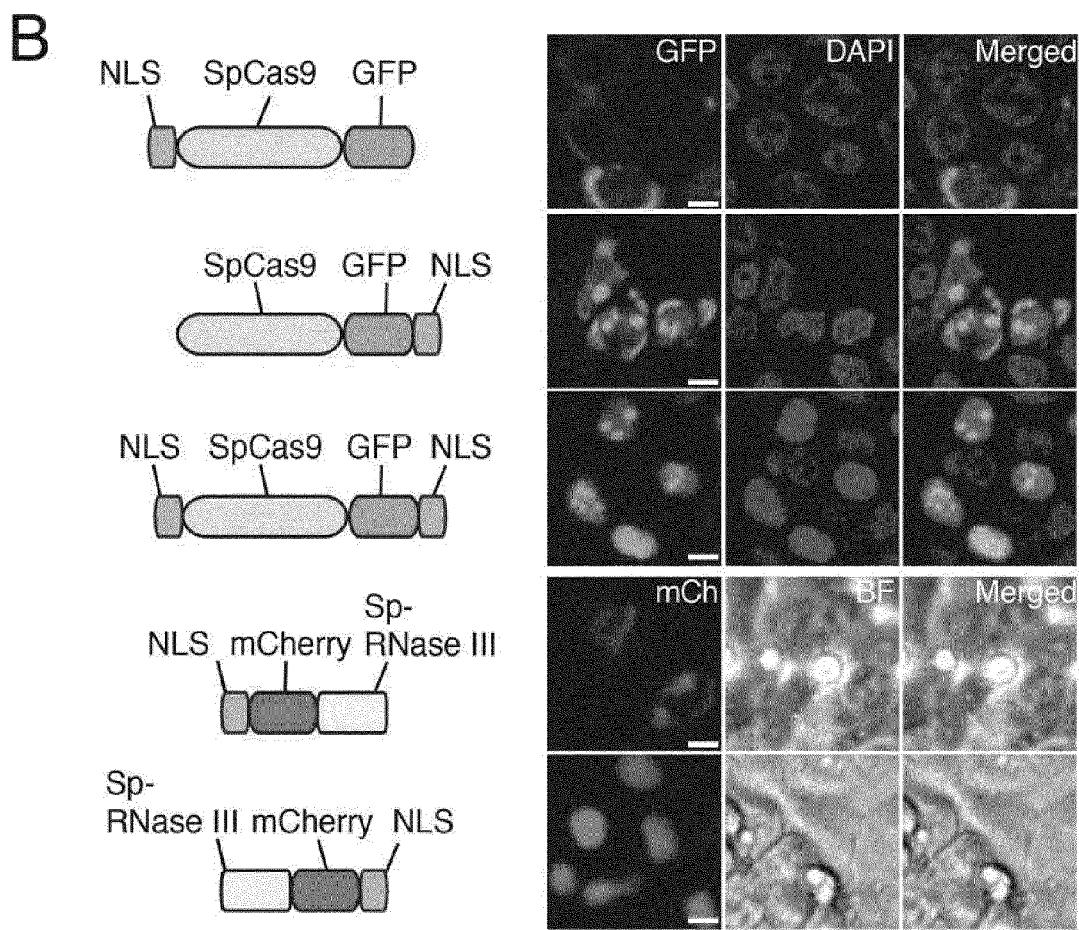
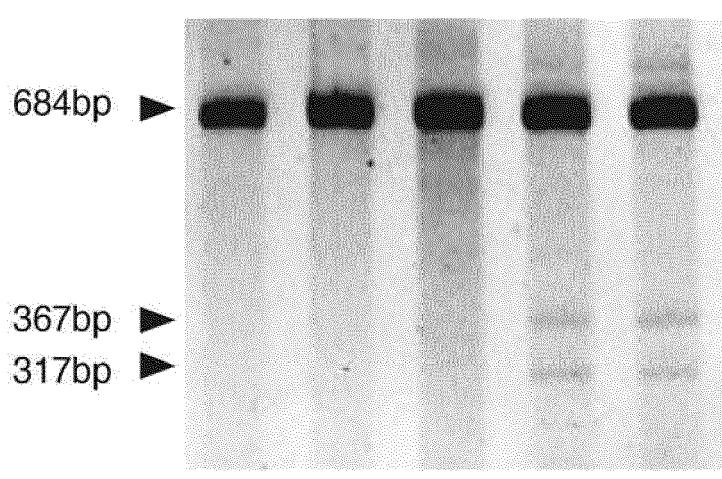
**FIG. 2B**

FIG. 2C

D

2xNLS-SpCas9	+	+	+	+	+
SpRNase III	-	+	+	-	+
short tracrRNA	-	+	-	+	+
DR-EMX1-DR	+	-	+	+	+



indel (%): 4.7 5.0

FIG. 2D

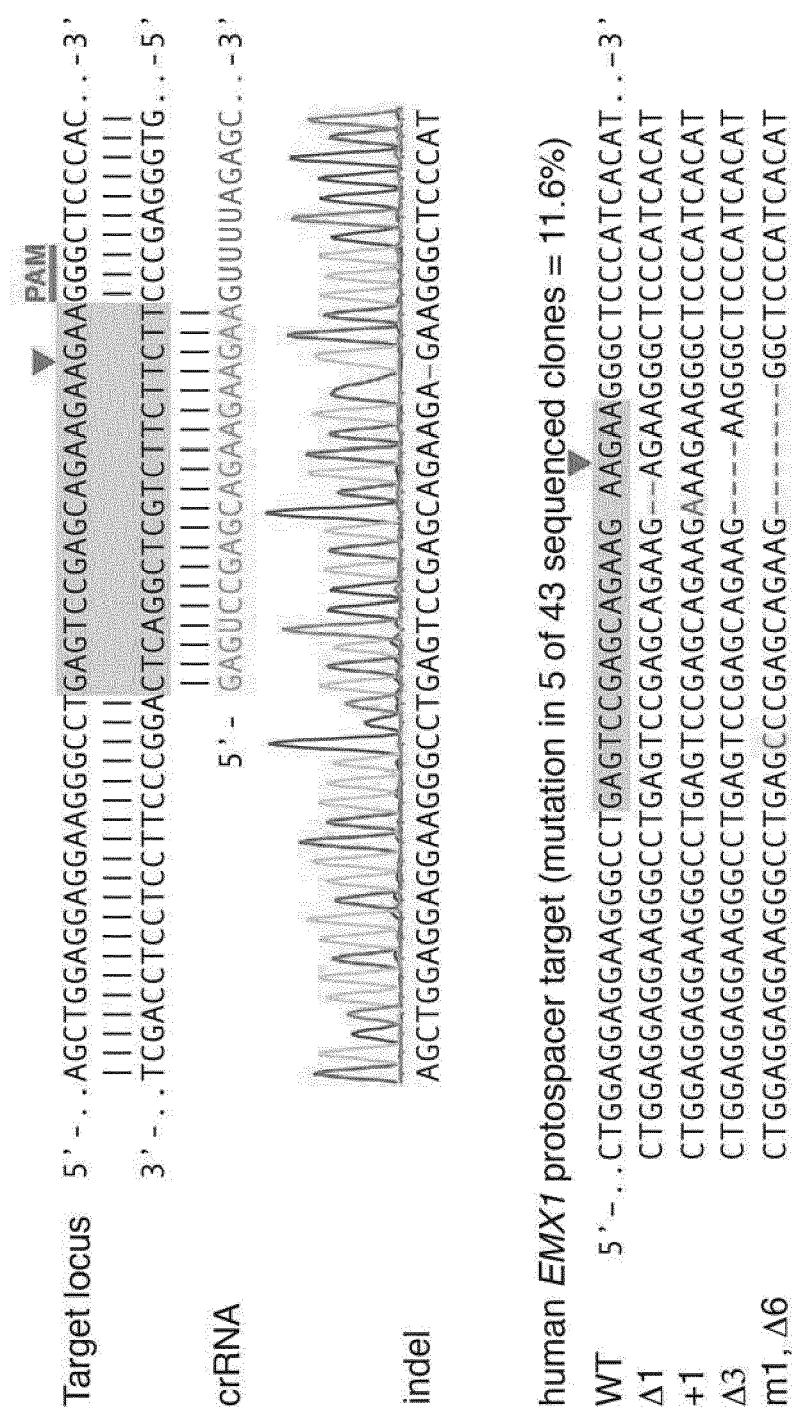


FIG. 2E-F

A

human *EMX1* locus

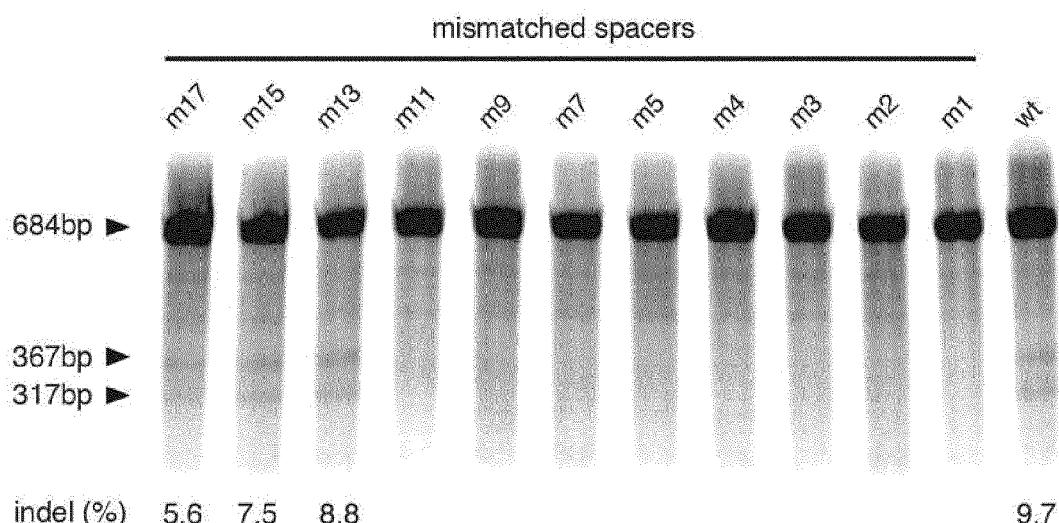
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 3' - ...GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCAGGGTA... -5'

protospacer (1) ▼ PAM

wt crRNA 5' - GAGUCCGAGCAGAAGAAGAAGUUUUAGAGC... -3'

m1 GAGUCCGAGCAGAAGAAGAU
 m2 GAGUCCGAGCAGAAGAAGUA
 m3 GAGUCCGAGCAGAAGAACAA
 m4 GAGUCCGAGCAGAAGAUGAA
 m5 GAGUCCGAGCAGAAGUAGAA
 m7 GAGUCCGAGCAGAUGAAGAA
 m9 GAGUCCGAGCACAAGAAGAA
 m11 GAGUCCGAGGAGAAGAAGAA
 m13 GAGUCCGAGCAGAAGAAGAA
 m15 GAGUCGGAGCAGAAGAAGAA
 m17 GAGACCGAGCAGAAGAAGAA

mismatch-containing guide sequences

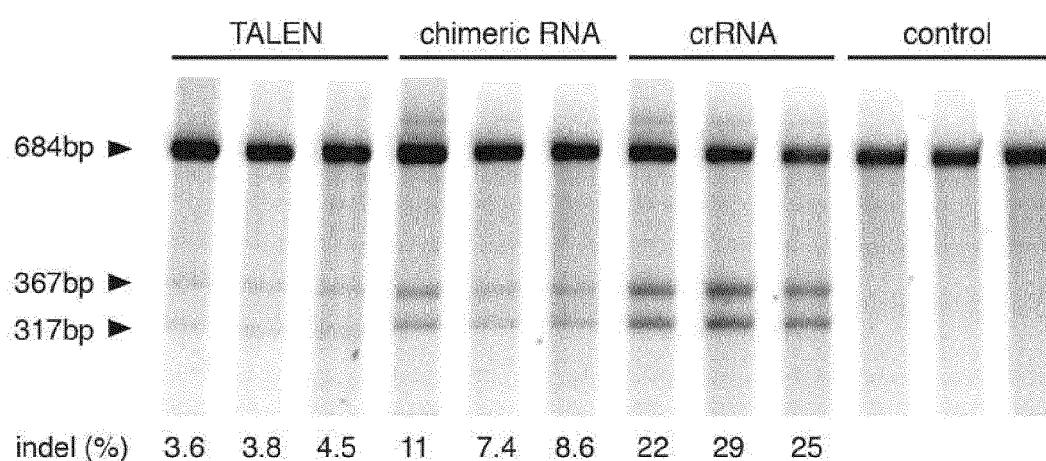
B**C**

left TALEN binding site protospacer (1) ▼ PAM right TALEN binding site

human *EMX1* locus

5' - ...CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCAT... -3'
 3' - ...GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCAGGGTA... -5'

