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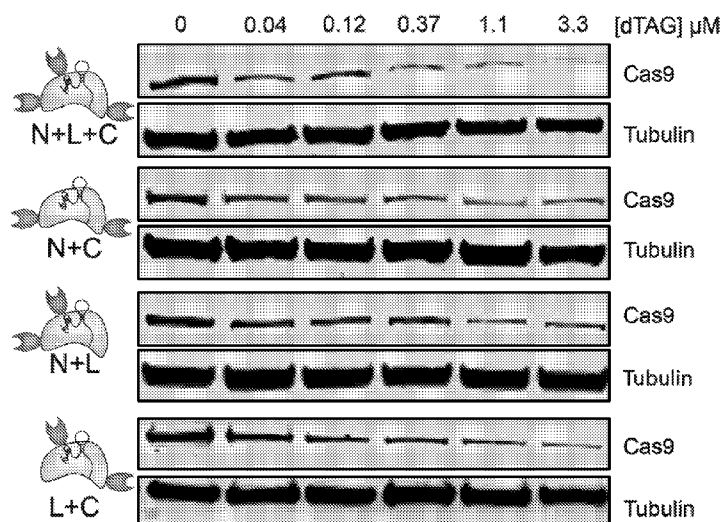


FIG. 2

(57) Abstract: The disclosure includes non-naturally occurring or engineered CRISPR Cas variant proteins comprising one or more functional domains and degrader compositions specifically targeting the CRISPR Cas variant proteins; compositions, systems and methods of using the CRISPR Cas variant proteins comprising one or more functional domains and degrader compounds for spatio-temporal control are also provided.



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DEGRADATION DOMAIN MODIFICATIONS FOR SPATIO-TEMPORAL CONTROL OF RNA-GUIDED NUCLEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/765,351 filed on August 20, 2018. The entire contents of the above-identified applications are hereby fully incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant No. AI126239 awarded by the National Institutes of Health, N66001-17-2-4055 awarded by the Defense Advanced Research Projects Agency, and Grant No. Q911NF1610586 awarded by the United States Department of the Army. The government has certain rights in the invention.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0003] The contents of the electronic sequence listing (BROD_3840_ST25.txt"; Size is 63,103 bytes and it was created on June 26, 2019) is herein incorporated by reference in its entirety.

TECHNICAL FIELD

[0004] The subject matter disclosed herein is generally directed to systems for target-specific protein degradation and methods of their use.

BACKGROUND

[0005] Recent advances in genome sequencing techniques and analysis methods have significantly accelerated the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. Precise genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology, biotechnological, and medical applications. Although genome-editing techniques are available for producing targeted genome perturbations, there remains a need for new genome

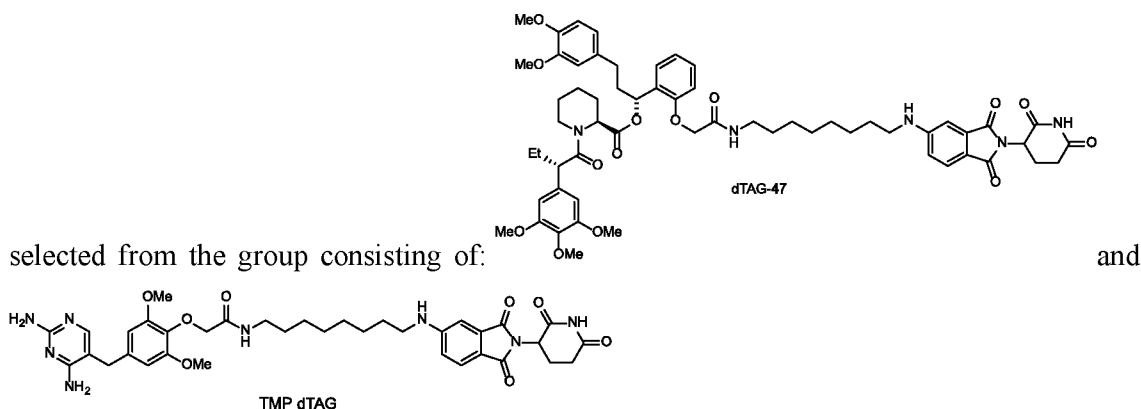
engineering technologies that employ novel strategies and molecular mechanisms and are affordable, easy to set up, scalable, and amenable to targeting multiple positions within the eukaryotic genome.

[0006] RNA-guided endonucleases, such as Cas9, are easily targeted to any desired DNA or RNA locus using guide RNAs (gRNA), which has provided new transformative technologies. For example, Cas9 has enabled facile and efficient induction of genomic alterations in cells and multiple organisms, and Cas9-based gene drives permit super-Mendelian self-propagation of such modifications (3). Furthermore, catalytically inactive CRISPR effectors, such as Cas9 (dCas9) can be fused to a wide range of effectors, including fluorescent proteins for genome imaging (4), enzymes that modify DNA or histones for epigenome editing (5), and transcription regulating domains for controlling endogenous gene expression (6). *Streptococcus pyogenes* and *Staphylococcus aureus* provide naturally occurring SpCas9 and SaCas9, respectively, that are commonly used in CRISPR approaches.

[0007] Despite such advances, a critical need still exists for methods to precisely and reversibly (switchably) regulate CRISPR effector activities across multiple dimensions, including dose, target, and time (7). Finely-tuned control of CRISPR effector proteins levels is important, as high concentrations result in elevated off-target DNA cleavage. Rapidly disabling activity after a desired genomic modification is also essential (8). However, the ability to control such systems is still needed. One method of control would be degradation of the Cas effector protein to effectively shut down systems after use. Typically, once proteins are no longer needed in a cell, they are tagged in the cell with ubiquitin utilizing an E3 ligase to designate the protein for degradation in the proteasome. Exploitation of a mechanism to target proteins for degradation in the proteasome would be one approach to degrade Cas effector protein after its use and provide a means of control after desired genomic modification or other uses of CRISPR Cas systems has been effected.

SUMMARY

[0008] In certain example embodiments, a compound is provided that can target and degrade a protein. Heterobifunctional degraders of FBKP12^{F36V} are provided herein, for example, compounds or a pharmaceutically acceptable salt thereof are provided that can be

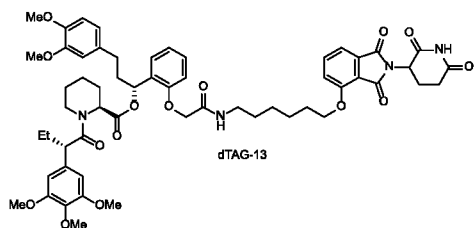
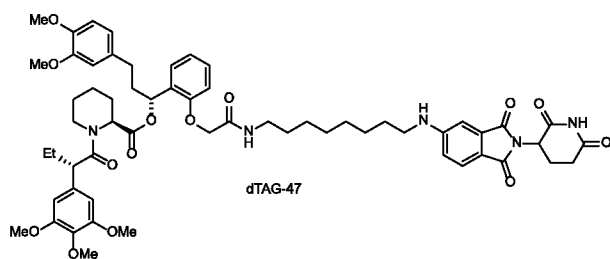


[0009] Also provided herein are variant CRISPR-Cas proteins that comprise one or more FK506 binding protein (FKBP) domains introduced into the CRISPR-Cas protein at one or more insertion sites. The CRISPR-Cas protein can be, in an embodiment, a Cas9 or a Cas12 variant protein. In one embodiment, the CRISPR-Cas protein is a Cas9 protein, and in a particular embodiment, SpCas9. The CRISPR-Cas protein can include one or more insertion sites for the FKBP domains, and, in some instances, the insertion sites are at the N-terminal (Nt), C-terminal (Ct) or at a position within the protein. In some embodiments, the insertion site can include an FKBP domain corresponding to position 231 (Lp) of the SpCas9 protein. In one embodiment, the one or more insertion sites are selected from: Nt and Ct; Nt and Lp; Lp and Ct; and Nt, Lp and Ct. In a particular embodiment, the FKBP domains are FKBP12^{F36V} domains.

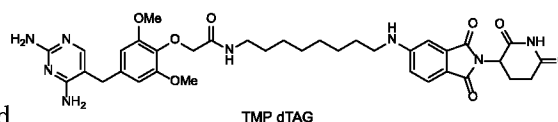
[0010] In some embodiments, the variant CRISPR-Cas protein is provided in a ribonucleoprotein (RNP). The variant CRISPR-Cas protein can also be provided in a plasmid or vector. In some embodiments, the plasmid comprises a sequence selected from sequences 1-4 of Table 1.

[0011] A cell transfected with a ribonucleoprotein containing the variant CRISPR-Cas protein or plasmid containing the variant CRISPR-Cas protein is provided herein. The cell can in some instances be a germline. In some instances, the cell is selected from HEK293FT, U2OS, NIH3T3 and S2.

[0012] Methods of using the variant CRISPR-Cas proteins and degraders are also disclosed herein. In one embodiment, a method of inducing degradation of a variant CRISPR-Cas protein is provided, including the step of exposing a CRISPR-Cas variant to a compound or a pharmaceutically acceptable salt thereof selected from



; and



. In

some embodiments, the compound is a heterobifunctional degrader of FBKP12^{F36V}. In some embodiments, the step of exposing a CRISPR-Cas variant to a compound includes contacting the CRISPR-Cas variant directly or indirectly with a degrader. In some embodiments, the exposing comprises incubating a cell comprising the CRISPR-Cas variant with the compound or pharmaceutically acceptable salt thereof.

[0013] In some embodiments, the method is performed *in vivo* or *in vitro*. The methods can be performed in a cell. In some instances, the cell is a germline cell. In an embodiment, the cell is selected from HEK293FT, U2OS, NIH3T3 and S2. In some embodiments, the cell is in an organism. In some embodiments, the cell is exposed to the degrader compound or pharmaceutically acceptable salt thereof at a concentration of about 1nM to about 3 μ M.

[0014] A method of degrading an activity of a variant CRISPR Cas protein-RNA complex is also provided, and comprises contacting the CRISPR Cas protein-RNA complex with a heterobifunctional degrader of FBKP12^{F36V}. In an embodiment, the CRISPR-Cas protein is CRISPR Cas9 or Cas 12. In an embodiment the CRISPR Cas9 is a SpCas9. In one embodiment, the degrading of the activity of a variant CRISPR Cas protein-RNA complex occurs in a cell, *in vitro* or *in vivo*.

[0015] Methods of treating a subject are also provided herein. In one embodiment, the method of treating a subject includes the steps of administering a CRISPR Cas protein-RNA complex or a reagent causing expression of a CRISPR-Cas protein-RNA complex to the subject; and administering an effective amount of a degrader compound disclosed herein.

[0016] Pharmaceutical formulations are also provided herein, and can include a compound of as disclosed herein and a pharmaceutically acceptable carrier. Kits including degrader compounds and/or the CRISPR-Cas proteins disclosed herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] An understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention may be utilized, and the accompanying drawings of which:

[0100] **FIG. 1A** depicts structures of dTAG-47, dTAG-13 and TMP dTAG small molecules; **FIG. 1B** shows insertion sites on spCas9; red triangles point out the insertion site on Cas9I used to introduce FKBP12 domain; **FIG. 1C** is a schematic of NtLpCt 3xFKBP Cas9, NtLp 2xFKBP Cas9, NtCt 2xFKBP Cas9, and LpCt 2xFKBP Cas9; Nt: N-terminal; Lp: Loop; Ct: C-terminal.

[0101] **FIG. 2** is a western blot analysis of different constructs of FKBP-SpCas9 degradation induced by indicated amount of dTAG-47 (3.3uM, 1.1uM, 0.37uM, 0.12uM, 0.04uM) in HEK293FT cells. With increasing amount of dTAG-47, less Cas9 protein was detected which confirmed the degradation of Cas9 by dTAG-47. Tubulin: loading control.

[0102] **FIG. 3A** eGFP disruption assay of 4 FKBP-Cas9 using plasmid delivery method, dTAG-47 was used to induce degradation. In **FIG. 3B**, dTAG-13 was used to induce degradation. The result showed that dTAG-13 is less potent than dTAG-47.

[0103] **FIG. 4A-4B** graphs eGFP disruption assay in U2OS cells using RNP delivery. NtLp 2xFKBP Cas9 and 3xFKBP Cas9 were overexpressed and purified. Cas9 activity was analyzed using eGFP disruption assay using RNP delivery method. **FIG. 4A** demonstrates that with increasing amount dTAG-47 (1.4nM, 4nM, 12nM, 37nM, 111nM, 333nM, 1000nM), NtLp 2xFKBP Cas9 activity (shown by % eGFP disruption) was reduced significantly compared to plasmid delivery method (Figure 3), however the activity of 3xFKBP is much lower than expected probably due to the fact that 3xFKBP Cas9 protein prone to precipitate. **FIG. 4B** demonstrated that with increasing amount dTAG-13 (1.4nM, 4nM, 12nM, 37nM, 111nM, 333nM, 1000nM), NtLp 2xFKBP Cas9 activity was reduced but not as significant as by dTAG-13, which suggests that dTAG-13 is less potent than dTAG-47.

[0104] **FIG. 5** provides results of eGFP disruption assay in S2 cells using RNP delivery. Upper panels of inactive apo Cas9 control show no disruption, with active Cas9 RNP control showing eGFP disruption. Lower panel: NtLp 2xFKBP Cas9 RNP, with indicated

amount of dTAG-47 (1000nM, 10nM, 0nM). With increasing amount of dTAG-47, less eGFP disrupted, confirming our Cas9-FKBP system also work in *Drosophila* S2 cell.

[0105] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE EXAMPLE EMBODIMENTS

General Definitions

[0018] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure pertains. Definitions of common terms and techniques in molecular biology may be found in *Molecular Cloning: A Laboratory Manual*, 2nd edition (1989) (Sambrook, Fritsch, and Maniatis); *Molecular Cloning: A Laboratory Manual*, 4th edition (2012) (Green and Sambrook); *Current Protocols in Molecular Biology* (1987) (F.M. Ausubel et al. eds.); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (1995) (M.J. MacPherson, B.D. Hames, and G.R. Taylor eds.); *Antibodies, A Laboratory Manual* (1988) (Harlow and Lane, eds.); *Antibodies A Laboratory Manual*, 2nd edition 2013 (E.A. Greenfield ed.); *Animal Cell Culture* (1987) (R.I. Freshney, ed.); Benjamin Lewin, *Genes IX*, published by Jones and Bartlet, 2008 (ISBN 0763752223); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0632021829); Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 9780471185710); Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* 2nd ed., J. Wiley & Sons (New York, N.Y. 1994), March, *Advanced Organic Chemistry Reactions, Mechanisms and Structure* 4th ed., John Wiley & Sons (New York, N.Y. 1992); and Marten H. Hofker and Jan van Deursen, *Transgenic Mouse Methods and Protocols*, 2nd edition (2011).

[0019] As used herein, the singular forms “a”, “an”, and “the” include both singular and plural referents unless the context clearly dictates otherwise.

[0020] The term “optional” or “optionally” means that the subsequent described event, circumstance or substituent may or may not occur, and that the description includes instances where the event or circumstance occurs and instances where it does not.

[0021] The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

[0022] The terms “about” or “approximately” as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, are meant to encompass variations of and from the specified value, such as variations of +/-10% or less, +/-5% or less, +/-1% or less, and +/-0.1% or less of and from the specified value, insofar such variations are appropriate to perform in the disclosed invention. It is to be understood that the value to which the modifier “about” or “approximately” refers is itself also specifically, and preferably, disclosed.

[0023] As used herein, a “biological sample” may contain whole cells and/or live cells and/or cell debris. The biological sample may contain (or be derived from) a “bodily fluid”. The present invention encompasses embodiments wherein the bodily fluid is selected from amniotic fluid, aqueous humour, vitreous humour, bile, blood serum, breast milk, cerebrospinal fluid, cerumen (earwax), chyle, chyme, endolymph, perilymph, exudates, feces, female ejaculate, gastric acid, gastric juice, lymph, mucus (including nasal drainage and phlegm), pericardial fluid, peritoneal fluid, pleural fluid, pus, rheum, saliva, sebum (skin oil), semen, sputum, synovial fluid, sweat, tears, urine, vaginal secretion, vomit and mixtures of one or more thereof. Biological samples include cell cultures, bodily fluids, cell cultures from bodily fluids. Bodily fluids may be obtained from a mammal organism, for example by puncture, or other collecting or sampling procedures.

[0024] 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.

[0025] Various embodiments are described hereinafter. It should be noted that the specific embodiments are not intended as an exhaustive description or as a limitation to the broader aspects discussed herein. One aspect described in conjunction with a particular embodiment is not necessarily limited to that embodiment and can be practiced with any other embodiment(s). Reference throughout this specification to “one embodiment,” “an embodiment,” “an example embodiment,” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment,” “in an embodiment,” or “an example embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore,

the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention. For example, in the appended claims, any of the claimed embodiments can be used in any combination.

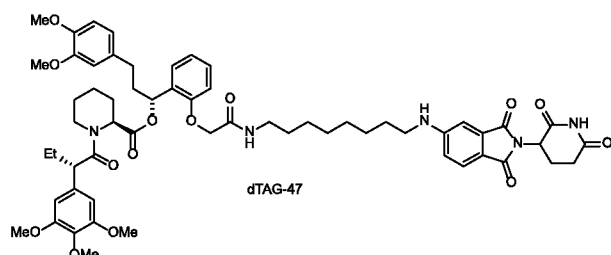
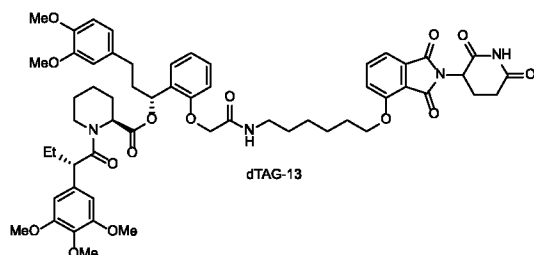
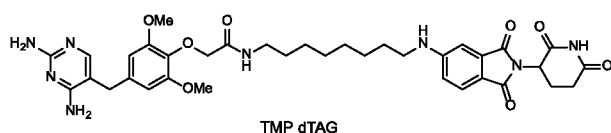
[0026] A degrader or degradation as used herein is a that modulates the activity of a protein or polypeptide. The degraders can specifically target a protein or polypeptide to the proteasome for degradation. In some instances, these degrader compositions for modulating activity target a variant CRISPR Cas protein. In one specific embodiment, the degraders target FKB12^{F36V} insertions in a variant CRISPR Cas protein.

[0027] All publications, published patent documents, and patent applications cited herein are hereby incorporated by reference to the same extent as though each individual publication, published patent document, or patent application was specifically and individually indicated as being incorporated by reference.

Overview

[0028] The presently disclosed subject matter provides compounds, systems and methods for target-specific protein degradation in CRISPR-Cas systems. In particular, the currently disclosed system provides small molecule degraders of Cas variant proteins containing one or more FKB12^{F36V} insertions.

[0029] In one embodiment, the degraders are heterobifunctional degraders. In one aspect, the degraders of the current system are cell-permeable. In a particular embodiment, the degrader is a dTAG. (11) In specific embodiments, the dTAG is selected from a compound or pharmaceutically acceptable salt selected from:



and

[0030] The degraders of the current system are utilized for target-specific protein degradation. In one aspect, the protein is a Cas effector protein. In some embodiments, the Cas effector protein is a Cas9, a Cas12a, Cas12b, Cas12c, Cas12d, Cas13a, Cas13b, Cas13c, or Cas13d system. In one embodiment the Cas protein is a Cas9 or Cas 12 protein, in a particular embodiment, the Cas protein is a SpCas9 protein. The Cas effector protein is provided as a variant that is disposed to degrade upon contact with the degrader compositions disclosed herein.

[0031] In one aspect, the invention provides an engineered, non-naturally occurring CRISPR-Cas system comprising a variant CRISPR Cas protein, and a guide RNA (or guide DNA) that targets a DNA or RNA molecule encoding a gene product in a cell, whereby the guide RNA/DNA targets the DNA/RNA molecule encoding the gene product and the Cas cleaves the DNA or RNA molecule encoding the gene product, whereby expression of the gene product is altered; and, wherein the Cas protein and the guide RNA (or DNA) do not naturally occur together. The Cas variant protein of the specific invention is engineered to contain insertions to which a degrader molecule of the instant invention targets. Once all copies of a gene in the genome of a cell have been edited, or the use of the CRISPR-Cas system is complete, continued CRISPR/Cas expression in that cell is no longer necessary. Indeed, sustained expression would be undesirable in case of off-target effects at unintended genomic sites, *etc.* Accordingly, in one aspect, the degrader molecule targets the Cas variant

protein at the insertions to degrade the Cas variant protein. In this manner, the degrader molecule will alter or decrease the enzymatic activity of the variant CRISPR Cas protein.

CRISPR-Cas Systems

[0032] In general, a CRISPR-Cas or CRISPR system as used herein and in documents, such as WO 2014/093622 (PCT/US2013/074667), refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated (“Cas”) genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a “direct repeat” and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a “spacer” in the context of an endogenous CRISPR system), or “RNA(s)” as that term is herein used (e.g., RNA(s) to guide Cas, such as Cas9, e.g. CRISPR RNA and transactivating (tracr) RNA or a single guide RNA (sgRNA) (chimeric RNA)) or other sequences and transcripts from a CRISPR locus. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). See, e.g., Shmakov et al. (2015) “Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems”, *Molecular Cell*, DOI: dx.doi.org/10.1016/j.molcel.2015.10.008. When the CRISPR protein is a Cpf1 protein, a tracrRNA is not required.

[0033] In certain embodiments, the CRISPR-Cas system is a class 2 CRISPR system, including Type II, Type V and Type VI systems. In certain example embodiments, the CRISPR system is a Cas9, a Cas12a, Cas12b, Cas12c, Cas12d, Cas13a, Cas13b, Cas13c, or Cas13d system.

[0034] As used herein, the term “Cas” can refer to a (modified) effector protein of the CRISPR/Cas system or complex, and can be without limitation a (modified) Cas9 or a (modified) Cas12 (e.g. Cas12a “Cpf1”, Cas12b “C2c1,” Cas12c “C2c3”), or, can be any other class 2 CRISPR system, for example, Cas 13a, Cas13b, Cas13c or Cas13d. The term “Cas” may be used herein interchangeably with the terms “CRISPR” protein, “CRISPR/Cas protein”, “CRISPR effector”, “CRISPR/Cas effector”, “CRISPR enzyme”, “CRISPR/Cas enzyme” and the like, unless otherwise apparent, such as by specific and exclusive reference to Cas9. It is to be understood that the term “CRISPR protein” may be used interchangeably with “CRISPR enzyme”, irrespective of whether the CRISPR protein has altered, such as

increased or decreased (or no) enzymatic activity, compared to the wild type CRISPR protein.

[0035] In some embodiments, the CRISPR Cas variant is based on a Type-II CRISPR effector protein such as Cas9. In some embodiments, the CRISPR Cas variant is based on a Type-V CRISPR effector protein such as Cas12a, Cas12b, or Cas12c. In some embodiments the CRISPR Cas variant is based on a Type-VI CRISPR effector protein such as Cas13a, Cas13b, Cas13c or Cas13d.

[0036] In some embodiments, the CRISPR Cas variant protein is a Cas9 CRISPR Cas variant, for instance SaCas9, SpCas9, StCas9, CjCas9 and so forth – any ortholog is envisaged. In some embodiments, the CRISPR Cas variant is a Cpf1 CRISPR Cas variant, for instance AsCpf1, LbCpf1, FnCpf1 and so forth – any ortholog is envisaged. Modifications to the location of insertion sites can be made according to the Cas effector protein.

[0037] In particular embodiments, the Cas variant protein comprise one, two three or more FKBP binding protein (FKBP) domains introduced into the Cas protein. The FKBP domains may be introduced at one or more insertion sites. In certain embodiments, the one or more insertion sites are at the N-terminal (Nt), C-terminal (Ct) or at a position corresponding to position 231 (Lp) of a SpCas9 protein. In particular embodiments, the FKBP domain is introduced at NT and Lp, at Nt and Ct, at Lp and Ct or at Nt, Ct and Lp sites on a Cas protein. In one aspect, the Cas variant is codon optimized for expression in eukaryotes. In an exemplary embodiment, one or more of the FKBP domains is an FKBP12^{F36V} domain.

[0038] In certain embodiments, a protospacer adjacent motif (PAM) or PAM-like motif directs binding of the effector protein complex as disclosed herein to the target locus of interest. In some embodiments, the PAM may be a 5' PAM (i.e., located upstream of the 5' end of the protospacer). In other embodiments, the PAM may be a 3' PAM (i.e., located downstream of the 5' end of the protospacer). The term “PAM” may be used interchangeably with the term “PFS” or “protospacer flanking site” or “protospacer flanking sequence”. In a preferred embodiment, the CRISPR effector protein may recognize a 3' PAM. In certain embodiments, the CRISPR effector protein may recognize a 3' PAM which is 5'H, wherein H is A, C or U.

[0039] In the context of formation of a CRISPR complex, “target sequence” refers to a sequence to which a guide sequence is designed to have complementarity, where

hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence may comprise RNA polynucleotides. The term “target RNA” refers to a RNA polynucleotide being or comprising the target sequence. In other words, the target RNA may be a RNA polynucleotide or a part of a RNA polynucleotide to which a part of the gRNA, i.e. the guide sequence, is designed to have complementarity and to which the effector function mediated by the complex comprising CRISPR effector protein and a gRNA is to be directed. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell.

[0040] In certain example embodiments, the CRISPR effector protein may be delivered using a nucleic acid molecule encoding the CRISPR effector protein. The nucleic acid molecule encoding a CRISPR effector protein, may advantageously be a codon optimized CRISPR effector protein. An example of a codon optimized sequence, is in this instance a sequence optimized for expression in eukaryote, e.g., humans (i.e. being optimized for expression in humans), or for another eukaryote, animal or mammal as herein discussed; see, e.g., SaCas9 human codon optimized sequence in WO 2014/093622 (PCT/US2013/074667). Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species other than human, or for codon optimization for specific organs is known. In some embodiments, an enzyme coding sequence encoding a CRISPR effector protein is a codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a plant or a mammal, including but not limited to human, or non-human eukaryote or animal or mammal as herein discussed, e.g., mouse, rat, rabbit, dog, livestock, or non-human mammal or primate. In some embodiments, processes for modifying the germ line genetic identity of human beings and/or processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes, may be excluded. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be

dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the “Codon Usage Database” available at kazusa.or.jp/codon/ and these tables can be adapted in a number of ways. See Nakamura, Y., et al. “Codon usage tabulated from the international DNA sequence databases: status for the year 2000” *Nucl. Acids Res.* 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a Cas correspond to the most frequently used codon for a particular amino acid.

[0041] In certain embodiments, the methods as described herein may comprise providing a Cas transgenic cell in which one or more nucleic acids encoding one or more guide RNAs are provided or introduced operably connected in the cell with a regulatory element comprising a promoter of one or more gene of interest. As used herein, the term “Cas transgenic cell” refers to a cell, such as a eukaryotic cell, in which a Cas gene has been genomically integrated. The nature, type, or origin of the cell are not particularly limiting according to the present invention. Also the way the Cas transgene is introduced in the cell may vary and can be any method as is known in the art. In certain embodiments, the Cas transgenic cell is obtained by introducing the Cas transgene in an isolated cell. In certain other embodiments, the Cas transgenic cell is obtained by isolating cells from a Cas transgenic organism. By means of example, and without limitation, the Cas transgenic cell as referred to herein may be derived from a Cas transgenic eukaryote, such as a Cas knock-in eukaryote. Reference is made to WO 2014/093622 (PCT/US13/74667), incorporated herein by reference. Methods of US Patent Publication Nos. 20120017290 and 20110265198 assigned to Sangamo BioSciences, Inc. directed to targeting the Rosa locus may be modified to utilize the CRISPR Cas system of the present invention. Methods of US Patent Publication No. 20130236946 assigned to Cellectis directed to targeting the Rosa locus may also be modified to utilize the CRISPR Cas system of the present invention. By means of further example reference is made to Platt et. al. (*Cell*; 159(2):440-455 (2014)), describing a Cas9 knock-in mouse, which is incorporated herein by reference. The Cas transgene can further

comprise a Lox-Stop-poly A-Lox(LSL) cassette thereby rendering Cas expression inducible by Cre recombinase. Alternatively, the Cas transgenic cell may be obtained by introducing the Cas transgene in an isolated cell. Delivery systems for transgenes are well known in the art. By means of example, the Cas transgene may be delivered in for instance eukaryotic cell by means of vector (e.g., AAV, adenovirus, lentivirus) and/or particle and/or nanoparticle delivery, as also described herein elsewhere.

[0042] It will be understood by the skilled person that the cell, such as the Cas transgenic cell, as referred to herein may comprise further genomic alterations besides having an integrated Cas gene or the mutations arising from the sequence specific action of Cas when complexed with RNA capable of guiding Cas to a target locus. In certain embodiments, the transfected cell is HEK293FT, U2OS, S2 or NIH3T3 cells. In one aspect the cell is a germline cell.

[0043] In certain aspects, the invention involves ribonucleoprotein comprising the variant CRISPR-Cas proteins disclosed herein. Pre-formed RNP comprising the variant CRISPR-Cas proteins can be used for nucleofection of cells. In embodiments, 5 pmol, 10 pmol, 15 pmol, 20 pmol 25 pmol or more of a preformed RNP can be utilized for nucleofection according to the invention disclosed herein.

[0044] In certain aspects the invention involves vectors, e.g. for delivering or introducing in a cell Cas and/or RNA capable of guiding Cas to a target locus (i.e. guide RNA), but also for propagating these components (e.g. in prokaryotic cells). In particular embodiments, a vector comprising the sequences of Table 1 or Table 2 are used for cell transfection according to the present invention. As used herein, a “vector” is a tool that allows or facilitates the transfer of an entity from one environment to another. It is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a vector is capable of replication when associated with the proper control elements. In general, the term “vector” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a “plasmid,” which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of

vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses (AAVs)). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as “expression vectors.” Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. Expression vectors may in some instances comprise the sequences disclosed in Table 2 of the instant disclosure.

[0045] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operably linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). With regards to recombination and cloning methods, mention is made of U.S. patent application 10/815,730, published September 2, 2004 as US 2004-0171156 A1, the contents of which are herein incorporated by reference in their entirety. Thus, the embodiments disclosed herein may also comprise transgenic cells comprising the CRISPR effector system. In certain example embodiments, the transgenic cell may function as an individual discrete volume. In other words samples comprising a masking construct may be delivered to a cell, for example in a suitable delivery vesicle and if the target is present in the delivery vesicle the CRISPR effector is activated and a detectable signal generated.

[0046] The vector(s) can include the regulatory element(s), e.g., promoter(s). The vector(s) can comprise Cas encoding sequences, and/or a single, but possibly also can comprise at least 3 or 8 or 16 or 32 or 48 or 50 guide RNA(s) (e.g., sgRNAs) encoding sequences, such as 1-2, 1-3, 1-4 1-5, 3-6, 3-7, 3-8, 3-9, 3-10, 3-8, 3-16, 3-30, 3-32, 3-48, 3-50

RNA(s) (e.g., sgRNAs). In a single vector there can be a promoter for each RNA (e.g., sgRNA), advantageously when there are up to about 16 RNA(s); and, when a single vector provides for more than 16 RNA(s), one or more promoter(s) can drive expression of more than one of the RNA(s), e.g., when there are 32 RNA(s), each promoter can drive expression of two RNA(s), and when there are 48 RNA(s), each promoter can drive expression of three RNA(s). By simple arithmetic and well established cloning protocols and the teachings in this disclosure one skilled in the art can readily practice the invention as to the RNA(s) for a suitable exemplary vector such as AAV, and a suitable promoter such as the U6 promoter. For example, the packaging limit of AAV is ~4.7 kb. The length of a single U6-gRNA (plus restriction sites for cloning) is 361 bp. Therefore, the skilled person can readily fit about 12-16, e.g., 13 U6-gRNA cassettes in a single vector. This can be assembled by any suitable means, such as a golden gate strategy used for TALE assembly (genome-engineering.org/talectors/). The skilled person can also use a tandem guide strategy to increase the number of U6-gRNAs by approximately 1.5 times, e.g., to increase from 12-16, e.g., 13 to approximately 18-24, e.g., about 19 U6-gRNAs. Therefore, one skilled in the art can readily reach approximately 18-24, e.g., about 19 promoter-RNAs, e.g., U6-gRNAs in a single vector, e.g., an AAV vector. A further means for increasing the number of promoters and RNAs in a vector is to use a single promoter (e.g., U6) to express an array of RNAs separated by cleavable sequences. And an even further means for increasing the number of promoter-RNAs in a vector, is to express an array of promoter-RNAs separated by cleavable sequences in the intron of a coding sequence or gene; and, in this instance it is advantageous to use a polymerase II promoter, which can have increased expression and enable the transcription of long RNA in a tissue specific manner. (see, e.g., nar.oxfordjournals.org/content/34/7/e53.short and nature.com/mt/journal/v16/n9/abs/mt2008144a.html). In an advantageous embodiment, AAV may package U6 tandem gRNA targeting up to about 50 genes. Accordingly, from the knowledge in the art and the teachings in this disclosure the skilled person can readily make and use vector(s), e.g., a single vector, expressing multiple RNAs or guides under the control or operatively or functionally linked to one or more promoters—especially as to the numbers of RNAs or guides discussed herein, without any undue experimentation.

[0047] The guide RNA(s) encoding sequences and/or Cas encoding sequences, can be functionally or operatively linked to regulatory element(s) and hence the regulatory element(s) drive expression. The promoter(s) can be constitutive promoter(s) and/or

conditional promoter(s) and/or inducible promoter(s) and/or tissue specific promoter(s). The promoter can be selected from the group consisting of RNA polymerases, pol I, pol II, pol III, T7, U6, H1, retroviral Rous sarcoma virus (RSV) LTR promoter, the cytomegalovirus (CMV) promoter, the SV40 promoter, the dihydrofolate reductase promoter, the β -actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 α promoter. An advantageous promoter is the promoter is U6.

[0048] Additional effectors for use according to the invention can be identified by their proximity to cas1 genes, for example, though not limited to, within the region 20 kb from the start of the cas1 gene and 20 kb from the end of the cas1 gene. In certain embodiments, the effector protein comprises at least one HEPN domain and at least 500 amino acids, and wherein the C2c2 effector protein is naturally present in a prokaryotic genome within 20 kb upstream or downstream of a Cas gene or a CRISPR array. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologues thereof, or modified versions thereof. In certain example embodiments, the C2c2 effector protein is naturally present in a prokaryotic genome within 20kb upstream or downstream of a Cas 1 gene. The terms “orthologue” (also referred to as “ortholog” herein) and “homologue” (also referred to as “homolog” herein) are well known in the art. By means of further guidance, a “homologue” of a protein as used herein is a protein of the same species which performs the same or a similar function as the protein it is a homologue of. Homologous proteins may but need not be structurally related, or are only partially structurally related. An “orthologue” of a protein as used herein is a protein of a different species which performs the same or a similar function as the protein it is an orthologue of. Orthologous proteins may but need not be structurally related, or are only partially structurally related.

[0049] The term “pharmaceutically acceptable salt” refers to those salts that are within the scope of proper medicinal assessment, suitable for use in contact with human tissues and organs and those of lower animals, without undue toxicity, irritation, allergic response or similar and are consistent with a reasonable benefit/risk ratio. In some embodiments, pharmaceutically acceptable salts can be formed by the reaction of a disclosed compound with an equimolar or excess amount of acid. Alternatively, hemi-salts can be formed by the

reaction of a compound with the desired acid in a 2:1 ratio, compound to acid. The reactants are generally combined in a mutual solvent such as diethyl ether, tetrahydrofuran, methanol, ethanol, iso-propanol, benzene, or the like. The salts normally precipitate out of solution within, e.g., about one hour to about ten days and can be isolated by filtration or other conventional methods.

Guide Molecules

[0050] The methods described herein may be used to modulate and/or screen modulation of CRISPR systems employing different types of guide molecules. As used herein, the term “guide sequence” and “guide molecule” in the context of a CRISPR-Cas system, comprises any polynucleotide sequence having sufficient complementarity with a target nucleic acid sequence to hybridize with the target nucleic acid sequence and direct sequence-specific binding of a nucleic acid-targeting complex to the target nucleic acid sequence. The guide sequences made using the methods disclosed herein may be a full-length guide sequence, a truncated guide sequence, a full-length sgRNA sequence, a truncated sgRNA sequence, or an E+F sgRNA sequence. In some embodiments, the degree of complementarity of the guide sequence to a given 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. In certain example embodiments, the guide molecule comprises a guide sequence that may be designed to have at least one mismatch with the target sequence, such that a RNA duplex formed between the guide sequence and the target sequence. Accordingly, the degree of complementarity is preferably less than 99%. For instance, where the guide sequence consists of 24 nucleotides, the degree of complementarity is more particularly about 96% or less. In particular embodiments, the guide sequence is designed to have a stretch of two or more adjacent mismatching nucleotides, such that the degree of complementarity over the entire guide sequence is further reduced. For instance, where the guide sequence consists of 24 nucleotides, the degree of complementarity is more particularly about 96% or less, more particularly, about 92% or less, more particularly about 88% or less, more particularly about 84% or less, more particularly about 80% or less, more particularly about 76% or less, more particularly about 72% or less, depending on whether the stretch of two or more mismatching nucleotides encompasses 2, 3, 4, 5, 6 or 7 nucleotides, etc. In some embodiments, aside from the stretch of one or more mismatching nucleotides, the degree of complementarity, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal

alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g., the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). The ability of a guide sequence (within a nucleic acid-targeting guide RNA) to direct sequence-specific binding of a nucleic acid -targeting complex to a target nucleic acid sequence may be assessed by any suitable assay. For example, the components of a nucleic acid-targeting CRISPR system sufficient to form a nucleic acid-targeting complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target nucleic acid sequence, such as by transfection with vectors encoding the components of the nucleic acid-targeting complex, followed by an assessment of preferential targeting (e.g., cleavage) within the target nucleic acid sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target nucleic acid sequence (or a sequence in the vicinity thereof) may be evaluated in a test tube by providing the target nucleic acid sequence, components of a nucleic acid-targeting 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 or in the vicinity of the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art. A guide sequence, and hence a nucleic acid-targeting guide RNA may be selected to target any target nucleic acid sequence.

[0051] In certain embodiments, the guide sequence or spacer length of the guide molecules is from 15 to 50 nt. In certain embodiments, the spacer length of the guide RNA is at least 15 nucleotides. In certain embodiments, the spacer length is from 15 to 17 nt, e.g., 15, 16, or 17 nt, from 17 to 20 nt, e.g., 17, 18, 19, or 20 nt, from 20 to 24 nt, e.g., 20, 21, 22, 23, or 24 nt, from 23 to 25 nt, e.g., 23, 24, or 25 nt, from 24 to 27 nt, e.g., 24, 25, 26, or 27 nt, from 27-30 nt, e.g., 27, 28, 29, or 30 nt, from 30-35 nt, e.g., 30, 31, 32, 33, 34, or 35 nt, or 35 nt or longer. In certain example embodiment, the guide sequence is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 nt.

[0052] In some embodiments, the guide sequence is an RNA sequence of between 10 to 50 nt in length, but more particularly of about 20-30 nt advantageously about 20 nt, 23-25 nt or 24 nt. The guide sequence is selected so as to ensure that it hybridizes to the target sequence. This is described more in detail below. Selection can encompass further steps which increase efficacy and specificity.

[0053] In some embodiments, the guide sequence has a canonical length (e.g., about 15-30 nt) is used to hybridize with the target RNA or DNA. In some embodiments, a guide molecule is longer than the canonical length (e.g., >30 nt) is used to hybridize with the target RNA or DNA, such that a region of the guide sequence hybridizes with a region of the RNA or DNA strand outside of the Cas-guide target complex. This can be of interest where additional modifications, such as deamination of nucleotides is of interest. In alternative embodiments, it is of interest to maintain the limitation of the canonical guide sequence length.

[0054] In some embodiments, the sequence of the guide molecule (direct repeat and/or spacer) is selected to reduce the degree secondary structure within the guide molecule. In some embodiments, about or less than about 75%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or fewer of the nucleotides of the nucleic acid-targeting guide RNA participate in self-complementary base pairing when optimally folded. Optimal folding may be determined by any suitable polynucleotide folding algorithm. Some programs are based on calculating the minimal Gibbs free energy. An example of one such algorithm is mFold, as described by Zuker and Stiegler (Nucleic Acids Res. 9 (1981), 133-148). Another example folding algorithm is the online webserver RNAfold, developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g., A.R. Gruber et al., 2008, Cell 106(1): 23-24; and PA Carr and GM Church, 2009, Nature Biotechnology 27(12): 1151-62). In some embodiments, it is of interest to reduce the susceptibility of the guide molecule to RNA cleavage, such as to cleavage by Cas13. Accordingly, in particular embodiments, the guide molecule is adjusted to avoid cleavage by Cas13 or other RNA-cleaving enzymes.

[0055] In certain embodiments, the guide molecule comprises non-naturally occurring nucleic acids and/or non-naturally occurring nucleotides and/or nucleotide analogs, and/or chemically modifications. Preferably, these non-naturally occurring nucleic acids and non-naturally occurring nucleotides are located outside the guide sequence. Non-naturally occurring nucleic acids can include, for example, mixtures of naturally and non-naturally

occurring nucleotides. Non-naturally occurring nucleotides and/or nucleotide analogs may be modified at the ribose, phosphate, and/or base moiety. In an embodiment of the invention, a guide nucleic acid comprises ribonucleotides and non-ribonucleotides. In one such embodiment, a guide comprises one or more ribonucleotides and one or more deoxyribonucleotides. In an embodiment of the invention, the guide comprises one or more non-naturally occurring nucleotide or nucleotide analog such as a nucleotide with phosphorothioate linkage, a locked nucleic acid (LNA) nucleotides comprising a methylene bridge between the 2' and 4' carbons of the ribose ring, or bridged nucleic acids (BNA). Other examples of modified nucleotides include 2'-O-methyl analogs, 2'-deoxy analogs, or 2'-fluoro analogs. Further examples of modified bases include, but are not limited to, 2-aminopurine, 5-bromo-uridine, pseudouridine, inosine, 7-methylguanosine. Examples of guide RNA chemical modifications include, without limitation, incorporation of 2' -O-methyl (M), 2' -O-methyl 3' phosphorothioate (MS), *S*-constrained ethyl(cEt), or 2' -O-methyl 3' thioPACE (MSP) at one or more terminal nucleotides. Such chemically modified guides can comprise increased stability and increased activity as compared to unmodified guides, though on-target vs. off-target specificity is not predictable. (See, Hendel, 2015, *Nat Biotechnol.* 33(9):985-9, doi: 10.1038/nbt.3290, published online 29 June 2015 Ragdarm et al., 0215, *PNAS*, E7110-E7111; Allerson et al., *J. Med. Chem.* 2005, 48:901-904; Bramsen et al., *Front. Genet.*, 2012, 3:154; Deng et al., *PNAS*, 2015, 112:11870-11875; Sharma et al., *MedChemComm.*, 2014, 5:1454-1471; Hendel et al., *Nat. Biotechnol.* (2015) 33(9): 985-989; Li et al., *Nature Biomedical Engineering*, 2017, 1, 0066 DOI:10.1038/s41551-017-0066). In some embodiments, the 5' and/or 3' end of a guide RNA is modified by a variety of functional moieties including fluorescent dyes, polyethylene glycol, cholesterol, proteins, or detection tags. (See Kelly et al., 2016, *J. Biotech.* 233:74-83). In certain embodiments, a guide comprises ribonucleotides in a region that binds to a target RNA and one or more deoxyribonucleotides and/or nucleotide analogs in a region that binds to Cas13. In an embodiment of the invention, deoxyribonucleotides and/or nucleotide analogs are incorporated in engineered guide structures, such as, without limitation, stem-loop regions, and the seed region. For Cas13 guide, in certain embodiments, the modification is not in the 5'-handle of the stem-loop regions. Chemical modification in the 5'-handle of the stem-loop region of a guide may abolish its function (see Li, et al., *Nature Biomedical Engineering*, 2017, 1:0066). In certain embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 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, or 75 nucleotides of a guide is chemically modified. In some embodiments, 3-5 nucleotides at either the 3' or the 5' end of a guide is chemically modified. In some embodiments, only minor modifications are introduced in the seed region, such as 2'-F modifications. In some embodiments, 2'-F modification is introduced at the 3' end of a guide. In certain embodiments, three to five nucleotides at the 5' and/or the 3' end of the guide are chemically modified with 2'-O-methyl (M), 2'-O-methyl 3' phosphorothioate (MS), *S*-constrained ethyl(cEt), or 2'-O-methyl 3' thioPACE (MSP). Such modification can enhance genome editing efficiency (see Hendel et al., *Nat. Biotechnol.* (2015) 33(9): 985-989). In certain embodiments, all of the phosphodiester bonds of a guide are substituted with phosphorothioates (PS) for enhancing levels of gene disruption. In certain embodiments, more than five nucleotides at the 5' and/or the 3' end of the guide are chemically modified with 2'-O-Me, 2'-F or *S*-constrained ethyl(cEt). Such chemically modified guide can mediate enhanced levels of gene disruption (see Ragdarm et al., 0215, *PNAS*, E7110-E7111). In an embodiment of the invention, a guide is modified to comprise a chemical moiety at its 3' and/or 5' end. Such moieties include, but are not limited to amine, azide, alkyne, thio, dibenzocyclooctyne (DBCO), or Rhodamine. In certain embodiment, the chemical moiety is conjugated to the guide by a linker, such as an alkyl chain. In certain embodiments, the chemical moiety of the modified guide can be used to attach the guide to another molecule, such as DNA, RNA, protein, or nanoparticles. Such chemically modified guide can be used to identify or enrich cells generically edited by a CRISPR system (see Lee et al., *eLife*, 2017, 6:e25312, DOI:10.7554).

[0056] In some embodiments, the modification to the guide is a chemical modification, an insertion, a deletion or a split. In some embodiments, the chemical modification includes, but is not limited to, incorporation of 2'-O-methyl (M) analogs, 2'-deoxy analogs, 2-thiouridine analogs, N6-methyladenosine analogs, 2'-fluoro analogs, 2-aminopurine, 5-bromo-uridine, pseudouridine (Ψ), N1-methylpseudouridine (me1 Ψ), 5-methoxyuridine(5moU), inosine, 7-methylguanosine, 2'-O-methyl 3'phosphorothioate (MS), *S*-constrained ethyl(cEt), phosphorothioate (PS), or 2'-O-methyl 3'thioPACE (MSP). In some embodiments, the guide comprises one or more of phosphorothioate modifications. In certain embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 25 nucleotides of the guide are chemically modified. In certain embodiments, one or more nucleotides in the seed region are chemically modified. In certain embodiments, one or more nucleotides in the 3'-terminus are chemically modified. In certain embodiments, none of the

nucleotides in the 5'-handle is chemically modified. In some embodiments, the chemical modification in the seed region is a minor modification, such as incorporation of a 2'-fluoro analog. In a specific embodiment, one nucleotide of the seed region is replaced with a 2'-fluoro analog. In some embodiments, 5 to 10 nucleotides in the 3'-terminus are chemically modified. Such chemical modifications at the 3'-terminus of the Cas13 CrRNA may improve Cas13 activity. In a specific embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides in the 3'-terminus are replaced with 2'-fluoro analogues. In a specific embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides in the 3'-terminus are replaced with 2'- O-methyl (M) analogs.

[0057] In some embodiments, the loop of the 5'-handle of the guide is modified. In some embodiments, the loop of the 5'-handle of the guide is modified to have a deletion, an insertion, a split, or chemical modifications. In certain embodiments, the modified loop comprises 3, 4, or 5 nucleotides. In certain embodiments, the loop comprises the sequence of UCUU, UUUU, UAUU, or UGUU.

[0058] In some embodiments, the guide molecule forms a stemloop with a separate non-covalently linked sequence, which can be DNA or RNA. In particular embodiments, the sequences forming the guide are first synthesized using the standard phosphoramidite synthetic protocol (Herdewijn, P., ed., *Methods in Molecular Biology* Col 288, *Oligonucleotide Synthesis: Methods and Applications*, Humana Press, New Jersey (2012)). In some embodiments, these sequences can be functionalized to contain an appropriate functional group for ligation using the standard protocol known in the art (Hermanson, G. T., *Bioconjugate Techniques*, Academic Press (2013)). Examples of functional groups include, but are not limited to, hydroxyl, amine, carboxylic acid, carboxylic acid halide, carboxylic acid active ester, aldehyde, carbonyl, chlorocarbonyl, imidazolylcarbonyl, hydrozide, semicarbazide, thio semicarbazide, thiol, maleimide, haloalkyl, sulfonyl, ally, propargyl, diene, alkyne, and azide. Once this sequence is functionalized, a covalent chemical bond or linkage can be formed between this sequence and the direct repeat sequence. Examples of chemical bonds include, but are not limited to, those based on carbamates, ethers, esters, amides, imines, amidines, aminotrizines, hydrozone, disulfides, thioethers, thioesters, phosphorothioates, phosphorodithioates, sulfonamides, sulfonates, fulfones, sulfoxides, ureas, thioureas, hydrazide, oxime, triazole, photolabile linkages, C-C bond forming groups such as Diels-Alder cyclo-addition pairs or ring-closing metathesis pairs, and Michael reaction pairs.

[0059] In some embodiments, these stem-loop forming sequences can be chemically synthesized. In some embodiments, the chemical synthesis uses automated, solid-phase oligonucleotide synthesis machines with 2'-acetoxylethyl orthoester (2'-ACE) (Scaringe et al., J. Am. Chem. Soc. (1998) 120: 11820-11821; Scaringe, Methods Enzymol. (2000) 317: 3-18) or 2'-thionocarbamate (2'-TC) chemistry (Dellinger et al., J. Am. Chem. Soc. (2011) 133: 11540-11546; Hendel et al., Nat. Biotechnol. (2015) 33:985-989).

[0060] In certain embodiments, the guide molecule comprises (1) a guide sequence capable of hybridizing to a target locus and (2) a tracr mate or direct repeat sequence whereby the direct repeat sequence is located upstream (i.e., 5') from the guide sequence. In a particular embodiment the seed sequence (i.e. the sequence essential critical for recognition and/or hybridization to the sequence at the target locus) of the guide sequence is approximately within the first 10 nucleotides of the guide sequence.

[0061] In a particular embodiment the guide molecule comprises a guide sequence linked to a direct repeat sequence, wherein the direct repeat sequence comprises one or more stem loops or optimized secondary structures. In particular embodiments, the direct repeat has a minimum length of 16 nts and a single stem loop. In further embodiments the direct repeat has a length longer than 16 nts, preferably more than 17 nts, and has more than one stem loops or optimized secondary structures. In particular embodiments the guide molecule comprises or consists of the guide sequence linked to all or part of the natural direct repeat sequence. A typical Type V or Type VI CRISPR-cas guide molecule comprises (in 3' to 5' direction or in 5' to 3' direction): a guide sequence a first complimentary stretch (the "repeat"), a loop (which is typically 4 or 5 nucleotides long), a second complimentary stretch (the "anti-repeat" being complimentary to the repeat), and a poly A (often poly U in RNA) tail (terminator). In certain embodiments, the direct repeat sequence retains its natural architecture and forms a single stem loop. In particular embodiments, certain aspects of the guide architecture can be modified, for example by addition, subtraction, or substitution of features, whereas certain other aspects of guide architecture are maintained. Preferred locations for engineered guide molecule modifications, including but not limited to insertions, deletions, and substitutions include guide termini and regions of the guide molecule that are exposed when complexed with the CRISPR-Cas protein and/or target, for example the stemloop of the direct repeat sequence.

[0062] In particular embodiments, the stem comprises at least about 4bp comprising complementary X and Y sequences, although stems of more, e.g., 5, 6, 7, 8, 9, 10, 11 or 12 or

fewer, e.g., 3, 2, base pairs are also contemplated. Thus, for example X₂-10 and Y₂-10 (wherein X and Y represent any complementary set of nucleotides) may be contemplated. In one aspect, the stem made of the X and Y nucleotides, together with the loop will form a complete hairpin in the overall secondary structure; and, this may be advantageous and the amount of base pairs can be any amount that forms a complete hairpin. In one aspect, any complementary X:Y basepairing sequence (e.g., as to length) is tolerated, so long as the secondary structure of the entire guide molecule is preserved. In one aspect, the loop that connects the stem made of X:Y basepairs can be any sequence of the same length (e.g., 4 or 5 nucleotides) or longer that does not interrupt the overall secondary structure of the guide molecule. In one aspect, the stemloop can further comprise, e.g. an MS2 aptamer. In one aspect, the stem comprises about 5-7bp comprising complementary X and Y sequences, although stems of more or fewer basepairs are also contemplated. In one aspect, non-Watson Crick basepairing is contemplated, where such pairing otherwise generally preserves the architecture of the stemloop at that position.

[0063] In particular embodiments the natural hairpin or stemloop structure of the guide molecule is extended or replaced by an extended stemloop. It has been demonstrated that extension of the stem can enhance the assembly of the guide molecule with the CRISPR-Cas protein (Chen et al. *Cell*. (2013); 155(7): 1479–1491). In particular embodiments the stem of the stemloop is extended by at least 1, 2, 3, 4, 5 or more complementary basepairs (i.e. corresponding to the addition of 2, 4, 6, 8, 10 or more nucleotides in the guide molecule). In particular embodiments these are located at the end of the stem, adjacent to the loop of the stemloop.

[0064] In particular embodiments, the susceptibility of the guide molecule to RNases or to decreased expression can be reduced by slight modifications of the sequence of the guide molecule which do not affect its function. For instance, in particular embodiments, premature termination of transcription, such as premature transcription of U6 Pol-III, can be removed by modifying a putative Pol-III terminator (4 consecutive U's) in the guide molecules sequence. Where such sequence modification is required in the stemloop of the guide molecule, it is preferably ensured by a basepair flip.

[0065] In a particular embodiment the direct repeat may be modified to comprise one or more protein-binding RNA aptamers. In a particular embodiment, one or more aptamers may be included such as part of optimized secondary structure. Such aptamers may be capable of binding a bacteriophage coat protein as detailed further herein.

[0066] In some embodiments, the guide molecule forms a duplex with a target RNA comprising at least one target cytosine residue to be edited. Upon hybridization of the guide RNA molecule to the target RNA, the cytidine deaminase binds to the single strand RNA in the duplex made accessible by the mismatch in the guide sequence and catalyzes deamination of one or more target cytosine residues comprised within the stretch of mismatching nucleotides.

A guide sequence, and hence a nucleic acid-targeting guide RNA may be selected to target any target nucleic acid sequence. The target sequence may be mRNA.

[0067] In certain embodiments, the target sequence should be associated with a PAM (protospacer adjacent motif) or PFS (protospacer flanking sequence or site); that is, a short sequence recognized by the CRISPR complex. Depending on the nature of the CRISPR-Cas protein, the target sequence should be selected such that its complementary sequence in the DNA duplex (also referred to herein as the non-target sequence) is upstream or downstream of the PAM. In the embodiments of the present invention where the CRISPR-Cas protein is a Cas13 protein, the complementary sequence of the target sequence is downstream or 3' of the PAM or upstream or 5' of the PAM. The precise sequence and length requirements for the PAM differ depending on the Cas13 protein used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence). Examples of the natural PAM sequences for different Cas13 orthologues are provided herein below and the skilled person will be able to identify further PAM sequences for use with a given Cas13 protein.

[0068] Further, engineering of the PAM Interacting (PI) domain may allow programming of PAM specificity, improve target site recognition fidelity, and increase the versatility of the CRISPR-Cas protein, for example as described for Cas9 in Kleinstiver BP et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature*. 2015 Jul 23;523(7561):481-5. doi: 10.1038/nature14592. As further detailed herein, the skilled person will understand that Cas13 proteins may be modified analogously.

[0069] In a particular embodiment, the guide is an escorted guide. By "escorted" is meant that the CRISPR-Cas system or complex or guide is delivered to a selected time or place within a cell, so that activity of the CRISPR-Cas system or complex or guide is spatially or temporally controlled. For example, the activity and destination of the CRISPR-Cas system or complex or guide may be controlled by an escort RNA aptamer sequence that has binding affinity for an aptamer ligand, such as a cell surface protein or other localized cellular component. Alternatively, the escort aptamer may for example be responsive to an aptamer

effector on or in the cell, such as a transient effector, such as an external energy source that is applied to the cell at a particular time.

The escorted CRISPR-Cas systems or complexes have a guide molecule with a functional structure designed to improve guide molecule structure, architecture, stability, genetic expression, or any combination thereof. Such a structure can include an aptamer.

[0070] Aptamers are biomolecules that can be designed or selected to bind tightly to other ligands, for example using a technique called systematic evolution of ligands by exponential enrichment (SELEX; Tuerk C, Gold L: "Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase." *Science* 1990, 249:505-510). Nucleic acid aptamers can for example be selected from pools of random-sequence oligonucleotides, with high binding affinities and specificities for a wide range of biomedically relevant targets, suggesting a wide range of therapeutic utilities for aptamers (Keefe, Anthony D., Supriya Pai, and Andrew Ellington. "Aptamers as therapeutics." *Nature Reviews Drug Discovery* 9.7 (2010): 537-550). These characteristics also suggest a wide range of uses for aptamers as drug delivery vehicles (Levy-Nissenbaum, Etgar, et al. "Nanotechnology and aptamers: applications in drug delivery." *Trends in biotechnology* 26.8 (2008): 442-449; and, Hicke BJ, Stephens AW. "Escort aptamers: a delivery service for diagnosis and therapy." *J Clin Invest* 2000, 106:923-928.). Aptamers may also be constructed that function as molecular switches, responding to a cue by changing properties, such as RNA aptamers that bind fluorophores to mimic the activity of green fluorescent protein (Paige, Jeremy S., Karen Y. Wu, and Samie R. Jaffrey. "RNA mimics of green fluorescent protein." *Science* 333.6042 (2011): 642-646). It has also been suggested that aptamers may be used as components of targeted siRNA therapeutic delivery systems, for example targeting cell surface proteins (Zhou, Jiehua, and John J. Rossi. "Aptamer-targeted cell-specific RNA interference." *Silence* 1.1 (2010): 4).

[0071] Accordingly, in particular embodiments, the guide molecule is modified, e.g., by one or more aptamer(s) designed to improve guide molecule delivery, including delivery across the cellular membrane, to intracellular compartments, or into the nucleus. Such a structure can include, either in addition to the one or more aptamer(s) or without such one or more aptamer(s), moiety(ies) so as to render the guide molecule deliverable, inducible or responsive to a selected effector. The invention accordingly comprehends an guide molecule that responds to normal or pathological physiological conditions, including without limitation pH, hypoxia, O₂ concentration, temperature, protein concentration, enzymatic concentration,

lipid structure, light exposure, mechanical disruption (e.g. ultrasound waves), magnetic fields, electric fields, or electromagnetic radiation.

[0072] Light responsiveness of an inducible system may be achieved via the activation and binding of cryptochrome-2 and CIB1. Blue light stimulation induces an activating conformational change in cryptochrome-2, resulting in recruitment of its binding partner CIB1. This binding is fast and reversible, achieving saturation in <15 sec following pulsed stimulation and returning to baseline <15 min after the end of stimulation. These rapid binding kinetics result in a system temporally bound only by the speed of transcription/translation and transcript/protein degradation, rather than uptake and clearance of inducing agents. Cryptochrome-2 activation is also highly sensitive, allowing for the use of low light intensity stimulation and mitigating the risks of phototoxicity. Further, in a context such as the intact mammalian brain, variable light intensity may be used to control the size of a stimulated region, allowing for greater precision than vector delivery alone may offer.

[0073] The invention contemplates energy sources such as electromagnetic radiation, sound energy or thermal energy to induce the guide. Advantageously, the electromagnetic radiation is a component of visible light. In a preferred embodiment, the light is a blue light with a wavelength of about 450 to about 495 nm. In an especially preferred embodiment, the wavelength is about 488 nm. In another preferred embodiment, the light stimulation is via pulses. The light power may range from about 0-9 mW/cm². In a preferred embodiment, a stimulation paradigm of as low as 0.25 sec every 15 sec should result in maximal activation.

[0074] The chemical or energy sensitive guide may undergo a conformational change upon induction by the binding of a chemical source or by the energy allowing it act as a guide and have the Cas13 CRISPR-Cas system or complex function. The invention can involve applying the chemical source or energy so as to have the guide function and the Cas13 CRISPR-Cas system or complex function; and optionally further determining that the expression of the genomic locus is altered.

There are several different designs of this chemical inducible system: 1. ABI-PYL based system inducible by Absciscic Acid (ABA) (see, e.g., <http://stke.sciencemag.org/cgi/content/abstract/sigtrans;4/164/rs2>), 2. FKBP-FRB based system inducible by rapamycin (or related chemicals based on rapamycin) (see, e.g., <http://www.nature.com/nmeth/journal/v2/n6/full/nmeth763.html>), 3. GID1-GAI based system inducible by Gibberellin (GA) (see, e.g., <http://www.nature.com/nchembio/journal/v8/n5/full/nchembio.922.html>).

[0075] A chemical inducible system can be an estrogen receptor (ER) based system inducible by 4-hydroxytamoxifen (4OHT) (see, e.g., <http://www.pnas.org/content/104/3/1027.abstract>). A mutated ligand-binding domain of the estrogen receptor called ERT2 translocates into the nucleus of cells upon binding of 4-hydroxytamoxifen. In further embodiments of the invention any naturally occurring or engineered derivative of any nuclear receptor, thyroid hormone receptor, retinoic acid receptor, estrogen receptor, estrogen-related receptor, glucocorticoid receptor, progesterone receptor, androgen receptor may be used in inducible systems analogous to the ER based inducible system.

[0076] Another inducible system is based on the design using Transient receptor potential (TRP) ion channel based system inducible by energy, heat or radio-wave (see, e.g., <http://www.sciencemag.org/content/336/6081/604>). These TRP family proteins respond to different stimuli, including light and heat. When this protein is activated by light or heat, the ion channel will open and allow the entering of ions such as calcium into the plasma membrane. This influx of ions will bind to intracellular ion interacting partners linked to a polypeptide including the guide and the other components of the Cas13 CRISPR-Cas complex or system, and the binding will induce the change of sub-cellular localization of the polypeptide, leading to the entire polypeptide entering the nucleus of cells. Once inside the nucleus, the guide protein and the other components of the Cas13 CRISPR-Cas complex will be active and modulating target gene expression in cells.

[0077] While light activation may be an advantageous embodiment, sometimes it may be disadvantageous especially for *in vivo* applications in which the light may not penetrate the skin or other organs. In this instance, other methods of energy activation are contemplated, in particular, electric field energy and/or ultrasound which have a similar effect.

[0078] Electric field energy is preferably administered substantially as described in the art, using one or more electric pulses of from about 1 Volt/cm to about 10 kVolts/cm under *in vivo* conditions. Instead of or in addition to the pulses, the electric field may be delivered in a continuous manner. The electric pulse may be applied for between 1 μ s and 500 milliseconds, preferably between 1 μ s and 100 milliseconds. The electric field may be applied continuously or in a pulsed manner for 5 about minutes.

[0079] As used herein, 'electric field energy' is the electrical energy to which a cell is exposed. Preferably the electric field has a strength of from about 1 Volt/cm to about 10 kVolts/cm or more under *in vivo* conditions (see WO97/49450).

[0080] As used herein, the term “electric field” includes one or more pulses at variable capacitance and voltage and including exponential and/or square wave and/or modulated wave and/or modulated square wave forms. References to electric fields and electricity should be taken to include reference the presence of an electric potential difference in the environment of a cell. Such an environment may be set up by way of static electricity, alternating current (AC), direct current (DC), etc, as known in the art. The electric field may be uniform, non-uniform or otherwise, and may vary in strength and/or direction in a time dependent manner.

[0081] Single or multiple applications of electric field, as well as single or multiple applications of ultrasound are also possible, in any order and in any combination. The ultrasound and/or the electric field may be delivered as single or multiple continuous applications, or as pulses (pulsatile delivery).

[0082] Electroporation has been used in both *in vitro* and *in vivo* procedures to introduce foreign material into living cells. With *in vitro* applications, a sample of live cells is first mixed with the agent of interest and placed between electrodes such as parallel plates. Then, the electrodes apply an electrical field to the cell/implant mixture. Examples of systems that perform *in vitro* electroporation include the Electro Cell Manipulator ECM600 product, and the Electro Square Porator T820, both made by the BTX Division of Genetronics, Inc (see U.S. Pat. No 5,869,326).

[0083] The known electroporation techniques (both *in vitro* and *in vivo*) function by applying a brief high voltage pulse to electrodes positioned around the treatment region. The electric field generated between the electrodes causes the cell membranes to temporarily become porous, whereupon molecules of the agent of interest enter the cells. In known electroporation applications, this electric field comprises a single square wave pulse on the order of 1000 V/cm, of about 100 μ s duration. Such a pulse may be generated, for example, in known applications of the Electro Square Porator T820.

[0084] Preferably, the electric field has a strength of from about 1 V/cm to about 10 kV/cm under *in vitro* conditions. Thus, the electric field may have a strength of 1 V/cm, 2 V/cm, 3 V/cm, 4 V/cm, 5 V/cm, 6 V/cm, 7 V/cm, 8 V/cm, 9 V/cm, 10 V/cm, 20 V/cm, 50 V/cm, 100 V/cm, 200 V/cm, 300 V/cm, 400 V/cm, 500 V/cm, 600 V/cm, 700 V/cm, 800 V/cm, 900 V/cm, 1 kV/cm, 2 kV/cm, 5 kV/cm, 10 kV/cm, 20 kV/cm, 50 kV/cm or more. More preferably from about 0.5 kV/cm to about 4.0 kV/cm under *in vitro* conditions. Preferably the electric field has a strength of from about 1 V/cm to about 10 kV/cm under *in*

vivo conditions. However, the electric field strengths may be lowered where the number of pulses delivered to the target site are increased. Thus, pulsatile delivery of electric fields at lower field strengths is envisaged.

Preferably the application of the electric field is in the form of multiple pulses such as double pulses of the same strength and capacitance or sequential pulses of varying strength and/or capacitance. As used herein, the term “pulse” includes one or more electric pulses at variable capacitance and voltage and including exponential and/or square wave and/or modulated wave/square wave forms.

[0085] Preferably the electric pulse is delivered as a waveform selected from an exponential wave form, a square wave form, a modulated wave form and a modulated square wave form.

[0086] A preferred embodiment employs direct current at low voltage. Thus, Applicants disclose the use of an electric field which is applied to the cell, tissue or tissue mass at a field strength of between 1V/cm and 20V/cm, for a period of 100 milliseconds or more, preferably 15 minutes or more.

[0087] Ultrasound is advantageously administered at a power level of from about 0.05 W/cm² to about 100 W/cm². Diagnostic or therapeutic ultrasound may be used, or combinations thereof.

[0088] As used herein, the term “ultrasound” refers to a form of energy which consists of mechanical vibrations the frequencies of which are so high they are above the range of human hearing. Lower frequency limit of the ultrasonic spectrum may generally be taken as about 20 kHz. Most diagnostic applications of ultrasound employ frequencies in the range 1 and 15 MHz' (From Ultrasonics in Clinical Diagnosis, P. N. T. Wells, ed., 2nd. Edition, Publ. Churchill Livingstone [Edinburgh, London & NY, 1977]).

[0089] Ultrasound has been used in both diagnostic and therapeutic applications. When used as a diagnostic tool ("diagnostic ultrasound"), ultrasound is typically used in an energy density range of up to about 100 mW/cm² (FDA recommendation), although energy densities of up to 750 mW/cm² have been used. In physiotherapy, ultrasound is typically used as an energy source in a range up to about 3 to 4 W/cm² (WHO recommendation). In other therapeutic applications, higher intensities of ultrasound may be employed, for example, HIFU at 100 W/cm up to 1 kW/cm² (or even higher) for short periods of time. The term "ultrasound" as used in this specification is intended to encompass diagnostic, therapeutic and focused ultrasound.

[0090] Focused ultrasound (FUS) allows thermal energy to be delivered without an invasive probe (see Morocz et al 1998 Journal of Magnetic Resonance Imaging Vol.8, No. 1, pp.136-142. Another form of focused ultrasound is high intensity focused ultrasound (HIFU) which is reviewed by Moussatov et al in Ultrasonics (1998) Vol.36, No.8, pp.893-900 and TranHuuHue et al in Acustica (1997) Vol.83, No.6, pp.1103-1106.

[0091] Preferably, a combination of diagnostic ultrasound and a therapeutic ultrasound is employed. This combination is not intended to be limiting, however, and the skilled reader will appreciate that any variety of combinations of ultrasound may be used. Additionally, the energy density, frequency of ultrasound, and period of exposure may be varied.

[0092] Preferably the exposure to an ultrasound energy source is at a power density of from about 0.05 to about 100 Wcm⁻². Even more preferably, the exposure to an ultrasound energy source is at a power density of from about 1 to about 15 Wcm⁻².

[0093] Preferably the exposure to an ultrasound energy source is at a frequency of from about 0.015 to about 10.0 MHz. More preferably the exposure to an ultrasound energy source is at a frequency of from about 0.02 to about 5.0 MHz or about 6.0 MHz. Most preferably, the ultrasound is applied at a frequency of 3 MHz.

[0094] Preferably the exposure is for periods of from about 10 milliseconds to about 60 minutes. Preferably the exposure is for periods of from about 1 second to about 5 minutes. More preferably, the ultrasound is applied for about 2 minutes. Depending on the particular target cell to be disrupted, however, the exposure may be for a longer duration, for example, for 15 minutes.

[0095] Advantageously, the target tissue is exposed to an ultrasound energy source at an acoustic power density of from about 0.05 Wcm⁻² to about 10 Wcm⁻² with a frequency ranging from about 0.015 to about 10 MHz (see WO 98/52609). However, alternatives are also possible, for example, exposure to an ultrasound energy source at an acoustic power density of above 100 Wcm⁻², but for reduced periods of time, for example, 1000 Wcm⁻² for periods in the millisecond range or less.

[0096] Preferably the application of the ultrasound is in the form of multiple pulses; thus, both continuous wave and pulsed wave (pulsatile delivery of ultrasound) may be employed in any combination. For example, continuous wave ultrasound may be applied, followed by pulsed wave ultrasound, or vice versa. This may be repeated any number of times, in any order and combination. The pulsed wave ultrasound may be applied against a background of

continuous wave ultrasound, and any number of pulses may be used in any number of groups.

[0097] Preferably, the ultrasound may comprise pulsed wave ultrasound. In a highly preferred embodiment, the ultrasound is applied at a power density of 0.7 Wcm⁻² or 1.25 Wcm⁻² as a continuous wave. Higher power densities may be employed if pulsed wave ultrasound is used.

[0098] Use of ultrasound is advantageous as, like light, it may be focused accurately on a target. Moreover, ultrasound is advantageous as it may be focused more deeply into tissues unlike light. It is therefore better suited to whole-tissue penetration (such as but not limited to a lobe of the liver) or whole organ (such as but not limited to the entire liver or an entire muscle, such as the heart) therapy. Another important advantage is that ultrasound is a non-invasive stimulus which is used in a wide variety of diagnostic and therapeutic applications. By way of example, ultrasound is well known in medical imaging techniques and, additionally, in orthopedic therapy. Furthermore, instruments suitable for the application of ultrasound to a subject vertebrate are widely available and their use is well known in the art.

[0099] In particular embodiments, the guide molecule is modified by a secondary structure to increase the specificity of the CRISPR-Cas system and the secondary structure can protect against exonuclease activity and allow for 5' additions to the guide sequence also referred to herein as a protected guide molecule.

[0100] In one aspect, the invention provides for hybridizing a "protector RNA" to a sequence of the guide molecule, wherein the "protector RNA" is an RNA strand complementary to the 3' end of the guide molecule to thereby generate a partially double-stranded guide RNA. In an embodiment of the invention, protecting mismatched bases (i.e. the bases of the guide molecule which do not form part of the guide sequence) with a perfectly complementary protector sequence decreases the likelihood of target RNA binding to the mismatched basepairs at the 3' end. In particular embodiments of the invention, additional sequences comprising an extended length may also be present within the guide molecule such that the guide comprises a protector sequence within the guide molecule. This "protector sequence" ensures that the guide molecule comprises a "protected sequence" in addition to an "exposed sequence" (comprising the part of the guide sequence hybridizing to the target sequence). In particular embodiments, the guide molecule is modified by the presence of the protector guide to comprise a secondary structure such as a hairpin. Advantageously there are three or four to thirty or more, e.g., about 10 or more, contiguous

base pairs having complementarity to the protected sequence, the guide sequence or both. It is advantageous that the protected portion does not impede thermodynamics of the CRISPR-Cas system interacting with its target. By providing such an extension including a partially double stranded guide molecule, the guide molecule is considered protected and results in improved specific binding of the CRISPR-Cas complex, while maintaining specific activity.

[0101] In particular embodiments, use is made of a truncated guide (tru-guide), i.e. a guide molecule which comprises a guide sequence which is truncated in length with respect to the canonical guide sequence length. As described by Nowak et al. (Nucleic Acids Res (2016) 44 (20): 9555-9564), such guides may allow catalytically active CRISPR-Cas enzyme to bind its target without cleaving the target RNA. In particular embodiments, a truncated guide is used which allows the binding of the target but retains only nickase activity of the CRISPR-Cas enzyme.

[0102] The present invention may be further illustrated and extended based on aspects of CRISPR-Cas development and use as set forth in the following articles and particularly as relates to delivery of a CRISPR protein complex and uses of an RNA guided endonuclease in cells and organisms:

- Multiplex genome engineering using CRISPR-Cas systems. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., & Zhang, F. Science Feb 15;339(6121):819-23 (2013);
- RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Jiang W., Bikard D., Cox D., Zhang F, Marraffini LA. Nat Biotechnol Mar;31(3):233-9 (2013);
- One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR-Cas-Mediated Genome Engineering. Wang H., Yang H., Shivalila CS., Dawlaty MM., Cheng AW., Zhang F., Jaenisch R. Cell May 9;153(4):910-8 (2013);
- Optical control of mammalian endogenous transcription and epigenetic states. Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM, Zhang F. Nature. Aug 22;500(7463):472-6. doi: 10.1038/Nature12466. Epub 2013 Aug 23 (2013);
- Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. Ran, FA., Hsu, PD., Lin, CY., Gootenberg, JS., Konermann, S., Trevino, AE., Scott, DA., Inoue, A., Matoba, S., Zhang, Y., & Zhang, F. Cell Aug 28. pii: S0092-8674(13)01015-5 (2013-A);

- DNA targeting specificity of RNA-guided Cas9 nucleases. Hsu, P., Scott, D., Weinstein, J., Ran, FA., Konermann, S., Agarwala, V., Li, Y., Fine, E., Wu, X., Shalem, O., Cradick, TJ., Marraffini, LA., Bao, G., & Zhang, F. Nat Biotechnol doi:10.1038/nbt.2647 (2013);
- Genome engineering using the CRISPR-Cas9 system. Ran, FA., Hsu, PD., Wright, J., Agarwala, V., Scott, DA., Zhang, F. Nature Protocols Nov;8(11):2281-308 (2013-B);
- Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells. Shalem, O., Sanjana, NE., Hartenian, E., Shi, X., Scott, DA., Mikkelsen, T., Heckl, D., Ebert, BL., Root, DE., Doench, JG., Zhang, F. Science Dec 12. (2013);
- Crystal structure of cas9 in complex with guide RNA and target DNA. Nishimasu, H., Ran, FA., Hsu, PD., Konermann, S., Shehata, SI., Dohmae, N., Ishitani, R., Zhang, F., Nureki, O. Cell Feb 27, 156(5):935-49 (2014);
- Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. Wu X., Scott DA., Kriz AJ., Chiu AC., Hsu PD., Dadon DB., Cheng AW., Trevino AE., Konermann S., Chen S., Jaenisch R., Zhang F., Sharp PA. Nat Biotechnol. Apr 20. doi: 10.1038/nbt.2889 (2014);
- CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F. Cell 159(2): 440-455 DOI: 10.1016/j.cell.2014.09.014(2014);
- Development and Applications of CRISPR-Cas9 for Genome Engineering, Hsu PD, Lander ES, Zhang F., Cell. Jun 5;157(6):1262-78 (2014).
- Genetic screens in human cells using the CRISPR-Cas9 system, Wang T, Wei JJ, Sabatini DM, Lander ES., Science. January 3; 343(6166): 80–84. doi:10.1126/science.1246981 (2014);
- Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation, Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ, Root DE., (published online 3 September 2014) Nat Biotechnol. Dec;32(12):1262-7 (2014);
- *In vivo* interrogation of gene function in the mammalian brain using CRISPR-Cas9, Swiech L, Heidenreich M, Banerjee A, Habib N, Li Y, Trombetta J, Sur M, Zhang F., (published online 19 October 2014) Nat Biotechnol. Jan;33(1):102-6 (2015);

- Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex, Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F., Nature. Jan 29;517(7536):583-8 (2015).
- A split-Cas9 architecture for inducible genome editing and transcription modulation, Zetsche B, Volz SE, Zhang F., (published online 02 February 2015) Nat Biotechnol. Feb;33(2):139-42 (2015);
- Genome-wide CRISPR Screen in a Mouse Model of Tumor Growth and Metastasis, Chen S, Sanjana NE, Zheng K, Shalem O, Lee K, Shi X, Scott DA, Song J, Pan JQ, Weissleder R, Lee H, Zhang F, Sharp PA. Cell 160, 1246–1260, March 12, 2015 (multiplex screen in mouse), and
- In vivo genome editing using *Staphylococcus aureus* Cas9, Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F., (published online 01 April 2015), Nature. Apr 9;520(7546):186-91 (2015).
- Shalem et al., “High-throughput functional genomics using CRISPR-Cas9,” Nature Reviews Genetics 16, 299-311 (May 2015).
- Xu et al., “Sequence determinants of improved CRISPR sgRNA design,” Genome Research 25, 1147-1157 (August 2015).
- Parnas et al., “A Genome-wide CRISPR Screen in Primary Immune Cells to Dissect Regulatory Networks,” Cell 162, 675-686 (July 30, 2015).
- Ramanan et al., CRISPR-Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus,” Scientific Reports 5:10833. doi: 10.1038/srep10833 (June 2, 2015)
- Nishimasu et al., Crystal Structure of *Staphylococcus aureus* Cas9,” Cell 162, 1113-1126 (Aug. 27, 2015)
- BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis, Canver et al., Nature 527(7577):192-7 (Nov. 12, 2015) doi: 10.1038/nature15521. Epub 2015 Sep 16.
- *Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System*, Zetsche et al., Cell 163, 759-71 (Sep 25, 2015).
- *Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems*, Shmakov et al., Molecular Cell, 60(3), 385–397 doi: 10.1016/j.molcel.2015.10.008 Epub October 22, 2015.

- *Rationally engineered Cas9 nucleases with improved specificity*, Slaymaker et al., Science 2016 Jan 1 351(6268): 84-88 doi: 10.1126/science.aad5227. Epub 2015 Dec 1.
- Gao *et al.*, “Engineered Cpf1 Enzymes with Altered PAM Specificities,” bioRxiv 091611; doi: <http://dx.doi.org/10.1101/091611> (Dec. 4, 2016).

each of which is incorporated herein by reference, may be considered in the practice of the instant invention, and discussed briefly below:

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

100% of cells that were recovered using the described approach contained the desired mutation, and in *E. coli*, 65% that were recovered contained the mutation.

- Wang *et al.* (2013) used the CRISPR-Cas system for the one-step generation of mice carrying mutations in multiple genes which were traditionally generated in multiple steps by sequential recombination in embryonic stem cells and/or time-consuming intercrossing of mice with a single mutation. The CRISPR-Cas system will greatly accelerate the *in vivo* study of functionally redundant genes and of epistatic gene interactions.
- Konermann *et al.* (2013) addressed the need in the art for versatile and robust technologies that enable optical and chemical modulation of DNA-binding domains based CRISPR Cas9 enzyme and also Transcriptional Activator Like Effectors
- Ran *et al.* (2013-A) described an approach that combined a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks. This addresses the issue of the Cas9 nuclease from the microbial CRISPR-Cas system being targeted to specific genomic loci by a guide sequence, which can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. Because individual nicks in the genome are repaired with high fidelity, simultaneous nicking *via* appropriately offset guide RNAs is required for double-stranded breaks and extends the number of specifically recognized bases for target cleavage. The authors demonstrated that using paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications that require high specificity.
- Hsu *et al.* (2013) characterized SpCas9 targeting specificity in human cells to inform the selection of target sites and avoid off-target effects. The study evaluated >700 guide RNA variants and SpCas9-induced indel mutation levels at >100 predicted genomic off-target loci in 293T and 293FT cells. The authors that SpCas9 tolerates mismatches between guide RNA and target DNA at different positions in a sequence-dependent manner, sensitive to the number, position and distribution of mismatches. The authors further showed that SpCas9-mediated cleavage is unaffected by DNA methylation and that the dosage of SpCas9 and guide RNA can be titrated to minimize off-target modification. Additionally, to facilitate mammalian genome

engineering applications, the authors reported providing a web-based software tool to guide the selection and validation of target sequences as well as off-target analyses.

- Ran *et al.* (2013-B) described a set of tools for Cas9-mediated genome editing *via* non-homologous end joining (NHEJ) or homology-directed repair (HDR) in mammalian cells, as well as generation of modified cell lines for downstream functional studies. To minimize off-target cleavage, the authors further described a double-nicking strategy using the Cas9 nickase mutant with paired guide RNAs. The protocol provided by the authors experimentally derived guidelines for the selection of target sites, evaluation of cleavage efficiency and analysis of off-target activity. The studies showed that beginning with target design, gene modifications can be achieved within as little as 1–2 weeks, and modified clonal cell lines can be derived within 2–3 weeks.
- Shalem *et al.* described a new way to interrogate gene function on a genome-wide scale. Their studies showed that delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeted 18,080 genes with 64,751 unique guide sequences enabled both negative and positive selection screening in human cells. First, the authors showed use of the GeCKO library to identify genes essential for cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, the authors screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic that inhibits mutant protein kinase BRAF. Their studies showed that the highest-ranking candidates included previously validated genes NF1 and MED12 as well as novel hits NF2, CUL3, TADA2B, and TADA1. The authors observed a high level of consistency between independent guide RNAs targeting the same gene and a high rate of hit confirmation, and thus demonstrated the promise of genome-scale screening with Cas9.
- Nishimasu *et al.* reported the crystal structure of *Streptococcus pyogenes* Cas9 in complex with sgRNA and its target DNA at 2.5 Å resolution. The structure revealed a bilobed architecture composed of target recognition and nuclease lobes, accommodating the sgRNA:DNA heteroduplex in a positively charged groove at their interface. Whereas the recognition lobe is essential for binding sgRNA and DNA, the nuclease lobe contains the HNH and RuvC nuclease domains, which are properly positioned for cleavage of the complementary and non-complementary strands of the target DNA, respectively. The nuclease lobe also contains a carboxyl-terminal domain

responsible for the interaction with the protospacer adjacent motif (PAM). This high-resolution structure and accompanying functional analyses have revealed the molecular mechanism of RNA-guided DNA targeting by Cas9, thus paving the way for the rational design of new, versatile genome-editing technologies.

- Wu *et al.* mapped genome-wide binding sites of a catalytically inactive Cas9 (dCas9) from *Streptococcus pyogenes* loaded with single guide RNAs (sgRNAs) in mouse embryonic stem cells (mESCs). The authors showed that each of the four sgRNAs tested targets dCas9 to between tens and thousands of genomic sites, frequently characterized by a 5-nucleotide seed region in the sgRNA and an NGG protospacer adjacent motif (PAM). Chromatin inaccessibility decreases dCas9 binding to other sites with matching seed sequences; thus 70% of off-target sites are associated with genes. The authors showed that targeted sequencing of 295 dCas9 binding sites in mESCs transfected with catalytically active Cas9 identified only one site mutated above background levels. The authors proposed a two-state model for Cas9 binding and cleavage, in which a seed match triggers binding but extensive pairing with target DNA is required for cleavage.
- Platt *et al.* established a Cre-dependent Cas9 knockin mouse. The authors demonstrated *in vivo* as well as *ex vivo* genome editing using adeno-associated virus (AAV)-, lentivirus-, or particle-mediated delivery of guide RNA in neurons, immune cells, and endothelial cells.
- Hsu *et al.* (2014) is a review article that discusses generally CRISPR-Cas9 history from yogurt to genome editing, including genetic screening of cells.
- Wang *et al.* (2014) relates to a pooled, loss-of-function genetic screening approach suitable for both positive and negative selection that uses a genome-scale lentiviral single guide RNA (sgRNA) library.
- Doench *et al.* created a pool of sgRNAs, tiling across all possible target sites of a panel of six endogenous mouse and three endogenous human genes and quantitatively assessed their ability to produce null alleles of their target gene by antibody staining and flow cytometry. The authors showed that optimization of the PAM improved activity and also provided an on-line tool for designing sgRNAs.
- Swiech *et al.* demonstrate that AAV-mediated SpCas9 genome editing can enable reverse genetic studies of gene function in the brain.

- Konermann *et al.* (2015) discusses the ability to attach multiple effector domains, e.g., transcriptional activator, functional and epigenomic regulators at appropriate positions on the guide such as stem or tetraloop with and without linkers.
- Zetsche *et al.* demonstrates that the Cas9 enzyme can be split into two and hence the assembly of Cas9 for activation can be controlled.
- Chen *et al.* relates to multiplex screening by demonstrating that a genome-wide *in vivo* CRISPR-Cas9 screen in mice reveals genes regulating lung metastasis.
- Ran *et al.* (2015) relates to SaCas9 and its ability to edit genomes and demonstrates that one cannot extrapolate from biochemical assays.
- Shalem *et al.* (2015) described ways in which catalytically inactive Cas9 (dCas9) fusions are used to synthetically repress (CRISPRi) or activate (CRISPRa) expression, showing advances using Cas9 for genome-scale screens, including arrayed and pooled screens, knockout approaches that inactivate genomic loci and strategies that modulate transcriptional activity.
- Xu *et al.* (2015) assessed the DNA sequence features that contribute to single guide RNA (sgRNA) efficiency in CRISPR-based screens. The authors explored efficiency of CRISPR-Cas9 knockout and nucleotide preference at the cleavage site. The authors also found that the sequence preference for CRISPRi/a is substantially different from that for CRISPR-Cas9 knockout.
- Parnas *et al.* (2015) introduced genome-wide pooled CRISPR-Cas9 libraries into dendritic cells (DCs) to identify genes that control the induction of tumor necrosis factor (Tnf) by bacterial lipopolysaccharide (LPS). Known regulators of Tlr4 signaling and previously unknown candidates were identified and classified into three functional modules with distinct effects on the canonical responses to LPS.
- Ramanan *et al.* (2015) demonstrated cleavage of viral episomal DNA (cccDNA) in infected cells. The HBV genome exists in the nuclei of infected hepatocytes as a 3.2kb double-stranded episomal DNA species called covalently closed circular DNA (cccDNA), which is a key component in the HBV life cycle whose replication is not inhibited by current therapies. The authors showed that sgRNAs specifically targeting highly conserved regions of HBV robustly suppresses viral replication and depleted cccDNA.
- Nishimasu *et al.* (2015) reported the crystal structures of SaCas9 in complex with a single guide RNA (sgRNA) and its double-stranded DNA targets, containing the 5'-

TTGAAT-3' PAM and the 5'-TTGGGT-3' PAM. A structural comparison of SaCas9 with SpCas9 highlighted both structural conservation and divergence, explaining their distinct PAM specificities and orthologous sgRNA recognition.

- Canver *et al.* (2015) demonstrated a CRISPR-Cas9-based functional investigation of non-coding genomic elements. The authors we developed pooled CRISPR-Cas9 guide RNA libraries to perform *in situ* saturating mutagenesis of the human and mouse BCL11A enhancers which revealed critical features of the enhancers.
- Zetsche *et al.* (2015) reported characterization of Cpf1, a class 2 CRISPR nuclease from *Francisella novicida* U112 having features distinct from Cas9. Cpf1 is a single RNA-guided endonuclease lacking tracrRNA, utilizes a T-rich protospacer-adjacent motif, and cleaves DNA via a staggered DNA double-stranded break.
- Shmakov *et al.* (2015) reported three distinct Class 2 CRISPR-Cas systems. Two system CRISPR enzymes (C2c1 and C2c3) contain RuvC-like endonuclease domains distantly related to Cpf1. Unlike Cpf1, C2c1 depends on both crRNA and tracrRNA for DNA cleavage. The third enzyme (C2c2) contains two predicted HEPN RNase domains and is tracrRNA independent.
- Slaymaker *et al.* (2016) reported the use of structure-guided protein engineering to improve the specificity of *Streptococcus pyogenes* Cas9 (SpCas9). The authors developed "enhanced specificity" SpCas9 (eSpCas9) variants which maintained robust on-target cleavage with reduced off-target effects.

[0103] The methods and tools provided herein are may be designed for use with or Cas13, a type II nuclease that does not make use of tracrRNA. Orthologs of Cas13 have been identified in different bacterial species as described herein. Further type II nucleases with similar properties can be identified using methods described in the art (Shmakov *et al.* 2015, 60:385–397; Abudayeh *et al.* 2016, *Science*, 5;353(6299)) . In particular embodiments, such methods for identifying novel CRISPR effector proteins may comprise the steps of selecting sequences from the database encoding a seed which identifies the presence of a CRISPR Cas locus, identifying loci located within 10 kb of the seed comprising Open Reading Frames (ORFs) in the selected sequences, selecting therefrom loci comprising ORFs of which only a single ORF encodes a novel CRISPR effector having greater than 700 amino acids and no more than 90% homology to a known CRISPR effector. In particular embodiments, the seed is a protein that is common to the CRISPR-Cas system, such as Cas1. In further embodiments, the CRISPR array is used as a seed to identify new effector proteins.

[0104] Also, “Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing”, Shengdar Q. Tsai, Nicolas Wyvekens, Cyd Khayter, Jennifer A. Foden, Vishal Thapar, Deepak Reyon, Mathew J. Goodwin, Martin J. Aryee, J. Keith Joung Nature Biotechnology 32(6): 569-77 (2014), relates to dimeric RNA-guided FokI Nucleases that recognize extended sequences and can edit endogenous genes with high efficiencies in human cells.

[0105] With respect to general information on CRISPR/Cas Systems, components thereof, and delivery of such components, including methods, materials, delivery vehicles, vectors, particles, and making and using thereof, including as to amounts and formulations, as well as CRISPR-Cas-expressing eukaryotic cells, CRISPR-Cas expressing eukaryotes, such as a mouse, reference is made to: US Patents Nos. 8,999,641, 8,993,233, 8,697,359, 8,771,945, 8,795,965, 8,865,406, 8,871,445, 8,889,356, 8,889,418, 8,895,308, 8,906,616, 8,932,814, and 8,945,839; US Patent Publications US 2014-0310830 (US App. Ser. No. 14/105,031), US 2014-0287938 A1 (U.S. App. Ser. No. 14/213,991), US 2014-0273234 A1 (U.S. App. Ser. No. 14/293,674), US2014-0273232 A1 (U.S. App. Ser. No. 14/290,575), US 2014-0273231 (U.S. App. Ser. No. 14/259,420), US 2014-0256046 A1 (U.S. App. Ser. No. 14/226,274), US 2014-0248702 A1 (U.S. App. Ser. No. 14/258,458), US 2014-0242700 A1 (U.S. App. Ser. No. 14/222,930), US 2014-0242699 A1 (U.S. App. Ser. No. 14/183,512), US 2014-0242664 A1 (U.S. App. Ser. No. 14/104,990), US 2014-0234972 A1 (U.S. App. Ser. No. 14/183,471), US 2014-0227787 A1 (U.S. App. Ser. No. 14/256,912), US 2014-0189896 A1 (U.S. App. Ser. No. 14/105,035), US 2014-0186958 (U.S. App. Ser. No. 14/105,017), US 2014-0186919 A1 (U.S. App. Ser. No. 14/104,977), US 2014-0186843 A1 (U.S. App. Ser. No. 14/104,900), US 2014-0179770 A1 (U.S. App. Ser. No. 14/104,837) and US 2014-0179006 A1 (U.S. App. Ser. No. 14/183,486), US 2014-0170753 (US App Ser No 14/183,429); US 2015-0184139 (U.S. App. Ser. No. 14/324,960); 14/054,414 European Patent Applications EP 2 771 468 (EP13818570.7), EP 2 764 103 (EP13824232.6), and EP 2 784 162 (EP14170383.5); and PCT Patent Publications WO2014/093661 (PCT/US2013/074743), WO2014/093694 (PCT/US2013/074790), WO2014/093595 (PCT/US2013/074611), WO2014/093718 (PCT/US2013/074825), WO2014/093709 (PCT/US2013/074812), WO2014/093622 (PCT/US2013/074667), WO2014/093635 (PCT/US2013/074691), WO2014/093655 (PCT/US2013/074736), WO2014/093712 (PCT/US2013/074819), WO2014/093701 (PCT/US2013/074800), WO2014/018423 (PCT/US2013/051418), WO2014/204723 (PCT/US2014/041790), WO2014/204724

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(PCT/US2015/051830), WO2016/094867 (PCT/US2015/065385), WO2016/094872
(PCT/US2015/065393), WO2016/094874 (PCT/US2015/065396), WO2016/106244
(PCT/US2015/067177).