FIG. 3A-C

D**FIG. 3D**

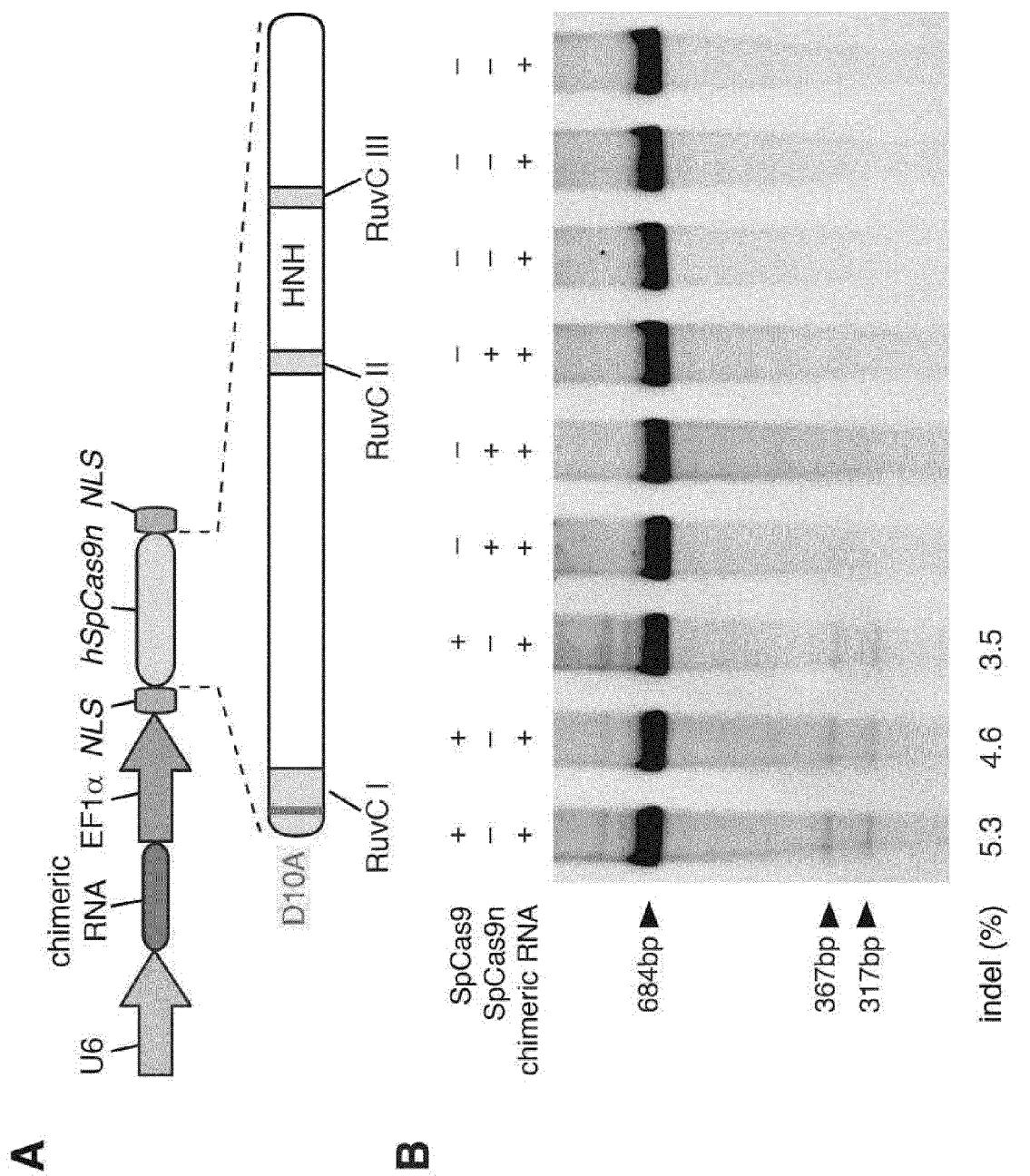


FIG. 4A-B

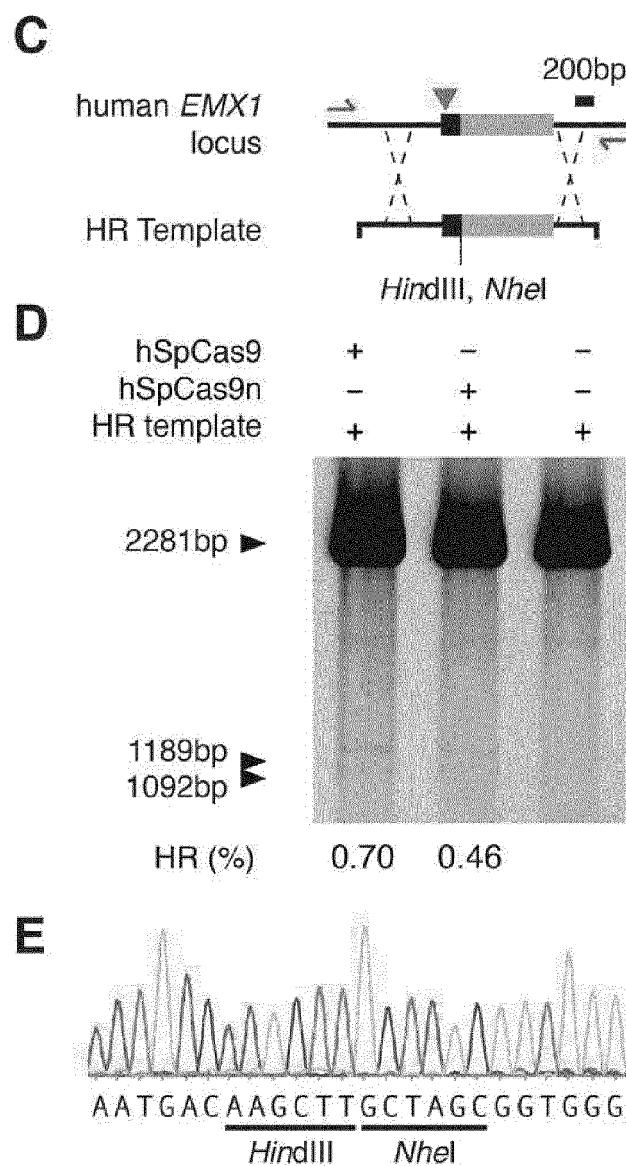


FIG. 4C-E

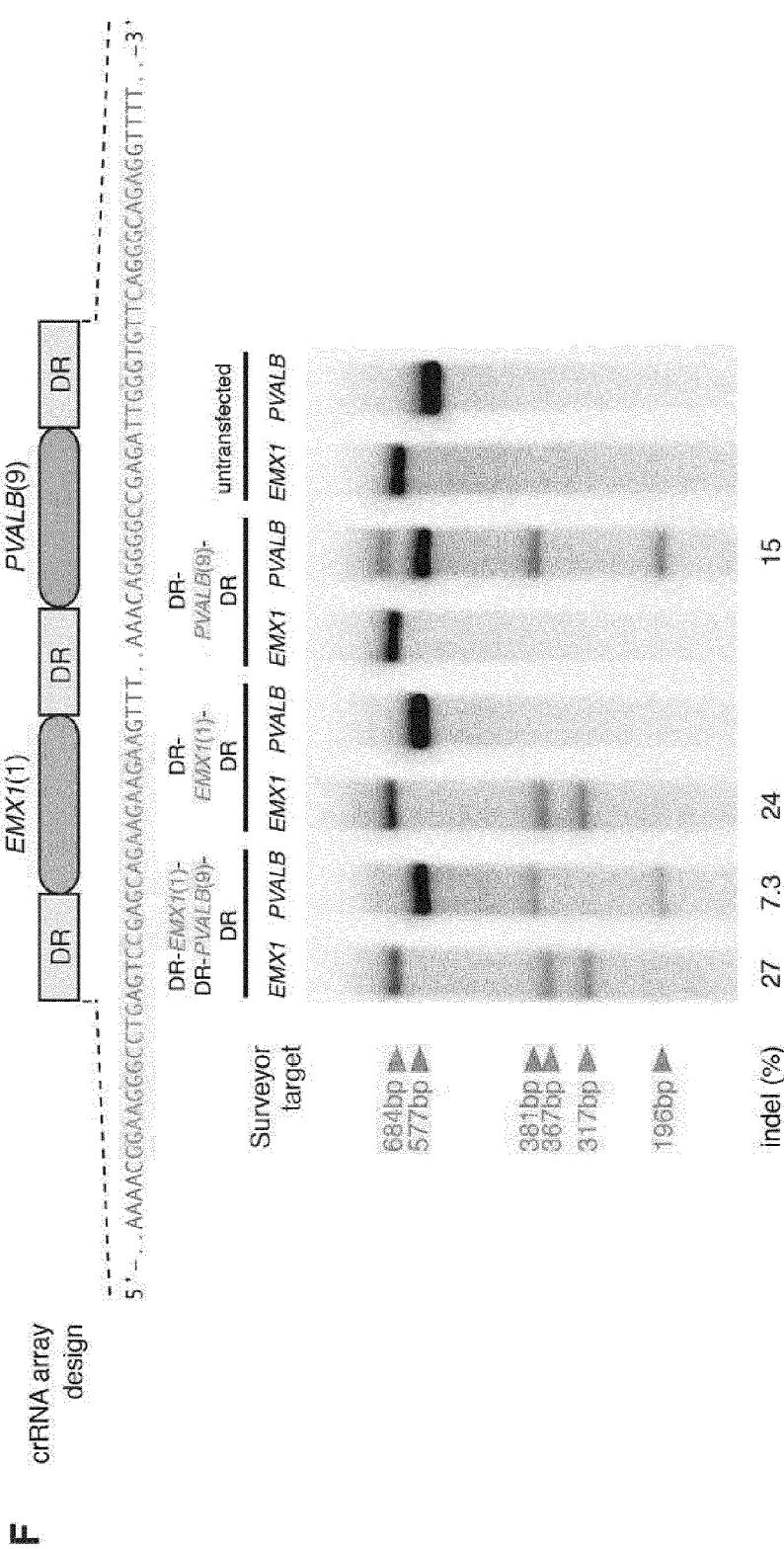


FIG. 4F

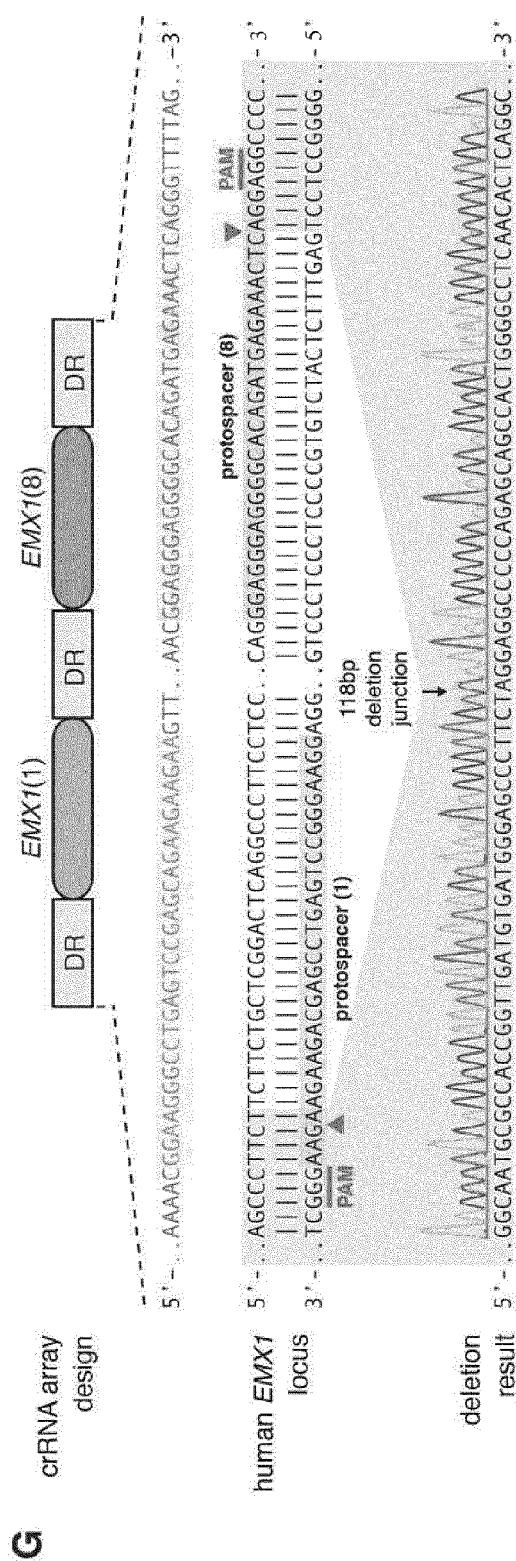
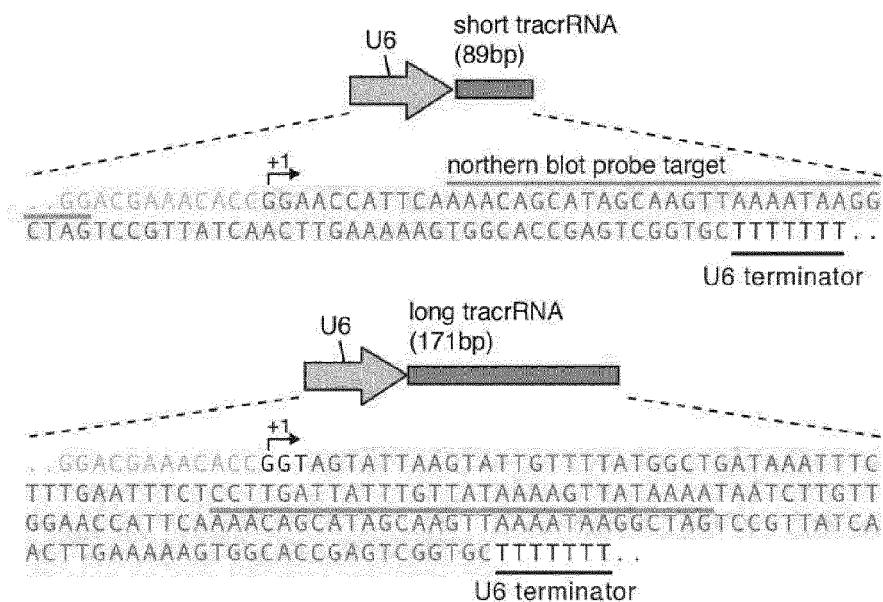
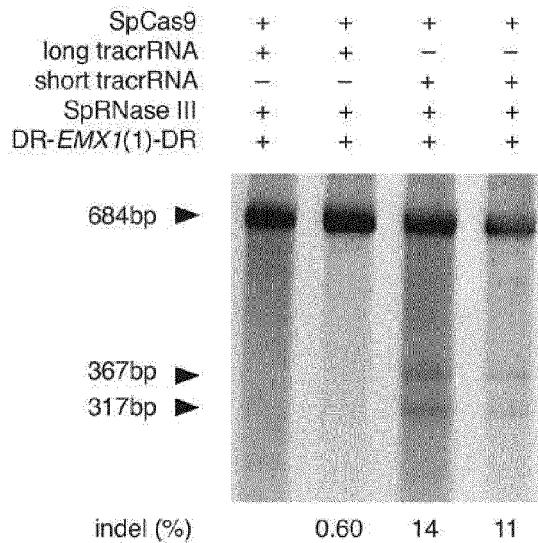


FIG. 4G

Cas9	target species	gene	protospacer ID	protospacer sequence (5' to 3')	PAM	strand	cell line tested	% indel (pre-crRNA + tracrRNA) (chimeric RNA)	% indel
Homo sapiens	EMX1	EMX1	1	GGAGGGCTGAGTCCGAGAGAGAA	GGG	+	293FT	20 ± 1.8	6.7 ± 0.62
			2	CATGGAGGTGACATCGATGTCCTCCAT	TGG	-	293FT	2.1 ± 0.31	N.D.
	EMX1	EMX1	3	GGACATCGATGTCACCTCAATGACTAGGG	TGG	+	293FT	14 ± 1.1	N.D.
			4	CATCGATGTCCTCCATGGCTGCTTG	TGG	-	293FT	11 ± 1.7	N.D.
	EMX1	EMX1	5	TTCGGCAATGGCCACCGGTTATGTTA	TGG	-	293FT	4.3 ± 0.46	2.1 ± 0.51
			6	TGTEGCAATGGCCACCGGTTATGTTA	GGG	-	293FT	4.0 ± 0.66	0.41 ± 0.25
	EMX1	EMX1	7	TCCAGCTTCGCGTTGACTTTGCTTC	GGG	-	293FT	1.5 ± 0.12	N.D.
			8	GGAGGGAGGGACAGATGAGAACTCAGG	AGG	-	293FT	7.8 ± 0.83	2.3 ± 1.2
S. pyogenes SF370 type II CRISPR	PVALB	PVALB	9	AGGGCCGAGATTGGGTGTCAGGGCAGAG	AGG	+	293FT	21 ± 2.6	6.5 ± 0.32
			10	ATGGAGGGGGGGGGGGGGGGGGGGGGAGAT	TGG	+	293FT	N.D.	N.D.
			11	GGGGGGAGGGGGGGGGGGGGGGGGGGGG	AGG	+	293FT	N.D.	N.D.
Mus musculus	Th	Th	12	CAAGCACTGAGTGGCATAGCTAAATGGAT	AGG	-	Neuro2A	27 ± 4.3	4.1 ± 2.2
			13	AATGCCATGGGTACCCACAGGTGGCAG	GGG	-	Neuro2A	4.8 ± 1.2	N.D.
			14	ACACACATGGGAAGCCCTGGGGCAAGGAA	AGG	+	Neuro2A	11.3 ± 1.3	N.D.
S. thermophilus LMD-9 CRISPR1	Homo sapiens	EMX1	15	GGAGGGAGGTAGTATACAGAAACAGAGAA	GTAGAA	-	293FT	14 ± 0.88	N.T.
			16	AGATGTAGGGAGTCAGAAACTCAGGA	CTAGAA	-	293FT	7.8 ± 0.77	N.T.