[0106] Mention is also made of US application 62/180,709, 17-Jun-15, PROTECTED GUIDE RNAS (PGRNAS); US application 62/091,455, filed, 12-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US application 62/096,708, 24-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US applications 62/091,462, 12-Dec-14, 62/096,324, 23-Dec-14, 62/180,681, 17-Jun-2015, and 62/237,496, 5-Oct-2015, DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS; US application 62/091,456, 12-Dec-14 and 62/180,692, 17-Jun-2015, ESCORTED AND FUNCTIONALIZED GUIDES FOR CRISPR-CAS SYSTEMS; US application 62/091,461, 12-Dec-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR GENOME EDITING AS TO HEMATOPOETIC STEM CELLS (HSCs); US application 62/094,903, 19-Dec-14, UNBIASED IDENTIFICATION OF DOUBLE-STRAND BREAKS AND GENOMIC REARRANGEMENT BY GENOME-WISE INSERT CAPTURE SEQUENCING; US application 62/096,761, 24-Dec-14, ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED ENZYME AND GUIDE SCAFFOLDS FOR SEQUENCE MANIPULATION; US application 62/098,059, 30-Dec-14, 62/181,641, 18-Jun-2015, and 62/181,667, 18-Jun-2015, RNA-TARGETING SYSTEM; US application 62/096,656, 24-Dec-14 and 62/181,151, 17-Jun-2015, CRISPR HAVING OR ASSOCIATED WITH DESTABILIZATION DOMAINS; US application 62/096,697, 24-Dec-14, CRISPR HAVING OR ASSOCIATED WITH AAV; US application 62/098,158,

30-Dec-14, ENGINEERED CRISPR COMPLEX INSERTIONAL TARGETING SYSTEMS; US application 62/151,052, 22-Apr-15, CELLULAR TARGETING FOR EXTRACELLULAR EXOSOMAL REPORTING; US application 62/054,490, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING PARTICLE DELIVERY COMPONENTS; US application 61/939,154, 12-FEB-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/055,484, 25-Sep-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,537, 4-Dec-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/054,651, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US application 62/067,886, 23-Oct-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US applications 62/054,675, 24-Sep-14 and 62/181,002, 17-Jun-2015, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN NEURONAL CELLS/TISSUES; US application 62/054,528, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN IMMUNE DISEASES OR DISORDERS; US application 62/055,454, 25-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING CELL PENETRATION PEPTIDES (CPP); US application 62/055,460, 25-Sep-14, MULTIFUNCTIONAL-CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; US application 62/087,475, 4-Dec-14 and 62/181,690, 18-Jun-2015, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/055,487, 25-Sep-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,546, 4-Dec-14 and 62/181,687, 18-Jun-2015,

MULTIFUNCTIONAL CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; and US application 62/098,285, 30-Dec-14, CRISPR MEDIATED IN VIVO MODELING AND GENETIC SCREENING OF TUMOR GROWTH AND METASTASIS.

[0107] Mention is made of US applications 62/181,659, 18-Jun-2015 and 62/207,318, 19-Aug-2015, ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS, ENZYME AND GUIDE SCAFFOLDS OF CAS9 ORTHOLOGS AND VARIANTS FOR SEQUENCE MANIPULATION. Mention is made of US applications 62/181,663, 18-Jun-2015 and 62/245,264, 22-Oct-2015, NOVEL CRISPR ENZYMES AND SYSTEMS, US applications 62/181,675, 18-Jun-2015, 62/285,349, 22-Oct-2015, 62/296,522, 17-Feb-2016, and 62/320,231, 8-Apr-2016, NOVEL CRISPR ENZYMES AND SYSTEMS, US application 62/232,067, 24-Sep-2015, US Application 14/975,085, 18-Dec-2015, European application No. 16150428.7, US application 62/205,733, 16-Aug-2015, US application 62/201,542, 5-Aug-2015, US application 62/193,507, 16-Jul-2015, and US application 62/181,739, 18-Jun-2015, each entitled NOVEL CRISPR ENZYMES AND SYSTEMS and of US application 62/245,270, 22-Oct-2015, NOVEL CRISPR ENZYMES AND SYSTEMS. Mention is also made of US application 61/939,256, 12-Feb-2014, and WO 2015/089473 (PCT/US2014/070152), 12-Dec-2014, each entitled ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED GUIDE COMPOSITIONS WITH NEW ARCHITECTURES FOR SEQUENCE MANIPULATION. Mention is also made of PCT/US2015/045504, 15-Aug-2015, US application 62/180,699, 17-Jun-2015, and US application 62/038,358, 17-Aug-2014, each entitled GENOME EDITING USING CAS9 NICKASES.

[0108] Each of these patents, patent publications, and applications, and all documents cited therein or during their prosecution (“appln cited documents”) and all documents cited or referenced in the appln cited documents, together with any instructions, descriptions, product specifications, and product sheets for any products mentioned therein or in any document therein and incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. All documents (e.g., these patents, patent publications and applications and the appln cited documents) are incorporated herein by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

[0109] Once intended alterations have been introduced, such as by editing intended copies of a gene in the genome of a cell, continued CRISPR-Cas expression in that cell is no longer necessary. Indeed, sustained expression would be undesirable in certain cases in case of off-target effects at unintended genomic sites, etc. Thus time-limited expression would be useful. Applicants have engineered a Cas protein comprising one or more degradation domains. Accordingly, degradation of the Cas protein, which may include some level of inactivation of nuclease activity, by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or all of the nuclease activity, or substantially inactivating the Cas protein inactivating the Cas protein with a degrader may be performed in the methods of the current invention.

Methods of Use

[0110] Methods of degrading an activity of a variant CRISPR Cas protein-RNA complex, the method comprising contacting the CRISPR Cas protein-RNA complex with a heterobifunctional degrader of FBKP12^{F36V} as detailed elsewhere herein. The method may be performed *in vitro* or *in vivo*. In an aspect, the method is performed in a cell. In particular embodiments, the methods are performed in a germline cell. Methods of degrading activity can be detected in a variety of ways, including measuring activity at a target molecule, via genomic disruption e.g. eGFP disruption as described in the examples herein. Varying levels of degrader agents may be utilized with eGFP disruption assayed versus an apoCas, and/or a Cas protein activity with no degrader.

[0111] Methods of treating a subject are also provided, comprising the steps of administering a CRISPR Cas protein-RNA complex or a reagent causing expression of a CRISPR-Cas protein-RNA complex to the subject; and administering a heterobifunctional degrader of FBKP12^{F36V}. Administering a heterobifunctional degrader of FBKP can be according to delivery methods as disclosed elsewhere herein.

Applications in non-animal organisms and animals

[0106] The degraders herein may be used to modulate the functions and activities of RNA-guided nuclease (e.g., Cas proteins), variants thereof, and fragments thereof in animals and non-animal organisms. In some examples, the animals and non-animal organisms may have been engineered to constitutively or inducibly express an RNA-guided nuclease (e.g., Cas protein) comprising one or more functional domains. In some examples, the degraders herein may modulate the activities of the RNA-guided nucleases comprising one or more

degradation domains or their interaction with other molecules, e.g., their binding with target polynucleotides.

Applications in non-animal organisms

[0107] The degraders herein can be used on non-animal organisms, such as plants and fungi. The degraders herein can be used to perform efficient and cost effective plant gene or genome interrogation or editing or manipulation—for instance, for rapid investigation and/or selection and/or interrogations and/or comparison and/or manipulations and/or transformation of plant genes or genomes; e.g., to create, identify, develop, optimize, or confer trait(s) or characteristic(s) to plant(s) or to transform a plant genome. There can accordingly be improved production of plants, new plants with new combinations of traits or characteristics or new plants with enhanced traits.

[0108] The degraders herein may be used on plants. In general, the term “plant” relates to any various photosynthetic, eukaryotic, unicellular or multicellular organism of the kingdom Plantae characteristically growing by cell division, containing chloroplasts, and having cell walls comprised of cellulose. The term plant encompasses monocotyledonous and dicotyledonous plants. Specifically, the plants are intended to comprise without limitation angiosperm and gymnosperm plants such as acacia, alfalfa, amaranth, apple, apricot, artichoke, ash tree, asparagus, avocado, banana, barley, beans, beet, birch, beech, blackberry, blueberry, broccoli, Brussel’s sprouts, cabbage, canola, cantaloupe, carrot, cassava, cauliflower, cedar, a cereal, celery, chestnut, cherry, Chinese cabbage, citrus, clementine, clover, coffee, corn, cotton, cowpea, cucumber, cypress, eggplant, elm, endive, eucalyptus, fennel, figs, fir, geranium, grape, grapefruit, groundnuts, ground cherry, gum hemlock, hickory, kale, kiwifruit, kohlrabi, larch, lettuce, leek, lemon, lime, locust, pine, maidenhair, maize, mango, maple, melon, millet, mushroom, mustard, nuts, oak, oats, oil palm, okra, onion, orange, an ornamental plant or flower or tree, papaya, palm, parsley, parsnip, pea, peach, peanut, pear, peat, pepper, persimmon, pigeon pea, pine, pineapple, plantain, plum, pomegranate, potato, pumpkin, radicchio, radish, rapeseed, raspberry, rice, rye, sorghum, safflower, sallow, soybean, spinach, spruce, squash, strawberry, sugar beet, sugarcane, sunflower, sweet potato, sweet corn, tangerine, tea, tobacco, tomato, trees, triticale, turf grasses, turnips, vine, walnut, watercress, watermelon, wheat, yams, yew, and zucchini. The term plant also encompasses Algae, which are mainly photoautotrophs unified primarily by their lack of roots, leaves and other organs that characterize higher plants.

[0109] The degraders herein can be used to confer desired traits on essentially any plant. A wide variety of plants and plant cell systems may be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the present disclosure and the various transformation methods mentioned above. In preferred embodiments, target plants and plant cells include, but are not limited to, those monocotyledonous and dicotyledonous plants, such as crops including grain crops (e.g., wheat, maize, rice, millet, barley), fruit crops (e.g., tomato, apple, pear, strawberry, orange), forage crops (e.g., alfalfa), root vegetable crops (e.g., carrot, potato, sugar beets, yam), leafy vegetable crops (e.g., lettuce, spinach); flowering plants (e.g., petunia, rose, chrysanthemum), conifers and pine trees (e.g., pine fir, spruce); plants used in phytoremediation (e.g., heavy metal accumulating plants); oil crops (e.g., sunflower, rape seed) and plants used for experimental purposes (e.g., *Arabidopsis*). Plant cells and tissues for engineering include, without limitation, roots, stems, leaves, flowers, and reproductive structures, undifferentiated meristematic cells, parenchyma, collenchyma, sclerenchyma, xylem, phloem, epidermis, and germplasm. Thus, the methods and systems can be used over a broad range of plants, such as for example with dicotyledonous plants belonging to the orders Magnoliales, Illiciales, Laurales, Piperales, Aristochiales, Nymphaeales, Ranunculales, Papaverales, Sarraceniaceae, Trochodendrales, Hamamelidales, Eucomiales, Leitneriales, Myricales, Fagales, Casuarinales, Caryophyllales, Batales, Polygonales, Plumbaginales, Dilleniales, Theales, Malvales, Urticales, Lecythidales, Violales, Salicales, Capparales, Ericales, Diapensales, Ebenales, Primulales, Rosales, Fabales, Podostemales, Haloragales, Myrtales, Cornales, Proteales, Santales, Rafflesiales, Celastrales, Euphorbiales, Rhamnales, Sapindales, Juglandales, Geraniales, Polygalales, Umbellales, Gentianales, Polemoniales, Lamiales, Plantaginales, Scrophulariales, Campanulales, Rubiales, Dipsacales, and Asterales; the methods and systems can be used with monocotyledonous plants such as those belonging to the orders Alismatales, Hydrocharitales, Najadales, Triuridales, Commelinales, Eriocaulales, Restionales, Poales, Juncales, Cyperales, Typhales, Bromeliales, Zingiberales, Arecales, Cyclanthales, Pandanales, Arales, Lilliales, and Orchidales, or with plants belonging to Gymnospermae, e.g. those belonging to the orders Pinales, Ginkgoales, Cycadales, Araucariales, Cupressales and Gnetales.

[0110] The degraders herein can be used over a broad range of plant species, included in the non-limitative list of dicot, monocot or gymnosperm genera hereunder: *Atropa*, *Alseodaphne*, *Anacardium*, *Arachis*, *Beilschmiedia*, *Brassica*, *Carthamus*, *Cocculus*, *Croton*,

Cucumis, Citrus, Citrullus, Capsicum, Catharanthus, Cocos, Coffea, Cucurbita, Daucus, Duguetia, Eschscholzia, Ficus, Fragaria, Glaucium, Glycine, Gossypium, Helianthus, Hevea, Hyoscyamus, Lactuca, Landolphia, Linum, Litsea, Lycopersicon, Lupinus, Manihot, Majorana, Malus, Medicago, Nicotiana, Olea, Parthenium, Papaver, Persea, Phaseolus, Pistacia, Pisum, Pyrus, Prunus, Raphanus, Ricinus, Senecio, Sinomenium, Stephania, Sinapis, Solanum, Theobroma, Trifolium, Trigonella, Vicia, Vinca, Vilis, and Vigna; and the genera Allium, Andropogon, Aragrostis, Asparagus, Avena, Cynodon, Elaeis, Festuca, Festulolium, Heterocallis, Hordeum, Lemna, Lolium, Musa, Oryza, Panicum, Pannesetum, Phleum, Poa, Secale, Sorghum, Triticum, Zea, Abies, Cunninghamia, Ephedra, Picea, Pinus, and Pseudotsuga.

[0111] The degraders herein can also be used over a broad range of "algae" or "algae cells"; including for example algae selected from several eukaryotic phyla, including the Rhodophyta (red algae), Chlorophyta (green algae), Phaeophyta (brown algae), Bacillariophyta (diatoms), Eustigmatophyta and dinoflagellates as well as the prokaryotic phylum Cyanobacteria (blue-green algae). The term "algae" includes for example algae selected from : Amphora, Anabaena, Anikstrodesmis, Botryococcus, Chaetoceros, Chlamydomonas, Chlorella, Chlorococcum, Cyclotella, Cylindrotheca, Dunaliella, Emiliana, Euglena, Hematococcus, Isochrysis, Monochrysis, Monoraphidium, Nannochloris, Nannochloropsis, Navicula, Nephrochloris, Nephroselmis, Nitzschia, Nodularia, Nostoc, Oochromonas, Oocystis, Oscillartoria, Pavlova, Phaeodactylum, Playtmonas, Pleurochrysis, Porphyra, Pseudoanabaena, Pyramimonas, Stichococcus, Synechococcus, Synechocystis, Tetraselmis, Thalassiosira, and Trichodesmium.

[0112] The degraders herein can also be used over a broad range of fungi. As used herein, a "fungal cell" refers to any type of eukaryotic cell within the kingdom of fungi. Phyla within the kingdom of fungi include Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Glomeromycota, Microsporidia, and Neocallimastigomycota. Fungal cells may include yeasts, molds, and filamentous fungi. In some embodiments, the fungal cell is a yeast cell. As used herein, the term "yeast" refers to any fungal cell within the phyla Ascomycota and Basidiomycota. Yeast cells may include budding yeast cells, fission yeast cells, and mold cells. Without being limited to these organisms, many types of yeast used in laboratory and industrial settings are part of the phylum Ascomycota. In some embodiments, the yeast cell is an *S. cerevisiae*, *Kluyveromyces marxianus*, or *Issatchenkia orientalis* cell. Other yeast cells may include without limitation *Candida* spp. (e.g., *Candida albicans*),

Yarrowia spp. (e.g., Yarrowia lipolytica), Pichia spp. (e.g., Pichia pastoris), Kluyveromyces spp. (e.g., Kluyveromyces lactis and Kluyveromyces marxianus), Neurospora spp. (e.g., Neurospora crassa), Fusarium spp. (e.g., Fusarium oxysporum), and Issatchenkia spp. (e.g., Issatchenkia orientalis, a.k.a. Pichia kudriavzevii and Candida acidothermophilum). In some embodiments, the fungal cell is a filamentous fungal cell. As used herein, the term "filamentous fungal cell" refers to any type of fungal cell that grows in filaments, i.e., hyphae or mycelia. Examples of filamentous fungal cells may include without limitation Aspergillus spp. (e.g., Aspergillus niger), Trichoderma spp. (e.g., Trichoderma reesei), Rhizopus spp. (e.g., Rhizopus oryzae), and Mortierella spp. (e.g., Mortierella isabellina).

Plant cultures and regeneration

[0113] In particular embodiments, plant cells which have a modified genome and that are produced or obtained may be treated with the degraders herein, and can be cultured to regenerate a whole plant which possesses the transformed or modified genotype and thus the desired phenotype. Conventional regeneration techniques are well known to those skilled in the art. Particular examples of such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, and typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. In further particular embodiments, plant regeneration is obtained from cultured protoplasts, plant callus, explants, organs, pollens, embryos or parts thereof (see e.g. Evans et al. (1983), Handbook of Plant Cell Culture, Klee et al (1987) Ann. Rev. of Plant Phys.).

[0114] In particular embodiments, transformed or improved plants to be treated with the degraders as described herein can be self-pollinated to provide seed for homozygous improved plants of the invention (homozygous for the DNA modification) or crossed with non-transgenic plants or different improved plants to provide seed for heterozygous plants. Where a recombinant DNA was introduced into the plant cell, the resulting plant of such a crossing is a plant which is heterozygous for the recombinant DNA molecule. Both such homozygous and heterozygous plants obtained by crossing from the improved plants and comprising the genetic modification (which can be a recombinant DNA) are referred to herein as "progeny". Progeny plants are plants descended from the original transgenic plant and containing the genome modification or recombinant DNA molecule introduced by the methods provided herein. Alternatively, genetically modified plants can be obtained by one of the methods described supra using the Cfp1 enzyme whereby no foreign DNA is incorporated into the genome. Progeny of such plants, obtained by further breeding may also

contain the genetic modification. Breedings are performed by any breeding methods that are commonly used for different crops (e.g., Allard, Principles of Plant Breeding, John Wiley & Sons, NY, U. of CA, Davis, CA, 50-98 (1960).

Generation of plants with enhanced agronomic traits

[0115] The degraders herein can be used to treat plants that are introduced with targeted double-strand or single-strand breaks and/or introduced gene activator and or repressor systems and without being limitative, can be used for gene targeting, gene replacement, targeted mutagenesis, targeted deletions or insertions, targeted inversions and/or targeted translocations. By co-expression of multiple targeting RNAs directed to achieve multiple modifications in a single cell, multiplexed genome modification can be ensured. This technology can be used to high-precision engineering of plants with improved characteristics, including enhanced nutritional quality, increased resistance to diseases and resistance to biotic and abiotic stress, and increased production of commercially valuable plant products or heterologous compounds.

[0116] In particular embodiments, the degraders herein is used to treat organisms that are introduced targeted double-strand breaks (DSB) in an endogenous DNA sequence. The DSB activates cellular DNA repair pathways, which can be harnessed to achieve desired DNA sequence modifications near the break site. This is of interest where the inactivation of endogenous genes can confer or contribute to a desired trait. In particular embodiments, homologous recombination with a template sequence is promoted at the site of the DSB, in order to introduce a gene of interest.

[0117] The degraders may be used to treat improved plants. Improved plants may have one or more desirable traits compared to the wildtype plant. The degraders herein may further modulate these traits. In particular embodiments, the plants, plant cells or plant parts obtained are transgenic plants, comprising an exogenous DNA sequence incorporated into the genome of all or part of the cells of the plant. In particular embodiments, non-transgenic genetically modified plants, plant parts or cells are obtained, in that no exogenous DNA sequence is incorporated into the genome of any of the plant cells of the plant. In such embodiments, the improved plants are non-transgenic. Where only the modification of an endogenous gene is ensured and no foreign genes are introduced or maintained in the plant genome, the resulting genetically modified crops contain no foreign genes and can thus basically be considered non-transgenic.

Modification of polyploid plants

[0118] Many plants are polyploid, which means they carry duplicate copies of their genomes—sometimes as many as six, as in wheat. The methods according to the present invention, which make use of the degraders can be “multiplexed” to affect all copies of a gene, or to target dozens of genes at once. For instance, in particular embodiments, the methods of the present invention are used to simultaneously ensure a loss of function mutation in different genes responsible for suppressing defenses against a disease. In particular embodiments, the methods of the present invention are used to simultaneously suppress the expression of the TaMLO-A1, TaMLO-B1 and TaMLO-D1 nucleic acid sequence in a wheat plant cell and regenerating a wheat plant therefrom, in order to ensure that the wheat plant is resistant to powdery mildew (see also WO2015109752).

Creating male sterile plants

[0119] Hybrid plants typically have advantageous agronomic traits compared to inbred plants. However, for self-pollinating plants, the generation of hybrids can be challenging. In different plant types, genes have been identified which are important for plant fertility, more particularly male fertility. For instance, in maize, at least two genes have been identified which are important in fertility (Amitabh Mohanty International Conference on New Plant Breeding Molecular Technologies Technology Development And Regulation, Oct 9-10, 2014, Jaipur, India; Svitashv et al. Plant Physiol. 2015 Oct;169(2):931-45; Djukanovic et al. Plant J. 2013 Dec;76(5):888-99). The degraders herein can be used to target genes required for male fertility so as to generate male sterile plants which can easily be crossed to generate hybrids. In particular embodiments, the system provided herein is used for targeted mutagenesis of the cytochrome P450-like gene (MS26) or the meganuclease gene (MS45) thereby conferring male sterility to the maize plant. Maize plants which are as such genetically altered can be used in hybrid breeding programs.

Increasing the fertility stage in plants

[0120] In particular embodiments, the degraders herein are used to prolong the fertility stage of a plant such as of a rice plant. For instance, a rice fertility stage gene such as Ehd3 can be targeted in order to generate a mutation in the gene and plantlets can be selected for a prolonged regeneration plant fertility stage (as described in CN 104004782)

Regulating fruit-ripening

[0121] Ripening is a normal phase in the maturation process of fruits and vegetables. Only a few days after it starts it renders a fruit or vegetable inedible. This process brings

significant losses to both farmers and consumers. In particular embodiments, the degraders herein are used to reduce ethylene production. This is ensured by ensuring one or more of the following: a. Suppression of ACC synthase gene expression. ACC (1-aminocyclopropane-1-carboxylic acid) synthase is the enzyme responsible for the conversion of S-adenosylmethionine (SAM) to ACC; the second to the last step in ethylene biosynthesis. Enzyme expression is hindered when an antisense (“mirror-image”) or truncated copy of the synthase gene is inserted into the plant’s genome; b. Insertion of the ACC deaminase gene. The gene coding for the enzyme is obtained from *Pseudomonas chlororaphis*, a common nonpathogenic soil bacterium. It converts ACC to a different compound thereby reducing the amount of ACC available for ethylene production; c. Insertion of the SAM hydrolase gene. This approach is similar to ACC deaminase wherein ethylene production is hindered when the amount of its precursor metabolite is reduced; in this case SAM is converted to homoserine. The gene coding for the enzyme is obtained from E. coli T3 bacteriophage and d. Suppression of ACC oxidase gene expression. ACC oxidase is the enzyme which catalyzes the oxidation of ACC to ethylene, the last step in the ethylene biosynthetic pathway. Using the methods described herein, down regulation of the ACC oxidase gene results in the suppression of ethylene production, thereby delaying fruit ripening. In particular embodiments, additionally or alternatively to the modifications described above, the methods described herein are used to modify ethylene receptors, so as to interfere with ethylene signals obtained by the fruit. In particular embodiments, expression of the ETR1 gene, encoding an ethylene binding protein is modified, more particularly suppressed. In particular embodiments, additionally or alternatively to the modifications described above, the methods described herein are used to modify expression of the gene encoding Polygalacturonase (PG), which is the enzyme responsible for the breakdown of pectin, the substance that maintains the integrity of plant cell walls. Pectin breakdown occurs at the start of the ripening process resulting in the softening of the fruit. Accordingly, in particular embodiments, the methods described herein are used to introduce a mutation in the PG gene or to suppress activation of the PG gene in order to reduce the amount of PG enzyme produced thereby delaying pectin degradation.

[0122] Thus in particular embodiments, the degraders herein may be used to ensure one or more modifications of the genome of a plant cell such as described above, and regenerating a plant therefrom. In particular embodiments, the plant is a tomato plant.

Increasing storage life of plants

[0123] In particular embodiments, degraders herein are used to modify genes involved in the production of compounds which affect storage life of the plant or plant part. More particularly, the modification is in a gene that prevents the accumulation of reducing sugars in potato tubers. Upon high-temperature processing, these reducing sugars react with free amino acids, resulting in brown, bitter-tasting products and elevated levels of acrylamide, which is a potential carcinogen. In particular embodiments, the methods provided herein are used to reduce or inhibit expression of the vacuolar invertase gene (VInv), which encodes a protein that breaks down sucrose to glucose and fructose (Clasen et al. DOI: 10.1111/pbi.12370).

The use of the system to ensure a value-added trait

[0124] In particular embodiments the degraders herein is used to produce nutritionally improved agricultural crops. In particular embodiments, the methods provided herein are adapted to generate “functional foods”, i.e. a modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains and or “nutraceutical”, i.e. substances that may be considered a food or part of a food and provides health benefits, including the prevention and treatment of disease. In particular embodiments, the nutraceutical is useful in the prevention and/or treatment of one or more of cancer, diabetes, cardiovascular disease, and hypertension.

Biofuel production

[0125] The degraders herein may be used to modulate biofuel production in plants, fungi, and other organism, e.g., those have been engineered by expressing an RNA- guide nucleases. The term “biofuel” as used herein is an alternative fuel made from plant and plant-derived resources. Renewable biofuels can be extracted from organic matter whose energy has been obtained through a process of carbon fixation or are made through the use or conversion of biomass. This biomass can be used directly for biofuels or can be converted to convenient energy containing substances by thermal conversion, chemical conversion, and biochemical conversion. This biomass conversion can result in fuel in solid, liquid, or gas form. There are two types of biofuels: bioethanol and biodiesel. Bioethanol is mainly produced by the sugar fermentation process of cellulose (starch), which is mostly derived from maize and sugar cane. Biodiesel on the other hand is mainly produced from oil crops such as rapeseed, palm, and soybean. Biofuels are used mainly for transportation.

Enhancing plant properties for biofuel production

[0126] In particular embodiments, the degraders herein are used to alter the properties of the cell wall in order to facilitate access by key hydrolyzing agents for a more efficient release of sugars for fermentation. In particular embodiments, the biosynthesis of cellulose and/or lignin are modified. Cellulose is the major component of the cell wall. The biosynthesis of cellulose and lignin are co-regulated. By reducing the proportion of lignin in a plant the proportion of cellulose can be increased. In particular embodiments, the methods described herein are used to downregulate lignin biosynthesis in the plant so as to increase fermentable carbohydrates. More particularly, the methods described herein are used to downregulate at least a first lignin biosynthesis gene selected from the group consisting of 4-coumarate 3-hydroxylase (C3H), phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl transferase (HCT), caffeic acid O-methyltransferase (COMT), caffeoyl CoA 3-O-methyltransferase (CCoAOMT), ferulate 5-hydroxylase (F5H), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl CoA-reductase (CCR), 4-coumarate-CoA ligase (4CL), monolignol-lignin-specific glycosyltransferase, and aldehyde dehydrogenase (ALDH) as disclosed in WO 2008064289 A2.

[0127] In particular embodiments, the degraders herein are used to produce plant mass that produces lower levels of acetic acid during fermentation (see also WO 2010096488). More particularly, the methods disclosed herein are used to generate mutations in homologs to CasL to reduce polysaccharide acetylation.

Modifying yeast for Biofuel production

[0128] In particular embodiments, the degraders herein may be used to modulate Cas enzymes used for bioethanol production by recombinant micro-organisms. For instance, Cas can be used to engineer micro-organisms, such as yeast, to generate biofuel or biopolymers from fermentable sugars and optionally to be able to degrade plant-derived lignocellulose derived from agricultural waste as a source of fermentable sugars. More particularly, the invention provides methods whereby the Cas CRISPR complex is used to introduce foreign genes required for biofuel production into micro-organisms and/or to modify endogenous genes which may interfere with the biofuel synthesis. More particularly the methods involve introducing into a micro-organism such as a yeast one or more nucleotide sequence encoding enzymes involved in the conversion of pyruvate to ethanol or another product of interest. In particular embodiments the methods ensure the introduction of one or more enzymes which allows the micro-organism to degrade cellulose, such as a cellulase. In yet further

embodiments, the Cas proteins may be those used to modify endogenous metabolic pathways which compete with the biofuel production pathway.

Modifying Algae and plants for production of vegetable oils or biofuels

[0129] 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.

[0130] According to particular embodiments of the invention, the system is used to generate lipid-rich diatoms which are useful in biofuel production.

[0131] In particular embodiments it is envisaged to specifically modify genes that are involved in the modification of the quantity of lipids and/or the quality of the lipids produced by the algal cell. Examples of genes encoding enzymes involved in the pathways of fatty acid synthesis can encode proteins having for instance acetyl-CoA carboxylase, fatty acid synthase, 3-ketoacyl_acyl- carrier protein synthase III, glycerol-3-phosphate deshydrogenase (G3PDH), Enoyl-acyl carrier protein reductase (Enoyl-ACP-reductase), glycerol-3-phosphate acyltransferase, lysophosphatidic acyl transferase or diacylglycerol acyltransferase, phospholipid:diacylglycerol acyltransferase, phosphatidate phosphatase, fatty acid thioesterase such as palmitoyl protein thioesterase, or malic enzyme activities. In further embodiments it is envisaged to generate diatoms that have increased lipid accumulation. This can be achieved by targeting genes that decrease lipid catabolisation. Of particular interest for use in the methods of the present invention are genes involved in the activation of both triacylglycerol and free fatty acids, as well as genes directly involved in β -oxidation of fatty acids, such as acyl-CoA synthetase, 3-ketoacyl-CoA thiolase, acyl-CoA oxidase activity and phosphoglucomutase. The system and methods described herein can be used to specifically activate such genes in diatoms as to increase their lipid content.

[0132] Organisms such as microalgae are widely used for synthetic biology. Stovicek et al. (Metab. Eng. Comm., 2015; 2:13 describes genome editing of industrial yeast, for example, *Saccharomyces cerevisiae*, to efficiently produce robust strains for industrial production. Stovicek used a CRISPR-Cas9 system codon-optimized for yeast to simultaneously disrupt both alleles of an endogenous gene and knock in a heterologous gene. Cas9 and gRNA were expressed from genomic or episomal 2 μ -based vector locations. The authors also showed that gene disruption efficiency could be improved by optimization of the levels of Cas9 and gRNA expression. Hlavová et al. (Biotechnol. Adv. 2015) discusses

development of species or strains of microalgae using techniques such as CRISPR to target nuclear and chloroplast genes for insertional mutagenesis and screening. The methods of Stovicek and Hlavová may be applied to the Cas effector protein system of the present invention.

[0133] US 8,945,839 describes a method for engineering Micro-Algae (*Chlamydomonas reinhardtii* cells) species) using Cas9. Using similar tools, the methods of the system described herein can be applied on *Chlamydomonas* species and other algae. In particular embodiments, Cas and guide RNA are introduced in algae expressed using a vector that expresses Cas 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. Alternatively, Cas mRNA and in vitro transcribed guide RNA can be delivered to algal cells. Electroporation protocol follows standard recommended protocol from the GeneArt *Chlamydomonas* Engineering kit.

The use of system in the generation of micro-organisms capable of fatty acid production

[0134] In particular embodiments, the methods of the invention are used for the generation of genetically engineered micro-organisms capable of the production of fatty esters, such as fatty acid methyl esters ("FAME") and fatty acid ethyl esters ("FAEE"),

[0135] Typically, host cells can be engineered to produce fatty esters from a carbon source, such as an alcohol, present in the medium, by expression or overexpression of a gene encoding a thioesterase, a gene encoding an acyl-CoA synthase, and a gene encoding an ester synthase. Accordingly, the methods provided herein are used to modify a micro-organisms so as to overexpress or introduce a thioesterase gene, a gene encoding an acyl-CoA synthase, and a gene encoding an ester synthase. In particular embodiments, the thioesterase gene is selected from *tesA*, *'tesA*, *tesB*, *fatB*, *fatB2*, *fatB3*, *fatA1*, or *fatA*. In particular embodiments, the gene encoding an acyl-CoA synthase is selected from *fadDJadK*, *BH3103*, *pfl-4354*, *EAV15023*, *fadDI*, *fadD2*, *RPC_4074*, *fadDD35*, *fadDD22*, *faa39*, or an identified gene encoding an enzyme having the same properties. In particular embodiments, the gene encoding an ester synthase is a gene encoding a synthase/acyl-CoA:diacylglycerol acyltransferase from *Simmondsia chinensis*, *Acinetobacter* sp. ADP, *Alcanivorax borkumensis*, *Pseudomonas aeruginosa*, *Fundibacter jadensis*, *Arabidopsis thaliana*, or *Alkaligenes eutrophus*, or a variant thereof.

[0136] Additionally or alternatively, the degraders herein are used to decrease expression in said micro-organism of at least one of a gene encoding an acyl-CoA dehydrogenase, a

gene encoding an outer membrane protein receptor, and a gene encoding a transcriptional regulator of fatty acid biosynthesis. In particular embodiments one or more of these genes is inactivated, such as by introduction of a mutation.

[0137] In particular embodiments, the gene encoding an acyl-CoA dehydrogenase is *fadE*. In particular embodiments, the gene encoding a transcriptional regulator of fatty acid biosynthesis encodes a DNA transcription repressor, for example, *fabR*.

[0138] Additionally, or alternatively, said micro-organism is modified to reduce expression of at least one of a gene encoding a pyruvate formate lyase, a gene encoding a lactate dehydrogenase, or both. In particular embodiments, the gene encoding a pyruvate formate lyase is *pflB*. In particular embodiments, the gene encoding a lactate dehydrogenase is *IdhA*. In particular embodiments one or more of these genes is inactivated, such as by introduction of a mutation therein.

[0139] In particular embodiments, the micro-organism is selected from the genus *Escherichia*, *Bacillus*, *Lactobacillus*, *Rhodococcus*, *Synechococcus*, *Synechocystis*, *Pseudomonas*, *Aspergillus*, *Trichoderma*, *Neurospora*, *Fusarium*, *Humicola*, *Rhizomucor*, *Kluyveromyces*, *Pichia*, *Mucor*, *Myceliophthora*, *Penicillium*, *Phanerochaete*, *Pleurotus*, *Trametes*, *Chrysosporium*, *Saccharomyces*, *Stenotrophomonas*, *Schizosaccharomyces*, *Yarrowia*, or *Streptomyces*.

[0140] The degraders herein are further used to engineer micro-organisms capable of organic acid production, more particularly from pentose or hexose sugars. In particular embodiments, the methods comprise introducing into a micro-organism an exogenous LDH gene. In particular embodiments, the organic acid production in said micro-organisms is additionally or alternatively increased by inactivating endogenous genes encoding proteins involved in an endogenous metabolic pathway which produces a metabolite other than the organic acid of interest and/or wherein the endogenous metabolic pathway consumes the organic acid. In particular embodiments, the modification ensures that the production of the metabolite other than the organic acid of interest is reduced. According to particular embodiments, the methods are used to introduce at least one engineered gene deletion and/or inactivation of an endogenous pathway in which the organic acid is consumed or a gene encoding a product involved in an endogenous pathway which produces a metabolite other than the organic acid of interest. In particular embodiments, the at least one engineered gene deletion or inactivation is in one or more gene encoding an enzyme selected from the group consisting of pyruvate decarboxylase (*pdc*), fumarate reductase, alcohol dehydrogenase

(adh), acetaldehyde dehydrogenase, phosphoenolpyruvate carboxylase (ppc), D-lactate dehydrogenase (d-ldh), L-lactate dehydrogenase (l-ldh), lactate 2-monooxygenase.

[0141] In further embodiments the at least one engineered gene deletion and/or inactivation is in an endogenous gene encoding pyruvate decarboxylase (pdc).

[0142] In further embodiments, the micro-organism is engineered to produce lactic acid and the at least one engineered gene deletion and/or inactivation is in an endogenous gene encoding lactate dehydrogenase. Additionally or alternatively, the micro-organism comprises at least one engineered gene deletion or inactivation of an endogenous gene encoding a cytochrome-dependent lactate dehydrogenase, such as a cytochrome B2-dependent L-lactate dehydrogenase.

The use of the degraders in the generation of improved xylose or cellobiose utilizing yeasts strains

[0143] In particular embodiments, the degraders herein may be applied to select for improved xylose or cellobiose utilizing yeast strains. Error-prone PCR can be used to amplify one (or more) genes involved in the xylose utilization or cellobiose utilization pathways. Examples of genes involved in xylose utilization pathways and cellobiose utilization pathways may include, without limitation, those described in Ha, S.J., et al. (2011) Proc. Natl. Acad. Sci. USA 108(2):504-9 and Galazka, J.M., et al. (2010) Science 330(6000):84-6. Resulting libraries of double-stranded DNA molecules, each comprising a random mutation in such a selected gene could be co-transformed with the components of the system into a yeast strain (for instance S288C) and strains can be selected with enhanced xylose or cellobiose utilization capacity, as described in WO2015138855.

[0144] The use of the degraders in the generation of improved yeasts strains for use in isoprenoid biosynthesis

[0145] Tadas Jakočiūnas et al. described the successful application of a multiplex CRISPR/Cas9 system for genome engineering of up to 5 different genomic loci in one transformation step in baker's yeast *Saccharomyces cerevisiae* (Metabolic Engineering Volume 28, March 2015, Pages 213–222) resulting in strains with high mevalonate production, a key intermediate for the industrially important isoprenoid biosynthesis pathway. In particular embodiments, the system may be applied in a multiplex genome engineering method as described herein for identifying additional high producing yeast strains for use in isoprenoid synthesis.

The use of the degraders in the generation of lactic acid producing yeasts strains

[0146] In another embodiment, successful application of a multiplex system is encompassed. In analogy with Vratislav Stovicek et al. (Metabolic Engineering Communications, Volume 2, December 2015, Pages 13–22), improved lactic acid-producing strains can be designed and obtained in a single transformation event. In a particular embodiment, the system is used for simultaneously inserting the heterologous lactate dehydrogenase gene and disruption of two endogenous genes PDC1 and PDC5 genes.

Further applications of the degraders in plants

[0147] In particular embodiments, the degraders herein, can be used for visualization of genetic element dynamics. For example, the degraders may regulate the CRISPR imaging that can visualize either repetitive or non-repetitive genomic sequences, report telomere length change and telomere movements and monitor the dynamics of gene loci throughout the cell cycle (Chen et al., Cell, 2013). These methods may also be applied to plants.

[0148] Other applications of the degraders herein, is the targeted gene disruption positive-selection screening in vitro and in vivo (Malina et al., Genes and Development, 2013). These methods may also be applied to plants.

[0149] In particular embodiments, fusion of inactive Cas endonucleases with histone-modifying enzymes can introduce custom changes in the complex epigenome (Rusk et al., Nature Methods, 2014). The degraders may also be applied to plants.

[0150] In particular embodiments, the degraders herein, can be used to purify a specific portion of the chromatin and identify the associated proteins, thus elucidating their regulatory roles in transcription (Waldrip et al., Epigenetics, 2014). These methods may also be applied to plants.

[0151] In particular embodiments, the degraders can be used as a therapy for virus removal in plant systems as it is able to cleave both viral DNA and RNA. Previous studies in human systems have demonstrated the success of utilizing CRISPR in targeting the single strand RNA virus, hepatitis C (A. Price, et al., Proc. Natl. Acad. Sci, 2015) as well as the double stranded DNA virus, hepatitis B (V. Ramanan, et al., Sci. Rep, 2015).

[0152] In particular embodiments, the degraders may be used to alter genome complexity. In further particular embodiment, the degraders herein, can be used to disrupt or alter chromosome number and generate haploid plants, which only contain chromosomes from one parent. Such plants can be induced to undergo chromosome duplication and converted into diploid plants containing only homozygous alleles (Karimi-Ashtiyani et al., PNAS, 2015; Anton et al., Nucleus, 2014). These methods may also be applied to plants.

[0153] In particular embodiments, the degraders can be used for regulating self-cleavage. In these embodiments, the promotor of the Cas enzyme and gRNA can be a constitutive promotor and a second gRNA is introduced in the same transformation cassette, but controlled by an inducible promoter. This second gRNA can be designated to induce site-specific cleavage in the Cas gene in order to create a non-functional Cas. In a further particular embodiment, the second gRNA induces cleavage on both ends of the transformation cassette, resulting in the removal of the cassette from the host genome. This system offers a controlled duration of cellular exposure to the Cas enzyme and further minimizes off-target editing. Furthermore, cleavage of both ends of a CRISPR/Cas cassette can be used to generate transgene-free T0 plants with bi-allelic mutations (as described for Cas9 e.g. Moore et al., *Nucleic Acids Research*, 2014; Schaeffer et al., *Plant Science*, 2015). The methods of Moore et al. may be applied to the systems described herein.

Applications in non-human animals

[0154] The degraders herein may be used on non-human animals, those expressing an RNA-guided nuclease (e.g., constitutively or inducibly). The degraders may regulate the functions and activities of the RNA-guided nucleases in the animals.

[0155] 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 present invention may also be extended to other agricultural applications such as, for example, farm and production animals. For example, pigs have many features that make them attractive as biomedical models, especially in regenerative medicine. In particular, pigs with severe combined immunodeficiency (SCID) may provide useful models for regenerative medicine, xenotransplantation (discussed also elsewhere herein), and tumor development and will aid in developing therapies for human SCID patients. Lee et al., (*Proc Natl Acad Sci U S A*. 2014 May 20;111(20):7260-5) utilized a reporter-guided transcription activator-like effector nuclease (TALEN) system to generate targeted modifications of recombination activating gene (RAG) 2 in somatic cells at high efficiency, including some that affected both alleles. The Type V effector protein may be applied to a similar system.

[0156] The degraders herein may be used (e.g., with an RNA-guided nuclease comprising one or more degradation domains) to create a platform to model a disease or disorder of an animal, in some embodiments a mammal, in some embodiments a human. In certain embodiments, such models and platforms are rodent based, in non-limiting examples rat or mouse. Such models and platforms can take advantage of distinctions among and comparisons between inbred rodent strains. In certain embodiments, such models and platforms primate, horse, cattle, sheep, goat, swine, dog, cat or bird-based, for example to directly model diseases and disorders of such animals or to create modified and/or improved lines of such animals. Advantageously, in certain embodiments, an animal-based platform or model is created to mimic a human disease or disorder. For example, the similarities of swine to humans make swine an ideal platform for modeling human diseases. Compared to rodent models, development of swine models has been costly and time intensive. On the other hand, swine and other animals are much more similar to humans genetically, anatomically, physiologically and pathophysiologically. The degraders herein may be used to provide a high efficiency platform for targeted gene and genome editing, gene and genome modification and gene and genome regulation to be used in such animal platforms and models. Though ethical standards block development of human models and in many cases models based on non-human primates, the present invention is used with in vitro systems, including but not limited to cell culture systems, three dimensional models and systems, and organoids to mimic, model, and investigate genetics, anatomy, physiology and pathophysiology of structures, organs, and systems of humans. The platforms and models provide manipulation of single or multiple targets.

[0157] In certain embodiments, the present invention is applicable to disease models like that of Schomberg et al. (FASEB Journal, April 2016; 30(1):Suppl 571.1). For example, degraders herein may be used to regulate the CRISPR systems used herein. To model the inherited disease neurofibromatosis type 1 (NF-1) Schomberg used CRISPR-Cas9 to introduce mutations in the swine neurofibromin 1 gene by cytosolic microinjection of CRISPR/Cas9 components into swine embryos. CRISPR guide RNAs (gRNA) were created for regions targeting sites both upstream and downstream of an exon within the gene for targeted cleavage by Cas9 and repair was mediated by a specific single-stranded oligodeoxynucleotide (ssODN) template to introduce a 2500 bp deletion. The degraders herein may also used to engineer swine with specific NF-1 mutations or clusters of mutations, and further can be used to engineer mutations that are specific to or representative

of a given human individual. The invention is similarly used to develop animal models, including but not limited to swine models, of human multigenic diseases. According to the invention, multiple genetic loci in one gene or in multiple genes are simultaneously targeted using multiplexed guides and optionally one or multiple templates.

[0158] The present invention is also applicable to modifying SNPs of other animals, such as cows. Tan et al. (Proc Natl Acad Sci U S A. 2013 Oct 8; 110(41): 16526–16531) expanded the livestock gene editing toolbox to include transcription activator-like (TAL) effector nuclease (TALEN)- and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9- stimulated homology-directed repair (HDR) using plasmid, rAAV, and oligonucleotide templates. Gene specific gRNA sequences were cloned into the Church lab gRNA vector (Addgene ID: 41824) according to their methods (Mali P, et al. (2013) RNA-Guided Human Genome Engineering via Cas9. Science 339(6121):823-826). The Cas9 nuclease was provided either by co-transfection of the hCas9 plasmid (Addgene ID: 41815) or mRNA synthesized from RCIScript-hCas9. This RCIScript-hCas9 was constructed by sub-cloning the XbaI-AgeI fragment from the hCas9 plasmid (encompassing the hCas9 cDNA) into the RCIScript plasmid.

[0159] Heo et al. (Stem Cells Dev. 2015 Feb 1;24(3):393-402. doi: 10.1089/scd.2014.0278. Epub 2014 Nov 3) reported highly efficient gene targeting in the bovine genome using bovine pluripotent cells and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 nuclease. First, Heo et al. generate induced pluripotent stem cells (iPSCs) from bovine somatic fibroblasts by the ectopic expression of yamanaka factors and GSK3 β and MEK inhibitor (2i) treatment. Heo et al. observed that these bovine iPSCs are highly similar to naïve pluripotent stem cells with regard to gene expression and developmental potential in teratomas. Moreover, CRISPR-Cas9 nuclease, which was specific for the bovine NANOG locus, showed highly efficient editing of the bovine genome in bovine iPSCs and embryos.

Livestock - Pigs

[0160] Viral targets in livestock may include, in some embodiments, porcine CD163, for example on porcine macrophages. CD163 is associated with infection (thought to be through viral cell entry) by PRRSV (Porcine Reproductive and Respiratory Syndrome virus, an arterivirus). Infection by PRRSV, especially of porcine alveolar macrophages (found in the lung), results in a previously incurable porcine syndrome (“Mystery swine disease” or “blue ear disease”) that causes suffering, including reproductive failure, weight loss and high

mortality rates in domestic pigs. Opportunistic infections, such as enzootic pneumonia, meningitis and ear oedema, are often seen due to immune deficiency through loss of macrophage activity. It also has significant economic and environmental repercussions due to increased antibiotic use and financial loss (an estimated \$660m per year).