FIG. 5

A**B****FIG. 6A-B**

C

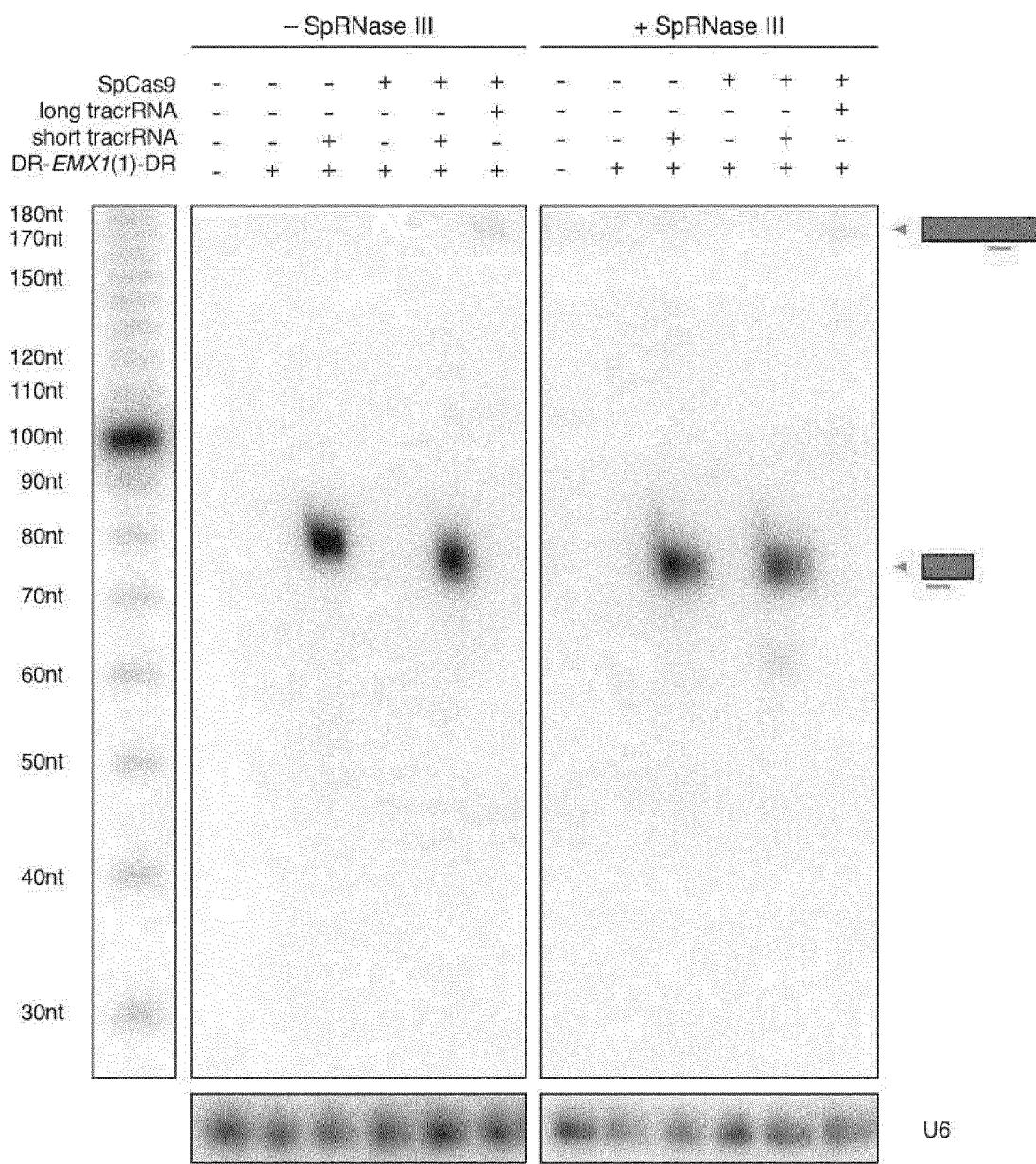
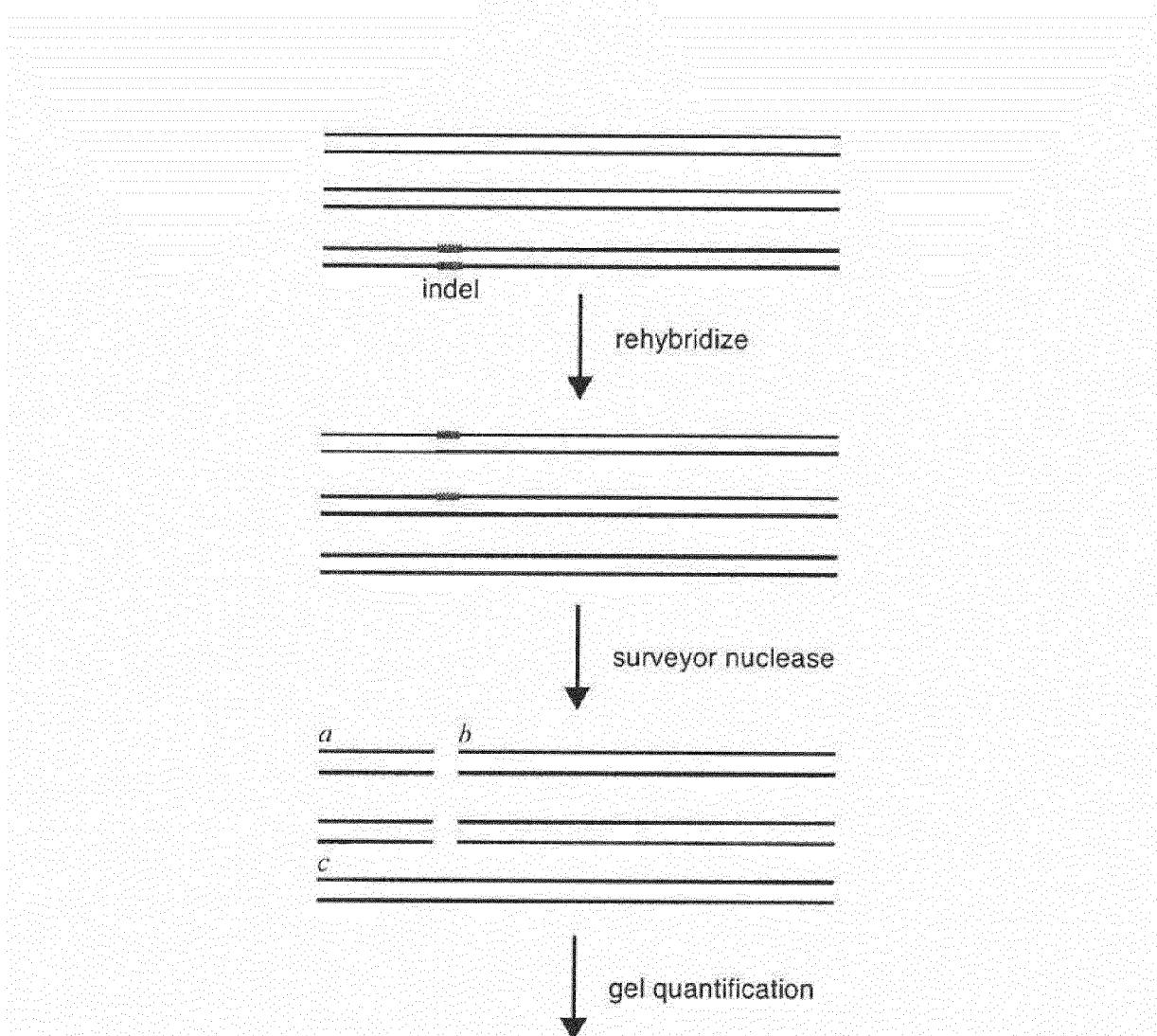


FIG. 6C



$$\% \text{ indel} = \left(1 - \sqrt{1 - (a + b)/(a + b + c)} \right) * 100$$

FIG. 7

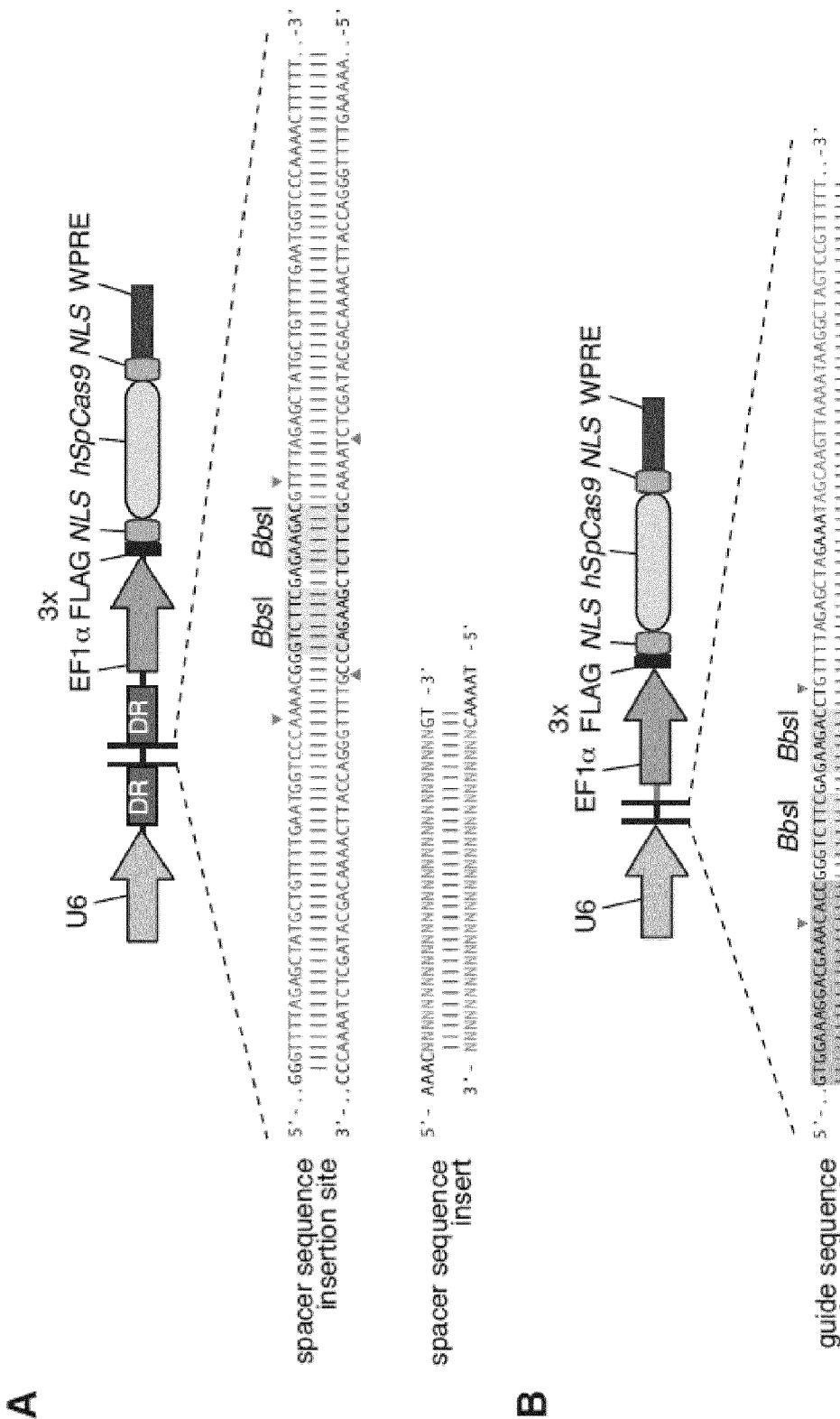


FIG. 8A-B

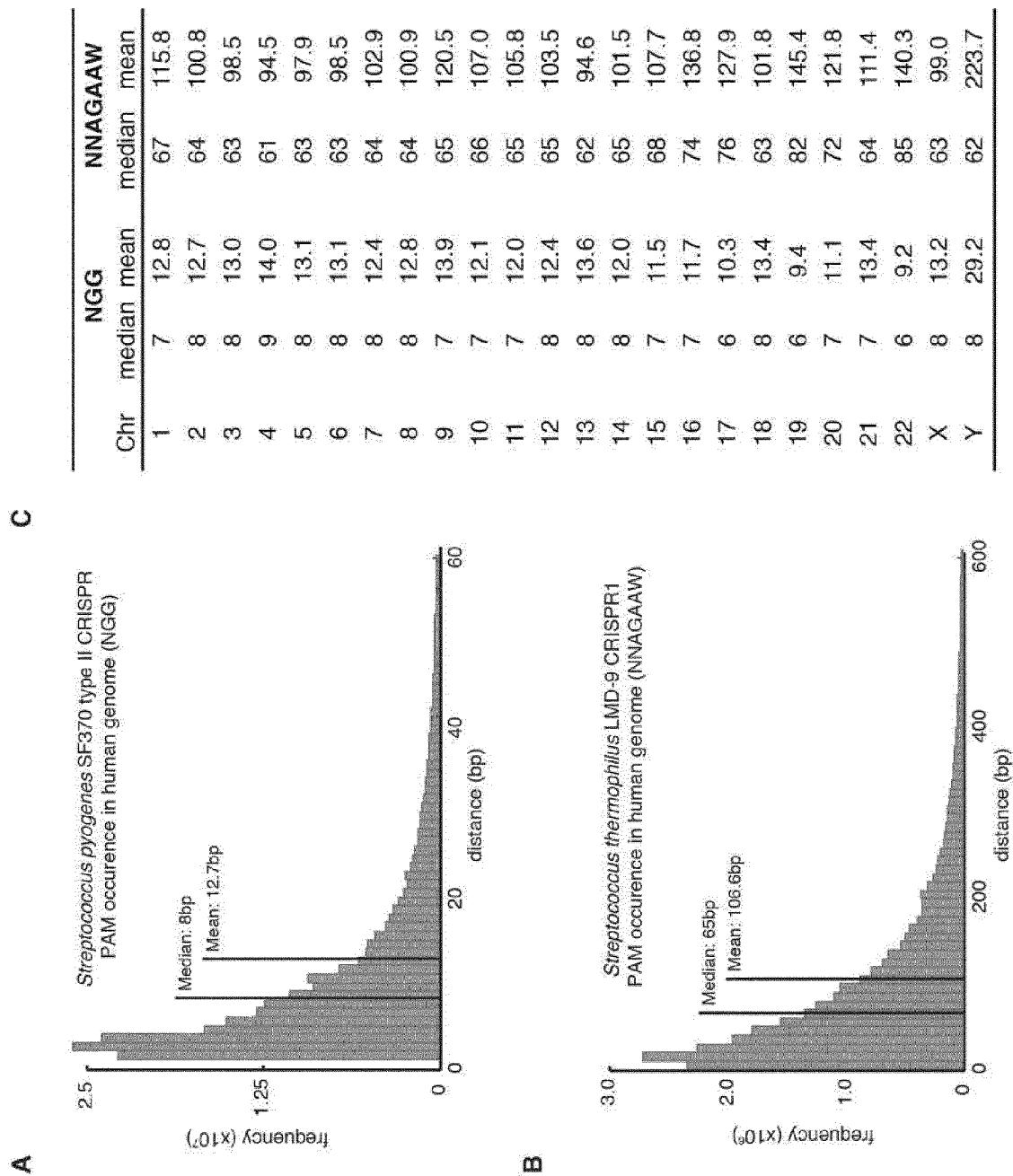


FIG. 9A-C

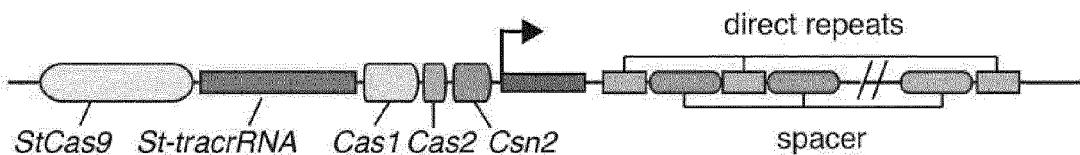
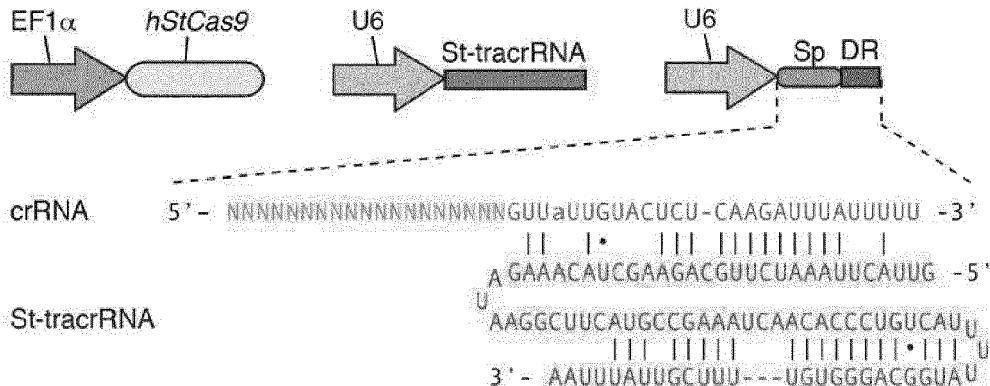
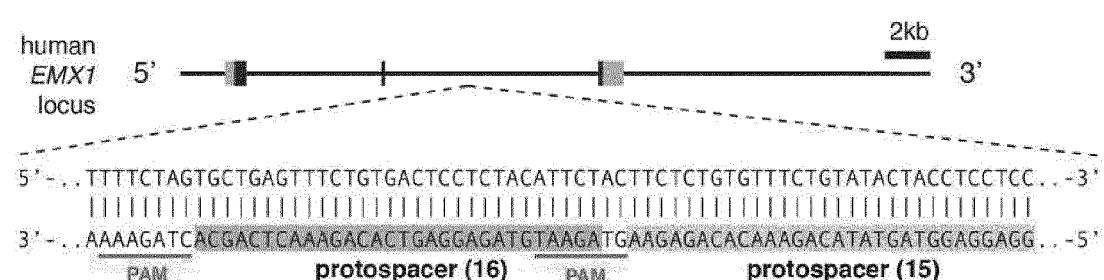
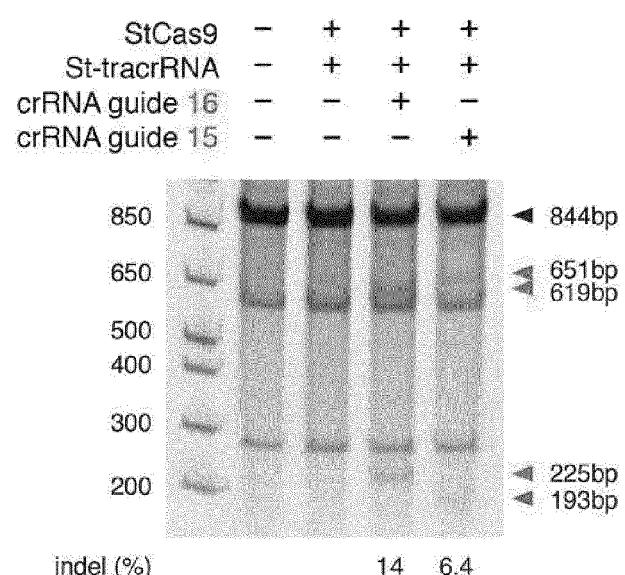
A *Streptococcus thermophilus* LMD-9 CRISPR1**B****C**