[0161] As reported by Kristin M Whitworth and Dr Randall Prather et al. (Nature Biotech 3434 published online 07 December 2015) at the University of Missouri and in collaboration with Genus Plc, CD163 was targeted using CRISPR-Cas9 and the offspring of edited pigs were resistant when exposed to PRRSV. One founder male and one founder female, both of whom had mutations in exon 7 of CD163, were bred to produce offspring. The founder male possessed an 11-bp deletion in exon 7 on one allele, which results in a frameshift mutation and missense translation at amino acid 45 in domain 5 and a subsequent premature stop codon at amino acid 64. The other allele had a 2-bp addition in exon 7 and a 377-bp deletion in the preceding intron, which were predicted to result in the expression of the first 49 amino acids of domain 5, followed by a premature stop code at amino acid 85. The sow had a 7 bp addition in one allele that when translated was predicted to express the first 48 amino acids of domain 5, followed by a premature stop codon at amino acid 70. The sow's other allele was unamplifiable. Selected offspring were predicted to be a null animal (CD163^{-/-}), i.e. a CD163 knock out.

[0162] Accordingly, in some embodiments, porcine alveolar macrophages may be targeted by the CRISPR protein and regulated by degraders herein. In some embodiments, porcine CD163 may be targeted by the CRISPR protein. In some embodiments, porcine CD163 may be knocked out through induction of a DSB or through insertions or deletions, for example targeting deletion or modification of exon 7, including one or more of those described above, or in other regions of the gene, for example deletion or modification of exon 5.

[0163] An edited pig and its progeny are also envisaged, for example a CD163 knock out pig. This may be for livestock, breeding or modelling purposes (i.e. a porcine model). Semen comprising the gene knock out is also provided.

[0164] CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily. Based on in vitro studies SRCR domain 5 of the protein is the domain responsible for unpackaging and release of the viral genome. As such, other members of the SRCR superfamily may also be targeted in order to assess resistance to other viruses. PRRSV is also a member of the mammalian arterivirus group, which also includes murine lactate

dehydrogenase-elevating virus, simian hemorrhagic fever virus and equine arteritis virus. The arteriviruses share important pathogenesis properties, including macrophage tropism and the capacity to cause both severe disease and persistent infection. Accordingly, arteriviruses, and in particular murine lactate dehydrogenase-elevating virus, simian hemorrhagic fever virus and equine arteritis virus, may be targeted, for example through porcine CD163 or homologues thereof in other species, and murine, simian and equine models and knockout also provided.

[0165] Indeed, this approach may be extended to viruses or bacteria that cause other livestock diseases that may be transmitted to humans, such as Swine Influenza Virus (SIV) strains which include influenza C and the subtypes of influenza A known as H1N1, H1N2, H2N1, H3N1, H3N2, and H2N3, as well as pneumonia, meningitis and edema mentioned above.

Generation of Models of Genetic and Epigenetic Conditions

[0166] The degraders herein may be used, e.g., with an RNA-guided nuclease, to create a plant, an animal or cell that may be used to model and/or study genetic or epigenetic conditions of interest, such as a through a model of mutations of interest or a disease model. In some embodiments, the models may be generated using the RNA-guided nuclease, and the characters of the models may be further modulated and controlled using the degraders herein.

[0167] As used herein, “disease” refers to a disease, disorder, or indication in a subject. For example, a method of the invention may be used to create an animal or cell that comprises a modification in one or more nucleic acid sequences associated with a disease, or a plant, animal or cell in which the expression of one or more nucleic acid sequences associated with a disease are altered. Such a nucleic acid sequence may encode a disease associated protein sequence or may be a disease associated control sequence. Accordingly, it is understood that in embodiments of the invention, a plant, subject, patient, organism or cell can be a non-human subject, patient, organism or cell. Thus, the invention provides a plant, animal or cell, produced by the present methods, or a progeny thereof. The progeny may be a clone of the produced plant or animal, or may result from sexual reproduction by crossing with other individuals of the same species to introgress further desirable traits into their offspring. The cell may be *in vivo* or *ex vivo* in the cases of multicellular organisms, particularly animals or plants. In the instance where the cell is in cultured, a cell line may be established if appropriate culturing conditions are met and preferably if the cell is suitably

adapted for this purpose (for instance a stem cell). Bacterial cell lines produced by the invention are also envisaged. Hence, cell lines are also envisaged.

[0168] In some methods, the disease model can be used to study the effects of mutations on the animal or cell and development and/or progression of the disease using measures commonly used in the study of the disease. Alternatively, such a disease model is useful for studying the effect of a pharmaceutically active compound on the disease.

[0169] In some methods, the disease model can be used to assess the efficacy of a potential gene therapy strategy. That is, a disease-associated gene or polynucleotide can be modified such that the disease development and/or progression is inhibited or reduced. In particular, the method comprises modifying a disease-associated gene or polynucleotide such that an altered protein is produced and, as a result, the animal or cell has an altered response. Accordingly, in some methods, a genetically modified animal may be compared with an animal predisposed to development of the disease such that the effect of the gene therapy event may be assessed.

[0170] In another embodiment, this invention provides a method of developing a biologically active agent that modulates a cell signaling event associated with a disease gene. The method comprises contacting a test compound with a cell comprising one or more vectors that drive expression of one or more of components of the system; and detecting a change in a readout that is indicative of a reduction or an augmentation of a cell signaling event associated with, e.g., a mutation in a disease gene contained in the cell.

[0171] A cell model or animal model can be constructed in combination with the method of the invention for screening a cellular function change. Such a model may be used to study the effects of a genome sequence modified by the systems and methods herein on a cellular function of interest. For example, a cellular function model may be used to study the effect of a modified genome sequence on intracellular signaling or extracellular signaling. Alternatively, a cellular function model may be used to study the effects of a modified genome sequence on sensory perception. In some such models, one or more genome sequences associated with a signaling biochemical pathway in the model are modified.

[0172] Several disease models have been specifically investigated. These include *de novo* autism risk genes CHD8, KATNAL2, and SCN2A; and the syndromic autism (Angelman Syndrome) gene UBE3A. These genes and resulting autism models are of course preferred, but serve to show the broad applicability of the invention across genes and corresponding models. An altered expression of one or more genome sequences associated with a signaling

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

[0173] To assay for an agent-induced alteration in the level of mRNA transcripts or corresponding polynucleotides, nucleic acid contained in a sample is first extracted according to standard methods in the art. For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook et al. (1989), or extracted by nucleic-acid-binding resins following the accompanying instructions provided by the manufacturers. The mRNA contained in the extracted nucleic acid sample is then detected by amplification procedures or conventional hybridization assays (e.g. Northern blot analysis) according to methods widely known in the art or based on the methods exemplified herein.

[0174] In practicing any of the methods disclosed herein, a suitable vector can be introduced to a cell or an embryo via one or more methods known in the art, including without limitation, microinjection, electroporation, sonoporation, biolistics, calcium phosphate-mediated transfection, cationic transfection, liposome transfection, dendrimer transfection, heat shock transfection, nucleofection transfection, magnetofection, lipofection, impalefection, optical transfection, proprietary agent-enhanced uptake of nucleic acids, and delivery via liposomes, immunoliposomes, virosomes, or artificial virions. In some methods, the vector is introduced into an embryo by microinjection. The vector or vectors may be microinjected into the nucleus or the cytoplasm of the embryo. In some methods, the vector or vectors may be introduced into a cell by nucleofection.

[0175] The degraders herein may regulate the effects (e.g., binding, cleavage) of a CRISPR-complex on a target polynucleotide. 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).

[0176] 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.

[0177] The target polynucleotide of the system herein 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. Further, engineering of the PAM Interacting (PI) domain may allow programming of PAM specificity, improve target site recognition fidelity, and increase the versatility of the Cas, e.g. Cas9, genome engineering platform. Cas proteins, such as Cas9 proteins may be engineered to alter their PAM specificity, for example as described in Kleinstiver BP et al. *Engineered CRISPR-Cas9 nucleases with altered PAM specificities*. Nature. 2015 Jul 23;523(7561):481-5. doi: 10.1038/nature14592.

[0178] The target polynucleotide of the system 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, and PCT Application PCT/US2013/074667, entitled DELIVERY, ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION AND THERAPEUTIC APPLICATIONS, filed December 12, 2013, the contents of all of which are herein incorporated by reference in their entirety.

[0179] 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.

THERAPEUTIC APPLICATIONS

[0180] The degraders herein may be used for treatment in a variety of diseases and disorders. The degraders may be used to modulate the function and activity of an RNA-guided nuclease (e.g., a Cas protein) used for treating a disease. For example, the degraders may be used for regulating the strength, efficacy, timing, dosage of the therapeutic RNA-guided nuclease.

[0181] In some cases, a small molecule inhibitor herein may be administered to a subject concurrently with an RNA-guided nuclease. Alternatively or additionally, a small molecule inhibitor herein may be administered to a subject prior to the administration of an RNA-guided nuclease. Alternatively or additionally, a small molecule inhibitor herein may be administered to a subject after the administration of an RNA-guided nuclease. In some examples, the degraders herein are used for modulating CRISPR gene editing (e.g., by modulating Cas protein of the CRISPR system).

[0182] The degraders herein may be administered as one or more doses as needed. In some examples, the degraders may be administered as a single dose. In certain examples, the

degraders may be administered as multiple doses, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more doses. The multi-dose regime may be used to achieve optimal efficacy and/or temporal control of the activity and function of the RNA-guided nuclease.

Exemplary Therapies

[0183] The degraders herein may be used for treatment in a variety of diseases and disorders. The degraders may be used to modulate the function and activity of an RNA-guided nuclease (e.g., a Cas protein) used for treating a disease.

[0184] In embodiments, the compounds can be used in method for therapy in which cells are edited *ex vivo*, *in vivo* or *in vitro* using CRISPR systems to modulate at least one gene. In embodiments, *in vitro* methods may include with subsequent administration of the edited cells to a patient in need thereof. In some embodiments, the CRISPR editing involves knocking in, knocking out or knocking down expression of at least one target gene in a cell. In particular embodiments, the degraders herein can modulate CRISPR editing when utilizing a CRISPR protein with one or more degradation domains inserts an exogenous, gene, minigene or sequence, which may comprise one or more exons and introns or natural or synthetic introns into the locus of a target gene, a hot-spot locus, a safe harbor locus of the gene genomic locations where new genes or genetic elements can be introduced without disrupting the expression or regulation of adjacent genes, or correction by insertions or deletions one or more mutations in DNA sequences that encode regulatory elements of a target gene.

[0185] In embodiments, the treatment is for disease/disorder of an organ, including liver disease, eye disease, muscle disease, heart disease, blood disease, brain disease, kidney disease, or may comprise treatment for an autoimmune disease, central nervous system disease, cancer and other proliferative diseases, neurodegenerative disorders, inflammatory disease, metabolic disorder, musculoskeletal disorder and the like.

[0186] Particular diseases/disorders include chondroplasia, achromatopsia, acid maltase deficiency, adrenoleukodystrophy, aicardi syndrome, alpha-1 antitrypsin deficiency, alpha-thalassemia, androgen insensitivity syndrome, apert syndrome, arrhythmogenic right ventricular, dysplasia, ataxia telangiectasia, barth syndrome, beta-thalassemia, blue rubber bleb nevus syndrome, canavan disease, chronic granulomatous diseases (CGD), cri du chat syndrome, cystic fibrosis, dercum's disease, ectodermal dysplasia, fanconi anemia, fibrodysplasia ossificans progressive, fragile X syndrome, galactosemia, Gaucher's disease, generalized gangliosidoses (e.g., GM1), hemochromatosis, the hemoglobin C mutation in the

6th codon of beta-globin (HbC), hemophilia, Huntington's disease, Hurler Syndrome, hypophosphatasia, Klinefelter syndrome, Krabbes Disease, Langer-Giedion Syndrome, leukodystrophy, long QT syndrome, Marfan syndrome, Moebius syndrome, mucopolysaccharidosis (MPS), nail patella syndrome, nephrogenic diabetes insipidus, neurofibromatosis, Neimann-Pick disease, osteogenesis imperfecta, porphyria, Prader- Willi syndrome, progeria, Proteus syndrome, retinoblastoma, Rett syndrome, Rubinstein-Taybi syndrome, Sanfilippo syndrome, severe combined immunodeficiency (SCID), Shwachman syndrome, sickle cell disease (sickle cell anemia), Smith-Magenis syndrome, Stickler syndrome, Tay-Sachs disease, Thrombocytopenia Absent Radius (TAR) syndrome, Treacher Collins syndrome, trisomy, tuberous sclerosis, Turner's syndrome, urea cycle disorder, von Hippel- Landau disease, Waardenburg syndrome, Williams syndrome, Wilson's disease, and Wiskott- Aldrich syndrome.

[0187] In embodiments, the disease is associated with expression of a tumor antigen, e.g., a proliferative disease, a precancerous condition, a cancer, or a non-cancer related indication associated with expression of the tumor antigen, which may in some embodiments comprise a target selected from B2M, CD247, CD3D, CD3E, CD3G, TRAC, TRBC1, TRBC2, HLA-A, HLA-B, HLA-C, DCK, CD52, FKBP1A, CIITA, NLRC5, RFXANK, RFX5, RFXAP, or NR3C1, HAVCR2, LAG3, PDCD1, PD-L2, CTLA4, CEACAM (CEACAM-1, CEACAM-3 and/or CEACAM-5), VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD113), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD107), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGF beta, or PTPN11 DCK, CD52, NR3C1, LILRB1, CD19; CD123; CD22; CD30; CD171; CS-1 (also referred to as CD2 subset 1, CRACC, SLAMF7, CD319, and 19A24); C-type lectin-like molecule-1 (CLL-1 or CLECL1); CD33; epidermal growth factor receptor variant III (EGFRvIII); ganglioside G2 (GD2); ganglioside GD3 (aNeu5Ac(2-8)aNeu5Ac(2-3)bDGalp(1-4)bDGlc(1-1)Cer); TNF receptor family member B cell maturation (BCMA); Tn antigen ((Tn Ag) or (GalNAc-Ser/Thr)); prostate-specific membrane antigen (PSMA); Receptor tyrosine kinase-like orphan receptor 1 (ROR1); Fms-Like Tyrosine Kinase 3 (FLT3); Tumor-associated glycoprotein 72 (TAG72); CD38; CD44v6; Carcinoembryonic antigen (CEA); Epithelial cell adhesion molecule (EPCAM); B7H3 (CD276); KIT (CD117); Interleukin-13 receptor subunit alpha-2 (IL-13Ra2 or CD213A2); Mesothelin; Interleukin 11 receptor alpha (IL-11Ra); prostate stem cell antigen (PSCA); Protease Serine 21 (Testisin or PRSS21); vascular endothelial growth factor receptor 2 (VEGFR2); Lewis(Y) antigen; CD24; Platelet-derived growth factor receptor beta

(PDGFR-beta); Stage-specific embryonic antigen-4 (SSEA-4); CD20; Folate receptor alpha; Receptor tyrosine-protein kinase ERBB2 (Her2/neu); n kinase ERBB2 (Her2/neu); Mucin 1, cell surface associated (MUC1); epidermal growth factor receptor (EGFR); neural cell adhesion molecule (NCAM); Prostase; prostatic acid phosphatase (PAP); elongation factor 2 mutated (ELF2M); Ephrin B2; fibroblast activation protein alpha (FAP); insulin-like growth factor 1 receptor (IGF-I receptor), carbonic anhydrase IX (CAIX); Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2); glycoprotein 100 (gp100); oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl); tyrosinase; ephrin type-A receptor 2 (EphA2); Fucosyl GM1; sialyl Lewis adhesion molecule (sLe); ganglioside GM3 (aNeu5Ac(2-3)bDGalp(1-4)bDGlcp(1-1)Cer); transglutaminase 5 (TGS5); high molecular weight-melanoma-associated antigen (HMWMAA); o-acetyl-GD2 ganglioside (OAcGD2); Folate receptor beta; tumor endothelial marker 1 (TEM1/CD248); tumor endothelial marker 7-related (TEM7R); claudin 6 (CLDN6); thyroid stimulating hormone receptor (TSHR); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); CD97; CD179a; anaplastic lymphoma kinase (ALK); Polysialic acid; placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycosphingolipid (GloboH); mammary gland differentiation antigen (NY-BR-1); uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 complex, locus K 9 (LY6K); Olfactory receptor 51E2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); Cancer/testis antigen 1 (NY-ESO-1); Cancer/testis antigen 2 (LAGE-1a); Melanoma-associated antigen 1 (MAGE-A1); ETS translocation-variant gene 6, located on chromosome 12p (ETV6-AML); sperm protein 17 (SPA17); X Antigen Family, Member 1A (XAGE1); angiopoietin-binding cell surface receptor 2 (Tie 2); melanoma cancer testis antigen-1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1; tumor protein p53 (p53); p53 mutant; prostate; surviving; telomerase; prostate carcinoma tumor antigen-1 (PCTA-1 or Galectin 8), melanoma antigen recognized by T cells 1 (MelanA or MART1); Rat sarcoma (Ras) mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N-Acetyl glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B1; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras

Homolog Family Member C (RhoC); Tyrosinase-related protein 2 (TRP-2); Cytochrome P450 1B1 (CYP1B1); CCCTC-Binding Factor (Zinc Finger Protein)-Like (BORIS or Brother of the Regulator of Imprinted Sites), Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OYTES1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Receptor for Advanced Glycation Endproducts (RAGE-1); renal ubiquitous 1 (RU1); renal ubiquitous 2 (RU2); legumain; human papilloma virus E6 (HPV E6); human papilloma virus E7 (HPV E7); intestinal carboxyl esterase; heat shock protein 70-2 mutated (mut hsp70-2); CD79a; CD79b; CD72; Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR or CD89); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF); C-type lectin domain family 12 member A (CLEC12A); bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor-like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); and immunoglobulin lambda-like polypeptide 1 (IGLL1), CD19, BCMA, CD70, G6PC, Dystrophin, including modification of exon 51 by deletion or excision, DMPK, CFTR (cystic fibrosis transmembrane conductance regulator). In embodiments, the targets comprise CD70, or a Knock-in of CD33 and Knock-out of B2M. In embodiments, the targets comprise a knockout of TRAC and B2M, or TRAC B2M and PD1, with or without additional target genes. In certain embodiments, the disease is cystic fibrosis with targeting of the SCNN1A gene, e.g., the non-coding or coding regions, e.g., a promoter region, or a transcribed sequence, e.g., intronic or exonic sequence, targeted knock-in at CFTR sequence within intron 2, into which, e.g., can be introduced CFTR sequence that codes for CFTR exons 3-27; and sequence within CFTR intron 10, into which sequence that codes for CFTR exons 11-27 can be introduced.

[0188] In embodiments, the disease is Metachromatic Leukodystrophy, and the target is Arylsulfatase A, the disease is Wiskott-Aldrich Syndrome and the target is Wiskott-Aldrich Syndrome protein, the disease is Adreno leukodystrophy and the target is ATP-binding cassette DI, the disease is Human Immunodeficiency Virus and the target is receptor type 5-C-C chemokine or CXCR4 gene, the disease is Beta-thalassemia and the target is Hemoglobin beta subunit, the disease is X-linked Severe Combined ID receptor subunit gamma and the target is interelukin-2 receptor subunit gamma, the disease is Multisystemic Lysosomal Storage Disorder cystinosis and the target is cystinosin, the disease is Diamon-

Blackfan anemia and the target is Ribosomal protein S19, the disease is Fanconi Anemia and the target is Fanconi anemia complementation groups (e.g. FNACA, FNACB, FANCC, FANCD1, FANCD2, FANCE, FANCF, RAD51C), the disease is Shwachman-Bodian-Diamond syndrome and the target is Shwachman syndrome gene, the disease is Gaucher's disease and the target is Glucocerebrosidase, the disease is Hemophilia A and the target is Anti-hemophilic factor OR Factor VIII, Christmas factor, Serine protease, Factor Hemophilia B IX, the disease is Adenosine deaminase deficiency (ADA-SCID) and the target is Adenosine deaminase, the disease is GM1 gangliosidosis and the target is beta-galactosidase, the disease is Glycogen storage disease type II, Pompe disease, the disease is acid maltase deficiency and the target is alpha-glucosidase, the disease is Niemann-Pick disease, SMPD1-associated (Types Sphingomyelin phosphodiesterase 1 OR A and B) and the target is sphingomyelinase, the disease is Krabbe disease, globoid cell leukodystrophy and the target is Galactosylceramidase or galactosylceramide lipidoses and the target is galactocerebrosidase, Human leukocyte antigens DR-15, DQ-6, the disease is Multiple Sclerosis (MS) DRB1, the disease is Herpes Simplex Virus 1 or 2 and the target is knocking down of one, two or three of RS1, RL2 and/or LAT genes. In embodiments, the disease is an HPV associated cancer with treatment including edited cells comprising binding molecules, such as TCRs or antigen binding fragments thereof and antibodies and antigen-binding fragments thereof, such as those that recognize or bind human papilloma virus. The disease can be Hepatitis B with a target of one or more of PreC, C, X, PreS1, PreS2, S, P and/or SP gene(s).

[0189] In embodiments, the immune disease is severe combined immunodeficiency (SCID), Omenn syndrome, and in one aspect the target is Recombination Activating Gene 1 (RAG1) or an interleukin-7 receptor (IL7R). In particular embodiments, the disease is Transthyretin Amyloidosis (ATTR), Familial amyloid cardiomyopathy, and in one aspect, the target is the TTR gene, including one or more mutations in the TTR gene. In embodiments, the disease is Alpha-1 Antitrypsin Deficiency (AATD) or another disease in which Alpha-1 Antitrypsin is implicated, for example GvHD, Organ transplant rejection, diabetes, liver disease, COPD, Emphysema and Cystic Fibrosis, in particular embodiments, the target is SERPINA1.

[0190] In embodiments, the disease is primary hyperoxaluria, which, in certain embodiments, the target comprises one or more of Lactate dehydrogenase A (LDHA) and hydroxy Acid Oxidase 1 (HAO 1). In embodiments, the disease is primary hyperoxaluria

type 1 (ph1) and other alanine-glyoxylate aminotransferase (agxt) gene related conditions or disorders, such as Adenocarcinoma, Chronic Alcoholic Intoxication, Alzheimer's Disease, Cooley's anemia, Aneurysm, Anxiety Disorders, Asthma, Malignant neoplasm of breast, Malignant neoplasm of skin, Renal Cell Carcinoma, Cardiovascular Diseases, Malignant tumor of cervix, Coronary Arteriosclerosis, Coronary heart disease, Diabetes, Diabetes Mellitus, Diabetes Mellitus Non- Insulin-Dependent, Diabetic Nephropathy, Eclampsia, Eczema, Subacute Bacterial Endocarditis, Glioblastoma, Glycogen storage disease type II, Sensorineural Hearing Loss (disorder), Hepatitis, Hepatitis A, Hepatitis B, Homocystinuria, Hereditary Sensory Autonomic Neuropathy Type 1, Hyperaldosteronism, Hypercholesterolemia, Hyperoxaluria, Primary Hyperoxaluria, Hypertensive disease, Inflammatory Bowel Diseases, Kidney Calculi, Kidney Diseases, Chronic Kidney Failure, leiomyosarcoma, Metabolic Diseases, Inborn Errors of Metabolism, Mitral Valve Prolapse Syndrome, Myocardial Infarction, Neoplasm Metastasis, Nephrotic Syndrome, Obesity, Ovarian Diseases, Periodontitis, Polycystic Ovary Syndrome, Kidney Failure, Adult Respiratory Distress Syndrome, Retinal Diseases, Cerebrovascular accident, Turner Syndrome, Viral hepatitis, Tooth Loss, Premature Ovarian Failure, Essential Hypertension, Left Ventricular Hypertrophy, Migraine Disorders, Cutaneous Melanoma, Hypertensive heart disease, Chronic glomerulonephritis, Migraine with Aura, Secondary hypertension, Acute myocardial infarction, Atherosclerosis of aorta, Allergic asthma, pineoblastoma, Malignant neoplasm of lung, Primary hyperoxaluria type I, Primary hyperoxaluria type 2, Inflammatory Breast Carcinoma, Cervix carcinoma, Restenosis, Bleeding ulcer, Generalized glycogen storage disease of infants, Nephrolithiasis, Chronic rejection of renal transplant, Urolithiasis, pricking of skin, Metabolic Syndrome X, Maternal hypertension, Carotid Atherosclerosis, Carcinogenesis, Breast Carcinoma, Carcinoma of lung, Nephronophthisis, Microalbuminuria, Familial Retinoblastoma, Systolic Heart Failure Ischemic stroke, Left ventricular systolic dysfunction, Cauda Equina Paraganglioma, Hepatocarcinogenesis, Chronic Kidney Diseases, Glioblastoma Multiforme, Non-Neoplastic Disorder, Calcium Oxalate Nephrolithiasis, Ablepharon-Macrostomia Syndrome, Coronary Artery Disease, Liver carcinoma, Chronic kidney disease stage 5, Allergic rhinitis (disorder), Crigler Najjar syndrome type 2, and Ischemic Cerebrovascular Accident. In certain embodiments, treatment is targeted to the liver. In embodiments, the gene is AGXT, with a a cytogenetic location of 2q37.3 and the genomic coordinate are on Chromosome 2 on the forward strand at position 240,868,479-240,880,502.

[0191] Treatment can also target collagen type vii alpha 1 chain (col7a1) gene related conditions or disorders, such as Malignant neoplasm of skin, Squamous cell carcinoma, Colorectal Neoplasms, Crohn Disease, Epidermolysis Bullosa, Indirect Inguinal Hernia, Pruritus, Schizophrenia, Dermatologic disorders, Genetic Skin Diseases, Teratoma, Cockayne-Touraine Disease, Epidermolysis Bullosa Acquisita, Epidermolysis Bullosa Dystrophica, Junctional Epidermolysis Bullosa, Hallopeau- Siemens Disease, Bullous Skin Diseases, Agenesis of corpus callosum, Dystrophia unguium, Vesicular Stomatitis, Epidermolysis Bullosa With Congenital Localized Absence Of Skin And Deformity Of Nails, Juvenile Myoclonic Epilepsy, Squamous cell carcinoma of esophagus, Poikiloderma of Kindler, pretibial Epidermolysis bullosa, Dominant dystrophic epidermolysis bullosa albopapular type (disorder), Localized recessive dystrophic epidermolysis bullosa, Generalized dystrophic epidermolysis bullosa, Squamous cell carcinoma of skin, Epidermolysis Bullosa Pruriginosa, Mammary Neoplasms, Epidermolysis Bullosa Simplex Superficialis, Isolated Toenail Dystrophy, Transient bullous dermolysis of the newborn, Autosomal Recessive Epidermolysis Bullosa Dystrophica Localisata Variant, and Autosomal Recessive Epidermolysis Bullosa Dystrophica Inversa.

[0192] In embodiments, the disease is acute myeloid leukemia (AML), targeting Wilms Tumor I (WTI) and HLA expressing cells. In embodiments, the therapy is T cell therapy, as described elsewhere herein, comprising engineered T cells with WTI specific TCRs. In certain embodiments, the target is CD157 in AML.

[0193] In embodiments, the disease is a blood disease. In certain embodiments, the disease is hemophilia, in one aspect the target is Factor XI. In other embodiments, the disease is a hemoglobinopathy, such as sickle cell disease, sickle cell trait, hemoglobin C disease, hemoglobin C trait, hemoglobin S/C disease, hemoglobin D disease, hemoglobin E disease, a thalassemia, a condition associated with hemoglobin with increased oxygen affinity, a condition associated with hemoglobin with decreased oxygen affinity, unstable hemoglobin disease, methemoglobinemia. Hemostasis and Factor X and XII deficiencies can also be treated. In embodiments, the target is BCL11A gene (e.g., a human BCL11a gene), a BCL11a enhancer (e.g., a human BCL11a enhancer), or a HFPH region (e.g., a human HPFH region), beta globulin, fetal hemoglobin, γ -globin genes (e.g., HBG1, HBG2, or HBG1 and HBG2), the erythroid specific enhancer of the BCL11A gene (BCL11Ae), or a combination thereof.

[0194] In embodiments, the target locus can be one or more of RAC, TRBC1, TRBC2, CD3E, CD3G, CD3D, B2M, CIITA, CD247, HLA-A, HLA-B, HLA-C, DCK, CD52, FKBP1A, NLRC5, RFXANK, RFX5, RFXAP, NR3C1, CD274, HAVCR2, LAG3, PDCD1, PD-L2, HCF2, PAI, TFPI, PLAT, PLAUI, PLG, RPOZ, F7, F8, F9, F2, F5, F7, F10, F11, F12, F13A1, F13B, STAT1, FOXP3, IL2RG, DCLRE1C, ICOS, MHC2TA, GALNS, HGSNAT, ARSB, RFXAP, CD20, CD81, TNFRSF13B, SEC23B, PKLR, IFNG, SPTB, SPTA, SLC4A1, EPO, EPB42, CSF2 CSF3, VFW, SERPINCA1, CTLA4, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD113), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD107), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGF beta, PTPN11, and combinations thereof. In embodiments, the target sequence within the genomic nucleic acid sequence at Chr1 1:5,250,094-5,250,237, - strand, hg38; Chr1 1:5,255,022-5,255,164, - strand, hg38; nondeletional HFPH region; Chr1 1:5,249,833 to Chr1 1:5,250,237, - strand, hg38; Chr1 1:5,254,738 to Chr1 1:5,255, 164, - strand, hg38; Chr1 1 : 5,249,833-5,249,927, - strand, hg3; Chr1 1 : 5,254,738-5,254,851, - strand, hg38; Chr1 1:5,250, 139-5,250,237, - strand, hg38.

[0195] In embodiments, the disease is associated with high cholesterol, and regulation of cholesterol is provided, in some embodiments, regulation is effected by modification in the target PCSK9. Other diseases in which PCSK9 can be implicated, and thus would be a target for the systems and methods described herein include Abetaipoproteinemia, Adenoma, Arteriosclerosis, Atherosclerosis, Cardiovascular Diseases, Cholelithiasis, Coronary Arteriosclerosis, Coronary heart disease, Non-Insulin-Dependent Diabetes Mellitus, Hypercholesterolemia, Familial Hypercholesterolemia, Hyperinsulinism, Hyperlipidemia, Familial Combined Hyperlipidemia, Hypobetalipoproteinemias, Chronic Kidney Failure, Liver diseases, Liver neoplasms, melanoma, Myocardial Infarction, Narcolepsy, Neoplasm Metastasis, Nephroblastoma, Obesity, Peritonitis, Pseudoxanthoma Elasticum, Cerebrovascular accident, Vascular Diseases, Xanthomatosis, Peripheral Vascular Diseases, Myocardial Ischemia, Dyslipidemias, Impaired glucose tolerance, Xanthoma, Polygenic hypercholesterolemia, Secondary malignant neoplasm of liver, Dementia, Overweight, Hepatitis C, Chronic, Carotid Atherosclerosis, Hyperlipoproteinemia Type Ha, Intracranial Atherosclerosis, Ischemic stroke, Acute Coronary Syndrome, Aortic calcification, Cardiovascular morbidity, Hyperlipoproteinemia Type lib, Peripheral Arterial Diseases, Familial Hyperaldosteronism Type II, Familial hypobetalipoproteinemia, Autosomal Recessive Hypercholesterolemia, Autosomal Dominant Hypercholesterolemia 3, Coronary

Artery Disease, Liver carcinoma, Ischemic Cerebrovascular Accident, and Arteriosclerotic cardiovascular disease NOS. In embodiments, the treatment can be targeted to the liver, the primary location of activity of PCSK9.

[0196] In embodiments, the disease or disorder is Hyper IGM syndrome or a disorder characterized by defective CD40 signaling. In certain embodiments, the insertion of CD40L exons are used to restore proper CD40 signaling and B cell class switch recombination. In particular embodiments, the target is CD40 ligand (CD40L)-edited at one or more of exons 2-5 of the CD40L gene, in cells, e.g., T cells or hematopoietic stem cells (HSCs).

[0197] In embodiments, the disease is merosin-deficient congenital muscular dystrophy (mdcmd) and other laminin, alpha 2 (lama2) gene related conditions or disorders. The therapy can be targeted to the muscle, for example, skeletal muscle, smooth muscle, and/or cardiac muscle. In certain embodiments, the target is Laminin, Alpha 2 (LAMA2) which may also be referred to as Laminin- 12 Subunit Alpha, Laminin-2 Subunit Alpha, Laminin-4 Subunit Alpha 3, Merosin Heavy Chain, Laminin M Chain, LAMM, Congenital Muscular Dystrophy and Merosin. LAMA2 has a cytogenetic location of 6q22.33 and the genomic coordinate are on Chromosome 6 on the forward strand at position 128,883, 141-129,516,563. In embodiments, the disease treated can be Merosin-Deficient Congenital Muscular Dystrophy (MDCMD), Amyotrophic Lateral Sclerosis, Bladder Neoplasm, Charcot-Marie-Tooth Disease, Colorectal Carcinoma, Contracture, Cyst, Duchenne Muscular Dystrophy, Fatigue, Hyperopia, Renovascular Hypertension, melanoma, Mental Retardation, Myopathy, Muscular Dystrophy, Myopia, Myositis, Neuromuscular Diseases, Peripheral Neuropathy, Refractive Errors, Schizophrenia, Severe mental retardation (I.Q. 20-34), Thyroid Neoplasm, Tobacco Use Disorder, Severe Combined Immunodeficiency, Synovial Cyst, Adenocarcinoma of lung (disorder), Tumor Progression, Strawberry nevus of skin, Muscle degeneration, Microdontia (disorder), Walker-Warburg congenital muscular dystrophy, Chronic Periodontitis, Leukoencephalopathies, Impaired cognition, Fukuyama Type Congenital Muscular Dystrophy, Scleroatonic muscular dystrophy, Eichsfeld type congenital muscular dystrophy, Neuropathy, Muscle eye brain disease, Limb-Muscular Dystrophies, Girdle, Congenital muscular dystrophy (disorder), Muscle fibrosis, cancer recurrence, Drug Resistant Epilepsy, Respiratory Failure, Myxoid cyst, Abnormal breathing, Muscular dystrophy congenital merosin negative, Colorectal Cancer, Congenital Muscular Dystrophy due to Partial LAMA2 Deficiency, and Autosomal Dominant Craniometaphyseal Dysplasia.

[0198] In certain embodiments, the target is an AAVS1 (PPPIR12C), an ALB gene, an Angptl3 gene, an ApoC3 gene, an ASGR2 gene, a CCR5 gene, a FIX (F9) gene, a G6PC gene, a Gys2 gene, an HGD gene, a Lp(a) gene, a Pcsk9 gene, a Serpinal gene, a TF gene, and a TTR gene). Assessment of efficiency of HDR/NHEJ mediated knock-in of cDNA into the first exon can utilize cDNA knock-in into “safe harbor” sites such as: single-stranded or double-stranded DNA having homologous arms to one of the following regions, for example: ApoC3 (chr11:116829908-116833071), Angptl3 (chr1:62,597,487-62,606,305), Serpinal (chr14:94376747-94390692), Lp(a) (chr6:160531483-160664259), Pcsk9 (chr1:55,039,475-55,064,852), FIX (chrX:139,530,736-139,563,458), ALB (chr4:73,404,254-73,421,411), TTR (chr1 8:31,591,766-31,599,023), TF (chr3:133,661,997-133,779,005), G6PC (chr17:42,900,796-42,914,432), Gys2 (chr12:21,536,188-21,604,857), AAVS1 (PPP1R12C) (chr19:55,090,912-55,117,599), HGD (chr3:120,628,167-120,682,570), CCR5 (chr3:46,370,854-46,376,206), or ASGR2 (chr17:7,101,322-7,114,310).

[0199] In one aspect, the target is superoxide dismutase 1, soluble (SOD1), which can aid in treatment of a disease or disorder associated with the gene. In particular embodiments, the disease or disorder is associated with SOD1, and can be, for example, Adenocarcinoma, Albuminuria, Chronic Alcoholic Intoxication, Alzheimer's Disease, Amnesia, Amyloidosis, Amyotrophic Lateral Sclerosis, Anemia, Autoimmune hemolytic anemia, Sickle Cell Anemia, Anoxia, Anxiety Disorders, Aortic Diseases, Arteriosclerosis, Rheumatoid Arthritis, Asphyxia Neonatorum, Asthma, Atherosclerosis, Autistic Disorder, Autoimmune Diseases, Barrett Esophagus, Behcet Syndrome, Malignant neoplasm of urinary bladder, Brain Neoplasms, Malignant neoplasm of breast, Oral candidiasis, Malignant tumor of colon, Bronchogenic Carcinoma, Non-Small Cell Lung Carcinoma, Squamous cell carcinoma, Transitional Cell Carcinoma, Cardiovascular Diseases, Carotid Artery Thrombosis, Neoplastic Cell Transformation, Cerebral Infarction, Brain Ischemia, Transient Ischemic Attack, Charcot-Marie-Tooth Disease, Cholera, Colitis, Colorectal Carcinoma, Coronary Arteriosclerosis, Coronary heart disease, Infection by *Cryptococcus neoformans*, Deafness, Cessation of life, Deglutition Disorders, Presenile dementia, Depressive disorder, Contact Dermatitis, Diabetes, Diabetes Mellitus, Experimental Diabetes Mellitus, Insulin-Dependent Diabetes Mellitus, Non-Insulin-Dependent Diabetes Mellitus, Diabetic Angiopathies, Diabetic Nephropathy, Diabetic Retinopathy, Down Syndrome, Dwarfism, Edema, Japanese Encephalitis, Toxic Epidermal Necrolysis, Temporal Lobe Epilepsy, Exanthema, Muscular fasciculation, Alcoholic Fatty Liver, Fetal Growth Retardation, Fibromyalgia, Fibrosarcoma,

Fragile X Syndrome, Giardiasis, Glioblastoma, Glioma, Headache, Partial Hearing Loss, Cardiac Arrest, Heart failure, Atrial Septal Defects, Helminthiasis, Hemochromatosis, Hemolysis (disorder), Chronic Hepatitis, HIV Infections, Huntington Disease, Hypercholesterolemia, Hyperglycemia, Hyperplasia, Hypertensive disease, Hyperthyroidism, Hypopituitarism, Hypoproteinemia, Hypotension, natural Hypothermia, Hypothyroidism, Immunologic Deficiency Syndromes, Immune System Diseases, Inflammation, Inflammatory Bowel Diseases, Influenza, Intestinal Diseases, Ischemia, Kearns-Sayre syndrome, Keratoconus, Kidney Calculi, Kidney Diseases, Acute Kidney Failure, Chronic Kidney Failure, Polycystic Kidney Diseases, leukemia, Myeloid Leukemia, Acute Promyelocytic Leukemia, Liver Cirrhosis, Liver diseases, Liver neoplasms, Locked-In Syndrome, Chronic Obstructive Airway Disease, Lung Neoplasms, Systemic Lupus Erythematosus, Non-Hodgkin Lymphoma, Machado- Joseph Disease, Malaria, Malignant neoplasm of stomach, Animal Mammary Neoplasms, Marfan Syndrome, Meningomyelocele, Mental Retardation, Mitral Valve Stenosis, Acquired Dental Fluorosis, Movement Disorders, Multiple Sclerosis, Muscle Rigidity, Muscle Spasticity, Muscular Atrophy, Spinal Muscular Atrophy, Myopathy, Mycoses, Myocardial Infarction, Myocardial Reperfusion Injury, Necrosis, Nephrosis, Nephrotic Syndrome, Nerve Degeneration, nervous system disorder, Neuralgia, Neuroblastoma, Neuroma, Neuromuscular Diseases, Obesity, Occupational Diseases, Ocular Hypertension, Oligospermia, Degenerative polyarthritis, Osteoporosis, Ovarian Carcinoma, Pain, Pancreatitis, Papillon-Lefevre Disease, Paresis, Parkinson Disease, Phenylketonurias, Pituitary Diseases, Pre-Eclampsia, Prostatic Neoplasms, Protein Deficiency, Proteinuria, Psoriasis, Pulmonary Fibrosis, Renal Artery Obstruction, Reperfusion Injury, Retinal Degeneration, Retinal Diseases, Retinoblastoma, Schistosomiasis, Schistosomiasis mansoni, Schizophrenia, Scrapie, Seizures, Age-related cataract, Compression of spinal cord, Cerebrovascular accident, Subarachnoid Hemorrhage, Progressive supranuclear palsy, Tetanus, Trisomy, Turner Syndrome, Unipolar Depression, Urticaria, Vitiligo, Vocal Cord Paralysis, Intestinal Volvulus, Weight Gain, HMN (Hereditary Motor Neuropathy) Proximal Type I, Holoprosencephaly, Motor Neuron Disease, Neurofibrillary degeneration (morphologic abnormality), Burning sensation, Apathy, Mood swings, Synovial Cyst, Cataract, Migraine Disorders, Sciatic Neuropathy, Sensory neuropathy, Atrophic condition of skin, Muscle Weakness, Esophageal carcinoma, Lingual-Facial-Buccal Dyskinesia, Idiopathic pulmonary hypertension, Lateral Sclerosis, Migraine with Aura, Mixed Conductive-Sensorineural Hearing Loss, Iron deficiency anemia, Malnutrition, Prion

Diseases, Mitochondrial Myopathies, MELAS Syndrome, Chronic progressive external ophthalmoplegia, General Paralysis, Premature aging syndrome, Fibrillation, Psychiatric symptom, Memory impairment, Muscle degeneration, Neurologic Symptoms, Gastric hemorrhage, Pancreatic carcinoma, Pick Disease of the Brain, Liver Fibrosis, Malignant neoplasm of lung, Age related macular degeneration, Parkinsonian Disorders, Disease Progression, Hypocupremia, Cytochrome-c Oxidase Deficiency, Essential Tremor, Familial Motor Neuron Disease, Lower Motor Neuron Disease, Degenerative myelopathy, Diabetic Polyneuropathies, Liver and Intrahepatic Biliary Tract Carcinoma, Persian Gulf Syndrome, Senile Plaques, Atrophic, Frontotemporal dementia, Semantic Dementia, Common Migraine, Impaired cognition, Malignant neoplasm of liver, Malignant neoplasm of pancreas, Malignant neoplasm of prostate, Pure Autonomic Failure, Motor symptoms, Spastic, Dementia, Neurodegenerative Disorders, Chronic Hepatitis C, Guam Form Amyotrophic Lateral Sclerosis, Stiff limbs, Multisystem disorder, Loss of scalp hair, Prostate carcinoma, Hepatopulmonary Syndrome, Hashimoto Disease, Progressive Neoplastic Disease, Breast Carcinoma, Terminal illness, Carcinoma of lung, Tardive Dyskinesia, Secondary malignant neoplasm of lymph node, Colon Carcinoma, Stomach Carcinoma, Central neuroblastoma, Dissecting aneurysm of the thoracic aorta, Diabetic macular edema, Microalbuminuria, Middle Cerebral Artery Occlusion, Middle Cerebral Artery Infarction, Upper motor neuron signs, Frontotemporal Lobar Degeneration, Memory Loss, Classical phenylketonuria, CADASIL Syndrome, Neurologic Gait Disorders, Spinocerebellar Ataxia Type 2, Spinal Cord Ischemia, Lewy Body Disease, Muscular Atrophy, Spinobulbar, Chromosome 21 monosomy, Thrombocytosis, Spots on skin, Drug-Induced Liver Injury, Hereditary Leber Optic Atrophy, Cerebral Ischemia, ovarian neoplasm, Tauopathies, Macroangiopathy, Persistent pulmonary hypertension, Malignant neoplasm of ovary, Myxoid cyst, Drusen, Sarcoma, Weight decreased, Major Depressive Disorder, Mild cognitive disorder, Degenerative disorder, Partial Trisomy, Cardiovascular morbidity, hearing impairment, Cognitive changes, Ureteral Calculi, Mammary Neoplasms, Colorectal Cancer, Chronic Kidney Diseases, Minimal Change Nephrotic Syndrome, Non-Neoplastic Disorder, X-Linked Bulbo- Spinal Atrophy, Mammographic Density, Normal Tension Glaucoma Susceptibility To Finding), Vitiligo-Associated Multiple Autoimmune Disease Susceptibility 1 (Finding), Amyotrophic Lateral Sclerosis And/Or Frontotemporal Dementia 1, Amyotrophic Lateral Sclerosis 1, Sporadic Amyotrophic Lateral Sclerosis, monomelic Amyotrophy, Coronary Artery Disease, Transformed migraine, Regurgitation, Urothelial Carcinoma, Motor

disturbances, Liver carcinoma, Protein Misfolding Disorders, TDP-43 Proteinopathies, Promyelocytic leukemia, Weight Gain Adverse Event, Mitochondrial cytopathy, Idiopathic pulmonary arterial hypertension, Progressive cGVHD, Infection, GRN-related frontotemporal dementia, Mitochondrial pathology, and Hearing Loss.

[0200] In particular embodiments, the disease is associated with the gene ATXN1, ATXN2, or ATXN3, which may be targeted for treatment. In some embodiments, the CAG repeat region located in exon 8 of ATXN1, exon 1 of ATXN2, or exon 10 of the ATXN3 is targeted. In embodiments, the disease is spinocerebellar ataxia 3 (sca3), sca1, or sca2 and other related disorders, such as Congenital Abnormality, Alzheimer's Disease, Amyotrophic Lateral Sclerosis, Ataxia, Ataxia Telangiectasia, Cerebellar Ataxia, Cerebellar Diseases, Chorea, Cleft Palate, Cystic Fibrosis, Mental Depression, Depressive disorder, Dystonia, Esophageal Neoplasms, Exotropia, Cardiac Arrest, Huntington Disease, Machado- Joseph Disease, Movement Disorders, Muscular Dystrophy, Myotonic Dystrophy, Narcolepsy, Nerve Degeneration, Neuroblastoma, Parkinson Disease, Peripheral Neuropathy, Restless Legs Syndrome, Retinal Degeneration, Retinitis Pigmentosa, Schizophrenia, Shy-Drager Syndrome, Sleep disturbances, Hereditary Spastic Paraplegia, Thromboembolism, Stiff-Person Syndrome, Spinocerebellar Ataxia, Esophageal carcinoma, Polyneuropathy, Effects of heat, Muscle twitch, Extrapyrmidal sign, Ataxic, Neurologic Symptoms, Cerebral atrophy, Parkinsonian Disorders, Protein S Deficiency, Cerebellar degeneration, Familial Amyloid Neuropathy Portuguese Type, Spastic syndrome, Vertical Nystagmus, Nystagmus End-Position, Antithrombin III Deficiency, Atrophic, Complicated hereditary spastic paraplegia, Multiple System Atrophy, Pallidolusian degeneration, Dystonia Disorders, Pure Autonomic Failure, Thrombophilia, Protein C, Deficiency, Congenital Myotonic Dystrophy, Motor symptoms, Neuropathy, Neurodegenerative Disorders, Malignant neoplasm of esophagus, Visual disturbance, Activated Protein C Resistance, Terminal illness, Myokymia, Central neuroblastoma, Dyssomnias, Appendicular Ataxia, Narcolepsy-Cataplexy Syndrome, Machado- Joseph Disease Type I, Machado- Joseph Disease Type II, Machado- Joseph Disease Type III, Dentatorubral-Pallidolusian Atrophy, Gait Ataxia, Spinocerebellar Ataxia Type 1, Spinocerebellar Ataxia Type 2, Spinocerebellar Ataxia Type 6 (disorder), Spinocerebellar Ataxia Type 7, Muscular Spinobulbar Atrophy, Genomic Instability, Episodic ataxia type 2 (disorder), Bulbo-Spinal Atrophy X-Linked, Fragile X Tremor/ Ataxia Syndrome, Thrombophilia Due to Activated Protein C Resistance (Disorder), Amyotrophic

Lateral Sclerosis 1, Neuronal Intranuclear Inclusion Disease, Hereditary Antithrombin Iii Deficiency, and Late-Onset Parkinson Disease.