FIG. 10A-C

D**FIG. 10D**

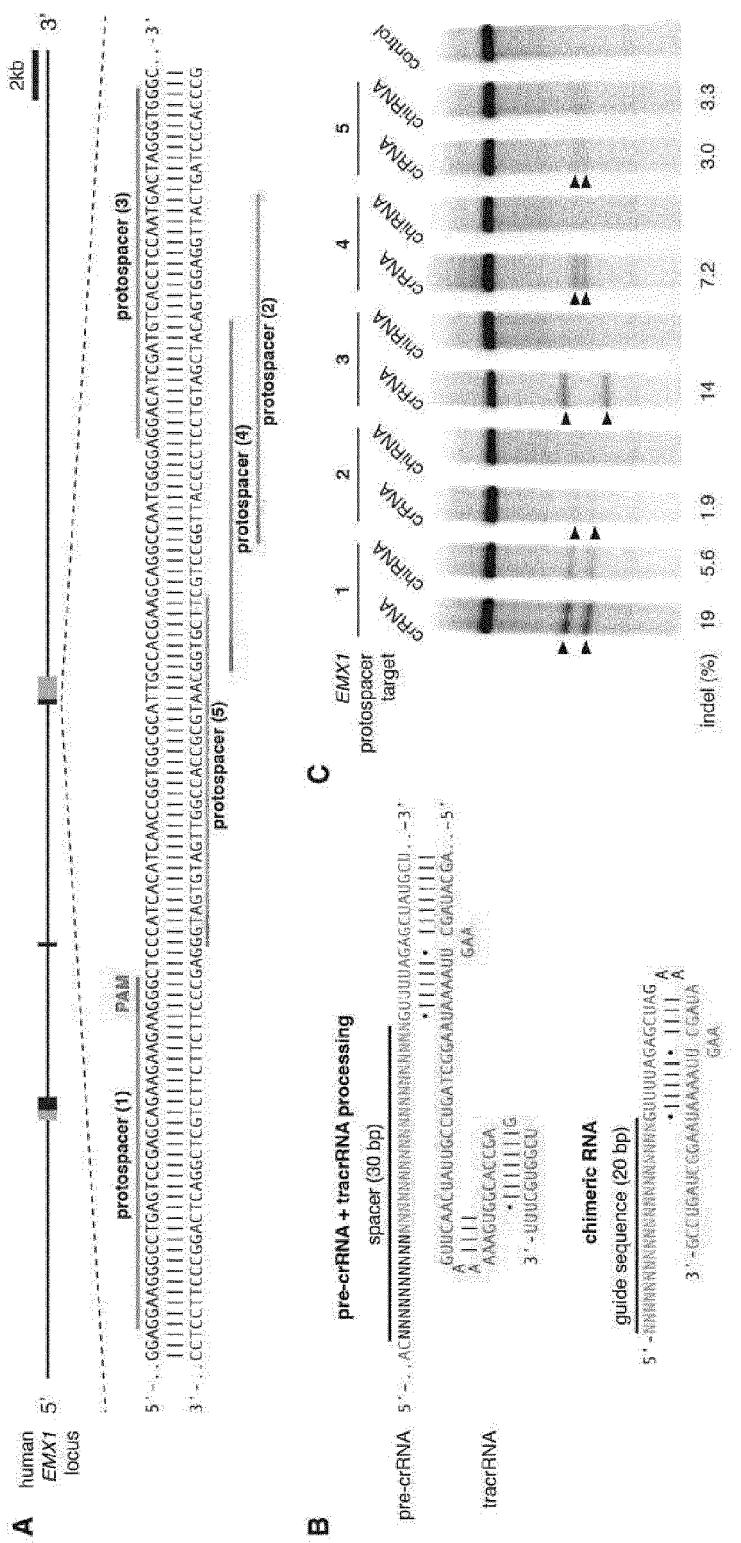
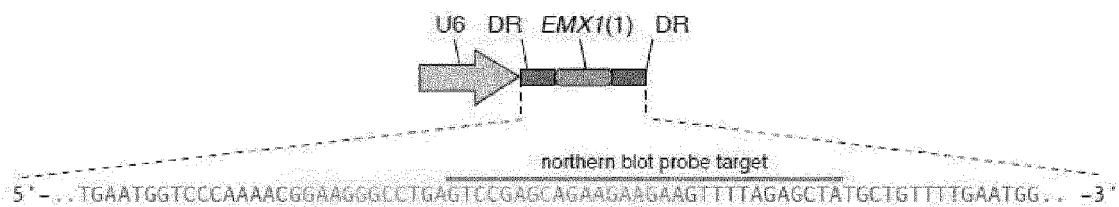
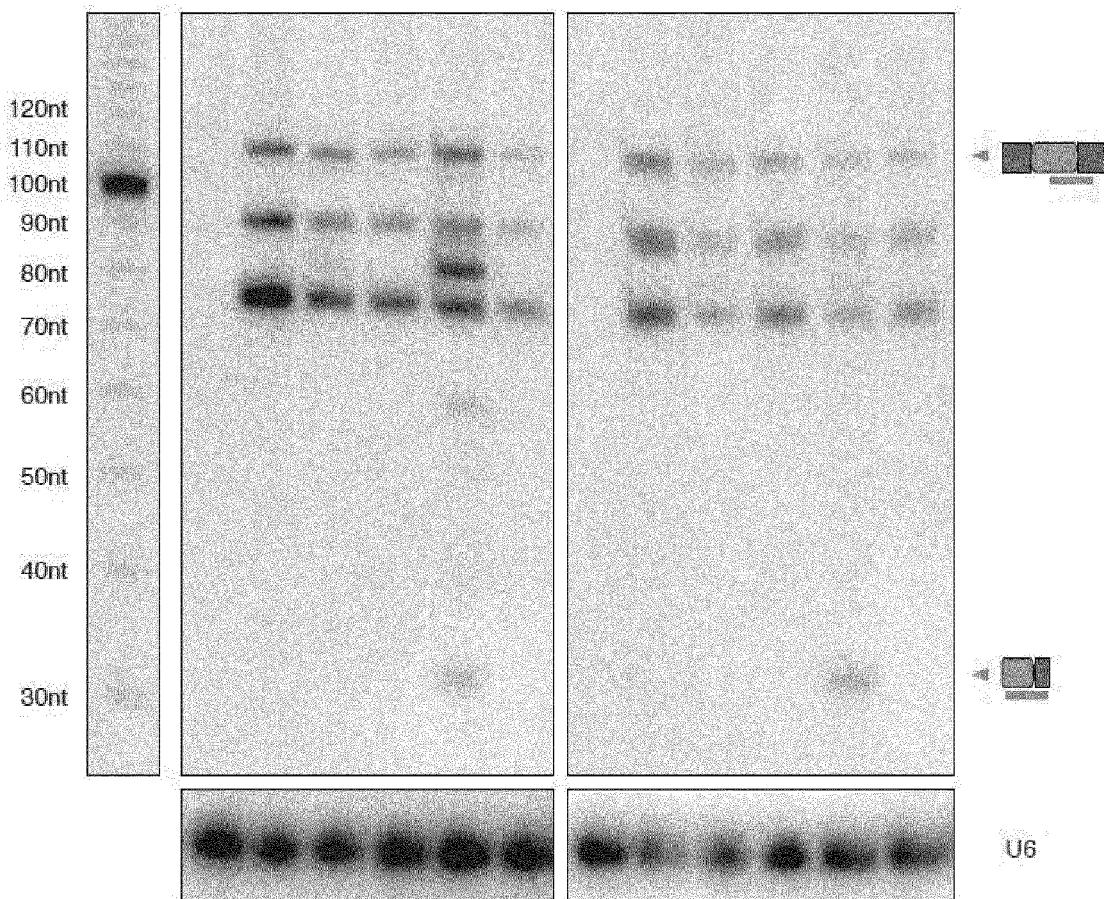


FIG. 11A-C

A**B**

	- SpRNase III						+ SpRNase III					
SpCas9	-	-	-	+	+	+	-	-	-	+	+	+
long tracrRNA	-	-	-	-	-	+	-	-	-	-	-	+
short tracrRNA	-	-	+	-	+	-	-	-	+	-	+	-
DR- <i>EMX1(1)</i> -DR	-	+	+	+	+	+	-	+	+	+	+	+

**FIG. 12A-B**

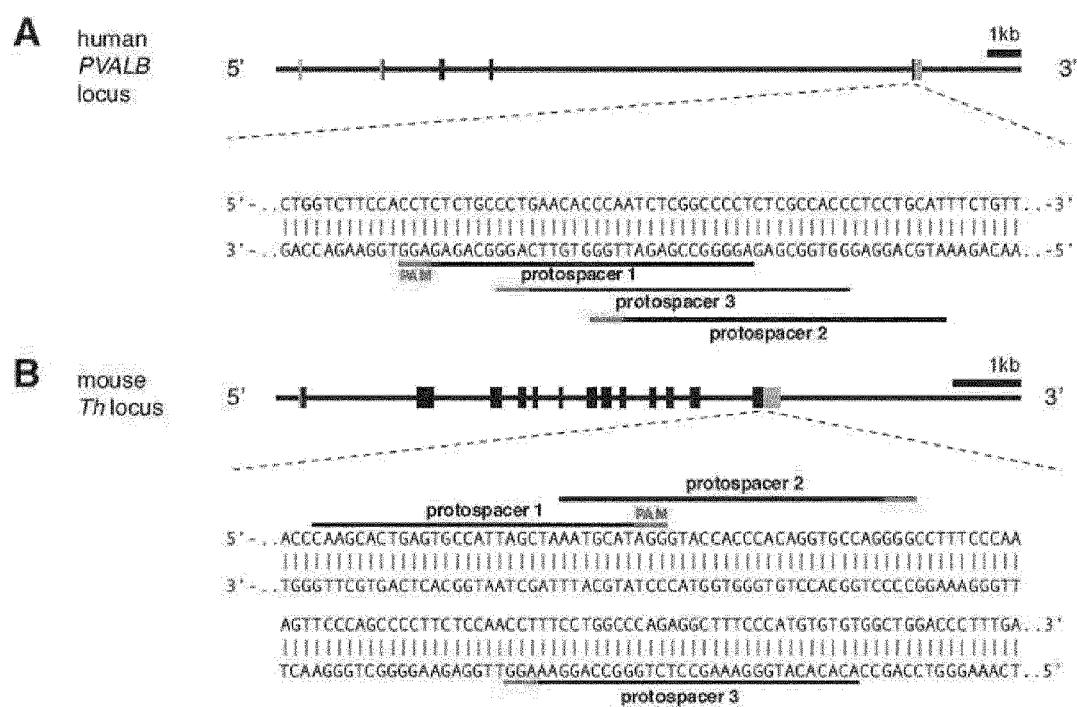


FIG. 13A-B

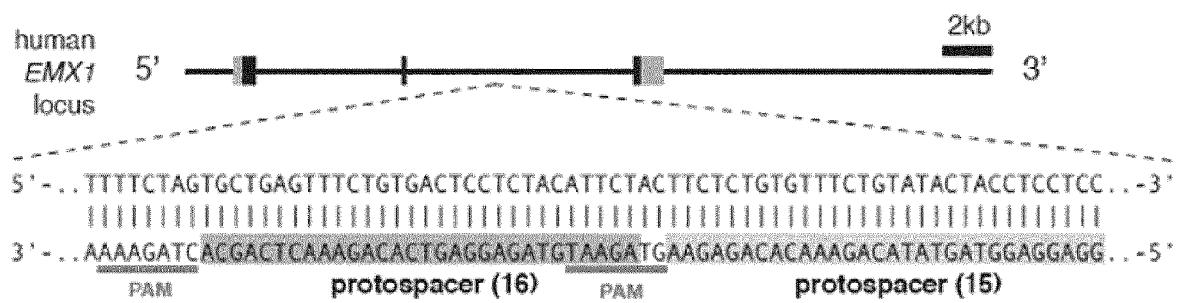


FIG. 14

Primer name	Assay	Genomic Target	Primer sequence
Sp-EMX1-F	SURVEYOR assay, sequencing	<i>EMX1</i>	AAAACCACCCCTCTCTCTGGC
Sp-EMX1-R	SURVEYOR assay, sequencing	<i>EMX1</i>	GGAGATTGGAGACACGGAGAG
Sp-PVALB-F	SURVEYOR assay, sequencing	<i>PVALB</i>	CTGGAAAGCCAATGCCTGAC
Sp-PVALB-R	SURVEYOR assay, sequencing	<i>PVALB</i>	GGCAGCAAACTCCTGTCCT
Sp-Th-F	SURVEYOR assay, sequencing	<i>Th</i>	GTGCTTGCAGAGGCCTACC
Sp-Th-R	SURVEYOR assay, sequencing	<i>Th</i>	CCTGGAGCGCATGCAGTAGT
St-EMX1-F	SURVEYOR assay, sequencing	<i>EMX1</i>	ACCTTCTGTGTTCCACCATTC
St-EMX1-R	SURVEYOR assay, sequencing	<i>EMX1</i>	TTGGGGAGTGCACAGACTTC
Sp-EMX1-RFLP-F	RFLP, sequencing	<i>EMX1</i>	GGCTCCCTGGGTTCAAAGTA
Sp-EMX1-RFLP-R	RFLP, sequencing	<i>EMX1</i>	AGAGGGGTCTGGATGTCGTA
Pb_EMX1_sp1	Northern Blot Probe	Not applicable	TAGCTCTAAACTCTTCTGCTCGGAC
Pb_tracrRNA	Northern Blot Probe	Not applicable	CTAGCCTTATTTAACTTGCTATGCTGTT