[0201] In embodiments, the disease is associated with expression of a tumor antigen-cancer or non-cancer related indication, for example acute lymphoid leukemia, diffuse large B cell lymphoma, follicular lymphoma, chronic lymphocytic leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma. In embodiments, the target can be TET2 intron, a TET2 intron-exon junction, a sequence within a genomic region of chr4.

[0202] In embodiments, neurodegenerative diseases can be treated. In particular embodiments, the target is Synuclein, Alpha (SNCA). In certain embodiments, the disorder treated is a pain related disorder, including congenital pain insensitivity, Compressive Neuropathies, Paroxysmal Extreme Pain Disorder, High grade atrioventricular block, Small Fiber Neuropathy, and Familial Episodic Pain Syndrome 2. In certain embodiments, the target is Sodium Channel, Voltage Gated, Type X Alpha Subunit (SCN10A).

[0203] In certain embodiments, hematopoietic stem cells and progenitor stem cells are edited, including knock-ins. In particular embodiments, the knock-in is for treatment of lysosomal storage diseases, glycogen storage diseases, mucopolysaccharoidoses, or any disease in which the secretion of a protein will ameliorate the disease. In one embodiment, the disease is sickle cell disease (SCD). In another embodiment, the disease is β -thalassemia.

[0204] In certain embodiments, the T cell or NK cell is used for cancer treatment and may include T cells comprising the recombinant receptor (e.g. CAR) and one or more phenotypic markers selected from CCR7+, 4-1BB+ (CD137+), TIM3+, CD27+, CD62L+, CD127+, CD45RA+, CD45RO-, t-bet^{hi}, IL-7R α +, CD95+, IL-2R β +, CXCR3+ or LFA-1+. In certain embodiments the editing of a T cell for cancer immunotherapy comprises altering one or more T-cell expressed gene, e.g., one or more of FAS, BID, CTLA4, PDCD1, CBLB, PTPN6, B2M, TRAC and TRBC gene. In some embodiments, editing includes alterations introduced into, or proximate to, the CBLB target sites to reduce CBLB gene expression in T cells for treatment of proliferative diseases and may include larger insertions or deletions at one or more CBLB target sites. T cell editing of TGFBR2 target sequence can be, for example, located in exon 3, 4, or 5 of the TGFBR2 gene and utilized for cancers and lymphoma treatment.

[0205] Cells for transplantation can be edited and may include allele-specific modification of one or more immunogenicity genes (e.g., an HLA gene) of a cell, e.g., HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3/4/5, HLA-DQ, and HLA-DP MHCs, and any

other MHC Class I or Class II genes or loci, which may include delivery of one or more matched recipient HLA alleles into the original position(s) where the one or more mismatched donor HLA alleles are located, and may include inserting one or more matched recipient HLA alleles into a “safe harbor” locus. In an embodiment, the method further includes introducing a chemotherapy resistance gene for in vivo selection in a gene.

[0206] Methods and systems can target Dystrophia Myotonica-Protein Kinase (DMPK) for editing, in particular embodiments, the target is the CTG trinucleotide repeat in the 3' untranslated region (UTR) of the DMPK gene. Disorders or diseases associated with DMPK include Atherosclerosis, Azoospermia, Hypertrophic Cardiomyopathy, Celiac Disease, Congenital chromosomal disease, Diabetes Mellitus, Focal glomerulosclerosis, Huntington Disease, Hypogonadism, Muscular Atrophy, Myopathy, Muscular Dystrophy, Myotonia, Myotonic Dystrophy, Neuromuscular Diseases, Optic Atrophy, Paresis, Schizophrenia, Cataract, Spinocerebellar Ataxia, Muscle Weakness, Adrenoleukodystrophy, Centronuclear myopathy, Interstitial fibrosis, myotonic muscular dystrophy, Abnormal mental state, X-linked Charcot- Marie-Tooth disease 1, Congenital Myotonic Dystrophy, Bilateral cataracts (disorder), Congenital Fiber Type Disproportion, Myotonic Disorders, Multisystem disorder, 3- Methylglutaconic aciduria type 3, cardiac event, Cardiogenic Syncope, Congenital Structural Myopathy, Mental handicap, Adrenomyeloneuropathy, Dystrophia myotonica 2, and Intellectual Disability.

[0207] In embodiments, the disease is an inborn error of metabolism. The disease may be selected from Disorders of Carbohydrate Metabolism (glycogen storage disease, G6PD deficiency), Disorders of Amino Acid Metabolism (phenylketonuria, maple syrup urine disease, glutaric acidemia type 1), Urea Cycle Disorder or Urea Cycle Defects (carbamoyl phosphate synthase I deficiency), Disorders of Organic Acid Metabolism (alkaptonuria, 2-hydroxyglutaric acidurias), Disorders of Fatty Acid Oxidation/Mitochondrial Metabolism (Medium-chain acyl-coenzyme A dehydrogenase deficiency), Disorders of Porphyrin metabolism (acute intermittent porphyria), Disorders of Purine/Pyrimidine Metabolism (Lesch-Nyhan syndrome), Disorders of Steroid Metabolism (lipoid congenital adrenal hyperplasia, congenital adrenal hyperplasia), Disorders of Mitochondrial Function (Kearns-Sayre syndrome), Disorders of Peroxisomal function (Zellweger syndrome), or Lysosomal Storage Disorders (Gaucher's disease, Niemann-Pick disease).

[0208] In embodiments, the target can comprise Recombination Activating Gene 1 (RAG1), BCL11 A, PCSK9, laminin, alpha 2 (lama2), ATXN3, alanine-glyoxylate

aminotransferase (AGXT), collagen type vii alpha 1 chain (COL7A1), spinocerebellar ataxia type 1 protein (ATXN1), Angiopoietin-like 3 (ANGPTL3), Frataxin (FXN), Superoxidase Dismutase 1, soluble (SOD1), Synuclein, Alpha (SNCA), Sodium Channel, Voltage Gated, Type X Alpha Subunit (SCN10A), Spinocerebellar Ataxia Type 2 Protein (ATXN2), Dystrophin Myotonic-Protein Kinase (DMPK), beta globin locus on chromosome 11, acyl-coenzyme A dehydrogenase for medium chain fatty acids (ACADM), long-chain 3-hydroxy-coenzyme A dehydrogenase for long chain fatty acids (HADHA), acyl-coenzyme A dehydrogenase for very long-chain fatty acids (ACADVL), Apolipoprotein C3 (APOCIII), Transthyretin (TTR), Angiopoietin-like 4 (ANGPTL4), Sodium Voltage-Gated Channel Alpha Subunit 9 (SCN9A), Interleukin-7 receptor (IL7R), glucose-6-phosphatase, catalytic (G6PC), haemochromatosis (HFE), SERPINA1, C9ORF72, β -globin, dystrophin, γ -globin.

[0209] In certain embodiments, the disease or disorder is associated with Apolipoprotein C3 (APOCIII), which can be targeted for editing. In embodiments, the disease or disorder may be Dyslipidemias, Hyperalphalipoproteinemia Type 2, Lupus Nephritis, Wilms Tumor 5, Morbid obesity and spermatogenic, Glaucoma, Diabetic Retinopathy, Arthrogryposis renal dysfunction cholestasis syndrome, Cognition Disorders, Altered response to myocardial infarction, Glucose Intolerance, Positive regulation of triglyceride biosynthetic process, Renal Insufficiency, Chronic, Hyperlipidemias, Chronic Kidney Failure, Apolipoprotein C-III Deficiency, Coronary Disease, Neonatal Diabetes Mellitus, Neonatal, with Congenital Hypothyroidism, Hypercholesterolemia Autosomal Dominant 3, Hyperlipoproteinemia Type III, Hyperthyroidism, Coronary Artery Disease, Renal Artery Obstruction, Metabolic Syndrome X, Hyperlipidemia, Familial Combined, Insulin Resistance, Transient infantile hypertriglyceridemia, Diabetic Nephropathies, Diabetes Mellitus (Type 1), Nephrotic Syndrome Type 5 with or without ocular abnormalities, and Hemorrhagic Fever with renal syndrome.

[0210] In certain embodiments, the target is Angiopoietin-like 4 (ANGPTL4). Diseases or disorders associated with ANGPTL4 that can be treated include ANGPTL4 is associated with dyslipidemias, low plasma triglyceride levels, regulator of angiogenesis and modulate tumorigenesis, and severe diabetic retinopathy. both proliferative diabetic retinopathy and non-proliferative diabetic retinopathy.

[0211] In embodiments, editing can be used for the treatment of fatty acid disorders. In certain embodiments, the target is one or more of ACADM, HADHA, ACADVL. In embodiments, the targeted edit is the activity of a gene in a cell selected from the acyl-

coenzyme A dehydrogenase for medium chain fatty acids (ACADM) gene, the long-chain 3-hydroxyl-coenzyme A dehydrogenase for long chain fatty acids (HADHA) gene, and the acyl-coenzyme A dehydrogenase for very long-chain fatty acids (ACADVL) gene. In one aspect, the disease is medium chain acyl-coenzyme A dehydrogenase deficiency (MCADD), long-chain 3-hydroxyl-coenzyme A dehydrogenase deficiency (LCHADD), and/or very long-chain acyl-coenzyme A dehydrogenase deficiency (VLCADD).

Adoptive Cell Therapies

[0212] The degraders herein may modulate a RNA-guided nuclease that modifies cells for adoptive therapies. Aspects of the invention accordingly involve the adoptive transfer of immune system cells, such as T cells, specific for selected antigens, such as tumor associated antigens (see Maus et al., 2014, Adoptive Immunotherapy for Cancer or Viruses, Annual Review of Immunology, Vol. 32: 189-225; Rosenberg and Restifo, 2015, Adoptive cell transfer as personalized immunotherapy for human cancer, Science Vol. 348 no. 6230 pp. 62-68; and, Restifo et al., 2015, Adoptive immunotherapy for cancer: harnessing the T cell response. Nat. Rev. Immunol. 12(4): 269-281; and Jenson and Riddell, 2014, Design and implementation of adoptive therapy with chimeric antigen receptor-modified T cells. Immunol Rev. 257(1): 127-144). Various strategies may for example be employed to genetically modify T cells by altering the specificity of the T cell receptor (TCR) for example by introducing new TCR α and β chains with selected peptide specificity (see U.S. Patent No. 8,697,854; PCT Patent Publications: WO2003020763, WO2004033685, WO2004044004, WO2005114215, WO2006000830, WO2008038002, WO2008039818, WO2004074322, WO2005113595, WO2006125962, WO2013166321, WO2013039889, WO2014018863, WO2014083173; U.S. Patent No. 8,088,379).

[0213] As an alternative to, or addition to, TCR modifications, chimeric antigen receptors (CARs) may be used in order to generate immunoresponsive cells, such as T cells, specific for selected targets, such as malignant cells, with a wide variety of receptor chimera constructs having been described (see U.S. Patent Nos. 5,843,728; 5,851,828; 5,912,170; 6,004,811; 6,284,240; 6,392,013; 6,410,014; 6,753,162; 8,211,422; and, PCT Publication WO9215322). Alternative CAR constructs may be characterized as belonging to successive generations. First-generation CARs typically consist of a single-chain variable fragment of an antibody specific for an antigen, for example comprising a VL linked to a VH of a specific antibody, linked by a flexible linker, for example by a CD8 α hinge domain and a CD8 α transmembrane domain, to the transmembrane and intracellular signaling domains of either

CD3 ζ or FcR γ (scFv-CD3 ζ or scFv-FcR γ ; see U.S. Patent No. 7,741,465; U.S. Patent No. 5,912,172; U.S. Patent No. 5,906,936). Second-generation CARs incorporate the intracellular domains of one or more costimulatory molecules, such as CD28, OX40 (CD134), or 4-1BB (CD137) within the endodomain (for example scFv-CD28/OX40/4-1BB-CD3 ζ ; see U.S. Patent Nos. 8,911,993; 8,916,381; 8,975,071; 9,101,584; 9,102,760; 9,102,761). Third-generation CARs include a combination of costimulatory endodomains, such a CD3 ζ -chain, CD97, GDI la-CD18, CD2, ICOS, CD27, CD154, CDS, OX40, 4-1BB, or CD28 signaling domains (for example scFv-CD28-4-1BB-CD3 ζ or scFv-CD28-OX40-CD3 ζ ; see U.S. Patent No. 8,906,682; U.S. Patent No. 8,399,645; U.S. Pat. No. 5,686,281; PCT Publication No. WO2014134165; PCT Publication No. WO2012079000). Alternatively, costimulation may be orchestrated by expressing CARs in antigen-specific T cells, chosen so as to be activated and expanded following engagement of their native $\alpha\beta$ TCR, for example by antigen on professional antigen-presenting cells, with attendant costimulation. In addition, additional engineered receptors may be provided on the immunoresponsive cells, for example to improve targeting of a T-cell attack and/or minimize side effects.

[0214] Alternative techniques may be used to transform target immunoresponsive cells, such as protoplast fusion, lipofection, transfection or electroporation. A wide variety of vectors may be used, such as retroviral vectors, lentiviral vectors, adenoviral vectors, adeno-associated viral vectors, plasmids or transposons, such as a Sleeping Beauty transposon (see U.S. Patent Nos. 6,489,458; 7,148,203; 7,160,682; 7,985,739; 8,227,432), may be used to introduce CARs, for example using 2nd generation antigen-specific CARs signaling through CD3 ζ and either CD28 or CD137. Viral vectors may for example include vectors based on HIV, SV40, EBV, HSV or BPV.

[0215] Cells that are targeted for transformation, and can be treated by the degraders herein, may for example include T cells, Natural Killer (NK) cells, cytotoxic T lymphocytes (CTL), regulatory T cells, human embryonic stem cells, tumor-infiltrating lymphocytes (TIL) or a pluripotent stem cell from which lymphoid cells may be differentiated. T cells expressing a desired CAR may for example be selected through co-culture with γ -irradiated activating and propagating cells (AaPC), which co-express the cancer antigen and co-stimulatory molecules. The engineered CAR T-cells may be expanded, for example by co-culture on AaPC in presence of soluble factors, such as IL-2 and IL-21. This expansion may for example be carried out so as to provide memory CAR⁺ T cells (which may for example be assayed by non-enzymatic digital array and/or multi-panel flow cytometry). In this way,

CAR T cells may be provided that have specific cytotoxic activity against antigen-bearing tumors (optionally in conjunction with production of desired chemokines such as interferon- γ). CAR T cells of this kind may for example be used in animal models, for example to treat tumor xenografts.

[0216] Approaches such as the foregoing may be adapted to provide methods of treating and/or increasing survival of a subject having a disease, such as a neoplasia, for example by administering an effective amount of an immunoresponsive cell comprising an antigen recognizing receptor that binds a selected antigen, wherein the binding activates the immunoreponsive cell, thereby treating or preventing the disease (such as a neoplasia, a pathogen infection, an autoimmune disorder, or an allogeneic transplant reaction). Dosing in CAR T cell therapies may for example involve administration of from 10^6 to 10^9 cells/kg, with or without a course of lymphodepletion, for example with cyclophosphamide.

[0217] In one embodiment, the treatment can be administered into patients undergoing an immunosuppressive treatment. The cells or population of cells, may be made resistant to at least one immunosuppressive agent due to the inactivation of a gene encoding a receptor for such immunosuppressive agent. Not being bound by a theory, the immunosuppressive treatment should help the selection and expansion of the immunoresponsive or T cells according to the invention within the patient.

[0218] The administration of the cells or population of cells according to the present invention may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The cells or population of cells may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous or intralymphatic injection, or intraperitoneally. In one embodiment, the cell compositions of the present invention are preferably administered by intravenous injection.

[0219] The administration of the cells or population of cells can consist of the administration of 10^4 - 10^9 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. Dosing in CAR T cell therapies may for example involve administration of from 10^6 to 10^9 cells/kg, with or without a course of lymphodepletion, for example with cyclophosphamide. The cells or population of cells can be administered in one or more doses. In another embodiment, the effective amount of cells are administered as a single dose. In another embodiment, the effective amount of cells are administered as more than one dose over a period time. Timing

of administration is within the judgment of managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained from any source, such as a blood bank or a donor. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions are within the skill of one in the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administered will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired.

[0220] In another embodiment, the effective number of cells or composition comprising those cells are administered parenterally. The administration can be an intravenous administration. The administration can be directly done by injection within a tumor.

[0221] To guard against possible adverse reactions, engineered immunoresponsive cells may be equipped with a transgenic safety switch, in the form of a transgene that renders the cells vulnerable to exposure to a specific signal. For example, the herpes simplex viral thymidine kinase (TK) gene may be used in this way, for example by introduction into allogeneic T lymphocytes used as donor lymphocyte infusions following stem cell transplantation (Greco, et al., Improving the safety of cell therapy with the TK-suicide gene. *Front. Pharmacol.* 2015; 6: 95). In such cells, administration of a nucleoside prodrug such as ganciclovir or acyclovir causes cell death. Alternative safety switch constructs include inducible caspase 9, for example triggered by administration of a small-molecule dimerizer that brings together two nonfunctional icasp9 molecules to form the active enzyme. A wide variety of alternative approaches to implementing cellular proliferation controls have been described (see U.S. Patent Publication No. 20130071414; PCT Patent Publication WO2011146862; PCT Patent Publication WO2014011987; PCT Patent Publication WO2013040371; Zhou et al. *BLOOD*, 2014, 123/25:3895 – 3905; Di Stasi et al., *The New England Journal of Medicine* 2011; 365:1673-1683; Sadelain M, *The New England Journal of Medicine* 2011; 365:1735-173; Ramos et al., *Stem Cells* 28(6):1107-15 (2010)).

[0222] In a further refinement of adoptive therapies, genome editing with a system as described herein may be used to tailor immunoresponsive cells to alternative implementations, for example providing edited CAR T cells (see Poirot et al., 2015, Multiplex genome edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies, *Cancer Res* 75 (18): 3853). For example, immunoresponsive cells may be edited to delete expression of some or all of the class of HLA type II and/or type I molecules,

or to knockout selected genes that may inhibit the desired immune response, such as the PD1 gene.

[0223] Cells may be edited using any system and method of use thereof as described herein. systems may be delivered to an immune cell by any method described herein. In preferred embodiments, cells are edited ex vivo and transferred to a subject in need thereof. Immunoresponsive cells, CAR T cells or any cells used for adoptive cell transfer may be edited. Editing may be performed to eliminate potential alloreactive T-cell receptors (TCR), disrupt the target of a chemotherapeutic agent, block an immune checkpoint, activate a T cell, and/or increase the differentiation and/or proliferation of functionally exhausted or dysfunctional CD8⁺ T-cells (see PCT Patent Publications: WO2013176915, WO2014059173, WO2014172606, WO2014184744, and WO2014191128). Editing may result in inactivation of a gene.

[0224] By inactivating a gene it is intended that the gene of interest is not expressed in a functional protein form. In a particular embodiment, the system specifically catalyzes cleavage in one targeted gene thereby inactivating said targeted gene. The nucleic acid strand breaks caused are commonly repaired through the distinct mechanisms of homologous recombination or non-homologous end joining (NHEJ). However, NHEJ is an imperfect repair process that often results in changes to the DNA sequence at the site of the cleavage. Repair via non-homologous end joining (NHEJ) often results in small insertions or deletions (Indel) and can be used for the creation of specific gene knockouts. Cells in which a cleavage induced mutagenesis event has occurred can be identified and/or selected by well-known methods in the art.

[0225] T cell receptors (TCR) are cell surface receptors that participate in the activation of T cells in response to the presentation of antigen. The TCR is generally made from two chains, α and β , which assemble to form a heterodimer and associates with the CD3-transducing subunits to form the T cell receptor complex present on the cell surface. Each α and β chain of the TCR consists of an immunoglobulin-like N-terminal variable (V) and constant (C) region, a hydrophobic transmembrane domain, and a short cytoplasmic region. As for immunoglobulin molecules, the variable region of the α and β chains are generated by V(D)J recombination, creating a large diversity of antigen specificities within the population of T cells. However, in contrast to immunoglobulins that recognize intact antigen, T cells are activated by processed peptide fragments in association with an MHC molecule, introducing an extra dimension to antigen recognition by T cells, known as MHC restriction. Recognition

of MHC disparities between the donor and recipient through the T cell receptor leads to T cell proliferation and the potential development of graft versus host disease (GVHD). The inactivation of TCR α or TCR β can result in the elimination of the TCR from the surface of T cells preventing recognition of alloantigen and thus GVHD. However, TCR disruption generally results in the elimination of the CD3 signaling component and alters the means of further T cell expansion.

[0226] Allogeneic cells are rapidly rejected by the host immune system. It has been demonstrated that, allogeneic leukocytes present in non-irradiated blood products will persist for no more than 5 to 6 days (Boni, Muranski et al. 2008 Blood 1;112(12):4746-54). Thus, to prevent rejection of allogeneic cells, the host's immune system usually has to be suppressed to some extent. However, in the case of adoptive cell transfer the use of immunosuppressive drugs also have a detrimental effect on the introduced therapeutic T cells. Therefore, to effectively use an adoptive immunotherapy approach in these conditions, the introduced cells would need to be resistant to the immunosuppressive treatment. Thus, in a particular embodiment, the present invention further comprises a step of modifying T cells to make them resistant to an immunosuppressive agent, preferably by inactivating at least one gene encoding a target for an immunosuppressive agent. An immunosuppressive agent is an agent that suppresses immune function by one of several mechanisms of action. An immunosuppressive agent can be, but is not limited to a calcineurin inhibitor, a target of rapamycin, an interleukin-2 receptor α -chain blocker, an inhibitor of inosine monophosphate dehydrogenase, an inhibitor of dihydrofolic acid reductase, a corticosteroid or an immunosuppressive antimetabolite. The present invention allows conferring immunosuppressive resistance to T cells for immunotherapy by inactivating the target of the immunosuppressive agent in T cells. As non-limiting examples, targets for an immunosuppressive agent can be a receptor for an immunosuppressive agent such as: CD52, glucocorticoid receptor (GR), a FKBP family gene member and a cyclophilin family gene member.

[0227] Immune checkpoints are inhibitory pathways that slow down or stop immune reactions and prevent excessive tissue damage from uncontrolled activity of immune cells. In certain embodiments, the immune checkpoint targeted is the programmed death-1 (PD-1 or CD279) gene (PDCD1). In other embodiments, the immune checkpoint targeted is cytotoxic T-lymphocyte-associated antigen (CTLA-4). In additional embodiments, the immune checkpoint targeted is another member of the CD28 and CTLA4 Ig superfamily such as

BTLA, LAG3, ICOS, PDL1 or KIR. In further additional embodiments, the immune checkpoint targeted is a member of the TNFR superfamily such as CD40, OX40, CD137, GITR, CD27 or TIM-3.

[0228] Additional immune checkpoints include Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) (Watson HA, et al., SHP-1: the next checkpoint target for cancer immunotherapy? *Biochem Soc Trans.* 2016 Apr 15;44(2):356-62). SHP-1 is a widely expressed inhibitory protein tyrosine phosphatase (PTP). In T-cells, it is a negative regulator of antigen-dependent activation and proliferation. It is a cytosolic protein, and therefore not amenable to antibody-mediated therapies, but its role in activation and proliferation makes it an attractive target for genetic manipulation in adoptive transfer strategies, such as chimeric antigen receptor (CAR) T cells. Immune checkpoints may also include T cell immunoreceptor with Ig and ITIM domains (TIGIT/Vstm3/WUCAM/VSIG9) and VISTA (Le Mercier I, et al., (2015) Beyond CTLA-4 and PD-1, the generation Z of negative checkpoint regulators. *Front. Immunol.* 6:418).

[0229] WO2014172606 relates to the use of MT1 and/or MT1 inhibitors to increase proliferation and/or activity of exhausted CD8⁺ T-cells and to decrease CD8⁺ T-cell exhaustion (e.g., decrease functionally exhausted or unresponsive CD8⁺ immune cells). In certain embodiments, metallothionenes are targeted by gene editing in adoptively transferred T cells.

[0230] In certain embodiments, targets of gene editing may be at least one targeted locus involved in the expression of an immune checkpoint protein. Such targets may include, but are not limited to CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, ICOS (CD278), PDL1, KIR, LAG3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244 (2B4), TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBR2, TGFBR1, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, VISTA, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, MT1, MT2, CD40, OX40, CD137, GITR, CD27, SHP-1 or TIM-3. In preferred embodiments, the gene locus involved in the expression of PD-1 or CTLA-4 genes is targeted. In other preferred embodiments, combinations of genes are targeted, such as but not limited to PD-1 and TIGIT.

[0231] In other embodiments, at least two genes are edited. Pairs of genes may include, but are not limited to PD1 and TCR α , PD1 and TCR β , CTLA-4 and TCR α , CTLA-4 and

TCR β , LAG3 and TCR α , LAG3 and TCR β , Tim3 and TCR α , Tim3 and TCR β , BTLA and TCR α , BTLA and TCR β , BY55 and TCR α , BY55 and TCR β , TIGIT and TCR α , TIGIT and TCR β , B7H5 and TCR α , B7H5 and TCR β , LAIR1 and TCR α , LAIR1 and TCR β , SIGLEC10 and TCR α , SIGLEC10 and TCR β , 2B4 and TCR α , 2B4 and TCR β .

[0232] Whether prior to or after genetic modification of the T cells, the T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and 7,572,631. T cells can be expanded in vitro or in vivo.

[0233] 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 MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989) (Sambrook, Fritsch and Maniatis); MOLECULAR CLONING: A LABORATORY MANUAL, 4th edition (2012) (Green and Sambrook); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1987) (F. M. Ausubel, et al. eds.); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR 2: A PRACTICAL APPROACH (1995) (M.J. MacPherson, B.D. Hames and G.R. Taylor eds.); ANTIBODIES, A LABORATORY MANUAL (1988) (Harlow and Lane, eds.); ANTIBODIES A LABORATORY MANUAL, 2nd edition (2013) (E.A. Greenfield ed.); and ANIMAL CELL CULTURE (1987) (R.I. Freshney, ed.).

[0234] The practice of the present invention employs, unless otherwise indicated, conventional techniques for generation of genetically modified mice. See Marten H. Hofker and Jan van Deursen, TRANSGENIC MOUSE METHODS AND PROTOCOLS, 2nd edition (2011).

[0235] In some embodiments, the invention described herein relates to a method for adoptive immunotherapy, in which T cells are edited ex vivo by CRISPR to modulate at least one gene and subsequently administered to a patient in need thereof. In some embodiments, the CRISPR editing comprising knocking-out or knocking-down the expression of at least one target gene in the edited T cells. In some embodiments, in addition to modulating the target gene, the T cells are also edited ex vivo by CRISPR to (1) knock-in an exogenous gene encoding a chimeric antigen receptor (CAR) or a T-cell receptor (TCR), (2) knock-out or knock-down expression of an immune checkpoint receptor, (3) knock-out or knock-down

expression of an endogenous TCR, (4) knock-out or knock-down expression of a human leukocyte antigen class I (HLA-I) proteins, and/or (5) knock-out or knock-down expression of an endogenous gene encoding an antigen targeted by an exogenous CAR or TCR.

[0236] In some embodiments, the T cells are contacted ex vivo with an adeno-associated virus (AAV) vector encoding a CRISPR effector protein, and a guide molecule comprising a guide sequence hybridizable to a target sequence, a tracr mate sequence, and a tracr sequence hybridizable to the tracr mate sequence. In some embodiments, the T cells are contacted ex vivo (e.g., by electroporation) with a ribonucleoprotein (RNP) comprising a CRISPR effector protein complexed with a guide molecule, wherein the guide molecule comprising a guide sequence hybridizable to a target sequence, a tracr mate sequence, and a tracr sequence hybridizable to the tracr mate sequence. See Rupp et al., *Scientific Reports* 7:737 (2017); Liu et al., *Cell Research* 27:154-157 (2017). In some embodiments, the T cells are contacted ex vivo (e.g., by electroporation) with an mRNA encoding a CRISPR effector protein, and a guide molecule comprising a guide sequence hybridizable to a target sequence, a tracr mate sequence, and a tracr sequence hybridizable to the tracr mate sequence. See Eyquem et al., *Nature* 543:113-117 (2017). In some embodiments, the T cells are not contacted ex vivo with a lentivirus or retrovirus vector.

[0237] In some embodiments, the degraders herein may modulate the editing of T cells ex vivo by CRISPR to knock-in an exogenous gene encoding a CAR, thereby allowing the edited T cells to recognize cancer cells based on the expression of specific proteins located on the cell surface. In some embodiments, T cells are edited ex vivo by CRISPR to knock-in an exogenous gene encoding a TCR, thereby allowing the edited T cells to recognize proteins derived from either the surface or inside of the cancer cells. In some embodiments, the method comprising providing an exogenous CAR-encoding or TCR-encoding sequence as a donor sequence, which can be integrated by homology-directed repair (HDR) into a genomic locus targeted by a CRISPR guide sequence. In some embodiments, targeting the exogenous CAR or TCR to an endogenous TCR α constant (TRAC) locus can reduce tonic CAR signaling and facilitate effective internalization and re-expression of the CAR following single or repeated exposure to antigen, thereby delaying effector T-cell differentiation and exhaustion. See Eyquem et al., *Nature* 543:113-117 (2017).

[0238] In some embodiments, the degraders herein may modulate the editing of T cells ex vivo by CRISPR. In some embodiments, T cells are edited ex vivo by CRISPR to knock-out or knock-down an endogenous gene involved in the programmed death-1 (PD-1)

signaling pathway, such as PD-1 and PD-L1. In some embodiments, T cells are edited ex vivo by CRISPR to mutate the *Pdcd1* locus or the *CD274* locus. In some embodiments, T cells are edited ex vivo by CRISPR using one or more guide sequences targeting the first exon of PD-1. See Rupp et al., *Scientific Reports* 7:737 (2017); Liu et al., *Cell Research* 27:154-157 (2017).

[0239] In some embodiments, the degraders herein may modulate the editing T cells ex vivo by CRISPR to eliminate potential alloreactive TCRs to allow allogeneic adoptive transfer. In some embodiments, T cells are edited ex vivo by CRISPR to knock-out or knock-down an endogenous gene encoding a TCR (e.g., an $\alpha\beta$ TCR) to avoid graft-versus-host-disease (GVHD). In some embodiments, T cells are edited ex vivo by CRISPR to mutate the TRAC locus. In some embodiments, T cells are edited ex vivo by CRISPR using one or more guide sequences targeting the first exon of TRAC. See Liu et al., *Cell Research* 27:154-157 (2017). In some embodiments, the method comprises use of CRISPR to knock-in an exogenous gene encoding a CAR or a TCR into the TRAC locus, while simultaneously knocking-out the endogenous TCR (e.g., with a donor sequence encoding a self-cleaving P2A peptide following the CAR cDNA). See Eyquem et al., *Nature* 543:113-117 (2017). In some embodiments, the exogenous gene comprises a promoter-less CAR-encoding or TCR-encoding sequence which is inserted operably downstream of an endogenous TCR promoter.

[0240] In some embodiments, the degraders herein may modulate the editing T cells ex vivo by CRISPR to knock-out or knock-down an endogenous gene encoding an HLA-I protein to minimize immunogenicity of the edited T cells. In some embodiments, T cells are edited ex vivo by CRISPR to mutate the beta-2 microglobulin (B2M) locus. In some embodiments, T cells are edited ex vivo by CRISPR using one or more guide sequences targeting the first exon of B2M. See Liu et al., *Cell Research* 27:154-157 (2017). In some embodiments, the method comprises use of CRISPR to knock-in an exogenous gene encoding a CAR or a TCR into the B2M locus, while simultaneously knocking-out the endogenous B2M (e.g., with a donor sequence encoding a self-cleaving P2A peptide following the CAR cDNA). See Eyquem et al., *Nature* 543:113-117 (2017). In some embodiments, the exogenous gene comprises a promoter-less CAR-encoding or TCR-encoding sequence which is inserted operably downstream of an endogenous B2M promoter.

[0241] In some embodiments, the degraders herein may modulate the editing T cells ex vivo by CRISPR to knock-out or knock-down an endogenous gene encoding an antigen targeted by an exogenous CAR or TCR. In some embodiments, the T cells are edited ex vivo

by CRISPR to knock-out or knock-down the expression of a tumor antigen selected from human telomerase reverse transcriptase (hTERT), survivin, mouse double minute 2 homolog (MDM2), cytochrome P450 1B 1 (CYP1B), HER2/neu, Wilms' tumor gene 1 (WT1), livin, alphafetoprotein (AFP), carcinoembryonic antigen (CEA), mucin 16 (MUC16), MUC1, prostate-specific membrane antigen (PSMA), p53 or cyclin (DI) (see WO2016/011210). In some embodiments, the T cells are edited ex vivo by CRISPR to knock-out or knock-down the expression of an antigen selected from B cell maturation antigen (BCMA), transmembrane activator and CAML Interactor (TACI), or B-cell activating factor receptor (BAFF-R), CD38, CD138, CS-1, CD33, CD26, CD30, CD53, CD92, CD100, CD148, CD150, CD200, CD261, CD262, or CD362 (see WO2017/011804).

Xenotransplantation

[0242] The degraders herein may modulate RNA-guided DNA nucleases that are adapted to be used to provide modified tissues for transplantation. For example, RNA-guided DNA nucleases may be used to knockout, knockdown or disrupt selected genes in an animal, such as a transgenic pig (such as the human heme oxygenase-1 transgenic pig line), for example by disrupting expression of genes that encode epitopes recognized by the human immune system, e.g., xenoantigen genes. Candidate porcine genes for disruption may for example include $\alpha(1,3)$ -galactosyltransferase and cytidine monophosphate-N-acetylneuraminic acid hydroxylase genes (see PCT Patent Publication WO 2014/066505). In addition, genes encoding endogenous retroviruses may be disrupted, for example the genes encoding all porcine endogenous retroviruses (see Yang et al., 2015, Genome-wide inactivation of porcine endogenous retroviruses (PERVs), Science 27 November 2015: Vol. 350 no. 6264 pp. 1101-1104). In addition, RNA-guided DNA nucleases may be used to target a site for integration of additional genes in xenotransplant donor animals, such as a human CD55 gene to improve protection against hyperacute rejection.

General Gene Therapy Considerations

[0243] Examples of disease-associated genes and polynucleotides AMD 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.

[0244] Mutations in these genes and pathways can result in production of improper proteins or proteins in improper amounts which affect function. Further examples of genes, diseases and proteins are hereby incorporated by reference from US Provisional application

61/736,527 filed December 12, 2012. Such genes, proteins and pathways may be the target polynucleotide of a CRISPR complex of the present invention. Examples of disease-associated genes and polynucleotides are listed in Tables 1 and 2. Examples of signaling biochemical pathway-associated genes and polynucleotides are listed in Table 3.

[0245] Table 1

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

Disorders	(Ncstn); PEN-2
Others	Nos1; Parp1; Nat1; Nat2
Prion - related disorders	Prp
ALS	SOD1; ALS2; STEX; FUS; TARDBP; VEGF (VEGF-a;
	VEGF-b; VEGF-c)
Drug addiction	Prkce (alcohol); Drd2; Drd4; ABAT (alcohol); GRIA2;
	Grm5; Grin1; Htr1b; Grin2a; Drd3; Pdyn; Gria1 (alcohol)
Autism	Mecp2; BZRAP1; MDGA2; Sema5A; Neurexin 1; Fragile X
	(FMR2 (AFF2); FXR1; FXR2; Mglur5)
Alzheimer's Disease	E1; CHIP; UCH; UBB; Tau; LRP; PICALM; Clusterin; PS1;
	SORL1; CR1; Vldlr; Uba1; Uba3; CHIP28 (Aqp1,
	Aquaporin 1); Uchl1; Uchl3; APP
Inflammation	IL-10; IL-1 (IL-1a; IL-1b); IL-13; IL-17 (IL-17a (CTLA8); IL-
	17b; IL-17c; IL-17d; IL-17f); IL-23; Cx3cr1; ptpn22; TNFa;
	NOD2/CARD15 for IBD; IL-6; IL-12 (IL-12a; IL-12b);
	CTLA4; Cx3cl1
Parkinson's Disease	x-Synuclein; DJ-1; LRRK2; Parkin; PINK1

[0112] Table 2:

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, FANCI, PHF9, FANCL, FANCM, KIAA1596); Hemophagocytic lymphohistiocytosis disorders (PRF1, HPLH2,
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	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).
Cell dysregulation and oncology diseases and disorders	B-cell non-Hodgkin lymphoma (BCL7A, BCL7); Leukemia (TAL1, TCL5, SCL, TAL2, FLT3, NBS1, NBS, ZNFN1A1, IK1, LYF1, HOXD4, HOX4B, BCR, CML, PHL, ALL, ARNT, KRAS2, RASK2, GMPS, AF10, ARHGEF12, LARG, KIAA0382, CALM, CLTH, CEBPA, CEBP, CHIC2, BTL, FLT3, KIT, PBT, LPP, NPM1, NUP214, D9S46E, CAN, CAIN, RUNX1, CBFA2, AML1, WHSC1L1, NSD3, FLT3, AF1Q, NPM1, NUMA1, ZNF145, PLZF, PML, MYL, STAT5B, AF10, CALM, CLTH, ARL11, ARLTS1, P2RX7, P2X7, BCR, CML, PHL, ALL, GRAF, NF1, VRNF, WSS, NFNS, PTPN11, PTP2C, SHP2, NS1, BCL2, CCND1, PRAD1, BCL1, TCRA, GATA1, GF1, ERYF1, NFE1, ABL1, NQO1, DIA4, NMOR1, NUP214, D9S46E, CAN, CAIN).
Inflammation and immune related diseases and disorders	AIDS (KIR3DL1, NKAT3, NKB1, AMB11, KIR3DS1, IFNG, CXCL12, SDF1); Autoimmune lymphoproliferative syndrome (TNFRSF6, APT1, FAS, CD95, ALPS1A); Combined immunodeficiency, (IL2RG, SCIDX1, SCIDX, IMD4); HIV-1 (CCL5, SCYA5, D17S136E, TCP228), HIV susceptibility or infection (IL10, CSIF, CMKBR2, CCR2, CMKBR5, CCKR5 (CCR5)); Immunodeficiencies (CD3E, CD3G, AICDA, AID, HIGM2, TNFRSF5, CD40, UNG, DGU, HIGM4, TNFSF5, CD40LG, HIGM1, IGM, FOXP3, IPEX, AIID, XPID, PIDX, TNFRSF14B, TACI); Inflammation (IL-10, IL-1 (IL-1a, IL-1b), IL-13, IL-17 (IL-17a (CTLA8), IL-17b, IL-17c, IL-17d, IL-17f), IL-23, Cx3cr1, ptpn22, TNFa, NOD2/CARD15 for IBD, IL-6, IL-12 (IL-12a, IL-12b), CTLA4, Cx3cl1); Severe combined immunodeficiencies (SCIDs)(JAK3, JAKL, DCLRE1C, ARTEMIS, SCIDA, RAG1, RAG2, ADA, PTPRC,

	CD45, LCA, IL7R, CD3D, T3D, IL2RG, SCIDX1, SCIDX, IMD4).
Metabolic, liver, kidney and protein diseases and disorders	Amyloid neuropathy (TTR, PALB); Amyloidosis (APOA1, APP, AAA, CVAP, AD1, GSN, FGA, LYZ, TTR, PALB); Cirrhosis (KRT18, KRT8, CIRH1A, NAIC, TEX292, KIAA1988); Cystic fibrosis (CFTR, ABCC7, CF, MRP7); Glycogen storage diseases (SLC2A2, GLUT2, G6PC, G6PT, G6PT1, GAA, LAMP2, LAMPB, AGL, GDE, GBE1, GYS2, PYGL, PFKM); Hepatic adenoma, 142330 (TCF1, HNF1A, MODY3), Hepatic failure, early onset, and neurologic disorder (SCOD1, SCO1), Hepatic lipase deficiency (LIPC), Hepatoblastoma, cancer and carcinomas (CTNNB1, PDGFRL, PDGRL, PRLTS, AXIN1, AXIN, CTNNB1, TP53, P53, LFS1, IGF2R, MPRI, MET, CASP8, MCH5; Medullary cystic kidney disease (UMOD, HNFJ, FJHN, MCKD2, ADMCKD2); Phenylketonuria (PAH, PKU1, QDPR, DHPR, PTS); Polycystic kidney and hepatic disease (FCYT, PKHD1, ARPKD, PKD1, PKD2, PKD4, PKDTS, PRKCSH, G19P1, PCLD, SEC63).
Muscular / Skeletal diseases and disorders	Becker muscular dystrophy (DMD, BMD, MYF6), Duchenne Muscular Dystrophy (DMD, BMD); Emery-Dreifuss muscular dystrophy (LMNA, LMN1, EMD2, FPLD, CMD1A, HGPS, LGMD1B, LMNA, LMN1, EMD2, FPLD, CMD1A); Facioscapulohumeral muscular dystrophy (FSHMD1A, FSHD1A); Muscular dystrophy (FKRP, MDC1C, LGMD2I, LAMA2, LAMM, LARGE, KIAA0609, MDC1D, FCMD, TTID, MYOT, CAPN3, CANP3, DYSF, LGMD2B, SGCG, LGMD2C, DMDA1, SCG3, SGCA, ADL, DAG2, LGMD2D, DMDA2, SGCB, LGMD2E, SGCD, SGD, LGMD2F, CMD1L, TCAP, LGMD2G, CMD1N, TRIM32, HT2A, LGMD2H, FKRP, MDC1C, LGMD2I, TTN, CMD1G, TMD, LGMD2J, POMT1, CAV3, LGMD1C, SEPNI, SELN, RSMD1, PLEC1, PLTN, EBS1); Osteopetrosis (LRP5, BMND1, LRP7, LR3, OPPG, VBCH2, CLCN7, CLC7, OPTA2, OSTM1, GL, TCIRG1, TIRC7, OC116, OPTB1); Muscular atrophy (VAPB, VAPC, ALS8, SMN1, SMA1, SMA2, SMA3, SMA4, BSCL2, SPG17, GARS, SMAD1,

	CMT2D, HEXB, IGHMBP2, SMUBP2, CATF1, SMARD1).
Neurological and neuronal diseases and disorders	ALS (SOD1, ALS2, STEX, FUS, TARDBP, VEGF (VEGF-a, VEGF-b, VEGF-c); Alzheimer disease (APP, AAA, CVAP, AD1, APOE, AD2, PSEN2, AD4, STM2, APBB2, FE65L1, NOS3, PLA2, URK, ACE, DCP1, ACE1, MPO, PACIP1, PAXIP1L, PTIP, A2M, BLMH, BMH, PSEN1, AD3); Autism (Mecp2, BZRAP1, MDGA2, Sema5A, Neurexin 1, GLO1, MECP2, RTT, PPMX, MRX16, MRX79, NLGN3, NLGN4, KIAA1260, AUTSX2); Fragile X Syndrome (FMR2, FXR1, FXR2, mGLUR5); Huntington's disease and disease like disorders (HD, IT15, PRNP, PRIP, JPH3, JP3, HDL2, TBP, SCA17); Parkinson disease (NR4A2, NURR1, NOT, TINUR, SNCAIP, TBP, SCA17, SNCA, NACP, PARK1, PARK4, DJ1, PARK7, LRRK2, PARK8, PINK1, PARK6, UCHL1, PARK5, SNCA, NACP, PARK1, PARK4, PRKN, PARK2, PDJ, DBH, NDUFV2); Rett syndrome (MECP2, RTT, PPMX, MRX16, MRX79, CDKL5, STK9, MECP2, RTT, PPMX, MRX16, MRX79, x-Synuclein, DJ-1); Schizophrenia (Neuregulin1 (Nrg1), Erb4 (receptor for Neuregulin), Complexin1 (Cplx1), Tph1 Tryptophan hydroxylase, Tph2, Tryptophan hydroxylase 2, Neurexin 1, GSK3, GSK3a, GSK3b, 5-HTT (Slc6a4), COMT, DRD (Drd1a), SLC6A3, DAOA, DTNBP1, Dao (Dao1)); Secretase Related Disorders (APH-1 (alpha and beta), Presenilin (Psen1), nicastrin, (Ncstn), PEN-2, Nos1, Parp1, Nat1, Nat2); Trinucleotide Repeat Disorders (HTT (Huntington's Dx), SBMA/SMAX1/AR (Kennedy's Dx), FXN/X25 (Friedrich's Ataxia), ATX3 (Machado- Joseph's Dx), ATXN1 and ATXN2 (spinocerebellar ataxias), DMPK (myotonic dystrophy), Atrophin-1 and Atn1 (DRPLA Dx), CBP (Creb-BP - global instability), VLDLR (Alzheimer's), Atxn7, Atxn10).
Occular diseases and disorders	Age-related macular degeneration (Abcr, Ccl2, Cc2, cp (ceruloplasmin), Timp3, cathepsinD, Vldlr, Ccr2); Cataract (CRYAA, CRYA1, CRYBB2, CRYB2, PITX3, BFSP2, CP49, CP47, CRYAA, CRYA1, PAX6, AN2,

	MGDA, CRYBA1, CRYB1, CRYGC, CRYG3, CCL, LIM2, MP19, CRYGD, CRYG4, BFSP2, CP49, CP47, HSF4, CTM, HSF4, CTM, MIP, AQP0, CRYAB, CRYA2, CTPP2, CRYBB1, CRYGD, CRYG4, CRYBB2, CRYB2, CRYGC, CRYG3, CCL, CRYAA, CRYA1, GJA8, CX50, CAE1, GJA3, CX46, CZP3, CAE3, CCM1, CAM, KRIT1); Corneal clouding and dystrophy (APOA1, TGFBI, CSD2, CDGG1, CSD, BIGH3, CDG2, TACSTD2, TROP2, M1S1, VSX1, RINX, PPCD, PPD, KTCN, COL8A2, FECD, PPCD2, PIP5K3, CFD); Cornea plana congenital (KERA, CNA2); Glaucoma (MYOC, TIGR, GLC1A, JOAG, GPOA, OPTN, GLC1E, FIP2, HYPL, NRP, CYP1B1, GLC3A, OPA1, NTG, NPG, CYP1B1, GLC3A); Leber congenital amaurosis (CRB1, RP12, CRX, CORD2, CRD, RPGRIP1, LCA6, CORD9, RPE65, RP20, AIPL1, LCA4, GUCY2D, GUC2D, LCA1, CORD6, RDH12, LCA3); Macular dystrophy (ELOVL4, ADMD, STGD2, STGD3, RDS, RP7, PRPH2, PRPH, AVMD, AOFMD, VMD2).
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[0246] Table 3:

CELLULAR FUNCTION	GENES
PI3K/AKT Signaling	PRKCE; ITGAM; ITGA5; IRAK1; PRKAA2; EIF2AK2;
	PTEN; EIF4E; PRKCZ; GRK6; MAPK1; TSC1; PLK1;
	AKT2; IKBKB; PIK3CA; CDK8; CDKN1B; NFKB2; BCL2;
	PIK3CB; PPP2R1A; MAPK8; BCL2L1; MAPK3; TSC2;
	ITGA1; KRAS; EIF4EBP1; RELA; PRKCD; NOS3;
	PRKAA1; MAPK9; CDK2; PPP2CA; PIM1; ITGB7;
	YWHAZ; ILK; TP53; RAF1; IKBKG; RELB; DYRK1A;
	CDKN1A; ITGB1; MAP2K2; JAK1; AKT1; JAK2; PIK3R1;
	CHUK; PDPK1; PPP2R5C; CTNNB1; MAP2K1; NFKB1;
	PAK3; ITGB3; CCND1; GSK3A; FRAP1; SFN; ITGA2;
	TTK; CSNK1A1; BRAF; GSK3B; AKT3; FOXO1; SGK;
	HSP90AA1; RPS6KB1
ERK/MAPK Signaling	PRKCE; ITGAM; ITGA5; HSPB1; IRAK1; PRKAA2;
	EIF2AK2; RAC1; RAP1A; TLN1; EIF4E; ELK1; GRK6;

	MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8; CREB1;
	PRKCI; PTK2; FOS; RPS6KA4; PIK3CB; PPP2R1A;
	PIK3C3; MAPK8; MAPK3; ITGA1; ETS1; KRAS; MYCN;
	EIF4EBP1; PPARG; PRKCD; PRKAA1; MAPK9; SRC;
	CDK2; PPP2CA; PIM1; PIK3C2A; ITGB7; YWHAZ;
	PPP1CC; KSR1; PXN; RAF1; FYN; DYRK1A; ITGB1;
	MAP2K2; PAK4; PIK3R1; STAT3; PPP2R5C; MAP2K1;
	PAK3; ITGB3; ESR1; ITGA2; MYC; TTK; CSNK1A1;
	CRKL; BRAF; ATF4; PRKCA; SRF; STAT1; SGK
Glucocorticoid Receptor	RAC1; TAF4B; EP300; SMAD2; TRAF6; PCAF; ELK1;
Signaling	MAPK1; SMAD3; AKT2; IKBKB; NCOR2; UBE2I;
	PIK3CA; CREB1; FOS; HSPA5; NFKB2; BCL2;
	MAP3K14; STAT5B; PIK3CB; PIK3C3; MAPK8; BCL2L1;
	MAPK3; TSC22D3; MAPK10; NRIP1; KRAS; MAPK13;
	RELA; STAT5A; MAPK9; NOS2A; PBX1; NR3C1;
	PIK3C2A; CDKN1C; TRAF2; SERPINE1; NCOA3;
	MAPK14; TNF; RAF1; IKBKG; MAP3K7; CREBBP;
	CDKN1A; MAP2K2; JAK1; IL8; NCOA2; AKT1; JAK2;
	PIK3R1; CHUK; STAT3; MAP2K1; NFKB1; TGFBR1;
	ESR1; SMAD4; CEBPB; JUN; AR; AKT3; CCL2; MMP1;
	STAT1; IL6; HSP90AA1
Axonal Guidance Signaling	PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; ADAM12;
	IGF1; RAC1; RAP1A; EIF4E; PRKCZ; NRP1; NTRK2;
	ARHGEF7; SMO; ROCK2; MAPK1; PGF; RAC2;
	PTPN11; GNAS; AKT2; PIK3CA; ERBB2; PRKCI; PTK2;
	CFL1; GNAQ; PIK3CB; CXCL12; PIK3C3; WNT11;
	PRKD1; GNB2L1; ABL1; MAPK3; ITGA1; KRAS; RHOA;
	PRKCD; PIK3C2A; ITGB7; GLI2; PXN; VASP; RAF1;
	FYN; ITGB1; MAP2K2; PAK4; ADAM17; AKT1; PIK3R1;
	GLI1; WNT5A; ADAM10; MAP2K1; PAK3; ITGB3;
	CDC42; VEGFA; ITGA2; EPHA8; CRKL; RND1; GSK3B;

	AKT3; PRKCA
Ephrin Receptor Signaling	PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; IRAK1;
	PRKAA2; EIF2AK2; RAC1; RAP1A; GRK6; ROCK2;
	MAPK1; PGF; RAC2; PTPN11; GNAS; PLK1; AKT2;
	DOK1; CDK8; CREB1; PTK2; CFL1; GNAQ; MAP3K14;
	CXCL12; MAPK8; GNB2L1; ABL1; MAPK3; ITGA1;
	KRAS; RHOA; PRKCD; PRKAA1; MAPK9; SRC; CDK2;
	PIM1; ITGB7; PXN; RAF1; FYN; DYRK1A; ITGB1;
	MAP2K2; PAK4; AKT1; JAK2; STAT3; ADAM10;
	MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2;
	EPHA8; TTK; CSNK1A1; CRKL; BRAF; PTPN13; ATF4;
	AKT3; SGK
Actin Cytoskeleton Signaling	ACTN4; PRKCE; ITGAM; ROCK1; ITGA5; IRAK1;
	PRKAA2; EIF2AK2; RAC1; INS; ARHGEF7; GRK6;
	ROCK2; MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8;
	PTK2; CFL1; PIK3CB; MYH9; DIAPH1; PIK3C3; MAPK8;
	F2R; MAPK3; SLC9A1; ITGA1; KRAS; RHOA; PRKCD;
	PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; ITGB7;
	PPP1CC; PXN; VIL2; RAF1; GSN; DYRK1A; ITGB1;
	MAP2K2; PAK4; PIP5K1A; PIK3R1; MAP2K1; PAK3;
	ITGB3; CDC42; APC; ITGA2; TTK; CSNK1A1; CRKL;
	BRAF; VAV3; SGK
Huntington's Disease Signaling	PRKCE; IGF1; EP300; RCOR1; PRKCZ; HDAC4; TGM2;
	MAPK1; CAPNS1; AKT2; EGFR; NCOR2; SP1; CAPN2;
	PIK3CA; HDAC5; CREB1; PRKCI; HSPA5; REST;
	GNAQ; PIK3CB; PIK3C3; MAPK8; IGF1R; PRKD1;
	GNB2L1; BCL2L1; CAPN1; MAPK3; CASP8; HDAC2;
	HDAC7A; PRKCD; HDAC11; MAPK9; HDAC9; PIK3C2A;
	HDAC3; TP53; CASP9; CREBBP; AKT1; PIK3R1;
	PDPK1; CASP1; APAF1; FRAP1; CASP2; JUN; BAX;
	ATF4; AKT3; PRKCA; CLTC; SGK; HDAC6; CASP3

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

	TNK2; MAP2K1; PAK3; ITGB3; CDC42; RND3; ITGA2;
	CRKL; BRAF; GSK3B; AKT3
Acute Phase Response	IRAK1; SOD2; MYD88; TRAF6; ELK1; MAPK1; PTPN11;
Signaling	AKT2; IKBKB; PIK3CA; FOS; NFKB2; MAP3K14;
	PIK3CB; MAPK8; RIPK1; MAPK3; IL6ST; KRAS;
	MAPK13; IL6R; RELA; SOCS1; MAPK9; FTL; NR3C1;
	TRAF2; SERPINE1; MAPK14; TNF; RAF1; PDK1;
	IKBKG; RELB; MAP3K7; MAP2K2; AKT1; JAK2; PIK3R1;
	CHUK; STAT3; MAP2K1; NFKB1; FRAP1; CEBPB; JUN;
	AKT3; IL1R1; IL6
PTEN Signaling	ITGAM; ITGA5; RAC1; PTEN; PRKCZ; BCL2L11;
	MAPK1; RAC2; AKT2; EGFR; IKBKB; CBL; PIK3CA;
	CDKN1B; PTK2; NFKB2; BCL2; PIK3CB; BCL2L1;
	MAPK3; ITGA1; KRAS; ITGB7; ILK; PDGFRB; INSR;
	RAF1; IKBKG; CASP9; CDKN1A; ITGB1; MAP2K2;
	AKT1; PIK3R1; CHUK; PDGFRA; PDPK1; MAP2K1;
	NFKB1; ITGB3; CDC42; CCND1; GSK3A; ITGA2;
	GSK3B; AKT3; FOXO1; CASP3; RPS6KB1
p53 Signaling	PTEN; EP300; BBC3; PCAF; FASN; BRCA1; GADD45A;
	BIRC5; AKT2; PIK3CA; CHEK1; TP53INP1; BCL2;
	PIK3CB; PIK3C3; MAPK8; THBS1; ATR; BCL2L1; E2F1;
	PMAIP1; CHEK2; TNFRSF10B; TP73; RB1; HDAC9;
	CDK2; PIK3C2A; MAPK14; TP53; LRDD; CDKN1A;
	HIPK2; AKT1; PIK3R1; RRM2B; APAF1; CTNNB1;
	SIRT1; CCND1; PRKDC; ATM; SFN; CDKN2A; JUN;
	SNAI2; GSK3B; BAX; AKT3
Aryl Hydrocarbon Receptor	HSPB1; EP300; FASN; TGM2; RXRA; MAPK1; NQO1;
Signaling	NCOR2; SP1; ARNT; CDKN1B; FOS; CHEK1;
	SMARCA4; NFKB2; MAPK8; ALDH1A1; ATR; E2F1;
	MAPK3; NRIP1; CHEK2; RELA; TP73; GSTP1; RB1;
	SRC; CDK2; AHR; NFE2L2; NCOA3; TP53; TNF;

	CDKN1A; NCOA2; APAF1; NFKB1; CCND1; ATM; ESR1;
	CDKN2A; MYC; JUN; ESR2; BAX; IL6; CYP1B1;
	HSP90AA1
Xenobiotic Metabolism	PRKCE; EP300; PRKCZ; RXRA; MAPK1; NQO1;
Signaling	NCOR2; PIK3CA; ARNT; PRKCI; NFKB2; CAMK2A;
	PIK3CB; PPP2R1A; PIK3C3; MAPK8; PRKD1;
	ALDH1A1; MAPK3; NRIP1; KRAS; MAPK13; PRKCD;
	GSTP1; MAPK9; NOS2A; ABCB1; AHR; PPP2CA; FTL;
	NFE2L2; PIK3C2A; PPARGC1A; MAPK14; TNF; RAF1;
	CREBBP; MAP2K2; PIK3R1; PPP2R5C; MAP2K1;
	NFKB1; KEAP1; PRKCA; EIF2AK3; IL6; CYP1B1;
	HSP90AA1
SAPK/JNK Signaling	PRKCE; IRAK1; PRKAA2; EIF2AK2; RAC1; ELK1;
	GRK6; MAPK1; GADD45A; RAC2; PLK1; AKT2; PIK3CA;
	FADD; CDK8; PIK3CB; PIK3C3; MAPK8; RIPK1;
	GNB2L1; IRS1; MAPK3; MAPK10; DAXX; KRAS;
	PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A;
	TRAF2; TP53; LCK; MAP3K7; DYRK1A; MAP2K2;
	PIK3R1; MAP2K1; PAK3; CDC42; JUN; TTK; CSNK1A1;
	CRKL; BRAF; SGK
PPAr/RXR Signaling	PRKAA2; EP300; INS; SMAD2; TRAF6; PPARA; FASN;
	RXRA; MAPK1; SMAD3; GNAS; IKBKB; NCOR2;
	ABCA1; GNAQ; NFKB2; MAP3K14; STAT5B; MAPK8;
	IRS1; MAPK3; KRAS; RELA; PRKAA1; PPARGC1A;
	NCOA3; MAPK14; INSR; RAF1; IKBKG; RELB; MAP3K7;
	CREBBP; MAP2K2; JAK2; CHUK; MAP2K1; NFKB1;
	TGFBR1; SMAD4; JUN; IL1R1; PRKCA; IL6; HSP90AA1;
	ADIPOQ
NF-KB Signaling	IRAK1; EIF2AK2; EP300; INS; MYD88; PRKCZ; TRAF6;
	TBK1; AKT2; EGFR; IKBKB; PIK3CA; BTRC; NFKB2;
	MAP3K14; PIK3CB; PIK3C3; MAPK8; RIPK1; HDAC2;
	KRAS; RELA; PIK3C2A; TRAF2; TLR4; PDGFRB; TNF;

	INSR; LCK; IKBKG; RELB; MAP3K7; CREBBP; AKT1;
	PIK3R1; CHUK; PDGFRA; NFKB1; TLR2; BCL10;
	GSK3B; AKT3; TNFAIP3; IL1R1
Neuregulin Signaling	ERBB4; PRKCE; ITGAM; ITGA5; PTEN; PRKCZ; ELK1;
	MAPK1; PTPN11; AKT2; EGFR; ERBB2; PRKCI;
	CDKN1B; STAT5B; PRKD1; MAPK3; ITGA1; KRAS;
	PRKCD; STAT5A; SRC; ITGB7; RAF1; ITGB1; MAP2K2;
	ADAM17; AKT1; PIK3R1; PDPK1; MAP2K1; ITGB3;
	EREG; FRAP1; PSEN1; ITGA2; MYC; NRG1; CRKL;
	AKT3; PRKCA; HSP90AA1; RPS6KB1
Wnt & Beta catenin	CD44; EP300; LRP6; DVL3; CSNK1E; GJA1; SMO;
Signaling	AKT2; PIN1; CDH1; BTRC; GNAQ; MARK2; PPP2R1A;
	WNT11; SRC; DKK1; PPP2CA; SOX6; SFRP2; ILK;
	LEF1; SOX9; TP53; MAP3K7; CREBBP; TCF7L2; AKT1;
	PPP2R5C; WNT5A; LRP5; CTNNB1; TGFBR1; CCND1;
	GSK3A; DVL1; APC; CDKN2A; MYC; CSNK1A1; GSK3B;
	AKT3; SOX2
Insulin Receptor	PTEN; INS; EIF4E; PTPN1; PRKCZ; MAPK1; TSC1;
Signaling	
	PTPN11; AKT2; CBL; PIK3CA; PRKCI; PIK3CB; PIK3C3;
	MAPK8; IRS1; MAPK3; TSC2; KRAS; EIF4EBP1;
	SLC2A4; PIK3C2A; PPP1CC; INSR; RAF1; FYN;
	MAP2K2; JAK1; AKT1; JAK2; PIK3R1; PDPK1; MAP2K1;
	GSK3A; FRAP1; CRKL; GSK3B; AKT3; FOXO1; SGK;
	RPS6KB1
IL-6 Signaling	HSPB1; TRAF6; MAPKAPK2; ELK1; MAPK1; PTPN11;
	IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK3;
	MAPK10; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1;
	MAPK9; ABCB1; TRAF2; MAPK14; TNF; RAF1; IKBKG;
	RELB; MAP3K7; MAP2K2; IL8; JAK2; CHUK; STAT3;
	MAP2K1; NFKB1; CEBPB; JUN; IL1R1; SRF; IL6
Hepatic Cholestasis	PRKCE; IRAK1; INS; MYD88; PRKCZ; TRAF6; PPARA;

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

	MAPK9; PIK3C2A; BTK; MAPK14; TNF; RAF1; FYN;
	MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; AKT3;
	VAV3; PRKCA
G-Protein Coupled	PRKCE; RAP1A; RGS16; MAPK1; GNAS; AKT2; IKBKB;
Receptor Signaling	PIK3CA; CREB1; GNAQ; NFKB2; CAMK2A; PIK3CB;
	PIK3C3; MAPK3; KRAS; RELA; SRC; PIK3C2A; RAF1;
	IKBKG; RELB; FYN; MAP2K2; AKT1; PIK3R1; CHUK;
	PDPK1; STAT3; MAP2K1; NFKB1; BRAF; ATF4; AKT3;
	PRKCA
Inositol Phosphate	PRKCE; IRAK1; PRKAA2; EIF2AK2; PTEN; GRK6;
Metabolism	MAPK1; PLK1; AKT2; PIK3CA; CDK8; PIK3CB; PIK3C3;
	MAPK8; MAPK3; PRKCD; PRKAA1; MAPK9; CDK2;
	PIM1; PIK3C2A; DYRK1A; MAP2K2; PIP5K1A; PIK3R1;
	MAP2K1; PAK3; ATM; TTK; CSNK1A1; BRAF; SGK
PDGF Signaling	EIF2AK2; ELK1; ABL2; MAPK1; PIK3CA; FOS; PIK3CB;
	PIK3C3; MAPK8; CAV1; ABL1; MAPK3; KRAS; SRC;
	PIK3C2A; PDGFRB; RAF1; MAP2K2; JAK1; JAK2;
	PIK3R1; PDGFRA; STAT3; SPHK1; MAP2K1; MYC;
	JUN; CRKL; PRKCA; SRF; STAT1; SPHK2
VEGF Signaling	ACTN4; ROCK1; KDR; FLT1; ROCK2; MAPK1; PGF;
	AKT2; PIK3CA; ARNT; PTK2; BCL2; PIK3CB; PIK3C3;
	BCL2L1; MAPK3; KRAS; HIF1A; NOS3; PIK3C2A; PXN;
	RAF1; MAP2K2; ELAVL1; AKT1; PIK3R1; MAP2K1; SFN;
	VEGFA; AKT3; FOXO1; PRKCA
Natural Killer Cell Signaling	PRKCE; RAC1; PRKCZ; MAPK1; RAC2; PTPN11;
	KIR2DL3; AKT2; PIK3CA; SYK; PRKCI; PIK3CB;
	PIK3C3; PRKD1; MAPK3; KRAS; PRKCD; PTPN6;
	PIK3C2A; LCK; RAF1; FYN; MAP2K2; PAK4; AKT1;
	PIK3R1; MAP2K1; PAK3; AKT3; VAV3; PRKCA
Cell Cycle: G1/S	HDAC4; SMAD3; SUV39H1; HDAC5; CDKN1B; BTRC;
Checkpoint Regulation	ATR; ABL1; E2F1; HDAC2; HDAC7A; RB1; HDAC11;

	HDAC9; CDK2; E2F2; HDAC3; TP53; CDKN1A; CCND1;
	E2F4; ATM; RBL2; SMAD4; CDKN2A; MYC; NRG1;
	GSK3B; RBL1; HDAC6
T Cell Receptor Signaling	RAC1; ELK1; MAPK1; IKBKB; CBL; PIK3CA; FOS;
	NFKB2; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS;
	RELA; PIK3C2A; BTK; LCK; RAF1; IKBKG; RELB; FYN;
	MAP2K2; PIK3R1; CHUK; MAP2K1; NFKB1; ITK; BCL10;
	JUN; VAV3
Death Receptor Signaling	CRADD; HSPB1; BID; BIRC4; TBK1; IKBKB; FADD;
	FAS; NFKB2; BCL2; MAP3K14; MAPK8; RIPK1; CASP8;
	DAXX; TNFRSF10B; RELA; TRAF2; TNF; IKBKG; RELB;
	CASP9; CHUK; APAF1; NFKB1; CASP2; BIRC2; CASP3;
	BIRC3
FGF Signaling	RAC1; FGFR1; MET; MAPKAPK2; MAPK1; PTPN11;
	AKT2; PIK3CA; CREB1; PIK3CB; PIK3C3; MAPK8;
	MAPK3; MAPK13; PTPN6; PIK3C2A; MAPK14; RAF1;
	AKT1; PIK3R1; STAT3; MAP2K1; FGFR4; CRKL; ATF4;
	AKT3; PRKCA; HGF
GM-CSF Signaling	LYN; ELK1; MAPK1; PTPN11; AKT2; PIK3CA; CAMK2A;
	STAT5B; PIK3CB; PIK3C3; GNB2L1; BCL2L1; MAPK3;
	ETS1; KRAS; RUNX1; PIM1; PIK3C2A; RAF1; MAP2K2;
	AKT1; JAK2; PIK3R1; STAT3; MAP2K1; CCND1; AKT3;
	STAT1
Amyotrophic Lateral	BID; IGF1; RAC1; BIRC4; PGF; CAPNS1; CAPN2;
Sclerosis Signaling	PIK3CA; BCL2; PIK3CB; PIK3C3; BCL2L1; CAPN1;
	PIK3C2A; TP53; CASP9; PIK3R1; RAB5A; CASP1;
	APAF1; VEGFA; BIRC2; BAX; AKT3; CASP3; BIRC3
JAK/Stat Signaling	PTPN1; MAPK1; PTPN11; AKT2; PIK3CA; STAT5B;
	PIK3CB; PIK3C3; MAPK3; KRAS; SOCS1; STAT5A;
	PTPN6; PIK3C2A; RAF1; CDKN1A; MAP2K2; JAK1;
	AKT1; JAK2; PIK3R1; STAT3; MAP2K1; FRAP1; AKT3;

	STAT1
Nicotinate and Nicotinamide	PRKCE; IRAK1; PRKAA2; EIF2AK2; GRK6; MAPK1;
Metabolism	PLK1; AKT2; CDK8; MAPK8; MAPK3; PRKCD; PRKAA1;
	PBEF1; MAPK9; CDK2; PIM1; DYRK1A; MAP2K2;
	MAP2K1; PAK3; NT5E; TTK; CSNK1A1; BRAF; SGK
Chemokine Signaling	CXCR4; ROCK2; MAPK1; PTK2; FOS; CFL1; GNAQ;
	CAMK2A; CXCL12; MAPK8; MAPK3; KRAS; MAPK13;
	RHOA; CCR3; SRC; PPP1CC; MAPK14; NOX1; RAF1;
	MAP2K2; MAP2K1; JUN; CCL2; PRKCA
IL-2 Signaling	ELK1; MAPK1; PTPN11; AKT2; PIK3CA; SYK; FOS;
	STAT5B; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS;
	SOCS1; STAT5A; PIK3C2A; LCK; RAF1; MAP2K2;
	JAK1; AKT1; PIK3R1; MAP2K1; JUN; AKT3
Synaptic Long Term	PRKCE; IGF1; PRKCZ; PRDX6; LYN; MAPK1; GNAS;
Depression	PRKCI; GNAQ; PPP2R1A; IGF1R; PRKD1; MAPK3;
	KRAS; GRN; PRKCD; NOS3; NOS2A; PPP2CA;
	YWHAZ; RAF1; MAP2K2; PPP2R5C; MAP2K1; PRKCA
Estrogen Receptor	TAF4B; EP300; CARM1; PCAF; MAPK1; NCOR2;
Signaling	SMARCA4; MAPK3; NRIP1; KRAS; SRC; NR3C1;
	HDAC3; PPARGC1A; RBM9; NCOA3; RAF1; CREBBP;
	MAP2K2; NCOA2; MAP2K1; PRKDC; ESR1; ESR2
Protein Ubiquitination	TRAF6; SMURF1; BIRC4; BRCA1; UCHL1; NEDD4;
Pathway	CBL; UBE2I; BTRC; HSPA5; USP7; USP10; FBXW7;
	USP9X; STUB1; USP22; B2M; BIRC2; PARK2; USP8;
	USP1; VHL; HSP90AA1; BIRC3
IL-10 Signaling	TRAF6; CCR1; ELK1; IKBKB; SP1; FOS; NFkB2;
	MAP3K14; MAPK8; MAPK13; RELA; MAPK14; TNF;
	IKBKG; RELB; MAP3K7; JAK1; CHUK; STAT3; NFkB1;
	JUN; IL1R1; IL6
VDR/RXR Activation	PRKCE; EP300; PRKCZ; RXRA; GADD45A; HES1;
	NCOR2; SP1; PRKCI; CDKN1B; PRKD1; PRKCD;

	RUNX2; KLF4; YY1; NCOA3; CDKN1A; NCOA2; SPP1;
	LRP5; CEBPB; FOXO1; PRKCA
TGF-beta Signaling	EP300; SMAD2; SMURF1; MAPK1; SMAD3; SMAD1;
	FOS; MAPK8; MAPK3; KRAS; MAPK9; RUNX2;
	SERPINE1; RAF1; MAP3K7; CREBBP; MAP2K2;
	MAP2K1; TGFBR1; SMAD4; JUN; SMAD5
Toll-like Receptor Signaling	IRAK1; EIF2AK2; MYD88; TRAF6; PPARA; ELK1;
	IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK13;
	RELA; TLR4; MAPK14; IKBKG; RELB; MAP3K7; CHUK;
	NFKB1; TLR2; JUN
p38 MAPK Signaling	HSPB1; IRAK1; TRAF6; MAPKAPK2; ELK1; FADD; FAS;
	CREB1; DDIT3; RPS6KA4; DAXX; MAPK13; TRAF2;
	MAPK14; TNF; MAP3K7; TGFBR1; MYC; ATF4; IL1R1;
	SRF; STAT1
Neurotrophin/TRK Signaling	NTRK2; MAPK1; PTPN11; PIK3CA; CREB1; FOS;
	PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; PIK3C2A;
	RAF1; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1;
	CDC42; JUN; ATF4
FXR/RXR Activation	INS; PPARA; FASN; RXRA; AKT2; SDC1; MAPK8;
	APOB; MAPK10; PPARG; MTPP; MAPK9; PPARGC1A;
	TNF; CREBBP; AKT1; SREBF1; FGFR4; AKT3; FOXO1
Synaptic Long Term Potentiation	PRKCE; RAP1A; EP300; PRKCZ; MAPK1; CREB1;
	PRKCI; GNAQ; CAMK2A; PRKD1; MAPK3; KRAS;
	PRKCD; PPP1CC; RAF1; CREBBP; MAP2K2; MAP2K1;
	ATF4; PRKCA
Calcium Signaling	RAP1A; EP300; HDAC4; MAPK1; HDAC5; CREB1;
	CAMK2A; MYH9; MAPK3; HDAC2; HDAC7A; HDAC11;
	HDAC9; HDAC3; CREBBP; CALR; CAMKK2; ATF4;
	HDAC6
EGF Signaling	ELK1; MAPK1; EGFR; PIK3CA; FOS; PIK3CB; PIK3C3;

	MAPK8; MAPK3; PIK3C2A; RAF1; JAK1; PIK3R1;
	STAT3; MAP2K1; JUN; PRKCA; SRF; STAT1
Hypoxia Signaling in the	EDN1; PTEN; EP300; NQO1; UBE2I; CREB1; ARNT;
Cardiovascular System	HIF1A; SLC2A4; NOS3; TP53; LDHA; AKT1; ATM;
	VEGFA; JUN; ATF4; VHL; HSP90AA1
LPS/IL-1 Mediated	IRAK1; MYD88; TRAF6; PPARA; RXRA; ABCA1;
Inhibition	
of RXR Function	MAPK8; ALDH1A1; GSTP1; MAPK9; ABCB1; TRAF2;
	TLR4; TNF; MAP3K7; NR1H2; SREBF1; JUN; IL1R1
LXR/RXR Activation	FASN; RXRA; NCOR2; ABCA1; NFKB2; IRF3; RELA;
	NOS2A; TLR4; TNF; RELB; LDLR; NR1H2; NFKB1;
	SREBF1; IL1R1; CCL2; IL6; MMP9
Amyloid Processing	PRKCE; CSNK1E; MAPK1; CAPNS1; AKT2; CAPN2;
	CAPN1; MAPK3; MAPK13; MAPT; MAPK14; AKT1;
	PSEN1; CSNK1A1; GSK3B; AKT3; APP
IL-4 Signaling	AKT2; PIK3CA; PIK3CB; PIK3C3; IRS1; KRAS; SOCS1;
	PTPN6; NR3C1; PIK3C2A; JAK1; AKT1; JAK2; PIK3R1;
	FRAP1; AKT3; RPS6KB1
Cell Cycle: G2/M DNA	EP300; PCAF; BRCA1; GADD45A; PLK1; BTRC;
Damage Checkpoint	CHEK1; ATR; CHEK2; YWHAZ; TP53; CDKN1A;
Regulation	PRKDC; ATM; SFN; CDKN2A
Nitric Oxide Signaling in	KDR; FLT1; PGF; AKT2; PIK3CA; PIK3CB; PIK3C3;
the	
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	RAP1A; MAPK1; GNAS; CREB1; CAMK2A; MAPK3;
Signaling	
	SRC; RAF1; MAP2K2; STAT3; MAP2K1; BRAF; ATF4
Mitochondrial	SOD2; MAPK8; CASP8; MAPK10; MAPK9; CASP9;

Dysfunction	
	PARK7; PSEN1; PARK2; APP; CASP3
Notch Signaling	HES1; JAG1; NUMB; NOTCH4; ADAM17; NOTCH2;
	PSEN1; NOTCH3; NOTCH1; DLL4
Endoplasmic Reticulum	HSPA5; MAPK8; XBP1; TRAF2; ATF6; CASP9; ATF4;
Stress Pathway	EIF2AK3; CASP3
Pyrimidine Metabolism	NME2; AICDA; RRM2; EIF2AK4; ENTPD1; RRM2B;
	NT5E; POLD1; NME1
Parkinson's Signaling	UCHL1; MAPK8; MAPK13; MAPK14; CASP9; PARK7;
	PARK2; CASP3
Cardiac & Beta Adrenergic	GNAS; GNAQ; PPP2R1A; GNB2L1; PPP2CA; PPP1CC;
Signaling	PPP2R5C
Glycolysis/Gluconeogenesis	HK2; GCK; GPI; ALDH1A1; PKM2; LDHA; HK1
Interferon Signaling	IRF1; SOCS1; JAK1; JAK2; IFITM1; STAT1; IFIT3
Sonic Hedgehog Signaling	ARRB2; SMO; GLI2; DYRK1A; GLI1; GSK3B; DYRK1B
Glycerophospholipid Metabolism	PLD1; GRN; GPAM; YWHAZ; SPHK1; SPHK2
Phospholipid Degradation	PRDX6; PLD1; GRN; YWHAZ; SPHK1; SPHK2
Tryptophan Metabolism	SIAH2; PRMT5; NEDD4; ALDH1A1; CYP1B1; SIAH1
Lysine Degradation	SUV39H1; EHMT2; NSD1; SETD7; PPP2R5C
Nucleotide Excision Repair	ERCC5; ERCC4; XPA; XPC; ERCC1
Pathway	
Starch and Sucrose Metabolism	UCHL1; HK2; GCK; GPI; HK1
Aminosugars Metabolism	NQO1; HK2; GCK; HK1
Arachidonic Acid Metabolism	PRDX6; GRN; YWHAZ; CYP1B1

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

Methane Metabolism	PRDX6; PRDX1
Phenylalanine Metabolism	PRDX6; PRDX1
Propanoate Metabolism	ALDH1A1; LDHA
Selenoamino Acid Metabolism	PRMT5; AHCY
Sphingolipid Metabolism	SPHK1; SPHK2
Aminophosphonate Metabolism	PRMT5
Androgen and Estrogen Metabolism	PRMT5
Ascorbate and Aldarate Metabolism	ALDH1A1
Bile Acid Biosynthesis	ALDH1A1
Cysteine Metabolism	LDHA
Fatty Acid Biosynthesis	FASN
Glutamate Receptor Signaling	GNB2L1
NRF2-mediated Oxidative Stress Response	PRDX1
Pentose Phosphate Pathway	GPI
Pentose and Glucuronate Interconversions	UCHL1
Retinol Metabolism	ALDH1A1
Riboflavin Metabolism	TYR
Tyrosine Metabolism	PRMT5, TYR
Ubiquinone Biosynthesis	PRMT5
Valine, Leucine and Isoleucine Degradation	ALDH1A1
Glycine, Serine and	CHKA

Threonine Metabolism	
Lysine Degradation	ALDH1A1
Pain/Taste	TRPM5; TRPA1
Pain	TRPM7; TRPC5; TRPC6; TRPC1; Cnr1; cnr2; Grk2;
	Trpa1; Pomc; Cgrp; Crf; Pka; Era; Nr2b; TRPM5; Prkaca;
	Prkacb; Prkar1a; Prkar2a
Mitochondrial Function	AIF; CytC; SMAC (Diablo); Aifm-1; Aifm-2
Developmental	BMP-4; Chordin (Chrd); Noggin (Nog); WNT (Wnt2;
Neurology	
	Wnt2b; Wnt3a; Wnt4; Wnt5a; Wnt6; Wnt7b; Wnt8b;
	Wnt9a; Wnt9b; Wnt10a; Wnt10b; Wnt16); beta-catenin;
	Dkk-1; Frizzled related proteins; Otx-2; Gbx2; FGF-8;
	Reelin; Dab1; unc-86 (Pou4f1 or Brn3a); Numb; Reln

[0247] Embodiments of the applications of the degraders also include those related to knocking out genes, amplifying genes and repairing particular mutations associated with DNA repeat instability and neurological disorders (Robert D. Wells, Tetsuo Ashizawa, Genetic Instabilities and Neurological Diseases, Second Edition, Academic Press, Oct 13, 2011 – Medical). Specific aspects of tandem repeat sequences have been found to be responsible for more than twenty human diseases (New insights into repeat instability: role of RNA•DNA hybrids. McIvor EI, Polak U, Napierala M. RNA Biol. 2010 Sep-Oct;7(5):551-8). The present effector protein systems may be harnessed to correct these defects of genomic instability.

[0248] 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.

SCREENING

[0249] Further disclosed herein include methods for screening, identifying, analyzing, and/or evaluating compounds that modulate (e.g., inhibit) RNA-guided nucleases. In some embodiments, such methods may comprise a combination of biochemical and cellular assays. The methods may be performed for screening, identifying, analyzing, and/or evaluating compounds that modulate (e.g., inhibit) any RNA-guided nucleases, such as RNA-guided nucleases in any CRISPR-Cas system. Examples of Cas proteins include those of Class 1 (e.g., Type I, Type III, and Type IV) and Class 2 (e.g., Type II, Type V, and Type VI) Cas proteins, e.g., Cas9, Cas12 (e.g., Cas12a, Cas12b, Cas12c, Cas12d), Cas13 (e.g., Cas13a, Cas13b, Cas13c, Cas13d), CasX, CasY, Cas14, variants thereof (e.g., mutated forms, truncated forms), homologs thereof, and orthologs thereof.

EGFP Disruption Assay

[0250] In some embodiments, a quantitative human cell-based reporter assay that enables rapid quantitation of targeted nuclease activities is used to characterize off-target cleavage of Cas9-based RNA guided endonucleases. In this assay, the activities of nucleases targeted to a single integrated EGFP reporter gene can be quantified by assessing loss of fluorescence signal in human U2OS.EGFP cells caused by inactivating frameshift insertion/deletion (indel) mutations introduced by error prone non-homologous end-joining (NHEJ) repair of nuclease-induced double-stranded breaks (DSBs).

[0251] In one protocol, U2OS.EGFP cells harboring a single integrated copy of an EGFP-PEST fusion gene are cultured (see e.g., Reyon et al., Nat Biotech 30, 460-465 (2012), which is herein incorporated by reference in its entirety). For transfections, 200,000 cells are nucleofected with gRNA expression plasmid and pJDS246 together with 30 ng of a Td-tomato-encoding plasmid using the SE Cell Line 4D-Nucleofector™ X Kit (Lonza) according to the manufacturer's protocol. Cells are analyzed 2 days post-transfection using a BD LSRII flow cytometer. Transfections for optimizing gRNA/Cas9 plasmid concentration are performed in triplicate and all other transfections are performed in duplicate.

[0252] PCR amplification is used for sequence verification of endogenous human genomic sites. PCR reactions are performed using Phusion Hot Start II high-fidelity DNA polymerase (NEB). Loci are amplified using touchdown PCR (98° C., 10 s; 72-62° C., -1° C./cycle, 15 s; 72° C., 30 s] 10 cycles, [98° C., 10 s; 62° C., 15 s; 72° C., 30 s] 25 cycles). Alternatively, PCR for other targets are performed with 35 cycles at a constant annealing temperature of 68° C. or 72° C. and 3% DMSO or 1M betaine, if necessary. PCR products

are analyzed on a QIAxCEL capillary electrophoresis system to verify both size and purity. Validated products are treated with ExoSap-IT (Affymetrix) and sequenced by the Sanger method (MGH DNA Sequencing Core) to verify each target site.

[0253] Degraders of RNA guided endonucleases can also be used to regulate genome editing technologies in other organisms, including invertebrates, plants, and unicellular organisms (e.g., bacteria). Potential uses include regulating gene drives for entomological and agricultural uses. In addition, it is anticipated that Cas9 degraders will be valuable probes to understand the role of Cas9 in CRISPR-mediated bacterial immunity (e.g., spacer acquisition) (Nunez et al., *Nature*. 2015 Mar 12;519(7542):193-8; Heler et al., *Nature* 2015, 519, 199-202). Along similar lines, Cas9 degraders can be deployed for directed evolution of Cas9. It is hypothesized that Cas9 degraders will disrupt bacterial immunity against bacteriophages (or toxic DNA) by interfering with the CRISPR-Cas9-based immune surveillance system in bacteria. Akin to the development of antibiotic resistance, bacteria will be forced to evolve Cas9 protein.

[0254] Degraders of the invention are useful to inhibit the nuclease activity of a CRISPR protein – guide complex. The guide need not be naturally occurring and can comprise, e.g., non-naturally occurring nucleotides, analogs, and/or chemical modifications.

[0255] The present invention also contemplates use of the systems described herein to provide RNA-guided gene drives, for example in systems analogous to gene drives described in PCT Patent Publication WO 2015/105928. Further reference can be found for instance in Esvelt et al. (*eLife* 2014; 3:e03401; DOI: 10.7554/eLife.03401.001); Webber et al. (*PNAS*; 2015; 112(34):10565-10567); DeFrancesco (*Nature Biotechnology*, 2015, 33(10):1019-1021); DiCarlo et al. (*Nature Biotechnology*, 2015; 33: 1250-1255); Gantz et al. (*PNAS*; 2015; 112(49):E6736-E6743). Systems of this kind may for example provide methods for altering eukaryotic germline cells, by introducing into the germline cell a nucleic acid sequence encoding an RNA or DNA-guided DNA or RNA nuclease and one or more guide RNAs or guide DNAs. The guide RNAs/DNAs may be designed to be complementary to one or more target locations on (genomic) DNA or RNA of the germline cell. The nucleic acid sequence encoding the DNA/RNA guided DNA/RNA nuclease and the nucleic acid sequence encoding the guide RNAs/DNAs may be provided on constructs between flanking sequences, with promoters arranged such that the germline cell may express the nuclease and the guides, together with any desired cargo-encoding sequences that are also situated between the flanking sequences. The flanking sequences will typically include a sequence which is

identical to a corresponding sequence on a selected target chromosome, so that the flanking sequences work with the components encoded by the construct to facilitate insertion of the foreign nucleic acid construct sequences into RNA or DNA at a target cut site by mechanisms such as homologous recombination, to render the germline cell homozygous for the foreign nucleic acid sequence. In this way, gene-drive systems are capable of introgressing desired cargo genes throughout a breeding population (Gantz et al., 2015, Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*, PNAS 2015, published ahead of print November 23, 2015, doi:10.1073/pnas.1521077112; Esvelt et al., 2014, Concerning DNA- or RNA-guided gene drives for the alteration of wild populations eLife 2014;3:e03401). In select embodiments, target sequences may be selected which have few potential off-target sites in a genome. Targeting multiple sites within a target locus, using multiple guide RNAs, may increase the cutting frequency and hinder the evolution of drive resistant alleles. Truncated guide RNAs may reduce off-target cutting. Paired nickases may be used instead of a single nuclease, to further increase specificity. Gene drive constructs may include cargo sequences encoding transcriptional regulators, for example to activate homologous recombination genes and/or repress non-homologous end-joining. Target sites may be chosen within an essential gene, so that non-homologous end-joining events may cause lethality rather than creating a drive-resistant allele. The gene drive constructs can be engineered to function in a range of hosts at a range of temperatures (Cho et al. 2013, Rapid and Tunable Control of Protein Stability in *Caenorhabditis elegans* Using a Small Molecule, PLoS ONE 8(8): e72393. doi:10.1371/journal.pone.0072393).

Modulation of gene editing mechanisms

[0256] The dgraders herein may be administered to cells or organisms at doses effective to impact gene editing outcomes, e.g., to control the gene editing mechanisms via NHEJ or HDR.

[0257] The activity of NHEJ and HDR DSB repair varies significantly by cell type and cell state. NHEJ is not highly regulated by the cell cycle and is efficient across cell types, allowing for high levels of gene disruption in accessible target cell populations. In contrast, HDR acts primarily during S/G2 phase, and is therefore restricted to cells that are actively dividing, limiting treatments that require precise genome modifications to mitotic cells [Ciccia, A. & Elledge, S.J. Molecular cell 40, 179-204 (2010); Chapman, J.R., et al. Molecular cell 47, 497-510 (2012)].

[0258] The degraders may affect the gene editing mechanisms by modulating the function and activity of the RNA-guided nuclease involved in the gene editing. The efficiency of correction via HDR may be controlled by the epigenetic state or sequence of the targeted locus, or the specific repair template configuration (single vs. double stranded, long vs. short homology arms) used [Hacein-Bey-Abina, S., et al. The New England journal of medicine 346, 1185-1193 (2002); Gaspar, H.B., et al. Lancet 364, 2181-2187 (2004); Beumer, K.J., et al. G3 (2013)]. The relative activity of NHEJ and HDR machineries in target cells may also affect gene correction efficiency, as these pathways may compete to resolve DSBs [Beumer, K.J., et al. Proceedings of the National Academy of Sciences of the United States of America 105, 19821-19826 (2008)]. HDR also imposes a delivery challenge not seen with NHEJ strategies, as it requires the concurrent delivery of nucleases and repair templates. In practice, these constraints have so far led to low levels of HDR in therapeutically relevant cell types. Clinical translation has therefore largely focused on NHEJ strategies to treat disease, although proof-of-concept preclinical HDR treatments have now been described for mouse models of haemophilia B and hereditary tyrosinemia [Li, H., et al. Nature 475, 217-221 (2011); Yin, H., et al. Nature biotechnology 32, 551-553 (2014)].

Formulations

[0259] Agents described herein, including analogs thereof, and/or agents discovered to have medicinal value using the methods described herein are useful as a drug. Compounds as disclosed herein, or pharmaceutically acceptable salts thereof may be provided, optionally with a variant CRISPR Cas protein. The CRISPR Cas protein, optionally with the compounds disclosed herein may be provided in a kit. For therapeutic uses, the compositions or agents identified using the methods disclosed herein may be administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline. Preferable routes of administration include, for example, subcutaneous, intravenous, interperitoneally, intramuscular, or intradermal injections that provide continuous, sustained levels of the drug in the patient. Treatment of human patients or other animals will be carried out using a therapeutically effective amount of a therapeutic identified herein in a physiologically-acceptable carrier. Suitable carriers and their formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin. The amount of the therapeutic agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the clinical symptoms.

Generally, amounts will be in the range of those used for other agents used in the treatment of other diseases associated with diabetes.

[0260] The disclosed compounds may be administered alone (e.g., in saline or buffer) or using any delivery vehicles known in the art. For instance, the following delivery vehicles have been described: Cochleates; Emulsomes, ISCOMs; Liposomes; Live bacterial vectors (e.g., Salmonella, Escherichia coli, Bacillus calmatte-guerin, Shigella, Lactobacillus); Live viral vectors (e.g., Vaccinia, adenovirus, Herpes Simplex); Microspheres; Nucleic acid vaccines; Polymers; Polymer rings; Proteosomes; Sodium Fluoride; Transgenic plants; Virosomes; Virus-like particles. Other delivery vehicles are known in the art and some additional examples are provided below.

[0261] The disclosed compounds may be administered by any route known, such as, for example, orally, transdermally, intravenously, cutaneously, subcutaneously, nasally, intramuscularly, intraperitoneally, intracranially, and intracerebroventricularly.

[0262] In certain embodiments, disclosed compounds are administered at dosage levels greater than about 0.001mg/kg, such as greater than about 0.01 mg/kg or greater than about 0.1 mg/kg. For example, the dosage level may be from about 0.001 mg/kg to about 50 mg/kg such as from about 0.01 mg/kg to about 25 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 5 mg/kg of subject body weight per day, one or more times a day, to obtain the desired therapeutic effect. It will also be appreciated that dosages smaller than about 0.001 mg/kg or greater than about 50 mg/kg (for example about 50-100 mg/kg) can also be administered to a subject.

[0263] In one embodiment, the compound is administered once-daily, twice-daily, or three-times daily. In one embodiment, the compound is administered continuously (i.e., every day) or intermittently (e.g., 3-5 days a week). In another embodiment, administration could be on an intermittent schedule.

[0264] Further, administration less frequently than daily, such as, for example, every other day may be chosen. In additional embodiments, administration with at least 2 days between doses may be chosen. By way of example only, dosing may be every third day, bi-weekly or weekly. As another example, a single, acute dose may be administered. Alternatively, compounds can be administered on a non-regular basis e.g., whenever symptoms begin. For any compound described herein the effective amount can be initially determined from animal models.

[0265] Toxicity and efficacy of the compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit large therapeutic indices may have a greater effect when practicing the methods as disclosed herein. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0266] Data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage of the compounds disclosed herein for use in humans. The dosage of such agents lies within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the disclosed methods, the effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography. In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, the active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Multiple doses of the compounds are also contemplated.

[0267] The formulations disclosed herein are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic ingredients.

[0113] For use in therapy, an effective amount of one or more disclosed compounds can be administered to a subject by any mode that delivers the compound(s) to the desired surface, e.g., mucosal, systemic. Administering the pharmaceutical composition of the present disclosure may be accomplished by any means known to the skilled artisan. Disclosed compounds may be administered orally, transdermally, intravenously, cutaneously,

subcutaneously, nasally, intramuscularly, intraperitoneally, intracranially, or intracerebroventricularly.

[0268] For oral administration, one or more compounds can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated.

[0269] Pharmaceutical preparations for oral use can be obtained as solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers, i.e. EDTA for neutralizing internal acid conditions or may be administered without any carriers.

[0270] Also specifically contemplated are oral dosage forms of one or more disclosed compounds. The compound(s) may be chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the compound itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the compound(s) and increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline. Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. In some aspects for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

[0271] The location of release may be the stomach, the small intestine (the duodenum, the jejunum, or the ileum), or the large intestine. One skilled in the art has available formulations which will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. In some aspects, the release will avoid the

deleterious effects of the stomach environment, either by protection of the compound or by release of the biologically active material beyond the stomach environment, such as in the intestine.

[0272] To ensure full gastric resistance a coating impermeable to at least pH 5.0 is important. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

[0273] A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic i.e. powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

[0274] The disclosed compounds can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The compound could be prepared by compression.

[0275] Colorants and flavoring agents may all be included. For example, the compound may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

[0276] One may dilute or increase the volume of compound delivered with an inert material. These diluents could include carbohydrates, especially mannitol, α -lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell. Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrates include but are not limited to starch, including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another

form of the disintegrants is the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

[0277] Binders may be used to hold the therapeutic together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

[0278] An anti-frictional agent may be included in the formulation of the compound to prevent sticking during the formulation process. Lubricants may be used as a layer between the compound and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000. Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

[0279] To aid dissolution of the compound into the aqueous environment a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential non-ionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the compound either alone or as a mixture in different ratios.

[0280] Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for

oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in dosages suitable for such administration.

[0281] For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

[0282] For administration by inhalation, the compounds for use according to the present disclosure may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0283] Also contemplated herein is pulmonary delivery of the compounds of the disclosure. The compound is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream using methods well known in the art.

[0284] Contemplated for use in the practice of methods disclosed herein are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Some specific examples of commercially available devices suitable for the practice of these methods are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

[0285] All such devices require the use of formulations suitable for the dispensing of compound. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified compound may also be prepared in different formulations depending on the type of chemical modification or the type of device employed. Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise compound dissolved in water at a

concentration of about 0.1 to about 25 mg of biologically active compound per mL of solution. The formulation may also include a buffer and a simple sugar (e.g., for stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the compound caused by atomization of the solution in forming the aerosol.

[0286] Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the compound suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

[0287] Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing compound and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, e.g., about 50 to about 90% by weight of the formulation. The compound should most advantageously be prepared in particulate form with an average particle size of less than 10 mm (or microns), such as about 0.5 to about 5 mm, for an effective delivery to the distal lung.

[0288] Nasal delivery of a disclosed compound is also contemplated. Nasal delivery allows the passage of a compound to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran.

[0289] For nasal administration, a useful device is a small, hard bottle to which a metered dose sprayer is attached. In one embodiment, the metered dose is delivered by drawing the pharmaceutical composition solution into a chamber of defined volume, which chamber has an aperture dimensioned to aerosolize and aerosol formulation by forming a spray when a liquid in the chamber is compressed. The chamber is compressed to administer the pharmaceutical composition. In a specific embodiment, the chamber is a piston arrangement. Such devices are commercially available.

[0290] Alternatively, a plastic squeeze bottle with an aperture or opening dimensioned to aerosolize an aerosol formulation by forming a spray when squeezed is used. The opening is usually found in the top of the bottle, and the top is generally tapered to partially fit in the

nasal passages for efficient administration of the aerosol formulation. In some aspects, the nasal inhaler will provide a metered amount of the aerosol formulation, for administration of a measured dose of the drug.

[0291] The compound, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[0292] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions.

[0293] Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0114] Alternatively, the active compounds may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[0294] The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

[0295] In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0296] The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

[0297] Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems.

[0298] The compounds may be administered per se (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

[0299] Suitable buffering agents include: acetic acid and a salt (about 1-2% w/v); citric acid and a salt (about 1-3% w/v); boric acid and a salt (about 0.5-2.5% w/v); and phosphoric acid and a salt (about 0.8-2% w/v). Suitable preservatives include benzalkonium chloride (about 0.003-0.03% w/v); chlorobutanol (about 0.3-0.9% w/v); parabens (about 0.01-0.25% w/v) and thimerosal (about 0.004- 0.02% w/v).

[0300] The pharmaceutical compositions contain an effective amount of a disclosed compound optionally included in a pharmaceutically acceptable carrier. The term pharmaceutically acceptable carrier means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term carrier denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

[0301] 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 (i.e. direct repeat) 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 if applicable; and/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence and/or (c) a third regulatory element operably linked to sequence encoding the fusion protein according to the invention as described herein, optionally comprising a nuclear localization sequence or NES. 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

[0302] The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1

[0303] Degraders targeting variant Cas9 proteins is explored in the following example. SpCas9 variants were prepared and transfected into several cell lines. The cells were incubated with dTAG, degrader compositions, and evaluated for SpCas9 activity via genomic eGFP-PEST disruption.

Reagents and plasmids

[0304] dTAG-47 was provided by our collaborator James Bradner and further resynthesized in our hand (>98% purity, validated by LC-MS). dTAG-13 was also utilized herein. Plasmid sequences for FKBP12^{F36V} fused Cas9 constructs are included in the Plasmid Sequences provided in Table 1.

Protein expression and purification

[0305] NtLp 2xFKBP Cas9 was cloned in pET21 vector using Gibson cloning assembly and overexpressed in *E. coli* Rosetta (DE3) in LB medium in the presence of 50ug/ml Ampicillin. Cells were grown at 37 °C and induced with 0.2mM isopropyl-1-thio-D-galactopyranoside (IPTG) at OD₆₀₀ 0.6 for 12hrs at 18 °C. Cell pellets were collected by centrifugation and lysed in lysis buffer (20mM Tris pH 8, 500mM NaCl, 5% glycerol, 1mM DTT, 20mM imidazole, 1mM PMSF, 1mg/ml lysozyme) and sonicated. Cell lysates were centrifuged at 16,000g for 30min at 4 °C. The Cas9 protein in the soluble lysates was purified using Ni-NTA column (Qiagen) followed by HiTrap column and Superdex size exclusion column.

Cell line

[0306] HEK293FT, U2OS, NIH3T3 cells were cultured at 37 °C in 5% CO₂ atmosphere in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine. S2 cells was cultured in room temperature in Schneider's Drosophila medium containing 10% heat-inactivated FBS.

Western blotting analysis

[0307] HEK293 or NIH3T3 were transfected with pX330 FKBP-Cas9 plasmid using Lipofectamine 2000 or Lipofectamine 3000 to the manufacturer's protocol accordingly. 24 hours after transfection, cells transiently expressing the different constructs (NtLp 2xFKBP, NtCt 2xFKBP, LpCt 2xFKBP, and 3xFKBP) were incubated in either the absence or the presence of dTAG 47 (3.3uM, 1.1uM, 0.37uM, 0.12uM, 0.042uM) for 24h before harvesting. Cells were washed with PBS and proceed to lysis in RIPA buffer. The cell suspensions were centrifuged at 16,000g for 30min at 4 °C. 20ug of supernatant were electrophoresed on 4-12% Bis-Tris gel with SDS-Tris running buffer. The protein bands were transferred to nitrocellulose membrane at 100V for 1h and probed with mouse anti-Cas9 antibody (Abcam) and rabbit anti-tubulin as loading control (Abcam). After blocking and incubation with primary antibodies, membranes were incubated with 680-nm or 800-nm fluorophore labeled secondary antibodies. Protein bands were detected using LI-COR BioSciences Odyssey Imager and analyzed by Image Studio Lite (LI-COR Biosciences).

Analysis of Cas9 nuclease activity via genomic eGFP-PEST disruption

[0308] In plasmid delivery, approximately 200,000 U2OS.eGFP-PEST cells were nucleofected with 300ng of Cas9 and sgRNA expression plasmids (NtLp 2xFKBP, NtCt 2xFKBP, LpCt 2xFKBP, and 3xFKBP) using the SE Cell Line 4D-Nucleofector X Kit (Lonza) according to the manufacturer's protocol. Approximately 12,000 transfected cells per

Table 1: Plasmid Sequences For Variant SpCas9

GAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGAC CGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTTCGACAGC GGCGAAACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAG ATACACCAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGC AACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGACTGGAAGAG TCCTTCCTGGTGGAAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCG GCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCACCATCTA CCACCTGAGAAAGAACTGGTGGACAGCACCGACAAGGCCGACCTGCG GCTGATCTATCTGGCCCTGGCCACATGATCAAGTTCCGGGGCCACTTC CTGATCGAGGGCGACCTGAACCCCGACAACAGCGACGTGGACAAGCTG TTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTTCGAGGAAAACCCCA TCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAG CAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGGAAG TGGCAGCgagtgagtgagtggaaccatctcccaggagacgggcgaccttcccaagcgcgccagacgtg cgtggtgcactacacgggatgcttgaagatggaaagaagtgattctctccgggacagaacaagcccttaagtta tgctaggcaagcaggagtgatccgaggctgggaagaagggttgcccagatgagtggtgcagagagccaaact gactatatctccagattatgcctatggtgccactgggcaccaggcatcatccaccacatgccactctcgtcttcgatgt ggagcttctaaaactggaaGGCAGTGGAAAGCGAGAAGAAGAATGGCCTGTTTCGGA AACCTGATTGCCCTGAGCCTGGGCCTGACCCCAACTTCAAGAGCAACT TCGACCTGGCCGAGGATGCCAACTGCAGCTGAGCAAGGACACCTACG ACGACGACCTGGACAACCTGCTGGCCAGATCGGCGACCAAGTACGCCG ACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGCTGAGCGA CATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCT ATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAA GCTCTCGTGCGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCG ACCAGAGCAAGAACGGCTACGCCGGCTACATTGACGGCGGAGCCAGCC AGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTGGAAAAGATGGACG GCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGGA AGCAGCGGACCTTCGACAACGGCAGCATCCCCACCAGATCCACCTGGG AGAGCTGCACGCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCTG AAGGACAACCGGGAAGATCGAGAAGATCCTGACCTTCCGCATCCCC TACTACGTGGGCCCTCTGGCCAGGGGAAACAGCAGATTTCGCTGGATGA CCAGAAAGAGCGAGGAAACCATCACCCCTGGAACCTCGAGGAAGTGG TGGACAAGGGCGCTTCCGCCAGAGCTTCATCGAGCGGATGACCAACTT CGATAAGAACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCT GTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATACGTG ACCGAGGGAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAG GCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGC AGCTGAAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGA AATCTCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCAC GATCTGCTGAAAATTATCAAGGACAAGGACTTCTGGACAATGAGGAA AACGAGGACATTCTGGAAGATATCGTGCTGACCTGACACTGTTTGAGG ACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCCACCTGTTTCG ACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGG GCAGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCG GCAAGACAATCCTGGATTTCTGAAGTCCGACGGCTTCGCCAACAGAAA CTTCATGCAGCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACATC CAGAAAGCCCAGGTGTCCGGCCAGGGCGATAGCCTGCACGAGCACATT
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Table 1: Plasmid Sequences For Variant SpCas9

GCCAATCTGGCCGCGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACA GTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCGGCACAAGCCC GAGAACATCGTGATCGAAATGGCCAGAGAGAACCAGACCACCCAGAAG GGACAGAAGAACAGCCGCGAGAGAATGAAGCGGATCGAAGAGGGCAT CAAAGAGCTGGGCAGCCAGATCCTGAAAGAACACCCCGTGGAACAC CCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGGGCG GGATATGTACGTGGACCAGGAAGCTGGACATCAACCGGCTGTCCGACTAC GATGTGGACCATATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCG ACAACAAGGTGCTGACCAGAAGCGACAAGAACCGGGGCAAGAGCGAC AACGTGCCCTCCGAAGAGGTCTGTGAAGAAGATGAAGAAGTACTGGCGG CAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTTCGACAATCTGA CCAAGGCCGAGAGAGGGCGGCTGAGCGAACTGGATAAGGCCGGCTTCA TCAAGAGACAGCTGGTGGAAACCCGGCAGATCACAAGCACGTGGCAC AGATCCTGGACTCCCGGATGAACACTAAGTACGACGAGAATGACAAGC TGATCCGGGAAGTGAAAGTGATCACCTGAAGTCCAAGCTGGTGTCCGA TTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAACACTAC CACCACGCCCACGACGCCTACCTGAACGCCGTCGTGGGAACCGCCCTGA TCAAAAAGTACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACA AGGTGTACGACGTGCGGAAGATGATCGCCAAGAGCGAGCAGGAAATCG GCAAGGCTACCGCCAAGTACTTCTTCTACAGCAACATCATGAACTTTTTC AAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGGCCTCTG ATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGG GATTTTGCCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATATCG TGAAAAAGACCGAGGTGCAGACAGGCGGCTTCAGCAAAGAGTCTATCC TGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTGGG ACCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGT GCTGGTGGTGGCCAAAGTGGAAGAGGGCAAGTCCAAGAACTGAAGAG TGTGAAAGAGCTGCTGGGGATCACCATCATGGAAAGAAGCAGCTTCGA GAAGAATCCCATCGACTTTCTGGAAGCCAAGGGCTACAAAGAAGTGAA AAAGGACCTGATCATCAAGCTGCCTAAGTACTCCCTGTTTCGAGCTGGAA AACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAACTGCAGAAGGGA AACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGGCCA GCCACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAAC AGCTGTTTGTGGAACAGCACAAAGCACTACCTGGACGAGATCATCGAGCA GATCAGCGAGTTCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTGGAC AAAGTGCTGTCCGCCTACAACAAGCACCCGGGATAAGCCCATCAGAGAG CAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCCC CTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACAC CAGCACCAAGAGAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCAC CGGCCTGTACGAGACACGGATCGACCTGTCTCAGCTGGGAGGCGACAA AAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAAAAAAGAAAAAGta agaattcCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGC CATCTGTTGTTTGCCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCC ACTCCCACTGTCCTTTTCTAATAAAATGAGGAAATTGCATCGCATTGTCT GAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAA GGGGGAGGATTGGGAAGAgAATAGCAGGCATGCTGGGGAgcggccgcaggaa cccctagtgtgagttggccactccctctctgcgcgctcgtcgtcactgaggccggcgaccaaaggtcgcccgga cgcccggtcttgcccgggcgccctcagtgcgcgagcgcgcagctgcctgcagggcgccctgatcgcggtatt
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Table 1: Plasmid Sequences For Variant SpCas9	
	ttctccttacgcatctgtgcggtatttcacaccgcatacgtcaaagcaaccatagtagcgccctgtagcggcgcatgaag cgccggcggtgtggtgtgttacgcgcagcgtgaccgtacacttgccagcgccctagcgcccgctccttctgctttctcc cttcttctcgcacggttcgcccgtttcccgctcaagctctaaatcggggctccctttaggggttcgatttagtgccttac ggcacctcgaccccaaaaaacttgatttgggtgatggttcacgtagtgggccatcgccctgatagacggttttgcctt tgacgttggagtcacggttcttaatagttgactctgttccaaactggacaacactcaaccctatctcgggctattctttg attataagggatttgcgatttcggcctattggttaaaaaatgagctgatttaacaaaaattaacgcgaattttaaaaaat attaacgtttacaatttatgtgcactctcagtacaatctgctctgatccgcatagttaagccagccccgacacccgcc acaccgctgacgcgccctgacgggcttctgctcctccggcatccgttacagacaagctgtgacgctcctgggagc tgcatgtgacagaggtttcacgcgtacacgaaacgcgcgagacgaaagggcctctgtatagcctattttataggtt aatgtcatgataataatggtttcttagacgtcaggtggcacttttcggggaaatgtgcgcgggaaccctattgtttatttct aaatacattcaaatatgtatccgctcatgagacaataacctgataaatgctcaataatattgaaaaggaagagtatgag tattcaacatttcgctgcgcccttattcccttttgcggcatttgccttcctgttttgcctcaccagaaacgctggtgaaag taaaagatgtgaagatcagttgggtgcacgagtggtgtacatcgaactggatctcaacagcggtaagatccttgagagt tttcgccccgaagaacgttttccaatgatgagcacttttaaaagtctgctatgtggcgcggtattatcccgattgacgcgg gcaagagcaactcggtcgcgcacatacactattctcagaatgacttggtgagtactcaccagtcacagaaaagcatctta cggatggcatgacagtaagagaattatgcagtgctgccataacctagtgatgataacactgcggccaacttactctgaca acgatcggaggaccgaaggagctaacgccttttgcacaacatgggggatcatgtaactgccttgatcgttgggaac cggagctgaatgaagccatacacaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacggtgcgcaaa ctattaactggcgaactacttactctagcttcccgcaacaattaatagactggatggagggcgataaagtgcaggacc acttctgcgtcggcccttcggctggctgttattgctgataaatctggagccggtgagcgtggaagccgcggtatca ttgcagcactggggccagatggtaagccctcccgatcgtatctacacgacggggagtcaggcaactatggatga acgaaatagacagatcgtgagataggtgcctcactgattaagcattggttaactgtgacaccaagttactcatataactt tagattgatttaaaacttcattttaatttaaaggatctaggtgaagatccttttgataatctcatgacaaaatccctaacg tgagtttctgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgatcctttttctgcgcgtaactctg ctgcttgcacaacaaaaaacaccgctaccagcgggtggtttgttccggatcaagagctaccaactcttttccgaaggt aactggctcagcagagcgcagatacacaactgtccttctagtgtagccgtagtaggaccacttcaagaactctgt agcaccgcctacatacctcgtctgctaatctgttaccagtggctgctgacagtgccgataaagctgtgtcttaccgggtt ggactcaagacgatagtttaccggataaggcgagcggctgggctgaacggggggttcgtgcacacagcccagcttg gagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaagcgccacgcttccgaaggagaa aggcggacaggtatccggaagcggcagggtcggaacaggagagcgcacgagggagctccaggggggaaacgcc tggtatctttatagtcctgtcgggttcgccacctctgactgagcgtcgattttgtgatgctcgtcagggggcgaggcct atggaaaaacgcagcaacgcggccttttacggttctggccttttgccttctgacatgt
2. pX330 NiCt 2xFKBP SpCas9 (SEQ ID NO:2)	gagggcctatttcccatgattccttcataattgcataacgatacaaggctgttagagagataattggaattaattgactgta aacacaaagatattagtacaaaatcgtgacgtagaaagtaataatttcttgggtagtttgagttttaaataattgtttaaaa tggactatcatatgcttaccgtaacttgaagatttctgatttcttggctttatatacttGTGGAAGGACGAA ACACCggGTCTTCgaGAAGACctgttttagagctaGAAAtagcaagttaaataaaggctagtcggtta tcaacttgaaaaagtggcaccgagtcggtgcTTTTTgttttagagctagaaatagcaagttaaataaaggctagtc cgtTTTTagcgcgtgcgccaatctgcagacaaatggctctagaggtacccgttacataacttacggttaaatggcccg cctggctgaccgccaacgacccccgccattgacgtcaatagtaacgccaatagggactttccattgacgtcaatggg tggagtatttacggttaactgcccacttggcagtagatcaagtgtatcatatgccaagtacgccccctattgacgtcaatga cggtaaatggcccgctggcattGtcccagtagatgacctatgggactttctacttggcagtagatctacgtatttagtc atcgctattaccatggctgaggtgagccccacgttctgcttacttccccatctccccccctccccacccccattttgt atttatttatttttaattattttgtgcagcgtatggggcgggggggggggggggggcgcgccagggcgggcgggggc ggggcgagggggcgggcgggggcgagggcgagaggtgcggcggcagccaatcagagcgcgcgctccgaaagt ttccttttatggcagggcgggcgggcgggcgccctataaaaagcgaagcgcgcgggcgggcgagtcgctgcgac gctgccttcgccccgtgccccgtccgcccgcctcgcgcgccccggcctgactgaccgcgttactccac

Table 1: Plasmid Sequences For Variant SpCas9

aggtgagcgggacggcccttctcctcgggctgtaattagctgagcaagaggttaagggatggttg ttgtgggtatgaatgttaattacctggagcacctgctgaaatcactttttcaggttGgaccggtgccaccATGG ACTATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTACA AAGACGATGACGATAAGATGGCCCCAAAGAAGAAGCGGAAGGTCGGTA TCCACGGAGTCCCAGCAGCCGGATCCggagtgcaggtggaaccatctccccaggagacgg gcgcaccttcccaagcgcggccagacctgcgtggtgcactacaccgggatgcttgaagatggaaagaagttgattc ctccgggacagaacaagcccttaagtattatgctaggcaagcaggaggtgatccgaggctgggaagaaggggtg cccagatgagtgtgggtcagagagccaaactgactatatcctcagattatgcctatggtgccactgggcacccaggcat catcccaccatgccactctcgtcttgatgtggagcttctaaactggaaGACAAGAAGTACAGCAT CGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTGATCACCGAC GAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGAC CGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTTCGACAGC GGCGAAACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAG ATACACCAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGC AACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGACTGGAAGAG TCCTTCCTGGTGGAAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCG GCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCACCATCTA CCACCTGAGAAAGAACTGGTGGACAGCACCGACAAGGCCGACCTGCG GCTGATCTATCTGGCCCTGGCCACATGATCAAGTTCCGGGGCCACTTC CTGATCGAGGGCGACCTGAACCCCGACAACAGCGACGTGGACAAGCTG TTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTTCGAGGAAAACCCCA TCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAG CAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGAGAA GAAGAATGGCCTGTTTCGGAAACCTGATTGCCCTGAGCCTGGGCCTGACC CCCAACTTCAAGAGCAACTTCGACCTGGCCGAGGATGCCAACTGCAGC TGAGCAAGGACACCTACGACGACGACCTGGACAACCTGCTGGCCCAGA TCGGCGACCAAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGA CGCCATCCTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAG GCCCCCTGAGCGCCTCTATGATCAAGAGATACGACGAGCACCACCAGG ACCTGACCCTGCTGAAAGCTCTCGTGCGGCAGCAGCTGCCTGAGAAGTA CAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCCGGCTACATT GACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATC CTGGAAGATGGACGGCACCGAGGAAGTCTGCTGTAAGCTGAACAGA GAGGACCTGCTGCGGAAGCAGCGGACCTTCGACAACGGCAGCATCCCC CACCAGATCCACCTGGGAGAGCTGCACGCCATTCTGCGGCGGCAGGAA GATTTTTACCCATTCTGAAGGACAACCGGGAAAAGATCGAGAAGATCC TGACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAG CAGATTCGCCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCCTG GAACTTCGAGGAAGTGGTGGACAAGGGCGCTTCCGCCAGAGCTTCATC GAGCGGATGACCAACTTCGATAAGAACCTGCCCAACGAGAAGGTGCTG CCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTGTATAACGAGCTGA CCAAAGTGAAATACGTGACCGAGGGAATGAGAAAGCCCGCCTTCCTGA GCGGCGAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAGACCAACC GGAAAGTGACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGAAAATCG AGTGCTTCGACTCCGTGGAAATCTCCGGCGTGGAAGATCGGTTCAACGC CTCCCTGGGCACATACCACGATCTGCTGAAAATTATCAAGGACAAGGAC TTCTTGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTGCTGA CCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAA
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Table 1: Plasmid Sequences For Variant SpCas9

	CCTATGCCACCTGTTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCG GAGATACACCGGCTGGGGCAGGCTGAGCCGGAAGCTGATCAACGGCAT CCGGGACAAGCAGTCCGGCAAGACAATCCTGGATTTCCTGAAGTCCGAC GGCTTCGCCAACAGAACTTCATGCAGCTGATCCACGACGACAGCCTGA CCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGCCAGGGCGATA GCCTGCACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGAA GGGCATCCTGCAGACAGTGAAGGTGGTGGACGAGCTCGTGAAAGTGAT GGGCCGGCACAAGCCCAGAAACATCGTGATCGAAATGGCCAGAGAGAA CCAGACCACCCAGAAGGGACAGAAGAACAGCCGCGAGAGAATGAAGC GGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAGATCCTGAAAGAAC ACCCCGTGGAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACT ACCTGCAGAATGGGCGGGATATGTACGTGGACCAGGAAGTGGACATCA ACCGGCTGTCCGACTACGATGTGGACCATATCGTGCCTCAGAGCTTTCT GAAGGACGACTCCATCGACAACAAGGTGCTGACCAGAAGCGACAAGAA CCGGGGCAAGAGCGACAACGTGCCCTCCGAAGAGGTCTGTGAAGAAGAT GAAGAACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAG AAAGTTCGACAATCTGACCAAGGCCGAGAGAGGCGGCCTGAGCGAACT GGATAAGGCCGGCTTCATCAAGAGACAGCTGGTGGAAACCCGGCAGAT CACAAAGCACGTGGCACAGATCCTGGACTCCCGGATGAACACTAAGTA CGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCTGAA GTCCAAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTACAAAGTG CGCGAGATCAACAACCTACCACCACGCCACGACGCCTACCTGAACGCCG TCGTGGGAACCGCCCTGATCAAAAAGTACCCTAAGCTGGAAAGCGAGTT CGTGACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCAA GAGCGAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGC AACATCATGAACTTTTTCAAGACCGAGATTACCCTGGCCAACGGCGAGA TCCGGAAGCGGCCTCTGATCGAGACAAACGGCGAAACCGGGGAGATCG TGTGGGATAAGGGCCGGGATTTTGCCACCGTGCGGAAAGTGCTGAGCAT GCCCCAAGTGAATATCGTGAAAAAGACCGAGGTGCAGACAGGCGGCTT CAGCAAAGAGTCTATCCTGCCAAGAGGAACAGCGATAAGCTGATCGC CAGAAAGAAGGACTGGGACCCTAAGAAGTACGGCGGCTTCGACAGCCC CACCGTGGCCTATTCTGTGCTGGTGGTGGCCAAAGTGGAAGGGCAAG TCCAAGAACTGAAGAGTGTGAAAGAGCTGCTGGGGATCACCATCATG GAAAGAAGCAGCTTCGAGAAGAATCCCATCGACTTTCTGGAAGCCAAG GGCTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCTAAGTAC TCCCTGTTTCGAGCTGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCG GCGAACTGCAGAAGGGAAACGAACTGGCCCTGCCCTCCAAATATGTGA ACTTCCTGTACCTGGCCAGCCACTATGAGAAGCTGAAGGGCTCCCCCGA GGATAATGAGCAGAAACAGCTGTTTGTGGAACAGCACAAGCACTACCT GGACGAGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTGATCCT GGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACAACAAGCACCGG GATAAGCCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCC TGACCAATCTGGGAGCCCCTGCCGCCTTCAAGTACTTTGACACCACCAT CGACCGGAAGAGGTACACCAGCACCAAGAGGTGCTGGACGCCACCCT GATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATCGACCTGTCT CAGCTGGGAGGCGACAAAAGGCCGGCGGCCACGAAAAAGGCCGGCCA GGCAAAAAAGAAAAAGggagtgagtggaaccatctccccaggagacggcgacactccccaa gcgcgccagacactgcgtggtgcactacacgggatgctgaagatggaagaaagttgattcctccgggacagaa
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Table 1: Plasmid Sequences For Variant SpCas9	
	acaagccctttaagtttatgctaggaagcaggaggtgatccgaggctgggaagaaggggtgcccagatgagtgtgg gtcagagagccaaactgactatctccagattatgctatggtgccactgggcacccaggcatcatccaccacatgcc actctcgtcttcgatgtggagcttctaaaactggaataagaattcCTAGAGCTCGCTGATCAGCCTC GACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCCTCCCCCGTGC CTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAAT GAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGG GTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAgAATAGCA GGCATGCTGGGGAgcggccgcaggaacccctagtgatggagttggccactccctctcgcgcgctcgc gctcactgaggccggcgaccaaaggtcgccgcacgcccgggctttgcccggcgccctcagtgagcgagcgagc gcgagctgctgcagggcgccgtatgcgggtattttctccttacgcatctgtgcgggtatttcacaccgcatacgtcaaag caaccatagtagcgccctgtagcgcgcatgaagcgcgggcggtgtgtgtgttacgcgcagcggtaccgctacactt gccagcgccctagcgccgctccttcgctttctccctcctttctcgcacgctcgccggctttcccgtaagctctaaa tcgggggctcccttaggggtccgatttagtgccttacggcacctcgacccaaaaaacttgattgggtgatgtgtacgt agtggggccatcgccctgatagacgggttttcgccccttgacgttggagtccacgttcttaataaggactctgttcaaac tggaacaacactcaaccctatctcgggctattctttgattataagggtatttgcgatttcggcctatttggttaaaaaatga gctgatttaacaaaaattaacgcgaatttaacaaaatattaacggttacaattttatggtgcactctcagtacaatctgctct gatgccgcatagttaagccagccccgacaccgccaacaccgctgacgcgcccctgacgggctgtctgtctccggc atccgcttacagacaagctgtgaccgctcgcgggagctgcatgtgtcagaggtttcacgcctacaccgaacgcgcg agacgaaaggcctcgtgatacgccctattttataggttaagtcatgataataatggtttcttagacgtcaggtggcactttt cggggaaatgtgcgcggaacccctatttggattttctaaatacattcaaatatgtatccgctcatgagacaataaccctg ataaatgtctcaataatattgaaaagggaagagtatgagtattcaacatttccgtgctgcccctattccctttttgcccatttt gccttctctgttttctcaccagaaacgctggtgaaagttaaagatgctgaagatcagttgggtgcacgagtggtgttac atcgaaactggatctcaacagcggtgaagatccttgagagtttcgccccgaagaacggtttccaatgatgacacttttaaa gttctgctatgtggcgcggtattatcccgtattgacgcccggcaagagcaactcggtcgccgcatacactattctcagaat gacttggttgagtactcaccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgtgccat aaccatgagtataactgcggccaacttactctgacaacgatcgaggaccgaaggagtaaccgctttttgcaca acatgggggatcatgtaactgccttgatcgttgggaaccggagctgaatgaagccataccaacgacgagcgtgaca ccacgatgcctgtagcaatggcaacaacggttgcgcaactattaactggcgaactacttactctagcttcccggcaacaa ttaatagactggatggagcggtgataaagttgcaggaccacttctgcgctcgcccttccgctggtgtgttattgctgat aaatctggagccggtgagcgtggaagccgcggtatcattgcagcactggggccagatggtgaagccctcccgtatcgta gttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagatagggtgctcactgatta agcattggttaactgtcagaccaagtttactcatatatactttagattgatttaaaacttcatttttaattaaaaggatcaggtg aagatcccttttgataatctcatgacaaaatcccttaacgtgagtttcttccactgagcgtcagaccccgtagaaaagat caaaggatcttcttgatccctttttctgcgcgtaatctgctgcttgcacacaaaaaaccaccgctaccagcggtggtt gtttgcccgatcaagagctaccaactcttttccgaaggtaactggcttcagcagagcgagataccaataactgtccttct agtgtagccgtagtttaggccaccacttcaagaactctgtagcaccgcctacatccctgctctgtaactctgttaccagt ggctgctgcccagtggcgataagtcgtgtcttaccgggttgactcaagacgatagttaccggataaggcgagcggtc gggctgaacggggggttcgtgcacacagcccagcttgagcggaacgacctacaccgaactgagatacctacagcgt gagctatgagaaagcgccacgcttcccgaaggagaaaggcgagaggtatccggtgaagcgaggggtcggaaca ggagagcgacgagggagcttccaggggaaacgctggtatctttatagtcctgtcggttttcgacactctgacttg agcgtcgattttgtgatgctcgtcagggggcgagcctatggaaaaacgccagcaacggcgctttttacgggtcctg gccttttgcctggttttgcacatgt
3. pX330 LpCt 2xFKBP SpCas 9	gagggcctatttcccatgattccctcatatttgcataacgatacaaggctgttagagagataattggaattaattgactgta aacacaaagatattagtacaaaactgtagcgtagaaagtaataattcttgggtagtttcagttttaaattatgttttaaaa tggaactatcatatgcttaccgtaacttgaagatttctgatttcttggctttatatacttGTGGAAAGGACGAA ACACCggGTCTTCgaGAAGACctgttttagagctaGAAAtagcaagttaaataaggctagtcggtta

Table 1: Plasmid Sequences For Variant SpCas9	
(SEQ ID NO:3)	tcaacttgaaaaagtggcaccgagtcggtgcTTTTTgttttagagctagaaatagcaagttaaaataaggctagtc cgtTTTTagcgcgtgcgccaattctgcagacaaatggctctagaggtaccggttacataaacttacggtaaatggcccg cctggctgaccgccaacgacccccgccattgacgtcaatagtaacgccaatagggactttccattgacgtcaatggg tggagtatttacggtaaaactgccacttggcagtagatcaagtgtatcatatgccaagtacgccccctattgacgtcaatga cggtaaatggcccgctggcattGtggccagtagatgacattatgggactttcctacttggcagtagatctacgtattagtc atcgctattaccatggtcgaggtgagccccacgttctgcttcaacttccccatctccccccctccccaccccccaatttgc atttatttttttaattatttgcagcagatggggcgggggggggggggggcgcgccaggcgggcgggcgggc ggggcgaggggcgggcgggcgaggcgagaggtgcgggcgagccaatcagagcgcgcgctccgaaagt ttcctttatggcgaggcgggcgggcgggcgccctataaaaagcgaagcgcgggcgggcgggagtcgctgcgac gctgccttcgccccgtgccccgctccgcccgcctcgccgccccgggctctgactgaccgcgttactccac aggtgagcgggcgggacggccttctcctcggggtgaattagctgagcaagaggttaagggttaagggtggttg ttggtgggtattaatgttaattacctggagcacctgcctgaaatcactttttcaggttGgaccggtgccaccATGG ACTATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTACA AAGACGATGACGATAAGATGGCCCCAAAGAAGAAGCGGAAGGTCGGTA TCCACGGAGTCCCAGCAGCCGGATCCGACAAGAAGTACAGCATCGGCC TGGACATCGGCACCAACTCTGTGGGCTGGGCCGTGATCACCGACGAGTA CAAGGTGCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGGCA CAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTTCGACAGCGGCGA AACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACAC CAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGCAACGA GATGGCCAAGGTGGACGACAGCTTCTTCCACAGACTGGAAGAGTCCTTC CTGGTGGAAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCGGCAAC ATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCACCATCTACCACC TGAGAAAGAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGA TCTATCTGGCCCTGGCCACATGATCAAGTTCCGGGGGCCACTTCCTGATC GAGGGCGACCTGAACCCCGACAACAGCGACGTGGACAAGCTGTTTCATC CAGCTGGTGCAGACCTACAACCAGCTGTTTCGAGGAAAACCCCATCAACG CCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGA GCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGGAAGTGGCA GCggagtgaggtggaaccatctcccaggagacgggcgaccttcccaagcgcgccagacctgcgtggtgc actacaccgggatgctgaagatggaagaaagttgattcctccgggacagaacaagcccttaagttatgctaggc aagcaggaggtgatccgaggtgggaagaagggttgccagatgagtggtgagagagccaaactgactatac tccagattatgcctatggtgccactgggacccaggcatcatccaccacatgccactctgcttctgatgtggagcttct aaaactgaaGGCAGTGGAAGCGAGAAGAAGAATGGCCTGTTTCGGAACCTG ATTGCCCTGAGCCTGGGCCTGACCCCCAAGTTCAGAGCAACTTCGACC TGGCCGAGGATGCCAACTGCAGCTGAGCAAGGACACCTACGACGACG ACCTGGACAACCTGCTGGCCAGATCGGCGACCAAGTACGCCGACCTGTT TCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGCTGAGCGACATCCTG AGAGTGAACACCGAGATCACCAAGGCCCCCTGAGCGCCTCTATGATCA AGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGT GCGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGC AAGAACGGCTACGCCGGCTACATTGACGGCGGAGCCAGCCAGGAAGAG TTCTACAAGTTCATCAAGCCATCCTGGAAAAGATGGACGGCACCGAGG AACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGGAAGCAGCGGA CCTTCGACAACGGCAGCATCCCCACCAGATCCACCTGGGAGAGCTGCA CGCCATTCTGCGGCGGCAGGAAGATTTTACCCATTCTGAAGGACAAC CGGGAAGATCGAGAAGATCCTGACCTTCCGCATCCCTACTACGTGG GCCCTCTGGCCAGGGGAAACAGCAGATTTCGCTGGATGACCAGAAAGA

Table 1: Plasmid Sequences For Variant SpCas9

GCGAGGAAACCATCACCCCTGGAACCTTCGAGGAAGTGGTGGACAAGG GCGCTTCCGCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAGAA CCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTACGAGTAC TTCACCGTGTATAACGAGCTGACCAAAGTGAAATACGTGACCGAGGGA ATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAGGCCATCGTG GACCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTGAAA GAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATCTCCG GCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCT GAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGA CATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGGACAGAGAG ATGATCGAGGAACGGCTGAAAACCTATGCCCACCTGTTTCGACGACAAA GTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGCAGGCTG AGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACA ATCCTGGATTTCTGAAGTCCGACGGCTTCGCCAACAGAACTTCATGC AGCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACATCCAGAAAG CCCAGGTGTCCGGCCAGGGCGATAGCCTGCACGAGCACATTGCCAATCT GGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACAGTGAAGGT GGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGAACAT CGTGATCGAAATGGCCAGAGAGAACCAGACCACCCAGAAGGGACAGAA GAACAGCCGCGAGAGAATGAAGCGGATCGAAGAGGGCATCAAAGAGCT GGGCAGCCAGATCCTGAAAGAACACCCCGTGGAAGAACACCCAGCTGCA GAACGAGAAGCTGTACCTGTACTACCTGCAGAATGGGCGGGATATGTAC GTGGACCAGGAACCTGGACATCAACCGGCTGTCCGACTACGATGTGGACC ATATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCGACAACAAGGT GCTGACCAGAAGCGACAAGAACCAGGGGCAAGAGCGACAACGTGCCCTC CGAAGAGGTCGTGAAGAAGATGAAGAACTACTGGCGGCAGCTGCTGAA CGCCAAGCTGATTACCCAGAGAAAAGTTCGACAATCTGACCAAGGCCGA GAGAGGCGGCCTGAGCGAACTGGATAAAGGCCGGCTTCATCAAGAGACA GCTGGTGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGA CTCCCGGATGAACACTAAGTACGACGAGAATGACAAGCTGATCCGGGA AGTGAAAGTGATCACCTGAAGTCCAAGCTGGTGTCCGATTTCCGGAAG GATTTCCAGTTTTACAAAGTGCGCGAGATCAACAACTACCACCACGCCC ACGACGCCTACCTGAACGCCGTCTGTGGGAACCGCCCTGATCAAAAAGTA CCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGAC GTGCGGAAGATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCTACC GCCAAGTACTTCTTCTACAGCAACATCATGAACTTTTTCAAGACCGAGA TTACCCTGGCCAACGGCGAGATCCGGAAGCGGCCTCTGATCGAGACAA ACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATTTTGCCA CCGTGCGGAAAGTGCTGAGCATGCCCAAGTGAATATCGTGAAAAAGA CCGAGGTGCAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCCAAGA GGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTGGGACCCTAAGA AGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGTGCTGGTGGT GGCCAAAGTGGAAGAGGGCAAGTCCAAGAACTGAAGAGTGTGAAAG AGCTGCTGGGGATCACCATCATGGAAAGAAGCAGCTTCGAGAAGAATC CCATCGACTTTCTGGAAGCCAAGGGCTACAAAGAAGTGAAAAAGGACC TGATCATCAAGCTGCCTAAGTACTCCCTGTTTCGAGCTGGAAAACGGCCG GAAGAGAATGCTGGCCTCTGCCGGCGAACTGCAGAAGGGGAAACGAACT GGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGGCCAGCCACTAT

Table 1: Plasmid Sequences For Variant SpCas9

	GAGAAGCTGAAGAGGCTCCCCGAGGATAATGAGCAGAAACAGCTGTTT GTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGC GAGTTCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTGGACAAAGTGC TGTCCGCCTACAACAAGCACCGGGATAAGCCCATCAGAGAGCAGGCCG AGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCCCCCTGCCGC CTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACC AAAGAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGT ACGAGACACGGATCGACCTGTCTCAGCTGGGAGGCGACAAAAGGCCGG CGGCCACGAAAAAGGCCGGCCAGGCAAAAAAGAAAAAGggagtgcaggtgaa accatctccccaggagacgggcgccactcccccaagcgcggccagacctgcgtggtgcactacacgggatgcttga agatggaaagaaagttgattctcccgggacagaacaagccctttaagtattatgctaggcaagcaggaggtgatccga ggctgggaagaaggggttggccagatgagtgtgggtcagagagccaaactgactatatctccagattatgcctatggtg ccactgggacaccaggcatcatccaccacatgccactctcgtcttcgatgtggagcttctaaaactggaataagaattc CTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCT GTTGTTTGCCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCC CACTGTCTTTTCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGT AGGTGTCATTCTATTCTGGGGGGGTGGGGTGGGGCAGGACAGCAAGGGG GAGGATTGGGAAGAgAATAGCAGGCATGCTGGGGAgcggccgcaggaacccctagt gatggagtgtggccactccctctctgcgcgctcgtcgtcactgaggccggggcgaccaaaaggtcgcccgacgcccgg gctttgcccggggcgccctcagtgagcgagcgagcgcgagctgcctgcaggggcgccgtgatcggtattttctcctta cgcactctgtcggttattcacaccgcatacgtcaaagcaaccatagtacgcgcctgtagcggcgcatlaagcgcggc gggtgtgtgtgttacgcgcagcgtgaccgtacacttgcagcgccctagcgcccgctcctttcgtttcttcccttcttt ctcgccacgttcgcccgtttccccgtcaagctctaaatcgggggctccctttagggttccgatttagtgccttacggcacc tcgaccccaaaaaacttgatttgggtgatggttcacgtagtggggccatcgccctgatagacggtttttcgccctttgacgtt ggagtccacgttctttaatagtggactcttgttccaaactggaacaacactcaaccctatctcgggctattcttttgattataa gggattttgccgatttcggcctattggttaaaaaatgagctgatttaacaaaaatttaacgcgaattttaacaaaatattaacg tttacaattttatggtgcactctcagtacaatctgctctgatgcccatagttaaagccagccccgacaccgccaacacc gctgacgcgcccgtacgggcttgcctgcctcccgcatccgcttacagacaagctgtgaccgtctccgggagctgcatgt gtcagagggtttaccgtcatcaccgaaacgcgcgagacgaaaggccctcgatagccctattttataggttaagtca tgataataatggtttcttagacgtcagggtggcacttttcggggaaatgtgcgcgggaaccctatttgtttatttttctaataca ttcaaatatgtatccgctcatgagacaataaacctgataaatgcttcaataatattgaaaaaggaagaglatgagtattcaa catttcgctgcgcccttattccctttttgcggcattttgccttctgttttgtcaccagaaacgctggtgaaagtaaaag atgctgaagatcagttgggtgcacgagtggtgttacatcgaactggatctcaacagcggttaagatccttgagagtttcgc cccgaagaacgttttcaatgatgagcacitttaaagttctgctatgtggcgcggtattatcccgtattgacgccgggcaa gagcaactcggtgcgcgcatacactattctcagaatgacttgggtgagtactaccagtcacagaaaagcatcttacgga tggcatgacagtaagagaattatgcagtgcctgcataaccatgagtataacactgcggccaacttactctgacaacga tcggaggaccgaaggagctaaccgctttttgcacaacatgggggatcatgtaactgccttgatcgttgggaaccgga gctgaatgaagccataccaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaactatt aactggcgaaacttactctagcttcccggcaacaattaatagactggatggaggcgataaaagtgcaggaccacttc tgctctcgcccttccggctggctggttattgtctgataaatctggagccggtgagcgtggaagccgcggtatcattgca gcaactggggccagatggttaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggtgaacga aatagacagatcgctgagataggtgcctcactgattaagcattggttaactgtcagaccaagtttactcataataactttagat tgatttaaaacttcaattttaatttaaaggatctaggtaagatccttttgataatctcatgaccaaatacccttaacgtgagt ttcgttccactgagcgtcagaccctgataaaagatcaaggatcttcttgagatccttttttctgcgcgtaattctgctgct tgcaaacaaaaaaaccaccgtaccagcggtggtttgtttgcccgatcaagagctaccaactcttttccgaaggttaact ggcttcagcagagcgcagataccaataactgtccttctagttagccgtagttagccaccacttcaagaactctgtagc accgctacataacctcgctctgtaactctgttaccagtggctgctgccagtggcgataagtctgtcttaccgggttgga ctcaagacgatagttaccggataaggcgccagcggtcgggctgaacggggggttcgtgcacacagcccagcttggag
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Table 1: Plasmid Sequences For Variant SpCas9	
	cgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcggcacgcttcccgaagggagaaagg cggacaggtatccggaagcggcagggcgggaacaggagagcgcacgagggagcttccagggggaacgcctgg tatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgattttgtgatgctcgtcagggggcggagcctatg gaaaaacgccagcaacgcggccttttacggttcctggccttttctgctggccttttctcacatgt
4. pX330 NtLpCt 3xFKBP SpCas9 (SEQ ID NO:4)	gagggcctatttcccatgattcctcatatttgcataacgatacgaaggctgttagagagataattggaattaattgactgta aacacaaagatattagtacaaaacgtgacgtagaaagtaataatttctgggtagtttcagttttaaattatgttttaaaa tggactatcatatgcttacgtaacttgaaagtatttcgatttcttggcctttatatacttGTGGAAAGGACGAA ACACCggGTCTTCgaGAAGACctgttttagagctaGAAAtagcaagttaaataaggctagtcctgta tcaactgaaaaagtggcaccgagtcggtgcTTTTTgttttagagctagaaatagcaagttaaataaggctagtc cgtTTTTagcgcgtgcgccaattctgcagacaaatggcctagaggtagccgttacataacttacggtaaatggccc cctggctgaccgccaacgacccccgccattgacgtcaatagtaacgccaatagggactttccattgacgtcaatggg tggagtatttacggtaaaactgcccacttggcagtagcatcaagtgtatcatatgccaagtacgccccctattgacgtcaatga cggtaaatggcccgcctggcattGtcccagtagcatgacctatgggactttcctacttggcagtagcatctacgtatttagtc atcgctattaccatggcgcaggtgagccccacgttctgcttcaacttccccatccccccccctccccaccccccaatttgt atttatttttttaattttttgtgcagcgatggggggcggggggggggggggggggcgcgcgccaggcgggggcggggc ggggcgaggggcgggggcgggggcgaggcggagaggtgcggcggcagccaatcagagcggcgcgctccgaaagt ttccttttatggcaggcggcgggcgggcgggccctataaaaagcgaagcgcggcgggcggggagtcgctgcgac gctgccttgcggcggtgccccgctccgcccgcctcgcggcgcccggcgctgactgaccgcttactccac aggtgagcggggcgggacggcccttctcctcgggctgtaattagctgagcaagaggttaagggttaagggtggttg ttggtgggttattaattgttaattacctggagcacctgctgaaatcacttttttcagggttGAccgggtgccaccATGG ACTATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTACA AAGACGATGACGATAAGATGGCCCCAAAGAAGAAGCGGAAGGTCTGGTA TCCACGGAGTCCCAGCAGCCGGATCCggagtgcaggtggaaccatctccccaggagacgg gcgcaccttccccaaagcggccagacctgcgtggtgcactacaccgggatgcttgaagatggaagaaagttgattc ctccgggacagaacaagccctttaagtttatgctaggcaagcaggaggtgatccgaggtgggaagaaggggttg cccagatgagtgtgggtcagagagccaaactgactatatctccagattatgcctatggtgccactgggcacccaggcat catccaccacatgccactctgcttctgatgtggagcttctaaactggaaGACAAGAAGTACAGCAT CGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTGATCACCGAC GAGTACAAGGTGCCAGCAAGAAATTCAGGTGCTGGGCAACACCGAC CGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGC GGCGAAACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAG ATACACCAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGC AACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGACTGGAAGAG TCCTTCCTGGTGGAAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCG GCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCACCATCTA CCACCTGAGAAAGAACTGGTGGACAGCACCGACAAGGCCGACCTGCG GCTGATCTATCTGGCCCTGGCCACATGATCAAGTTCCGGGGCCACTTC CTGATCGAGGGCGACCTGAACCCCGACAACAGCGACGTGGACAAGCTG TTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTTCGAGGAAAACCCCA TCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAG CAAGAGCAGACGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGGAAG TGGCAGCggagtgcaggtggaaccatctccccaggagacggcgccaccttccccaaagcggccagacctg cgtgtgtgcactacaccgggatgcttgaagatggaaagaaagttgattcctccgggacagaacaagccctttaagttta tgctaggcaagcaggaggtgatccgaggtgggaagaaggggtgcccagatgagtgtgggtcagagagccaaact gactatatctccagattatgcctatggtgccactgggcaccagagcatcatccaccacatgccactctgcttctgatgt ggagcttctaaaactggaaGGCAGTGGAAAGCGAGAAGAAGAATGGCCTGTTCGGA