FIG. 15

2

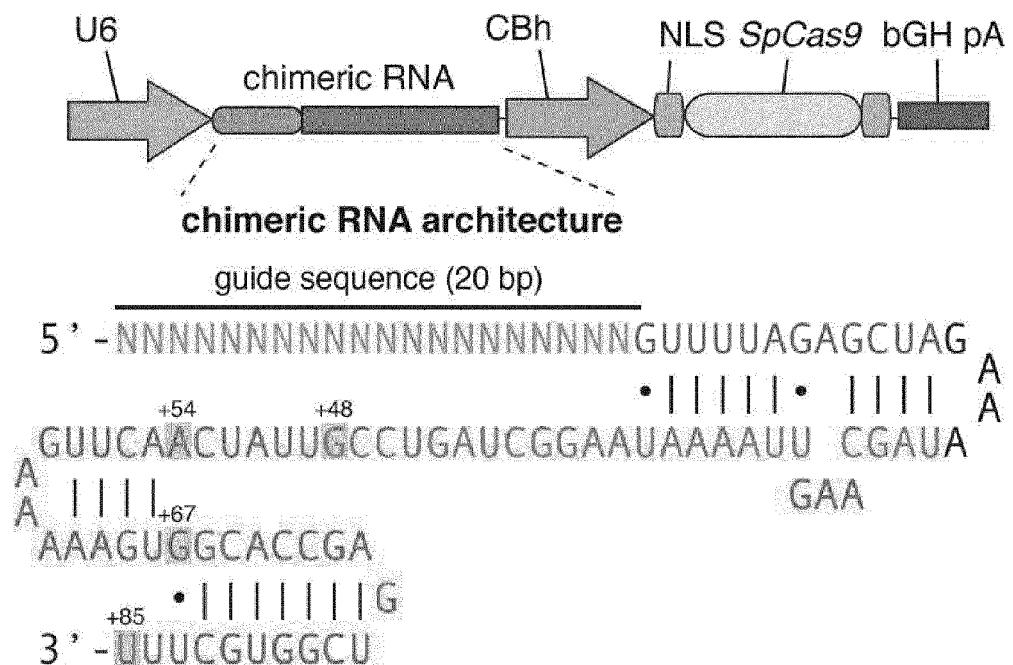


FIG. 16A

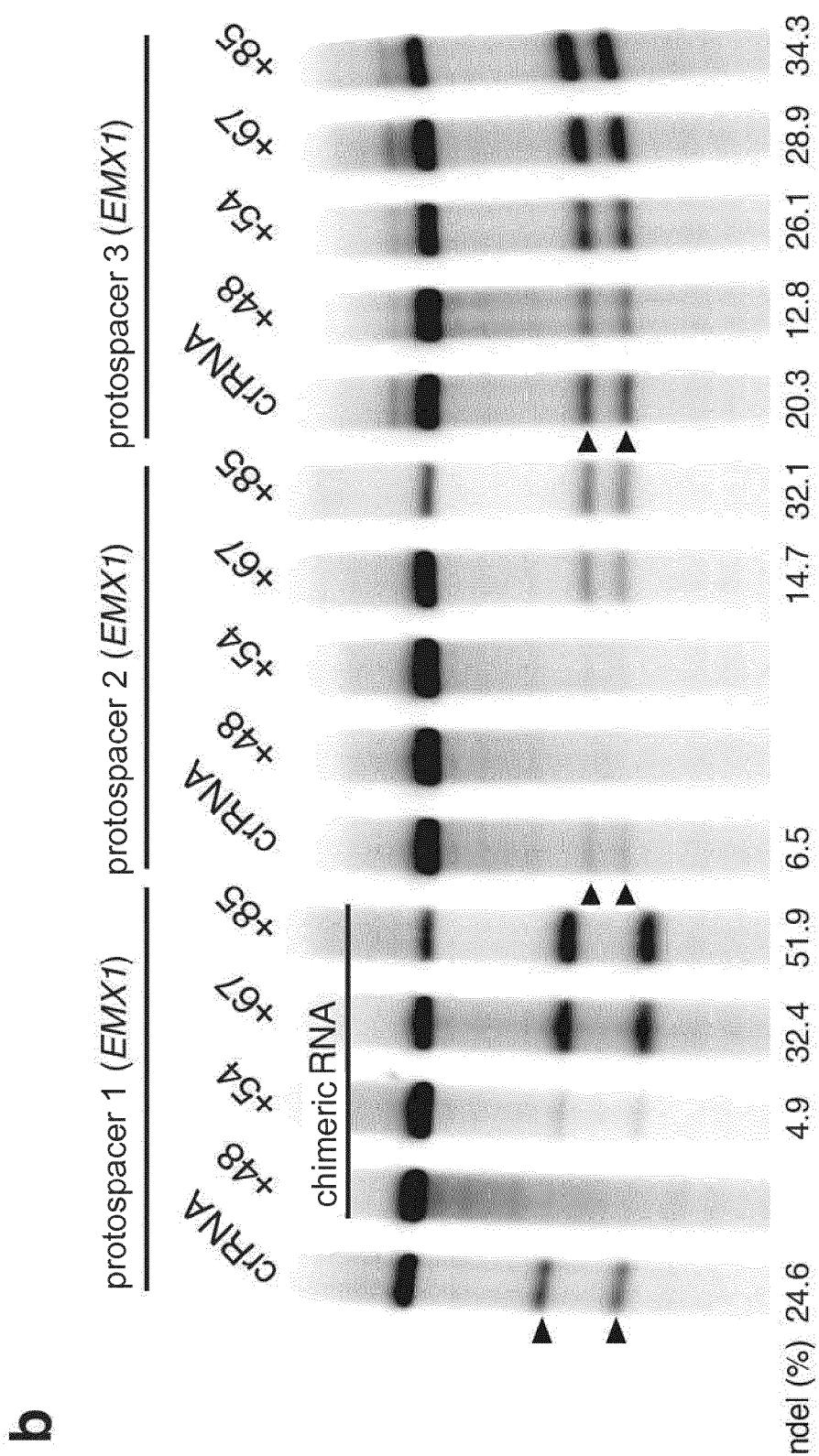


FIG. 16B

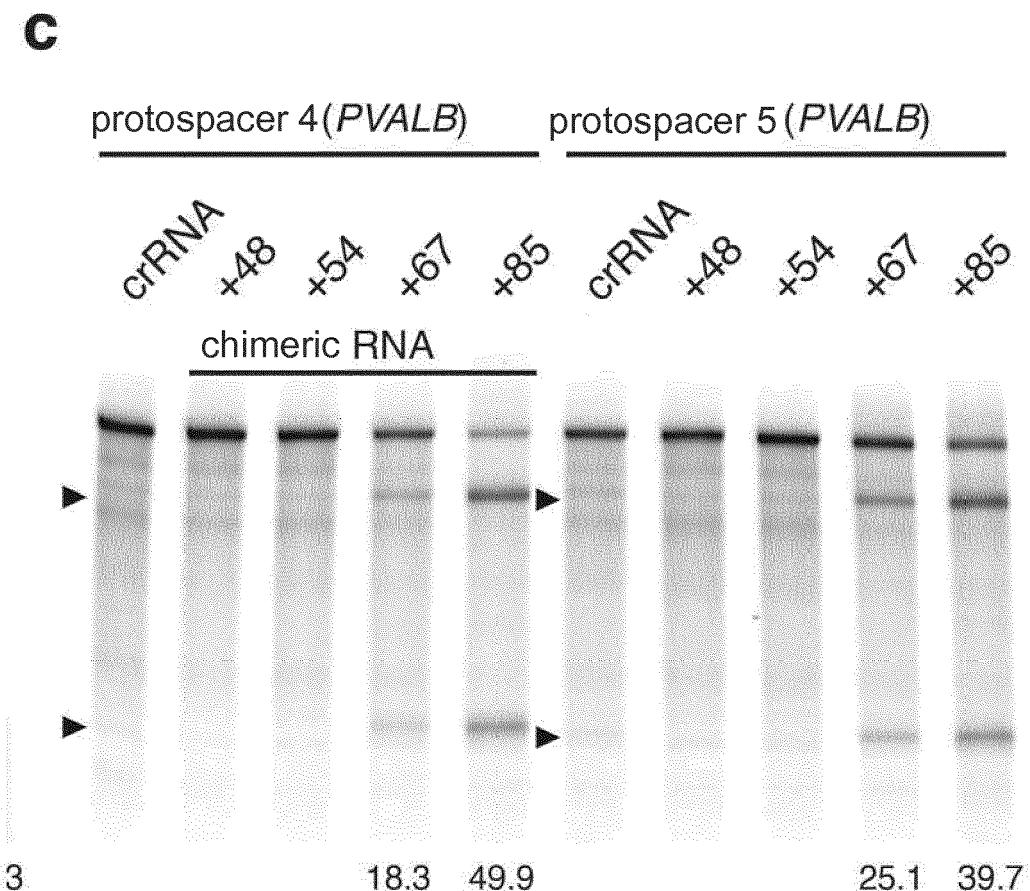


FIG. 16C

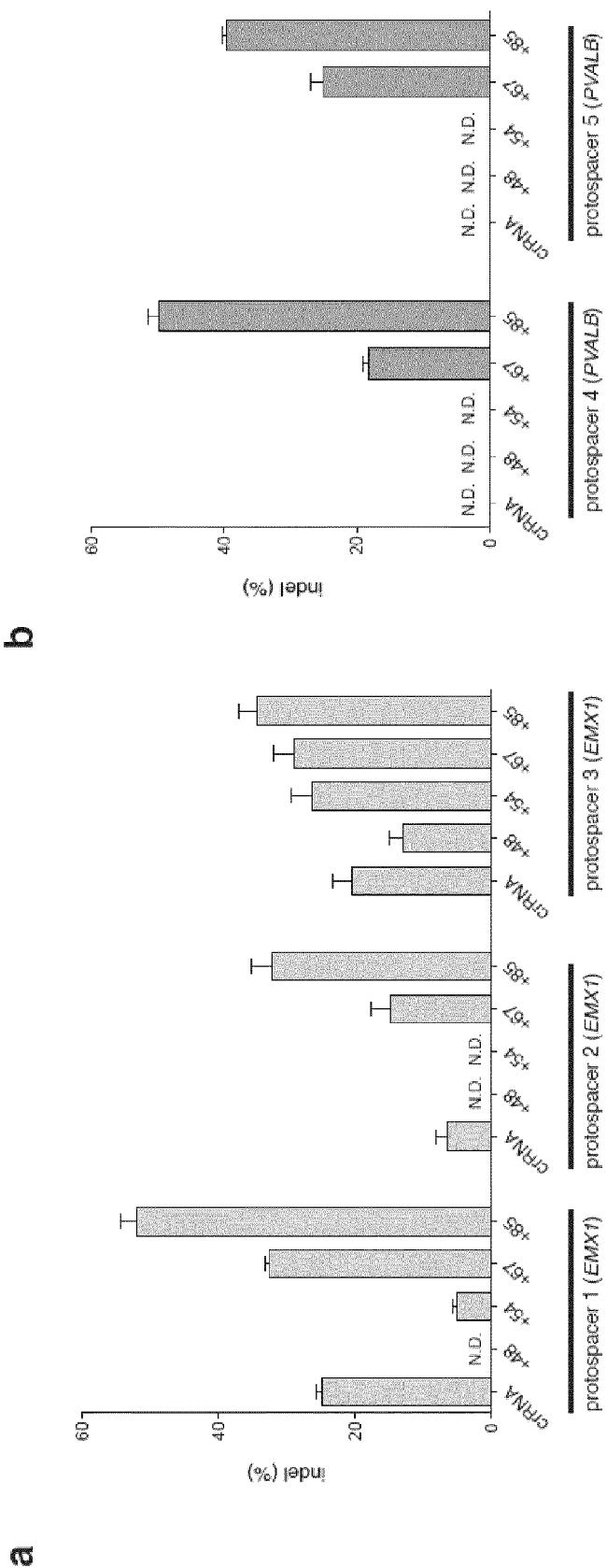
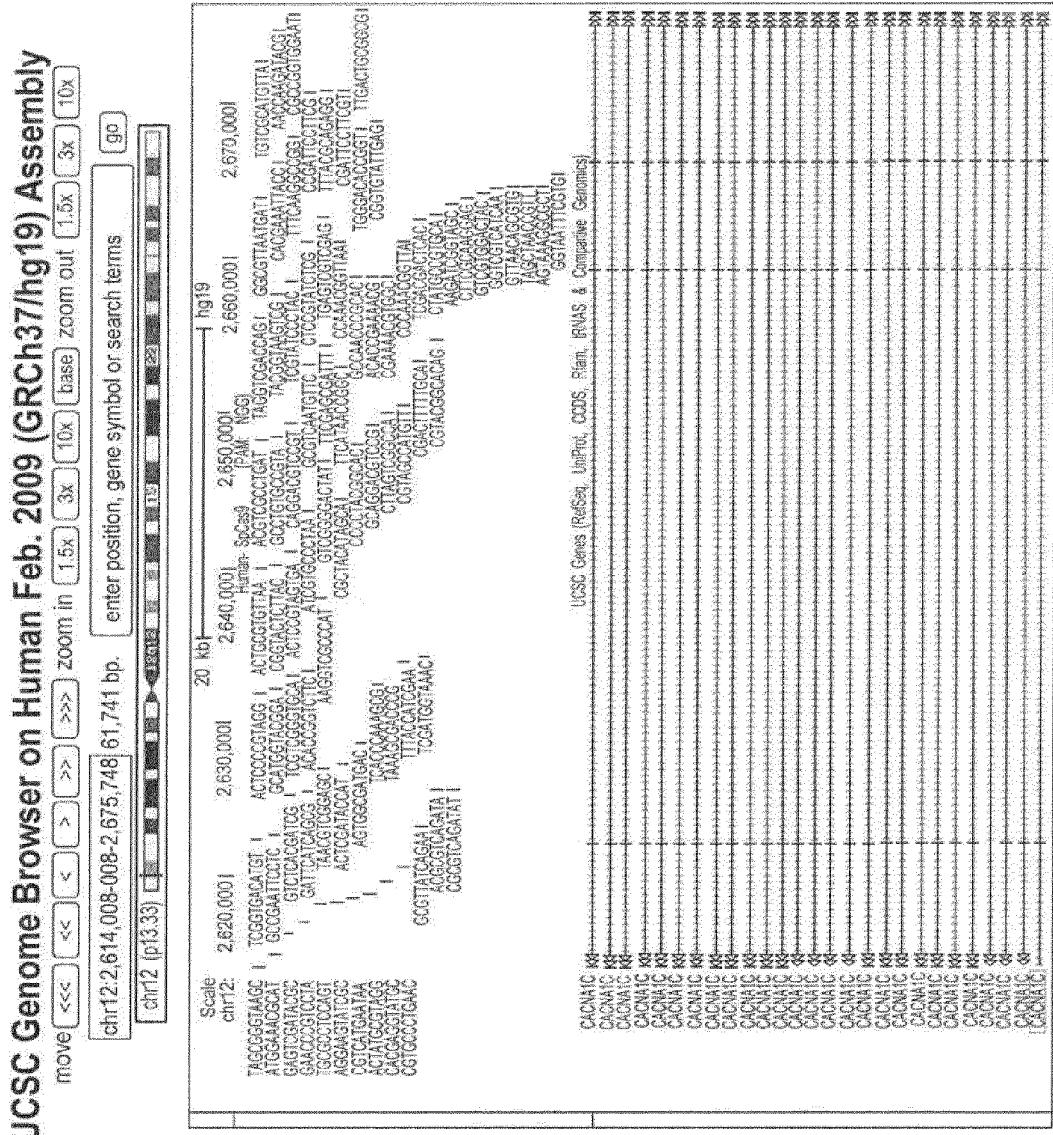


FIG. 17A-B

88
1
E
G



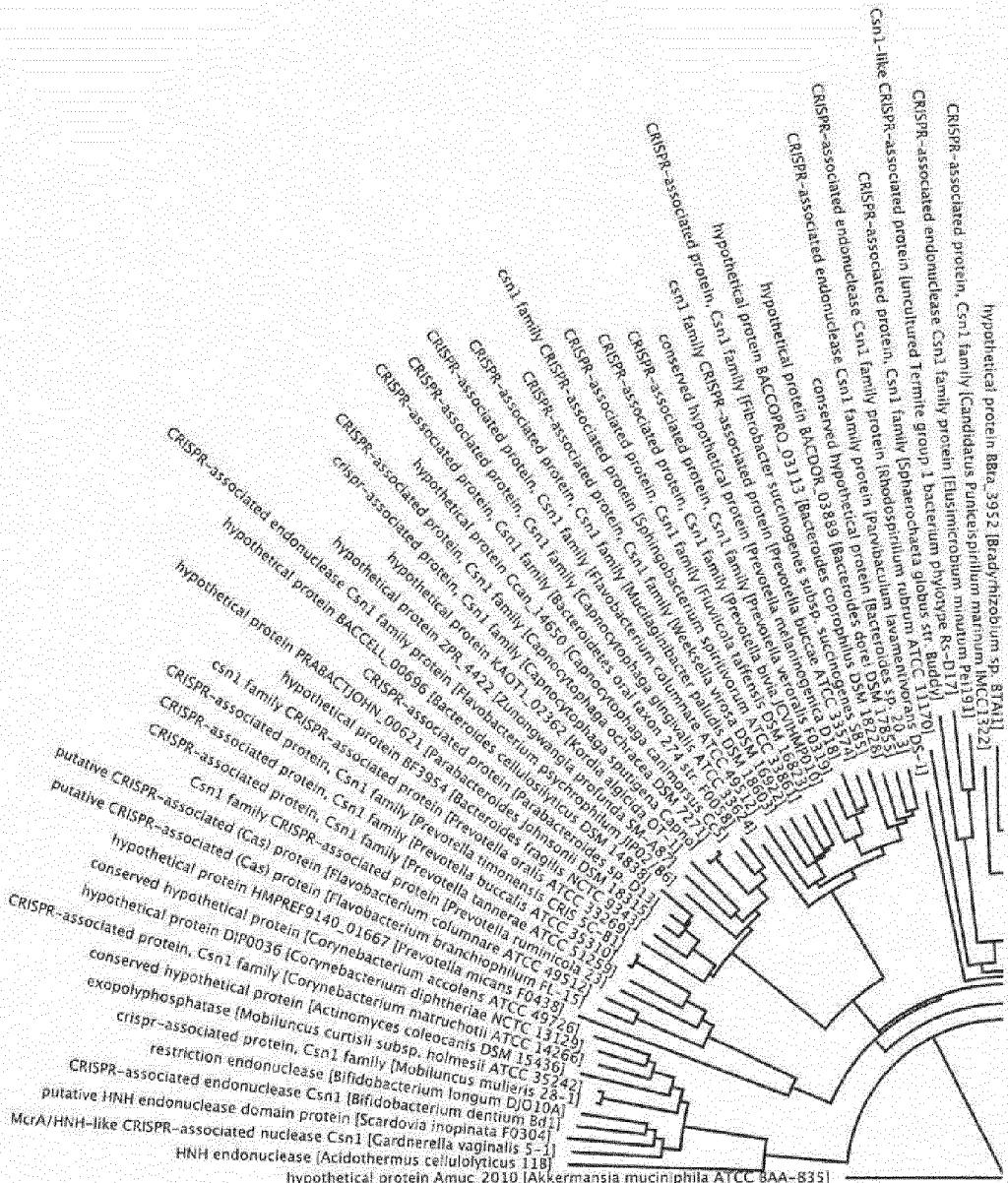


FIG. 19A

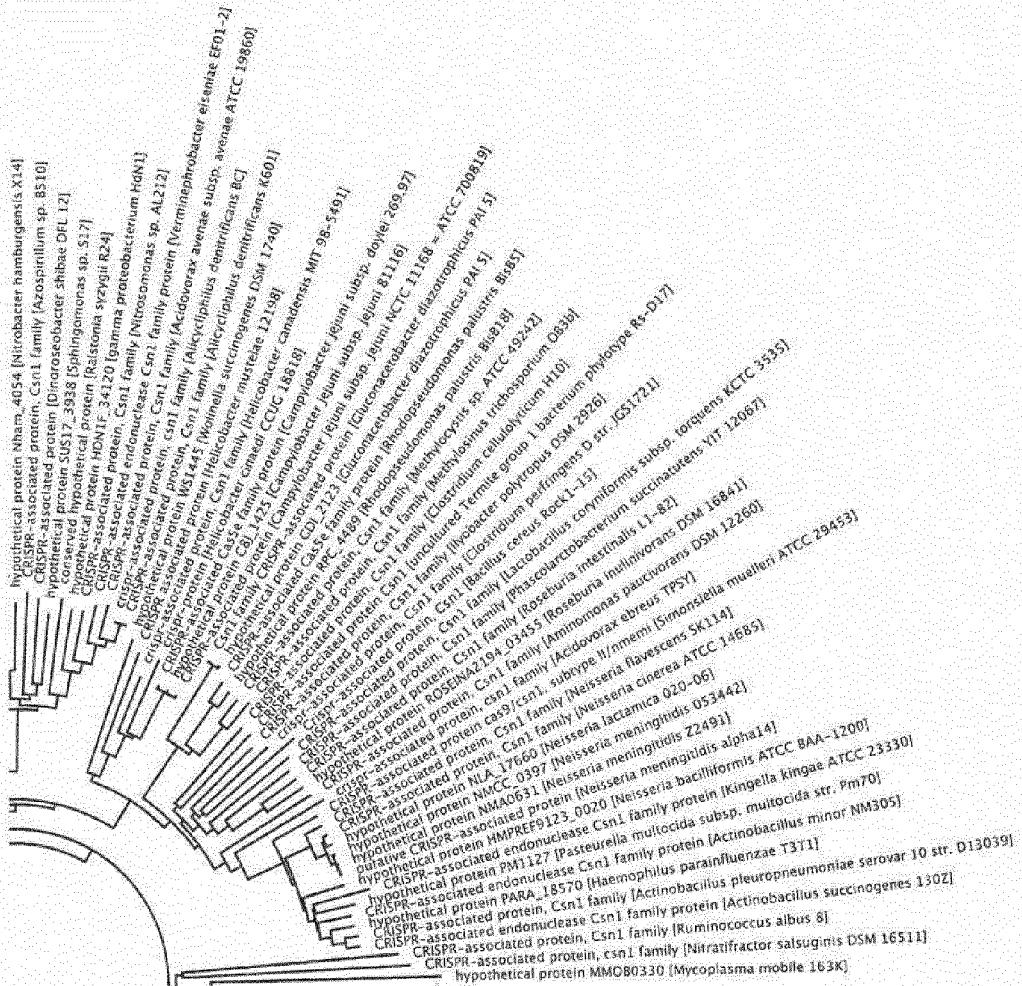


FIG. 19B

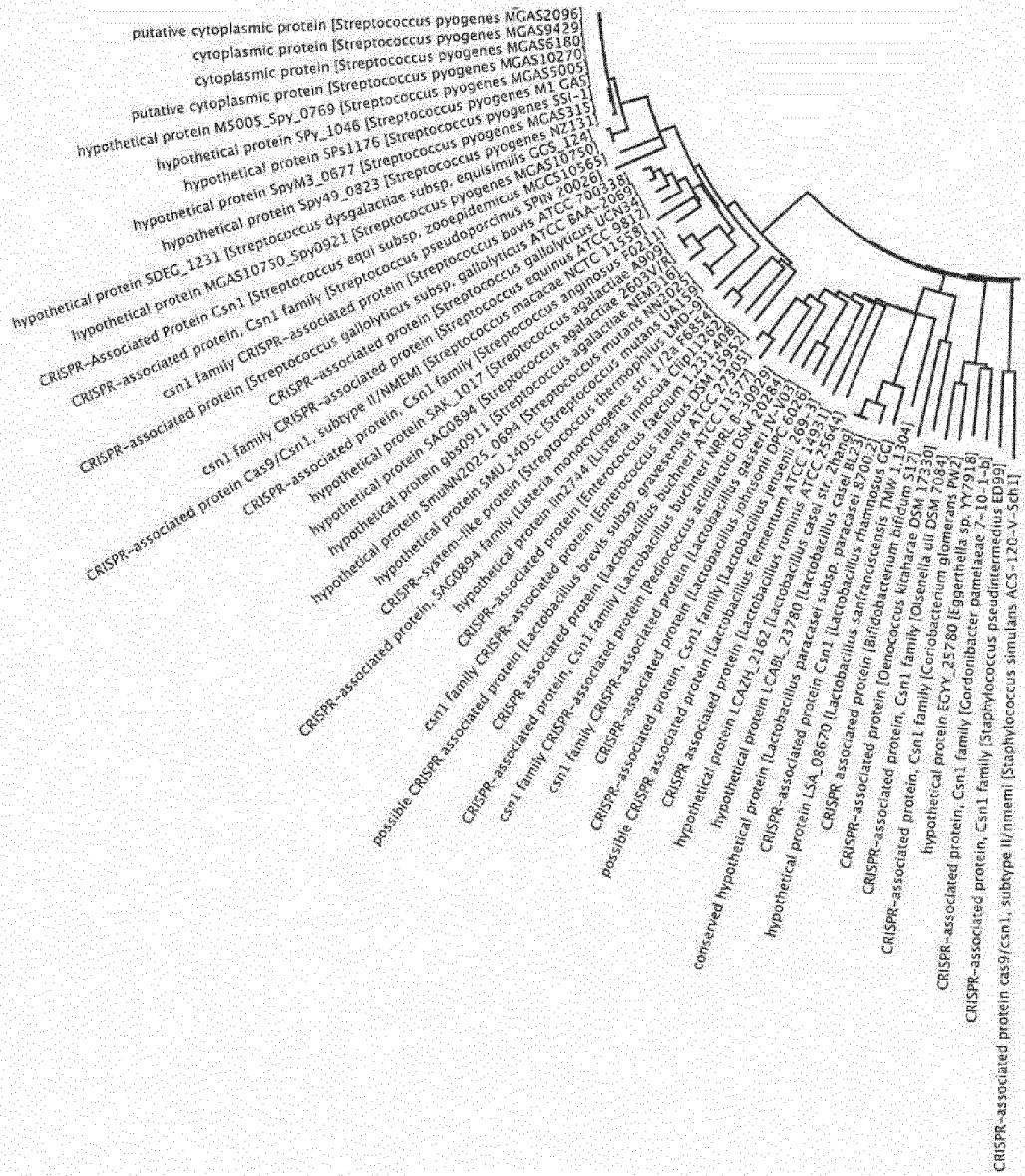


FIG. 19C

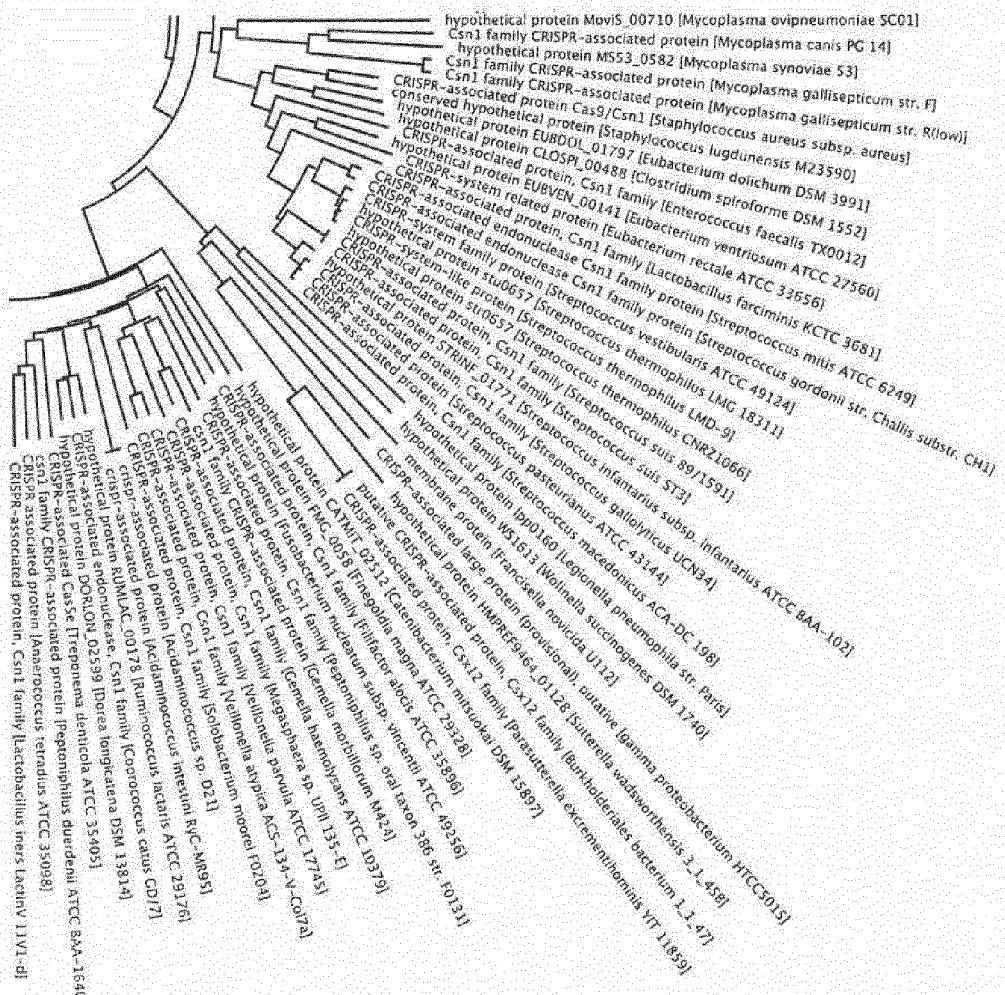


FIG. 19D

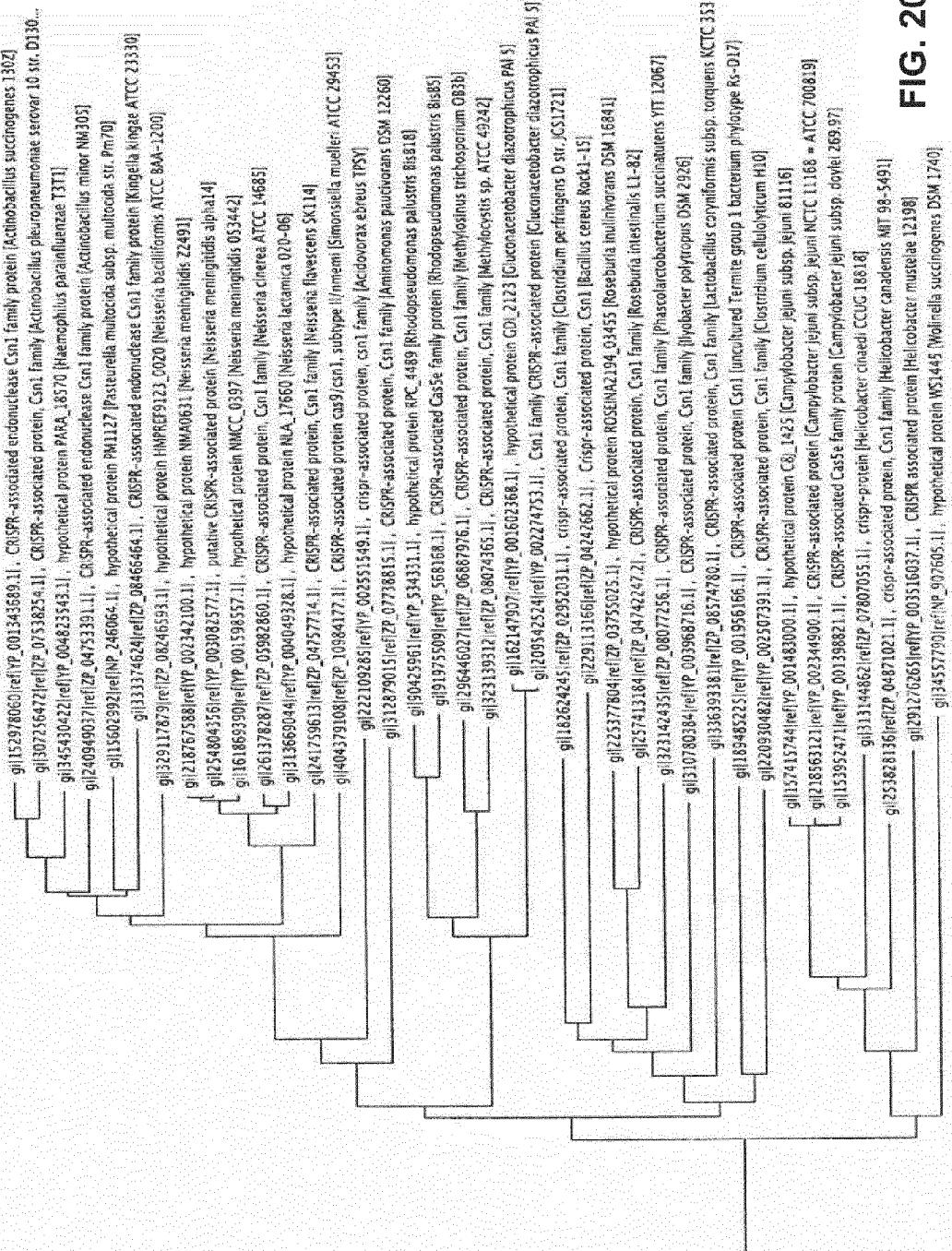


FIG. 20A

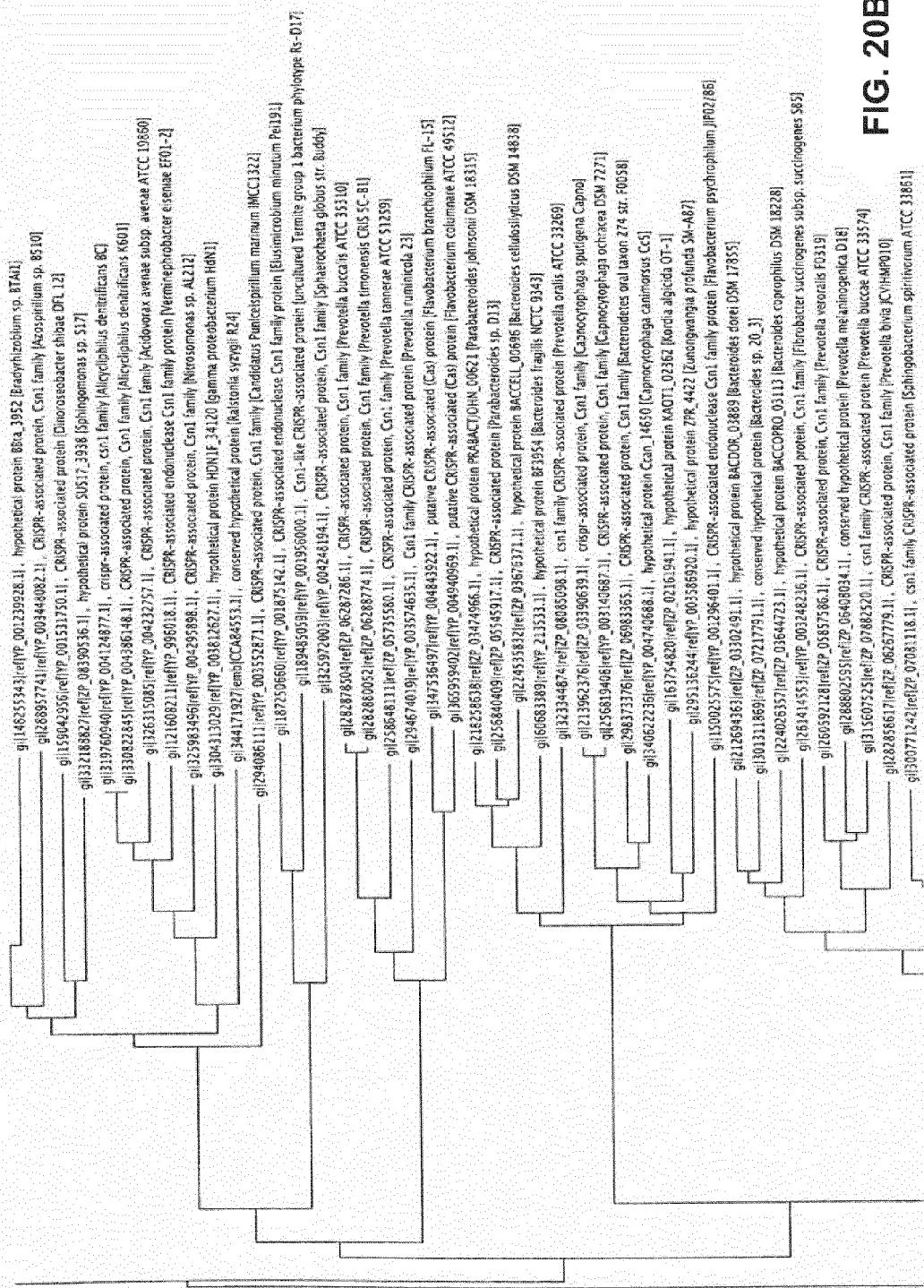


FIG. 20B

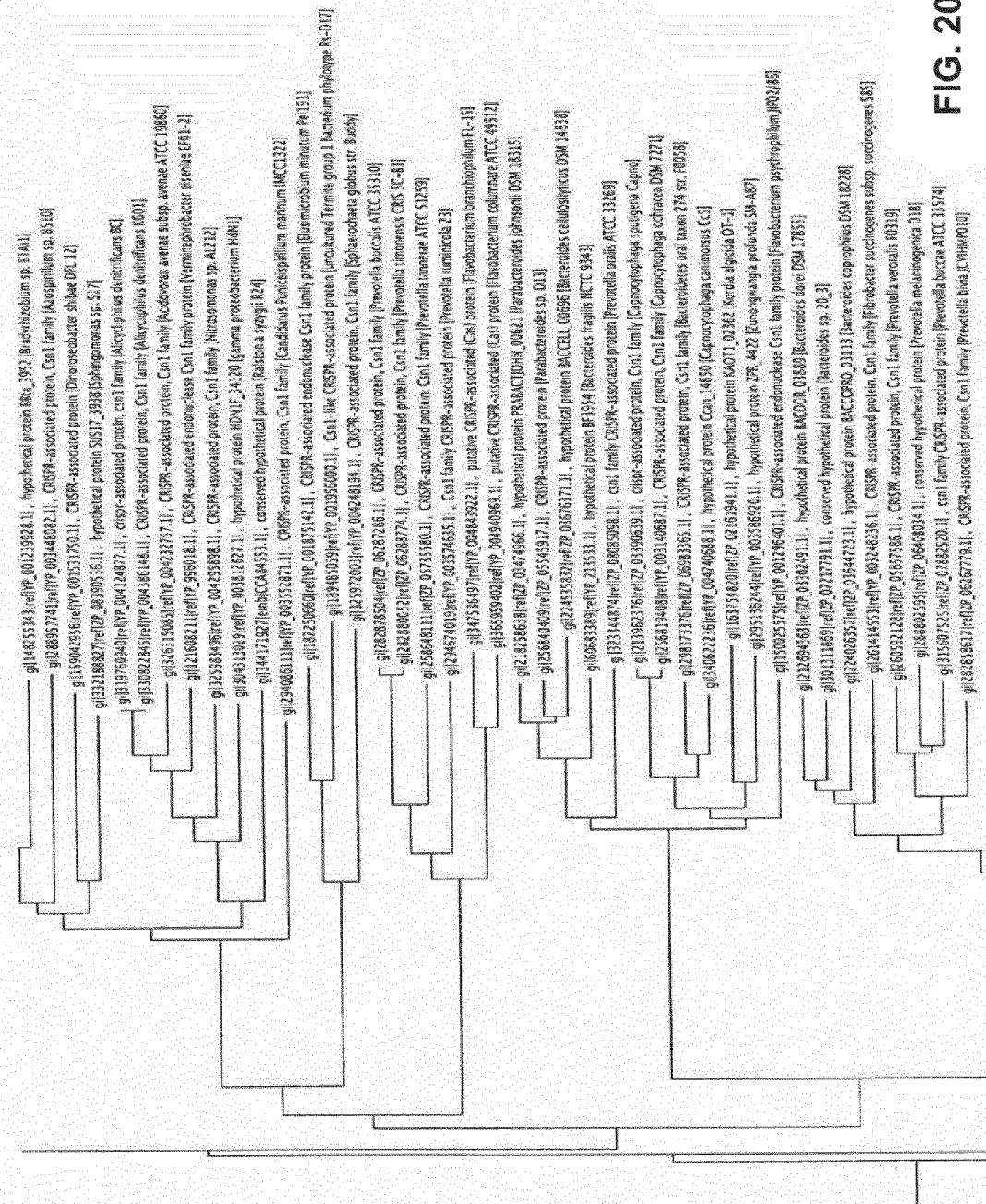


FIG. 20C

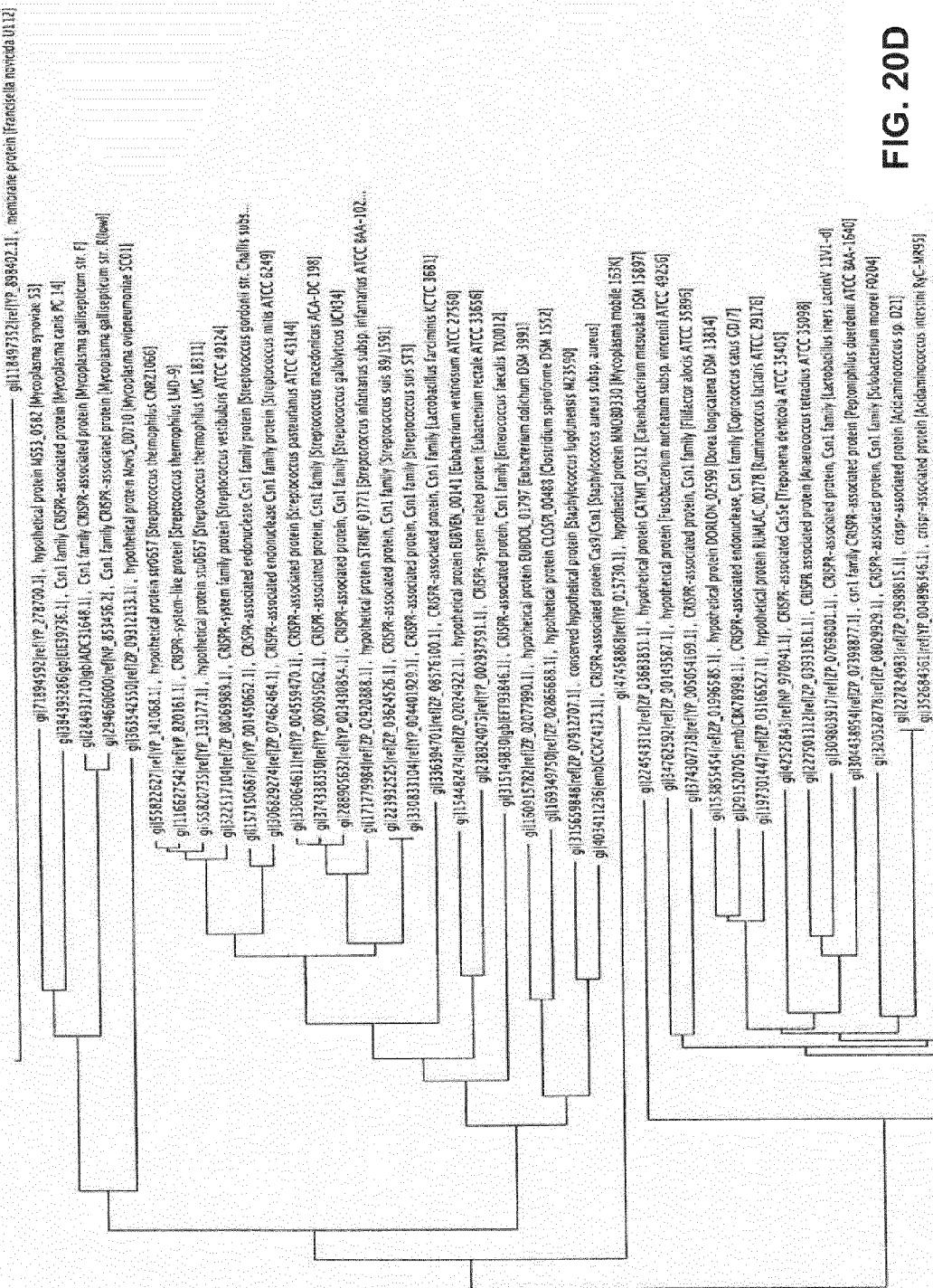


FIG. 20D

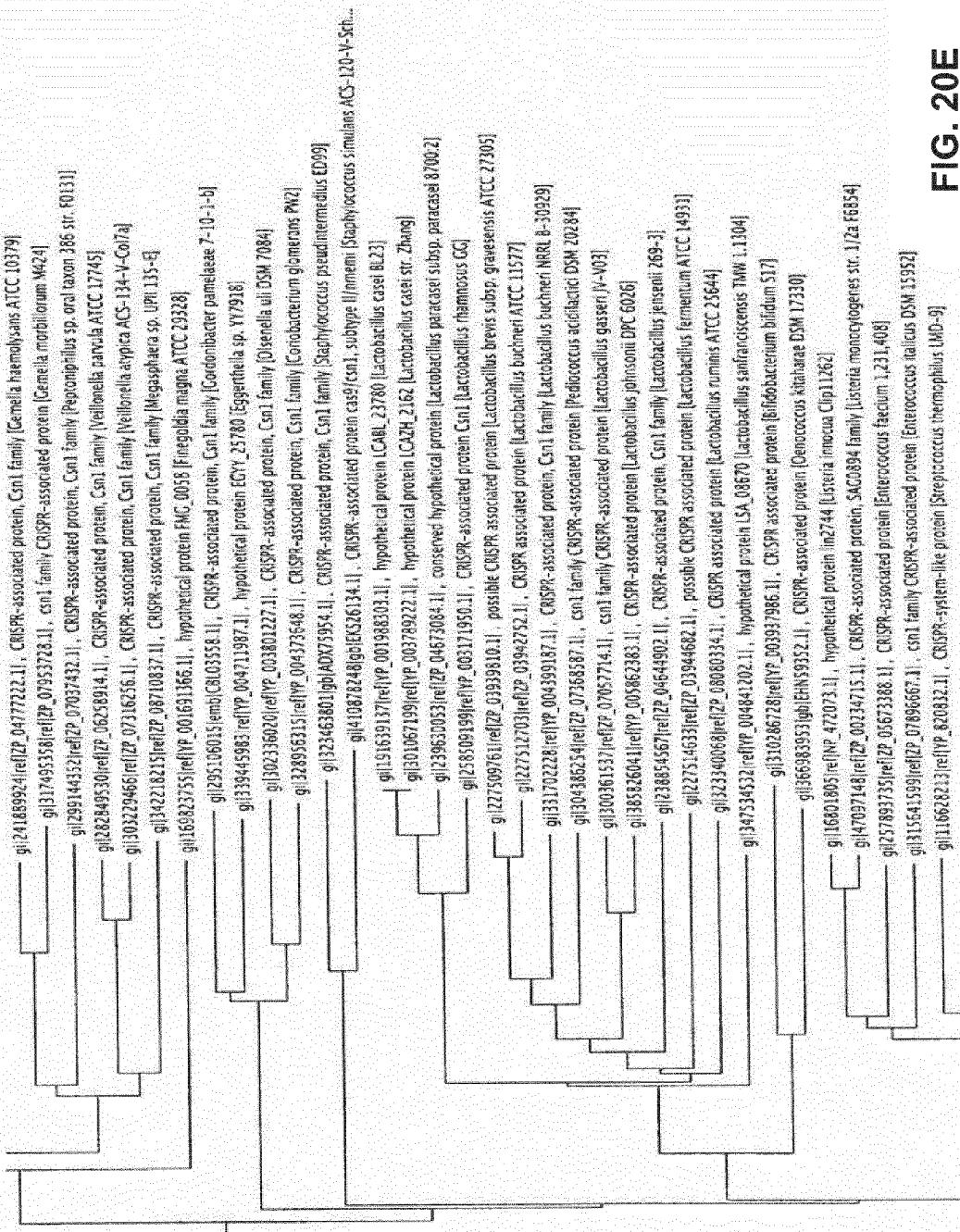


FIG. 20E

FIG. 20F

a

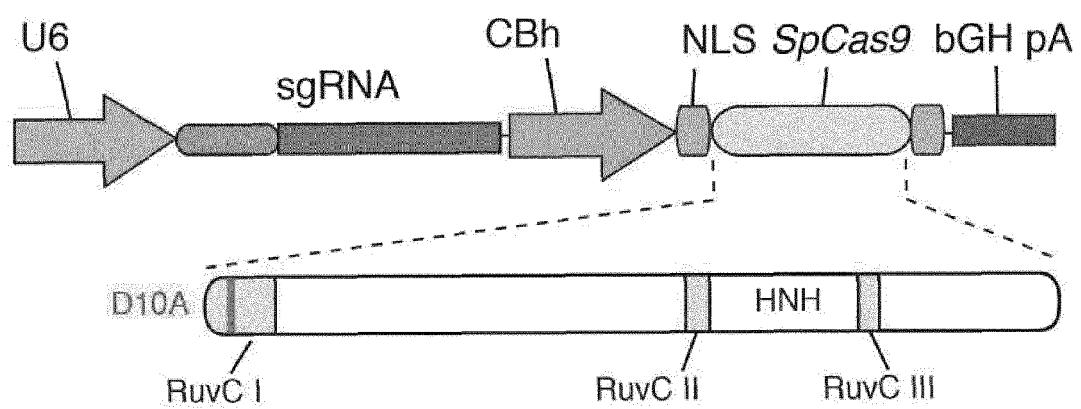


FIG. 21A

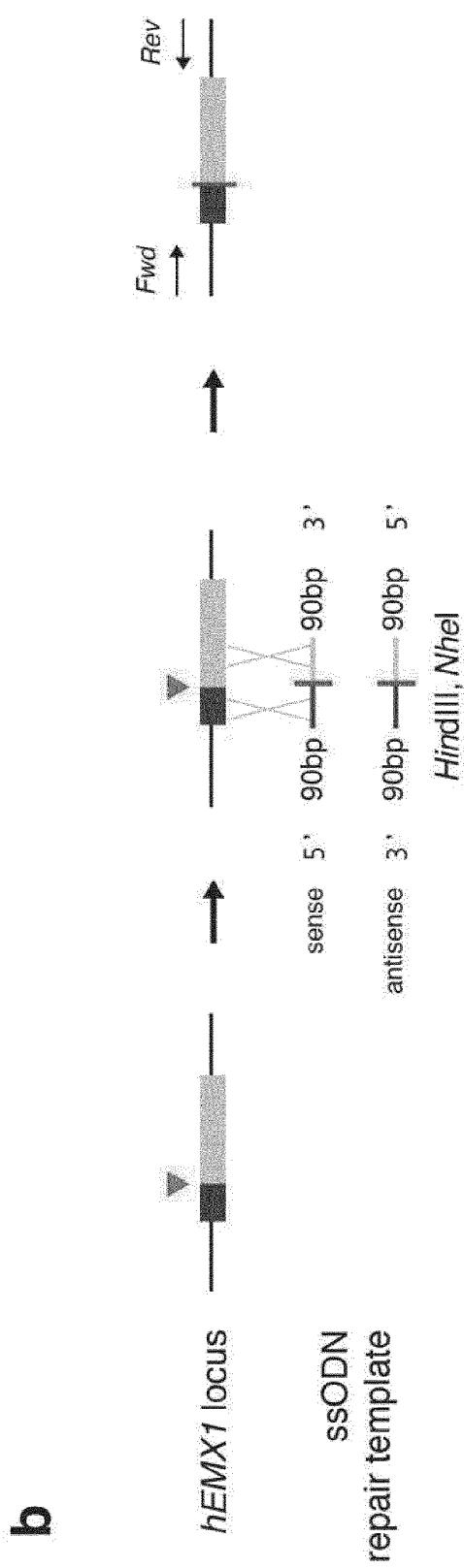


FIG. 21B

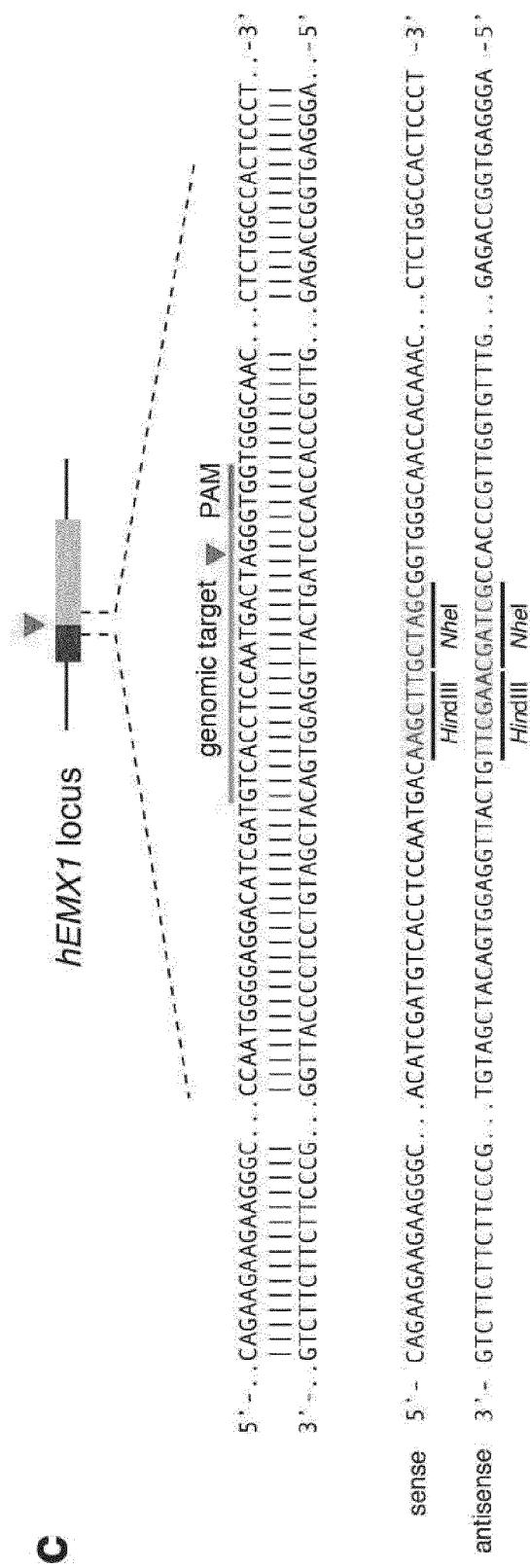
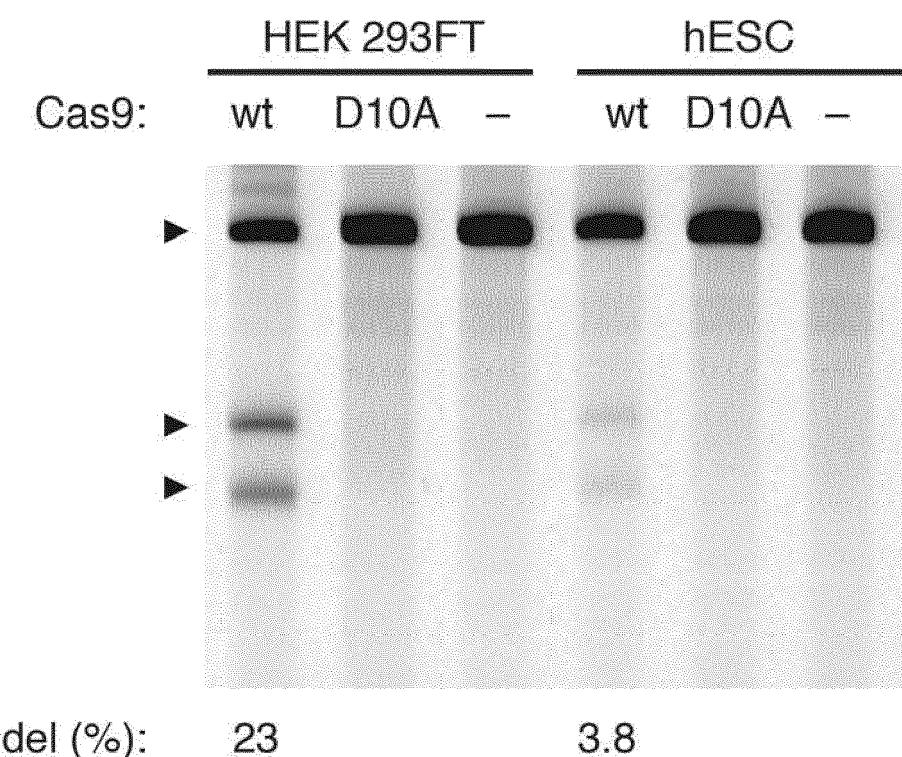


FIG. 21C

d**FIG. 21D**

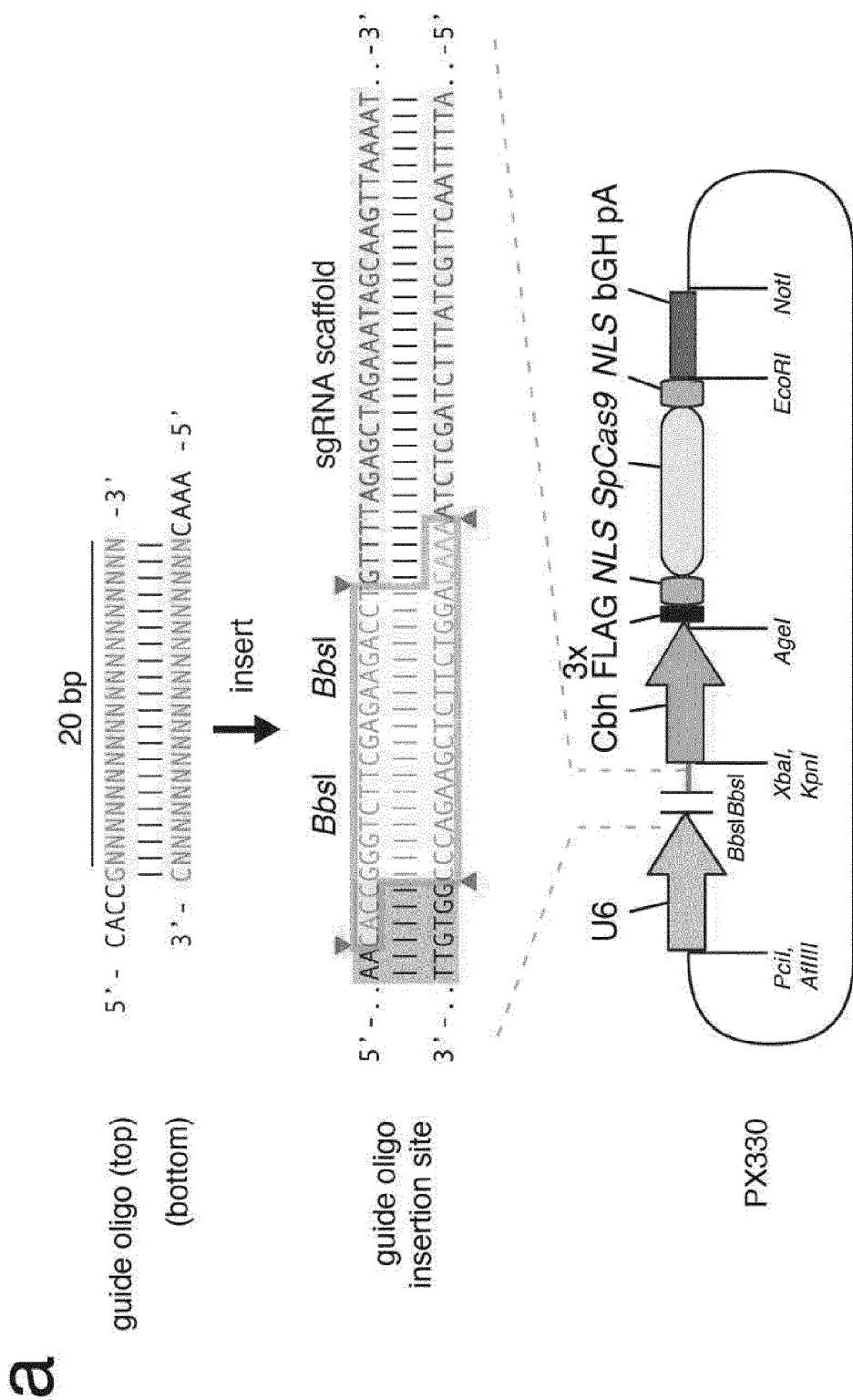


FIG. 22A

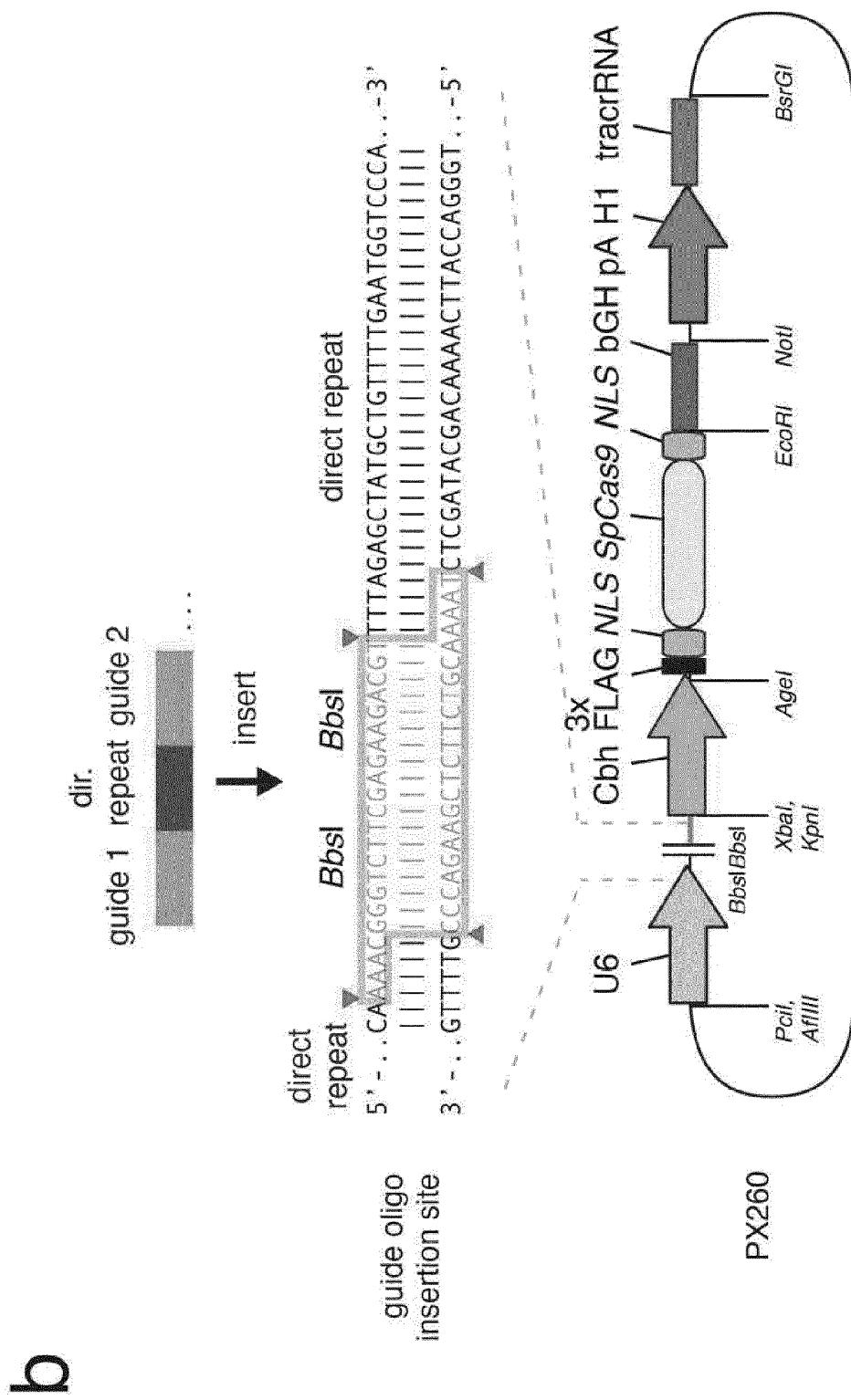


FIG. 22B



EUROPEAN SEARCH REPORT

Application Number

EP 15 17 6051

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15	E WO 2014/065596 A1 (TOOLGEN INC [KR]) 1 May 2014 (2014-05-01) * See the whole document, and more especially: * * claims 1-57; examples 1-9 *	1-3,7-9, 12-18, 20-24	
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25			C12N
30			
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45		-/-	
50	1 The present search report has been drawn up for all claims		
55	Place of search Munich	Date of completion of the search 7 January 2016	Examiner Seroz, Thierry
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30	A G. GASIUNAS ET AL: "PNAS Plus: Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 109, no. 39, 25 September 2012 (2012-09-25), pages E2579-E2586, XP055068588, ISSN: 0027-8424, DOI: 10.1073/pnas.1208507109 * abstract; figures 4, S9 * * page E2579 * * page E2583 *	1-27	TECHNICAL FIELDS SEARCHED (IPC)