Table 1: Plasmid Sequences For Variant SpCas9

AACCTGATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACT TCGACCTGGCCGAGGATGCCAACTGCAGCTGAGCAAGGACACCTACG ACGACGACCTGGACAACCTGCTGGCCAGATCGGCGACCAGTACGCCG ACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGCTGAGCGA CATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCCTGAGCGCCTCT ATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAA GCTCTCGTGCGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCG ACCAGAGCAAGAACGGCTACGCCGGCTACATTGACGGCGGAGCCAGCC AGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTGGAAAAGATGGACG GCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGGA AGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGG AGAGCTGCACGCCATTCTGCGGCGGCAGGAAGATTTTTTACCCATTCTG AAGGACAACCGGGAAAAGATCGAGAAGATCCTGACCTTCCGCATCCCC TACTACGTGGGCCCTCTGGCCAGGGGAAACAGCAGATTCGCCTGGATGA CCAGAAAAGAGCGAGGAAACCATCACCCCTGGAACCTCGAGGAAGTGG TGGACAAGGGCGCTTCCGCCAGAGCTTCATCGAGCGGATGACCAACTT CGATAAGAACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCT GTACGAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATACGTG ACCGAGGGAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAG GCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGC AGCTGAAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGA AATCTCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCAC GATCTGCTGAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAA AACGAGGACATTCTGGAAGATATCGTGCTGACCCTGACACTGTTTGAGG ACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCCACCTGTTCG ACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGG GCAGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCG GCAAGACAATCCTGGATTTCTGAAGTCCGACGGCTTCGCCAACAGAAA CTTCATGCAGCTGATCCACGACGACAGCCTGACCTTTAAAGAGGACATC CAGAAAGCCCAGGTGTCCGGCCAGGGCGATAGCCTGCACGAGCACATT GCCAATCTGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACA GTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCC GAGAACATCGTGATCGAAATGGCCAGAGAGAACCAGACCACCCAGAAG GGACAGAAGAACAGCCGCGAGAGAATGAAGCGGATCGAAGAGGGCAT CAAAGAGCTGGGCAGCCAGATCCTGAAAGAACACCCCGTGGAACACAC CCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAAATGGGCG GGATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTAC GATGTGGACCATATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCG ACAACAAGGTGCTGACCAGAAGCGACAAGAACCGGGGCAAGAGCGAC AACGTGCCCTCCGAAGAGGTCTGTGAAGAAGATGAAGAACTACTGGCGG CAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTGACAATCTGA CCAAGGCCGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCA TCAAGAGACAGCTGGTGGAACCCGGCAGATCACAAAGCACGTGGCAC AGATCCTGGACTCCCGGATGAACACTAAGTACGACGAGAATGACAAGC TGATCCGGGAAGTGAAAGTGATCACCTGAAGTCCAAGCTGGTGTCCGA TTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAACCTAC CACCACGCCCACGACGCCTACCTGAACGCCGTCTGGGAACCGCCCTGA TCAAAAAGTACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACA

Table 1: Plasmid Sequences For Variant SpCas9

AGGTGTACGACGCTGCGGGAAGATGATCGCCAAAGACGAGCAGGAAATCG
GCAAGGCTACCGCCAAGTACTTCTTCTACAGCAACATCATGAACTTTTTTC
AAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGGCCTCTG
ATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGG
GATTTTGGCACCCTGCGGAAAGTGCTGAGCATGCCCAAGTGAATATCG
TGAAAAAGACCGAGGTGCAGACAGGCGGCTTCAGCAAAGAGTCTATCC
TGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGACTGGG
ACCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTCTGT
GCTGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCCAAGAACTGAAGAG
TGTGAAAGAGCTGCTGGGGATCACCATCATGGAAAGAAGCAGCTTCGA
GAAGAATCCCATCGACTTTCTGGAAGCCAAGGGCTACAAAGAAGTGAA
AAAGGACCTGATCATCAAGCTGCCTAAGTACTCCCTGTTCGAGCTGGAA
AACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAACTGCAGAAGGGA
AACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGGCCA
GCCACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAAC
AGCTGTTTGTGGAACAGCACAAGCACTACCTGGACGAGATCATCGAGCA
GATCAGCGAGTTCTCCAAGAGAGTGATCCTGGCCGACGCTAATCTGGAC
AAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAGCCCATCAGAGAG
CAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCCC
CTGCCGCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACAC
CAGCACCAAGAGGGTGCTGGACGCCACCCTGATCCACCAGAGCATCAC
CGGCCTGTACGAGACACGGATCGACCTGTCTCAGCTGGGAGGCGACAA
AAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAAAAAAGAAAAAGgg
agtgcaggtggaaccatctccccaggagacgggcgaccltccccaaagcgcgccagacctcggtggtgcactac
accgggatgctgaagatggaagaaggttgattcctccgggacagaacaagcccttaagtftatgctaggcaagc
aggaggtgatccgaggtcggaagaaggggtgcccagatgagtgtgggtcagagagccaaactgactatatctcca
gattatgcctatggtgccactgggcaccaggaatcatccaccacatgccactctcgtcttcgatgtggagcttctaaaa
ctggaataagaattcCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGC
CAGCCATCTGTTGTTTGGCCCCCCCCGTGCCTTCCTTGACCCTGGAAGG
TGCCACTCCCCTGTCTTTTCTAATAAAATGAGGAAATTGCATCGCATT
GTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAG
CAAGGGGGAGGATTGGGAAGAgAATAGCAGGCATGCTGGGGAGcgggccgca
ggaaccctagtgatggagttggccactccctctctgcgcgtctgctgcctcactgaggccggggcgaccaaggtcgc
ccgacgccccgggctttgccccggggcgccctcagtgagcgagcgagcgcgagctgcctgcagggggcgccctgatgcg
gtattttctccttacgcatctgtgcggtatttcacaccgcatacgtcaaaagcaaccatagtagcgccctgtagcggcgcat
taagcgcgggcggtgtggtgttacgcgcgagcgtgaccgctacacttgccagcgccctagcgccccgtcctttcgcttt
cttccctctcttctcgccacgttcgcgggctttccccgtcaagctctaaatcgggggctccctttagggttccgatttagtg
ctttacggcacctcgaccccaaaaaacttgatttgggtgatggttcacgttagtgggcatcgccctgatagacggtttttc
gccccttgacgttgagtcacgttctttaatagtggactctgttccaaactggaacaacactcaaccctatctcgggctat
tcttttgattataagggtatttgcgatttcggcctattggttaaaaaatgagctgatftaacaataatttaacgcgaattftaa
caaaatftaacgtttacaattttatggtgcactctcagtacaatctgctctgatgccgcatagtttaagccagccccgacac
ccgccaacaccgctgacgcgcccctgacgggcttctgctctccgggcatccgcttacagacaagctgtgaccgtctcc
gggagctgcatgtgtcagaggtttaccgctcatcaccgaaacgcgcgagacgaaagggcctcgtagacgcctattttt
atagggttaatgtcatgataataatggtttcttagacgtcaggtggcacttttggggaaatgtgcgcggaaccctatttggtt
atttttctaaatacattcaaatatgtatccgctcatgagacaataaaccttgataaatgcttcaataatattgaaaaaggaaga
gtatgagtattcaacatttccgtgtcgcccttattcccttttttgcggcattttgccttctgtttttgtcaccagaaacgctg
gtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggaatctcaacagcggttaagatcc
ttgagagttttgcggccgaagaacgcttttccaatgatgagcacttttaaaagtctgctatgtggcgcggtattatccccgtattg

Table 1: Plasmid Sequences For Variant SpCas9	
	acgccgggcaagagcaactcggctcgccgcatacactattctcagaatgacttgggtgagtactcaccagtcacagaaaa gcatcttacggatggcatgacagtaagagaattatgcagtgctgccataacctagtgataaacactcgggccaacttac ttctgacaacgatcggaggaccgaaggagctaaccgctttttgcacaacatgggggatcatgtaactcgcccttgatcgtt gggaaccggagctgaatgaagccatacacaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgtt gcgcaactattaactggcgaaactacttactctagcttcccggcaacaattaatagactggatggaggcggataaagtgtg caggaccacttctgcgtcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtggaagccg cggatcattgcagcactggggccagatggtaagccctcccgtatcgtatctacacgacggggagtcaggcaact atggtatgaacgaaatagacagatcgctgagatagggtcctcactgattaagcattggtaactgtcagaccaagttaactca tatatacttttagattgatttaaaacttcatttttaatttaaaggatctagggtgaagatccttttgataatctcatgacaaaatc ccttaacgtgagtttctgctccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttctgcg cgtaatctgctgcttcaaacaaaaaaccaccgctaccagcgggtggttgggttgcgggatcaagagctaccaactctttt ccgaaggttaactggcttcagcagagcgcagatacacaactgtccttctagtgtagccgtagtagccaccacttcaa gaactctgtagcaccgctacatacctcgtctgtaactctgttaccagtggctgctgagtgagtgataagtcgtgtctt accgggttgactcaagacgatagttaccggataaggcgcagcggctgggctgaacgggggggtcgtgcacacagc ccagcttgagcgaacgacacacacgaactgagatctacagcgtgagctatgagaagcgccacgcttcccgaa gggagaaaggcggacaggtatccggtaagcggcagggctcggaacaggagagcgcacgagggagcttccagggg gaaacgccttggtatctttatagtcctgtcgggttcgacacctctgacttgagcgtcgatttttgtgatgtcgtcaggggg gcggagcctatggaaaaacgccagcaacgcggccttttacgggtccttggccttttgcctggccttttgcctcacatgt

Table 2: Expression Vector	
pET NtLp 2xFKBP SpCas9 (S1) ID NO:5)	GAATGAAGCCATACCAACGACGAGCGTGACACCACGATGCCTGCAGCA ATGGCAACAACGTTGCGCAAACTATTAAGTGGCGAACTACTTACTCTAGC TTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGA CCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCT GGAGCCGGTGAGCGTGGTAGTCGCGGTATCATTGCAGCACTGGGGCCAG ATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCA ACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGA TTAAGCATTGGTAAGTGTGACACCAAGTTTACTCATATATACTTTAGATTG ATTTAAAACTTCATTTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTG ATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTTCGTTCCACTGAGCGT CAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTG CGCGTAATCTGCTGCTTGCAAACAAAAAACCACCGCTACCAGCGGTGGT TTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAGTGGCTT CAGCAGAGCGCAGATACCAAAATACTGTCTTCTAGTGTAGCCGTAGTTAG GCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTA ATCCTGTTACCAAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGG GTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGCTGA ACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCG AACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGA AGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGG AGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGT CCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCG TCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTAC GGTTCCTGGCCTTTTTGCTGGCCTTTTTGCTCACATGTTCTTTCTGCGTTATC CCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCG CTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGC GGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTT

ACACCGCAATGGTGCACCTCTCAGTACAATCTGCTCTGATGCCGCATAGTT
AAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCC
CCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCC
CGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGT
CAGAGGTTTTACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTA
GCTCATCAGCGTGGTCGTGAAGCGATTACAGATGTCTGCCTGTTTCATCC
GCGTCCAGCTCGTTGAGTTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATA
AAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCC
GTGTAAGGGGGGATTTCTGTTTCATGGGGGTAATGATACCGATGAAACGAG
AGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTG
GAACGTTGTGAGGGTAACAACCTGGCGGTATGGATGCGGCGGGACCAGA
GAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGT
GTTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAAT
GGTGCAGGGCGCTGACTTCCGCGTTTTCCAGACTTTACGAAACACGGAAAC
CGAAGACCATTTCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAGCAG
TCGCTTCACGTTTCGCTCGCGTATCGGTGATTCACTCTGCTAACCAGTAAGG
CAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGC
GCACCCGTGGGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAA
CGTTTGGTGGCGGGACCAGTGACGAAGGCTTGAGCGAGGGCGTGCAAGA
TTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGCGCTCCAGCGAAA
GCGGTCTTCGCCGAAAATGACCCAGAGCGCTGCCGGCACCTGTCCTACGA
GTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGATAGTCATGCC
CCGCGCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATC
GGTCGAGATCCCGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTT
GCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCTGTCGAGCTGCATT
AATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTTCGTATTGGGCGCCA
GGGTGGTTTTTCTTTTACCAGTGAGACGGGCAACAGCTGATTGCCCTTC
ACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGGCC
CAGCAGGCGAAAATCCTGTTTGATGGTGGTTAACGGCGGGATATAACATG
AGCTGTCTTCGGTATCGTCGTATCCCACTACCGAGATATCCGCACCAACG
CGCAGCCCGGACTCGGTAATGGCGCGCATTGCGCCCAGCGCCATCTGATC
GTTGGCAACCAGCATCGCAGTGGGAACGATGCCCTCATTACGATTTGCA
TGGTTTGTGAAAACCGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCT
ATCGGCTGAATTTGATTGCGAGTGAGATATTTATGCCAGCCAGCCAGACG
CAGACGCGCCGAGACAGAACTTAATGGGCCCCGCTAACAGCGCGATTTGC
TGGTGACCCAATGCGACCAGATGCTCCACGCCCAGTCGCGTACCGTCTTC
ATGGGAGAAAATAACTGTTGATGGGTGTCTGGTCAGAGACATCAAGA
AATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGGCATCCTG
GTCATCCAGCGGATAGTTAATGATCAGCCCACTGACGCGTTGCGCGAGAA
GATTGTGCACCGCCGCTTTACAGGCTTCGACGCCGCTTCGTTCTACCATCG
ACACCACCACGCTGGCACCCAGTTGATCGGCGCGAGATTTAATCGCCGCG
ACAATTTGCGACGGCGCGTGCAGGGCCAGACTGGAGGTGGCAACGCCAA
TCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGCGGTTGGGAATG
TAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTTTTCGAGAA
ACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACAC
CGGCATACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCC
TGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTG
CGCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTG
CATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCC

GCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCA
 CGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAA
 GTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAG
 CAACCGCACCTGTGGCGCCGGTGTATGCCGGCCACGATGCGTCCGGCGTAG
 AGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGA
 ATTGTGAGCGGATAACAATTCCTCTAGAAATAATTTTGGTTTAACTTTA
 AGAAGGAGATATACatATGGGTCATCACCATCATCATCACGGGTCCCTGCA
 GGAATCAGAAGTCAATCAAGAAGCTAAGCCAGAGGTCAAGCCAGAAGTC
 AAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGAT
 CTTCTTCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGATGGAAGCGT
 TCGCTAAAAGACAGGGTAAGGAAATGGACTCCTTAAGATTCTTGTACGAC
 GGTATTAGAATTCAAGCTGATCAGGCCCTGAAGATTGACATGGAGGA
 TAACGATATTATTGAGGCTCACCGCGAACAGATTGGAGGTgctagcgaatctgt
 atttcagggaATGggagtgcaggtggaaccatctcccaggagacgggcgaccttcccaagcgcggccagac
 ctgctggtgcactacaccgggatgctgaagatggaagaaagttgattcctccgggacagaacaagcccttaagtt
 tatgctaggcaagcaggaggtgatccgaggtggaagaagggttggccagatgagtgtggtcagagagccaaact
 gactatatctccagattatgctatggtgccactgggacccaggcatcatccaccacatgccactctcgtcttcgatgtg
 agcttctaaaactggaaGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAAC
 TCTGTGGGCTGGGCGGTGATCACCGACGAGTACAAGGTGCCCAGCAAGA
 AATTCAAGGTGCTGGGCAACACCGACCGGCACAGCATCAAGAAGAACCT
 GATCGGAGCCCTGCTGTTTCGACAGCGGCGAAACAGCCGAGGCCACCCGG
 CTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCT
 GCTATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAG
 CTTCTTCCACAGACTGGAAGAGTCTTCTGTTGGAAGAGGATAAGAAGC
 ACGAGCGGCACCCCATCTTCGGCAACATCGTGGACGAGGTGGCCTACCAC
 GAGAAGTACCCACCATCTACCACCTGAGAAAGAACTGGTGGACAGCA
 CCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCACATGATC
 AAGTTCCGGGGCCACTTCTGATCGAGGGCGACCTGAACCCCGACAACA
 GCGACGTGGACAAGCTGTTTCATCCAGCTGGTGCAGACCTACAACCAGCTG
 TTCGAGGAAAACCCCATCAACGCCAGCGGCGTGGACGCCAAGGCCATCC
 TGTCTGCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTGATCGCCCA
 GCTGCCCGGCGGAAGTGGCAGCggagtgcaggtggaaccatctcccaggagacgggcgac
 tcccaagcgcggccagacctgctggtgcactacaccgggatgctgaagatggaagaaagttgattcctccggg
 acagaacaagcccttaagtttatgctaggcaagcaggaggtgatccgaggtggaagaagggttggccagatgag
 tgtgggtcagagagccaaactgactatatctccagattatgctatggtgccactgggacccaggcatcatccaccaca
 tgccactctcgtcttcgatgtggagcttctaaaactggaaGGCAGTGGAAAGCGAGAAGAAGAATG
 GCCTGTTCGGAAACCTGATTGCCCTGAGCCTGGGCCTGACCCCCAACTTC
 AAGAGCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGAGCAAGG
 ACACCTACGACGACGACCTGGACAACCTGCTGGCCAGATCGGCGACCA
 GTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTGC
 TGAGCGACATCCTGAGAGTGAACACCGAGATACCAAGGCCCCCTGAG
 CGCCTCTATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGC
 TGAAAGCTCTCGTGCGGCAGCAGCTGCCTGAGAAGTACAAAGAGATTTTC
 TTCGACCAGAGCAAGAACGGCTACGCCGGCTACATTGACGGCGGAGCCA
 GCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTGGAAAAGATGGA
 CGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGG
 AAGCAGCGGACCTTCGACAACGGCAGCATCCCCACCAGATCCACCTGG
 GAGAGCTGCACGCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCTG
 AAGGACAACCGGGAAAAGATCGAGAAGATCCTGACCTTCCGCATCCCCCT

ACTACGTGGGCCCCTCTGGCCAGGGGAAACAGCAGATTTCGCCTGGATGAC
CAGAAAGAGCGAGGAAACCATCACCCCCTGGAACCTTCGAGGAAGTGGTG
GACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGA
TAAGAACCTGCCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTAC
GAGTACTTCACCGTGTATAACGAGCTGACCAAAGTGAAATACGTGACCG
AGGGAATGAGAAAGCCCGCCTTCCTGAGCGGCGAGCAGAAAAAGGCCAT
CGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACCGTGAAGCAGCTG
AAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATCTC
CGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCACGATCTGC
TGAAAATTATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGA
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AGCT

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[0310] The structures of several dTAG small molecules that can be used to specifically degrade FKBP domains of Cas variant proteins are provided in FIG. 1A. The insertion sites of FKBP domain on SpCas9 is provided in FIG. 1B with FKBP inserted in varying numbers and sites on SpCas9 (FIG. 1C). A western blot analysis of different constructs of FKBP-SpCas9 degradation in HEK239FT cells indicates that with an increasing amount of dTAG-47, less Cas protein was detected, confirming degradation by the small molecule degrader dTAG-47. eGFP disruption assays were also performed using dTAG-47 and dTAG-13 using both a

plasmid delivery method (FIG. 3A and 3B) and RNP delivery (FIG. 4A-4B). Increasing amounts of the degraders reduced Cas9 activity, although the dTAG-47 appears more potent in reducing Cas9 activity.

[0311] Figure 5 provides confirmation that the SpCas9-FKBP variants disclosed herein operate in *Drosophila* S2 cells. An eGFP disruption assay was performed in S2 cells using RNP delivery. Upper panels of inactive apo Cas9 control show no disruption, with active Cas9 RNP control showing eGFP disruption. In the lower panel NtLp 2xFKBP SpCas9 RNP was disrupted with indicated amounts of dTAG-47 (1000nM, 10nM, 0nM), and shows that with increasing amount of dTAG-47, less eGFP disrupted.

[0312] Various modifications and variations of the described methods, pharmaceutical compositions, and kits of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it will be understood that it is capable of further modifications and that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art are intended to be within the scope of the invention. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known customary practice within the art to which the invention pertains and may be applied to the essential features herein before set forth.

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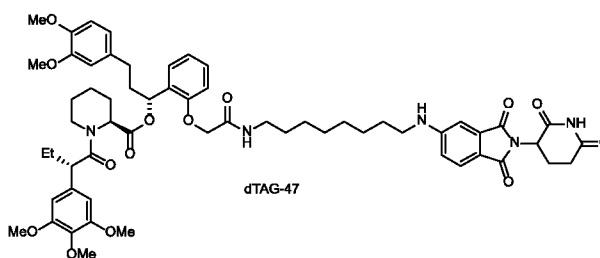
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CLAIMS

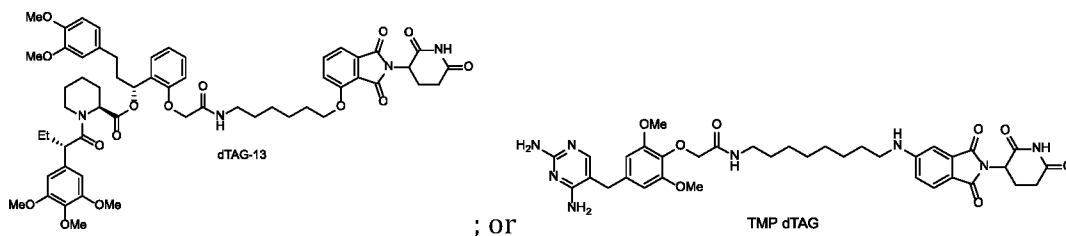
What is claimed is:

1. A variant CRISPR-Cas protein comprising one or more FK506 binding protein (FKBP) domains introduced into the CRISPR-Cas protein at one or more insertion sites.
2. The variant CRISPR-Cas protein of claim 1, wherein the CRISPR-Cas protein is a Cas9, a Cas12a, Cas12b, Cas12c, Cas12d, Cas13a, Cas13b, Cas13c, or Cas13d protein.
3. The variant CRISPR-Cas polypeptide of claim 1 that is codon optimized for expression in eukaryotes.
4. The variant of claim 2, wherein the CRISPR-Cas protein is a Cas9 or a Cas12a, Cas12b, Cas12c or Cas12d protein.
5. The variant CRISPR-Cas protein of claim 3, wherein the one or more insertion sites are at the N-terminal (Nt), C-terminal (Ct) or at a position corresponding to position 231 (Lp) of a SpCas9 protein.
6. The variant CRISPR-Cas protein any one of claims 2 to 4, wherein the one or more insertion sites are selected from: Nt and Ct; Nt and Lp; Lp and Ct; and Nt, Lp and Ct.
7. The variant CRISPR-Cas protein of anyone of claims 2 to 5, wherein the FKBP domains are FKBP12^{F36V} domains.
8. A ribonucleoprotein comprising the variant CRISPR-Cas protein of any one of claims 2 to 6.
9. A plasmid comprising the variant CRISPR-Cas protein of any one of claims 2 to 6.

10. The plasmid of claim 8, comprising a sequence selected from SEQ ID NOs: 1-4.
11. A cell transfected with the ribonucleoprotein of claim 7 or the plasmid of claims 8 or 9.
12. The cell of claim 10, wherein the cell is selected from HEK293FT, U2OS, NIH3T3 and S2.
13. A method of inducing degradation of a variant CRISPR-Cas protein, comprising:
exposing the cell of claim 11 with a compound or a pharmaceutically acceptable salt

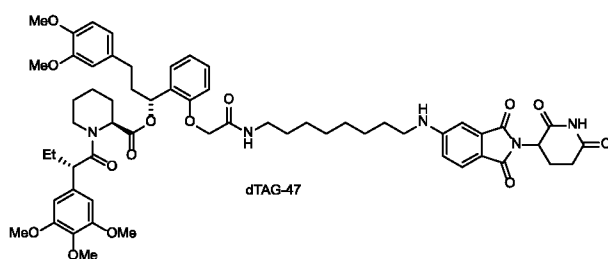


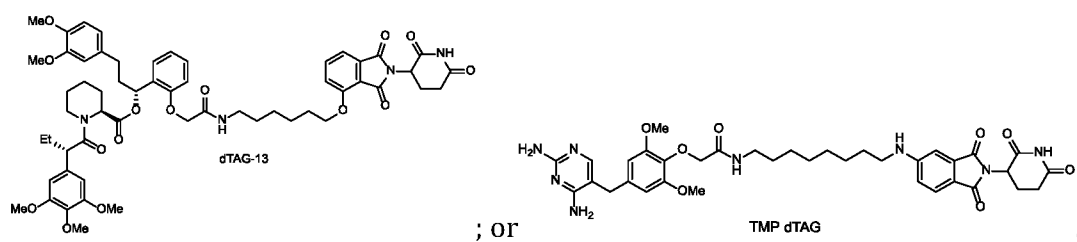
thereof according to the formula:



14. The method of claim 12, wherein the cell is a germline cell.
15. The method of claim 12, wherein the cell is in an organism.

16. The method of claim 13, wherein the exposing comprises incubating the cell with the compound or pharmaceutically acceptable salt thereof.
17. The method of claim 13, wherein the cell is exposed to the compound or pharmaceutically acceptable salt thereof at a concentration of about 1nM to about 3 μ M.
18. A method of degrading an activity of a variant CRISPR Cas protein-RNA complex, the method comprising contacting the CRISPR Cas protein-RNA complex with a heterobifunctional degrader of FBKP12^{F36V}.
19. The method of claim 17, wherein the method is performed *in vitro* or *in vivo*.
20. The method of claim 18, wherein the method is performed in a cell.
21. The method of claim 17, wherein the variant CRISPR Cas protein is CRISPR Cas 9.
22. A method of treating a subject comprising:
 - a. administering a CRISPR Cas protein-RNA complex or a reagent causing expression of a CRISPR-Cas protein-RNA complex to the subject; and
 - b. administering a heterobifunctional degrader of FBKP12^{F36V}.
23. A pharmaceutical formulation comprising the variant CRISPR Cas protein of claim 1 and a pharmaceutically acceptable carrier.
24. A kit comprising the variant CRISPR Cas protein of claim 1, and optionally a compound or pharmaceutically acceptable salt thereof according to the formula:





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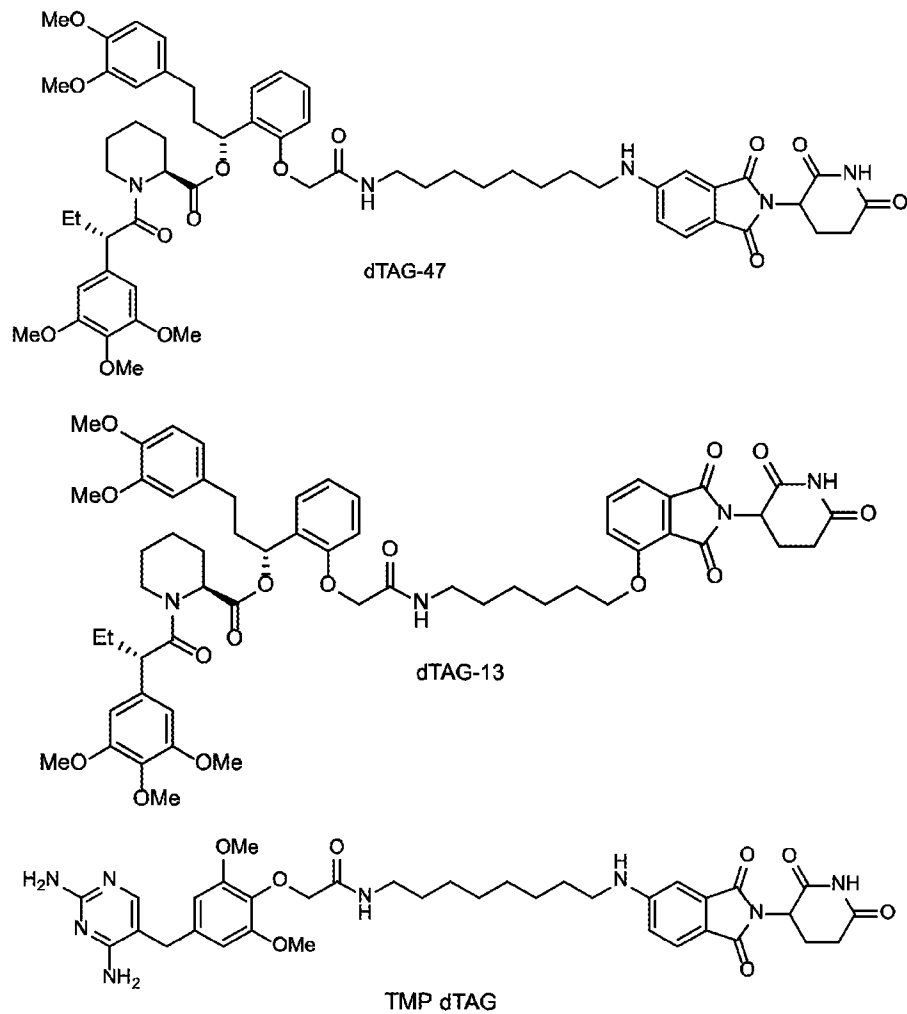


FIG. 1A

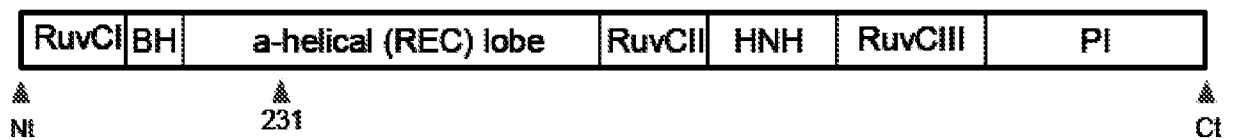


FIG. 1B



FIG. 1C

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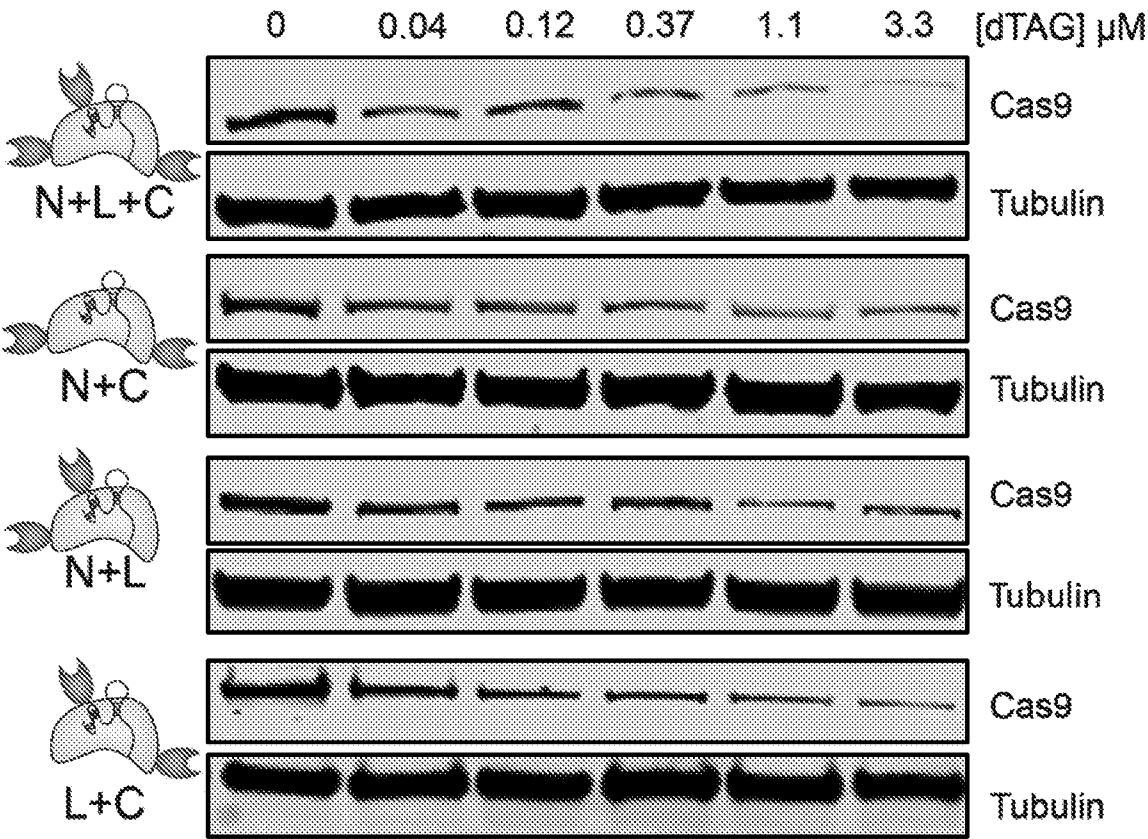


FIG. 2

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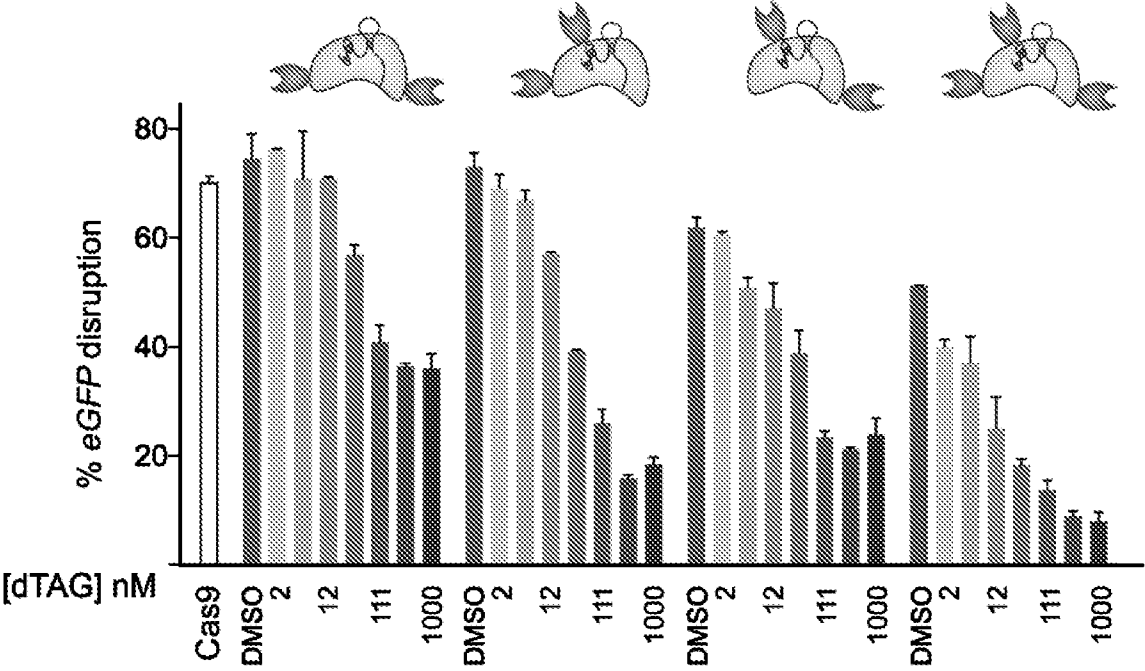


FIG. 3A

dTAG13

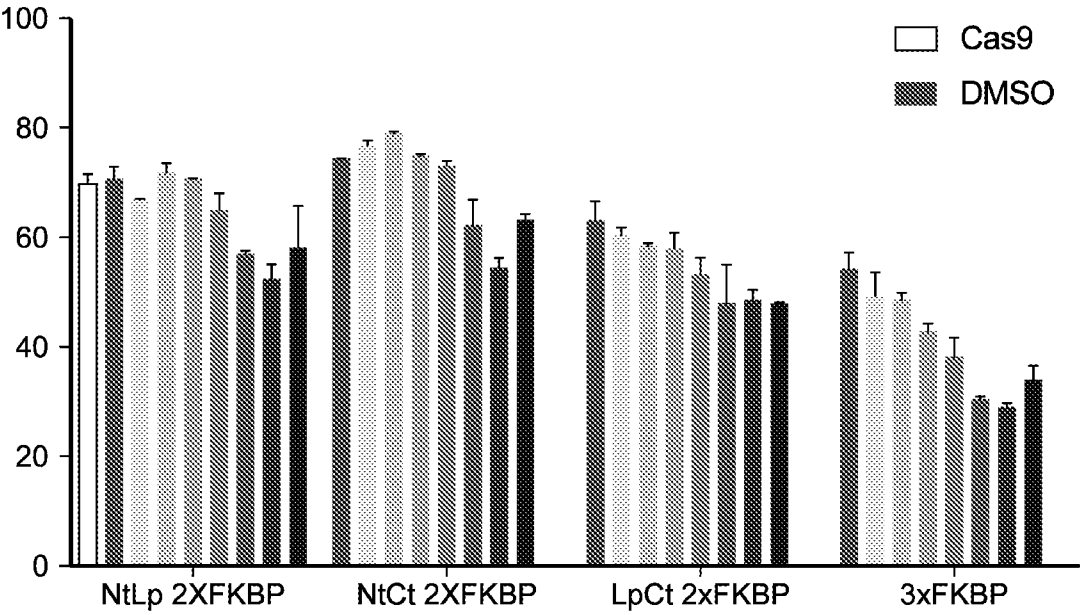


FIG. 3B

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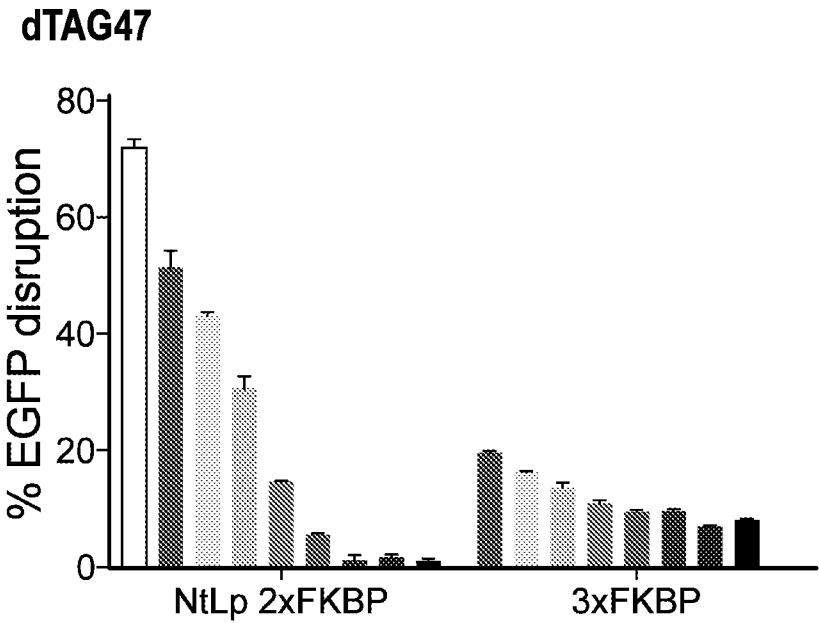


FIG. 4A

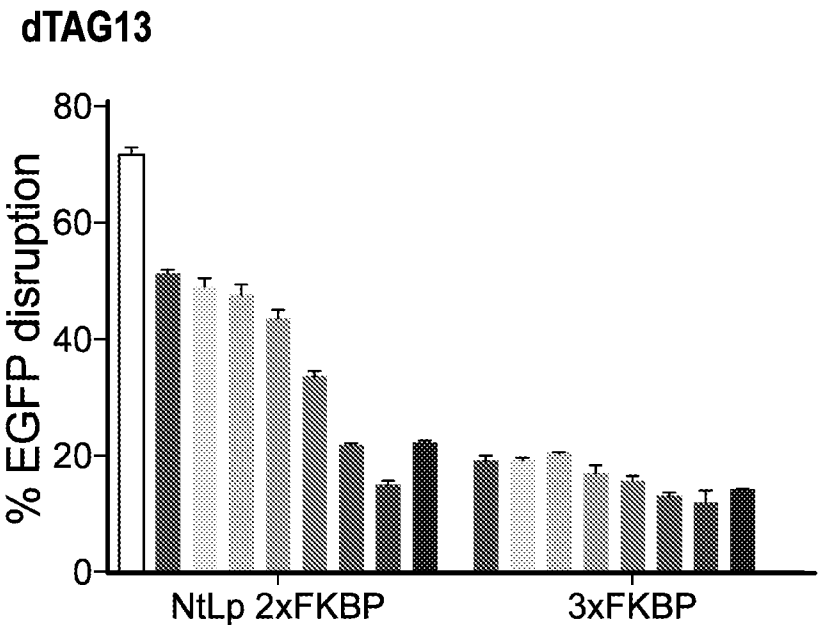
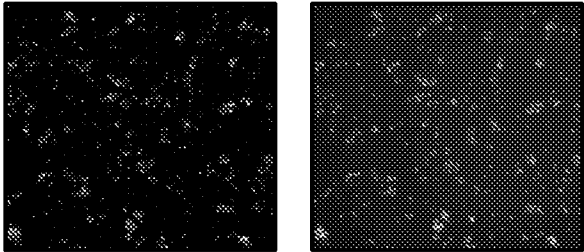


FIG. 4B

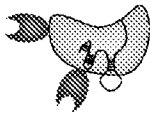
5/5

Apo Cas9

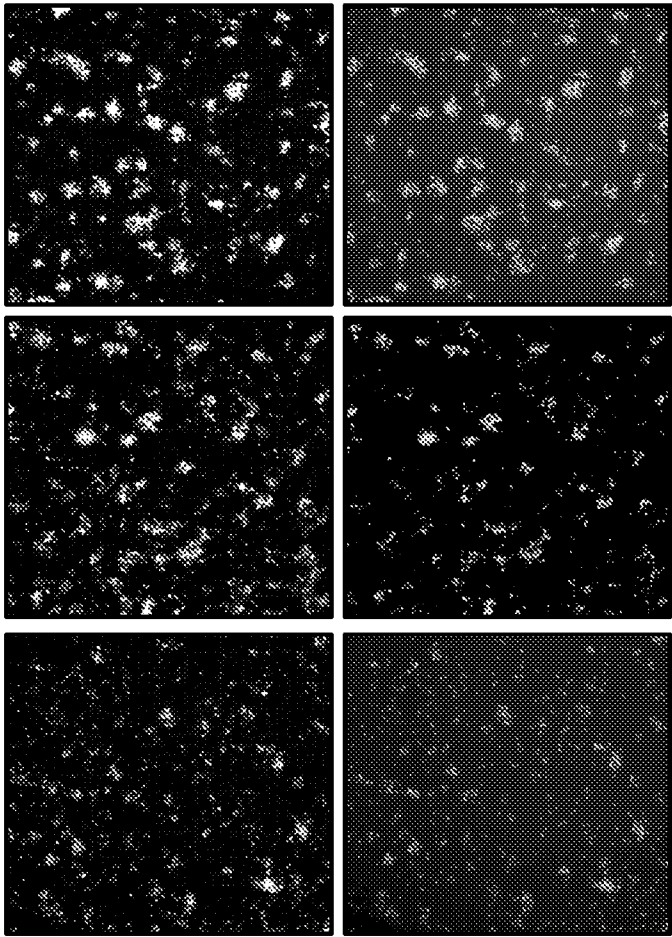

Cas9 RNP



NtLp 2xFKBP RNP



[dTAG 47]



1000nM

10nM

0nM

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/047366

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/22
ADD. C07D209/48 C07D401/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/024317 A2 (DANA-FARBER CANCER INST INC [US]) 9 February 2017 (2017-02-09) page 16, line 27 - page 17, line 4; claims 5-8, 14-18 the whole document	1-24
X	WO 2016/106244 A1 (BROAD INST INC [US]; MASSACHUSETTS INST TECHNOLOGY [US] ET AL.) 30 June 2016 (2016-06-30) cited in the application	1-9,11, 12,23,24
A	paragraph [0140]; claims 1-43 the whole document	10,13-22
X	WO 2017/048969 A1 (UNIV CALIFORNIA [US]) 23 March 2017 (2017-03-23) paragraph [0529]; claims 1, 11, 12, 18, 21, 27 the whole document	1-9,11, 12,23,24 10,13-22
A		
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Further documents are listed in the continuation of Box C.



See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

14 November 2019

Date of mailing of the international search report

22/11/2019

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European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Spindler, Mark-Peter

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/047366

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SERIF SENTURK ET AL: "Rapid and tunable method to temporally control gene editing based on conditional Cas9 stabilization", NATURE COMMUNICATIONS, vol. 8, no. 1, 22 February 2017 (2017-02-22), XP055631869, DOI: 10.1038/ncomms14370	1-9,11, 12,23,24
A	abstract; figure 1 the whole document	10,13-22
X	BERND ZETSCHE ET AL: "A split-Cas9 architecture for inducible genome editing and transcription modulation", NATURE BIOTECHNOLOGY, vol. 33, no. 2, 2 February 2015 (2015-02-02), pages 139-142, XP055227889, New York	1-9,11, 12,23,24
A	ISSN: 1087-0156, DOI: 10.1038/nbt.3149 abstract; figure 1 the whole document	10,13-22
X	WO 2015/089427 A1 (BROAD INST INC; MASSACHUSETTS INST TECHNOLOGY [US]) 18 June 2015 (2015-06-18) cited in the application	1-9,11, 12,23,24
A	claims 1, 3, 7, 8, 18, 19, 23; figure 1 the whole document	10,13-22
A	Behnam Nabet: "dTAG - You're it!", addgene blog, 21 June 2018 (2018-06-21), XP055642640, Retrieved from the Internet: URL:https://blog.addgene.org/dtag-youre-it [retrieved on 2019-11-14] the whole document	1-24
X,P	WO 2019/028426 A1 (UNIV NORTH CAROLINA CHAPEL HILL [US]) 7 February 2019 (2019-02-07)	1-12,23, 24
A,P	claim 25; figures 21, 22, 30 the whole document	13-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2019/047366

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