35			
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45		-/-	
50	1 The present search report has been drawn up for all claims		
55	Place of search Munich	Date of completion of the search 7 January 2016	Examiner Seroz, Thierry
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T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			



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EP 15 17 6051

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15 A	BLAKE WIEDENHEFT ET AL: "RNA-guided genetic silencing systems in bacteria and archaea", NATURE, vol. 482, no. 7385, 15 February 2012 (2012-02-15), pages 331-338, XP055116249, ISSN: 0028-0836, DOI: 10.1038/nature10886 * the whole document *	1-27	
20 A	ELITZA DELTCHEVA ET AL: "CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III", NATURE, vol. 471, no. 7340, 31 March 2011 (2011-03-31), pages 602-607, XP055068535, ISSN: 0028-0836, DOI: 10.1038/nature09886 * abstract; figures 1-6 *	1-27	
25 A	MICHAEL P TERNS ET AL: "CRISPR-based adaptive immune systems", CURRENT OPINION IN MICROBIOLOGY, vol. 14, no. 3, 1 June 2011 (2011-06-01), pages 321-327, XP055097823, ISSN: 1369-5274, DOI: 10.1016/j.mib.2011.03.005 * the whole document *	1-27	
30 A			TECHNICAL FIELDS SEARCHED (IPC)
35 A			
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1	The present search report has been drawn up for all claims		
50	Place of search Munich	Date of completion of the search 7 January 2016	Examiner Seroz, Thierry
55	CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document	T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	



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20	X, P WANG HAOYI ET AL: "One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering", CELL, vol. 153, no. 4, 9 May 2013 (2013-05-09), pages 910-918, XP028538358, ISSN: 0092-8674, DOI: 10.1016/J.CELL.2013.04.025 * abstract *	1-27	
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30	X, P LUKE A. GILBERT ET AL: "CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes", CELL, vol. 154, no. 2, 1 July 2013 (2013-07-01), pages 442-451, XP055115843, ISSN: 0092-8674, DOI: 10.1016/j.cell.2013.06.044 * abstract *	1-27	TECHNICAL FIELDS SEARCHED (IPC)
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40	X, P KRZYSZTOF CHYLINSKI ET AL: "The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems", RNA BIOLOGY, vol. 10, no. 5, 1 May 2013 (2013-05-01), pages 726-737, XP055116068, ISSN: 1547-6286, DOI: 10.4161/rna.24321 * the whole document *	1-27	
45			
50	1 The present search report has been drawn up for all claims		
55	Place of search Munich	Date of completion of the search 7 January 2016	Examiner Seroz, Thierry
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T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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5 This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

07-01-2016

10	Patent document cited in search report	Publication date	Patent family member(s)		Publication date	
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摘要

本發明提供出某些系統、方法以及組成物，以供改變標的基因序列及相關基因產物的表達。所提供的是一種載體與載體系統，其中一些編碼出CRISPR複合體的一個或多個組份，亦提供出設計並使用此等載體的方法。此外，所提供的亦包括用於引導CRISPR複合體於真核細胞中形成的方法，以及利用該CRISPR-Cas系統的方法。